



- (51) International Patent Classification:
A61K 47/14 (2006.01) *C12Q 1/68* (2006.01)
- (21) International Application Number:
PCT/US2015/029993
- (22) International Filing Date:
8 May 2015 (08.05.2015)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
61/990,518 8 May 2014 (08.05.2014) US
62/031,530 31 July 2014 (31.07.2014) US
- (71) Applicant: BIOGEN MA INC. [US/US]; 250 Binney Street, Cambridge, MA 02142 (US).
- (72) Inventors: SCANNEVIN, Robert, H.; 26 Huckleberry Road, Hopkinton, MA 01748 (US). RHODES, Kenneth, J.; 86 Juniper Road, Belmont, MA 02478 (US). BRENNAN, Melanie; 7 Linden Street Apt 2, Brookline, MA 02445 (US).
- (74) Agent: MYERS, Louis, P.; Lando & Anastasi LLP, Riverfront Office Park, One Main Street, Suite 1100, Cambridge, MA 02142 (US).
- (81) Designated States (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.
- (84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

[Continued on next page]

- (54) Title: DIMETHYLFUMARATE AND PRODRUGS FOR TREATMENT OF MULTIPLE SCLEROSIS

(57) Abstract: Methods and systems to evaluate prodrugs, drugs, or drug metabolites are provided.

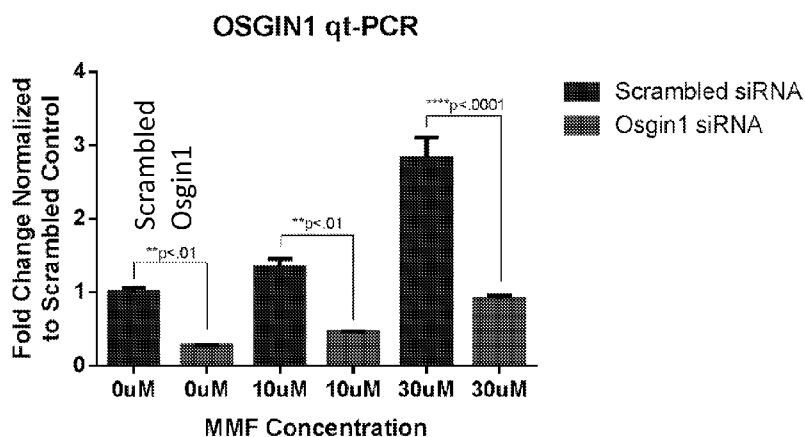


FIGURE 30

**Declarations under Rule 4.17:**

- *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
- *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

- *with international search report (Art. 21(3))*
- *with sequence listing part of description (Rule 5.2(a))*

DIMETHYLFUMARATE AND PRODRUGS FOR TREATMENT OF MULTIPLE SCLEROSIS

The invention relates, *inter alia*, to the use and activity of drugs and their metabolites, *e.g.*, dimethyl fumarate (DMF) and monomethyl fumarate (MMF), *e.g.*, in the treatment of multiple sclerosis (MS) and other disorders.

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application Nos. 61/990,518, filed May 8, 2014, and 62/031,530, filed July 31, 2014, the entire contents of each of which are incorporated herein by reference.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on May 7, 2015, is named B2047-7114WO_SL.txt and is 8,466 bytes in size.

BACKGROUND OF THE INVENTION

The relationship between a drug and its metabolite and their contribution to overall pharmacologic effect is often poorly understood.

Tecfidera® (BG-12, dimethyl fumarate, DMF) is a methyl ester of fumaric acid. Tecfidera® is an oral therapeutic approved in the U.S. for relapsing multiple sclerosis (MS). MS is an inflammatory disease of the brain and spinal cord characterized by recurrent foci of inflammation that lead to destruction of the myelin sheath. In many areas, nerve fibers are also damaged.

Preclinical studies indicate that activation of the nuclear factor (erythroid-derived 2)-like 2(Nrf2) pathway is thought to be involved in the clinical effects of Tecfidera®. *In vivo*, DMF is rapidly metabolized to monomethyl fumarate (MMF), and both compounds are

pharmacologically active. *In vitro*, DMF and MMF share some common effects, but also have divergent pharmacological properties.

Given the destructive effects of inflammatory MS lesions and the distinct effects of therapies such as DMF and MMF, the need exists for evaluating or monitoring a subject undergoing an MS therapy, or identifying a subject that would benefit from an MS therapy.

SUMMARY OF THE INVENTION

The present invention provides, at least in part, methods, devices, reaction mixtures and kits for evaluating, identifying, and/or treating a subject, *e.g.*, a subject having multiple sclerosis (MS) (*e.g.*, a subject with relapsing MS). In certain embodiments, responsiveness of a subject to a treatment (*e.g.*, an MS therapy that includes dimethyl fumarate) is evaluated by detecting a differential expression (*e.g.*, level and/or expression), of a gene (*e.g.*, a gene or a gene product) in response to a treatment that includes DMF and/or monomethyl fumarate (MMF). In certain embodiments, the gene is oxidative stress induced growth inhibitor 1 (*OSGIN1*). Applicants have identified both specific and common responses to DMF treatment and to MMF treatment in selected tissues and blood, *e.g.*, whole blood, in a subject. Without being bound by theory, the specific responses, *e.g.*, transcriptional signatures, induced by DMF and MMF indicate that not all the DMF *in vivo* effects are mediated through MMF, thus suggesting that DMF can directly mediate unique biological responses, not captured by MMF alone. Thus, the invention can, therefore, be used, for example: To evaluate responsiveness to, or monitor, a therapy or treatment that includes DMF; identify a subject as likely to benefit from a therapy or treatment that includes DMF; stratify a subject or a patient populations (*e.g.*, stratify a subject or patients as being likely or unlikely to respond to a therapy or treatment that includes DMF); and/or more effectively monitor, treat a disorder, *e.g.*, MS, or prevent worsening of disease and/or relapse. Many of the methods, devices, reaction mixtures and other inventions provided herein are described for use with DMF and its active metabolite MMF. However, it should be understood that the methods, devices, reaction mixtures and other inventions can be used with, or apply generically to, dialkyl fumarate prodrugs, *e.g.*, as shown in Formula A below, and other prodrugs, *e.g.*, as shown in Formulas I-X, and their active metabolites (*e.g.*, MMF).

Accordingly, in one aspect, the invention features a method of evaluating, monitoring, stratifying, or treating, a subject, *e.g.*, a subject having or at risk for MS. The method includes:

- a) acquiring a value for the expression of the *OSGIN1* gene;
 - b) responsive to said value,
 - i) classifying said subject, *e.g.*, classifying said subject as in need of treatment with DMF, MMF, or a prodrug of MMF,
 - ii) selecting said subject for treatment with DMF, MMF, or a prodrug of MMF, or with a treatment other than DMF, MMF, or a prodrug of MMF, or
 - iii) administering DMF, MMF, or a prodrug of MMF, or a treatment other than DMF, MMF, or a prodrug of MMF, to said subject,
- provided that the method comprises one of treating the subject, directly acquiring the value, or directly acquiring a sample from which the value is acquired.

In certain embodiments, the method comprises classifying said subject as in need of treatment with DMF, MMF, or a prodrug of MMF. In other embodiments, the method comprises selecting said subject for treatment with DMF, MMF, or a prodrug of MMF, or with a treatment other than DMF, MMF, or a prodrug of MMF. In certain embodiments, the method comprises administering DMF, MMF, or a prodrug of MMF, or a treatment other than DMF, MMF, or a prodrug of MMF, to said subject.

In certain embodiments, acquiring a value for the expression of the *OSGIN1* gene comprises hybridization of a probe specific for *OSGIN1* with an *OSGIN1* mRNA or an *OSGIN1* cDNA. In other embodiments, acquiring a value for the expression of the *OSGIN1* gene comprises amplifying, *e.g.*, with PCR amplification, an *OSGIN1* mRNA or an *OSGIN1* cDNA.

In certain embodiments, the value for expression of the gene comprises a value for a transcriptional parameter, *e.g.*, the level of an mRNA encoded by the gene. In other embodiments, acquiring a value for the expression of the *OSGIN1* gene comprises normalizing a value for an *OSGIN1* mRNA or an *OSGIN1* cDNA with the level of a second gene, *e.g.*, gene the expression of which is not affected by MS. In certain embodiments, the value for expression of the gene comprises a value for a translational parameter, *e.g.*, the level of a protein encoded by the gene, *e.g.*, by an antibody based measurement.

In certain embodiments, the subject, *e.g.*, a human subject, has an autoimmune disorder, *e.g.*, MS. In other embodiments, the subject with MS has a relapsing form of MS. In other embodiments, the subject has been administered DMF, MMF, or a prodrug of MMF, *e.g.*, prior to, or at the time of, acquiring the value.

In certain embodiments, a tissue of the subject, *e.g.*, the peripheral blood, comprises, greater than background levels, *e.g.*, therapeutic levels, of DMF, MMF, or a prodrug of MMF, or a combination thereof, *e.g.*, prior to, or at the time of, acquiring the value. In other embodiments, the value for expression of the gene is for cerebral spinal fluid or blood, *e.g.*, whole blood. In certain embodiments, the value for expression of the gene comprises a value for a translational parameter, *e.g.*, the level of a protein encoded by the gene, in cerebral spinal fluid or blood, *e.g.*, whole blood. In other embodiments, the value for expression of the gene is for a cerebral spinal fluid sample, a blood sample, or a blood derived sample, *e.g.*, serum, or an NK-cell containing fraction, from the subject. In certain embodiments, the sample is blood, and comprises, greater than background levels, *e.g.*, therapeutic levels, of DMF, MMF, or a prodrug of MMF, or a combination thereof.

In a related aspect, the invention features a method of evaluating, or monitoring, an MS treatment, *e.g.*, an MS treatment with DMF, MMF, or a prodrug of MMF, in a subject having MS, or at risk for developing MS. The method includes:

administering an MS treatment with DMF, MMF, or a prodrug of MMF to the subject;
and

acquiring from said subject a value for the expression of the *OSGIN1* gene,
wherein a change in the gene expression is indicative of a differential response to DMF, MMF, or a prodrug of MMF.

In certain embodiments, the method further comprises, responsive to said value, treating, selecting and/or altering one or more of: the course of the MS treatment, the dosing of the MS treatment, the schedule or time course of the MS treatment, or administration of a treatment other than DMF, MMF, or a prodrug of MMF.

In another related aspect, the invention features a method of treating a subject having, or at risk of having, MS, said method comprising:

administering DMF, MMF, or a prodrug of MMF to the subject in an amount sufficient to treat MS, provided that the subject is identified for treatment with the DMF, MMF, or a prodrug of MMF, on the basis of a value for the expression of a the *OSGIN1* gene.

In certain embodiments, the subject, *e.g.*, a human subject, has an autoimmune disorder, *e.g.*, MS. In other embodiments, the subject with MS has a relapsing form of MS. In certain embodiments, the subject has been administered DMF, MMF, or a prodrug of MMF, *e.g.*, prior to, or at the time of, acquiring the value.

In certain embodiments, a tissue of the subject, *e.g.*, the peripheral blood, comprises, greater than background levels, *e.g.*, therapeutic levels, of DMF, MMF, or a prodrug of MMF, or a combination thereof, *e.g.*, prior to, or at the time of, acquiring the value.

In certain embodiments, the value for expression of the gene comprises a value for a transcriptional parameter, *e.g.*, the level of an mRNA encoded by the gene. In other embodiments, the value for expression of the gene comprises a value for a translational parameter, *e.g.*, the level of a protein encoded by the gene.

In certain embodiments, the value for expression of the gene is for cerebral spinal fluid or blood, *e.g.*, whole blood. In other embodiments, the value for expression of the gene comprises a value for a translational parameter, *e.g.*, the level of a protein encoded by the gene, in cerebral spinal fluid or blood, *e.g.*, whole blood. In certain embodiments, the value for expression of the gene is for a cerebral spinal fluid sample, a blood sample, or a blood derived sample, *e.g.*, serum, or an NK-cell containing fraction, from the subject. In other embodiments, the sample is blood, and comprises, greater than background levels, *e.g.*, therapeutic levels, of DMF, MMF, or a prodrug of MMF, or a combination thereof.

Accordingly, in one aspect, the invention features a method of evaluating, monitoring, stratifying, or treating, a subject. The method includes:

a) acquiring a value for the expression of a gene (*e.g.*, a gene or a gene product), wherein said gene is chosen from one, two or all of:

- i) a dimethyl fumarate (DMF)-differentially expressed gene,
- ii) a monomethyl fumarate (MMF)-differentially expressed gene, or
- iii) a DMF/MMF-differentially expressed gene;

b) responsive to said value, performing one, two or all of:

- i) classifying said subject,
- ii) selecting or identifying said subject for treatment with DMF, or with a treatment other than DMF, or
- iii) administering DMF, or a treatment other than DMF, to said subject,

provided that the method comprises one of treating the subject, directly acquiring the value, or directly acquiring a sample from which the value is acquired.

In a related aspect, the invention features a method of evaluating, or monitoring, a treatment (*e.g.*, an MS treatment, *e.g.*, an MS treatment with a DMF) in a subject (*e.g.*, a subject, a patient, a patient group or population, having MS, or at risk for developing MS). The method includes:

administering to the subject, *e.g.*, a subject in need of treatment (*e.g.*, an MS treatment), a DMF;

acquiring from said subject a value for the expression of a gene (*e.g.*, a gene or a gene product), wherein said gene is chosen from one, two or all of:

- i) a dimethyl fumarate (DMF)-differentially expressed gene,
- ii) a monomethyl fumarate (MMF)-differentially expressed gene, or
- iii) a DMF/MMF-differentially expressed gene,

wherein a change in (i) or (ii) is indicative of a differential response to DMF or MMF, respectively, and a change in (iii) is indicative of a response to both DMF and MMF.

In certain embodiments, the method further comprises, responsive to said value, treating, selecting and/or altering one or more of: the course of the treatment (*e.g.*, MS treatment), the dosing of the treatment (*e.g.*, MS treatment), the schedule or time course of the treatment (*e.g.*,

MS treatment), or administration of a second, alternative treatment (*e.g.*, a treatment other than DMF).

In another related aspect, the invention features a method of treating a subject, *e.g.*, a subject having, or at risk of having, MS. The method includes:

administering to the subject a DMF in an amount sufficient to treat MS, provided that the subject is identified for treatment with the DMF on the basis of a value for the expression of a gene, wherein said gene is chosen from one, two or all of:

- i) a dimethyl fumarate (DMF)-differentially expressed gene,
- ii) a monomethyl fumarate (MMF)-differentially expressed gene, or
- iii) a DMF/MMF-differentially expressed gene.

Additional embodiments or features of any of the above aspects are as follows:

Acquiring a Value

In certain embodiments, the method includes acquiring a value for the expression of *OSGIN1*.

In certain embodiments, the method includes acquiring a value for the expression of *PADI4*. In other embodiments, the method includes acquiring a value for the expression of *p53*.

In certain embodiments, the method comprises acquiring a value for the expression of a plurality, *e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more, genes, and, optionally, any step responsive thereto can be responsive to one, some, or all, of the acquired values.

In certain embodiments, the gene used in acquiring the value is chosen from one, two or all of: a DMF-differentially expressed gene, an MMF-differentially expressed gene, or a gene expressed in response to both DMF and MMF (*e.g.*, a DMF/MMF-differentially expressed gene).

In certain embodiments, the plurality of genes comprise *OSGIN1*. In other embodiments, the plurality of genes comprise *PADI4*. In certain embodiments, the plurality of genes comprise *p53*.

In one embodiment, the value for expression of the gene includes a value for a transcriptional parameter, *e.g.*, the level of an mRNA encoded by the gene.

In another embodiment, the value for expression of the gene includes a value for a translational parameter, *e.g.*, the level of a protein encoded by the gene.

In certain embodiments, the method includes acquiring a value for the expression of a plurality of genes. In certain embodiments, said plurality includes two, three, four or more of:

- a) a plurality, *e.g.*, 2, 3, 4, 5, 6, 7, 8, 9 or 10, or more, DMF-differentially expressed genes;
- b) a plurality, *e.g.*, 2, 3, 4, 5, 6, 7, 8, 9 or 10, or more, MMF-differentially expressed genes;
- c) a DMF-differentially expressed gene and an MMF-differentially expressed gene;
- d) a DMF-differentially expressed gene and a gene that is both DMF-differentially expressed and MMF-differentially expressed; and
- e) an MMF-differentially expressed gene and a gene that is both DMF-differentially expressed and MMF-differentially expressed.

Blood Genes

In certain embodiments, the value for expression of the gene acquired is from blood, *e.g.*, whole blood (*e.g.*, a gene expressed in blood or a blood sample).

In one embodiment, the value for expression of the gene includes a value for a transcriptional parameter, *e.g.*, the level of an mRNA encoded by the gene, in blood, *e.g.*, whole blood. In certain embodiments, the gene is selected from one or more of the genes in Table 1 or Table 9. In embodiments, the gene is a gene from Table 9 that shows differential expression as measured by mRNA levels. In one embodiment, the differential expression is detected prior to or after (*e.g.*, 2, 3, 5, 7, 10, 12, 15 or 24 hours after) administration of a treatment (*e.g.*, a DMF or an MMF). In one embodiment, the gene is chosen from one, two, three, four or all of: Granzyme A (Gzma), Natural cytotoxicity triggering receptor 1 (Ncr1), Killer cell lectin-like receptor subfamily C member 1 (Klrc1), Killer cell lectin-like receptor subfamily B member 1B (Klrb1b), or Killer cell lectin-like receptor family E member 1 (Klre1). In one embodiment, the gene is chosen from one, two, three or all of: Granzyme A (Gzma), Natural cytotoxicity triggering receptor 1 (Ncr1), Killer cell lectin-like receptor subfamily C member 1 (Klrc1), or Killer cell lectin-like receptor subfamily B member 1B (Klrb1b).

In one embodiment, the value for expression of the gene comprises a value for a transcriptional parameter, *e.g.*, the level of an mRNA encoded by the gene, in blood, for 1, 2, 3, 4, or all of, Gzma, Ncr1, Klrc1, Klr1b, and Klre1. In one embodiment, the value for expression of the gene comprises a value for a transcriptional parameter, *e.g.*, the level of an mRNA encoded by the gene, in blood, for 1, 2, 3, or all of, Gzma, Ncr1, Klrc1, and Klr1b.

In other embodiments, the value for expression of the gene comprises a value for a translational parameter, *e.g.*, the level of a protein encoded by the gene, in blood, *e.g.*, whole blood. In certain embodiments, the gene is selected from one or more of the genes in Table 1 or Table 9. In embodiments, the gene is a gene from Table 9 that shows differential expression as measured by protein levels. In one embodiment, the differential expression is detected prior to or after (*e.g.*, 2, 3, 5, 7, 10, 12, 15 or 24 hours after) administration of a treatment (*e.g.*, a DMF or an MMF). In certain embodiments, the gene is chosen from one, two, three, or all of: Killer cell lectin-like receptor subfamily C member 1 (Klrc1), Killer cell lectin-like receptor subfamily B member 1B (Klr1b), NKKG2d (Klrk1), or Natural killer cells (CD94) (Klr1).

In one embodiment, a value for expression of the gene comprises a value for a translational parameter, *e.g.*, the level of a protein encoded by the gene, in blood, for 1, 2, 3, or all of, Klrc1, Klr1b, Klrk1, and Klr1.

In certain embodiments, the value for expression of the gene is for a blood sample, or a blood derived sample, *e.g.*, serum or plasma, or an NK-cell containing fraction, from the subject.

In certain embodiments, the blood comprises, greater than background levels, *e.g.*, therapeutic levels, of DMF, MMF, or both.

Other Tissues

In certain embodiments, the value for expression of the gene is for a tissue selected from cortical tissue, hippocampus, striatum, jejunum, kidney, liver, or spleen. In certain embodiments, the value for expression of the gene is for cerebral spinal fluid.

In certain embodiments, said gene is selected from the genes in Table 2, Table 3, Table 4, Table 5a, Table 5b, Table 6, Table 7, Table 8, or Tables 18-26.

Timing of Evaluation or Administration

In some embodiments, the value is acquired at one or more of the following periods: prior to beginning of treatment; during the treatment; or after the treatment has been administered. In embodiments, the treatment is an MS treatment (*e.g.*, a treatment that includes a DMF).

In certain embodiments, the subject has been administered the treatment, *e.g.*, the DMF, *e.g.*, prior to, at the time of, or after, acquiring the value. In one embodiment, the value is acquired after (*e.g.*, 2, 3, 5, 7, 10, 12, 15 or 24 hours after) administration of a treatment (*e.g.*, a DMF).

In one embodiment, the methods described herein include the step of comparing the value (*e.g.*, level) of one or more genes described herein to a specified parameter (*e.g.*, a reference value or sample; a sample obtained from a healthy subject; a sample obtained from the subject at different treatment intervals). For example, a sample can be analyzed at any stage of treatment, but preferably, prior to, during, or after terminating, administration of the therapy, *e.g.*, the MS therapy.

In certain embodiments, the methods include the step of detecting the level of one or more genes in the subject, prior to, or after, administering the therapy (*e.g.*, MS therapy), to the subject. In an embodiment, a change in gene expression indicates that the subject from whom the sample was obtained is responding to the therapy, *e.g.*, the MS therapy.

Tissue/Sample

In certain embodiments, a tissue (*e.g.*, cerebral spinal fluid) or blood (*e.g.*, a tissue or blood sample) of the subject, *e.g.*, the peripheral blood, comprises, greater than background levels, *e.g.*, therapeutic levels, of DMF, MMF, or both, *e.g.*, prior to, or at the time of, acquiring the value.

In certain embodiments, the sample is chosen from a non-cellular body fluid; or a cellular or tissue fraction. In one embodiment, the non-cellular fraction is chosen from blood, *e.g.*, whole blood, plasma or serum. In other embodiments, the cellular fraction comprises one or more of: T cells, B cells or myeloid cells. For example, the cellular fraction can include one or more of: natural killer (NK) cells, peripheral blood mononuclear cells (PBMC), CD8+ T cells, or Regulatory T cells.

In certain embodiments, the methods described herein, further includes the step of acquiring the sample, *e.g.*, a biological sample, from the subject.

A sample can include any material obtained and/or derived from a biological sample, including a polypeptide, and nucleic acid (*e.g.*, genomic DNA, cDNA, RNA) purified or processed from the sample.

Subjects

For any of the methods, devices or kits disclosed herein, the subject treated, or the subject from which the value or sample is acquired, is a subject having, or at risk of having MS at any stage of treatment. In certain embodiments, the MS patient is chosen from a patient having one or more of: Benign MS, relapsing MS, *e.g.*, relapsing-remitting MS (RRMS) (*e.g.*, quiescent RRMS, active RRMS), primary progressive MS, or secondary progressive MS. In other embodiments, the subject has MS-like symptoms, such as those having clinically isolated syndrome (CIS) or clinically defined MS (CDMS). In one embodiment, the subject is an MS patient (*e.g.*, a patient with relapsing MS) prior to administration of an MS therapy described herein (*e.g.*, prior to administration of a DMF). In another embodiment, the subject is an MS patient (*e.g.*, a relapsing MS patient) after administration of an MS therapy described herein (*e.g.*, a DMF). In other embodiments, the subject is an MS patient after administration of the MS therapy for one, two, five, ten, twenty, twenty four hours; one week, two weeks, one month, two months, three months, four months, six months, one year or more.

In one embodiment, the subject has a relapsing form of MS, *e.g.*, RRMS.

Treatment/Other Therapies

Alternatively, or in combination with the methods described herein, the invention features a method of treating a subject having one or more symptoms associated with MS. In one embodiment, the subject is identified as responding or not responding to a therapy, using the methods, devices, or kits described herein.

In an embodiment the method comprises treating the subject with DMF, MMF, or a combination thereof.

In certain embodiments, the treatment includes reducing, retarding or preventing, a relapse, or the worsening of a disability, in the MS patients.

In one embodiment, the method includes administering to a subject (*e.g.*, a subject described herein) a therapy for MS (*e.g.*, a DMF), in an amount sufficient to reduce one or more symptoms associated with MS.

In embodiments where a first therapy (*e.g.*, the DMF therapy) is not detectably effective, an alternative or other MS therapy can be chosen. Exemplary other therapies include, but are not limited to, an IFN- β agent (*e.g.*, an IFN- β 1a molecule or an IFN- β 1b molecule, including analogues and derivatives thereof (*e.g.*, pegylated variants thereof)). In one embodiment, the other MS therapy includes an IFN- β 1a agent (*e.g.*, Avonex®, Rebif®). In another embodiment, the other MS therapy includes an IFN- β 1b agent (*e.g.*, Betaseron®, Betaferon®). In other embodiments, the other MS therapy includes a polymer of four amino acids found in myelin basic protein, *e.g.*, a polymer of glutamic acid, lysine, alanine and tyrosine (*e.g.*, glatiramer (Copaxone®)); an antibody or fragment thereof against α -4 integrin (*e.g.*, natalizumab (Tysabri®)); an anthracenedione molecule (*e.g.*, mitoxantrone (Novantrone®)); fingolimod (FTY720; Gilenya®); Daclizumab; alemtuzumab (Lemtrada®); or an anti-LINGO-1 antibody. In certain embodiments, the methods include the use of one or more symptom management therapies, such as antidepressants, analgesics, anti-tremor agents, among others.

Detection Methods

In certain embodiments, the gene or gene product detected is, *e.g.*, nucleic acid, cDNA, RNA (*e.g.*, mRNA), or a polypeptide.

A nucleic acid can be detected, or the level determined, by any means of nucleic acid detection, or detection of the expression level of the nucleic acids, including but not limited to, nucleic acid hybridization assay, amplification-based assays (*e.g.*, polymerase chain reaction), sequencing, and/or *in situ* hybridization.

In certain embodiments, a probe is a nucleic acid that specifically hybridizes with a transcription product of the gene or genes. In other embodiments, the detection includes amplification of a transcription product of the gene or genes. In other embodiments, the detection includes reverse transcription and amplification of a transcription product of the gene or genes.

In other embodiments, a translation product of the gene or genes, *e.g.*, a polypeptide, is detected. The polypeptide can be detected, or the level determined, by any means of polypeptide

detection, or detection of the expression level of the polypeptides. For example, the polypeptide can be detected using a probe or reagent which specifically binds with the polypeptides. In another embodiment, the reagent is selected from the group consisting of an antibody, an antibody derivative, and an antibody fragment, *e.g.*, a labeled antibody (*e.g.*, a fluorescent or a radioactive label), or fragment thereof, that specifically binds with a translation product of the gene or genes. In one embodiment, the polypeptide is detected using antibody-based detection techniques, such as enzyme-based immunoabsorbent assay, immunofluorescence cell sorting (FACS), immunohistochemistry, immunofluorescence (IF), antigen retrieval and/or microarray detection methods. Polypeptide detection methods can be performed in any other assay format, including but not limited to, ELISA, RIA, and mass spectrometry.

In certain embodiments, the probe is an antibody. In one embodiment, the method of detection includes a sandwich-based detection, *e.g.*, ELISA based sandwich assay detection, of a translation product of the gene or genes.

Other embodiments:

The methods of the invention can further include the step of monitoring the subject, *e.g.*, for a change (*e.g.*, an increase or decrease) in one or more of: levels of one or more MS biomarkers; the rate of appearance of new lesions, *e.g.*, in an MRI scan; the appearance of new disease-related symptoms; a change in EDSS score; a change in quality of life; or any other parameter related to clinical outcome. The subject can be monitored in one or more of the following periods: prior to beginning of treatment; during the treatment; or after the treatment has been administered. Monitoring can be used to evaluate the need for further treatment with the same MS therapy, or for additional MS treatment. Generally, a decrease in one or more of the parameters described above is indicative of the improved condition of the subject. In certain embodiments, the methods described herein further include: performing a neurological examination, evaluating the subject's status on the Expanded Disability Status Scale (EDSS), or detecting the subject's lesion status as assessed using an MRI.

Devices

In an aspect, the invention features a device comprising:

one, or a plurality of, probes, each probe being specific for a product, *e.g.*, a translational product or transcriptional product, of the *OSGIN1* gene, wherein the device includes less than 10, 25, 50, 100, 200, 250, 300, or 500 probes specific for products, *e.g.*, a translational product or transcriptional product, of a gene other than *OSGIN1*.

In certain embodiments, the device further comprises, a sample from a tissue of a subject, *e.g.*, the peripheral blood, which comprises greater than background levels, *e.g.*, therapeutic levels, of DMF, MMF, or a prodrug of MMF, or a combination thereof.

In another aspect, the invention features a device comprising:

one, or a plurality of, *e.g.*, 2, 3, 4, 5, 6, 7, 8, 9 or 10, or more, probes, each probe being specific for a product, *e.g.*, a translational product or transcriptional product, of a gene selected independently from:

- i) a dimethyl fumarate (DMF)-differentially expressed gene,
- ii) a monomethyl fumarate (MMF)-differentially expressed gene, or
- iii) a DMF/MMF-differentially expressed gene.

In certain embodiments, the gene is *OSGIN1*.

In certain embodiments, the gene is *PADI4*. In other embodiments, the gene is *p53*.

In one embodiment, the device includes one, or a plurality of, *e.g.*, 2, 3, 4, 5, 6, 7, 8, 9 or 10, or more, probes, each probe being specific for a product, *e.g.*, a translational product or transcriptional product, of a dimethyl fumarate (DMF)-differentially expressed gene.

In certain embodiments, the plurality of genes comprise *OSGIN1*.

In certain embodiments, the plurality of genes comprise *PADI4*. In other embodiments, the plurality of genes comprise *p53*.

In one embodiment, the probe or probes of the device are specific for a gene or genes selected from the genes in Table 9.

In other embodiments, the probe or probes of the device are specific for a gene or genes selected from the genes in Table 9 that shows differential expression as measured by mRNA levels.

In yet other embodiments, the device includes a probe specific for a transcriptional product of 1, 2, 3, 4, or all of, *Gzma*, *Ncr1*, *Klrc1*, *Klrb1b*, and *Klre1*. In yet other embodiments,

the device includes a probe specific for a transcriptional product of 1, 2, 3, or all of, *Gzma*, *Ncr1*, *Klrc1*, and *Klrb1b*.

In other embodiments, the device includes a probe specific for a gene or genes from the genes in Table 9 that shows differential expression as measured by protein levels.

In other embodiments, the device includes a probe specific for a translational product of 1, 2, 3, or all of, *Klrc1*, *Klrb1b*, *Klrk1*, and *Klrd1*.

In one embodiment, the device further comprises a sample, *e.g.*, a sample as described herein. In one embodiment, the sample is from a subject having an autoimmune disorder, *e.g.*, MS, relapsing MS. In other embodiments, the sample is from a subject that has been administered DMF. In yet other embodiments, the sample is from a tissue of the subject, *e.g.*, the peripheral blood, which comprises greater than background levels, *e.g.*, therapeutic levels, of DMF, MMF, or both.

In other embodiments, the device further comprises a sample, *e.g.*, a blood sample, or a substance derived from blood, *e.g.*, serum, or an NK-cell containing fraction.

In yet other embodiments, the probe or probes of the device are specific for a gene or genes that are selected independently from the genes in Table 2, Table 3, Table 4, Table 5a, Table 5b, Table 6, Table 7, Table 8, or Tables 18-26

In other embodiments, the probe is a nucleic acid that specifically hybridizes with a transcription product of the gene or genes.

In embodiments, the device is configured to allow amplification of a transcription product of the gene or genes.

In other embodiments, the device is configured to allow reverse transcription and amplification of a transcription product of the gene or genes.

In other embodiments, a probe is an antibody, *e.g.*, a labeled antibody, or fragment thereof, that specifically binds with a translation product of the gene or genes.

In other embodiments, the device is configured to allow sandwich-based detection, *e.g.*, ELISA based sandwich assay detection, of a translation product of the gene or genes.

In yet other embodiments, the device has less than 10, 25, 50, 100, 200, 250, 300, or 500 probes specific for products, *e.g.*, a translational product or transcriptional product, of genes that are not

- i) a dimethyl fumarate (DMF)-differentially expressed gene,
- ii) a monomethyl fumarate (MMF)-differentially expressed gene, or
- iii) a DMF/MMF-differentially expressed gene.

In one embodiment, the device has less than 10, 25, 50, 100, 200, 250, 300, or 500 probes specific for products, *e.g.*, a translational product or transcriptional product, of genes that are not a dimethyl fumarate (DMF)-differentially expressed gene.

In yet other embodiments, the device has less than 10, 25, 50, 100, 200, 250, 300, or 500 probes specific for products, *e.g.*, a translational product or transcriptional product, of genes that are not listed in Table 9.

In other embodiments, the device has at least 10, 20, 30, 40, 50, 60, 70, 80, or 90 % of the probes of the device are specific for a product, *e.g.*, a translational product or transcriptional product, of:

- i) a dimethyl fumarate (DMF)-differentially expressed gene,
- ii) a monomethyl fumarate (MMF)-differentially expressed gene, or
- iii) a DMF/MMF-differentially expressed gene.

In other embodiments, the device has at least 10, 20, 30, 40, 50, 60, 70, 80, or 90 % of the probes of the device are specific for a product, *e.g.*, a translational product or transcriptional product, of a dimethyl fumarate (DMF)-differentially expressed gene.

In other embodiments, the device has at least 10, 20, 30, 40, 50, 60, 70, 80, or 90 % of the probes of the device are specific for a product, *e.g.*, a translational product or transcriptional product, of a gene listed in Table 9.

In certain embodiments, the probe or probes are disposed on a surface of the device.

In another aspect, the invention features a method of using a device described herein. The method includes:

- providing a device described herein; and
- contacting the device with a sample described herein,
- thereby using the device.

In one embodiment, the method includes a step of capturing a signal, *e.g.*, an electronic, or visual signal, to evaluate the sample.

Reaction Mixtures

In an aspect, the invention features a reaction mixture comprising:

a sample from a tissue of a subject, *e.g.*, the peripheral blood, *e.g.*, tissue which comprises greater than background levels, *e.g.*, therapeutic levels, of DMF, MMF, or a prodrug of MMF, or a combination thereof; and

one, or a plurality of, probes each probe being specific for a product, *e.g.*, a translational product or transcriptional product, of the *OSGIN1* gene,

wherein the reaction mixture includes less than 10, 25, 50, 100, 200, 250, 300, or 500 probes specific for products, *e.g.*, a translational product or transcriptional product, of genes other than *OSGIN1*.

In another aspect, the invention features, a reaction mixture comprising:

a sample; and

one, or a plurality of, *e.g.*, 2, 3, 4, 5, 6, 7, 8, 9 or 10, or more, probes, each probe being specific for a product, *e.g.*, a translational product or transcriptional product, of a gene selected independently from:

- i) a dimethyl fumarate (DMF)-differentially expressed gene,
- ii) a monomethyl fumarate (MMF)-differentially expressed gene, or
- iii) a DMF/MMF-differentially expressed gene.

In certain embodiments, the gene is *OSGIN1*.

In certain embodiments, the gene is *PADI4*. In other embodiments, the gene is *p53*.

In an embodiment the reaction mixture comprises one, or a plurality of, *e.g.*, 2, 3, 4, 5, 6, 7, 8, 9 or 10, or more, probes, each probe being specific for a product, *e.g.*, a translational product or transcriptional product, of a dimethyl fumarate (DMF)-differentially expressed gene.

In another embodiment, the probe or probes are specific for a gene or genes selected from the genes in Table 9.

In another embodiment, the probe or probes are specific for a gene or genes selected from the genes in Table 9 that shows differential expression as measured by mRNA levels.

In one embodiment, the reaction mixture comprises probes specific for a transcriptional product of 1, 2, 3, 4, or all of, *Gzma*, *Ncr1*, *Klrc1*, *Klrb1b*, and *Klre1*. In one embodiment, the

reaction mixture comprises probes specific for a transcriptional product of 1, 2, 3, or all of, Gzma, Nrcl, Klrc1, and Klrb1b.

In another embodiment, the probe or probes are specific for a gene or genes from the genes in Table 9 that shows differential expression as measured by protein levels.

In other embodiments, the reaction mixture comprises probes specific for a translational product of 1, 2, 3, or all of, Klrc1, Klrb1b, Klrk1, and Klrd1.

In an embodiment, said sample is from a subject having an autoimmune disorder, *e.g.*, MS, *e.g.*, relapsing MS.

In one embodiment, said sample is from a subject that has been administered DMF.

In an embodiment, said sample is from a tissue of the subject, *e.g.*, the peripheral blood, which comprises greater than background levels, *e.g.*, therapeutic levels, of DMF, MMF, or both.

In an embodiment, said sample comprises blood, or a substance derived from blood, *e.g.*, serum, or an NK-cell containing fraction.

In other embodiments, the probe or probes are specific for a gene or genes that are selected independently from the genes in Table 2, Table 3, Table 4, Table 5a, Table 5b, Table 6, Table 7, Table 8, or Tables 18-26.

In an embodiment, a probe is a nucleic acid that specifically hybridizes with a transcription product of the gene or genes.

In an embodiment, the reaction mixture further comprises reagents to allow for amplification of a transcription product of the gene or genes.

In an embodiment, the reaction mixture further comprises reagents to allow for reverse transcription and amplification of a transcription product of the gene or genes.

In an embodiment, a probe is an antibody, *e.g.*, a labeled antibody, or fragment thereof, that specifically binds with a translation product of the gene or genes.

In an embodiment, the reaction mixture comprises reagents to allow sandwich-based detection, *e.g.*, ELISA based sandwich assay detection, of a translation product of the gene or genes.

In other embodiments, the reaction mixture has less than 10, 25, 50, 100, 200, 250, 300, or 500 probes specific for products, *e.g.*, a translational product or transcriptional product, of genes that are not

i) a dimethyl fumarate (DMF)-differentially expressed gene,

- ii) a monomethyl fumarate (MMF)-differentially expressed gene, or
- iii) a DMF/MMF-differentially expressed gene.

In other embodiments, the reaction mixture has less than 10, 25, 50, 100, 200, 250, 300, or 500 probes specific for products, *e.g.*, a translational product or transcriptional product, of genes that are not a dimethyl fumarate (DMF)-differentially expressed gene.

In other embodiments, the reaction mixture has less than 10, 25, 50, 100, 200, 250, 300, or 500 probes specific for products, *e.g.*, a translational product or transcriptional product, of genes that are not listed in Table 9.

In other embodiments, the reaction mixture has at least 10, 20, 30, 40, 50, 60, 70, 80, or 90 % of the probes of the device are specific for a product, *e.g.*, a translational product or transcriptional product, of:

- i) a dimethyl fumarate (DMF)-differentially expressed gene,
- ii) a monomethyl fumarate (MMF)-differentially expressed gene, or
- iii) a DMF/MMF-differentially expressed gene.

In other embodiments, at least 10, 20, 30, 40, 50, 60, 70, 80, or 90 % of the probes are specific for a product, *e.g.*, a translational product or transcriptional product, of a dimethyl fumarate (DMF)-differentially expressed gene.

In other embodiments of the reaction mixture at least 10, 20, 30, 40, 50, 60, 70, 80, or 90 % of the probes of the device are specific for a product, *e.g.*, a translational product or transcriptional product, of a gene listed in Table 9.

The reaction mixture of can comprise a surface on which the probe or probes are disposed.

In another aspect, the invention features a method of making a reaction mixture comprising:

providing a sample from a tissue of a subject, *e.g.*, the peripheral blood, *e.g.*, tissue which comprises greater than background levels, *e.g.*, therapeutic levels, of DMF, MMF, or a prodrug of MMF, or a combination thereof;

contacting the sample with one or a plurality of probes specific for *OSGIN1*,

wherein the reaction mixture includes less than 10, 25, 50, 100, 200, 250, 300, or 500 probes specific for products, *e.g.*, a translational product or transcriptional product, of genes other than *OSGIN1*,

thereby making a reaction mixture.

In another aspect, the invention features a method of making a reaction mixture comprising:

providing the a sample described herein;

contacting the sample with one or a plurality of probes described herein, or with a device described herein,

thereby making a reaction mixture.

In embodiments, the method of making includes capturing a signal, *e.g.*, an electronic, or visual signal, to evaluate the sample.

Kits

In another aspect, the invention features kits for evaluating a sample, *e.g.*, a sample from an MS patient, to detect or determine the level of one or more genes as described herein. In certain embodiments, the one or more genes comprise *OSGIN1*. In certain embodiments, the one or more genes comprise *PADI4*. In other embodiments, the one or more genes comprise *p53*. The kit includes a means for detection of (*e.g.*, a reagent that specifically detects) one or more genes as described herein. In certain embodiments, the kit includes an MS therapy. In one another embodiment, the kit comprises an antibody, an antibody derivative, or an antibody fragment to a protein produce of the gene. In one embodiment, the kit includes an antibody-based detection technique, such as immunofluorescence cell sorting (FACS), immunohistochemistry, antigen retrieval and/or microarray detection reagents. In one embodiment, at least one of the reagents in the kit is an antibody that binds to a gene product (optionally) with a detectable label (*e.g.*, a fluorescent or a radioactive label). In certain embodiments, the kit is an ELISA or an immunohistochemistry (IHC) assay for detection of the gene.

The methods, devices, reaction mixtures, kits, and other inventions described herein can further include providing or generating, and/or transmitting information, *e.g.*, a report, containing data of the evaluation or treatment determined by the methods, assays, and/or kits as described herein. The information can be transmitted to a report-receiving party or entity (*e.g.*, a patient, a health care provider, a diagnostic provider, and/or a regulatory agency, *e.g.*, the FDA), or otherwise submitting information about the methods, assays and kits disclosed herein to another party. The method can relate to compliance with a regulatory requirement, *e.g.*, a pre- or post approval requirement of a regulatory agency, *e.g.*, the FDA. In one embodiment, the report-receiving party or entity can determine if a predetermined requirement or reference value is met by the data, and, optionally, a response from the report-receiving entity or party is received, *e.g.*, by a physician, patient, diagnostic provider.

In a related aspect, the invention features a method of evaluating, or monitoring, a prodrug, in a subject, *e.g.*, a human or a non-human mammal. The method includes:

administering the prodrug to the subject;

acquiring from said subject a value for the expression of a gene (*e.g.*, a gene or a gene product), wherein said gene is chosen from one, two or all of:

- i) a prodrug-differentially expressed gene,
- ii) a drug-differentially expressed gene, or
- iii) a prodrug/drug-differentially expressed gene,

wherein a change in (i) or (ii) is indicative of a differential response to prodrug or drug, respectively, and a change in (iii) is indicative of a response to both prodrug and drug.

In certain embodiments, the gene is *OSGIN1*.

In certain embodiments, the gene is *PADI4*. In other embodiments, the gene is *p53*.

In certain embodiments, the method further comprises comparing the value with a reference value.

In a related aspect, the invention features a method of evaluating, or monitoring, a drug, in a subject, *e.g.*, a human or a non-human mammal. The method includes:

administering the drug to the subject;

acquiring from said subject a value for the expression of a gene (*e.g.*, a gene or a gene product), wherein said gene is chosen from one, two or all of:

- i) a drug-differentially expressed gene,
- ii) a drug metabolite-differentially expressed gene, or
- iii) a drug/drug metabolite-differentially expressed gene,

wherein a change in (i) or (ii) is indicative of a differential response to drug or drug metabolite, respectively, and a change in (iii) is indicative of a response to both drug and drug metabolite.

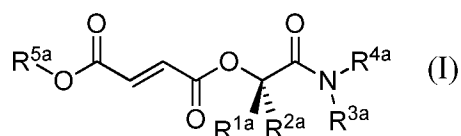
In certain embodiments, the gene is *OSGIN1*.

In certain embodiments, the gene is *PADI4*. In other embodiments, the gene is *p53*.

In certain embodiments, the method further comprises comparing the value with a reference value.

In an embodiment the drug is DMF and the drug metabolite is MMF.

The present invention contemplates treatment with DMF and its active metabolite MMF. However, it should be understood that the methods and other inventions can be used with, or apply generically to, dialkyl fumarate prodrugs, *e.g.*, as shown in Formula A below, and other prodrugs, *e.g.*, as shown in Formulas I-X, and their active metabolites (*e.g.*, MMF), and monoalkyl fumarate drugs, *e.g.*, as shown in Formula B below. In an embodiment the drug metabolite is MMF and the drug is DMF. In an embodiment the drug metabolite is MMF and the drug or prodrug is a compound of Formula I:



or a pharmaceutically acceptable salt thereof, wherein

R^{1a} and R^{2a} are independently chosen from hydrogen, C_{1-6} alkyl, and substituted C_{1-6} alkyl;

R^{3a} and R^{4a} are independently chosen from hydrogen, C_{1-6} alkyl, substituted C_{1-6} alkyl, C_{1-6} heteroalkyl, substituted C_{1-6} heteroalkyl, C_{4-12} cycloalkylalkyl, substituted C_{4-12} cycloalkylalkyl, C_{7-12} arylalkyl, and substituted C_{7-12} arylalkyl; or R^{3a} and R^{4a} together with the nitrogen to which they are bonded form a ring chosen from a C_{5-10} heteroaryl,

substituted C₅₋₁₀ heteroaryl, C₅₋₁₀ heterocycloalkyl, and substituted C₅₋₁₀ heterocycloalkyl;
and

R^{5a} is chosen from methyl, ethyl, and C₃₋₆ alkyl;

wherein each substituent group is independently chosen from halogen, -OH,
-CN, -CF₃, =O, -NO₂, benzyl, -C(O)NR^{11a}₂, -R^{11a}, -OR^{11a}, -C(O)R^{11a}, -COOR^{11a}, and
-NR^{11a}₂ wherein each R^{11a} is independently chosen from hydrogen and C₁₋₄ alkyl;

with the proviso that when R^{5a} is ethyl; then R^{3a} and R^{4a} are independently chosen from
hydrogen, C₁₋₆ alkyl, and substituted C₁₋₆ alkyl.

In certain embodiments of a compound of Formula (I), each substituent group is
independently chosen from halogen, -OH, -CN, -CF₃, -R^{11a}, -OR^{11a}, and
-NR^{11a}₂ wherein each R^{11a} is independently chosen from hydrogen and C₁₋₄ alkyl. In certain
embodiments, each substituent group is independently chosen from -OH, and
-COOH.

In certain embodiments of a compound of Formula (I), each substituent group is
independently chosen from =O, C₁₋₄ alkyl, and -COOR^{11a} wherein R^{11a} is chosen from hydrogen
and C₁₋₄ alkyl.

In certain embodiments of a compound of Formula (I), each of R^{1a} and R^{2a} is hydrogen.

In certain embodiments of a compound of Formula (I), one of R^{1a} and R^{2a} is hydrogen
and the other of R^{1a} and R^{2a} is C₁₋₄ alkyl.

In certain embodiments of a compound of Formula (I), one of R^{1a} and R^{2a} is hydrogen
and the other of R^{1a} and R^{2a} is chosen from methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl,
sec-butyl, and tert-butyl.

In certain embodiments of a compound of Formula (I), one of R^{1a} and R^{2a} is hydrogen
and the other of R^{1a} and R^{2a} is methyl.

In certain embodiments of a compound of Formula (I), R^{3a} and R^{4a} are independently
chosen from hydrogen and C₁₋₆ alkyl.

In certain embodiments of a compound of Formula (I), R^{3a} and R^{4a} are independently
chosen from hydrogen and C₁₋₄ alkyl.

In certain embodiments of a compound of Formula (I), R^{3a} and R^{4a} are independently
chosen from hydrogen, methyl, and ethyl.

In certain embodiments of a compound of Formula (I), each of R^{3a} and R^{4a} is hydrogen; in certain embodiments, each of R^{3a} and R^{4a} is methyl; and in certain embodiments, each of R^{3a} and R^{4a} is ethyl.

In certain embodiments of a compound of Formula (I), R^{3a} is hydrogen; and R^{4a} is chosen from C_{1-4} alkyl, substituted C_{1-4} alkyl wherein the substituent group is chosen from $=O$, $-OR^{11a}$, $-COOR^{11a}$, and $-NR^{11a}_2$, wherein each R^{11a} is independently chosen from hydrogen and C_{1-4} alkyl. In certain embodiments of a compound of Formula (I), R^{3a} is hydrogen; and R^{4a} is chosen from C_{1-4} alkyl, benzyl, 2-methoxyethyl, carboxymethyl, carboxypropyl, 1,2,4-thiadioxolyl, methoxy, 2-methoxycarbonyl, 2-oxo(1,3-oxazolidinyl), 2-(methylethoxy)ethyl, 2-ethoxyethyl, (tert-butyloxycarbonyl)methyl, (ethoxycarbonyl)methyl, carboxymethyl, (methylethyl)oxycarbonylmethyl, and ethoxycarbonylmethyl.

In certain embodiments of a compound of Formula (I), R^{3a} and R^{4a} together with the nitrogen to which they are bonded form a ring chosen from a C_{5-6} heterocycloalkyl, substituted C_{5-6} heterocycloalkyl, C_{5-6} heteroaryl, and substituted C_{5-6} heteroaryl ring. In certain embodiments of a compound of Formula (I), R^{3a} and R^{4a} together with the nitrogen to which they are bonded form a ring chosen from a C_5 heterocycloalkyl, substituted C_5 heterocycloalkyl, C_5 heteroaryl, and substituted C_5 heteroaryl ring. In certain embodiments of a compound of Formula (I), R^{3a} and R^{4a} together with the nitrogen to which they are bonded form a ring chosen from a C_6 heterocycloalkyl, substituted C_6 heterocycloalkyl, C_6 heteroaryl, and substituted C_6 heteroaryl ring. In certain embodiments of a compound of Formula (I), R^{3a} and R^{4a} together with the nitrogen to which they are bonded form a ring chosen from piperazine, 1,3-oxazolidinyl, pyrrolidine, and morpholine ring.

In certain embodiments of a compound of Formula (I), R^{3a} and R^{4a} together with the nitrogen to which they are bonded form a C_{5-10} heterocycloalkyl ring.

In certain embodiments of a compound of Formula (I), R^{5a} is methyl.

In certain embodiments of a compound of Formula (I), R^{5a} is ethyl.

In certain embodiments of a compound of Formula (I), R^{5a} is C_{3-6} alkyl.

In certain embodiments of a compound of Formula (I), R^{5a} is chosen from methyl, n-propyl, isopropyl, n-butyl, sec-butyl, isobutyl, and tert-butyl.

In certain embodiments of a compound of Formula (I), R^{5a} is chosen from methyl, ethyl, n-propyl, isopropyl, n-butyl, sec-butyl, isobutyl, and tert-butyl.

In certain embodiments of a compound of Formula (I), one of R^{1a} and R^{2a} is hydrogen and the other of R^{1a} and R^{2a} is C_{1-6} alkyl; R^{3a} is hydrogen; R^{4a} is chosen from hydrogen, C_{1-6} alkyl, and benzyl.

In certain embodiments of a compound of Formula (I), one of R^{1a} and R^{2a} is hydrogen and the other of R^{1a} and R^{2a} is C_{1-6} alkyl; R^{3a} is hydrogen; R^{4a} is chosen from hydrogen, C_{1-6} alkyl, and benzyl; and R^{5a} is methyl.

In certain embodiments of a compound of Formula (I), one of R^{1a} and R^{2a} is hydrogen and the other of R^{1a} and R^{2a} is chosen from hydrogen and C_{1-6} alkyl; and each of R^{3a} and R^{4a} is C_{1-6} alkyl.

In certain embodiments of a compound of Formula (I), one of R^{1a} and R^{2a} is hydrogen and the other of R^{1a} and R^{2a} is chosen from hydrogen and C_{1-6} alkyl; each of R^{3a} and R^{4a} is C_{1-6} alkyl; and R^{5a} is methyl. In certain embodiments of a compound of Formula (I), each of R^{1a} and R^{2a} is hydrogen; each of R^{3a} and R^{4a} is C_{1-6} alkyl; and R^{5a} is methyl.

In certain embodiments of a compound of Formula (I), one of R^{1a} and R^{2a} is hydrogen and the other of R^{1a} and R^{2a} is chosen from hydrogen and C_{1-4} alkyl; R^{3a} is hydrogen; R^{4a} is chosen from C_{1-4} alkyl, substituted C_{1-4} alkyl wherein the substituent group is chosen from =O, -OR^{11a}, -COOR^{11a}, and -NR^{11a}₂, wherein each R^{11a} is independently chosen from hydrogen and C_{1-4} alkyl; and R^{5a} is methyl. In certain embodiments of a compound of Formula (I), one of R^{1a} and R^{2a} is hydrogen and the other of R^{1a} and R^{2a} is methyl; R^{3a} is hydrogen; R^{4a} is chosen from C_{1-4} alkyl, substituted C_{1-4} alkyl wherein the substituent group is chosen from =O, -OR^{11a}, -COOR^{11a}, and -NR^{11a}₂, wherein each R^{11a} is independently chosen from hydrogen and C_{1-4} alkyl; and R^{5a} is methyl. In certain embodiments of a compound of Formula (I), each of R^{1a} and R^{2a} is hydrogen; R^{3a} is hydrogen; R^{4a} is chosen from C_{1-4} alkyl, substituted C_{1-4} alkyl wherein the substituent group is chosen from =O, -OR^{11a}, -COOR^{11a}, and -NR^{11a}₂, wherein each R^{11a} is independently chosen from hydrogen and C_{1-4} alkyl; and R^{5a} is methyl.

In certain embodiments of a compound of Formula (I), R^{3a} and R^{4a} together with the nitrogen to which they are bonded form a C_{5-10} heterocycloalkyl ring.

In certain embodiments of a compound of Formula (I), one of R^{1a} and R^{2a} is hydrogen and the other of R^{1a} and R^{2a} is chosen from hydrogen and C_{1-6} alkyl; R^{3a} and R^{4a} together with the nitrogen to which they are bonded form a ring chosen from a C_{5-6} heterocycloalkyl,

substituted C₅₋₆ heterocycloalkyl, C₅₋₆ heteroaryl, and substituted C₅₋₆ heteroaryl ring; and R^{5a} is methyl. In certain embodiments of a compound of Formula (I), one of R^{1a} and R^{2a} is hydrogen and the other of R^{1a} and R^{2a} is methyl; R^{3a} and R^{4a} together with the nitrogen to which they are bonded form a ring chosen from a C₅₋₆ heterocycloalkyl, substituted C₅₋₆ heterocycloalkyl, C₅₋₆ heteroaryl, and substituted C₅₋₆ heteroaryl ring; and R^{5a} is methyl. In certain embodiments of a compound of Formula (I), each of R^{1a} and R^{2a} is hydrogen; R^{3a} and R^{4a} together with the nitrogen to which they are bonded form a ring chosen from a C₅₋₆ heterocycloalkyl, substituted C₅₋₆ heterocycloalkyl, C₅₋₆ heteroaryl, and substituted C₅₋₆ heteroaryl ring; and R^{5a} is methyl.

In certain embodiments of a compound of Formula (I), one of R^{1a} and R^{2a} is hydrogen and the other of R^{1a} and R^{2a} is chosen from hydrogen and C₁₋₆ alkyl; and R^{3a} and R^{4a} together with the nitrogen to which they are bonded form a ring chosen from morpholine, piperazine, and N-substituted piperazine.

In certain embodiments of a compound of Formula (I), one of R^{1a} and R^{2a} is hydrogen and the other of R^{1a} and R^{2a} is chosen from hydrogen and C₁₋₆ alkyl; R^{3a} and R^{4a} together with the nitrogen to which they are bonded form a ring chosen from morpholine, piperazine, and N-substituted piperazine; and R^{5a} is methyl.

In certain embodiments of a compound of Formula (I), R^{5a} is not methyl.

In certain embodiments of a compound of Formula (I), R^{1a} is hydrogen, and in certain embodiments, R^{2a} is hydrogen.

In certain embodiments of a compound of Formula (I), the compound is chosen from: (N,N-diethylcarbamoyl)methyl methyl(2E)but-2-ene-1,4-dioate; methyl[N-benzylcarbamoyl]methyl(2E)but-2-ene-1,4-dioate; methyl 2-morpholin-4-yl-2-oxoethyl(2E)but-2-ene-1,4-dioate; (N-butylcarbamoyl)methyl methyl(2E)but-2-ene-1,4-dioate; [N-(2-methoxyethyl)carbamoyl]methyl methyl(2E)but-2-ene-1,4-dioate; 2-{2-[(2E)-3-(methoxycarbonyl)prop-2-enoyloxy]acetylamino}acetic acid; 4-{2-[(2E)-3-(methoxycarbonyl)prop-2-enoyloxy]acetylamino}butanoic acid; methyl(N-(1,3,4-thiadiazol-2-yl)carbamoyl)methyl(2E)but-2-ene-1,4-dioate; (N,N-dimethylcarbamoyl)methyl methyl(2E)but-2-ene-1,4-dioate; (N-methoxy-N-methylcarbamoyl)methyl methyl(2E)but-2-ene-1,4-dioate; bis-(2-methoxyethylamino)carbamoyl]methyl methyl(2E)but-2-ene-1,4-dioate; [N-(methoxycarbonyl)carbamoyl]methyl methyl(2E)but-2-ene-1,4-dioate; 4-{2-[(2E)-3-(methoxycarbonyl)prop-2-enoyloxy]acetylamino}butanoic acid, sodium salt; methyl 2-oxo-2-

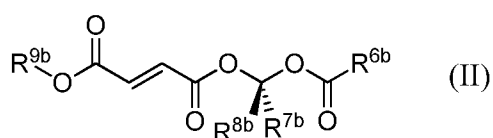
piperazinylethyl(2E)but-2-ene-1,4-dioate; methyl 2-oxo-2-(2-oxo(1,3-oxazolidin-3-yl)ethyl(2E)but-2-ene-1,4-dioate; {N-[2-(dimethylamino)ethyl]carbamoyl}methyl methyl(2E)but-2-ene-1,4 dioate; methyl 2-(4-methylpiperazinyl)-2-oxoethyl(2E)but-2-ene-1,4-dioate; methyl {N-[(propylamino)carbonyl]carbamoyl}methyl(2E)but-2-ene-1,4-dioate; 2-(4-acetylpiperazinyl)-2-oxoethyl methyl(2E)but-2-ene-1,4-dioate; {N,N-bis[2-(methylethoxy)ethyl]carbamoyl}methyl methyl(2E)but-2-ene-1,4-dioate; methyl 2-(4-benzylpiperazinyl)-2-oxoethyl(2E)but-2-ene-1,4-dioate; [N,N-bis(2-ethoxyethyl)carbamoyl]methyl methyl(2E)but-2-ene-1,4-dioate; 2-{(2S)-2-[(tert-butyl)oxycarbonyl]pyrrolidinyl}-2-oxoethyl methyl(2E)but-2-ene-1,4-dioate; 1-{2-{(2E)-3-(methoxycarbonyl)prop-2-enoyloxy}acetyl}(2S)pyrrolidine-2-carboxylic acid; (N-{[(tert-butyl)oxycarbonyl]methyl}-N-methylcarbamoyl)methyl methyl(2E)but-2-ene-1,4-dioate; {N-(ethoxycarbonyl)methyl]-N-methylcarbamoyl}methyl methyl(2E)but-2-ene-1,4-dioate; methyl 1-methyl-2-morpholin-4-yl-2-oxoethyl(2E)but-2-ene-1,4-dioate; [N,N-bis(2-methoxyethyl)carbamoyl]ethyl methyl(2E)but-2-ene-1,4-dioate; (N,N-dimethylcarbamoyl)ethyl methyl(2E)but-2-ene-1,4-dioate; 2-{2-[(2E)-3-(methoxycarbonyl)prop-2-enoyloxy]-N-methylacetylamin}acetic acid; (N-{[(tert-butyl)oxycarbonyl]methyl}carbamoyl)methyl methyl(2E)but-2-ene-1,4-dioate; (2E)but-methyl-N-{[(methylethyl)oxycarbonyl]methyl}carbamoyl)methyl(2E)but-2-ene-1,4-dioate; {N-[(ethoxycarbonyl)methyl]-N-benzylcarbamoyl}methyl methyl(2E)but-2-ene-1,4-dioate; {N-[(ethoxycarbonyl)methyl]-N-benzylcarbamoyl}ethyl methyl(2E)but-2-ene-1,4-dioate; {N-[(ethoxycarbonyl)methyl]-N-methylcarbamoyl}ethyl methyl(2E)but-2-ene-1,4-dioate; (1S)-1-methyl-2-morpholin-4-yl-2-oxo ethyl methyl(2E)but-2-ene-1,4-dioate; (1S)-1-[N,N-bis(2-methoxyethyl)carbamoyl]ethyl methyl(2E)but-2-ene-1,4-dioate; (1R)-1-(N,N-diethylcarbamoyl)ethyl methyl(2E)but-2-ene-1,4-dioate; and a pharmaceutically acceptable salt of any of the foregoing.

In certain embodiments of a compound of Formula (I), the compound is chosen from: (N,N-diethylcarbamoyl)methyl methyl(2E)but-2-ene-1,4-dioate; methyl[N-benzylcarbamoyl]methyl(2E)but-2-ene-1,4-dioate; methyl 2-morpholin-4-yl-2-oxoethyl(2E)but-2-ene-1,4-dioate; (N-butylcarbamoyl)methyl methyl(2E)but-2-ene-1,4-dioate; [N-(2-methoxyethyl)carbamoyl]methyl methyl(2E)but-2-ene-1,4-dioate; 2-{2-[(2E)-3-(methoxycarbonyl)prop-2-enoyloxy]acetylamin}acetic acid; {2-[(2E)-3-

(methoxycarbonyl)prop-2-enoyloxy]acetyl amino}butanoic acid; methyl(N-(1,3,4-thiadiazol-2-yl)carbamoyl)methyl(2E)but-2-ene-1,4-dioate; (N,N-dimethylcarbamoyl)methyl methyl(2E)but-2-ene-1,4-dioate; (N-methoxy-N-methylcarbamoyl)methyl methyl(2E)but-2-ene-1,4-dioate; bis-(2-methoxyethylamino)carbamoyl]methyl methyl(2E)but-2-ene-1,4-dioate; [N-(methoxycarbonyl)carbamoyl]methyl methyl(2E)but-2-ene-1,4-dioate; methyl 2-oxo-2-piperazinyethyl(2E)but-2-ene-1,4-dioate; methyl 2-oxo-2-(2-oxo(1,3-oxazolidin-3-yl)ethyl(2E)but-2-ene-1,4-dioate; {N-[2-(dimethylamino)ethyl]carbamoyl}methyl methyl(2E)but-2-ene-1,4-dioate; (N-[(methoxycarbonyl)ethyl]carbamoyl)methyl methyl(2E)but-2-ene-1,4-dioate; 2-{2-[(2E)-3-(methoxycarbonyl)prop-2-enoyloxy]acetyl amino}propanoic acid; and a pharmaceutically acceptable salt of any of the foregoing.

In certain embodiments of a compound of Formula (I), R^{3a} and R^{4a} are independently chosen from hydrogen, C₁₋₆ alkyl, substituted C₁₋₆ alkyl, C₆₋₁₀ aryl, substituted C₆₋₁₀ aryl, C₄₋₁₂ cycloalkylalkyl, substituted C₄₋₁₂ cycloalkylalkyl, C₇₋₁₂ arylalkyl, substituted C₇₋₁₂ arylalkyl, C₁₋₆ heteroalkyl, substituted C₁₋₆ heteroalkyl, C₆₋₁₀ heteroaryl, substituted C₆₋₁₀ heteroaryl, C₄₋₁₂ heterocycloalkylalkyl, substituted C₄₋₁₂ heterocycloalkylalkyl, C₇₋₁₂ heteroarylalkyl, substituted C₇₋₁₂ heteroarylalkyl; or R^{3a} and R^{4a} together with the nitrogen to which they are bonded form a ring chosen from a C₅₋₁₀ heteroaryl, substituted C₅₋₁₀ heteroaryl, C₅₋₁₀ heterocycloalkyl, and substituted C₅₋₁₀ heterocycloalkyl.

In some embodiments, the compound that metabolizes to MMF is a compound of Formula II:



or a pharmaceutically acceptable salt thereof, wherein

R^{6b} is chosen from C₁₋₆ alkyl, substituted C₁₋₆ alkyl, C₁₋₆ heteroalkyl, substituted C₁₋₆ heteroalkyl, C₃₋₈ cycloalkyl, substituted C₃₋₈ cycloalkyl, C₆₋₈ aryl, substituted C₆₋₈ aryl, and -OR^{10b} wherein R^{10b} is chosen from C₁₋₆ alkyl, substituted C₁₋₆ alkyl, C₃₋₁₀ cycloalkyl, substituted C₃₋₁₀ cycloalkyl, C₆₋₁₀ aryl, and substituted C₆₋₁₀ aryl;

R^{7b} and R^{8b} are independently chosen from hydrogen, C₁₋₆ alkyl, and substituted C₁₋₆ alkyl; and

R^{9b} is chosen from C₁₋₆ alkyl and substituted C₁₋₆ alkyl;

wherein each substituent group is independently chosen from halogen, -OH, -CN, -CF₃, =O, -NO₂, benzyl, -C(O)NR^{11b}₂, -R^{11b}, -OR^{11b}, -C(O)R^{11b}, -COOR^{11b}, and -NR^{11b}₂

wherein each R^{11b} is independently chosen from hydrogen and C₁₋₄ alkyl.

In certain embodiments of a compound of Formula (II), each substituent group is independently chosen from halogen, -OH, -CN, -CF₃, -R^{11b}, -OR^{11b}, and -NR^{11b}₂ wherein each R^{11b} is independently chosen from hydrogen and C₁₋₄ alkyl.

In certain embodiments of a compound of Formula (I), each substituent group is independently chosen from =O, C₁₋₄ alkyl, and -COOR^{11b} wherein R^{11b} is chosen from hydrogen and C₁₋₄ alkyl.

In certain embodiments of a compound of Formula (II), one of R^{7b} and R^{8b} is hydrogen and the other of R^{7b} and R^{8b} is C₁₋₆ alkyl. In certain embodiments of a compound of Formula (II), one of R^{7b} and R^{8b} is hydrogen and the other of R^{7b} and R^{8b} is C₁₋₄ alkyl.

In certain embodiments of a compound of Formula (II), one of R^{7b} and R^{8b} is hydrogen and the other of R^{7b} and R^{8b} is chosen from methyl, ethyl, n-propyl, and isopropyl. In certain embodiments of a compound of Formula (II), each of R^{7b} and R^{8b} is hydrogen.

In certain embodiments of a compound of Formula (II), R^{9b} is chosen from substituted C₁₋₆ alkyl and -OR^{11b} wherein R^{11b} is independently C₁₋₄ alkyl.

In certain embodiments of a compound of Formula (II), R^{9b} is C₁₋₆ alkyl, in certain embodiments, R^{9b} is C₁₋₃ alkyl; and in certain embodiments, R^{9b} is chosen from methyl and ethyl.

In certain embodiments of a compound of Formula (II), R^{9b} is methyl.

In certain embodiments of a compound of Formula (II), R^{9b} is chosen from ethyl, n-propyl, isopropyl, n-butyl, sec-butyl, isobutyl, and tert-butyl.

In certain embodiments of a compound of Formula (II), R^{9b} is chosen from methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, and tert-butyl.

In certain embodiments of a compound of Formula (II), R^{6b} is C₁₋₆ alkyl; one of R^{7b} and R^{8b} is hydrogen and the other of R^{7b} and R^{8b} is C₁₋₆ alkyl; and R^{9b} is chosen from C₁₋₆ alkyl and substituted C₁₋₆ alkyl.

In certain embodiments of a compound of Formula (II), R^{6b} is -OR^{10b}.

In certain embodiments of a compound of Formula (II), R^{10b} is chosen from C₁₋₄ alkyl, cyclohexyl, and phenyl.

In certain embodiments of a compound of Formula (II), R^{6b} is chosen from methyl, ethyl, n-propyl, and isopropyl; one of R^{7b} and R^{8b} is hydrogen and the other of R^{7b} and R^{8b} is chosen from methyl, ethyl, n-propyl, and isopropyl.

In certain embodiments of a compound of Formula (II), R^{6b} is substituted C₁₋₂ alkyl, wherein each of the one or more substituent groups are chosen from -COOH, -NHC(O)CH₂NH₂, and -NH₂.

In certain embodiments of a compound of Formula (II), R^{6b} is chosen from ethoxy, methylethoxy, isopropyl, phenyl, cyclohexyl, cyclohexyloxy, -CH(NH₂CH₂COOH, -CH₂CH(NH₂)COOH, -CH(NHC(O)CH₂NH₂)-CH₂COOH, and -CH₂CH(NHC(O)CH₂NH₂)-COOH.

In certain embodiments of a compound of Formula (II), R^{9b} is chosen from methyl and ethyl; one of R^{7b} and R^{8b} is hydrogen and the other of R^{7b} and R^{8b} is chosen from hydrogen, methyl, ethyl, n-propyl, and isopropyl; and R^{6b} is chosen from C₁₋₃ alkyl, substituted C₁₋₂ alkyl wherein each of the one or more substituent groups are chosen -COOH, -NHC(O)CH₂NH₂, and -NH₂, -OR^{10b} wherein R^{10b} is chosen from C₁₋₃ alkyl and cyclohexyl, phenyl, and cyclohexyl.

In certain embodiments of a compound of Formula (II), the compound is chosen from:

ethoxycarbonyloxyethyl methyl(2E)but-2-ene-1,4-dioate;
methyl(methylethoxycarbonyloxy)ethyl(2E)but-2-ene-1,4-dioate;
(cyclohexyloxycarbonyloxy)ethyl methyl(2E)but-2-ene-1,4-dioate; and a pharmaceutically acceptable salt of any of the foregoing.

In certain embodiments of a compound of Formula (II), the compound is chosen from:

methyl(2-methylpropanoyloxy)ethyl(2E)but-2-ene-1,4-dioate; methyl phenylcarbonyloxyethyl(2E)but-2-ene-1,4-dioate; cyclohexylcarbonyloxybutyl methyl(2E)but-2-ene-1,4-dioate; [(2E)-3-(methoxycarbonyl)prop-2-enoyloxy]ethyl methyl(2E)but-2-ene-1,4-dioate; methyl 2-methyl-1-phenylcarbonyloxypropyl(2E)but-2-ene-1,4-dioate; and a pharmaceutically acceptable salt of any of the foregoing.

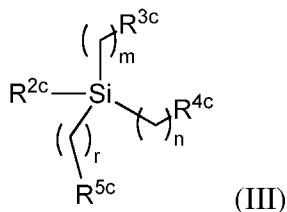
In certain embodiments of a compound of Formula (II), the compound is chosen from:

ethoxycarbonyloxyethyl methyl(2E)but-2-ene-1,4-dioate;
methyl(methylethoxycarbonyloxy)ethyl(2E)but-2-ene-1,4-dioate; methyl(2-methylpropanoyloxy)ethyl(2E)but-2-ene-1,4-dioate; methyl phenylcarbonyloxyethyl(2E)but-2-ene-1,4-dioate; cyclohexylcarbonyloxybutyl methyl(2E)but-2-ene-1,4-dioate; [(2E)-3-

(methoxycarbonyl)prop-2-enoyloxy]ethyl methyl(2E)but-2-ene-1,4-dioate;
 (cyclohexyloxycarbonyloxy)ethyl methyl(2E)but-2-ene-1,4-dioate; methyl 2-methyl-1-phenylcarbonyloxypropyl(2E)but-2-ene-1,4-dioate; 3-({[(2E)-3-(methoxycarbonyl)prop-2-enoyloxy]methyl}oxycarbonyl)(3S)-3-aminopropanoic acid, 2,2,2-trifluoroacetic acid; 3-({[(2E)-3-(methoxycarbonyl)prop-2-enoyloxy]methyl}oxycarbonyl)(2S)-2-aminopropanoic acid, 2,2,2-trifluoroacetic acid; 3-({[(2E)-3-(methoxycarbonyl)prop-2-enoyloxy]methyl}oxycarbonyl)(3S)-3-(2- -aminoacetyl amino)propanoic acid, 2,2,2-trifluoroacetic acid; 3-({[(2E)-3-(methoxycarbonyl)prop-2-enoyloxy]methyl}oxycarbonyl)(2S)-2-aminopropanoic acid, 2,2,2-trifluoroacetic acid; 3-{{[(2E)-3-(methoxycarbonyl)prop-2-enoyloxy]ethoxycarbonyloxy}(2S)-2-aminopropanoic acid, chloride; and a pharmaceutically acceptable salt of any of the foregoing. The compounds of Formulae (I)-(II) may be prepared using methods known to those skilled in the art, or the methods disclosed in U.S. Pat. No. 8,148,414 B2.

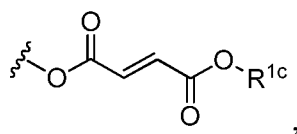
In another embodiment is provided silicon-containing compounds, which like DMF and the compounds of Formulae (I)-(II), can metabolize into MMF upon administration.

In some embodiments, the compound that metabolizes to MMF is a compound of Formula (III):



or a pharmaceutically acceptable salt thereof, wherein:

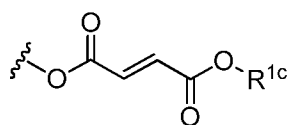
R^{2c} is C_1 - C_{10} alkyl, C_5 - C_{15} aryl, hydroxyl, $-O$ - C_1 - C_{10} alkyl, or $-O$ - C_5 - C_{15} aryl;
 each of R^{3c} , R^{4c} , and R^{5c} , independently, is C_1 - C_{10} alkyl, C_5 - C_{15} aryl, hydroxyl, $-O$ - C_1 - C_{10} alkyl, $-O$ - C_5 - C_{15} aryl, or



wherein R^{1c} is C_1 - C_{24} alkyl or C_5 - C_{50} aryl; each of which can be optionally substituted; and

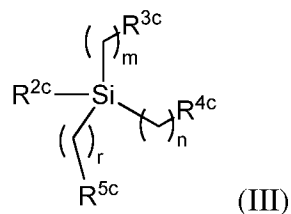
each of m , n , and r , independently, is 0-4;

provided that at least one of R^{3c} , R^{4c} , and R^{5c} is



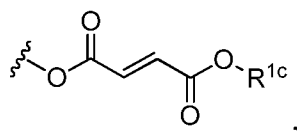
Another group of compounds of Formula III include compounds wherein R^{1c} is optionally substituted C_1 - C_{24} alkyl. Another group of compounds of Formula III include compounds wherein R^{1c} is optionally substituted C_1 - C_6 alkyl. Another group of compounds of Formula III include compounds wherein R^{1c} is optionally substituted methyl, ethyl, or isopropyl. Another group of compounds of Formula III include compounds wherein R^{1c} is optionally substituted C_5 - C_{50} aryl. Another group of compounds of Formula III include compounds wherein R^{1c} is optionally substituted C_5 - C_{10} aryl. Another group of compounds of Formula III include compounds wherein R^{2c} is C_1 - C_{10} alkyl. Another group of compounds of Formula III include compounds wherein R^{2c} is optionally substituted C_1 - C_6 alkyl. Another group of compounds of Formula III include compounds wherein R^{2c} is optionally substituted methyl, ethyl, or isopropyl. Another group of compounds of Formula III include compounds wherein R^{2c} is optionally substituted C_5 - C_{15} aryl. Another group of compounds of Formula III include compounds wherein R^{2c} is optionally substituted C_5 - C_{10} aryl.

In a further embodiment, the compound that metabolizes to MMF is a compound of Formula (III):



or a pharmaceutically acceptable salt thereof, wherein

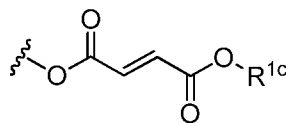
R^{2c} is C_1 - C_{10} alkyl, C_6 - C_{10} aryl, hydroxyl, $-O$ - C_1 - C_{10} alkyl, or $-O$ - C_6 - C_{10} aryl; each of R^{3c} , R^{4c} , and R^{5c} , independently, is C_1 - C_{10} alkyl, C_6 - C_{10} aryl, hydroxyl, $-O$ - C_1 - C_{10} alkyl, $-O$ - C_6 - C_{10} aryl, or



wherein R^{1c} is C_1 - C_{24} alkyl or C_6 - C_{10} aryl; each of which can be optionally substituted;
and

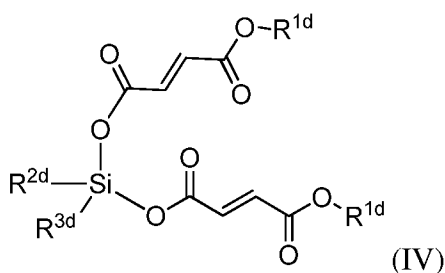
each of m , n , and r , independently, is 0-4;

provided that at least one of R^{3c} , R^{4c} , and R^{5c} is



In some embodiments, the compound that metabolizes to MMF is chosen from (dimethylsilanediyl)dimethyl difumarate; methyl ((trimethoxysilyl)methyl) fumarate; methyl ((trihydroxysilyl)methyl) fumarate; trimethyl (methylsilanetriyl) trifumarate; and a pharmaceutically acceptable salt of any of the foregoing.

In some embodiments, the compound that metabolizes to MMF is a compound of Formula (IV):



or a pharmaceutically acceptable salt thereof, wherein:

each R^{1d} is independently optionally substituted C_1 - C_{24} alkyl or C_5 - C_{50} aryl;

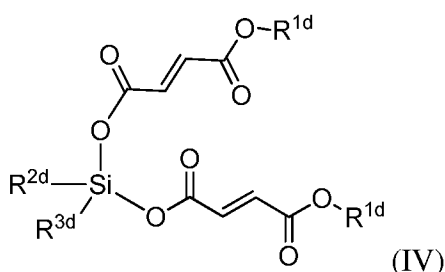
each of, independently, R^{2d} and R^{3d} , is C_1 - C_{10} alkyl or C_5 - C_{15} aryl.

R^{2d} and R^{3d} can be the same or different, can be optionally substituted, and independently can be selected from the group consisting of C_1 - C_{10} alkyl or C_5 - C_{15} aryl.

In another embodiment, compounds of Formula IV include compounds wherein each R^{1d} is independently optionally substituted C_1 - C_{24} alkyl or C_6 - C_{10} aryl. In another embodiment, compounds of Formula IV include compounds wherein R^{1d} is optionally substituted C_1 - C_{24} alkyl. Another group of compounds of Formula IV include compounds wherein R^{1d} is optionally substituted C_1 - C_6 alkyl. Another group of compounds of Formula IV include compounds wherein R^{1d} is optionally substituted methyl, ethyl, or isopropyl. Another group of compounds of Formula IV include compounds wherein R^{1d} is optionally substituted C_5 - C_{50} aryl. Another group of compounds of Formula IV include compounds wherein R^{1d} is optionally substituted C_5 - C_{10} aryl. Another group of compounds of Formula IV include compounds wherein each of R^{2d}

and R^{3d} is, independently, optionally substituted C_1 - C_{10} alkyl. Another group of compounds of Formula IV include compounds wherein each of R^{2d} and R^{3d} is, independently, optionally substituted C_1 - C_6 alkyl. Another group of compounds of Formula IV include compounds wherein each of R^{2d} and R^{3d} is, independently, optionally substituted methyl, ethyl, or isopropyl. Another group of compounds of Formula IV include compounds wherein each of R^{2d} and R^{3d} is, independently, optionally substituted C_5 - C_{15} aryl. Another group of compounds of Formula IV include compounds wherein each of R^{2d} and R^{3d} is, independently, optionally substituted C_5 - C_{10} aryl.

In a further embodiment, the compound that metabolizes to MMF is a compound of Formula (IV):

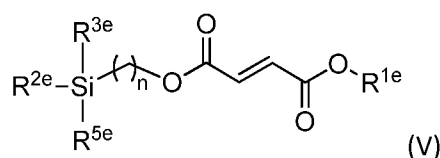


or a pharmaceutically acceptable salt thereof, wherein:

R^{1d} is C_1 - C_{24} alkyl or C_6 - C_{10} aryl; and

each of, independently, R^{2d} and R^{3d} , is C_1 - C_{10} alkyl or C_6 - C_{10} aryl.

In some embodiments, the compound that metabolizes to MMF is a compound of Formula (V):



or a pharmaceutically acceptable salt thereof, wherein:

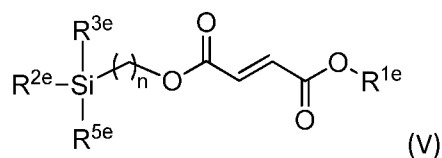
R^{1e} is C_1 - C_{24} alkyl or C_5 - C_{50} aryl;

each of R^{2e} , R^{3e} , and R^{5e} , independently, is hydroxyl, C_1 - C_{10} alkyl, C_5 - C_{15} aryl, $-O$ - C_1 - C_{10} alkyl, or $-O$ - C_5 - C_{15} aryl; and

n is 1 or 2.

In another embodiment, compounds of Formula V include compounds wherein R^{1e} is optionally substituted C₁-C₂₄ alkyl. Another group of compounds of Formula V include compounds wherein R^{1e} is optionally substituted C₁-C₆ alkyl. Another group of compounds of Formula V include compounds wherein R^{1e} is optionally substituted methyl, ethyl, or isopropyl. Another group of compounds of Formula V include compounds wherein R^{1e} is optionally substituted C₅-C₅₀ aryl. Another group of compounds of Formula V include compounds wherein R^{1e} is optionally substituted C₅-C₁₀ aryl. Another group of compounds of Formula V include compounds wherein each of R^{2e}, R^{3e}, and R^{5e} is, independently, hydroxyl. Another group of compounds of Formula V include compounds wherein each of R^{2e}, R^{3e}, and R^{5e} is, independently, optionally substituted C₁-C₁₀ alkyl. Another group of compounds of Formula V include compounds wherein each of R^{2e}, R^{3e}, and R^{5e} is, independently, optionally substituted C₁-C₆ alkyl. Another group of compounds of Formula V include compounds wherein each of R^{2e}, R^{3e}, and R^{5e} is, independently, optionally substituted methyl, ethyl, or isopropyl. Another group of compounds of Formula V include compounds wherein each of R^{2e}, R^{3e}, and R^{5e} is, independently, optionally substituted C₅-C₁₅ aryl. Another group of compounds of Formula V include compounds wherein each of R^{2e}, R^{3e}, and R^{5e} is, independently, optionally substituted C₅-C₁₀ aryl.

In a further embodiment, the compound that metabolizes to MMF is a compound of Formula (V):



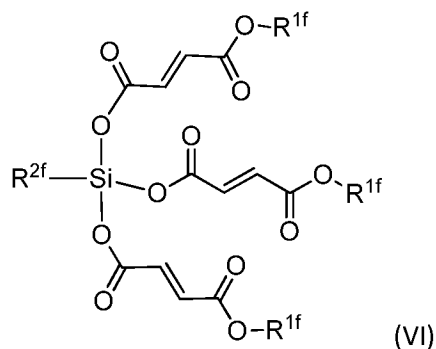
or a pharmaceutically acceptable salt thereof, wherein:

R^{1e} is C₁-C₂₄ alkyl or C₆-C₁₀ aryl;

each of R^{2e}, R^{3e}, and R^{5e}, independently, is hydroxyl, C₁-C₁₀ alkyl, C₆-C₁₀ aryl, -O-C₁-C₁₀ alkyl, or -O-C₆-C₁₀ aryl; and

n is 1 or 2.

In some embodiments, the compound that metabolizes to MMF is a compound of Formula (VI):



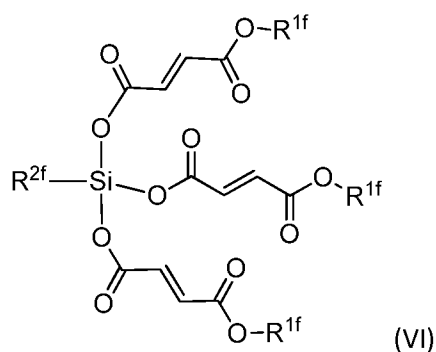
or a pharmaceutically acceptable salt thereof, wherein:

R^{1f} is C_1 - C_{24} alkyl or C_5 - C_{50} aryl; and

R^{2f} is C_1 - C_{10} alkyl.

In another embodiment, compounds of Formula VI include compounds wherein R^{1f} is optionally substituted C_1 - C_{24} alkyl. Another group of compounds of Formula VI include compounds wherein R^{1f} is optionally substituted C_1 - C_6 alkyl. Another group of compounds of Formula VI include compounds wherein R^{1f} is optionally substituted methyl, ethyl, or isopropyl. Another group of compounds of Formula VI include compounds wherein R^{1f} is optionally substituted C_5 - C_{50} aryl. Another group of compounds of Formula VI include compounds wherein R^{1f} is optionally substituted C_5 - C_{10} aryl. Another group of compounds of Formula VI include compounds wherein R^{2f} is optionally substituted C_1 - C_6 alkyl. Another group of compounds of Formula VI include compounds wherein R^{2f} is optionally substituted methyl, ethyl, or isopropyl.

In a further embodiment, the compound that metabolizes to MMF is a compound of Formula (VI):

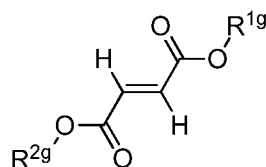


or a pharmaceutically acceptable salt thereof, wherein:

R^{1f} is C_1 - C_{24} alkyl or C_6 - C_{10} aryl; and

R^{2f} is C_1 - C_{10} alkyl.

In an embodiment, the dialkyl fumarate is:



Formula A

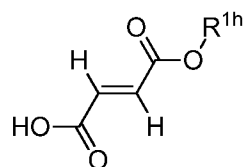
wherein R^{1g} and R^{2g} , which may be the same or different, independently represent a linear, branched or cyclic, saturated or unsaturated C_{1-20} alkyl radical which may be optionally substituted with halogen (Cl, F, I, Br), hydroxy, C_{1-4} alkoxy, nitro or cyano.

In an embodiment, R^{1g} and R^{2g} , which may be the same or different, independently are methyl, ethyl, n-propyl, isopropyl, n-butyl, sec-butyl, t-butyl, pentyl, cyclopentyl, 2-ethyl hexyl, hexyl, cyclohexyl, heptyl, cycloheptyl, octyl, vinyl, allyl, 2-hydroxy ethyl, 2 or 3-hydroxy propyl, 2-methoxy ethyl, methoxy methyl or 2- or 3-methoxy propyl.

In an embodiment, R^{1g} and R^{2g} are identical and are methyl or ethyl.

In an embodiment, R^{1g} and R^{2g} are methyl.

In an embodiment, the compound is a monoalkyl fumarate. In an embodiment, the monoalkyl fumarate is:



Formula B

wherein R^{1h} represents a linear, branched or cyclic, saturated or unsaturated C_{1-20} alkyl radical which may be optionally substituted with halogen (Cl, F, I, Br), hydroxy, C_{1-4} alkoxy, nitro or cyano;

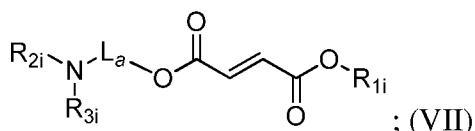
or a pharmaceutically acceptable salt thereof.

In an embodiment, R^{1h} is methyl, ethyl, n-propyl, isopropyl, n-butyl, sec-butyl, t-butyl, pentyl, cyclopentyl, 2-ethyl hexyl, hexyl, cyclohexyl, heptyl, cycloheptyl, octyl, vinyl, allyl, 2-hydroxy ethyl, 2 or 3-hydroxy propyl, 2-methoxy ethyl, methoxy methyl or 2- or 3-methoxy propyl.

In an embodiment, R^{1h} is methyl or ethyl.

In an embodiment, R^{1h} is methyl.

In a further embodiment, the compound that metabolizes to MMF is a compound of Formula (VII):



wherein:

R_{1i} is unsubstituted C_1-C_6 alkyl;

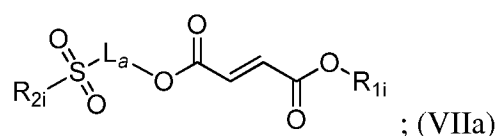
L_a is substituted or unsubstituted C_1-C_6 alkyl linker, substituted or unsubstituted C_3-C_{10} carbocycle, substituted or unsubstituted C_6-C_{10} aryl, substituted or unsubstituted heterocycle comprising one or two 5- or 6-member rings and 1-4 heteroatoms selected from N, O and S, or substituted or unsubstituted heteroaryl comprising one or two 5- or 6-member rings and 1-4 heteroatoms selected from N, O and S; and

R_{2i} and R_{3i} are each, independently, H, substituted or unsubstituted C_1-C_6 alkyl, substituted or unsubstituted C_2-C_6 alkenyl, substituted or unsubstituted C_2-C_6 alkynyl, substituted or unsubstituted C_6-C_{10} aryl, substituted or unsubstituted C_3-C_{10} carbocycle, substituted or unsubstituted heterocycle comprising one or two 5- or 6-member rings and 1-4 heteroatoms selected from N, O and S, or substituted or unsubstituted heteroaryl comprising one or two 5- or

6-member rings and 1-4 heteroatoms selected from N, O and S;

or alternatively, R_{2i} and R_{3i}, together with the nitrogen atom to which they are attached, form a substituted or unsubstituted heteroaryl comprising one or two 5- or 6-member rings and 1-4 heteroatoms selected from N, O and S or a substituted or unsubstituted heterocycle comprising one or two 5- or 6-member rings and 1-4 heteroatoms selected from N, O and S.

In some embodiments, the compound of Formula (VII) is selected from a compound of Formula (VIIa):



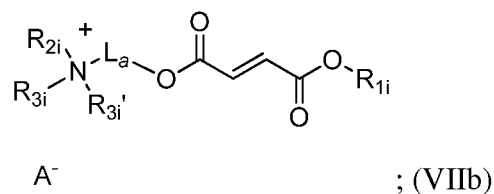
wherein:

R_{1i} is unsubstituted C₁-C₆ alkyl;

L_a is substituted or unsubstituted C₁-C₆ alkyl linker, substituted or unsubstituted C₃-C₁₀ carbocycle, substituted or unsubstituted C₆-C₁₀ aryl, substituted or unsubstituted heterocycle comprising one or two 5- or 6-member rings and 1-4 heteroatoms selected from N, O and S, or substituted or unsubstituted heteroaryl comprising one or two 5- or 6-member rings and 1-4 heteroatoms selected from N, O and S; and

R_{2i} is H, substituted or unsubstituted C₁-C₆ alkyl, substituted or unsubstituted C₂-C₆ alkenyl, substituted or unsubstituted C₂-C₆ alkynyl, substituted or unsubstituted C₆-C₁₀ aryl, substituted or unsubstituted C₃-C₁₀ carbocycle, substituted or unsubstituted heterocycle comprising one or two 5- or 6-member rings and 1-4 heteroatoms selected from N, O and S, or substituted or unsubstituted heteroaryl comprising one or two 5- or 6-member rings and 1-4 heteroatoms selected from N, O and S.

In some embodiments, the compound of Formula (VII) is selected from a compound of Formula (VIIb):



A^- is a pharmaceutically acceptable anion;

R_{1i} is unsubstituted C_1 - C_6 alkyl;

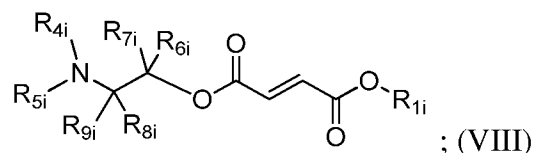
L_a is substituted or unsubstituted C_1 - C_6 alkyl linker, substituted or unsubstituted C_3 - C_{10} carbocycle, substituted or unsubstituted C_6 - C_{10} aryl, substituted or unsubstituted heterocycle comprising one or two 5- or 6-member rings and 1-4 heteroatoms selected from N, O and S, or substituted or unsubstituted heteroaryl comprising one or two 5- or 6-member rings and 1-4 heteroatoms selected from N, O and S;

R_{3i}' is substituted or unsubstituted C_1 - C_6 alkyl; and

R_{2i} and R_{3i} are each, independently, H, substituted or unsubstituted C_1 - C_6 alkyl, substituted or unsubstituted C_2 - C_6 alkenyl, substituted or unsubstituted C_2 - C_6 alkynyl, substituted or unsubstituted C_6 - C_{10} aryl, substituted or unsubstituted C_3 - C_{10} carbocycle, substituted or unsubstituted heterocycle comprising one or two 5- or 6-member rings and 1-4 heteroatoms selected from N, O and S, or substituted or unsubstituted heteroaryl comprising one or two 5- or 6-member rings and 1-4 heteroatoms selected from N, O and S;

or alternatively, R_{2i} and R_{3i} , together with the nitrogen atom to which they are attached, form a substituted or unsubstituted heteroaryl comprising one or two 5- or 6-member rings and 1-4 heteroatoms selected from N, O and S, or a substituted or unsubstituted heterocycle comprising one or two 5- or 6-member rings and 1-4 heteroatoms selected from N, O and S.

In some embodiments, the compound of Formula (VII) is selected from a compound of Formula (VIII):



wherein:

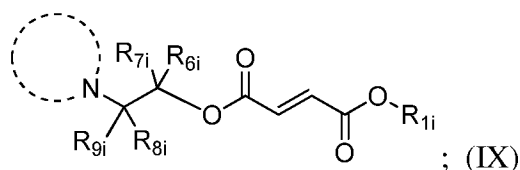
R_{1i} is unsubstituted C₁-C₆ alkyl;

R_{4i} and R_{5i} are each, independently, H, substituted or unsubstituted C₁-C₆ alkyl, substituted or unsubstituted C₂-C₆ alkenyl, substituted or unsubstituted C₂-C₆ alkynyl, substituted or unsubstituted C₆-C₁₀ aryl, substituted or unsubstituted C₃-C₁₀ carbocycle, substituted or unsubstituted heterocycle comprising one or two 5- or 6-member rings and 1-4 heteroatoms selected from N, O and S, or substituted or unsubstituted heteroaryl comprising one or two 5- or 6-member rings and 1-4 heteroatoms selected from N, O and S;

R_{6i}, R_{7i}, R_{8i} and R_{9i} are each, independently, H, substituted or unsubstituted C₁-C₆ alkyl, substituted or unsubstituted C₂-C₆ alkenyl, substituted or unsubstituted C₂-C₆ alkynyl or C(O)OR_a; and

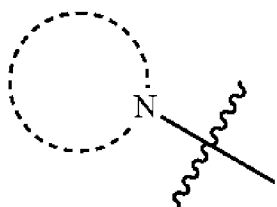
R_a is H or substituted or unsubstituted C₁-C₆ alkyl.

In some embodiments, the compound of Formula (VII) is selected from a compound of Formula (IX):

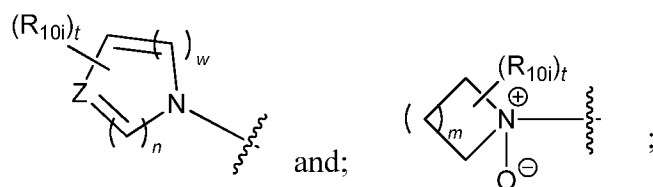
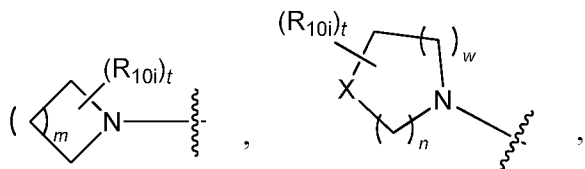


wherein:

R_{1i} is unsubstituted C₁-C₆ alkyl;



is selected from the group consisting of:



X is N, O, S or SO₂;

Z is C or N;

m is 0, 1, 2, or 3;

n is 1 or 2;

w is 0, 1, 2 or 3;

t is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10;

R_{6i}, R_{7i}, R_{8i} and R_{9i} are each, independently, H, substituted or unsubstituted C₁-C₆ alkyl, substituted or unsubstituted C₂-C₆ alkenyl, substituted or unsubstituted C₂-C₆ alkynyl or C(O)OR_a; and

R_a is H or substituted or unsubstituted C₁-C₆ alkyl; and

each R_{10i} is, independently, H, halogen, substituted or unsubstituted C₁-C₆ alkyl, substituted or unsubstituted C₂-C₆ alkenyl, substituted or unsubstituted C₂-C₆ alkynyl, substituted or unsubstituted C₃-C₁₀ carbocycle, substituted or unsubstituted heterocycle comprising one or two 5- or 6-member rings and 1-4 heteroatoms selected from N, O and S, or substituted or unsubstituted heteroaryl comprising one or two 5- or 6-member rings and 1-4 heteroatoms selected from N, O and S;

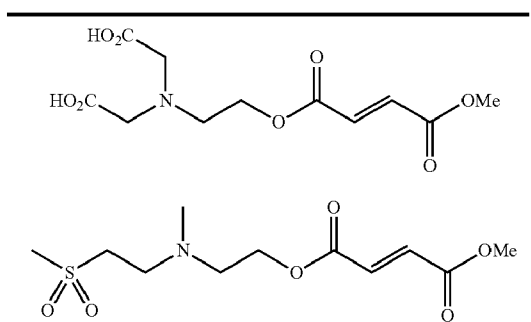
or, alternatively, two R_{10i}'s attached to the same carbon atom, together with the carbon atom to which they are attached, form a carbonyl, substituted or unsubstituted C₃-C₁₀ carbocycle, substituted or unsubstituted heterocycle comprising one or two 5- or 6-member rings and 1-4 heteroatoms selected from N, O and S, or substituted or unsubstituted heteroaryl comprising one or two 5- or 6-member rings and 1-4 heteroatoms selected from N, O and S;

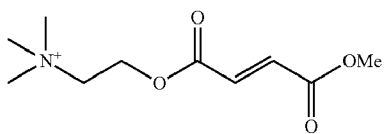
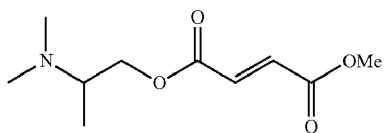
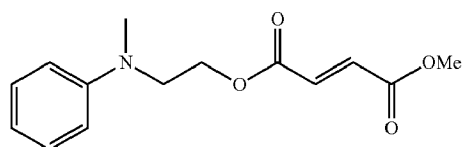
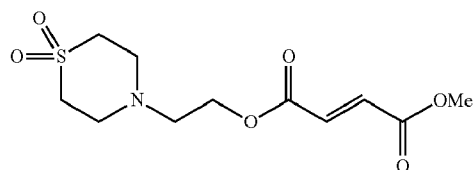
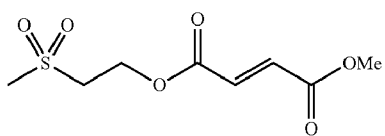
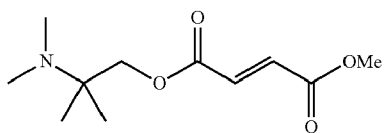
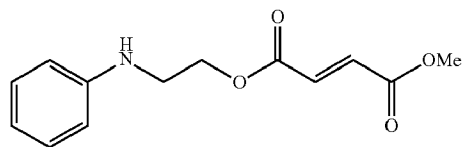
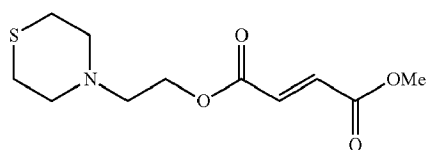
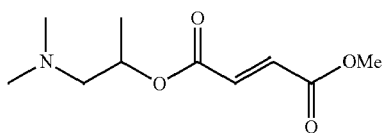
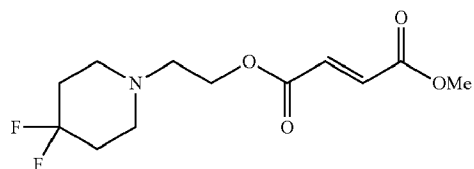
or, alternatively, two R_{10i}'s attached to different atoms, together with the atoms to which they are attached, form a substituted or unsubstituted C₃-C₁₀ carbocycle, substituted or unsubstituted heterocycle comprising one or two 5- or 6-member rings and 1-4 heteroatoms selected from N, O and S, or substituted or unsubstituted heteroaryl comprising one or two 5- or 6-member rings and 1-4 heteroatoms selected from N, O and S.

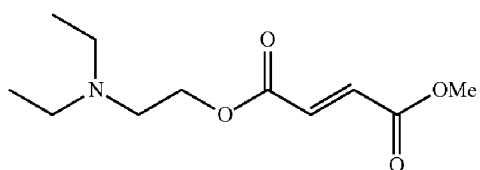
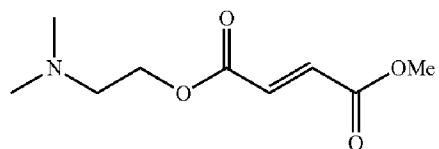
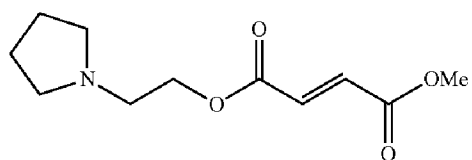
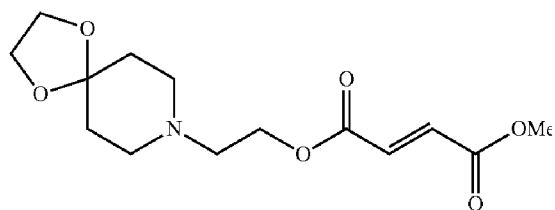
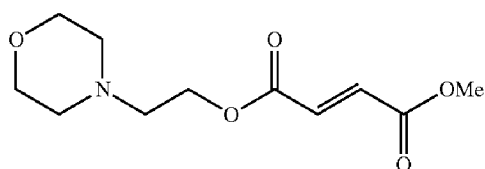
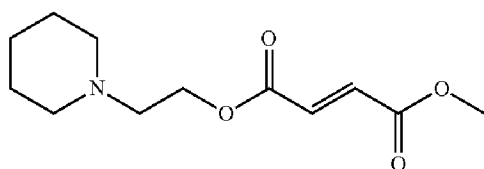
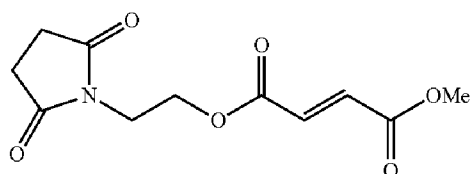
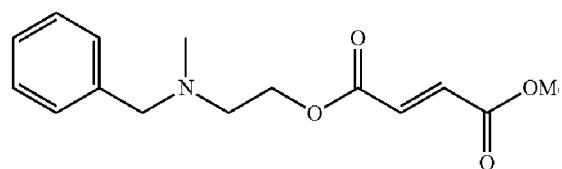
In some embodiments, the compound is a compound listed in Table A herein.

Representative compounds of the present invention include compounds listed in Table A.

TABLE A.

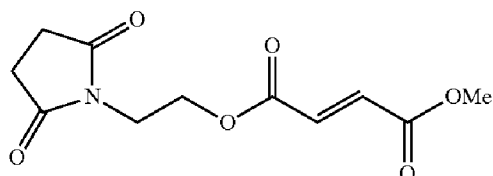


 A^{-} 



A⁻ is a pharmaceutically acceptable anion.

In some embodiments, the compound of Formula (VII) is the compound (X):



(X), or a pharmaceutically acceptable salt thereof.

In an embodiment, the disorder or condition is cancer.

In an embodiment, the disorder or condition is a hematological malignancy.

In an embodiment, the hematological malignancy is selected from lymphocytic leukemia, chronic lymphocytic leukemia, and lymphoma.

In an embodiment, the disorder or condition is a solid tumor.

In an embodiment, the solid tumor is selected from gastrointestinal sarcoma, neuroblastoma, and kidney cancer.

In an embodiment, the disorder or condition is a viral infection.

In another aspect, the invention features, a method of treating a disorder or condition described herein by administering to the subject: a dialkyl fumarate, *e.g.*, DMF, MMF; or a combination thereof.

In another aspect, the invention features a method of modulating an immune response in a subject, comprising antagonizing expression of OSGIN1 in the subject, thereby promoting an immune response in the subject. In some embodiments, the level and/or activity of TNF- α is increased by antagonizing expression of OSGIN1 in the subject. In some embodiments, antagonizing expression of OSGIN1 comprises administering to the subject an antagonist selected from the group consisting of an anti-OSGIN1 antibody or OSGIN1-binding fragment thereof, siRNA, shRNA, antisense RNA, miRNA, and combinations thereof.

In another aspect, the invention features a method of modulating an immune response in a subject, comprising agonizing expression of OSGIN1 in the subject, thereby inhibiting an immune response in the subject. In some embodiments, the level and/or activity of TNF- α is decreased by agonizing expression of OSGIN1 in the subject. In some embodiments, agonizing expression of OSGIN1 comprises administering to the subject an agonist selected from the group consisting of an expression vector encoding OSGIN1 (*e.g.*, retroviral, lentiviral, among

other expression vectors), mRNA encoding OSGIN1, OSGIN1 translation product, and combinations thereof.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

BRIEF DESCRIPTION OF DRAWINGS

FIGURE 1 depicts exemplary monomethyl fumarate (MMF) exposures after oral dimethyl fumarate (DMF) or MMF dosing. Satellite 5 animals per group were dosed orally with DMF or MMF (100 mg/kg) and sacrificed 30 minutes post-dosing. MMF exposures were determined and compared in various compartments. MMF levels were highly comparable between DMF and MMF dosing, suggesting any subsequent differences in pharmacodynamic responses would not simply be due to different exposures.

FIGURE 2A depicts an exemplary overview of Venn diagrams comparing differentially expressed genes in each tissue. Data are presented as an aggregation of three time points for each tissue.

FIGURE 2B depicts exemplary Venn diagrams comparing differentially expressed genes in whole blood, cortex, hippocampus, striatum, jejunum, kidney, liver and spleen.

FIGURE 3 depicts exemplary analysis of whole blood DMF differentially expressed genes (DEGs) in various pathways. Common effects were observed on NK cell function.

FIGURE 4 depicts exemplary analysis of cortical MMF DEGs in various pathways.

FIGURE 5 depicts exemplary analysis of hippocampal MMF DEGs in various pathways.

FIGURE 6 depicts exemplary analysis of striatal MMF DEGs in various pathways.

FIGURES 7A and 7B depict exemplary analysis of jejunum DEGs in various pathways. **Figure 7A** depicts DMF and MMF common DEGs. **Figure 7B** depicts MMF DEGs.

FIGURES 8A-8C depict exemplary analysis of kidney DEGs in various pathways. **Figure 8A** depicts DMF and MMF common DEGs. DMF and MMF common DEGs showed good representation of Nrf2 pathway activation and xenobiotic metabolism, with particular emphasis on glutathione biosynthesis. **Figure 8B** depicts DMF specific DEGs. **Figure 8C** depicts MMF specific DEGs.

FIGURE 9 depicts exemplary analysis of liver DMF and MMF common DEGs in various pathways.

FIGURE 10 depicts an exemplary flow cytometry gating strategy for comparative analysis of DMF and MMF on Natural Killer (NK) cell phenotype. Protein expression is quantified by mean fluorescent intensity (MFI).

FIGURE 11 depicts an exemplary comparative analysis of DMF and MMF on NK cell phenotype using markers: NK1.1 (klrb1b), Nkg2d (klrk1), NKp46 (ncr1), Nkg2a (klrc1), and CD94 (klrd1). Study design: Naïve C57Bl/6 mice were dosed PO with 100 mpk dimethyl fumarate (DMF) or molar equivalency of monomethyl fumarate (MMF). 12-hours post-dose, mice were sacrificed and blood, spleen, and inguinal lymph node were collected for analysis by flow cytometry.

FIGURE 12 depicts an exemplary immunophenotyping panel for analysis of immune cell subsets in EAE mice treated with DMF or MMF.

FIGURE 13 depicts exemplary NK cell analysis in blood and spleen and EAE clinical score analysis for a chronic dosing experiment in EAE mice.

FIGURE 14 depicts exemplary NK cell protein expression in spleen and blood for a chronic dosing experiment in EAE mice.

FIGURE 15 depicts exemplary NK cell subset and protein expression analysis in spleen and blood for a chronic dosing experiment in EAE mice.

FIGURE 16 depicts exemplary NK cell analysis in spleen, iLN, and blood for a single dose experiment in EAE mice.

FIGURE 17 depicts exemplary NK cell protein expression in spleen, iLN and blood for a single dose experiment in EAE mice.

FIGURE 18 depicts exemplary NK cell subset and protein expression analysis in spleen, iLN and blood for a single dose experiment in EAE mice.

FIGURE 19 depicts an exemplary flow cytometry gating strategy for comparative analysis of DMF and MMF on T cell phenotype. Protein expression is quantified by mean fluorescent intensity (MFI).

FIGURE 20 depicts exemplary T regulatory cell analysis in spleen, iLN and blood for a chronic dosing experiment in EAE mice.

FIGURE 21 depicts exemplary CD4⁺ cell subset and protein expression analysis in spleen, iLN and blood for a chronic dosing experiment in EAE mice.

FIGURE 22 depicts exemplary CD8⁺ cell subset and protein expression analysis in spleen, iLN and blood for a chronic dosing experiment in EAE mice.

FIGURE 23 depicts exemplary CD4⁺ T cell subset vs. EAE clinical score analysis in spleen for a chronic dosing experiment in EAE mice.

FIGURE 24 depicts exemplary CD8⁺ T cell subset vs. EAE clinical score analysis in spleen for a chronic dosing experiment in EAE mice.

FIGURE 25 depicts exemplary B cell analysis in naïve, vehicle, MMF or DMF treated EAE mice.

FIGURE 26 depicts an exemplary myeloid cell gating strategy for comparative analysis of DMF and MMF on myeloid cell phenotype. (Swirski, F. K. et al. Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science* 325, 612–616 (2009))

FIGURE 27 depicts exemplary myeloid cell subset analysis in spleen and iLN for a chronic dosing experiment in EAE mice.

FIGURE 28 depicts the changes in OSGIN1 expression in mouse cortex, cerebellum, hippocampus and striatum after DMF administration. The levels of OSGIN1 gene expression were measured by qPCR. The fold changes were normalized to vehicle control.

FIGURE 29A depicts the changes in OSGIN1 expression in hippocampus from wild-type mice and Nrf2^{-/-} mice after DMF administration. The levels of OSGIN1 gene expression were measured by qPCR. The fold changes were normalized to wild-type control.

FIGURE 29B depicts the changes in OSGIN1 expression in striatum from wild-type mice and Nrf2^{-/-} mice after DMF administration. The levels of OSGIN1 gene expression were measured by qPCR. The fold changes were normalized to wild-type control.

FIGURE 30 depicts the levels of OSGIN1 expression in human spinal cord astrocytes treated with MMF (0, 10 or 30 μ M) and OSGIN1 siRNA (10 μ M) or scrambled siRNA (10 μ M). The levels of OSGIN1 gene expression were measured by qPCR. The fold changes were normalized to scrambled siRNA control. Left bars: scrambled siRNA; right bars: OSGIN1 siRNA.

FIGURE 31A depicts the number of live human spinal cord astrocytes after treatment with MMF (0, 10 or 30 μ M), H₂O₂ (200 μ M), and OSGIN1 siRNA (10 μ M) or scrambled siRNA (10 μ M). The number of live cells was quantified by Calcein AM fluorescence intensity.

FIGURE 31B depicts the percentage of viable nuclear count in human spinal cord astrocytes treated with MMF (0, 10 or 30 μ M), H₂O₂ (200 μ M), and OSGIN1 siRNA (10 μ M) or scrambled siRNA (10 μ M). The percentage of viable nuclear count was measured by DAPI staining and normalized to 0 μ M H₂O₂ control.

FIGURE 31C depicts exemplary images of live and dead human spinal cord astrocytes treated with MMF (0 or 30 μ M), H₂O₂ (200 μ M), and OSGIN1 siRNA (10 μ M) or scrambled siRNA (10 μ M). The live and dead cells are shown by calcein AM staining and ethidium homodimer staining, respectively.

FIGURE 32A depicts the levels of PADI4 gene expression in human spinal cord astrocytes treated with MMF (0, 10 or 30 μ M), H₂O₂ (200 μ M), and OSGIN1 siRNA (10 μ M), PADI4 siRNA (10 μ M) or scrambled siRNA (10 μ M). The levels of PADI4 expression were measured by qPCR and normalized to scramble control. Left bars: scrambled siRNA; middle bars: OSGIN1 siRNA; right bars: PADI4 siRNA.

FIGURE 32B depicts the levels of OSGIN1 gene expression in human spinal cord astrocytes treated with MMF (0, 10 or 30 μ M), H₂O₂ (200 μ M), and OSGIN1 siRNA (10 μ M), PADI4 siRNA (10 μ M) or scrambled siRNA (10 μ M). The levels of OSGIN1 expression were measured by qPCR and normalized to scramble control. Left bars: scrambled siRNA; middle bars: OSGIN1 siRNA; right bars: PADI4 siRNA.

FIGURE 33A depicts the percentage of viable nuclear count in human spinal cord astrocytes treated with MMF (0, 10 or 30 μ M), H₂O₂ (300 μ M), and PADI4 siRNA (10 μ M) or scrambled siRNA (10 μ M). The percentage of viable nuclear count was measured by DAPI staining and normalized to 0 μ M H₂O₂ control.

FIGURE 33B depicts exemplary images of live and dead human spinal cord astrocytes treated with MMF (0 or 30 μ M), H₂O₂ (200 μ M), and PADI4 siRNA (10 μ M) or scrambled siRNA (10 μ M). The live and dead cells are shown by calcein AM staining and ethidium homodimer staining, respectively.

FIGURE 34 depicts the percentage of viable nuclear count in human spinal cord astrocytes treated with MMF (0, 10 or 30 μ M), H₂O₂ (300 μ M), and p53 siRNA or scrambled siRNA. The percentage of live cells was measured by nuclear counting and normalized to 0 μ M H₂O₂ control.

FIGURE 35 depicts gene selections for Fluidigm real-time PCR as described in Example 4.

FIGURE 36 depicts epitope locations of OSGIN1 isoform-specific antibodies. Boxes represent translational start sites of each OSGIN1 isoform labeled with the corresponding amino acid length protein; 560aa (OSGIN1~61kDa), 477aa (OSGIN1~52kDa) and 375aa (OSGIN1~38kDa). Dashed underlined amino acids indicate region of peptide generation for antibody creation; *:OSGIN1~61kDa, *:OSGIN1~52kDa and ***:OSGIN1~38kDa.

FIGURE 37A depicts the structure and basic Properties of dimethyl fumarate (DMF) and monomethyl fumarate (MMF).

FIGURE 37B depicts MMF exposure in peripheral and CNS tissues following a single dose of DMF. Drug exposure levels were measured in mouse tissue samples collected 30 minutes post-dose of DMF and were analyzed for levels of MMF, the active metabolite of DMF.

FIGURES 38A and **38B** depict DMF transcriptional profiling time course in peripheral tissues: kidney/jejunum. Transcriptional changes following single 100mg/kg oral dose of DMF in kidney (**Figure 38A**) and jejunum (**Figure 38B**), graphed as changes in selected genes over time. Data are graphed as fold change values normalized to vehicle treated animals, with the starting value

of 1 equivalent to the normalized baseline of vehicle animals. Graphed genes represent only those that were found to be regulated in peripheral tissues, with Vegfa as a baseline control gene.

FIGURES 39A and 39B depict DMF transcriptional profiling time course in peripheral tissues: spleen/liver. Transcriptional changes following single 100mg/kg oral dose of DMF in spleen (**Figure 39A**) and liver (**Figure 39B**), graphed as changes in selected genes over time. Data are graphed as fold change values normalized to vehicle treated animals, with the starting value of 1 equivalent to the normalized baseline of vehicle animals. Graphed genes represent only those that were found to be regulated in peripheral tissues, with Vegfa as a baseline control gene.

FIGURES 40A and 40B depict DMF transcriptional profiling time course in CNS tissues: OSGIN1/BDNF. Transcriptional changes following single 100mg/kg oral dose of DMF for two genes found to be regulated in the brain: Osgin1 (**Figure 40A**) and Bdnf (**Figure 40B**). Data are graphed as fold change values normalized to vehicle treated animals, with the dashed line at 1 equivalent to the normalized baseline of vehicle animals. Later time points are not included since regulation of the graphed genes is consistent with the graphed 16.5 hour time point.

FIGURE 41 depicts DMF transcriptional profiling time course in CNS tissues: NQO1. Transcriptional changes following single 100mg/kg oral dose of DMF Nqo1 in the brain. Data are graphed as fold change values normalized to vehicle treated animals, with the dashed line at 1 equivalent to the normalized baseline of vehicle animals. Later time points are not included since regulation of the graphed genes is consistent with the graphed 16.5 hour time point.

FIGURES 42A and 42B depict DMF transcriptional profiling dose-response in peripheral tissues: kidney/jejunum. Transcriptional changes following multiple doses of DMF in kidney (**Figure 42A**) and jejunum (**Figure 42B**), graphed as changes in selected genes across doses. Data are graphed as fold change values normalized to vehicle treated animals, with the starting value of 0 equivalent to the normalized baseline of vehicle animals. Graphed genes represent only those that were found to be regulated in peripheral tissues, with Vegfa as a baseline control gene.

FIGURES 43A and 43B depict DMF transcriptional profiling dose-response in peripheral tissues: spleen/liver. Transcriptional changes following multiple doses of DMF in spleen

(**Figure 43A**) and liver (**Figure 43B**), graphed as changes in selected genes across doses. Data are graphed as fold change values normalized to vehicle treated animals, with the starting value of 0 equivalent to the normalized baseline of vehicle animals. Graphed genes represent only those that were found to be regulated in peripheral tissues, with Vegfa as a baseline control gene.

FIGURES 44A and **44B** depict DMF transcriptional profiling dose-response in CNS tissues: cortex/cerebellum. Transcriptional changes following multiple doses of DMF in Cortex (**Figure 44A**) and Cerebellum (**Figure 44B**), graphed as changes in selected genes across doses. Data are graphed as fold change values normalized to vehicle treated animals, with the starting value of 0 equivalent to the normalized baseline of vehicle animals. Graphed genes represent only those that were found to be regulated in CNS tissues, with Vegfa as a baseline control gene.

FIGURES 45A and **45B** depict DMF transcriptional profiling dose-response in CNS tissues: hippocampus/striatum. Transcriptional changes following multiple doses of DMF in Hippocampus (**Figure 45A**), Striatum (**Figure 45B**), graphed as changes in selected genes across doses. Data are graphed as fold change values normalized to vehicle treated animals, with the starting value of 0 equivalent to the normalized baseline of vehicle animals. Graphed genes represent only those that were found to be regulated in CNS tissues, with Vegfa as a baseline control gene.

FIGURES 46A and **46B** depict DMF transcriptional profiling dose-response in whole blood. Transcriptional changes following a single dose of 100mg/kg DMF across 8 time points in whole blood samples (**Figure 46A**). Data are graphed as fold change values normalized to vehicle treated animals, with the dashed line at 1 equivalent to the normalized baseline of vehicle animals. Transcriptional changes following multiple doses of DMF in whole blood (**Figure 46B**). Data are graphed as fold change values normalized to vehicle treated animals, with the starting value of 0 equivalent to the normalized baseline of vehicle animals. Graphed genes represent only those that were found to be regulated in whole blood.

FIGURES 47A and **47B** depict transcriptional time course with 100mg/kg DMF in Nrf2^{-/-} and wild type mice: peripheral tissues. Transcriptional changes following single 100mg/kg oral dose of DMF in peripheral tissues: Jejunum (**Figure 47A**) and Kidney (**Figure 47B**), graphed as changes in selected genes over time. Data are graphed as fold change values normalized to

vehicle treated animals, with the starting value of 1 equivalent to the normalized baseline of vehicle animals. Graphed genes represent a select panel determined based on gene regulation from the time course study.

FIGURES 48A and 48B depict transcriptional time course with 100mg/kg DMF in Nrf2^{-/-} and wild type mice: CNS tissues. Transcriptional changes following single 100mg/kg oral dose of DMF in CNS tissues: Hippocampus (**Figure 48A**) and Striatum (**Figure 48B**), graphed as changes in selected genes over time. Data are graphed as fold change values normalized to vehicle treated animals, with the starting value of 1 equivalent to the normalized baseline of vehicle animals. Graphed genes represent a select panel determined based on gene regulation from the time course study.

FIGURES 49A and 49B depict DMF-induced Nrf2-dependent protein expression in peripheral tissues. Nrf2^{-/-} and wild type mice were treated with vehicle or 100mg/kg DMF in triplicate and tissues harvested at 6 h after compound addition. Relative protein levels of Txnrd1, Sqstm1, Nqo1 and GCLC were assessed and actin control levels measured as an internal control. Protein expression following a single 100mg/kg oral dose of DMF in Kidney (**Figure 49A**) and Jejunum (**Figure 49B**).

FIGURES 50A and 50B depicts DMF-induced protein expression in CNS tissues. Nrf2^{-/-} and wild type mice were treated with vehicle or 100mg/kg DMF in triplicate and tissues harvested at 6 h after compound addition. Relative protein levels of Nqo1 and BDNF were assessed and actin control levels measured as an internal control. Protein expression following a single 100mg/kg oral dose of DMF in Striatum (**Figure 50A**) and Cortex (**Figure 50B**).

FIGURES 51A and 51B depict MMF-induced Nrf2 target gene expression in human astrocytes. Human astrocytes were treated with either DMSO or a titration of MMF for 24 hours before RNA extraction and q-PCR analysis. The graph represents triplicate samples; error bars indicate S.D. **Figure 51A**: q-PCR analysis of classical Nrf2 target genes. **Figure 51B**: OSGIN1 q-PCR analysis.

FIGURES 52A-52D depict MMF protection of human astrocytes from oxidative challenge. Human astrocytes were treated with DMSO or 30uM MMF for 24 hours followed by oxidative

challenge. **Figures 52A-Figure 52C** show live imaging of LIVE/DEAD labeled cells pretreated with MMF and then challenged with H₂O₂. LIVE calcein AM (green) and DEAD ethidium homodimer (red) labeling. **Figure 52A** shows positive control cells treated with DMSO. **Figure 52B** shows control DMSO-treated and 300uM H₂O₂-challenged cells. **Figure 52C** shows astrocytes pretreated with 30uM MMF and challenged with 300uM H₂O₂. **Figure 52D** shows quantification of calcein AM fluorescence intensity in LIVE/DEAD labeled cells from A-C.

FIGURES 53A and 53B depict Nrf2 knockdown experiments. **Figure 53A** shows q-PCR analysis of human astrocytes treated with 10nM of control or Nrf2 siRNA and either DMSO or 30uM MMF. *, $p < .001$ as compared to control siRNA using two-way ANOVA and Tukey's multiple comparison test. **Figure 53B** shows western blot data correlating with Figure 53A. Actin is shown as a loading control.

FIGURE 54 depicts loss of Nrf2 and reduction in OSGIN1 expression. Human astrocytes were transfected with scrambled (control) or Nrf2 siRNA followed by treatment with a titration of MMF. q-PCR analysis was conducted for OSGIN1 transcription. Error bars represent SD and p values based on two-way ANOVA with Tukey's post-test for multiple-samples comparisons.

FIGURES 55A and 55B depict the abolishment of MMF-mediated cytoprotection with the loss of Nrf2. Human astrocytes were transfected with scrambled (control) or Nrf2 siRNA and treated with either DMSO or MMF for 24 hours then challenged with H₂O₂. A, cells fixed and stained with DAPI nuclear dye. **Figure 55A** shows a graph representing average cell nuclei counts in 20 fields per well (2 wells averaged in graph). Error bars represent SD and p values based on two-way ANOVA with Sidak's post-test for multiple-samples comparisons. **Figure 55B** shows live imaging of LIVE/DEAD labeled cells pretreated with MMF and then challenged with H₂O₂. LIVE calcein AM (green) and DEAD ethidium homodimer (red) labeling.

FIGURES 56A and 56B depict OSGIN1 siRNA knockdown experiments. Human astrocytes were transfected with scrambled (control) or OSGIN1 siRNA and treated with either DMSO, 10uM MMF or 30uM MMF for 24 hours before RNA extraction and q-PCR analysis. **Figure 56A** shows q-PCR analysis for OSGIN1 transcriptional regulation. **Figure 56B** shows q-PCR analysis for Nrf2 transcriptional regulation. The graphs represent duplicate samples; error bars

indicate S.D. *p* values based on two-way ANOVA with Tukey's post-test for multiple-samples comparisons.

FIGURES 57A-57C depict that OSGIN1 siRNA knockdown inhibits MMF-mediated cytoprotection. Human astrocytes were transfected with scrambled (control) or OSGIN1 siRNA and treated with MMF for 24 hours then challenged with H₂O₂. **Figure 57A:** imaging of LIVE/DEAD labeled cells pretreated with MMF and then challenged with H₂O₂. LIVE calcein AM (green) and DEAD ethidium homodimer (red) labeling. **Figure 57B:** quantification of calcein AM fluorescence intensity in LIVE/DEAD labeled cells from **Figure 57A**. **Figure 57C:** replicate plates as in **Figure 57A** fixed and stained with DAPI nuclear dye. Graph represents average cell nuclei counts in 20 fields per well (2 wells averaged in graph). Error bars represent SD and *p* value based on one-way ANOVA with Tukey's post-test for multiple-samples comparisons.

FIGURES 58A and 58B depict optimization of OSGIN1-52kDa and OSGIN1-61kDa antibodies. Human astrocyte cell lysates were probed with three antibody condition: antibody alone (NP), antibody pre-incubated with epitope specific peptide (P) or non-specific control peptide (CP). **Figure 58A:** OSGIN1-52kDa antibody conditions. **Figure 58B:** OSGIN1-61kDa antibody conditions.

FIGURE 59 depicts that OSGIN1 knockdown depletes OSGIN1 isoform specific immunoreactivity. Human astrocytes were transfected with 10nM of control or OSGIN1 specific siRNA and cell lysates were probed with OSGIN1 isoform-specific antibodies. β -actin is included as a loading control.

FIGURES 60A-60C depict the regulation of OSGIN1 61kDa encoding isoform via MMF and Nrf2. Human astrocytes were transfected with either control or OSGIN1 specific siRNA followed by treatment with 30uM MMF for 24 hours. **Figure 60A:** transfected cell lysates probed with OSGIN1-52kDa antibody. **Figure 60B:** transfected cell lysates probed with OSGIN1-61kDa antibody. **Figure 60C:** human astrocytes transfected with either control or Nrf2 specific siRNA and probed with OSGIN1-61kDa antibody. β -actin is included in all figures as a loading control.

FIGURE 61 depicts immunocytochemical analysis of OSGIN1-52kDa and OSGIN1-61kDa antibodies. Human astrocytes were treated with MMF for 24 hours, fixed and then probed with antibodies against OSGIN1-52kDa and OSGIN1-61kDa isoforms of OSGIN1. Total fluorescent spot count was accomplished using Thermo HCS Arrayscan technology. *p* values based on one-way ANOVA with Tukey's post-test for multiple sample comparisons.

FIGURES 62A and 62B depict 3' and 5' RACE of OSGIN1 transcript. RNA extracted from human astrocytes treated with a titration of MMF were subjected to 3' and 5' RACE. **Figure 62A** shows resulting RACE products analyzed via gel electrophoresis. **Figure 62B**: sequencing of 5' RACE products identified two transcript variants of OSGIN1 within the 5' UTR of the OSGIN1-52kDa transcript that differed in two nucleotide substitutions (indicated by arrows). WT=canonical sequence; ALT=identified 5' RACE sequence with nucleotide substitutions. Dash lined indicates where RACE was terminated.

FIGURE 63 depicts the q-PCR analysis of identified 5'RACE transcripts. RNA was extracted from human astrocytes treated with DMSO or MMF for 24 hours and subjected to q-PCR using primer/probe sets specific to identified 5'RACE transcripts. WT=canonical sequence; ALT=identified 5' RACE sequence with nucleotide substitutions. *, *p*<.05 and ****, *p*<.0001 based on one-way ANOVA with Tukey's post-test for multiple comparisons.

FIGURES 64A-64C depict q-PCR of p53 siRNA knockdown experiments. Human astrocytes transfected with either control (scrambled) or p53 siRNA followed by treatment with DMSO or MMF for 24 hours. **Figure 64A**: q-PCR analysis of p53 mRNA levels. *, *p*<.01 based on two-way ANOVA with Tukey's post-test for multiple comparisons. **Figure 64B**, human astrocytes transfected with either control (scrambled), p53 or Nrf2 siRNA and analyzed for Nrf2 mRNA levels. **Figure 64C**, same as Figure 64B but q-PCR directed against OSGIN1 transcript levels. B/C, *, *p*<.01 based on one-way ANOVA with Dunnett's post-test for multiple comparisons.

FIGURES 65A-65C depict p53 protein regulation by MMF in an Nrf2- and OSGIN1-dependent manner. Human astrocytes were transfected with 10nM control (scrambled), p53, OSGIN1 or Nrf2 siRNA followed by treatment with DMSO or MMF for 24 hours. **Figure 65A** shows p53 protein measure in scrambled (control), p53 and OSGIN1 siRNA knockdown samples treated with MMF. **Figure 65B** shows Nrf2 protein measure in scrambled (control) and p53 siRNA

knockdown samples treated with MMF. **Figure 65C** shows Scrambled (control) and Nrf2 siRNA knockdown samples treated with MMF and probed for p53 protein. β -actin is included in all figures as a loading control.

FIGURES 66A and 66B depict MMF induction of nuclear translocation of p53: ICC. Human astrocytes were treated with a titration of MMF for 24 hours followed by fixation and ICC analysis of p53. **Figure 66A:** immunocytochemistry images acquired using the Thermo HCS Arrayscan and algorithm analysis overlaid. **Figure 66B:** Quantification of images in Figure 66A. Each point represents 20 images from one well; n=6 wells per condition. Statistical analysis was performed using one-way ANOVA with Dunett's multiple comparison post-test (*, $p < .05$)

FIGURES 67A and 67B depict MMF induction of nuclear translocation of p53: p53 TransAM assay. Human astrocytes were treated with a titration of MMF for 24 hours followed by analysis of nuclear and cytoplasmic cell extracts for p53 expression. **Figure 67A:** nuclear extract quantification. **Figure 67B:** cytoplasmic extract quantification. Statistical analysis was performed using one-way ANOVA with Dunett's multiple comparison post-test (*, $p < .05$)

FIGURES 68A and 68B depict that p53 contributes to MMF-mediated cytoprotection. Human astrocytes were transfected with scrambled (control) or p53 siRNA and treated with MMF for 24 hours then challenged with H_2O_2 . **Figure 68A:** imaging of LIVE/DEAD labeled cells pretreated with MMF and then challenged with H_2O_2 . LIVE calcein AM (green) and DEAD ethidium homodimer (red) labeling. **Figure 68B:** quantification of calcein AM fluorescence intensity in LIVE/DEAD labeled cells from Figure 68A. Error bars represent SD and p value based on one-way ANOVA with Tukey's post-test for multiple-samples comparisons.

FIGURES 69A and 69B depict the q-PCR time course of NQO1 and OSGIN1. Human astrocytes were treated with 0, 10 or 30uM of MMF and analyzed for transcript regulation of OSGIN1 and NQO1 over various time points. **Figure 69A:** OSGIN1 time course. **Figure 69B:** NQO1 time course.

FIGURE 70 depicts the protein time course of MMF-regulated proteins. Human astrocytes were treated with 0, 10 or 30uM of MMF and analyzed for protein regulation of OSGIN1, Nrf2, p53 and NQO1 over various time points. β -actin is included as a loading control.

FIGURES 71A and 71B depict MMF inhibition of cell proliferation. Human astrocytes were transfected with scrambled (control) or OSGIN1 siRNA followed by treatment with 0, 10 or 30uM MMF and pulse incorporation of EdU. Cells were fixed and EdU incorporated cells were imaged and quantified using a Thermo HCS Arrayscan and HCS Studio software, as shown in **Figure 71B**. **Figure 71A** shows quantification of four wells are averaged in groups (25 fields/well). *, $p < .05$; **, $p < .01$; ***, $p < .001$ based on two-way ANOVA with Tukey's post-test for multiple comparisons.

FIGURES 72A and 72B depicts the loss of OSGIN1 and PADI4 does not significantly induce apoptosis. Human astrocytes transfected with 10nM scrambled (control), OSGIN1, PADI4 or p53 siRNA for 48 hours followed by analysis of apoptotic cells using the TiterTACSTM assay. **Figure 72A**: siRNA transfection alone. **Figure 72B**: siRNA transfection in the presence of MMF treatment for 24 hours.

FIGURE 73 depicts MMF-mediation of PADI4 in a similar manner to NQO1. Human astrocytes were treated with 0, 10 or 30uM of MMF and analyzed for transcript regulation of PADI4 over various time points.

FIGURES 74A and 74B depict that PADI4 is regulated by OSGIN1 and p53. Human astrocytes were transfected with either scrambled (control), PADI4, OSGIN1, Nrf2 or p53 siRNA followed by q-PCR analysis. **Figure 74A** shows PADI4 q-PCR. *, $p < .0001$ based on one-way ANOVA with Dunnett's post-test for multiple comparisons. **Figure 74B** shows transfected cells treated with MMF for 24 hours followed by q-PCR for PADI4. p values based on two-way ANOVA with Tukey's post-test for multiple comparisons.

FIGURES 75A and 75B depict that PADI4 does not regulate OSGIN1 or p53. Human astrocytes were transfected with either scrambled (control), PADI4, OSGIN1 or p53 siRNA followed by q-PCR analysis. **Figure 75A** shows transfected cells treated with MMF for 24 hours followed by q-PCR for OSGIN1. **Figure 75B** shows q-PCR for p53. p values based on two-way ANOVA with Tukey's post-test for multiple comparisons.

FIGURE 76 depicts that OSGIN1 knockdown induces expression of TNF- α . Human astrocytes were transfected with scrambled (control) or OSGIN1 specific siRNA. Samples were analyzed for alterations in TNF- α transcript levels. *, $p < .05$ based on student's t test.

FIGURES 77A and 77B depict a potential mechanism of action of OSGIN1 mediated cellular protection. Interaction of MMF with cysteine residues on Keap1 results in allosteric conformational change in the Keap1 protein, resulting in the inability of Nrf2 to be targeted for ubiquitination and subsequent proteosomal degradation. This allows Nrf2 to accumulate in the cytoplasm and translocate to the nucleus where it can regulate the transcription of various genes including OSGIN1 (**Figure 77A**). OSGIN1 transcript expression is then translated to a 61kDa protein that can induce the accumulation of p53 in an unknown mechanism. p53 protein can then translocate to the nucleus and induce gene transcription (**Figure 77B**).

DETAILED DESCRIPTION

The invention is based, at least in part, on the discovery that the DMF makes a contribution to pharmacologic effect that is distinct from that of its metabolite, MMF.

Definitions

As used herein, the articles "a" and "an" refer to one or to more than one (*e.g.*, to at least one) of the grammatical object of the article.

"About" and "approximately" shall generally mean an acceptable degree of error for the quantity measured given the nature or precision of the measurements. Exemplary degrees of error are within 20 percent (%), typically, within 10%, and more typically, within 5% of a given value or range of values.

"Acquire" or "acquiring" as the terms are used herein, refer to obtaining possession of a physical entity, or a value, *e.g.*, a numerical value, by "directly acquiring" or "indirectly acquiring" the physical entity or value. "Directly acquiring" means performing a physical process (*e.g.*, performing a synthetic or analytical method) to obtain the physical entity or value. "Indirectly acquiring" refers to receiving the physical entity or value from another party or source (*e.g.*, a third party laboratory that directly acquired the physical entity or value). Directly

acquiring a physical entity includes performing a process that includes a physical change in a physical substance, *e.g.*, a starting material. Exemplary changes include making a physical entity from two or more starting materials, shearing or fragmenting a substance, separating or purifying a substance, combining two or more separate entities into a mixture, performing a chemical reaction that includes breaking or forming a covalent or non covalent bond. Directly acquiring a value includes performing a process that includes a physical change in a sample or another substance, *e.g.*, performing an analytical process which includes a physical change in a substance, *e.g.*, a sample, analyte, or reagent (sometimes referred to herein as “physical analysis”), performing an analytical method, *e.g.*, a method which includes one or more of the following: separating or purifying a substance, *e.g.*, an analyte, or a fragment or other derivative thereof, from another substance; combining an analyte, or fragment or other derivative thereof, with another substance, *e.g.*, a buffer, solvent, or reactant; or changing the structure of an analyte, or a fragment or other derivative thereof, *e.g.*, by breaking or forming a covalent or non covalent bond, between a first and a second atom of the analyte; or by changing the structure of a reagent, or a fragment or other derivative thereof, *e.g.*, by breaking or forming a covalent or non covalent bond, between a first and a second atom of the reagent.

“Acquiring a sample” as the term is used herein, refers to obtaining possession of a sample, *e.g.*, a tissue sample or nucleic acid sample, by “directly acquiring” or “indirectly acquiring” the sample. “Directly acquiring a sample” means performing a process (*e.g.*, performing a physical method such as a surgery or extraction) to obtain the sample. “Indirectly acquiring a sample” refers to receiving the sample from another party or source (*e.g.*, a third party laboratory that directly acquired the sample). Directly acquiring a sample includes performing a process that includes a physical change in a physical substance, *e.g.*, a starting material, such as a tissue, *e.g.*, a tissue in a human patient or a tissue that has was previously isolated from a patient. Exemplary changes include making a physical entity from a starting material, dissecting or scraping a tissue; separating or purifying a substance (*e.g.*, a sample tissue or a nucleic acid sample); combining two or more separate entities into a mixture; performing a chemical reaction that includes breaking or forming a covalent or non-covalent bond. Directly acquiring a sample includes performing a process that includes a physical change in a sample or another substance, *e.g.*, as described above.

A dimethyl fumarate (DMF)-differentially expressed gene, as the term is used herein, is a gene, the expression of which differs in a subject that has been administered DMF, as compared to a subject not administered DMF. Differential expression can be manifest at the transcriptional or translation level, *e.g.*, at the level of mRNA or protein, or at both. By way of example, a gene is DMF-differentially expressed if the levels of the RNA or protein product, or both, of the gene are higher, in a subject administered DMF, as compared to a subject not administered DMF. A DMF-differentially expressed gene can be characterized by differential expression at one or both of the transcriptional and translational levels. In an embodiment a DMF-differentially expressed gene will not also be MMF-differentially expressed, or the differential expression for DMF will differ from the differential expression seen for MMF.

A prodrug (PD)-differentially expressed gene, as the term is used herein, is a gene, the expression of which differs in a subject that has been administered a prodrug, as compared to a subject not administered a prodrug. Differential expression can be manifest at the transcriptional or translation level, *e.g.*, at the level of mRNA or protein, or at both. By way of example, a gene is PD-differentially expressed if the levels of the RNA or protein product, or both, of the gene are higher, in a subject administered prodrug, as compared to a subject not administered prodrug, *e.g.*, DMF. A PD-differentially expressed gene can be characterized by differential expression at one or both of the transcriptional and translational levels. In an embodiment a PD-differentially expressed gene will not also be drug, *e.g.*, MMF-differentially expressed, or the differential expression for PD will differ from the differential expression seen for drug, *e.g.*, MMF.

A monomethyl fumarate (MMF)-differentially expressed gene, as the term is used herein, is a gene, the expression of which differs in a subject that has been administered MMF, as compared to a subject not administered MMF. Differential expression can be manifest at the transcriptional or translation level, *e.g.*, at the level of mRNA or protein, or at both. By way of example, a gene is MMF-differentially expressed if the levels of the RNA or protein product, or both, of the gene are higher, in a subject administered MMF, as compared to a subject not administered MMF. An MMF-differentially expressed gene can be characterized by differential expression at one or both of the transcriptional and translational levels. In an embodiment an MMF-differentially expressed gene will not also be DMF-differentially expressed, or the differential expression for MMF will differ from the differential expression seen for DMF.

A DMF/MMF-differentially expressed gene, as the term is used herein, is a gene that is differentially expressed for both DMF and MMF.

A drug-differentially expressed gene, as the term is used herein, is a gene, the expression of which differs in a subject that has been administered drug, *e.g.*, MMF, as compared to a subject not administered the drug. Differential expression can be manifest at the transcriptional or translation level, *e.g.*, at the level of mRNA or protein, or at both. By way of example, a gene is drug-differentially expressed if the levels of the RNA or protein product, or both, of the gene are higher, in a subject administered drug, as compared to a subject not administered drug. A drug-differentially expressed gene can be characterized by differential expression at one or both of the transcriptional and translational levels. In an embodiment a drug-differentially expressed gene will not also be PD-differentially expressed, or the differential expression for drug will differ from the differential expression seen for prodrug.

A Drug/PD-differentially expressed gene, as the term is used herein, is a gene that is differentially expressed for both prodrug and drug.

As used herein, the “Expanded Disability Status Scale” or “EDSS” is intended to have its customary meaning in the medical practice. EDSS is a rating system that is frequently used for classifying and standardizing MS. The accepted scores range from 0 (normal) to 10 (death due to MS). Typically patients having an EDSS score of about 6 will have moderate disability (*e.g.*, walk with a cane), whereas patients having an EDSS score of about 7 or 8 will have severe disability (*e.g.*, will require a wheelchair). More specifically, EDSS scores in the range of 1-3 refer to an MS patient who is fully ambulatory, but has some signs in one or more functional systems; EDSS scores in the range higher than 3 to 4.5 show moderate to relatively severe disability; an EDSS score of 5 to 5.5 refers to a disability impairing or precluding full daily activities; EDSS scores of 6 to 6.5 refer to an MS patient requiring intermittent to constant, or unilateral to bilateral constant assistance (cane, crutch or brace) to walk; EDSS scores of 7 to 7.5 means that the MS patient is unable to walk beyond five meters even with aid, and is essentially restricted to a wheelchair; EDSS scores of 8 to 8.5 refer to patients that are restricted to bed; and EDSS scores of 9 to 10 mean that the MS patient is confined to bed, and progressively is unable to communicate effectively or eat and swallow, until death due to MS.

As used herein, the term "probe" refers to any molecule which is capable of selectively binding to a specifically intended target molecule, for example, a transcription product, *e.g.*, an mRNA or cDNA, or a translation product, *e.g.*, a polypeptide or protein. Probes can be either synthesized by one skilled in the art, or derived from appropriate biological preparations. For purposes of detection of the target molecule, probes can be specifically designed to be labeled, as described herein. Examples of molecules that can be utilized as probes include, but are not limited to, RNA, DNA, proteins, antibodies, and organic monomers.

As used herein, the term prodrug, or pro-drug, refers to a compound that is processed, in the body of a subject, into a drug. In an embodiment the processing comprises the breaking or formation of a bond, *e.g.*, a covalent bond. Typically, breakage of a covalent bond releases the drug.

"Sample," "tissue sample," "subject or patient sample," "subject or patient cell or tissue sample" or "specimen" each refers to a biological sample obtained from a tissue, *e.g.*, a bodily fluid, of a subject or patient. The source of the tissue sample can be solid tissue as from a fresh, frozen and/or preserved organ, tissue sample, biopsy, or aspirate; blood or any blood constituents (*e.g.*, serum, plasma); bodily fluids such as cerebral spinal fluid, whole blood, plasma and serum. The sample can include a non-cellular fraction (*e.g.*, plasma, serum, or other non-cellular body fluid). In one embodiment, the sample is a serum sample. In other embodiments, the body fluid from which the sample is obtained from an individual comprises blood (*e.g.*, whole blood). In certain embodiments, the blood can be further processed to obtain plasma or serum. In another embodiment, the sample contains a tissue, cells (*e.g.*, peripheral blood mononuclear cells (PBMC)). In an embodiment the sample includes NK cells. For example, the sample can be a fine needle biopsy sample, an archival sample (*e.g.*, an archived sample with a known diagnosis and/or treatment history), a histological section (*e.g.*, a frozen or formalin-fixed section, *e.g.*, after long term storage), among others. The term sample includes any material obtained and/or derived from a biological sample, including a polypeptide, and nucleic acid (*e.g.*, genomic DNA, cDNA, RNA) purified or processed from the sample. Purification and/or processing of the sample can involve one or more of extraction, concentration, antibody isolation, sorting, concentration, fixation, addition of reagents and the like. The sample can contain compounds

that are not naturally intermixed with the tissue in nature such as preservatives, anticoagulants, buffers, fixatives, nutrients, antibiotics or the like.

The term "alkyl" as employed herein by itself or as part of another group refers to both straight and branched chain radicals of up to 24 carbons. Alkyl groups include straight-chained and branched C₁-C₂₄ alkyl groups, *e.g.*, C₁-C₁₀ alkyl groups. C₁-C₁₀ alkyl groups include methyl, ethyl, propyl, isopropyl, butyl, isobutyl, sec-butyl, tert-butyl, pentyl, isopentyl, neopentyl, hexyl, isohexyl, 3-methylpentyl, 2,2-dimethylbutyl, 2,3-dimethylbutyl, heptyl, 1-methylhexyl, 2-ethylhexyl, 1,4-dimethylpentyl, octyl, nonyl, and decyl. Unless otherwise indicated, all alkyl groups described herein include both unsubstituted and substituted alkyl groups. Further, each alkyl group can include its deuterated counterparts.

The term "heteroalkyl" is an alkyl group in which one to five carbons in the alkyl chain are replaced by an independently selected oxygen, nitrogen or sulfur atom.

The term "aryl" as employed herein by itself or as part of another group refers to monocyclic, bicyclic, or tricyclic aromatic hydrocarbon containing from 5 to 50 carbons in the ring portion. Aryl groups include C₅₋₁₅ aryl, *e.g.*, phenyl, p-tolyl, 4-methoxyphenyl, 4-(tert-butoxy)phenyl, 3-methyl-4-methoxyphenyl, 4-fluorophenyl, 4-chlorophenyl, 3-nitrophenyl, 3-aminophenyl, 3-acetamidophenyl, 4-acetamidophenyl, 2-methyl-3-acetamidophenyl, 2-methyl-3-aminophenyl, 3-methyl-4-aminophenyl, 2-amino-3-methylphenyl, 2,4-dimethyl-3-aminophenyl, 4-hydroxyphenyl, 3-methyl-4-hydroxyphenyl, 1-naphthyl, 3-amino-naphthyl, 2-methyl-3-amino-naphthyl, 6-amino-2-naphthyl, 4,6-dimethoxy-2-naphthyl, indanyl, biphenyl, phenanthryl, anthryl, and acenaphthyl. Unless otherwise indicated, all aryl groups described herein include both unsubstituted and substituted aryl groups.

The term "arylalkyl" refers to an alkyl group which is attached to another moiety through an aryl group.

"Halogen" or "halo" may be fluoro, chloro, bromo or iodo.

The term "cycloalkyl" refers to completely saturated monocyclic, bicyclic or tricyclic hydrocarbon groups of 3-12 carbon atoms, preferably 3-9, or more preferably 3-8 carbon atoms.

Exemplary monocyclic cycloalkyl groups include, but are not limited to, cyclopropyl, cyclobutyl, cyclopentyl, and cyclohexyl. Exemplary bicyclic cycloalkyl groups include bornyl, decahydronaphthyl, bicyclo[2.1.1]hexyl, bicyclo[2.2.1]heptyl, 6,6-dimethylbicyclo[3.1.1]heptyl, 2,6,6-trimethylbicyclo[3.1.1]heptyl, or bicyclo[2.2.2]octyl. Exemplary tricyclic carbocyclyl groups include adamantyl.

The term "cycloalkylalkyl" refers to a cycloalkyl group which is attached to another moiety through an alkyl group.

The term "heterocycloalkyl" refers to completely saturated monocyclic, bicyclic or tricyclic heterocyclyl comprising 3-15 ring members, at least one of which is a heteroatom, and up to 10 of which may be heteroatoms, wherein the heteroatoms are independently selected from O, S and N, and wherein N and S can be optionally oxidized to various oxidation states. Examples of heterocycloalkyl groups include [1,3]dioxolane, 1,4-dioxane, 1,4-dithiane, piperazinyl, 1,3-dioxolane, imidazolidinyl, imidazolinyl, pyrrolidine, dihydropyran, oxathiolane, dithiolane, 1,3-dioxane, 1,3-dithianyl, oxathianyl, thiomorpholinyl, oxiranyl, aziridinyl, oxetanyl, azetidiny, tetrahydrofuranyl, pyrrolidinyl, tetrahydropyranyl, piperidinyl, morpholinyl, and piperazinyl.

As used herein, the term "heteroaryl" refers to a 5-14 membered monocyclic-, bicyclic-, or tricyclic-ring system, having 1 to 10 heteroatoms independently selected from N, O or S, wherein N and S can be optionally oxidized to various oxidation states, and wherein at least one ring in the ring system is aromatic. In one embodiment, the heteroaryl is monocyclic and has 5 or 6 ring members. Examples of monocyclic heteroaryl groups include pyridyl, thienyl, furanyl, pyrrolyl, pyrazolyl, imidazolyl, oxazolyl, isoxazolyl, thiazolyl, isothiazolyl, triazolyl, oxadiazolyl, thiadiazolyl and tetrazolyl. In another embodiment, the heteroaryl is bicyclic and has from 8 to 10 ring members. Examples of bicyclic heteroaryl groups include indolyl, benzofuranyl, quinolyl, isoquinolyl, indazolyl, indolyl, isoindolyl, indolizyl, benzamidazolyl, quinolyl, 5,6,7,8-tetrahydroquinoline and 6,7-dihydro-5H-pyrrolo[3,2-d]pyrimidine.

The term "heteroarylalkyl" refers to an alkyl group which is attached to another moiety through an alkyl group.

Multiple Sclerosis and Methods of Diagnosis

Multiple sclerosis (MS) is a central nervous system disease that is characterized by inflammation and loss of myelin sheaths.

Patients having MS can be identified by clinical criteria establishing a diagnosis of clinically definite MS as defined by Poser et al., *Ann. Neurol.* 13:227, 1983. Briefly, an individual with clinically definite MS has had two attacks and clinical evidence of either two lesions or clinical evidence of one lesion and paraclinical evidence of another, separate lesion. Definite MS may also be diagnosed by evidence of two attacks and oligoclonal bands of IgG in cerebrospinal fluid or by combination of an attack, clinical evidence of two lesions and oligoclonal band of IgG in cerebrospinal fluid. The McDonald criteria can also be used to diagnose MS. (McDonald et al., 2001, Recommended diagnostic criteria for Multiple sclerosis: guidelines from the International Panel on the Diagnosis of Multiple Sclerosis, *Ann Neurol* 50:121-127). The McDonald criteria include the use of MRI evidence of CNS impairment over time to be used in diagnosis of MS, in the absence of multiple clinical attacks. Effective treatment of multiple sclerosis may be evaluated in several different ways. The following parameters can be used to gauge effectiveness of treatment. Two exemplary criteria include: EDSS (extended disability status scale), and appearance of exacerbations on MRI (magnetic resonance imaging).

The EDSS is a means to grade clinical impairment due to MS (Kurtzke, *Neurology* 33:1444, 1983). Eight functional systems are evaluated for the type and severity of neurologic impairment. Briefly, prior to treatment, patients are evaluated for impairment in the following systems: pyramidal, cerebella, brainstem, sensory, bowel and bladder, visual, cerebral, and other. Follow-ups are conducted at defined intervals. The scale ranges from 0 (normal) to 10 (death due to MS). A decrease of one full step indicates an effective treatment (Kurtzke, *Ann. Neurol.* 36:573-79, 1994), while an increase of one full step will indicate the progression or worsening of disease (e.g., exacerbation). Typically patients having an EDSS score of about 6 will have moderate disability (e.g., walk with a cane), whereas patients having an EDSS score of about 7 or 8 will have severe disability (e.g., will require a wheelchair).

Exacerbations are defined as the appearance of a new symptom that is attributable to MS and accompanied by an appropriate new neurologic abnormality (IFNB MS Study Group, *supra*). In addition, the exacerbation must last at least 24 hours and be preceded by stability or improvement for at least 30 days. Briefly, patients are given a standard neurological examination by clinicians. Exacerbations are mild, moderate, or severe according to changes in a Neurological Rating Scale (Sipe et al., *Neurology* 34:1368, 1984). An annual exacerbation rate and proportion of exacerbation-free patients are determined.

Therapy can be deemed to be effective using a clinical measure if there is a statistically significant difference in the rate or proportion of exacerbation-free or relapse-free patients between the treated group and the placebo group for either of these measurements. In addition, time to first exacerbation and exacerbation duration and severity may also be measured. A measure of effectiveness as therapy in this regard is a statistically significant difference in the time to first exacerbation or duration and severity in the treated group compared to control group. An exacerbation-free or relapse-free period of greater than one year, 18 months, or 20 months is particularly noteworthy. Clinical measurements include the relapse rate in one and two-year intervals, and a change in EDSS, including time to progression from baseline of 1.0 unit on the EDSS that persists for six months. On a Kaplan-Meier curve, a delay in sustained progression of disability shows efficacy. Other criteria include a change in area and volume of T2 images on MRI, and the number and volume of lesions determined by gadolinium enhanced images.

MRI can be used to measure active lesions using gadolinium-DTPA-enhanced imaging (McDonald et al., *Ann. Neurol.* 36:14, 1994) or the location and extent of lesions using T2-weighted techniques. Briefly, baseline MRIs are obtained. The same imaging plane and patient position are used for each subsequent study. Positioning and imaging sequences can be chosen to maximize lesion detection and facilitate lesion tracing. The same positioning and imaging sequences can be used on subsequent studies. The presence, location and extent of MS lesions can be determined by radiologists. Areas of lesions can be outlined and summed slice by slice for total lesion area. Three analyses may be done: evidence of new lesions, rate of appearance of active lesions, percentage change in lesion area (Paty et al., *Neurology* 43:665, 1993). Improvement due to therapy can be established by a statistically significant improvement in an individual patient compared to baseline or in a treated group versus a placebo group.

Exemplary symptoms associated with multiple sclerosis, which can be treated with the methods described herein or managed using symptom management therapies, include: optic neuritis, diplopia, nystagmus, ocular dysmetria, internuclear ophthalmoplegia, movement and sound phosphenes, afferent pupillary defect, paresis, monoparesis, paraparesis, hemiparesis, quadraparesis, plegia, paraplegia, hemiplegia, tetraplegia, quadraplegia, spasticity, dysarthria, muscle atrophy, spasms, cramps, hypotonia, clonus, myoclonus, myokymia, restless leg syndrome, footdrop, dysfunctional reflexes, paraesthesia, anaesthesia, neuralgia, neuropathic and neurogenic pain, l'hermitte's, proprioceptive dysfunction, trigeminal neuralgia, ataxia, intention tremor, dysmetria, vestibular ataxia, vertigo, speech ataxia, dystonia, dysdiadochokinesia, frequent micturation, bladder spasticity, flaccid bladder, detrusor-sphincter dyssynergia, erectile dysfunction, anorgasmy, frigidity, constipation, fecal urgency, fecal incontinence, depression, cognitive dysfunction, dementia, mood swings, emotional lability, euphoria, bipolar syndrome, anxiety, aphasia, dysphasia, fatigue, Uhthoff's symptom, gastroesophageal reflux, and sleeping disorders.

Each case of MS displays one of several patterns of presentation and subsequent course. Most commonly, MS first manifests itself as a series of attacks followed by complete or partial remissions as symptoms mysteriously lessen, only to return later after a period of stability. This is called relapsing-remitting MS (RRMS). Primary-progressive MS (PPMS) is characterized by a gradual clinical decline with no distinct remissions, although there may be temporary plateaus or minor relief from symptoms. Secondary-progressive MS (SPMS) begins with a relapsing-remitting course followed by a later primary-progressive course. Rarely, patients may have a progressive-relapsing (PRMS) course in which the disease takes a progressive path punctuated by acute attacks. PPMS, SPMS, and PRMS are sometimes lumped together and called chronic progressive MS.

A few patients experience malignant MS, defined as a swift and relentless decline resulting in significant disability or even death shortly after disease onset. This decline may be arrested or decelerated by determining the likelihood of the patient to respond to a therapy early in the therapeutic regime and switching the patient to an agent that they have the highest likelihood of responding to.

Differentially Expressed Genes

As described in the Examples, transcriptional profiling of mouse genes was performed on C57BL/6 mice that were administered vehicle, DMF or MMF (100 mg/kg). Treated mice were sacrificed at 2, 7, or 12 hours. Tissues (liver, spleen, kidney, jejunum, cortex, hippocampus, striatum and whole blood) were collected and analyzed by transcriptional profiling on mouse Affymetrix GeneChips. Differentially expressed genes were identified by comparing DMF or MMF treated mice to matched vehicle controls and exemplary genes that were identified are provided in **TABLES 1-8** below. While the experiments were performed in mice, the human homolog of the identified murine gene transcript is included in the tables. Additional genes that were identified in the study are provided in **Tables 18-26**.

Table 1. Murine Whole Blood DEG's

Probe Set	Gene Title	Gene	ENTREZ ID	DMF 2 hr	DMF 7 hr	DMF 12 hr	MMF 2 hr	MMF 7 hr	MMF 12 hr	Human Homolog Entrez ID
1420937_PM_at	cleavage and polyadenylation specific factor 2	Cpsf2		-1.65						
1417898_PM_a_at	granzyme A	Gzma	14938			1.92				3001
1422089_PM_at	natural cytotoxicity triggering receptor 1	Ncr1	17086			1.86				9437
1425005_PM_at	killer cell lectin-like receptor subfamily C, member 1	Klrc1	16641			1.85				3821
1445399_PM_at	killer cell lectin-like receptor subfamily B member 1B	Klrb1b	80782			1.65				
	killer cell lectin-like receptor family E member 1	Klre1	243655			1.85				
1418133_PM_at	B-cell leukemia/lymphoma 3	Bcl3					-1.23			
1415943_PM_at	syndecan 1	Sdc1							-1.96	

Table 2. Murine Cortex DEGs

Probe Set	Gene Title	Gene	ENTREZ ID	DMF 2 hr	DMF 7 hr	DMF 12 hr	MMF 2 hr	MMF 7 hr	MMF 12 hr	Human Homolog
-----------	------------	------	-----------	----------	----------	-----------	----------	----------	-----------	---------------

											Entrez ID
1419086_PM_at	fibroblast growth factor binding protein 1	Fgfbp1	14181		1.54				1.62		9982
1448140_PM_at	cytokine induced apoptosis inhibitor 1	Ciapi1	109006	-1.02							57019
1423627_PM_at	NAD(P)H dehydrogenase, quinone 1	Nqo1	18104		1.50						1728
1416734_PM_at	muskelin 1, intracellular mediator containing kelch motifs	Mkln1	27418					-1.85			4289
1420670_PM_at	aryl hydrocarbon receptor nuclear translocator 2	Arnt2	11864					-1.66			9915
1421166_PM_at	attractin	Atrn	11990					-1.62			8455
1421339_PM_at	exostoses (multiple)-like 3	Extl3	54616					-1.57			2137
1423594_PM_a_at	endothelin receptor type B	Ednrb	13618					-1.53			1910
1429607_PM_at	trafficking protein, kinesin binding 2	Trak2	70827					-1.57			66008
1429992_PM_at	spermatogenesis associated glutamate (E)-rich protein 4b	Speer4b	73526					-1.60			NA
1430827_PM_a_at	PTK2 protein tyrosine kinase 2	Ptk2	14083					-1.53			5747
1438108_PM_at	pleckstrin homology domain containing, family M, member 3	Plekhn3	241075					-1.50			389072
1442277_PM_at	choline kinase alpha	Chka	12660					-1.53			1119
1443123_PM_at	tetratricopeptide repeat, ankyrin repeat and coiled-coil containing 2	Tanc2	77097					-1.72			26115
1448458_PM_at	topoisomerase (DNA) II beta	Top2b	21974					-1.51			7155
1456070_PM_at	protein tyrosine phosphatase, receptor type, G	Ptprg	19270					-1.83			5793
1419435_PM_at	aldehyde oxidase 1	Aox1	11761						1.65	1.54	316

Table 3. Murine Hippocampus DEGs

Probe Set	Gene Title	Gene	ENTREZ ID	DMF 2 hr	DMF 7 hr	DMF 12 hr	MMF 2 hr	MMF 7 hr	MMF 12 hr
-----------	------------	------	-----------	----------	----------	-----------	----------	----------	-----------

1458305_PM_at	transmembrane and tetratricopeptide repeat containing 3	Tmtc3	237500		-2.38				-2.40	
1417975_PM_at	karyopherin (importin) alpha 4	Kpna4	16649					-1.51		
1425077_PM_at	DnaJ (Hsp40) homolog, subfamily C, member 18	Dnajc18	76594					-1.58		
1426029_PM_a_at	nuclear factor of activated T-cells 5	Nfat5	54446					-1.51		
1434407_PM_at	SLIT-ROBO Rho GTPase activating protein 2	Srgap2	14270					-1.50		
1438085_PM_at	HEAT repeat containing 5B	Heatr5b	320473					-1.56		
1458421_PM_at	potassium voltage-gated channel, subfamily Q, member 3	Kcnq3	110862					-1.50		

Table 4. Murine Striatum DEGs

Probe Set	Gene Title	Gene	ENTREZ ID	DMF 2 hr	DMF 7 hr	DMF 12 hr	MMF 2 hr	MMF 7 hr	MMF 12 hr	Human Homolog Entrez ID
1419435_PM_at	aldehyde oxidase 1	Aox1	11761		1.64			1.95		316
1428320_PM_at	KDM3B lysine (K)-specific demethylase 3B	Kdm3b	277250	-1.54			-1.56			51780
1434030_PM_at	GTP-binding protein 10 (putative)	Gtpbp10	207704	-1.52						85865
1456070_PM_at	protein tyrosine phosphatase, receptor type, G	Ptprg	19270	-1.54						5793
1416734_PM_at	muskelin 1, intracellular mediator containing kelch motifs	Mkln1	27418	-1.97						4289
1438236_PM_at	nuclear factor I/A	Nfia	18027			-1.53				4774
1422748_PM_at	zinc finger E-box binding homeobox 2	Zeb2	24136			-1.66				9839
1427125_PM_s_at	leucine rich repeat containing 41	Lrrc41	230654				-1.54			10489

1448458_ PM_at	topoisomera se (DNA) II beta	Top2b	21974					-1.56			7155
1449616_ PM_s_at	golgi autoantigen, golgin subfamily a, 3	Golga3	269682					-1.63			2802
1422556_ PM_at	guanine nucleotide binding protein, alpha 13	Gna13	14674					-1.63			10672
1421616_ PM_at	glutamate receptor, ionotropic, NMDA2A (epsilon 1)	Grin2a	14811					-1.78			2903
1428676_ PM_at	transmembra ne serine protease 6	Tmprss 6	71753							1.68	164656
1448807_ PM_at	histamine receptor H3	Hrh3	99296							1.59	11255
1437760_ PM_at	UDP-N- acetyl-alpha- D- galactosamin e:polypeptid e N- acetylgalacto saminyltrans ferase 12	Galnt12	230145							1.50	79695
1435272_ PM_at	inositol 1,4,5- trisphosphat e 3-kinase B	Itpkb	320404							-1.56	3707
1455123_ PM_at	suppression of tumorigenici ty 18	St18	240690							-1.69	9705
1457257_ PM_x_at	poliovirus receptor- related 3	Pvr13	58998							-1.80	25945
1437785_ PM_at	a disintegrin- like and metallopepti dase (reprolysin type) with thrombospon din type 1 motif, 9	Adamts 9	101401							-2.58	56999

Table 5a. Murine Jejunum DMF-specific DEGs

Probe Set	Gene Title	Gene	ENTREZ ID	DMF 2 hr	DMF 7 hr	DMF 12 hr	Human Homolog
-----------	------------	------	--------------	-------------	-------------	--------------	------------------

							Entrez ID
1440230_PM_at	tsukushin	Tsku	244152	1.61			25987
1453421_PM_at	serine racemase	Srr	27364	1.99			63826
1428834_PM_at	dual specificity phosphatase 4	Dusp4	319520	1.51			1846
1429950_PM_at	unc-5 homolog C (C. elegans)-like	Unc5cl	76589	-1.53			222643
1420393_PM_at	nitric oxide synthase 2, inducible	Nos2	18126	-2.39			4843
1442923_PM_at	PTK6 protein tyrosine kinase 6	Ptk6	20459	-2.42			5753
1426501_PM_a_at	TRAF-interacting protein with forkhead-associated domain	Tifa	211550	-2.43			92610
1428397_PM_at	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 5	B3galt5	93961	-2.77			10317
1456611_PM_at	family with sequence similarity 13, member A	Fam13a	58909	1.54			10144

Table 5b. Murine Jejunum MMF-specific DEGs

Probe Set	Gene Title	Gene	ENTREZ ID	DMF 2 hr	DMF 7 hr	DMF 12 hr	MMF 2 hr	MMF 7 hr	MMF 12 hr	Human Homolog Entrez ID
1440882_PM_at	low density lipoprotein receptor-related protein 8, apolipoprotein e receptor	Lrp8	16975				2.23			7804
1448239_PM_at	heme oxygenase (decycling) 1	Hmox1	15368				1.81			3162
1436605_PM_at	transketolase	Tkt	21881				1.73			7086
1422645_PM_at	hemochromatosis	Hfe	15216				1.73			3077
1426600_PM_at	solute carrier family 2 (facilitated glucose transporter), member 1	Slc2a1	20525				1.68			6513
1438953_PM_at	c-fos induced growth factor	Figf	14205				1.63			2277
1450410_PM_a_at	solute carrier family 48 (heme transporter), member 1	Slc48a1	67739				1.62			55652
1452837_PM_at	lipin 2	Lpin2	64898				1.61			9663
1424181_PM_at	septin 6	6-Sep	56526				1.56			23157
1449078_PM_at	ST3 beta-galactoside alpha-2,3-sialyltransferase 6	St3gal6	54613				1.52			10402
1416418_PM_at	gamma-aminobutyric acid (GABA) A receptor-associated protein-like 1	Gabara p11	57436				1.52			23710

1423635_PM_at	bone morphogenetic protein 2	Bmp2	12156					-1.55			650
1421886_PM_at	son of sevenless homolog 1 (Drosophila)	Sos1	20662					-1.57			6654
1452834_PM_at	proline rich 5 like	Prr51	72446					-1.64			79899
1433699_PM_at	tumor necrosis factor, alpha-induced protein 3	Tnfaip3	21929					-1.67			7128
1450165_PM_at	schlafen 2	Slfn2	20556					-1.82			NA
1416632_PM_at	malic enzyme 1, NADP(+)-dependent, cytosolic	Me1	17436						2.01		4199
1419072_PM_at	glutathione S-transferase, mu 7	Gstm7	68312						1.97		2946
1451385_PM_at	family with sequence similarity 162, member A	Fam162a	70186						1.67		26355
1451095_PM_at	asparagine synthetase	Asns	27053						1.60		440
1419442_PM_at	matrilin 2	Matn2	17181						1.59		4147
1423706_PM_a_at	phosphogluconate dehydrogenase	Pgd	110208						1.55		5226
1450109_PM_s_at	ATP-binding cassette, sub-family C (CFTR/MRP), member 2	Abcc2	12780						1.51		1244
1428960_PM_at	enkurin, TRPC channel interacting protein	Enkur	71233						-1.56		219670
1418580_PM_at	receptor transporter protein 4	Rtp4	67775						-1.61		64108
1435529_PM_at	predicted gene 14446	Gm14446	667373						-1.65		3434
1437960_PM_at	calpain 13	Capn13	381122						-1.75		92291
1436058_PM_at	radical S-adenosyl methionine domain containing 2	Rsad2	58185						-1.81		91543
1450783_PM_at	interferon-induced protein with tetratricopeptide repeats 1	Ifit1	15957						-1.95		439996
1422438_PM_at	epoxide hydrolase 1, microsomal	Ephx1	13849							2.17	2052
1434458_PM_at	folliculin	Fst	14313							1.94	10468
1422000_PM_at	aldo-keto reductase family 1, member C12	Akr1c12	622402							1.52	622402
1417991_PM_at	deiodinase, iodothyronine,	Dio1	13370							-1.51	1733

	type I										
1451642_PM_at	kinesin family member 1B	Kif1b	16561							-1.51	23095
1457554_PM_at	apolipoprotein B	Apob	238055							-1.54	338

Table 6. Murine Kidney DMF specific DEGs

Probe Set	Gene Title	Gene	ENTREZ ID	DMF 2 hr	DMF 7 hr	DMF 12 hr	Human Homolog Entrez ID
1436521_PM_at	solute carrier family 36 (proton/amino acid symporter), member 2	Slc36a2	246049	4.63			153201
1422612_PM_at	hexokinase 2	Hk2	15277	2.72			3099
1438664_PM_at	protein kinase, cAMP dependent regulatory, type II beta	Prkar2b	19088	2.43			5577
1434893_PM_at	ATPase, Na+/K+ transporting, alpha 2 polypeptide	Atp1a2	98660	2.25			477
1423405_PM_at	tissue inhibitor of metalloproteinase 4	Timp4	110595	1.91			7079
1453596_PM_at	inhibitor of DNA binding 2	Id2	15902	1.83			3398
1456041_PM_at	sorting nexin 16	Snx16	74718	1.75			64089
1436366_PM_at	protein phosphatase 1, regulatory (inhibitor) subunit 15b	Ppp1r15b	108954	1.72			84919
1448170_PM_at	seven in absentia 2	Siah2	20439	1.71			6478
1441190_PM_at	actin related protein 2/3 complex, subunit 5-like	Arpc5l	74192	1.70			81873
1455657_PM_at	SMG1 homolog, phosphatidylinositol 3-kinase-related kinase (C. elegans)	Smg1	233789	1.69			23049
1418203_PM_at	phorbol-12-myristate-13-acetate-induced protein 1	Pmaip1	58801	1.68			NA
1433944_PM_at	HECT domain containing 2	Hectd2	226098	1.67			143279
1423170_PM_at	TAF7 RNA polymerase II, TATA box binding protein (TBP)-associated factor	Taf7	24074	1.66			6879
1452394_PM_at	cysteinyl-tRNA synthetase	Cars	27267	1.63			833
1441546_PM_at	Membrane protein, palmitoylated 6 (MAGUK p55 subfamily member 6)	Mpp6	56524	1.62			51678
1441955_PM_s_at	polyadenylate binding protein-interacting protein 1	Paip1	218693	1.61			10605
1438578_PM_a_at	BTB (POZ) domain containing 10	Btbd10	68815	1.60			84280
1418025_PM_at	basic helix-loop-helix family, member e40	Bhlhe40	20893	1.59			8553
1459585_PM_at	growth arrest specific 6	Gas6	14456	1.59			2621

1417479_PM_at	protein phosphatase 2, regulatory subunit B", gamma	Ppp2r3c	59032	1.59			55012
1455185_PM_s_at	PHD finger protein 16	Phf16	382207	1.58			9767
1436871_PM_at	serine/arginine-rich splicing factor 7	Srsf7	225027	1.57			6432
1452094_PM_at	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha 1 polypeptide	P4ha1	18451	1.56			5033
1443116_PM_at	proteasome (prosome, macropain) activator subunit 4	Psme4	103554	1.56			23198
1424380_PM_at	vacuolar protein sorting 37B (yeast)	Vps37b	330192	1.56			79720
1456810_PM_at	vacuolar protein sorting 54 (yeast)	Vps54	245944	1.54			51542
1452218_PM_at	coiled-coil domain containing 117	Ccdc117	104479	1.54			150275
1435904_PM_at	eukaryotic translation initiation factor 2C, 3	Eif2c3	214150	1.53			192669
1442071_PM_at	ATP-binding cassette, sub-family E (OABP), member 1	Abce1	24015	1.53			6059
1440346_PM_at	KDM1 lysine (K)-specific demethylase 6B	Kdm6b	216850	1.52			23135
1423549_PM_at	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	Slc1a4	55963	1.52			6509
1435912_PM_at	UBX domain protein 7	Ubxn7	224111	1.51			26043
1423605_PM_a_at	transformed mouse 3T3 cell double minute 2	Mdm2	17246	1.51			4193
1425656_PM_a_at	brain-specific angiogenesis inhibitor 1-associated protein 2	Baiap2	108100	1.51			10458
1425287_PM_at	zinc finger protein 189	Zfp189	230162	1.51			7743
1455904_PM_at	growth arrest specific 5 /// small nucleolar RNA, C/D box 47	Gas5 /// Snord47	100217446 /// 14455	1.51			NA // NA
1438535_PM_at	pleckstrin homology domain interacting protein	Phip	83946	1.50			55023
1419140_PM_at	activin receptor IIB	Acvr2b	11481	-1.51			93
1428975_PM_at	sushi domain containing 3	Susd3	66329	-1.51			203328
1437231_PM_at	SLIT and NTRK-like family, member 6	Slitrk6	239250	-1.52			84189
1452962_PM_at	transmembrane protein 25	Tmem25	71687	-1.52			84866
1438784_PM_at	B-cell leukemia/lymphoma 11B	Bcl11b	58208	-1.52			64919
1427369_PM_at	NLR family, pyrin domain containing 6	Nlrp6	101613	-1.52			171389
1455087_PM_at	DNA segment, Chr 7, ERATO Doi 715, expressed	D7Ertd715e	52480	-1.53			NA
1449813_PM_at	zinc finger protein 30	Zfp30	22693	-1.55			22835
1451692_PM_at	transmembrane and	Tmco6	71983	-1.57			55374

	coiled-coil domains 6						
1419051_PM_at	OVO homolog-like 1 (Drosophila)	Ovol1	18426	-1.60			5017
1424213_PM_at	UbiA prenyltransferase domain containing 1	Ubiad1	71707	-1.61			29914
1441198_PM_at	zinc finger protein 39	Zfp39	22698	-1.64			100131827
1446303_PM_at	insulin-like growth factor I receptor	Igf1r	16001	-1.66			3480
1429456_PM_a_at	polymerase (RNA) III (DNA directed) polypeptide E	Polr3e	26939	-1.69			55718
1457548_PM_at	A disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 6	Adamts6	108154	-1.83			11174
1449981_PM_a_at	N-acetyltransferase 2 (arylamine N-acetyltransferase)	Nat2	17961	-2.01			9
1452975_PM_at	alanine-glyoxylate aminotransferase 2-like 1	Agxt211	71760	-2.10			64850
1427056_PM_at	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 15	Adamts15	235130	-2.26			170689
1427372_PM_at	cytochrome P450, family 27, subfamily b, polypeptide 1	Cyp27b1	13115	-3.22			1594
1427094_PM_at	polymerase (DNA directed), epsilon 2 (p59 subunit)	Pole2	18974		1.75		5427
1440899_PM_at	flavin containing monooxygenase 5	Fmo5	14263		-1.51		2330
1460196_PM_at	carbonyl reductase 1	Cbr1	12408			1.80	873
1421108_PM_at	camello-like 2	Cml2	93673			1.60	NA
1421603_PM_a_at	carcinoembryonic antigen-related cell adhesion molecule 2	Ceacam2	26367			-1.68	NA

Table 7. Murine Liver DEGs

Probe Set	Gene Title	Gene	ENTREZ ID	DMF 2 hr	DMF 7 hr	DMF 12 hr	MMF 2 hr	MMF 7 hr	MMF 12 hr	Human Homolog Entrez ID
1448894_PM_at	aldo-keto reductase family 1, member B8	Akr1b8	14187	2.18	1.58		2.27	1.75		57016
1419627_PM_s_at	C-type lectin domain family 4, member n	Clec4n	56620	2.34	2.01			2.40		93978
1419942_PM_at	Sulfiredoxin 1 homolog (S. cerevisiae)	Srxn1	76650	1.69			1.68	1.52		140809

1417801_PM_a_at	PTPRF interacting protein, binding protein 2 (liprin beta 2)	Ppfibp2	19024	2.38			2.55			8495
1448239_PM_at	heme oxygenase (decycling) 1	Hmox1	15368	2.37			2.41			3162
1420804_PM_s_at	C-type lectin domain family 4, member d	Clec4d	17474	2.30			2.05			338339
1438953_PM_at	c-fos induced growth factor	Figf	14205	2.23			2.25			2277
1452233_PM_at	ATP-binding cassette, sub-family C (CFTR/MRP), member 1	Abcc1	17250	1.75			1.86			4363
1436771_PM_x_at	phosphogluc onate dehydrogenase	Pgd	110208	1.59			1.61			5226
1423627_PM_at	NAD(P)H dehydrogenase, quinone 1	Nqo1	18104		1.81			2.20		1728
1450759_PM_at	bone morphogenetic protein 6	Bmp6	12161		1.57			1.65		654
1435495_PM_at	adenosine A1 receptor	Adora1	11539		1.50					134
1424835_PM_at	glutathione S-transferase, mu 4	Gstm4	14865			1.12				2948
1424296_PM_at	glutamate-cysteine ligase, catalytic subunit	Gclc	14629				1.72			2729
1422573_PM_at	adenosine monophosphate deaminase 3	Ampd3	11717				1.70			272
1455016_PM_at	PRP38 pre-mRNA processing factor 38 (yeast) domain containing B	Prpf38b	66921				-1.53			55119
1452247_PM_at	fragile X mental retardation gene 1, autosomal homolog	Fxr1	14359				-1.53			8087
1447019_PM_at	cytidine monophosph	Cmah	12763				-1.55			NA

	o-N-acetylneuraminic acid hydroxylase									
1448348_PM_at	cell cycle associated protein 1	Caprin1	53872				-1.56			4076
1419038_PM_a_at	casein kinase 2, alpha 1 polypeptide	Csnk2a1	12995				-1.62			1457
1440091_PM_at	Meis homeobox 2	Meis2	17536				-1.67			4212
1416734_PM_at	muskelin 1, intracellular mediator containing kelch motifs	Mkln1	27418				-1.69			4289
1453908_PM_at	protein tyrosine phosphatase, receptor type, B	Ptpnb	19263				-1.76			5787
1456070_PM_at	protein tyrosine phosphatase, receptor type, G	Ptpng	19270				-1.85			5793
1449498_PM_at	macrophage receptor with collagenous structure	Marco	17167					2.44		8685
1427357_PM_at	cytidine deaminase	Cda	72269					1.92		978
1448354_PM_at	glucose-6-phosphate dehydrogenase X-linked	G6pdx	14381					1.61		2539
1435040_PM_at	interleukin-1 receptor-associated kinase 3	Irak3	73914					1.53		11213
1429001_PM_at	pirin	Pir	69656					1.50		8544
1445815_PM_at	frizzled homolog 8 (Drosophila)	Fzd8	14370					-1.81		8325
1434582_PM_at	ELKS/RAB6 - interacting/C AST family member 2	Erc2	238988					-1.95		26059
1419669_PM_at	proteinase 3	Prtn3	19152						4.46	5657
1431214_PM_at	predicted gene 3579	Gm3579	100041932						2.74	NA
1417441_PM_at	DnaJ (Hsp40) homolog, subfamily C, member 12	Dnajc12	30045						2.53	56521
1428154_PM_s_at	phosphatidic acid phosphatase	Ppapdc1b	71910						1.87	84513

	type 2 domain containing 1B										
1444176_PM_at	ATPase, H+ transporting, lysosomal V0 subunit D2	Atp6v0d 2	242341							1.84	245972
1423290_PM_at	hypoxia up-regulated 1	Hyou1	12282							1.82	10525
1433833_PM_at	fibronectin type III domain containing 3B	Fndc3b	72007							1.66	64778
1416235_PM_at	leucine rich repeat containing 59	Lrrc59	98238							1.65	55379
1448471_PM_a_at	cytotoxic T lymphocyte-associated protein 2 alpha	Ctla2a	13024							1.63	NA
1448574_PM_at	non-metastatic cells 6, protein expressed in (nucleoside-diphosphate kinase)	Nme6	54369							1.57	10201
1443807_PM_x_at	cyclin F	Ccnf	12449							1.57	899
1450971_PM_at	growth arrest and DNA-damage-inducible 45 beta	Gadd45b	17873							1.50	4616
1453103_PM_at	actin-binding LIM protein 1	Ablim1	226251							-1.51	3983
1439117_PM_at	calmin	Clmn	94040							-1.54	79789
1441988_PM_at	protein phosphatase 1K (PP2C domain containing)	Ppmlk	243382							-1.56	152926
1460256_PM_at	carbonic anhydrase 3	Car3	12350							-1.57	761

Table 8. Murine Spleen DEGs

Probe Set	Gene Title	Gene	ENTREZ ID	DMF 2 hr	DMF 7 hr	DMF 12 hr	MMF 2 hr	MMF 7 hr	MMF 12 hr	Human Homolog Entrez ID
1419942_PM_at	Sulfiredoxin 1 homolog (S. cerevisiae)	Srxn1	76650	2.16	1.67		2.02	1.87		140809

1419435_PM_at	aldehyde oxidase 1	Aox1	11761	2.61			2.35			316
1434797_PM_at	kin of IRRE like (Drosophila)	Kirrel	170643	-1.54			-1.65			55243
1424022_PM_at	oxidative stress induced growth inhibitor 1	Osgin1	71839	1.78			1.83			29948
1416497_PM_at	protein disulfide isomerase associated 4	Pdia4	12304	-1.60			-1.59			9601
1424486_PM_a_at	thioredoxin reductase 1	Txnrd1	50493	2.41			2.14			7296
1424296_PM_at	glutamate-cysteine ligase, catalytic subunit	Gclc	14629	1.76						2729
1448916_PM_at	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein G (avian)	Mafg	17134	1.58						4097
1434951_PM_at	armadillo repeat containing 8	Armc8	74125				-1.54			25852
1425521_PM_at	polyadenylate binding protein-interacting protein 1	Paip1	218693				-1.56			10605
1424084_PM_at	ROD1 regulator of differentiation 1 (S. pombe)	Rod1	230257				-1.54			9991

Table 9. Murine Transcript and protein level change in DEGs from blood.

Gene Title	Murine Gene	Murine Entrez ID	Human Homolog Entrez ID	DMF 12hr FC (transcriptional fold change)	DMF 12hr MFI difference (compared to MMF 12 hr or vehicle control) (protein expression change)
Granzyme A	Gzma	14938	3001	1.92	Not tested
Natural cytotoxicity triggering receptor 1	Ncr1	17086	9437	1.86	Not significant (<i>ns</i> ; $p=0.02$)
Killer cell lectin-like receptor subfamily C, member 1	Klrc1	16641	3821	1.85	Significant ($p<0.0001$; $p=0.003$)
Killer cell lectin-like receptor subfamily B, member 1B	Klrb1b	80782	3820	1.65	Significant ($p=0.0006$; $p<0.0001$)
Killer cell lectin-like receptor family E, member 1	Klre1	243655		1.85	Not tested

NKG2d	Klrk1	27007	22914	n/a	Significant ($p=0.014$; $p=0.0009$)
Natural killer cells CD94	Klr1d1	16643	3824	n/a	Significant ($p=0.0005$; $p=0.014$)

Table 18:

Tissue	Common Genes	DMF Only	MMF Only
Blood	0	6	2
Cortex	1	2	14
Hippocampus	1	0	6
Striatum	2	5	12
Jejunum	51	9	35
Kidney	222	61	94
Liver	11	2	34
Spleen	6	2	3

Table 19: Blood

Probe Set	Gene Title	Gene	Entrez	DMF 2 hr	DMF 7 hr	DMF 12 hr	MMF 2 hr	MMF 7 hr	MMF 12 hr
1420937_PM_at	cleavage and polyadenylation specific factor 2	Cpsf2		-1.65					
1417898_PM_a_at	granzyme A	Gzma	14938			1.92			
1422089_PM_at	natural cytotoxicity triggering receptor 1	Ncr1	17086			1.86			
1425005_PM_at	killer cell lectin-like receptor subfamily C, member 1	Klrc1	16641			1.85			
1445399_PM_at	killer cell lectin-like receptor subfamily B member 1B	Klrb1b	80782			1.65			

1458642_PM_at	killer cell lectin-like receptor family E member 1	Klre1	243655			1.85				
1418133_PM_at	B-cell leukemia/lymphoma 3	Bcl3					-1.23			
1415943_PM_at	syndecan 1	Sdc1								-1.96
		Common	DMF	MMF						
		0	6	2						

Table 20: Cortex:

Probe Set	Gene Title	Gene	Entrez	DMF 2 hr	DMF 7 hr	DMF 12 hr	MMF 2 hr	MMF 7 hr	MMF 12 hr
1419086_PM_at	fibroblast growth factor binding protein 1	Fgfbp1	14181		1.54			1.62	
1448140_PM_at	cytokine induced apoptosis inhibitor 1	Ciapin1	109006	-1.02					
1423627_PM_at	NAD(P)H dehydrogenase, quinone 1	Nqo1	18104		1.50				
1416734_PM_at	muskelin 1, intracellular mediator containing kelch motifs	Mkln1	27418				-1.85		
1420670_PM_at	aryl hydrocarbon receptor nuclear translocator 2	Arnt2	11864				-1.66		
1421166_PM_at	attractin	Atrn	11990				-1.62		
1421339_PM_at	exostoses (multiple)-like 3	Extl3	54616				-1.57		
1423594_PM_a_at	endothelin receptor type B	Ednrb	13618				-1.53		

1429607_PM_at	trafficking protein, kinesin binding 2	Trak2	70827					-1.57		
1429992_PM_at	spermatogenesis associated glutamate (E)-rich protein 4b	Speer4b	73526					-1.60		
1430827_PM_a_at	PTK2 protein tyrosine kinase 2	Ptk2	14083					-1.53		
1438108_PM_at	pleckstrin homology domain containing, family M, member 3	Plekhn3	241075					-1.50		
1442277_PM_at	choline kinase alpha	Chka	12660					-1.53		
1443123_PM_at	tetratricopeptide repeat, ankyrin repeat and coiled-coil containing 2	Tanc2	77097					-1.72		
1448458_PM_at	topoisomerase (DNA) II beta	Top2b	21974					-1.51		
1456070_PM_at	protein tyrosine phosphatase, receptor type, G	Ptprg	19270					-1.83		
1419435_PM_at	aldehyde oxidase 1	Aox1	11761						1.65	1.54
				Common	DMF	MMF				
				1	2	14				

Table 21: Hippocampus

Probe Set	Gene Title	Gene	Entrez	DMF 2 hr	DMF 7 hr	DMF 12 hr		MMF 2 hr	MMF 7 hr	MMF 12 hr
1458305_PM_at	transmembrane and tetratricopeptide repeat containing 3	Tmtc3	237500		-2.38				-2.40	
1417975_PM_at	karyopherin (importin) alpha 4	Kpna4	16649					-1.51		
1425077_PM_at	DnaJ (Hsp40) homolog, subfamily C, member 18	Dnajc18	76594					-1.58		

1426029_PM_a_at	nuclear factor of activated T-cells 5	Nfat5	54446					-1.51		
1434407_PM_at	SLIT-ROBO Rho GTPase activating protein 2	Srgap2	14270					-1.50		
1438085_PM_at	HEAT repeat containing 5B	Heatr5b	320473					-1.56		
1458421_PM_at	potassium voltage-gated channel, subfamily Q, member 3	Kcnq3	110862					-1.50		

Table 22: Striatum

Probe Set	Gene Title	Gene	Entrez	DMF 2 hr	DM F 7 hr	DM F 12 hr		MM F 2 hr	MM F 7 hr	MM F 12 hr
1419435_PM_at	aldehyde oxidase 1	Aox1	11761		1.64				1.95	
1428320_PM_at	KDM3B lysine (K)-specific demethylase 3B	Kdm3b	277250	-1.54				-1.56		
1434030_PM_at	GTP-binding protein 10 (putative)	Gtpbp10	207704	-1.52						
1456070_PM_at	protein tyrosine phosphatase, receptor type, G	Ptprg	19270	-1.54						
1416734_PM_at	muskelin 1, intracellular mediator containing kelch motifs	Mkln1	27418	-1.97						
1438236_PM_at	nuclear factor I/A	Nfia	18027			-1.53				
1422748_PM_at	zinc finger E-box binding homeobox 2	Zeb2	24136			-1.66				
1427125_PM_s_at	leucine rich repeat containing 41	Lrrc41	230654					-1.54		
1448458_PM_at	topoisomerase (DNA) II beta	Top2b	21974					-1.56		
1449616_PM_s_at	golgi autoantigen, golgin subfamily a, 3	Golga3	269682					-1.63		
1422556_PM_at	guanine nucleotide binding protein, alpha 13	Gna13	14674					-1.63		

1421616_PM_at	glutamate receptor, ionotropic, NMDA2A (epsilon 1)	Grin2a	14811					-1.78		
1428676_PM_at	transmembrane serine protease 6	Timprss 6	71753							1.68
1448807_PM_at	histamine receptor H3	Hrh3	99296							1.59
1437760_PM_at	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 12	Galnt12	230145							1.50
1435272_PM_at	inositol 1,4,5-trisphosphate 3-kinase B	Itpkb	320404							-1.56
1455123_PM_at	suppression of tumorigenicity 18	St18	240690							-1.69
1457257_PM_x_at	poliovirus receptor-related 3	Pvr13	58998							-1.80
1437785_PM_at	a disintegrin-like and metalloproteinase (reprolysin type) with thrombospondin type 1 motif, 9	Adamts 9	101401							-2.58
				Comm on	DM F	MM F				
				2	5	12				

Table 23: Jejunum

Probe Set	Gene Title	Gene	Entrez	DMF 2 hr	DMF 7 hr	DMF 12 hr	MMF 2 hr	MMF 7 hr	MMF 12 hr
1427912_PM_at	carbonyl reductase 3	Cbr3	109857	54.72	22.39	25.95	55.49	40.98	32.24
1448894_PM_at	aldo-keto reductase family 1, member B8	Akr1b8	14187	7.35	8.53	17.57	8.24	14.16	18.78
1449486_PM_at	carboxylesterase 1G	Ces1g	12623	4.06	4.67	7.40	3.68	9.24	9.45
1423436_PM_at	glutathione S-transferase, alpha 3	Gsta3	14859	4.03	4.27	10.36	3.64	5.71	10.80

1419622_PM_at	UDP glucuronosyltransferase 2 family, polypeptide B5	Ugt2b5	22238	3.40	3.42	4.92		3.13	4.50	5.00
1439624_PM_at	UDP glucuronosyltransferase 2 family, polypeptide B35	Ugt2b35	243085	3.22	2.66	2.37		3.08	3.31	2.45
1424266_PM_s_at	carboxylesterase 1F	Ces1f	234564	2.48	3.58	6.17		2.71	5.19	6.54
1455454_PM_at	aldo-keto reductase family 1, member C19	Akr1c19	432720	2.45	2.07	3.20		2.33	2.39	3.53
1427473_PM_at	glutathione S-transferase, mu 3	Gstm3	14864	2.37	2.82	2.03		2.51	3.46	2.14
1416416_PM_x_at	glutathione S-transferase, mu 1	Gstm1	14862	2.30	3.19	4.15		2.38	4.80	3.70
1421816_PM_at	glutathione reductase	Gsr	14782	2.23	2.83	2.20		2.19	3.13	2.67
1423627_PM_at	NAD(P)H dehydrogenase, quinone 1	Nqo1	18104	2.16	2.31	2.30		2.16	2.63	2.34
1441931_PM_x_at	glutathione synthetase	Gss	14854	1.95	1.97	1.53		1.92	2.20	1.55
1435405_PM_at	SET domain containing 4	Setd4	224440	1.91	1.90	1.66		2.11	2.27	1.60
1416368_PM_at	glutathione S-transferase, alpha 4	Gsta4	14860	1.72	2.09	2.58		1.73	2.52	2.66
1421040_PM_a_at	glutathione S-transferase, alpha 2 (Yc2)	Gsta2	14858	3.82	5.81	5.65			9.84	6.66
1455595_PM_at	UDP glucuronosyltransferase 2 family, polypeptide B36	Ugt2b36	231396	2.07	2.23	2.94			2.50	3.03
1419510_PM_at	carboxylesterase 1E	Ces1e	13897	1.85	2.10	2.70			2.44	2.77
1446542_PM_at	acyl-CoA synthetase short-chain family member 2	Acss2	60525	1.68	1.71			1.84	2.06	1.52
1418672_PM_at	aldo-keto reductase family 1, member C13	Akr1c13	27384	1.62	1.61	1.96			1.86	2.01

1422327_PM_s_at	glucose-6-phosphate dehydrogenase 2 /// glucose-6-phosphate dehydrogenase X-linked	G6pd2 /// G6pdx	14380 /// 14381		1.82	1.74		1.74	2.06	1.83
1424296_PM_at	glutamate-cysteine ligase, catalytic subunit	Gclc	14629	4.25	2.06			3.99	2.87	
1460196_PM_at	carbonyl reductase 1	Cbr1	12408	1.55	1.65			1.53	1.83	
1424835_PM_at	glutathione S-transferase, mu 4	Gstm4	14865		3.83	5.03			5.75	5.43
1429001_PM_at	pirin	Pir	69656		3.79	3.79			5.12	4.19
1422072_PM_a_at	glutathione S-transferase, mu 6	Gstm6	14867		1.94	1.95			2.23	2.19
1428988_PM_at	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	Abcc3	76408		1.88	2.02			2.26	2.10
1421041_PM_s_at	predicted gene 3776 /// glutathione S-transferase, alpha 1 (Ya) /// glutathione S-transferase, alpha 2 (Yc2)	Gm3776 /// Gsta1 /// Gsta2	100042295 /// 14857 /// 14858		1.72	2.04			1.90	2.09
1422757_PM_at	solute carrier family 5 (neutral amino acid transporters, system A), member 4b	Slc5a4b	64454		1.67	2.21			1.79	2.16
1437662_PM_at	acyl-CoA synthetase medium-chain family member 5	Acsm5	272428		1.60	1.86			1.71	1.95
1416411_PM_at	glutathione S-transferase, mu 2	Gstm2	14863		1.58	2.04			2.09	2.20
1441604_PM_at	Esterase D/formylglutathione hydrolase	Esd	13885	1.71				1.52	2.15	
1437240_PM_at	phosphoglucomutase 2	Pgm2	72157	2.15				2.09	1.54	
1421134_PM_at	amphiregulin	Areg	11839	3.58				2.71	2.93	

1416552_PM_at	developmental pluripotency associated 5A	Dppa5a	434423	3.07				2.64	1.88	
1418320_PM_at	protease, serine, 8 (prostasin)	Prss8	76560	2.60				2.41	1.75	
1451814_PM_a_a t	HIV-1 tat interactive protein 2, homolog (human)	Htatip2	53415		1.51			1.53	1.68	
1450455_PM_s_at	aldo-keto reductase family 1, member C12 /// aldo-keto reductase family 1, member C13	Akr1c12 /// Akr1c13	27384 /// 622402			1.72			1.58	1.76
1449279_PM_at	glutathione peroxidase 2	Gpx2	14776			1.67			1.53	1.54
1418601_PM_at	aldehyde dehydrogenase family 1, subfamily A7	Aldh1a7	26358			1.65			1.86	1.74
1451386_PM_at	biliverdin reductase B (flavin reductase (NADPH))	Blvrb	233016			1.53		1.57	1.55	1.54
1419431_PM_at	epiregulin	Ereg	13874	2.50				2.00		
1417061_PM_at	solute carrier family 40 (iron-regulated transporter), member 1	Slc40a1	53945	2.33				2.73		
1448916_PM_at	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein G (avian)	Mafg	17134	2.20				1.95		
1424022_PM_at	oxidative stress induced growth inhibitor 1	Osgin1	71839	2.08				1.98		
1419030_PM_at	ERO1-like (S. cerevisiae)	Ero1l	50527	1.92				1.90		
1430135_PM_at	deoxyribonuclease II alpha	Dnase2a	13423	1.77				1.79		
1425351_PM_at	sulfiredoxin 1 homolog (S. cerevisiae)	Srxn1	76650	1.71				1.77		

1436600_PM_at	TOX high mobility group box family member 3	Tox3	244579	1.70				1.81		
1433763_PM_at	ectonucleoside triphosphate diphosphohydrolase 5	Entpd5	12499	1.53					1.60	
1428805_PM_at	solute carrier family 35, member E3	Slc35e3	215436	1.53					1.67	
1426215_PM_at	dopa decarboxylase	Ddc	13195		1.74				2.03	
1424487_PM_x_at	thioredoxin reductase 1	Txnrd1	50493		1.60				2.07	
1448354_PM_at	glucose-6-phosphate dehydrogenase X-linked	G6pdx	14381		1.54				1.76	
1440230_PM_at	tsukushin	Tsku	244152	1.61						
1453421_PM_at	serine racemase	Srr	27364	1.99						
1428834_PM_at	dual specificity phosphatase 4	Dusp4	319520	1.51						
1429950_PM_at	unc-5 homolog C (C. elegans)-like	Unc5cl	76589	-1.53						
1420393_PM_at	nitric oxide synthase 2, inducible	Nos2	18126	-2.39						
1442923_PM_at	PTK6 protein tyrosine kinase 6	Ptk6	20459	-2.42						
1426501_PM_a_at	TRAF-interacting protein with forkhead-associated domain	Tifa	211550	-2.43						
1428397_PM_at	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 5	B3galt5	93961	-2.77						
1456611_PM_at	family with sequence similarity 13, member A	Fam13a	58909	1.54						

1440882_PM_at	low density lipoprotein receptor-related protein 8, apolipoprotein e receptor	Lrp8	16975				2.23		
1448239_PM_at	heme oxygenase (decycling) 1	Hmox1	15368				1.81		
1436605_PM_at	transketolase	Tkt	21881				1.73		
1422645_PM_at	hemochromatosis	Hfe	15216				1.73		
1426600_PM_at	solute carrier family 2 (facilitated glucose transporter), member 1	Slc2a1	20525				1.68		
1438953_PM_at	c-fos induced growth factor	Figf	14205				1.63		
1450410_PM_a_a t	solute carrier family 48 (heme transporter), member 1	Slc48a1	67739				1.62		
1452837_PM_at	lipin 2	Lpin2	64898				1.61		
1424181_PM_at	septin 6	6-Sep	56526				1.56		
1449078_PM_at	ST3 beta-galactoside alpha-2,3-sialyltransferase 6	St3gal6	54613				1.52		
1416418_PM_at	gamma-aminobutyric acid (GABA) A receptor-associated protein-like 1	Gabarapl1	57436				1.52		
1423635_PM_at	bone morphogenetic protein 2	Bmp2	12156				-1.55		
1421886_PM_at	son of sevenless homolog 1 (Drosophila)	Sos1	20662				-1.57		
1452834_PM_at	proline rich 5 like	Prr5l	72446				-1.64		
1433699_PM_at	tumor necrosis factor, alpha-induced protein 3	Tnfaip3	21929				-1.67		
1450165_PM_at	schlafen 2	Slfn2	20556				-1.82		

1416632_PM_at	malic enzyme 1, NADP(+)-dependent, cytosolic	Me1	17436						2.01	
1419072_PM_at	glutathione S- transferase, mu 7	Gstm7	68312						1.97	
1451385_PM_at	family with sequence similarity 162, member A	Fam162a	70186						1.67	
1451095_PM_at	asparagine synthetase	Asns	27053						1.60	
1419442_PM_at	matrilin 2	Matn2	17181						1.59	
1423706_PM_a_a t	phosphogluconate dehydrogenase	Pgd	110208						1.55	
1450109_PM_s_at	ATP-binding cassette, sub-family C (CFTR/MRP), member 2	Abcc2	12780						1.51	
1428960_PM_at	enkurin, TRPC channel interacting protein	Enkur	71233						-1.56	
1418580_PM_at	receptor transporter protein 4	Rtp4	67775						-1.61	
1435529_PM_at	predicted gene 14446	Gm14446	667373						-1.65	
1437960_PM_at	calpain 13	Capn13	381122						-1.75	
1436058_PM_at	radical S-adenosyl methionine domain containing 2	Rsad2	58185						-1.81	
1450783_PM_at	interferon-induced protein with tetratricopeptide repeats 1	Ifit1	15957						-1.95	
1422438_PM_at	epoxide hydrolase 1, microsomal	Ephx1	13849							2.17
1434458_PM_at	folliculin	Fst	14313							1.94
1422000_PM_at	aldo-keto reductase family 1, member C12	Akr1c12	622402							1.52
1417991_PM_at	deiodinase, iodothyronine, type I	Dio1	13370							-1.51
1451642_PM_at	kinesin family member 1B	Kif1b	16561							-1.51

1457554_PM_at	apolipoprotein B	Apob	238055							-1.54
---------------	------------------	------	--------	--	--	--	--	--	--	-------

Table 24: Kidney

Probe Set	Gene Title	Gene	Entrez	DMF 2 hr	DMF 7 hr	DMF 12 hr		MMF 2 hr	MMF 7 hr	MMF 12 hr
1418949_PM_at	growth differentiation factor 15	Gdf15	23886	5.39	1.75	1.77		5.50	1.98	2.01
1427912_PM_at	carbonyl reductase 3	Cbr3	109857	3.04	5.88	5.67		2.96	9.54	7.02
1451095_PM_at	asparagine synthetase	Asns	27053	3.03	1.92	2.65		2.46	2.13	2.49
1419253_PM_at	methylenetetrahydrofolate dehydrogenase (NAD+ dependent), methenyltetrahydrofolate cyclohydrolase	Mthfd2	17768	2.06	1.65	1.60		2.03	1.89	1.65
1423436_PM_at	glutathione S-transferase, alpha 3	Gsta3	14859	2.01	2.14	2.66		1.93	2.95	2.85
1424296_PM_at	glutamate-cysteine ligase, catalytic subunit	Gclc	14629	1.70	1.63	1.60		1.66	1.87	1.64
1419942_PM_at	Sulfiredoxin 1 homolog (S. cerevisiae)	Srxn1	76650	1.62	2.01	1.92		1.57	2.92	2.09
1423706_PM_at	phosphogluconate dehydrogenase	Pgd	110208	1.61	1.91	2.20		1.51	2.21	2.27
1455454_PM_at	aldo-keto reductase family 1, member C19	Akr1c19	432720	1.53	1.64	1.92		1.56	1.97	2.00
1419435_PM_at	aldehyde oxidase 1	Aox1	11761	2.46	1.95			2.31	2.50	2.23
1421529_PM_at	thioredoxin reductase 1	Txnrd1	50493	1.53	2.82	2.31			3.08	1.74
1429813_PM_at	pantothenate kinase 1	Pank1	75735	-1.59	-1.64	-1.53			-1.79	-1.73
1443797_PM_at	alkylglycerone phosphate synthase	Agps	228061	-1.62		-1.55		-1.65	-1.68	-1.80
1441042_PM_at	fibroblast growth factor 1	Fgf1	14164	-2.91	-1.82			-2.48	-1.71	-1.58
1448239_PM_at	heme oxygenase (decycling) 1	Hmox1	15368	6.27	5.61			5.43	7.48	

1451612_PM_at	metallothionein 1	Mt1	17748	2.79	1.66			2.47	1.89	
1418571_PM_at	tumor necrosis factor receptor superfamily, member 12a	Tnfrsf12a	27279	2.37	1.94			2.32	2.40	
1438824_PM_at	solute carrier family 20, member 1	Slc20a1	20515	1.89	1.55			2.14	1.70	
1426645_PM_at	heat shock protein 90, alpha (cytosolic), class A member 1	Hsp90aa1	15519	1.64		1.57		1.53	1.50	
1435311_PM_s_at	synapsin III	Syn3	27204	-1.73	-1.67			-2.07	-1.68	
1445089_PM_at	DNA segment, Chr 16, ERATO Doi 778, expressed	D16Erd778e	52714	-2.60				-2.41	-1.87	-2.25
1430744_PM_at	napsin A aspartic peptidase	Napsa	16541	-4.53	-1.60			-4.07	-1.79	
1429001_PM_at	pirin	Pir	69656		2.35	2.89			2.63	3.08
1426300_PM_at	activated leukocyte cell adhesion molecule	Alcam	11658		2.08	2.05			2.66	2.83
1423186_PM_at	T-cell lymphoma invasion and metastasis 2	Tiam2	24001		1.84	1.56			2.27	1.84
1443870_PM_at	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	Abcc4	239273		1.78	1.81			2.09	2.07
1423627_PM_at	NAD(P)H dehydrogenase, quinone 1	Nqo1	18104		1.71	2.06			1.96	2.11
1430135_PM_at	deoxyribonuclease II alpha	Dnase2a	13423		1.69	2.12			2.20	2.15
1442291_PM_at	lysophosphatidic acid receptor 2	Lpar2	53978		1.66	1.61			1.84	1.93
1421209_PM_s_at	inhibitor of kappaB kinase gamma	Ikbkg	16151		1.64	1.50			1.77	1.57
1448894_PM_at	aldo-keto reductase family 1, member B8	Akr1b8	14187		1.62	1.81			1.86	1.68
1422327_PM_s_at	glucose-6-phosphate dehydrogenase 2 /// glucose-6-phosphate dehydrogenase X-linked	G6pd2 /// G6pdx	14380 /// 14381		1.61	1.77			1.86	1.91
1451386_PM_at	biliverdin reductase B (flavin reductase (NADPH))	Blvrb	233016		1.55	1.94			1.83	2.23

1416368_PM_at	glutathione S-transferase, alpha 4	Gsta4	14860		1.51	1.89			1.85	1.95
1448354_PM_at	glucose-6-phosphate dehydrogenase X-linked	G6pdx	14381		1.50	1.55			1.75	1.70
1430111_PM_at	branched chain aminotransferase 1, cytosolic	Beat1	12035		-1.56	-1.72			-1.59	-2.07
1452733_PM_at	pantothenate kinase 2	Pank2	74450		-1.77	-1.65			-1.94	-1.97
1418358_PM_at	sperm mitochondria-associated cysteine-rich protein	Smcp	17235		-1.84	-1.87			-1.96	-2.49
1436101_PM_at	ring finger protein 24	Rnf24	51902		-1.88	-1.68			-2.03	-2.01
1437199_PM_at	dual specificity phosphatase 5	Dusp5	240672	3.68				4.07	1.74	
1419086_PM_at	fibroblast growth factor binding protein 1	Fgfbp1	14181	1.81				1.94	1.73	
1448566_PM_at	solute carrier family 40 (iron-regulated transporter), member 1	Slc40a1	53945	1.71				1.57	1.51	
1450957_PM_at	sequestosome 1	Sqstm1	18412	1.69				1.67	1.62	
1418627_PM_at	glutamate-cysteine ligase, modifier subunit	Gclm	14630	1.51		1.50			1.64	
1435005_PM_at	centromere protein E	Cenpe	229841	-2.20				-2.08	-1.60	
1428223_PM_at	major facilitator superfamily domain containing 2A	Mfsd2a	76574	-2.59	-3.07				-3.38	
1421398_PM_at	tripartite motif-containing 7	Trim7	94089	-3.34				-2.74	-1.66	
1444138_PM_at	cytochrome P450, family 2, subfamily r, polypeptide 1	Cyp2r1	244209		1.74				1.67	1.58
1415983_PM_at	lymphocyte cytosolic protein 1	Lcp1	18826		1.53				1.59	1.54
1434716_PM_at	hepatitis A virus cellular receptor 1	Havcr1	171283			4.46			3.22	4.79
1440330_PM_at	Histone cluster 1, H2be	Hist1h2be	319179			3.25			1.98	2.92

1418601_PM_at	aldehyde dehydrogenase family 1, subfamily A7	Aldh1a7	26358			2.48			2.16	3.02
1424126_PM_at	aminolevulinic acid synthase 1	Alas1	11655			1.88			1.51	2.02
1449937_PM_at	endonuclease, polyU-specific	Endou	19011			1.75			1.52	1.89
1442145_PM_at	ATPase type 13A3	Atp13a3	224088			1.64			1.52	1.62
1450298_PM_at	tumor necrosis factor (ligand) superfamily, member 14	Tnfsf14	50930			1.62			1.50	1.63
1450702_PM_at	hemochromatosis	Hfe	15216			1.60			1.57	1.56
1448766_PM_at	gap junction protein, beta 1	Gjb1	14618			1.59			1.50	1.81
1435663_PM_at	estrogen receptor 1 (alpha)	Esr1	13982			-1.54			-1.54	-1.75
1452975_PM_at	alanine-glyoxylate aminotransferase 2-like 1	Agxt2l1	71760			-1.65	-1.88		-1.74	
1454865_PM_at	solute carrier family 9 (sodium/hydrogen exchanger), member 8	Slc9a8	77031			-1.98			-1.68	-2.40
1422533_PM_at	cytochrome P450, family 51	Cyp51	13121			-2.05			-1.54	-1.95
1451382_PM_at	ChaC, cation transport regulator-like 1 (E. coli)	Chac1	69065	31.00				30.40		
1449363_PM_at	activating transcription factor 3	Atf3	11910	5.86				6.56		
1424022_PM_at	oxidative stress induced growth inhibitor 1	Osgin1	71839	4.63				3.97		
1417065_PM_at	early growth response 1	Egr1	13653	4.60				5.51		
1416756_PM_at	DnaJ (Hsp40) homolog, subfamily B, member 1	Dnajb1	81489	4.36				3.73		
1429863_PM_at	LON peptidase N-terminal domain and ring finger 3	Lonrf3	74365	4.13				3.58		
1417516_PM_at	DNA-damage inducible transcript 3	Ddit3	13198	3.97				4.07		
1427126_PM_at	heat shock protein 1B	Hspa1b	15511	3.65				3.25		

1434496_PM_at	polo-like kinase 3 (Drosophila)	Plk3	12795	3.14				3.55		
1418936_PM_at	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein F (avian)	Maff	17133	2.83				3.80		
1428694_PM_at	MIR17 host gene 1 (non-protein coding)	Mir17hg	75957	2.82				2.23		
1417930_PM_at	Ngfi-A binding protein 2	Nab2	17937	2.74				2.33		
1425305_PM_at	zinc finger protein 295	Zfp295	114565	2.68				2.46		
1438133_PM_a_at	cysteine rich protein 61	Cyr61	16007	2.64				2.19		
1431182_PM_at	heat shock protein 8 /// hypothetical LOC624853	Hspa8 /// LOC624853	15481 /// 624853	2.63				2.43		
1447930_PM_at	bromodomain adjacent to zinc finger domain 1A /// bromodomain adjacent to zinc finger domain protein 1A-like	Baz1a /// LOC100505185	100505185 /// 217578	2.56				2.24		
1449311_PM_at	BTB and CNC homology 1	Bach1	12013	2.55				2.26		
1428963_PM_at	RWD domain containing 2A	Rwdd2a	69519	2.54				2.10		
1434901_PM_at	zinc finger and BTB domain containing 2	Zbtb2	381990	2.52				2.39		
1433599_PM_at	bromodomain adjacent to zinc finger domain 1A	Baz1a	217578	2.51				2.12		
1451177_PM_at	DnaJ (Hsp40) homolog, subfamily B, member 4	Dnajb4	67035	2.48				2.32		
1421262_PM_at	lipase, endothelial	Lipg	16891	2.42				2.25		
1423566_PM_a_at	heat shock 105kDa/110kDa protein 1	Hsph1	15505	2.40				2.22		
1452388_PM_at	heat shock protein 1A	Hspa1a	193740	2.35				2.41		
1418932_PM_at	nuclear factor, interleukin 3, regulated	Nfil3	18030	2.29				2.29		

1424974_PM_at	zinc finger protein 418	Zfp418	232854	2.24				2.08		
1448135_PM_at	activating transcription factor 4	Atf4	11911	2.12				1.94		
1428834_PM_at	dual specificity phosphatase 4	Dusp4	319520	2.09				1.93		
1420056_PM_s_at	jumonji domain containing 6	Jmjd6	107817	2.08				2.00		
1418334_PM_at	DBF4 homolog (S. cerevisiae)	Dbf4	27214	2.08				2.04		
1439094_PM_at	clathrin, heavy polypeptide (Hc)	Cltc	67300	2.07				1.89		
1437210_PM_a_at	bromodomain containing 2	Brd2	14312	2.07				1.87		
1417406_PM_at	SERTA domain containing 1	Sertad1	55942	2.06				1.92		
1426722_PM_at	solute carrier family 38, member 2	Slc38a2	67760	2.05				1.78		
1418591_PM_at	DnaJ (Hsp40) homolog, subfamily A, member 4	Dnaja4	58233	2.05				1.87		
1418349_PM_at	heparin-binding EGF-like growth factor	Hbegf	15200	2.03				2.27		
1422452_PM_at	BCL2-associated athanogene 3	Bag3	29810	2.03				1.86		
1438725_PM_at	mediator complex subunit 13	Med13	327987	2.02				1.83		
1427375_PM_at	RNA (guanine-9-) methyltransferase domain containing 2	Rg9mt2	108943	1.98				1.67		
1453137_PM_at	F-box protein 30	Fbxo30	71865	1.97				1.94		
1455665_PM_at	LON peptidase N-terminal domain and ring finger 1	Lonrf1	244421	1.96				1.98		
1425185_PM_at	PPPDE peptidase domain containing 1	Pppde1	78825	1.94				1.94		
1456909_PM_at	glucose-6-phosphate isomerase-like	LOC676974	676974	1.94				1.57		
1437884_PM_at	ADP-ribosylation factor-like 5B	Ar15b	75869	1.93				1.69		
1418370_PM_at	troponin C, cardiac/slow skeletal	Tnnc1	21924	1.89				1.94		

1438764_PM_at	annexin A7	Anxa7	11750	1.88				1.76		
1450716_PM_at	a disintegrin-like and metallopeptidase (repolysin type) with thrombospondin type 1 motif, 1	Adamts1	11504	1.86				1.92		
1429350_PM_at	EP300 interacting inhibitor of differentiation 3	Eid3	66341	1.84				1.64		
1431140_PM_at	thioesterase superfamily member 4	Them4	75778	1.80				1.62		
1418966_PM_a_at	discoidin, CUB and LCCL domain containing 1	Dcbld1	66686	1.78				1.76		
1420990_PM_at	chromodomain helicase DNA binding protein 1	Chd1	12648	1.76				1.53		
1416442_PM_at	immediate early response 2	Ier2	15936	1.75				1.86		
1449519_PM_at	growth arrest and DNA-damage-inducible 45 alpha	Gadd45a	13197	1.75				1.76		
1426381_PM_at	peroxisome proliferative activated receptor, gamma, coactivator-related 1	Pprc1	226169	1.74				1.68		
1428997_PM_at	PHD finger protein 23	Phf23	78246	1.72				1.56		
1429056_PM_at	N(alpha)-acetyltransferase 16, NatA auxiliary subunit	Naa16	66897	1.71				1.56		
1440867_PM_at	sprouty homolog 4 (Drosophila)	Spry4	24066	1.71				1.85		
1450724_PM_at	family with sequence similarity 126, member A	Fam126a	84652	1.70				1.55		
1437410_PM_at	aldehyde dehydrogenase 2, mitochondrial	Aldh2	11669	1.69				1.75		
1416288_PM_at	DnaJ (Hsp40) homolog, subfamily A, member 1	Dnaja1	15502	1.69				1.59		
1418640_PM_at	sirtuin 1 (silent mating type information regulation 2, homolog) 1 (S. cerevisiae)	Sirt1	93759	1.68				1.53		
1438842_PM_at	mitochondrial carrier homolog 2 (C. elegans)	Mtch2	56428	1.67				1.51		
1439093_PM_at	heat shock protein 4 like	Hspa4l	18415	1.67				1.60		

1455658_PM_at	CGG triplet repeat binding protein 1	Cggbp1	106143	1.67				1.51		
1433398_PM_at	FYVE, RhoGEF and PH domain containing 3	Fgd3	30938	1.66				1.60		
1460511_PM_at	plakophilin 2	Pkp2	67451	1.66				1.67		
1451463_PM_at	proline rich 5 (renal)	Prr5	109270	1.65				1.86		
1435160_PM_at	AHA1, activator of heat shock protein ATPase homolog 2 (yeast)	Ahsa2	268390	1.64				1.64		
1434967_PM_at	zinc finger, SWIM domain containing 6	Zswim6	67263	1.63				1.52		
1416084_PM_at	zinc finger, AN1-type domain 5	Zfand5	22682	1.63				1.70		
1423481_PM_at	RIO kinase 2 (yeast)	Riok2	67045	1.62				1.58		
1433674_PM_a_at	small nucleolar RNA host gene (non-protein coding) 1	Snhg1	83673	1.62				1.58		
1441604_PM_at	Esterase D/formylglutathione hydrolase	Esd	13885	1.62				1.57		
1422054_PM_a_at	SKI-like	Skil	20482	1.62				1.64		
1416042_PM_s_at	nuclear autoantigenic sperm protein (histone-binding)	Nasp	50927	1.62				1.52		
1452239_PM_at	gene trap ROSA 26, Philippe Soriano	Gt(ROS A)26Sor	14910	1.62				1.51		
1454826_PM_at	zinc finger and BTB domain containing 11	Zbtb11	271377	1.60				1.53		
1438130_PM_at	TAF15 RNA polymerase II, TATA box binding protein (TBP)-associated factor	Taf15	70439	1.59				1.71		
1435632_PM_at	nuclear fragile X mental retardation protein interacting protein 2	Nufip2	68564	1.57				1.52		
1440255_PM_at	histone H4 transcription factor	Hinfp	102423	1.57				1.68		
1455175_PM_at	PHD finger protein 13	Phf13	230936	1.56				1.51		

1416077_PM_at	adrenomedullin	Adm	11535	1.56				1.59		
1415940_PM_at	zinc finger, AN1-type domain 2A	Zfand2a	100494	1.55				1.51		
1423862_PM_at	pleckstrin homology domain containing, family F (with FYVE domain) member 2	Plekhf2	71801	1.54				1.50		
1451083_PM_s_at	alanyl-tRNA synthetase	Aars	234734	1.53				1.53		
1434660_PM_at	alkB, alkylation repair homolog 1 (E. coli)	Alkbh1	211064	1.53				1.60		
1416067_PM_at	interferon-related developmental regulator 1	Ifrd1	15982	1.52				1.53		
1420342_PM_at	ganglioside-induced differentiation-associated-protein 10	Gdap10	14546	1.52				1.63		
1415834_PM_at	dual specificity phosphatase 6	Dusp6	67603	1.52				1.57		
1416600_PM_a_at	regulator of calcineurin 1	Rcan1	54720	1.51				1.51		
1458452_PM_at	Ankyrin repeat domain 11	Ankrd11	77087	1.51				1.56		
1418401_PM_a_at	dual specificity phosphatase 16	Dusp16	70686	1.50				1.50		
1448694_PM_at	Jun oncogene	Jun	16476	1.50				1.58		
1417639_PM_at	solute carrier family 22 (organic cation transporter), member 4	Slc22a4	30805	-1.51				-1.52		
1439680_PM_at	tumor necrosis factor (ligand) superfamily, member 10	Tnfsf10	22035	-1.52				-1.60		
1450184_PM_s_at	thyrotroph embryonic factor	Tef	21685	-1.56				-1.61		
1447818_PM_x_at	Ras homolog enriched in brain like 1	Rheb11	69159	-1.57				-1.74		
1458503_PM_at	B-cell CLL/lymphoma 7A	Bcl7a	77045	-1.59				-1.54		
1442436_PM_at	fructosamine 3 kinase	Fn3k	63828	-1.60				-1.73		
1441988_PM_at	protein phosphatase 1K (PP2C domain containing)	Ppm1k	243382	-1.62				-1.72		

1456922_PM_at	sorting nexin 29	Snx29	74478	-1.64				-1.74		
1452623_PM_at	zinc finger protein 759	Zfp759	268670	-1.65				-1.67		
1434583_PM_at	transmembrane protein 26	Tmem26	327766	-1.66				-1.51		
1450512_PM_at	netrin 4	Ntn4	57764	-1.67				-1.57		
1424988_PM_at	myosin regulatory light chain interacting protein	Mylip	218203	-1.70				-1.67		
1415997_PM_at	thioredoxin interacting protein	Txnip	56338	-1.73				-1.71		
1440765_PM_at	Fraser syndrome 1 homolog (human)	Fras1	231470	-1.73				-1.76		
1418955_PM_at	zinc finger protein 93	Zfp93	22755	-1.74				-1.62		
1421224_PM_a_at	HNF1 homeobox B	Hnf1b	21410	-1.77				-1.59		
1431254_PM_at	kelch repeat and BTB (POZ) domain containing 11	Kbtbd11	74901	-1.77				-1.52		
1433699_PM_at	tumor necrosis factor, alpha-induced protein 3	Tnfaip3	21929	-1.83				-1.74		
1445485_PM_at	DNA segment, Chr 7, ERATO Doi 187, expressed	D7Ert187e	52196	-1.83				-1.70		
1430593_PM_at	coiled-coil domain containing 30	Ccdc30	73332	-2.01				-1.78		
1436974_PM_at	transmembrane protein 88B	Tmem88b	320587	-2.24				-2.68		
1459132_PM_at	basonuclin 2	Bnc2	242509	-2.24				-2.38		
1457770_PM_at	solute carrier family 39 (zinc transporter), member 14	Slc39a14	213053	-2.52				-2.26		
1431740_PM_at	solute carrier family 7, (cationic amino acid transporter, y+ system) member 13	Slc7a13	74087	-2.60				-2.14		
1418918_PM_at	insulin-like growth factor binding protein 1	Igfbp1	16006	-2.89				-2.27		
1449545_PM_at	fibroblast growth factor 18	Fgf18	14172		2.44				3.30	

1428942_PM_at	metallothionein 2	Mt2	17750		2.34				2.71	
1440882_PM_at	low density lipoprotein receptor-related protein 8, apolipoprotein e receptor	Lrp8	16975		2.13				2.19	
1451751_PM_at	DNA-damage-inducible transcript 4-like	Ddit4l	73284		2.11				1.85	
1420696_PM_at	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C	Sema3c	20348		1.62				1.66	
1460632_PM_at	retinol dehydrogenase 10 (all-trans)	Rdh10	98711		1.59				1.68	
1454992_PM_at	solute carrier family 7 (cationic amino acid transporter, y+ system), member 1	Slc7a1	11987		1.57				1.62	
1436791_PM_at	wingless-related MMTV integration site 5A	Wnt5a	22418		1.57				1.69	
1456524_PM_at	neuregulin 1	Nrg1	211323		1.56				1.81	
1453474_PM_at	abhydrolase domain containing 15	Abhd15	67477		1.56				1.64	
1456888_PM_at	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	Pfkfb4	270198		1.55				1.69	
1426887_PM_at	nudix (nucleoside diphosphate linked moiety X)-type motif 11	Nudt11	58242		1.55				1.62	
1434456_PM_at	RUN domain containing 3B	Runde3b	242819		1.55				1.54	
1451041_PM_at	Rho-associated coiled-coil containing protein kinase 2	Rock2	19878		1.54				1.60	
1427320_PM_at	coatamer protein complex, subunit gamma 2, antisense 2	Copg2as 2	1E+08		1.53				1.56	
1451828_PM_a_at	acyl-CoA synthetase long-chain family member 4	Acs14	50790		1.53				1.78	
1436210_PM_at	glycerol kinase 5 (putative)	Gk5	235533		1.51				1.55	

1426399_PM_at	von Willebrand factor A domain containing 1	Vwa1	246228		-1.51				-1.69	
1421609_PM_a_at	camello-like 3	Cml3	93674		-1.56				-1.64	
1425009_PM_at	t-complex-associated testis expressed 2	Tcte2	21646		-1.59				-1.61	
1428427_PM_at	F-box and leucine-rich repeat protein 2	Fbxl2	72179		-1.59				-1.68	
1451548_PM_at	uridine phosphorylase 2	Upp2	76654		-3.28				-4.00	
1418847_PM_at	arginase type II	Arg2	11847			2.39				2.70
1417828_PM_at	aquaporin 8	Aqp8	11833			2.15				3.41
1424638_PM_at	cyclin-dependent kinase inhibitor 1A (P21)	Cdkn1a	12575			2.02				2.73
1450562_PM_at	lymphocyte antigen 6 complex, locus F	Ly6f	17071			1.91				1.93
1423954_PM_at	complement component 3	C3	12266			1.90				2.34
1452934_PM_at	transmembrane channel-like gene family 5	Tmc5	74424			1.88				1.91
1459661_PM_at	doublecortin domain containing 2a	Dcdc2a	195208			1.76				1.69
1458504_PM_at	zinc finger CCCH type containing 12D	Zc3h12d	237256			1.69				1.58
1428988_PM_at	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	Abcc3	76408			1.67				1.64
1444487_PM_at	lecithin-retinol acyltransferase (phosphatidylcholine-retinol-O-acyltransferase)	Lrat	79235			1.67				1.72
1418649_PM_at	EGL nine homolog 3 (C. elegans)	Egln3	112407			1.63				1.71
1426047_PM_a_at	protein tyrosine phosphatase, receptor type, R	Ptprr	19279			1.61				1.69
1416101_PM_a_at	histone cluster 1, H1c	Hist1h1c	50708			1.60				1.82

1440058_PM_at	solute carrier family 22 (organic cation transporter), member 14	Slc22a14	382113			1.59				1.54
1449002_PM_at	pleckstrin homology-like domain, family A, member 3	Phlda3	27280			1.57				1.81
1448330_PM_at	glutathione S-transferase, mu 1	Gstm1	14862			1.54				1.51
1424835_PM_at	glutathione S-transferase, mu 4	Gstm4	14865			1.52				1.70
1418215_PM_at	meprin 1 beta	Mep1b	17288			-1.50				-1.55
1434050_PM_at	vacuolar protein sorting 8 homolog (S. cerevisiae)	Vps8	209018			-1.56				-1.84
1422606_PM_at	C1q and tumor necrosis factor related protein 3	C1qtnf3	81799			-1.67				-1.57
1456722_PM_at	chordin-like 1	Chrdl1	83453			-1.71				-1.55
1437870_PM_at	solute carrier organic anion transporter family, member 4C1	Slco4c1	227394			-1.90				-1.69
1419754_PM_at	myosin VA	Myo5a	17918			-2.68				-3.09
1436521_PM_at	solute carrier family 36 (proton/amino acid symporter), member 2	Slc36a2	246049	4.63						
1422612_PM_at	hexokinase 2	Hk2	15277	2.72						
1438664_PM_at	protein kinase, cAMP dependent regulatory, type II beta	Prkar2b	19088	2.43						
1434893_PM_at	ATPase, Na ⁺ /K ⁺ transporting, alpha 2 polypeptide	Atp1a2	98660	2.25						
1423405_PM_at	tissue inhibitor of metalloproteinase 4	Timp4	110595	1.91						
1453596_PM_at	inhibitor of DNA binding 2	Id2	15902	1.83						
1456041_PM_at	sorting nexin 16	Snx16	74718	1.75						

1436366_PM_at	protein phosphatase 1, regulatory (inhibitor) subunit 15b	Ppp1r15b	108954	1.72						
1448170_PM_at	seven in absentia 2	Siah2	20439	1.71						
1441190_PM_at	actin related protein 2/3 complex, subunit 5-like	Arpc5l	74192	1.70						
1455657_PM_at	SMG1 homolog, phosphatidylinositol 3-kinase-related kinase (C. elegans)	Smg1	233789	1.69						
1418203_PM_at	phorbol-12-myristate-13-acetate-induced protein 1	Pmaip1	58801	1.68						
1433944_PM_at	HECT domain containing 2	Hectd2	226098	1.67						
1423170_PM_at	TAF7 RNA polymerase II, TATA box binding protein (TBP)-associated factor	Taf7	24074	1.66						
1452394_PM_at	cysteinyl-tRNA synthetase	Cars	27267	1.63						
1441546_PM_at	Membrane protein, palmitoylated 6 (MAGUK p55 subfamily member 6)	Mpp6	56524	1.62						
1441955_PM_s_at	polyadenylate binding protein-interacting protein 1	Paip1	218693	1.61						
1438578_PM_a_at	BTB (POZ) domain containing 10	Btbd10	68815	1.60						
1418025_PM_at	basic helix-loop-helix family, member e40	Bhlhe40	20893	1.59						
1459585_PM_at	growth arrest specific 6	Gas6	14456	1.59						
1417479_PM_at	protein phosphatase 2, regulatory subunit B", gamma	Ppp2r3c	59032	1.59						
1455185_PM_s_at	PHD finger protein 16	Phf16	382207	1.58						
1436871_PM_at	serine/arginine-rich splicing factor 7	Srsf7	225027	1.57						

1452094_PM_at	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha 1 polypeptide	P4ha1	18451	1.56						
1443116_PM_at	proteasome (prosome, macropain) activator subunit 4	Psme4	103554	1.56						
1424380_PM_at	vacuolar protein sorting 37B (yeast)	Vps37b	330192	1.56						
1456810_PM_at	vacuolar protein sorting 54 (yeast)	Vps54	245944	1.54						
1452218_PM_at	coiled-coil domain containing 117	Ccdc117	104479	1.54						
1435904_PM_at	eukaryotic translation initiation factor 2C, 3	Eif2c3	214150	1.53						
1442071_PM_at	ATP-binding cassette, sub-family E (OABP), member 1	Abce1	24015	1.53						
1440346_PM_at	KDM1 lysine (K)-specific demethylase 6B	Kdm6b	216850	1.52						
1423549_PM_at	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	Slc1a4	55963	1.52						
1435912_PM_at	UBX domain protein 7	Ubxn7	224111	1.51						
1423605_PM_at	transformed mouse 3T3 cell double minute 2	Mdm2	17246	1.51						
1425656_PM_at	brain-specific angiogenesis inhibitor 1-associated protein 2	Baiap2	108100	1.51						
1425287_PM_at	zinc finger protein 189	Zfp189	230162	1.51						
1455904_PM_at	growth arrest specific 5 /// small nucleolar RNA, C/D box 47	Gas5 /// Snord47	1002174 46 /// 14455	1.51						
1438535_PM_at	pleckstrin homology domain interacting protein	Phip	83946	1.50						
1419140_PM_at	activin receptor IIB	Acvr2b	11481	-1.51						
1428975_PM_at	sushi domain containing 3	Susd3	66329	-1.51						

1437231_PM_at	SLIT and NTRK-like family, member 6	Slitrk6	239250	-1.52						
1452962_PM_at	transmembrane protein 25	Tmem25	71687	-1.52						
1438784_PM_at	B-cell leukemia/lymphoma 11B	Bcl11b	58208	-1.52						
1427369_PM_at	NLR family, pyrin domain containing 6	Nlrp6	101613	-1.52						
1455087_PM_at	DNA segment, Chr 7, ERATO Doi 715, expressed	D7Ert715e	52480	-1.53						
1449813_PM_at	zinc finger protein 30	Zfp30	22693	-1.55						
1451692_PM_at	transmembrane and coiled-coil domains 6	Tmco6	71983	-1.57						
1419051_PM_at	OVO homolog-like 1 (Drosophila)	Ovo11	18426	-1.60						
1424213_PM_at	UbiA prenyltransferase domain containing 1	Ubiad1	71707	-1.61						
1441198_PM_at	zinc finger protein 39	Zfp39	22698	-1.64						
1446303_PM_at	insulin-like growth factor I receptor	Igf1r	16001	-1.66						
1429456_PM_a_at	polymerase (RNA) III (DNA directed) polypeptide E	Polr3e	26939	-1.69						
1457548_PM_at	A disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 6	Adamts6	108154	-1.83						
1449981_PM_a_at	N-acetyltransferase 2 (arylamine N-acetyltransferase)	Nat2	17961	-2.01						
1452975_PM_at	alanine-glyoxylate aminotransferase 2-like 1	Agxt211	71760	-2.10						
1427056_PM_at	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 15	Adamts15	235130	-2.26						
1427372_PM_at	cytochrome P450, family 27, subfamily b, polypeptide 1	Cyp27b1	13115	-3.22						

1427094_PM_at	polymerase (DNA directed), epsilon 2 (p59 subunit)	Pole2	18974		1.75					
1440899_PM_at	flavin containing monooxygenase 5	Fmo5	14263		-1.51					
1460196_PM_at	carbonyl reductase 1	Cbr1	12408			1.80				
1421108_PM_at	camello-like 2	Cml2	93673			1.60				
1421603_PM_a_at	carcinoembryonic antigen-related cell adhesion molecule 2	Ceacam2	26367			-1.68				
1439348_PM_at	S100 calcium binding protein A10 (calpactin)	S100a10	20194				1.89			
1453775_PM_at	RPTOR independent companion of MTOR, complex 2	Rictor	78757				1.66			
1438073_PM_at	sprouty homolog 3 (Drosophila)	Spry3	236576				1.61			
1432108_PM_at	polycomb group ring finger 6	Pcgf6	71041				1.56			
1443049_PM_at	Transmembrane protein 19	Tmem19	67226				1.56			
1456225_PM_x_at	tribbles homolog 3 (Drosophila)	Trib3	228775				1.56			
1442959_PM_at	baculoviral IAP repeat-containing 6	Birc6	12211				1.52			
1429204_PM_at	calcium/calmodulin-dependent protein kinase II inhibitor 2	Camk2n2	73047				1.51			
1423672_PM_at	tetratricopeptide repeat domain 30B	Ttc30b	72421				-1.51			
1426029_PM_a_at	nuclear factor of activated T-cells 5	Nfat5	54446				-1.51			
1429943_PM_at	chitinase, di-N-acetyl-	Ctbs	74245				-1.51			
1443054_PM_at	potassium inwardly-rectifying channel, subfamily J, member 15	Kcnj15	16516				-1.51			
1440779_PM_s_at	solute carrier family 5 (sodium/glucose cotransporter), member 9	Slc5a9	230612				-1.52			

1431726_PM_a_at	transmembrane protein 80	Tmem80	71448					-1.52		
1439300_PM_at	cysteine-rich hydrophobic domain 1	Chic1	12212					-1.53		
1418500_PM_at	nucleosome assembly protein 1-like 3	Nap113	54561					-1.53		
1424508_PM_at	tetratricopeptide repeat domain 5	Ttc5	219022					-1.55		
1458176_PM_at	Period homolog 3 (Drosophila)	Per3	18628					-1.57		
1448494_PM_at	growth arrest specific 1	Gas1	14451					-1.57		
1422138_PM_at	plasminogen activator, urokinase	Plau	18792					-1.58		
1436870_PM_s_at	actin filament associated protein 1-like 2	Afap112	226250					-1.62		
1430834_PM_at	GPRIN family member 3	Gprin3	243385					-1.62		
1416497_PM_at	protein disulfide isomerase associated 4	Pdia4	12304					-1.66		
1456070_PM_at	protein tyrosine phosphatase, receptor type, G	Ptprg	19270					-2.31		
1421816_PM_at	glutathione reductase	Gsr	14782						1.83	
1455902_PM_x_at	Ras homolog gene family, member f	Rhof	23912						1.66	
1429262_PM_at	Ras association (RalGDS/AF-6) domain family member 6	Rassf6	73246						1.65	
1442051_PM_at	histone cluster 2, H3c1	Hist2h3c1	15077						1.65	
1423723_PM_s_at	TAR DNA binding protein	Tardbp	230908						1.62	
1442018_PM_at	B-cell translocation gene 1, anti-proliferative	Btg1	12226						1.60	
1416563_PM_at	cytidine 5'-triphosphate synthase	Ctps	51797						1.57	
1434976_PM_x_at	eukaryotic translation initiation factor 4E binding protein 1	Eif4ebp1	13685						1.56	

1417884_PM_at	solute carrier family 16 (monocarboxylic acid transporters), member 6	Slc16a6	104681						1.55	
1457707_PM_at	multiple C2 domains, transmembrane 2	Mctp2	244049						1.54	
1442116_PM_at	G protein-coupled receptor 176	Gpr176	381413						1.53	
1434775_PM_at	par-3 (partitioning defective 3) homolog (C. elegans)	Pard3	93742						1.53	
1427347_PM_s_at	tubulin, beta 2A	Tubb2a	22151						1.52	
1423413_PM_at	N-myc downstream regulated gene 1	Ndrp1	17988						1.52	
1426275_PM_a_at	UDP-glucuronate decarboxylase 1	Uxs1	67883						1.50	
1448700_PM_at	G0/G1 switch gene 2	G0s2	14373						1.50	
1449220_PM_at	GTPase, IMAF family member 3	Gimap3	83408						-1.51	
1425778_PM_at	indoleamine 2,3-dioxygenase 2	Ido2	209176						-1.52	
1417932_PM_at	interleukin 18	Il18	16173						-1.52	
1439866_PM_at	cullin 9	Cul9	78309						-1.52	
1430530_PM_s_at	NmrA-like family domain containing 1	NmrA1	67824						-1.52	
1434990_PM_at	protein phosphatase 1E (PP2C domain containing)	Ppm1e	320472						-1.53	
1426230_PM_at	sphingosine kinase 2	Sphk2	56632						-1.54	
1428758_PM_at	transmembrane protein 86A	Tmem86a	67893						-1.56	
1417700_PM_at	RAB38, member of RAS oncogene family	Rab38	72433						-1.57	
1426767_PM_at	WD repeat domain 90	Wdr90	106618						-1.58	
1435994_PM_at	potassium voltage-gated channel, subfamily H (eag-related), member 1	Kcnh1	16510						-1.61	
1457038_PM_at	Fras1 related extracellular matrix	Frem2	242022						-1.66	

	protein 2									
1418174_PM_at	D site albumin promoter binding protein	Dbp	13170						-1.73	
1435918_PM_at	family with sequence similarity 107, member A	Fam107a	268709						-1.77	
1416468_PM_at	aldehyde dehydrogenase family 1, subfamily A1	Aldh1a1	11668							2.21
1418706_PM_at	solute carrier family 38, member 3	Slc38a3	76257							1.81
1424279_PM_at	fibrinogen alpha chain	Fga	14161							1.79
1423596_PM_at	NIMA (never in mitosis gene a)-related expressed kinase 6	Nek6	59126							1.69
1443964_PM_at	transmembrane inner ear	Tmie	20776							1.65
1444242_PM_at	Solute carrier organic anion transporter family, member 2a1	Slco2a1	24059							1.63
1421040_PM_a_at	glutathione S-transferase, alpha 2 (Yc2)	Gsta2	14858							1.61
1435121_PM_at	deiodinase, iodothyronine type III, opposite strand	Dio3os	353504							1.60
1449065_PM_at	acyl-CoA thioesterase 1	Acot1	26897							1.59
1417150_PM_at	solute carrier family 6 (neurotransmitter transporter, serotonin), member 4	Slc6a4	15567							1.58
1451607_PM_at	kallikrein 1-related peptidase b21	Klk1b21	16616							1.57
1437932_PM_a_at	claudin 1	Cldn1	12737							1.57
1449575_PM_a_at	glutathione S-transferase, pi 1	Gstp1	14870							1.54
1440965_PM_at	phosphatidylinositol glycan anchor biosynthesis, class L	Pigl	327942							1.52
1430128_PM_a_at	receptor accessory protein 6	Reep6	70335							1.51

1422997_PM_s_at	acyl-CoA thioesterase 1 /// acyl-CoA thioesterase 2	Acot1 /// Acot2	171210 /// 26897							1.51
1418746_PM_at	paroxysmal nonkinesigenic dyskinesia	Pnkd	56695							1.51
1420654_PM_a_at	glucan (1,4-alpha-), branching enzyme 1	Gbe1	74185							1.51
1422966_PM_a_at	transferrin receptor	Tfrc	22042							1.51
1426502_PM_s_at	glutamic pyruvic transaminase, soluble	Gpt	76282							1.51
1436291_PM_a_at	dihydropyrimidinase	Dpys	64705							1.51
1460629_PM_at	tripartite motif-containing 16	Trim16	94092							1.51
1421756_PM_a_at	G protein-coupled receptor 19	Gpr19	14760							1.50
1431172_PM_at	origin recognition complex, subunit 4	Orc4	26428							1.50
1440227_PM_at	solute carrier family 5 (inositol transporters), member 3	Slc5a3	53881							-1.50
1433617_PM_s_at	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 5	B4galt5	56336							-1.51
1428012_PM_at	complement component 8, alpha polypeptide	C8a	230558							-1.51
1428343_PM_at	REST corepressor 3	Rcor3	214742							-1.51
1427224_PM_at	acyl-CoA synthetase medium-chain family member 2	Acsn2	233799							-1.52
1448482_PM_at	solute carrier family 39 (metal ion transporter), member 8	Slc39a8	67547							-1.55
1440688_PM_at	Rho GTPase activating protein 26	Arhgap26	71302							-1.57
1452333_PM_at	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2	Smarca2	67155							-1.60

1421167_PM_at	ATPase, class VI, type 11A	Atp11a	50770							-1.62
1449610_PM_at	E1A binding protein p400	Ep400	75560							-1.67
1446742_PM_at	Nuclear factor I/A	Nfia	18027							-1.74
1437675_PM_at	solute carrier family 8 (sodium/calcium exchanger), member 1	Slc8a1	20541							-1.86
1437473_PM_at	avian musculoaponeurotic fibrosarcoma (v-maf) AS42 oncogene homolog	Maf	17132					-1.56		1.53
1441944_PM_s_at	G protein-coupled receptor 135	Gpr135	238252						1.66	1.59
1460196_PM_at	carbonyl reductase 1	Cbr1	12408						1.58	2.10
1450410_PM_a_at	solute carrier family 48 (heme transporter), member 1	Slc48a1	67739						1.58	1.53
1439695_PM_a_at	kinesin family member 20B	Kif20b	240641						-2.61	-2.85

Table 25: Liver

Probe Set	Gene Title	Gene		DMF 2 hr	DMF 7 hr	DMF 12 hr	MMF 2 hr	MMF 7 hr	MMF 12 hr
1448894_PM_at	aldo-keto reductase family 1, member B8	Akr1b8	14187	2.18	1.58		2.27	1.75	
1419627_PM_s_at	C-type lectin domain family 4, member n	Clec4n	56620	2.34	2.01			2.40	
1419942_PM_at	Sulfiredoxin 1 homolog (S. cerevisiae)	Srxn1	76650	1.69			1.68	1.52	
1417801_PM_a_at	PTPRF interacting protein, binding protein 2 (liprin beta 2)	Ppfibp2	19024	2.38			2.55		
1448239_PM_at	heme oxygenase (decycling) 1	Hmox1	15368	2.37			2.41		
1420804_PM_s_at	C-type lectin domain family 4, member d	Clec4d	17474	2.30			2.05		

1438953_PM_at	c-fos induced growth factor	Figf	14205	2.23			2.25		
1452233_PM_at	ATP-binding cassette, sub-family C (CFTR/MRP), member 1	Abcc1	17250	1.75			1.86		
1436771_PM_x_at	phosphogluconate dehydrogenase	Pgd	110208	1.59			1.61		
1423627_PM_at	NAD(P)H dehydrogenase, quinone 1	Nqo1	18104		1.81			2.20	
1450759_PM_at	bone morphogenetic protein 6	Bmp6	12161		1.57			1.65	
1435495_PM_at	adenosine A1 receptor	Adora1	11539		1.50				
1424835_PM_at	glutathione S-transferase, mu 4	Gstm4	14865			1.12			
1424296_PM_at	glutamate-cysteine ligase, catalytic subunit	Gclc	14629				1.72		
1422573_PM_at	adenosine monophosphate deaminase 3	Ampd3	11717				1.70		
1455016_PM_at	PRP38 pre-mRNA processing factor 38 (yeast) domain containing B	Prpf38b	66921				-1.53		
1452247_PM_at	fragile X mental retardation gene 1, autosomal homolog	Fxr1	14359				-1.53		
1447019_PM_at	cytidine monophospho-N-acetylneuraminic acid hydroxylase	Cmah	12763				-1.55		
1448348_PM_at	cell cycle associated protein 1	Caprin1	53872				-1.56		
1419038_PM_a_at	casein kinase 2, alpha 1 polypeptide	Csnk2a1	12995				-1.62		
1440091_PM_at	Meis homeobox 2	Meis2	17536				-1.67		
1416734_PM_at	muskelin 1, intracellular mediator containing kelch motifs	Mkln1	27418				-1.69		

1453908_PM_at	protein tyrosine phosphatase, receptor type, B	Ptprb	19263					-1.76		
1456070_PM_at	protein tyrosine phosphatase, receptor type, G	Ptprg	19270					-1.85		
1449498_PM_at	macrophage receptor with collagenous structure	Marco	17167						2.44	
1427357_PM_at	cytidine deaminase	Cda	72269						1.92	
1448354_PM_at	glucose-6-phosphate dehydrogenase X-linked	G6pdx	14381						1.61	
1435040_PM_at	interleukin-1 receptor-associated kinase 3	Irak3	73914						1.53	
1429001_PM_at	pirin	Pir	69656						1.50	
1445815_PM_at	frizzled homolog 8 (Drosophila)	Fzd8	14370						-1.81	
1434582_PM_at	ELKS/RAB6-interacting/CAST family member 2	Erc2	238988						-1.95	
1419669_PM_at	proteinase 3	Prtn3	19152							4.46
1431214_PM_at	predicted gene 3579	Gm3579	1E+08							2.74
1417441_PM_at	DnaJ (Hsp40) homolog, subfamily C, member 12	Dnajc12	30045							2.53
1428154_PM_s_at	phosphatidic acid phosphatase type 2 domain containing 1B	Ppapdc1b	71910							1.87
1444176_PM_at	ATPase, H ⁺ transporting, lysosomal V0 subunit D2	Atp6v0d2	242341							1.84
1423290_PM_at	hypoxia up-regulated 1	Hyou1	12282							1.82
1433833_PM_at	fibronectin type III domain containing 3B	Fndc3b	72007							1.66
1416235_PM_at	leucine rich repeat containing 59	Lrrc59	98238							1.65

1448471_PM_a_at	cytotoxic T lymphocyte-associated protein 2 alpha	Ctla2a	13024						1.63
1448574_PM_at	non-metastatic cells 6, protein expressed in (nucleoside-diphosphate kinase)	Nme6	54369						1.57
1443807_PM_x_at	cyclin F	Ccnf	12449						1.57
1450971_PM_at	growth arrest and DNA-damage-inducible 45 beta	Gadd45b	17873						1.50
1453103_PM_at	actin-binding LIM protein 1	Ablim1	226251						-1.51
1439117_PM_at	calmin	Clmn	94040						-1.54
1441988_PM_at	protein phosphatase 1K (PP2C domain containing)	Ppm1k	243382						-1.56
1460256_PM_at	carbonic anhydrase 3	Car3	12350						-1.57

Table 26: Spleen

Probe Set	Gene Title	Gene	Entrez	DMF 2 hr	DMF 7 hr	DMF 12 hr	MMF 2 hr	MMF 7 hr	MMF 12 hr
1419942_PM_at	Sulfiredoxin 1 homolog (S. cerevisiae)	Srxn1	76650	2.16	1.67		2.02	1.87	
1419435_PM_at	aldehyde oxidase 1	Aox1	11761	2.61			2.35		
1434797_PM_at	kin of IRRE like (Drosophila)	Kirrel	170643	-1.54			-1.65		
1424022_PM_at	oxidative stress induced growth inhibitor 1	Osgin1	71839	1.78			1.83		
1416497_PM_at	protein disulfide isomerase associated 4	Pdia4	12304	-1.60			-1.59		
1424486_PM_a_at	thioredoxin reductase 1	Txnrd1	50493	2.41			2.14		
1424296_PM_at	glutamate-cysteine ligase, catalytic subunit	Gclc	14629	1.76					

1448916_PM_at	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein G (avian)	Mafg	17134	1.58						
1434951_PM_at	armadillo repeat containing 8	Armc8	74125					-1.54		
1425521_PM_at	polyadenylate binding protein-interacting protein 1	Paip1	218693					-1.56		
1424084_PM_at	ROD1 regulator of differentiation 1 (S. pombe)	Rod1	230257					-1.54		

Probes and Methods for Detection

Probes and Methods for Detection of Translation Products

Probe-based methods, include, but are not limited to: Western blot, immunoblot, enzyme-linked immunosorbant assay (ELISA), radioimmunoassay (RIA), immunoprecipitation, surface plasmon resonance, chemiluminescence, fluorescent polarization, phosphorescence, immunohistochemical analysis, liquid chromatography mass spectrometry (LC-MS), matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, microcytometry, microarray, microscopy, fluorescence activated cell sorting (FACS), flow cytometry, laser scanning cytometry, hematology analyzer and assays based on a property of the protein including but not limited to DNA binding, ligand binding, or interaction with other protein partners.

The translation product or polypeptide can be detected and quantified by any of a number of means well known to those of skill in the art. These can include analytic biochemical methods such as electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), hyperdiffusion chromatography, and the like, or various immunological methods such as fluid or gel precipitin reactions, immunodiffusion (single or double), immunoelectrophoresis, radioimmunoassay (RIA), enzyme-linked immunosorbent assays (ELISAs), immunofluorescent assays, Western blotting, immunohistochemistry and the like. A skilled artisan can readily adapt known protein/antibody detection methods for use in determining the expression level of one or more biomarkers in a serum sample.

A useful probe for detecting a polypeptide is an antibody capable of binding to the polypeptide, *e.g.*, an antibody with a detectable label. Antibodies can be polyclonal or monoclonal. An intact antibody, or a fragment thereof (*e.g.*, Fab or F(ab')₂) can be used. The term "labeled", with regard to the probe or antibody, is intended to encompass direct labeling of the probe or antibody by coupling (*i.e.*, physically linking) a detectable substance to the probe or antibody, as well as indirect labeling of the probe or antibody by reactivity with another reagent that is directly labeled. Examples of indirect labeling include detection of a primary antibody using a fluorescently labeled secondary antibody and end-labeling of a DNA probe with biotin such that it can be detected with fluorescently labeled streptavidin.

An antibody probe can be labeled, *e.g.*, a radio-labeled, chromophore-labeled, fluorophore-labeled, or enzyme-labeled antibody. In another embodiment, an antibody derivative (*e.g.*, an antibody conjugated with a substrate or with the protein or ligand of a protein-ligand pair {*e.g.*, biotin-streptavidin}), or an antibody fragment (*e.g.*, a single-chain antibody, an isolated antibody hypervariable domain, *etc.*) which binds specifically with a protein corresponding to the marker, such as the protein encoded by the open reading frame corresponding to the marker or such a protein which has undergone all or a portion of its normal post-translational modification, is used.

Immunohistochemistry or IHC refers to the process of localizing antigens (*e.g.* proteins), *e.g.*, in cells of a tissue section or other sample, exploiting the principle of antibodies binding specifically to antigens in biological tissues. Specific molecular markers are characteristic of particular cellular events such as proliferation or cell death (apoptosis). Visualizing an antibody-antigen interaction can be accomplished in a number of ways. In the most common instance, an antibody is conjugated to an enzyme, such as peroxidase, that can catalyze a color-producing reaction. Alternatively, the antibody can also be tagged to a fluorophore, such as fluorescein, rhodamine, DyLight Fluor or Alexa Fluor.

Proteins from cells can be isolated using techniques that are well known to those of skill in the art. The protein isolation methods employed can, for example, be such as those described in Harlow and Lane (Harlow and Lane, 1988, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York).

In one format, antibodies, or antibody fragments, can be used as probes in methods such as Western blots or immunofluorescence techniques to detect the expressed proteins. In such

uses, one can immobilize either the antibody or proteins on a solid support. Suitable solid phase supports or carriers include any support capable of binding an antigen or an antibody. Well-known supports or carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, gabbros, and magnetite.

One skilled in the art will know other suitable carriers for binding antibody or antigen, and will be able to adapt such support for use with the present invention. For example, protein isolated from cells can be run on a polyacrylamide gel electrophoresis and immobilized onto a solid phase support such as nitrocellulose. The support can then be washed with suitable buffers followed by treatment with the detectably labeled antibody. The solid phase support can then be washed with the buffer a second time to remove unbound antibody. The amount of bound label on the solid support can then be detected by conventional means. Means of detecting proteins using electrophoretic techniques are well known to those of skill in the art (see generally, R. Scopes (1982) *Protein Purification*, Springer-Verlag, N.Y.; Deutscher, (1990) *Methods in Enzymology* Vol. 182: *Guide to Protein Purification*, Academic Press, Inc., N.Y.).

In another embodiment, Western blot (immunoblot) analysis is used to detect and quantify the presence of a polypeptide in the sample. This technique generally comprises separating sample proteins by gel electrophoresis on the basis of molecular weight, transferring the separated proteins to a suitable solid support, (such as a nitrocellulose filter, a nylon filter, or derivatized nylon filter), and incubating the sample with the antibodies that specifically bind a polypeptide. The anti-polypeptide antibodies specifically bind to the polypeptide on the solid support. These antibodies can be directly labeled or alternatively can be subsequently detected using labeled antibodies (*e.g.*, labeled sheep anti-human antibodies) that specifically bind to the anti-polypeptide.

In another embodiment, the polypeptide is detected using an immunoassay. As used herein, an immunoassay is an assay that utilizes an antibody to specifically bind to the analyte. The immunoassay is thus characterized by detection of specific binding of a polypeptide to an anti-antibody as opposed to the use of other physical or chemical properties to isolate, target, and quantify the analyte.

The polypeptide is detected and/or quantified using any of a number of well recognized immunological binding assays (see, *e.g.*, U.S. Patent Nos. 4,366,241; 4,376,110; 4,517,288; and 4,837,168). For a review of the general immunoassays, see also Asai (1993) *Methods in Cell*

Biology Volume 37: *Antibodies in Cell Biology*, Academic Press, Inc. New York; Stites & Terr (1991) *Basic and Clinical Immunology* 7th Edition.

In another embodiment, the polypeptide is detected and/or quantified using Luminex™ assay technology. The Luminex™ assay separates tiny color-coded beads into *e.g.*, distinct sets that are each coated with a reagent for a particular bioassay, allowing the capture and detection of specific analytes from a sample in a multiplex manner. The Luminex™ assay technology can be compared to a multiplex ELISA assay using bead-based fluorescence cytometry to detect analytes such as biomarkers.

Immunological binding assays (or immunoassays) typically utilize a "capture agent" to specifically bind to and often immobilize the analyte (polypeptide or subsequence). The capture agent is a moiety that specifically binds to the analyte. In another embodiment, the capture agent is an antibody that specifically binds a polypeptide. The antibody (anti-peptide) can be produced by any of a number of means well known to those of skill in the art.

Immunoassays also often utilize a labeling agent to specifically bind to and label the binding complex formed by the capture agent and the analyte. The labeling agent can itself be one of the moieties comprising the antibody/analyte complex. Thus, the labeling agent can be a labeled polypeptide or a labeled anti-antibody. Alternatively, the labeling agent can be a third moiety, such as another antibody, that specifically binds to the antibody/polypeptide complex.

In one embodiment, the labeling agent is a second human antibody bearing a label. Alternatively, the second antibody can lack a label, but it can, in turn, be bound by a labeled third antibody specific to antibodies of the species from which the second antibody is derived. The second can be modified with a detectable moiety, *e.g.*, as biotin, to which a third labeled molecule can specifically bind, such as enzyme-labeled streptavidin.

Other proteins capable of specifically binding immunoglobulin constant regions, such as protein A or protein G can also be used as the label agent. These proteins are normal constituents of the cell walls of streptococcal bacteria. They exhibit a strong non-immunogenic reactivity with immunoglobulin constant regions from a variety of species (see, generally Kronval, *et al.* (1973) *J. Immunol.*, 111: 1401-1406, and Akerstrom (1985) *J. Immunol.*, 135: 2589-2542).

As indicated above, immunoassays for the detection and/or quantification of a polypeptide can take a wide variety of formats well known to those of skill in the art.

Exemplary immunoassays for detecting a polypeptide can be competitive or noncompetitive. Noncompetitive immunoassays are assays in which the amount of captured analyte is directly measured. In one "sandwich" assay, for example, the capture agent (anti-peptide antibodies) can be bound directly to a solid substrate where they are immobilized. These immobilized antibodies then capture polypeptide present in the test sample. The polypeptide thus immobilized is then bound by a labeling agent, such as a second human antibody bearing a label.

In competitive assays, the amount of analyte (polypeptide) present in the sample is measured indirectly by measuring the amount of an added (exogenous) analyte (polypeptide) displaced (or competed away) from a capture agent (anti-peptide antibody) by the analyte present in the sample. In one competitive assay, a known amount of, in this case, a polypeptide is added to the sample and the sample is then contacted with a capture agent. The amount of polypeptide bound to the antibody is inversely proportional to the concentration of polypeptide present in the sample.

In another embodiment, the antibody is immobilized on a solid substrate. The amount of polypeptide bound to the antibody can be determined either by measuring the amount of polypeptide present in a polypeptide/antibody complex, or alternatively by measuring the amount of remaining uncomplexed polypeptide. The amount of polypeptide can be detected by providing a labeled polypeptide.

The assays described herein are scored (as positive or negative or quantity of polypeptide) according to standard methods well known to those of skill in the art. The particular method of scoring will depend on the assay format and choice of label. For example, a Western Blot assay can be scored by visualizing the colored product produced by the enzymatic label. A clearly visible colored band or spot at the correct molecular weight is scored as a positive result, while the absence of a clearly visible spot or band is scored as a negative. The intensity of the band or spot can provide a quantitative measure of polypeptide.

In another embodiment, level (activity) is assayed by measuring the enzymatic activity of the gene product. Methods of assaying the activity of an enzyme are well known to those of skill in the art.

In vivo techniques for detection of a marker protein include introducing into a subject a labeled antibody directed against the protein. For example, the antibody can be labeled with a radioactive marker whose presence and location in a subject can be detected by standard imaging techniques.

Certain markers identified by the methods of the invention can be secreted proteins. It is a simple matter for the skilled artisan to determine whether any particular marker protein is a secreted protein. In order to make this determination, the marker protein is expressed in, for example, a mammalian cell, *e.g.*, a human cell line, extracellular fluid is collected, and the presence or absence of the protein in the extracellular fluid is assessed (*e.g.*, using a labeled antibody which binds specifically with the protein).

Antibodies can be used as probes for translation products. The terms "antibody" and "antibody substance" as used interchangeably herein refer to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain an antigen binding site which specifically binds an antigen, such as a polypeptide of the invention. A molecule which specifically binds to a given polypeptide is a molecule which binds the polypeptide, but does not substantially bind other molecules in a sample, *e.g.*, a biological sample, which naturally contains the polypeptide. Examples of immunologically active portions of immunoglobulin molecules include F(ab) and F(ab')₂ fragments which can be generated by treating the antibody with an enzyme such as pepsin. Probes can be polyclonal or monoclonal antibodies. The term "monoclonal antibody" or "monoclonal antibody composition", as used herein, refers to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope.

An antibody directed against a polypeptide can be used to isolate the polypeptide by standard techniques, such as affinity chromatography or immunoprecipitation. Moreover, such an antibody can be used to detect the marker (*e.g.*, in a cellular lysate or cell supernatant) in order to evaluate the level and pattern of expression of the marker. The antibodies can also be used diagnostically to monitor protein levels in tissues or body fluids (*e.g.*, in a tumor cell-containing body fluid) as part of a clinical testing procedure, *e.g.*, to, for example, determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling the antibody to a detectable substance. Examples of detectable substances include, but are not limited to, various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent

materials, and radioactive materials. Examples of suitable enzymes include, but are not limited to, horseradish peroxidase, alkaline phosphatase, β -galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include, but are not limited to, streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include, but are not limited to, umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes, but is not limited to, luminol; examples of bioluminescent materials include, but are not limited to, luciferase, luciferin, and aequorin, and examples of suitable radioactive materials include, but are not limited to, ^{125}I , ^{131}I , ^{35}S or ^3H .

Probes and Methods for Detection of Transcription Products

Translational expression can be assessed by any of a wide variety of well known methods for detecting expression. Non-limiting examples of such methods include nucleic acid hybridization methods, nucleic acid reverse transcription methods, and nucleic acid amplification methods.

In certain embodiments, activity of a particular gene is characterized by a measure of gene transcript (*e.g.*, mRNA). Detection can involve quantification of the level of gene expression (*e.g.*, cDNA, mRNA), or, alternatively, can be a qualitative assessment of the level of gene expression, in particular in comparison with a control level. The type of level being detected will be clear from the context.

Methods of detecting and/or quantifying the gene transcript (mRNA or cDNA made therefrom) using nucleic acid hybridization techniques are known to those of skill in the art (see *e.g.*, Sambrook *et al. supra*). For example, one method for evaluating the presence, absence, or quantity of cDNA involves a Southern transfer as described above. Briefly, the mRNA is isolated (*e.g.*, using an acid guanidinium-phenol-chloroform extraction method, Sambrook *et al. supra*.) and reverse transcribed to produce cDNA. The cDNA is then optionally digested and run on a gel in buffer and transferred to membranes. Hybridization is then carried out using the nucleic acid probes specific for the target cDNA.

A general principle of such diagnostic and prognostic assays involves preparing a sample or reaction mixture that can contain a marker, and a probe, under appropriate conditions and for a time sufficient to allow the marker and probe to interact and bind, thus forming a complex that

can be removed and/or detected in the reaction mixture. These assays can be conducted in a variety of ways.

For example, one method to conduct such an assay would involve anchoring the marker or probe onto a solid phase support, also referred to as a substrate, and detecting target marker/probe complexes anchored on the solid phase at the end of the reaction. In one embodiment of such a method, a sample from a subject, which is to be assayed for presence and/or concentration of marker, can be anchored onto a carrier or solid phase support. In another embodiment, the reverse situation is possible, in which the probe can be anchored to a solid phase and a sample from a subject can be allowed to react as an unanchored component of the assay.

There are many established methods for anchoring assay components to a solid phase. These include, without limitation, marker or probe molecules which are immobilized through conjugation of biotin and streptavidin. Such biotinylated assay components can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques known in the art (*e.g.*, biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). In certain embodiments, the surfaces with immobilized assay components can be prepared in advance and stored.

Other suitable carriers or solid phase supports for such assays include any material capable of binding the class of molecule to which the marker or probe belongs. Well-known supports or carriers include, but are not limited to, glass, polystyrene, nylon, polypropylene, polyethylene, dextran, amyloses, natural and modified celluloses, polyacrylamides, gabbros, and magnetite.

In order to conduct assays with the above-mentioned approaches, the non-immobilized component is added to the solid phase upon which the second component is anchored. After the reaction is complete, uncomplexed components can be removed (*e.g.*, by washing) under conditions such that any complexes formed will remain immobilized upon the solid phase. The detection of marker/probe complexes anchored to the solid phase can be accomplished in a number of methods outlined herein.

In another embodiment, the probe, when it is the unanchored assay component, can be labeled for the purpose of detection and readout of the assay, either directly or indirectly, with detectable labels discussed herein and which are well-known to one skilled in the art.

It is also possible to directly detect marker/probe complex formation without further manipulation or labeling of either component (marker or probe), for example by utilizing the technique of fluorescence energy transfer (see, for example, Lakowicz *et al.*, U.S. Patent No. 5,631,169; Stavrianopoulos, *et al.*, U.S. Patent No. 4,868,103). A fluorophore label on the first, 'donor' molecule is selected such that, upon excitation with incident light of appropriate wavelength, its emitted fluorescent energy will be absorbed by a fluorescent label on a second 'acceptor' molecule, which in turn is able to fluoresce due to the absorbed energy. Alternately, the 'donor' protein molecule can simply utilize the natural fluorescent energy of tryptophan residues. Labels are chosen that emit different wavelengths of light, such that the 'acceptor' molecule label can be differentiated from that of the 'donor'. Since the efficiency of energy transfer between the labels is related to the distance separating the molecules, spatial relationships between the molecules can be assessed. In a situation in which binding occurs between the molecules, the fluorescent emission of the 'acceptor' molecule label in the assay should be maximal. An FET binding event can be conveniently measured through standard fluorometric detection means well known in the art (*e.g.*, using a fluorimeter).

In another embodiment, determination of the ability of a probe to recognize a marker can be accomplished without labeling either assay component (probe or marker) by utilizing a technology such as real-time Biomolecular Interaction Analysis (BIA) (see, *e.g.*, Sjolander, S. and Urbaniczky, C., 1991, *Anal. Chem.* 63:2338-2345 and Szabo *et al.*, 1995, *Curr. Opin. Struct. Biol.* 5:699-705). As used herein, "BIA" or "surface plasmon resonance" is a technology for studying biospecific interactions in real time, without labeling any of the interactants (*e.g.*, BIAcore). Changes in the mass at the binding surface (indicative of a binding event) result in alterations of the refractive index of light near the surface (the optical phenomenon of surface plasmon resonance (SPR)), resulting in a detectable signal which can be used as an indication of real-time reactions between biological molecules.

Alternatively, in another embodiment, analogous diagnostic and prognostic assays can be conducted with marker and probe as solutes in a liquid phase. In such an assay, the complexed marker and probe are separated from uncomplexed components by any of a number of standard techniques, including but not limited to: differential centrifugation, chromatography, electrophoresis and immunoprecipitation. In differential centrifugation, marker/probe complexes can be separated from uncomplexed assay components through a series of centrifugal steps, due

to the different sedimentation equilibria of complexes based on their different sizes and densities (see, for example, Rivas, G., and Minton, A.P., 1993, *Trends Biochem Sci.* 18(8):284-7). Standard chromatographic techniques can also be utilized to separate complexed molecules from uncomplexed ones. For example, gel filtration chromatography separates molecules based on size, and through the utilization of an appropriate gel filtration resin in a column format, for example, the relatively larger complex can be separated from the relatively smaller uncomplexed components. Similarly, the relatively different charge properties of the marker/probe complex as compared to the uncomplexed components can be exploited to differentiate the complex from uncomplexed components, for example, through the utilization of ion-exchange chromatography resins. Such resins and chromatographic techniques are well known to one skilled in the art (see, *e.g.*, Heegaard, N.H., 1998, *J. Mol. Recognit.* Winter 11(1-6):141-8; Hage, D.S., and Tweed, S.A. *J Chromatogr B Biomed Sci Appl* 1997 Oct 10;699(1-2):499-525). Gel electrophoresis can also be employed to separate complexed assay components from unbound components (see, *e.g.*, Ausubel *et al.*, ed., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, 1987-1999). In this technique, protein or nucleic acid complexes are separated based on size or charge, for example. In order to maintain the binding interaction during the electrophoretic process, non-denaturing gel matrix materials and conditions in the absence of reducing agent are typical. Appropriate conditions to the particular assay and components thereof will be well known to one skilled in the art.

In a particular embodiment, the level of mRNA corresponding to the marker can be determined both by *in situ* and by *in vitro* formats in a biological sample using methods known in the art. The term "biological sample" is intended to include tissues, cells, biological fluids and isolates thereof, isolated from a subject, as well as tissues, cells and fluids present within a subject. Many expression detection methods use isolated RNA. For *in vitro* methods, any RNA isolation technique that does not select against the isolation of mRNA can be utilized for the purification of RNA from cells (see, *e.g.*, Ausubel *et al.*, ed., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York 1987-1999). Additionally, large numbers of tissue samples can readily be processed using techniques well known to those of skill in the art, such as, for example, the single-step RNA isolation process of Chomczynski (1989, U.S. Patent No. 4,843,155).

The isolated nucleic acid can be used in hybridization or amplification assays that include, but are not limited to, Southern or Northern analyses, polymerase chain reaction analyses and probe arrays. One diagnostic method for the detection of mRNA levels involves contacting the isolated mRNA with a nucleic acid molecule (probe) that can hybridize to the mRNA encoded by the gene being detected. The nucleic acid probe can be, for example, a full-length cDNA, or a portion thereof, such as an oligonucleotide of at least 7, 15, 30, 50, 100, 250 or 500 nucleotides in length and sufficient to specifically hybridize under stringent conditions to a mRNA or genomic DNA encoding a marker of the present invention. Other suitable probes for use in the diagnostic assays of the invention are described herein. Hybridization of an mRNA with the probe indicates that the marker in question is being expressed.

In one format, the mRNA is immobilized on a solid surface and contacted with a probe, for example by running the isolated mRNA on an agarose gel and transferring the mRNA from the gel to a membrane, such as nitrocellulose. In an alternative format, the probe(s) are immobilized on a solid surface and the mRNA is contacted with the probe(s), for example, in an Affymetrix gene chip array. A skilled artisan can readily adapt known mRNA detection methods for use in detecting the level of mRNA encoded by the markers of the present invention.

The probes can be full length or less than the full length of the nucleic acid sequence encoding the protein. Shorter probes are empirically tested for specificity. Exemplary nucleic acid probes are 20 bases or longer in length (See, *e.g.*, Sambrook *et al.* for methods of selecting nucleic acid probe sequences for use in nucleic acid hybridization). Visualization of the hybridized portions allows the qualitative determination of the presence or absence of cDNA.

An alternative method for determining the level of a transcript involves the process of nucleic acid amplification, *e.g.*, by rtPCR (the experimental embodiment set forth in Mullis, 1987, U.S. Patent No. 4,683,202), ligase chain reaction (Barany, 1991, *Proc. Natl. Acad. Sci. USA*, 88:189-193), self sustained sequence replication (Guatelli *et al.*, 1990, *Proc. Natl. Acad. Sci. USA* 87:1874-1878), transcriptional amplification system (Kwoh *et al.*, 1989, *Proc. Natl. Acad. Sci. USA* 86:1173-1177), Q-Beta Replicase (Lizardi *et al.*, 1988, *Bio/Technology* 6:1197), rolling circle replication (Lizardi *et al.*, U.S. Patent No. 5,854,033) or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well known to those of skill in the art. Fluorogenic rtPCR can also be used in the methods of the invention. In fluorogenic rtPCR, quantitation is based on amount of fluorescence signals, *e.g.*,

TaqMan and sybr green. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers. As used herein, amplification primers are defined as being a pair of nucleic acid molecules that can anneal to 5' or 3' regions of a gene (plus and minus strands, respectively, or *vice-versa*) and contain a short region in between. In general, amplification primers are from about 10 to 30 nucleotides in length and flank a region from about 50 to 200 nucleotides in length. Under appropriate conditions and with appropriate reagents, such primers permit the amplification of a nucleic acid molecule comprising the nucleotide sequence flanked by the primers.

For *in situ* methods, mRNA does not need to be isolated from the cells prior to detection. In such methods, a cell or tissue sample is prepared/processed using known histological methods. The sample is then immobilized on a support, typically a glass slide, and then contacted with a probe that can hybridize to mRNA that encodes the marker.

As an alternative to making determinations based on the absolute expression level of the marker, determinations can be based on the normalized expression level of the marker. Expression levels are normalized by correcting the absolute expression level of a marker by comparing its expression to the expression of a gene that is not a marker, *e.g.*, a housekeeping gene that is constitutively expressed. Suitable genes for normalization include housekeeping genes such as the actin gene, or epithelial cell-specific genes. This normalization allows the comparison of the expression level in one sample, *e.g.*, a subject sample, to another sample, *e.g.*, a healthy subject, or between samples from different sources.

Alternatively, the expression level can be provided as a relative expression level. To determine a relative expression level of a marker, the level of expression of the marker is determined for 10 or more samples of normal versus MS isolates, or even 50 or more samples, prior to the determination of the expression level for the sample in question. The mean expression level of each of the genes assayed in the larger number of samples is determined and this is used as a baseline expression level for the marker. The expression level of the marker determined for the test sample (absolute level of expression) is then divided by the mean expression value obtained for that marker. This provides a relative expression level.

In certain embodiments, the samples used in the baseline determination will be from samples derived from a subject having multiple sclerosis versus samples from a healthy subject of the same tissue type. The choice of the cell source is dependent on the use of the relative

expression level. Using expression found in normal tissues as a mean expression score aids in validating whether the marker assayed is specific to the tissue from which the cell was derived (*versus* normal cells). In addition, as more data is accumulated, the mean expression value can be revised, providing improved relative expression values based on accumulated data. Expression data from normal cells provides a means for grading the severity of the multiple sclerosis disease state.

In another embodiment, expression of a marker is assessed by preparing mRNA/cDNA (*i.e.*, a transcribed polynucleotide) from cells in a subject sample, and by hybridizing the genomic DNA or mRNA/cDNA with a reference polynucleotide which is a complement of a polynucleotide comprising the marker, and fragments thereof. cDNA can, optionally, be amplified using any of a variety of polymerase chain reaction methods prior to hybridization with the reference polynucleotide. Expression of one or more markers can likewise be detected using quantitative PCR (QPCR) to assess the level of expression of the marker(s). Alternatively, any of the many known methods of detecting mutations or variants (*e.g.*, single nucleotide polymorphisms, deletions, *etc.*) of a marker of the invention can be used to detect occurrence of a mutated marker in a subject.

In a related embodiment, a mixture of transcribed polynucleotides obtained from the sample is contacted with a substrate having fixed thereto a polynucleotide complementary to or homologous with at least a portion (*e.g.*, at least 7, at least 10, at least 15, at least 20, at least 25, at least 30, at least 40, at least 50, at least 100, at least 500, or more nucleotide residues) of a marker of the invention. If polynucleotides complementary to or homologous with a marker of the invention are differentially detectable on the substrate (*e.g.*, detectable using different chromophores or fluorophores, or fixed to different selected positions), then the levels of expression of a plurality of markers can be assessed simultaneously using a single substrate (*e.g.*, a "gene chip" microarray of polynucleotides fixed at selected positions). When a method of assessing marker expression is used which involves hybridization of one nucleic acid with another, the hybridization can be performed under stringent hybridization conditions.

In another embodiment, a combination of methods to assess the expression of a marker is utilized.

Because the compositions, kits, and methods of the invention rely on detection of a difference in expression levels of one or more markers of the invention, in certain embodiments

the level of expression of the marker is significantly greater than the minimum detection limit of the method used to assess expression in at least one of a biological sample from a subject with MS or a healthy control.

A nucleic acid molecule of the invention can be amplified using cDNA, mRNA, or genomic DNA as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. The nucleic acid molecules so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. Furthermore, oligonucleotides corresponding to all or a portion of a nucleic acid molecule of the invention can be prepared by standard synthetic techniques, *e.g.*, using an automated DNA synthesizer.

Probes based on the sequence of a nucleic acid molecule of the invention can be used to detect transcripts (*e.g.*, mRNA) or genomic sequences corresponding to one or more markers of the invention. The probe comprises a label group attached thereto, *e.g.*, a radioisotope, a fluorescent compound, an enzyme, or an enzyme co-factor. Such probes can be used as part of a diagnostic test kit for identifying cells or tissues which mis-express the protein, such as by measuring levels of a nucleic acid molecule encoding the protein in a sample of cells from a subject, *e.g.*, detecting mRNA levels or determining whether a gene encoding the protein has been mutated or deleted.

The methods described herein can also include molecular beacon nucleic acid molecules having at least one region which is complementary to a nucleic acid molecule of the invention, such that the molecular beacon is useful for quantitating the presence of the nucleic acid molecule of the invention in a sample. A "molecular beacon" nucleic acid is a nucleic acid molecule comprising a pair of complementary regions and having a fluorophore and a fluorescent quencher associated therewith. The fluorophore and quencher are associated with different portions of the nucleic acid in such an orientation that when the complementary regions are annealed with one another, fluorescence of the fluorophore is quenched by the quencher. When the complementary regions of the nucleic acid molecules are not annealed with one another, fluorescence of the fluorophore is quenched to a lesser degree. Molecular beacon nucleic acid molecules are described, for example, in U.S. Patent 5,876,930.

Kits

A kit is any manufacture (*e.g.*, a package or container) comprising at least one reagent, *e.g.*, a probe, *e.g.*, a nucleic acid probe or an antibody, for specifically detecting a translation or transcription product described herein.

The invention also encompasses kits having probes for detecting the presence of a polypeptide or nucleic acid in a biological sample, *e.g.*, a sample containing tissue, whole blood, serum, plasma, buccal scrape, saliva, cerebrospinal fluid, urine, stool, and bone marrow. For example, the kit can comprise a labeled compound or agent capable of detecting a polypeptide or an mRNA encoding a polypeptide in a biological sample and means for determining the amount of the polypeptide or mRNA in the sample (*e.g.*, an antibody which binds the polypeptide or an oligonucleotide probe which binds to DNA or mRNA encoding the polypeptide). Kits can also include instructions for interpreting the results obtained using the kit.

A kit can include a plurality of probes for detecting a plurality of translation or transcription products. If a plurality of expression products are to be analysed the kit can comprise a probe for each.

The kit can comprise one or more probes capable of identifying one or more of gene products described herein, *e.g.*, gene products identified herein (*e.g.*, the markers set forth in Table 9). Suitable probes for a polypeptide include antibodies, antibody derivatives, antibody fragments, and the like. Suitable probes for a transcription product include a nucleic acid include complementary nucleic acids. For example, a kit can include oligonucleotides (labeled or non-labeled) fixed to a substrate, labeled oligonucleotides not bound with a substrate, pairs of PCR primers, molecular beacon probes, and the like.

The kit of the invention can optionally comprise additional components useful for performing the methods of the invention. By way of example, the kit can comprise fluids (*e.g.*, SSC buffer) suitable for annealing complementary nucleic acids or for binding an antibody with a protein with which it specifically binds, one or more sample compartments, an instructional material which describes performance of a method of the invention, a reference sample for comparison of expression levels of the biomarkers described herein, and the like.

A kit can include a device described herein.

For antibody-based kits, the kit can comprise, for example: (1) a first antibody (*e.g.*, attached to a solid support) which binds to a polypeptide corresponding to a marker of the

invention; and, optionally, (2) a second, different antibody which binds to either the polypeptide or the first antibody and is conjugated to a detectable label.

For oligonucleotide-based kits, the kit can comprise, for example: (1) an oligonucleotide, *e.g.*, a detectably labeled oligonucleotide, which hybridizes to a nucleic acid sequence encoding a polypeptide corresponding to a marker of the invention or (2) a pair of primers useful for amplifying a nucleic acid molecule corresponding to a marker of the invention. The kit can also comprise, *e.g.*, a buffering agent, a preservative, or a protein stabilizing agent. The kit can further comprise components necessary for detecting the detectable label (*e.g.*, an enzyme or a substrate). The kit can also contain a control sample or a series of control samples which can be assayed and compared to the test sample. Each component of the kit can be enclosed within an individual container and all of the various containers can be within a single package, along with instructions for interpreting the results of the assays performed using the kit.

regions.

MS Therapeutic Agents, Compositions and Administration

There are several medications presently used to modify the course of multiple sclerosis. Such agents include, but are not limited to, dialkyl fumarates (*e.g.*, DMF or others of Formula A herein), Beta interferons (*e.g.*, Avonex®, Rebif®, Betaseron®, Betaferon®, among others), glatiramer (Copaxone®), natalizumab (Tysabri®), and mitoxantrone (Novantrone®).

"Treat," "treatment," and other forms of this word refer to the administration of an IFN agent, alone or in combination with one or more symptom management agents, to a subject, *e.g.*, an MS patient, to impede progression of multiple sclerosis, to induce remission, to extend the expected survival time of the subject and or reduce the need for medical interventions (*e.g.*, hospitalizations). In those subjects, treatment can include, but is not limited to, inhibiting or reducing one or more symptoms such as numbness, tingling, muscle weakness; reducing relapse rate, reducing size or number of sclerotic lesions; inhibiting or retarding the development of new lesions; prolonging survival, or prolonging progression-free survival, and/or enhanced quality of life.

As used herein, unless otherwise specified, the terms "prevent," "preventing" and "prevention" contemplate an action that occurs before a subject begins to suffer from the a multiple sclerosis relapse and/or which inhibits or reduces the severity of the disease.

As used herein, and unless otherwise specified, the terms "manage," "managing" and "management" encompass preventing the progression of MS symptoms in a patient who has already suffered from the disease, and/or lengthening the time that a patient who has suffered from MS remains in remission. The terms encompass modulating the threshold, development and/or duration of MS, or changing the way that a patient responds to the disease.

As used herein, and unless otherwise specified, a "therapeutically effective amount" of a compound is an amount sufficient to provide a therapeutic benefit in the treatment or management of multiple sclerosis, or to delay or minimize one or more symptoms associated with MS. A therapeutically effective amount of a compound means an amount of therapeutic agent, alone or in combination with other therapeutic agents, which provides a therapeutic benefit in the treatment or management of MS. The term "therapeutically effective amount" can encompass an amount that improves overall therapy, reduces or avoids symptoms or causes of the disease, or enhances the therapeutic efficacy of another therapeutic agent.

As used herein, and unless otherwise specified, a "prophylactically effective amount" of a compound is an amount sufficient to prevent relapse of MS, or one or more symptoms associated with the disease, or prevent its recurrence. A prophylactically effective amount of a compound means an amount of the compound, alone or in combination with other therapeutic agents, which provides a prophylactic benefit in the prevention of MS relapse. The term "prophylactically effective amount" can encompass an amount that improves overall prophylaxis or enhances the prophylactic efficacy of another prophylactic agent.

As used herein, the term "patient" or "subject" refers to an animal, typically a human (*i.e.*, a male or female of any age group, *e.g.*, a pediatric patient (*e.g.*, infant, child, adolescent) or adult patient (*e.g.*, young adult, middle-aged adult or senior adult) or other mammal, such as a primate (*e.g.*, cynomolgus monkey, rhesus monkey); commercially relevant mammals such as cattle, pigs, horses, sheep, goats, cats, and/or dogs; and/or birds, including commercially relevant birds such as chickens, ducks, geese, and/or turkeys, that will be or has been the object of treatment, observation, and/or experiment. When the term is used in conjunction with administration of a compound or drug, then the patient has been the object of treatment, observation, and/or administration of the compound or drug.

The methods described herein permit one of skill in the art to identify a monotherapy that an MS patient is most likely to respond to, thus eliminating the need for administration of

multiple therapies to the patient to ensure that a therapeutic effect is observed. However, in one embodiment, combination treatment of an individual with MS is contemplated.

It will be appreciated that the MS therapies, as described above and herein, can be administered in combination with one or more additional therapies to treat and/or reduce the symptoms of MS described herein, particularly to treat patients with moderate to severe disability (*e.g.*, EDSS score of 5.5 or higher). The pharmaceutical compositions can be administered concurrently with, prior to, or subsequent to, one or more other additional therapies or therapeutic agents. In general, each agent will be administered at a dose and/or on a time schedule determined for that agent. It will further be appreciated that the additional therapeutic agent utilized in this combination can be administered together in a single composition or administered separately in different compositions. The particular combination to employ in a regimen will take into account compatibility of the pharmaceutical composition with the additional therapeutically active agent and/or the desired therapeutic effect to be achieved. In general, it is expected that additional therapeutic agents utilized in combination be utilized at levels that do not exceed the levels at which they are utilized individually. In some embodiments, the levels utilized in combination will be lower than those utilized individually.

Alternative or Other Therapies

In other embodiments, alternative therapies to the DMF can be administered.

In one embodiment, the alternative therapy includes an interferon beta, a polymer of four amino acids found in myelin basic protein, *e.g.*, a polymer of glutamic acid, lysine, alanine and tyrosine (*e.g.*, glatiramer (Copaxone®)). In other embodiments, the alternative therapy includes an antibody or fragment thereof against alpha-4 integrin (*e.g.*, natalizumab (Tysabri®)). In yet other embodiments, the alternative therapy includes an anthracenedione molecule (*e.g.*, mitoxantrone (Novantrone®)). In yet another embodiment, the alternative therapy includes a fingolimod (*e.g.*, FTY720; Gilenya®). In other embodiments, the alternative therapy is an antibody to the alpha subunit of the IL-2 receptor of T cells (*e.g.*, Daclizumab; described in, *e.g.*, Rose, J.W. et al. (2007) *Neurology* 69 (8): 785–789). In yet other embodiments, the alternative therapy is an antibody against CD52 (*e.g.*, alemtuzumab (Lemtrada®)). In yet another

embodiment, the alternative therapy includes an anti-LINGO-1 antibody (described in, *e.g.*, US 8,058,406, entitled “Composition comprising antibodies to LINGO or fragments thereof.”).

Steroids, *e.g.*, corticosteroid, and ACTH agents can be used to treat acute relapses in relapsing-remitting MS or secondary progressive MS. Such agents include, but are not limited to, Depo-Medrol®, Solu-Medrol®, Deltasone®, Delta-Cortef®, Medrol®, Decadron®, and Acthar®.

Treatment of Other Disorders

Dialkyl fumarates, *e.g.*, those of Formula A, can be used to treat NK function related disorders and conditions. While not wishing to be bound by theory it is believed that these disorders are ameliorated by NK cells. Such disorders include: cancer, *e.g.*, hematopoietic malignancies, *e.g.*, acute lymphocytic leukemia, chronic lymphocytic leukemia, and lymphoma; solid tumors, *e.g.*, gastrointestinal sarcoma, neuroblastoma, and kidney cancer; viral infection; autoimmune disorders; and inflammation. Such conditions also include transplantation, *e.g.*, solid organ transplantation, and GVHD.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, figures, sequence listing, patents and published patent applications cited throughout this application are hereby incorporated by reference.

EXAMPLES

Example 1: Transcriptional Profiling of Pharmacodynamic Effects of DMF and MMF

Tecfidera (BG-12, dimethyl fumarate, DMF) is an oral therapeutic approved in the U.S. for relapsing multiple sclerosis (MS). The mechanism by which Tecfidera exerts clinical effects is unknown, but preclinical studies indicate activation of the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathway is involved. In vivo, DMF is rapidly metabolized to monomethyl fumarate (MMF), and both compounds are pharmacologically active. In vitro, DMF and MMF share some common effects, but also have divergent pharmacological properties. To understand if *in vitro* differences translate into differential *in vivo* biology, DMF and MMF pharmacodynamic responses were characterized and compared in mice. This example describes the discovery and evaluation of differential transcriptional responses in multiple tissues and whole blood after oral dosing of DMF or MMF.

C57BL/6 mice were dosed with vehicle, DMF or MMF (100 mg/kg) and sacrificed at 2, 7, and 12 hours. Tissues (liver, spleen, kidney, jejunum, cortex, hippocampus, striatum and whole blood) were collected and analyzed by transcriptional profiling on mouse Affymetrix GeneChips. Differentially expressed genes were identified by comparing DMF or MMF treated mice to matched vehicle controls.

A separate cohort was sacrificed 30 minutes after dosing to evaluate MMF exposure. More specifically, satellite 5 animals per group were dosed orally with DMF or MMF (100 mg/kg) and sacrificed 30 minutes post-dosing. MMF exposures were determined and compared in various compartments. These analyses demonstrate that in mice receiving DMF or MMF, no significant differences in MMF exposure was observed, and this was consistent across tissues. As shown in **FIGURE 1**, MMF levels were highly comparable between DMF and MMF dosing, suggesting any subsequent differences in pharmacodynamic responses were not simply due to different exposures.

A specific transcriptional response to DMF and MMF treatment was observed in all tissues and whole blood. The overall number and identity of differentially regulated transcripts varied between tissues and treatment (**FIGURE 2**). In most tissues, a similar trend was observed comparing genes regulated by DMF to those by MMF; a common set of transcripts induced by both treatments was identified, with a strong association with Nrf2 pathway activation. Additionally, treatment-specific transcripts were also identified. For example, differentially expressed genes (DEGs) from liver, spleen, kidney, jejunum, cortex, hippocampus, striatum and whole blood are shown in **TABLES 1-8**, above. Furthermore, DEGs identified from these tissues were analyzed to identify various pathways and cell types that may be involved in DMF and MMF pharmacological activity (**FIGURES 3-9**). Transcriptional differences were observed, for example, in NK cell markers identified in blood (**FIGURE 3**), including Granzyme A (Gzma), natural cytotoxicity triggering receptor 1 (Ncr1), killer cell lectin-like receptor subfamily C, member 1 (Klrc1), killer cell lectin-like receptor subfamily B, member 1B (Klrb1b), and killer cell lectin-like receptor family E, member 1 (Klre1) (**TABLE 1**). These data demonstrate that several DMF differentially expressed genes identified in the transcriptional analysis effect NK cell function, and may indicate a link to macrophage signaling.

The incomplete overlap of transcriptional signatures induced by DMF and MMF indicates that not all DMF pharmacodynamic effects are conveyed through MMF, as may have been predicted due to the rapid in vivo metabolism of DMF to MMF. This suggests DMF may directly drive unique pharmacology not captured by MMF alone. Characterizing the potential biological consequences of these responses, such as effects on NK cells, and how they may contribute to the therapeutic benefit derived from oral administration of DMF, will be further investigated.

Example 2: Evaluation of NK Cell Surface Markers in Naïve Mice Receiving DMF or MMF

Transcriptional differences in NK cell markers were confirmed at the protein level by flow cytometry. **FIGURE 10** depicts an exemplary flow cytometry gating strategy for comparative analysis of DMF and MMF on Natural Killer (NK) cell phenotype. Protein

expression was quantified by mean fluorescent intensity (MFI). A comparative analysis of DMF and MMF on NK cell phenotype was performed using the following markers: NK1.1 (klrb1b), Nkg2d (klrk1), NKp46 (ncr1), Nkg2a (klrc1), and CD94 (klrd1). Naïve C57Bl/6 mice were dosed PO with 100 mpk dimethyl fumarate (DMF) or molar equivalency of monomethyl fumarate (MMF). 12-hours post-dose, mice were sacrificed and blood, spleen, and inguinal lymph node (iLN) were collected for analysis by flow cytometry. **FIGURE 11** shows protein expression by MFI on total splenic NK cells (top) and on CD94+NKG2a+ splenic NK cells (bottom). **TABLE 9** summarizes protein expression level differences between DMF 12 hour timepoint as compared to MMF 12 hour time point or vehicle-treated controls. DMF differentially expressed proteins as determined by MFI included Klrc1, Klrb1b, Klrk1 and Klrd1.

Among other things, these data confirm and expand the findings described in Example 1. For example, flow cytometry analysis confirmed transcriptional data identifying a number of DMF specific transcriptional changes related to NK cell function in blood. Furthermore, the data demonstrate that DMF exerts effects on NK cells in the spleen that were not observed with MMF.

Example 3: Evaluation of EAE Mice Receiving DMF or MMF

As shown in Example 1, a transcriptional comparison of single-dose MMF vs DMF in naïve mice revealed an NK cell “signature” in DMF-treated naïve mice. Example 2 describes FACS analysis which confirmed and extended the findings of an NK cell signature. The present example describes immunophenotyping analysis of immune cell subsets in Experimental Autoimmune Encephalomyelitis (EAE) mice treated with a single dose or chronic administration of DMF or MMF.

EAE induction is generally performed by immunization with brain extracts, CNS proteins (such as myelin basic protein), or peptides from such protein emulsified in an adjuvant such as complete Freund's adjuvant., *e.g.*, as described in Linker et al., Brain. 2011 Mar 134(Pt3): 678-92. Vehicle, MMF or DMF was administered to EAE mice by a chronic or single dose administration, as described below. Immune cells were obtained from various mouse tissues and analyzed by flow cytometry. **FIGURE 12** depicts exemplary immunophenotyping panels used

to analyze various immune cell populations (*e.g.*, T cells, T regulatory cells, NK cells, B cells, myeloid cells).

In the first study (chronic dose study), EAE mice were chronically dosed with vehicle, MMF, or DMF beginning at day 4 post-immunization. Mice were sacrificed on day 17 post-immunization at 12-hours after receiving the last dose. **FIGURES 13-15** depict exemplary NK cell analysis in blood and spleen and EAE clinical score analysis for the chronic dosing experiment in EAE mice.

In the second study (single dose study), EAE mice were treated with a single dose of vehicle, MMF, or DMF on day 17 post-immunization. Animals were sacrificed 12 hours after receiving the dose. **FIGURES 16-18** depict exemplary NK cell and NK subpopulation analysis in spleen, iLN, and blood for the single dose experiment in EAE mice.

Additional immune cell types were analyzed, including T cells, B cells, and myeloid cells. In particular, T cells from EAE mice treated with vehicle, MMF, or DMF were analyzed by flow cytometry. **FIGURE 19** depicts an exemplary flow cytometry gating strategy for comparative analysis of DMF and MMF on T cell phenotype. Protein expression was quantified by mean fluorescent intensity (MFI). **FIGURES 20-24** depict exemplary T cell and T cell subpopulation analysis in spleen, iLN and blood and EAE clinical score analyses for a chronic dosing experiment in EAE mice.

B cells from EAE mice treated with vehicle, MMF, or DMF were also analyzed by flow cytometry. **FIGURE 25** depicts exemplary B cell analysis in naïve, vehicle, MMF or DMF treated EAE mice.

Myeloid cells from EAE mice treated with vehicle, MMF, or DMF were also analyzed by flow cytometry. **FIGURE 26** depicts an exemplary myeloid cell gating strategy for comparative analysis of DMF and MMF on myeloid cell phenotype. (Swirski, F. K. et al. Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science* 325, 612–616 (2009)). **FIGURE 27** depicts exemplary myeloid cell subset analysis in spleen and iLN for a chronic dosing experiment in EAE mice.

Example 4: In Vivo Evaluation of OSGIN1 Regulation via DMF

This example describes the regulation of OSGIN1 gene expression following DMF treatment *in vivo*.

Methods

Time course in wild type mice: Wild type C57BL/6 mice were dosed with a single suspension of 100 mg/kg DMF in 0.8% HPMC or vehicle solution alone. 100 mg/kg was selected based on previous animal studies with DMF. Drug was delivered by oral gavage at 10 μ L/gram and dosed at 5 minute intervals to ensure proper timing and reduction of RNA variability following tissue collection. Tissues were harvested 2, 4, 6, 8, 12.5, 16.5, 23 or 37.5 hours after dosing with each time point including a vehicle control group and DMF group with an n of 6 animals. Animals were exposed to CO₂ and whole blood collected via cardiac puncture. Central Nervous System (CNS) brain (cerebellum, hippocampus, striatum and cortex) tissues were harvested and snap frozen in liquid nitrogen with special care taken to collect tissues of similar size and from the same location. All samples were stored at -80°C until RNA extraction was conducted.

Tissue RNA extraction: For RNA preparation, frozen tissues were placed in 2 mL RNase-free 96-well blocks with 1.5mL QIAzol Lysis Reagent (QIAGEN) and a 3.2 mm stainless steel bead (BioSpec Products, Bartlesville, OK). Tissues were disrupted for four cycles of 45 seconds in a Mini-Beadbeater (BioSpec Products). RNA was extracted in chloroform and the aqueous phase was mixed with an equal volume of 70% ethanol. Extracted RNA was applied to RNeasy 96 plates and purified by the spin method according to the manufacturer's protocol (RNeasy 96 Universal Tissue Protocol, QIAGEN, Hilden, Germany).

Real-Time PCR: RNA was analyzed for purity and integrity by capillary electrophoresis using the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). Total RNA (125 ng) was DNase-treated for 15 min at room temperature and EDTA heat inactivated at 65°C for 10 min using DNase I Amp Grade from Invitrogen. Samples were then reverse transcribed using the High Capacity cDNA Reverse Transcription Kit according to the manufacturer's protocol (Applied Biosystems).

High-throughput qPCR was conducted to study gene expression in 5 ng cDNA using 96.96 Dynamic Arrays for the microfluidic BioMark™ System (Fluidigm Corporation, CA, USA). A panel of 48 genes measured in duplicate, were selected based on previous DMF gene profiling studies conducted *in vivo* and *in vitro*, as well as published experimental and computational literature of identified Nrf2 regulated genes (**FIGURE 35**). Real time PCR probe sets were designed using Primer Express 3.0 (Applied Biosystems) with TaqMan MGB Quantification default settings. A 20X Assay stock of the primer probe set of each gene to be investigated was made (900nM for primers and 200nM for Probe in 1X TE Buffer [10 mM Tris pH8 (Ambion), 1 mM EDTA pH8 (Sigma)]) and then diluted to a 0.2X assay pool. 2 µl of 0.2X assay pool was combined with 2ul of the reverse-transcribed total RNA and 4ul of 2x TaqMan® PreAmp Master Mix (Applied Biosystems) for a final reaction volume of 8ul. Pre-amplification of cDNA was performed in the Tetrad thermocycler [at 95°C for a 10 min activation step followed by 10 cycles: 95°C, (15 s), 60°C, (4 min)]. Following pre-amplification, 32 µl 0.1X TE Buffer was added to each sample and then mixed at an 11:9 ratio of 2X TaqMan® Gene Expression Master Mix (Applied Biosystems) with 1% 20X GE Sample Loading Reagent (Fluidigm) to create the Sample Mix. In a separate mixture, 2X of Assay Loading Reagent (Fluidigm) was mixed with each pre-made 20X Assay at a 1:1 ratio to yield a 10X Assay mix.

5 µl of each Sample Mix and 5 µl of each 10X Assay Mix were loaded and mixed into dedicated wells of a 96.96 Dynamic Array using the Integrated Fluidic Circuits (IFC) Controller HX (Fluidigm). The loaded Dynamic Array was then transferred to the BioMark™ real-time PCR instrument and the Fluidigm GE 96x96 Standard v1 PCR Thermal Protocol was run [Thermal Mix at 50°C (2 min), 70°C (30 min), and 25°C (10 min); UNG and Hot Start at 50°C (2 min), and 95°C (10 min); cycling was performed using 95°C (15 s), and 60°C (1 min) for 40 cycles]. Samples were measured in duplicate and normalized to Gapdh, Actb and Ubc housekeeping controls. Final analysis was performed using the comparative CT method to calculate fold changes and samples were normalized relative to vehicle control conditions within each data set.

The results indicate that OSGIN1 is transcriptional regulated in the brain following DMF treatment. *In vivo* administration of DMF led to increased transcriptional regulation of OSGIN1 in the brain. As shown in **FIGURE 28**, DMF administration to C57/BL6 mice resulted in

significant increases in *OSGIN1* gene expression in all brain regions, with the highest regulation in the cortex, hippocampus and cerebellum.

Example 5: In Vivo Evaluation of OSGIN1 Regulation as an NRF2 Transcriptional Target

This example describes the regulation of *OSGIN1* gene as an NRF2 transcriptional target *in vivo*.

Methods

Modified time course in transgenic Nrf2^{-/-} mice: Wild type and *Nrf2^{-/-}* transgenic mice were dosed with a single suspension of 100 mg/kg DMF in 0.8% HPMC or vehicle solution alone. 100 mg/kg was selected based on previous animal studies with DMF. Drug was delivered by oral gavage at 10 μ L/gram and dosed at 5 minute intervals to ensure proper timing and reduction of RNA variability following tissue collection. Tissues were harvested at 4, 8, 16 and 32 hours post-dose with an n of 6 per group. The time points for the *Nrf2^{-/-}* study were selected following analysis of the first time course study in wild type mice. Tissue samples were also collected for protein analysis at 6 hours post DMF administration in *Nrf2^{-/-}* and wild type mice based upon gene regulation changes identified in the latter study. Tissue harvest and RNA extraction was conducted in the same way as described for the time course study in Example 1.

Quantitative Real-Time PCR: Quantitative Real-Time PCR (qtPCR) was performed from total mRNA isolated from tissues and reverse-transcribed into cDNA according to manufacturer protocols (Life Technologies, Carlsbad, CA). Target gene mouse primers for Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*): Mm99999915_g1; oxidative stress induced growth inhibitor 1 (*Osgin1*): Mm00660947_m1 and 6-FAM[™] dye-labeled TaqMan[®] MGB[™] probes (Applied Biosystems) were custom dried onto 384-well PCR plates and mixed with 10 μ l of cDNA and 10 μ l 20X TaqMan Universal Master Mix II, no UNG (Applied Biosystems) to yield a final reaction volume of 20 μ l. Final reactions contained 100ng of cDNA, 900 nM of each primer, and 250-nM TaqMan[®] probes and were cycled on a QuantStudio[™] 12K Flex system (Life Technologies) once for 10 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. All samples were measured in duplicate using GAPDH as a normalizing

gene. Final analysis was performed using the comparative CT method to calculate fold changes and samples were normalized relative to vehicle control conditions within each data set.

Results

In the CNS, the Nrf2 transcriptional target gene, OSGIN1, was identified to be significantly upregulated following DMF treatment *in vivo*. To determine if OSGIN1 was an Nrf2 regulated target following DMF treatment, the expression of OSGIN1 in the presence of DMF was analyzed in Nrf2 ^{-/-} mice. As shown in **FIGURES 29A-29B**, the loss of Nrf2 resulted in an inability to induce OSGIN1 in the hippocampus (**FIGURE 29A**) and the striatum (**FIGURE 29B**) following treatment with DMF, suggesting that this gene is strongly regulated via Nrf2. These results indicate that DMF-induced transcriptional regulation of OSGIN1 is Nrf2 dependent. The Nrf2 pathway is a major pathway targeted by DMF. OSGIN1 is a transcriptional target of the transcription factor Nrf2 and OSGIN1 is induced in the presence of DMF.

Example 6: In Vitro Evaluation of OSGIN1 as a Mediator of Cytoprotection in the CNS Following MMF Treatment

This example describes the cytoprotective properties of dimethyl fumarate, contributed by OSGIN1, a transcriptional target of Nrf2. The experiments disclosed in this example characterize the *in vitro* effect of DMF and MMF on OSGIN1 gene expression and evaluate the role of OSGIN1 in mediating cytoprotective effects against toxic oxidative stress.

Methods

Primary human astrocyte cultures were transfected with siRNA targeted against OSGIN1 or Nrf2 and treated with MMF, followed by toxic oxidative challenge with hydrogen peroxide. Cell viability was measured after treatment/insult and RT-PCR was conducted to determine transcriptional levels of OSGIN1, as well as the OSGIN1 associated genes PADI4 and p53. The potential involvement of PADI4 and p53 in MMF-mediated cytoprotection was also analyzed via

siRNA knockdown. Furthermore, the importance of OSGIN1 and its associated genes on cell proliferation was also analyzed. The methods are described below in more detail.

Reverse transfection of human spinal cord astrocytes: Human spinal cord astrocytes were transfected with X-tremeGENE siRNA transfection reagent (Roche) using the reverse transfection method. siRNA complexes were created according to the manufacturer's protocol and consisted of 1.5ul of X-tremeGENE transfection reagent and 10uM of either OSGIN1 siRNA (Origene), PADI4 siRNA (Origene), p53 siRNA, or scrambled siRNA (Origene). Using the reverse transfection method, complexes were added to poly-d-lysine coated 24-well tissue culture plates (BioCoat) and astrocytes were added on top of the complex at a concentration of 60,000 cells/well in human spinal cord astrocyte medium (ScienCell). Cells were incubated with complex for 12 hours at 37°C followed by replacement of complex media with fresh human astrocyte media.

Addition of MMF to transfected human astrocyte cultures: 24 hours post-transfection, human astrocytes were treated with 0, 10 or 30uM of MMF compound in DMSO for 20 hours at 37°C with 5% CO₂. Since DMF is rapidly metabolized to its bioactive metabolite, MMF, following oral administration, cells will likely be exposed to MMF rather than DMF. Therefore, MMF was used in these *in vitro* studies.

Quantitative real-time PCR: Following 20 hours of MMF addition to transfected human spinal cord astrocytes, quantitative real-time PCR was conducted to confirm siRNA knockdown and determine the effect on MMF induced transcriptional regulation of the Nrf2 target gene, OSGIN1, as well as the OSGIN1 associated gene PADI4.

Total mRNA was isolated from cells extracted using an RNeasy kit (QIAGEN) and reverse-transcribed into cDNA according to the manufacturer's protocols (Applied Biosystems, Carlsbad, CA). qPCR was performed using target gene human primers for beta-actin (ActinB): Hs01060665_g1; oxidative stress induced growth inhibitor 1 (Osgin1): Hs00203539_m1; peptidyl arginine deiminase, type IV (PADI4): Hs00202612_m1 and 6-FAM™ dye-labeled TaqMan® MGB™ probes (Applied Biosystems). Reactions containing 100 ng of cDNA, 900 nM of each primer, and 250 nM TaqMan probes were cycled on a QuantStudio 12k-flex system (Life Technologies) once for 10 min at 95°C, followed by 40 cycles of 95°C for 10 s and 60°C

for 1 min. All samples were measured in duplicate by using beta-actin as a normalizing gene. Final analysis was performed by using the comparative CT method to calculate fold changes. Samples were normalized relative to vehicle or dimethyl sulfoxide (DMSO) control conditions within each data set.

Oxidative challenge: Following 20-hour treatment with MMF compound, media was removed from human astrocytes and replaced with 0, 200 or 300uM of hydrogen peroxide (H₂O₂) diluted in Hanks balanced salt solution plus 20 mM HEPES, pH 7.4. Astrocytes were challenged with hydrogen peroxide for 2 hours at 37°C with 5% CO₂ followed by recovery for 20 hours in human astrocyte media.

LIVE/DEAD viability assay and nuclear quantification: Following 20 hour recovery from oxidative challenge, cellular viability was assessed using a LIVE/DEAD viability stain according to the manufacturer's protocol (Invitrogen). LIVE stain was quantified by fluorescence intensity from calcein AM fluorescence in live cells (excitation wavelength, 488 nM; emission wavelength, 525 nM) and in parallel by counting Hoechst-labeled nuclei using automated imaging and counting. In the LIVE/DEAD assay, dead cells were labeled with ethidium homodimer and appear red in fluorescent images (excitation wavelength, 550nM; emission wavelength, 575 nM). Live images from LIVE/DEAD labeled cells were obtained. Cell nuclei from Hoechst dye labeled cells were quantitated in an automated fashion on the Cellomics ArrayScan VTi platform (Thermo Fisher Scientific).

Results

As discussed in more detail below, siRNA knockdown of OSGIN1 significantly reduced the ability of MMF to protect human astrocytes against a toxic oxidative stress, as well as reducing the expression levels of PADI4. Additionally, the loss of PADI4 and p53 via siRNA knockdown also significantly reduced the cytoprotective effects of MMF in human astrocytes against oxidative stress.

As shown in **FIGURE 30**, siRNA knockdown in human spinal cord astrocytes significantly reduced MMF-induced expression of the Nrf2-target gene OSGIN1 compared to the scrambled siRNA control.

OSGIN1 siRNA knockdown led to a reduction in MMF-mediated cytoprotection against H₂O₂ insult in human spinal cord astrocytes. The reduction was measured by fluorescence intensity of calcein AM positive LIVE stained cells (**FIGURE 31A**), nuclear DAPI count (**FIGURE 31B**), and LIVE/DEAD imaging of live versus dead cells (**FIGURE 31C**).

FIGURE 32A indicates that siRNA knockdown of PADI4 resulted in significant loss of PADI4 expression and the inability of this gene to be induced in the presence of MMF. As shown in **FIGURE 32A**, siRNA knockdown of OSGIN1 resulted in significant loss of PADI4 expression. However, loss of PADI4 expression had no effect on OSGIN1 expression levels (**FIGURE 32B**). This suggests that PADI4 is a downstream target of OSGIN1.

As shown in **FIGURES 33A-33B**, siRNA knockdown of the OSGIN1-regulated gene, PADI4, significantly decreased MMF-induced cytoprotection in human spinal cord astrocytes. The MMF-induced cytoprotection was measured by nuclear counting of live cells (**FIGURE 33A**) and LIVE/DEAD imaging (**FIGURE 33B**).

FIGURE 34 shows that siRNA knockdown of the p53 gene significantly decreased MMF-induced cytoprotection in human spinal cord astrocytes as measured by nuclear counting of live cells following 300 μ M H₂O₂ insult.

Conclusions

Loss of OSGIN1 expression resulted in a decreased ability of MMF to induce OSGIN1 and to protect cells in the presence of oxidative insult. Further, loss of OSGIN1 expression resulted in decreased expression of the OSGIN1-associated gene, PADI4, as well as its ability to be induced by MMF. In addition, loss of PADI4 and p53 expression also led to a decreased ability of MMF to protect cells in the presence of oxidative insult. These results indicate the importance of OSGIN1, PADI4 and p53 in MMF-induced cytoprotection in human spinal cord astrocytes.

In sum, these data demonstrate a role for the Nrf2 transcriptional target, OSGIN1, as an important mediator of cytoprotection in the CNS following DMF/MMF administration. Furthermore, these data identified a mechanism for DMF/MMF-mediated cytoprotection in human astrocytes that functions in an Nrf2-dependent manner. These data supports activation of

the Nrf2 pathway is a primary pharmacodynamic response to DMF or MMF treatment, and these responses may be differentially regulated in distinct cell types.

Example 7

Animals

All procedures involving animals were performed in accordance with standards established in the Guide for the Care and Use of Laboratory Animals as adopted by the U.S. National Institutes of Health.

C57BL/6 wild type mice: 6-8 week-old male C57BL/6 wild-type mice were used for experimentation. Mice were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained on a 12-hour light/dark cycle and given access to food and water ad libitum.

Transgenic Nrf2 -/- mice: Transgenic *Nrf2*^{-/-} knockout mice were obtained from Masayuki Yamamoto at the University of Tsukuba (Tsukuba, Japan). Generation of these mice was obtained by a target vector designed to replace the b-Zip region of the *Nrf2* gene with a SV40 nuclear localization signal (NSL)- β -galactosidase (*lacZ*) recombinant gene. To select for transformants a neomycin resistance (*neo*) gene was inserted downstream of the NSL-*lacZ* gene. The diphtheria toxin gene was placed upstream of the *Nrf2* gene for negative selection against non-homologous recombinants (Itoh et al., 1997). Heterozygote males were shipped to Jackson Laboratories (Bar Harbor, ME), bred to female C57BL/6J and maintained on the 6J background. 6-8 week-old male *Nrf2*^{-/-} and aged matched C57BL/6 wild-type mice from the same colony were used for all experimentation.

Antibodies

All purchased and custom generated antibodies used for experimentation are listed and described in **Table 10**.

Table 10. *Antibodies*

ANTIBODY	MANUFACTURER	CATALOG NUMBER	DILUTION
β -ACTIN	MP Biomedicals	69100	1:5000
BDNF	Abcam	ab108383	1:5000
GCLC	Abcam	ab41463	1:500
NQO1	Epitomics	2618-1	1:1000
NRF2	Epitomics	2178-1	1:1000
OSGIN1	NEP	Custom*	1:200
OSGIN1-1a/2a	NEP	Custom*	1:1000
OSGIN1-2b	NEP	Custom*	1:500
OSGIN1 C-terminal	Abcam	ab173766	1:250
OSGIN1 N-terminal	Abcam	ab90128	1:1000
PADI4	Abcam	ab96758	1:500
P53	Cell Signaling	9282	1:1000
SQSTM1	Abcam	ab56416	1:250
TXNRD1	Abcam	ab124954	1:1000

**please see Generation of OSGIN1 isoform-specific antibodies section below*

Generation of OSGIN1 isoform-specific antibodies: Three isoform specific rabbit polyclonal antibodies were generated by New England Peptide (NEP) based on the sequences identified and cloned by Ong et al. (Ong 2004). Peptides were generated against human specific sequences in the OSGIN1 38kDa, 52kDa and 61kDa protein regions (**FIGURE 36**). Based on the methods of NEP, antibodies were generated and affinity purified using OSGIN1 isoform-specific peptides. Due to the overlapping homology of the sequences only the longest 61kDa peptide sequence resulted in a unique antibody (**FIGURE 36**).

Human siRNA constructs

Human siRNA constructs were purchased from OriGene technologies (Rockville, MD) or Invitrogen (Grand Island, NY) and are listed and described in **Table 11**. For all siRNA transfection studies, constructs were transfected into cells at a final concentration of 10nM. If multiple duplexes were used, the total addition of combined duplexes equaled 10nM.

Table 11. *siRNA constructs.*

siRNA	MANUFACTORER	CATALOG NUMBER	DUPLEX USED
OSGIN1	OriGene	SR309301	A and C (5nM each)
PADI4	OriGene	SR308375	A,B,C (3.3nM each)
Trilencer-27 Scrambled Negative Control	OriGene	SR30004	10nM
Scrambled Negative Control Stealth RNA	Invitrogen	13750-047	10nM
p53 Pos Control Stealth RNA	Invitrogen	13750-047	10nM
NRF2	OriGene	SR303156	A,B,C (3.3nM each)

Primers and Probes

Purchased primer/probe sets: All manufactured primer/probe sets were purchased from Life Technologies (Grand Island, NY) at a concentration of 20X and are listed in **Table 12**.

Table 12. *Taqman Primer/Probe Assays*

Gene	Species	Taqman Assay ID		Gene	Species	Taqman Assay ID
AATF	Mouse	Mm00498160_m1		NINJ1	Mouse	Mm00479014_m1
ACTB	Mouse	Mm00442646_m1		NOS1	Mouse	Mm00435175_m1
ACTIN	Human	Hs01060665_g1		NQO1	Human	Hs02512143_s1
AKR1B8	Mouse	Mm00484314_m1			Mouse	Mm01253561_m1
ALS2	Mouse	Mm01255664_m1		NR2F6	Mouse	Mm01340321_m1
APOE	Mouse	Mm01307193_g1		NSG2	Mouse	Mm00476554_m1
ATG2A	Mouse	Mm01212087_m1		NUDT7	Mouse	Mm01240093_m1
BDNF	Mouse	Mm04230607_s1		OLIG1	Mouse	Mm00497537_s1
CCS	Mouse	Mm00444148_m1		OSGIN1	Human	Hs00203539_m1
CDKN1A	Mouse	Mm04205640_g1			Mouse	Mm00660947_m1
DNAJA3	Mouse	Mm00469723_m1		OXNAD1	Mouse	Mm00520913_m1
EGR2	Mouse	Mm00456650_m1		PADI4	Human	Hs00202612_m1
GAB1	Mouse	Mm00491216_m1		P21	Human	Hs00355782_m1
GAPDH	Human	Hs02758991_g1		PRDX1	Mouse	Mm01621996_s1
	Mouse	Mm99999915_g1		PTGS2	Mouse	Mm00478374_m1
GCLC	Human	Hs00155249_m1		P53	Human	Hs01034249_m1
	Mouse	Mm00802655_m1		RBP4	Mouse	Mm00803264_g1
GCLM	Mouse	Mm00514996_m1		SIRT1	Mouse	Mm00490758_m1
GDF15	Mouse	Mm00442228_m1		SLC1A2	Mouse	Mm00441457_m1

GFAP	Mouse	Mm01253033_m1		SNAI1	Mouse	Mm00441533_g1
GLUL	Mouse	Mm00725701_s1		SOD1	Mouse	Mm01344233_g1
GSTA2	Mouse	Mm03019257_g1		SQSTM1	Mouse	Mm00448091_m1
HMOX1	Human	Hs01110250_m1		SRXN1	Human	Hs00607800_m1
	Mouse	Mm00516005_m1			Mouse	Mm00769566_m1
IGFBP	Mouse	Mm01187817_m1		STRA6	Mouse	Mm00486457_m1
MMP11	Mouse	Mm00485048_m1		TNF-α	Human	Hs01113624_g1
NETO2	Mouse	Mm01245002_m1		TXNRD1	Human	Hs00182418_m1
NFKBIB	Mouse	Mm00456849_m1			Mouse	Mm00443675_m1
NGFG	Mouse	Mm01203825_gH		UBC	Mouse	Mm02525934_g1
				VEGFA	Mouse	Mm01281449_m1

Custom primer/probes sets: Custom primers for 5'UTR analysis were generated using primer express software to distinguish between two nucleotide substitutions identified within the 5'UTR of two human OSGIN1 transcripts (**Table 13**). These transcripts were identified from RACE analysis and encode for the same protein. Generated primers and probes were analyzed using BLAST (Basic Logical Alignment Search Tool) to confirm specificity.

Table 13. *Primer and probe sets for 5'UTR transcript variants of OSGIN1.*

	Forward Primer	SEQ ID NO.	Reverse Primer	SEQ ID NO.	Probe	SEQ ID NO.
WT	CTTCCCTCTGGCCTCT	1	GAGATCGGGACACC	3	CCTCTTGGATCCC	5

	CAGA		CATTACC		C	
ALT	AATGGGTGTCCCGAT GTCA	2	CCGGCCAAGTTGTG CACTA	4	ACTCTGTGATCCG TGTTC	6

WT-Represents canonical sequence; ALT-represents nucleotide alteration identified in 5'RACE

Primers for 3' and 5' RACE: All primers for RACE were generated against the human OSGIN1 sequence and were analyzed using BLAST (Basic Logical Alignment Search Tool) to confirm specificity.

Table 14. *Primers for 3' and 5' RACE.*

3' RACE Primers		
Primer	Sequence	SEQ ID NO
GSP1	GCTCCCGGACCTGGAGGT	7
Nested GSP2	ACTGGATGCAGAAGAAGCGA	8
5' RACE Primers		
Primer	Sequence	SEQ ID NO
GSP1	CGCTTCTTCTGCATCCAGTCC	9
GSP2	GCATCCAGTCCTTGACCTCCA	10
Nested GSP3	TGACCTCCAGGTCCGGGAGC	11

Methods

Pharmacokinetic Measure of MMF Exposure

Whole blood and tissues (brain, liver, kidney, jejunum and spleen) were collected in a separate cohort of mice 30 minutes after dosing. Whole blood was collected via cardiac puncture and collected in lithium heparin tubes (Fisher Scientific, Cambridge MA) with an addition of 10 mg sodium fluoride. Tubes were immediately inverted several times and stored on wet ice until processed (no longer than 30 min). Whole blood was then centrifuged (15 minutes @ 1500xg, 4 °C), and plasma was immediately collected and stored on dry ice until transfer to -80°C for storage. Tissues of interest were also collected at 30 minutes after dosing, snap frozen and stored at -80°C. A liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS) based bioanalytical assay was used to determine MMF concentration levels in plasma and tissue samples. Stable isotope labeled ¹⁴C-MMF was employed as an internal standard (IS). Study samples were thawed on ice and MMF, as well as the IS, were extracted from plasma samples using protein precipitation followed by separation from endogenous components using a liquid chromatography column. Detection was conducted by mass spectrometry using negative electrospray and the multiple reaction monitoring mode. Concentrations of MMF in study samples were calculated using peak area ratios of MMF to the IS against the standard curve relating the peak area ratios to spiked MMF concentrations.

Acute Oral Administration of DMF in vivo

Time course in wild type C57BL/6 mice: Wild type mice were dosed with a single suspension of 100 mg/kg DMF in 0.8% HPMC or vehicle solution alone. 100 mg/kg was selected based on previous animal studies with DMF. Drug was delivered by oral gavage at 10µL/gram and dosed at 5 minute intervals to ensure proper timing and reduction of RNA variability following tissue collection. Tissues were harvested 2, 4, 6, 8, 12.5, 16.5, 23 or 37.5 hours after dosing with each time point including a vehicle control group and DMF group with an n of 6 animals.

Dose response in wild type C57BL/6 mice: Experimental procedures for the DMF dose

response study parallel those stated for the time course study unless noted otherwise. Wild type C57BL/6 mice were dosed with a suspension of 50, 100, 200, 400 or 600 mg/kg DMF in 0.8% HPMC or vehicle solution alone. Drug was delivered by oral gavage and tissues harvested 7 hours after dosing. This time point was selected following analysis of the DMF time course data.

Modified time course in transgenic Nrf2^{-/-} mice: Wild type and Nrf2^{-/-} mice were dosed with a single suspension of 100 mg/kg DMF in 0.8% HPMC or vehicle solution alone. 100 mg/kg was selected based on previous animal studies with DMF. Drug was delivered by oral gavage at 10 μ L/gram and dosed at 5 minute intervals to ensure proper timing and reduction of RNA variability following tissue collection. Tissues were harvested at 4, 8, 16 and 32 hours post-dose with an n of 6 per group. The time points for the Nrf2^{-/-} study were selected following analysis of the first time course study in wild type mice. Tissue samples were also collected for protein analysis at 6 hours post DMF administration in Nrf2^{-/-} and wild type mice based upon gene regulation changes identified in the latter study.

Tissue harvest: Animals were exposed to CO₂ and whole blood collected via cardiac puncture. Two 100 μ L aliquots of whole blood were collected in 1.5 mL microcentrifuge tubes and snap frozen in liquid nitrogen. Peripheral (liver, spleen, kidney and jejunum) and CNS brain (cerebellum, hippocampus, striatum and cortex) tissues were harvested and snap frozen in liquid nitrogen with special care taken to collect tissues of similar size and from the same location. Two samples were collected for each tissue, one for RNA extraction and one for protein extraction, and protein tissue samples were weighed in pre-tared tubes before snap freezing. All samples were stored at -80°C until RNA and protein extraction was conducted.

Cell Culture

Primary human spinal cord astrocytes: Primary cultures of human spinal cord astrocytes were purchased from ScienCell Research Laboratories (Carlsbad, CA) and grown in Astrocyte Medium (AM; ScienCell). Cultures were maintained according to the supplier specifications. Cells for plate based assays were seeded into clear-bottom poly-D-lysine tissue culture 12-or 24-well plates (BD Biosciences, San Jose, CA).

Reverse transient transfection : Human spinal cord astrocytes were transfected with X-

tremeGENE siRNA transfection reagent (Roche, Indianapolis, IN) using the reverse transfection method. siRNA complexes were created according to the manufacturer's protocol and consisted of 1.5ul of X-tremeGENE transfection reagent and 10nM of either OSGIN1 siRNA (Origene), PADI4 siRNA (Origene), Nrf2 siRNA (Origene), p53 siRNA (Invitrogen) or scrambled siRNA (Origene, Invitrogen). Using the reverse transfection method, complexes were added to poly-d-lysine coated 24-well tissue culture plates (BioCoat) and astrocytes were added on top of the complex at a concentration of 60,000 cells/well in human spinal cord astrocyte medium (ScienCell). Cells were incubated with complex for 12 hours at 37°C followed by replacement of complex media with fresh human astrocyte media. Knockdown was assessed at 48 hours post-transfection for q-PCR analysis and western blotting. For analysis with MMF, cells were treated with a titration of MMF 24 hours post-transfection and analyzed in plate-based assays as described herein.

Plate-based Cellular Assays

Compound handling: MMF was prepared in 100mM solutions of DMSO, titrated in DMSO, and then diluted into normal growth media for cell treatments. The final concentration of DMSO (0.03%) was consistent for all treated cells.

H₂O₂ in vitro stress response assay: 24 hours post-transfection, human astrocytes were treated with 0, 10 or 30uM of MMF compound in DMSO for 20 hours at 37°C with 5% CO₂. Since DMF is rapidly metabolized to its bioactive metabolite, MMF, following oral administration, cells will likely be exposed to MMF rather than DMF. Therefore, we used MMF in these in vitro studies. Following 20 hour treatment with MMF compound, media was removed from human astrocytes and replaced with 0, 200 or 300uM of hydrogen peroxide (H₂O₂) diluted in Hanks balanced salt solution plus 20 mM HEPES, pH 7.4. Astrocytes were challenged with hydrogen peroxide for 2 hours at 37°C with 5% CO₂ followed by recovery for 20 hours in human astrocyte media. Following 20 hour recovery from oxidative challenge, cellular viability was assessed using a LIVE/DEAD viability stain according to the manufacturer's protocol (Invitrogen). LIVE stain was quantified by fluorescence intensity from calcein AM fluorescence in live cells (excitation wavelength, 488 nM; emission wavelength, 525 nM) and in parallel by counting Hoechst-labeled nuclei using automated imaging and counting. In the LIVE/DEAD

assay, dead cells were labeled with ethidium homodimer and appear red in fluorescent images (excitation wavelength, 550nm; emission wavelength, 575 nm). Live images from LIVE/DEAD labeled cells were imaged as above. Cell nuclei from Hoechst dye labeled cells were quantitated in an automated fashion on the Thermo ArrayScan VTI platform (Thermo Fisher Scientific).

EdU proliferation assay: Human astrocytes were treated with a titration of MMF and incubated for 20-24 hours at 37°C with 5% CO₂. Cells were incubated with EdU to measure proliferation according to the manufacturer protocol outlined for the Click-iT[®] EdU HCS Assay (Invitrogen). EdU was added to cells at a 1:1000 fold dilution in growth media and pulse labeled for 1 hour. Following EdU incorporation, cells were fixed in 4% paraformaldehyde (PFA)/4% sucrose in PBS and EdU detected by immunostaining according the manufacturer. Immunostained plates were quantitated for EdU incorporation using an automated fashion on the Cellomics ArrayScan VTI platform and correlating algorithm analysis.

TiterTACSTM Assay for Apoptosis: Human astrocytes were transfected with siRNA according to the protocol in section 2.3.2 of this chapter. Following 20-24 hours incubation with MMF, cells were fixed in 4% PFA/4% sucrose and the HT TiterTACSTM Assay Kit (Trevigen, Gaithersburg, MD) was used to detect cellular apoptosis according to the manufacturer's protocol.

Cellular extract preparation and Nrf2/p53 activity assays: For Nrf2 and p53 activity assays, human astrocytes were treated for 6 or 24 hours with MMF. Cytosolic and nuclear extracts were prepared by using a nuclear extract kit from Active Motif Inc. (Carlsbad, CA). TransAM Nrf2 and TransAM p53 assays (Active Motif Inc.) were used according to the manufacturer's instructions.

Immunocytochemistry : Localization of p53 signal and OSGIN1 positive staining were analyzed via immunocytochemistry following treatment with MMF after 24 hours. Cells were fixed in 4% PFA/4% sucrose, permeabilized in .1% Triton X-100 and labeled with primary anti-p53 antibody or NEP antibodies generated against the 52kDa and 61kDa isoforms of OSGIN1. Primary labeled cells were tagged with a secondary fluorescence antibody and DAPI nuclear stain. Images were acquired using the Thermo HCS Arrayscan VTI platform and an algorithm generated to measure p53 nuclear versus cytoplasmic signal and total OSGIN1 positive puncta.

RNA Extraction

Tissue RNA extraction: For RNA preparation, frozen tissues were placed in 2 mL RNase-free 96-well blocks with 1.5mL QIAzol Lysis Reagent (QIAGEN) and a 3.2 mm stainless steel bead (BioSpec Products, Bartlesville, OK). Tissues were disrupted for four cycles of 45 seconds in a Mini-Beadbeater (Biospec Products). RNA was extracted in chloroform and the aqueous phase was mixed with an equal volume of 70% ethanol. Extracted RNA was applied to RNeasy 96 plates and purified by the spin method according to the manufacturer's protocol (RNeasy 96 Universal Tissue Protocol, QIAGEN, Hilden Germany).

Whole blood RNA extraction (RBC and PBMC): RNA extraction from whole blood was conducted using the Agencourt RNAdvance Blood kit according to the manufacturer's protocol (Beckman Coulter, CA). To begin, frozen whole blood samples were arranged on dry ice in a 96-well tube rack and 520ul Proteinase K/Lysis buffer was added to each blood aliquot. Samples were then simultaneously inverted at room temperature until all were mixed directly into solution. Agencourt RNAdvance Blood protocol was then followed using the Agencourt SPRIPlate 96R Super Magnet Plate (Beckman Coulter) for RNA extraction.

RNA extraction from whole cells: Total mRNA was isolated from cells using an RNeasy kit (QIAGEN) according to the manufacturer's protocol for spin technology.

Measure of RNA integrity, purity and quantity: For initial optimization of RNA extraction and all transcriptional profiling studies, RNA was analyzed for purity and integrity by capillary electrophoresis on an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA).

Quantitative Real-Time Polymerase Chain Reaction (PCR)

Quantitative Real-Time PCR using the Qiagen QuantStudio 12K-Flex: qPCR was performed from total mRNA isolated from tissues and reverse-transcribed into cDNA according to manufacturer protocols (Life Technologies, Carlsbad, CA). For *in vivo* *Nrf2*^{-/-} analysis, target gene mouse primers and 6-FAMTM dye-labeled TaqMan® MGBTM probes (Applied Biosystems) were custom dried onto 384-well PCR plates and mixed with 10ul of cDNA and 10ul 2X Taqman Universal Master Mix II, no ung (Applied Biosystems), to yield a final reaction volume of 20ul. For all other studies, qPCR was performed using 20X Taqman target gene human

primer/probe sets mixed with cDNA and 2X Taqman Universal Master Mix II, no ung, to a final volume of 20 μ l. All final reactions contained 100ng of cDNA, 900 nM of each primer, and 250-nM TaqMan® probes and were cycled on a QuantStudio™ 12K Flex system (Life Technologies) once for 10 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. All samples were measured in duplicate using GAPDH or β -Actin as a normalizing gene. Final analysis was performed using the comparative CT method to calculate fold changes and samples were normalized relative to vehicle control conditions within each data set.

Fluidigm BioMark™ Real-Time PCR: RNA was analyzed for purity and integrity by capillary electrophoresis using the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). Total RNA (125 ng) was DNase-treated for 15 min at room temperature and EDTA heat inactivated at 65°C for 10 min using Dnase I Amp Grade from Invitrogen. Samples were then reverse transcribed using the High Capacity cDNA Reverse Transcription Kit according to the manufacturer's protocol (Applied Biosystems). High-throughput qtPCR was conducted to study gene expression in 5 ng cDNA using 96.96 Dynamic Arrays for the microfluidic BioMark™ System (Fluidigm Corporation, CA, USA). A panel of 48 genes measured in duplicate, were selected based on previous DMF gene profiling studies conducted in vivo and in vitro, as well as published experimental and computational literature of identified Nrf2 regulated genes (**Table 15**). Real time PCR probe sets were designed using Primer Express 3.0 (Applied Biosystems) with TaqMan MGB Quantification default settings. A 20X Assay stock of the primer probe set of each gene to be investigated was made (900nM for primers and 200nM for Probe in 1X TE Buffer [10 mM Tris pH8 (Ambion), 1 mM EDTA pH8 (Sigma)]) and then diluted to a 0.2X assay pool. 2 μ l of 0.2X assay pool was combined with 2 μ l of the reverse-transcribed total RNA and 4 μ l of 2x Taqman® PreAmp Master Mix (Applied Biosystems) for a final reaction volume of 8 μ l. Pre-amplification of cDNA was performed in the Tetrad thermocycler [at 95°C for a 10 min activation step followed by 10 cycles: 95°C, (15 s), 60°C, (4 min)]. Following pre-amplification, 32 μ l 0.1X TE Buffer was added to each sample and then mixed at an 11:9 ratio of 2X Taqman® Gene Expression Master Mix (Applied Biosystems) with 1% 20X GE Sample Loading Reagent (Fluidigm) to create the Sample Mix. In a separate mixture, 2X of Assay Loading Reagent (Fluidigm) was mixed with each pre-made 20X Assay at a 1:1 ratio to yield a 10X Assay mix. 5 μ l of each Sample Mix and 5 μ l of each 10X Assay Mix were loaded and

mixed into dedicated wells of a 96.96 Dynamic Array using the Integrated Fluidic Circuits (IFC) Controller HX (Fluidigm). The loaded Dynamic Array was then transferred to the BioMark™ real-time PCR instrument and the Fluidigm GE 96x96 Standard v1 PCR Thermal Protocol was run [Thermal Mix at 50°C (2 min), 70°C (30 min), and 25°C (10 min); UNG and Hot Start at 50°C (2 min), and 95°C (10 min); cycling was performed using 95°C (15 s), and 60°C (1 min) for 40 cycles]. Samples were measured in duplicate and normalized to Gapdh, Actb and Ubc housekeeping controls. Final analysis was performed using the comparative CT method to calculate fold changes and samples were normalized relative to vehicle control conditions within each data set.

Northern Blotting

RNA probe generation

RNA probes were generated using primers that covered the primer/probe sequence of the OSGIN1 Taqman assay used for q-PCR analysis. PCR was conducted using human spinal cord astrocyte cDNA as a template and PfuUltra II Hotstart PCR Master Mix according to the manufacturer's protocol (Agilent). PCR products were separated on 2% SeaKem® LE Agarose DNA gels (Lonza), appropriate bands excised and purified using QIAquick Gel Extraction Kit (Qiagen). Purified DNA was digested, re-purified and inserted into a pBluescript II KS+ Vector using a Quick Ligation Kit (Invitrogen). The vector-DNA ligation was then transformed into One Shot Chemically Competent *E. coli* (Invitrogen) by heat shock at 42°C for 30 seconds. Transformed cells were spread on ampicillin resistant selection plates and incubated overnight at 37°C. Following overnight incubation, eight colonies were collected in lysogeny broth (LB) medium plus ampicillin and shaken for eight hours at 37°C. Plasmid DNA was then purified using the Nucleospin 8 Plasmid Kit according to the manufacturer's protocol (Machery-Nagel, Bethlehem PA). Purified plasmid was checked for correct insertion by restriction digest and submitted for DNA sequencing. Plasmid DNA containing the appropriate insert was linearized and transcribed into RNA probes using a MAXIscript *in vitro* Transcription Kit (Invitrogen) according to the manufacturer's protocol. Unincorporated nucleotides were removed by column purification using NucAway Spin Columns (Ambion) and biotin labeled using a BrightStar Psoralen-Biotin Kit (Ambion).

Northern blot analysis: Northern blot analysis was conducted using the Ambion[®] NorthernMax[®]-Gly Kit (Life Technologies) according to the manufacturer's protocol. RNA was transferred to Ambion BrightStar Plus Membranes (Ambion) using an iBlot Transfer System (Invitrogen) and detection was accomplished using the BrightStat BioDetect Kit (Ambion).

Rapid Amplification of cDNA Ends (RACE)

3' RACE: 3' RACE was conducted using the Invitrogen 3' RACE Kit (Invitrogen) with the primers listed herein. The manufacturer's protocol was followed other than for target cDNA amplification which was accomplished using Platinum PCR Supermix (Invitrogen). 3' RACE products were separated by gel electrophoresis, excised and inserted into a TOPO vector. Generated plasmids were transformed into bacteria and purified according to the protocol mentioned herein. Purified DNA was checked by restriction digest and submitted for DNA sequencing.

5' RACE: 5' RACE was conducted using the Invitrogen 5' RACE Kit (Invitrogen) with the primers listed herein. 5' RACE primer starting points were determined based on the sequence identified from 3' RACE. 5' RACE products were separated by gel electrophoresis, excised and inserted into a TOPO vector. Generated plasmids were transformed into bacteria and purified according to the protocol mentioned herein. Purified DNA was checked by restriction digest and submitted for DNA sequencing.

Protein Isolation

Protein isolation from tissue: For protein extraction, frozen tissues were placed on wet ice and Radio Immuno Precipitation Assay (RIPA) buffer containing protease inhibitors was immediately added to samples at a volume of ~300ul per 5mg of tissue. Tissues were homogenized in buffer for 20 seconds using an F60 Sonic Dismembrator (Fisher Scientific) at setting 8 in a 4°C walk-in fridge. Homogenized samples were incubated for 2 hours at 4°C with constant agitation and then centrifuged for 20 minutes at 13,000 rpm in a microcentrifuge maintained at 4°C. Following centrifugation, the supernatant was collected and the pellet discarded. Supernatants were analyzed for total protein concentration using the Thermo

Scientific Pierce BCA Protein Assay Kit (Thermo Scientific) and diluted to yield equal concentrations of 2ug/uL. Samples were diluted 1:1 in 2X Laemmli denaturing buffer and boiled at 100°C for 5 minutes.

Protein isolation from cells: For protein isolation from whole cells, cells were scraped directly in 1X Laemmli denaturing buffer, disrupted using an F60 Sonic Dismembrator (Fisher Scientific) and boiled at 100°C for 5 minutes.

Protein quantification: Protein was quantified using the Pierce[®] BCA Protein Assay Kit (Pierce) and samples diluted to equal loading volumes.

Western Blot Analysis

30ug of total denatured protein was loaded on Criterion™ TGX™ precast gels (Bio-Rad Laboratories) and ran at a constant voltage of 200V for ~45 minutes. Gels were transferred to nitrocellulose membranes using the Invitrogen iBlot system (Invitrogen). Membranes were blocked in 5% milk in TBST for 1 hour followed by incubation with primary (overnight at 4°C) and secondary (1 hour at room temperature) antibodies in 5% milk in TBST. Signal was developed using SuperSignal[®] Chemiluminescent Substrate (Thermo Scientific). Primary antibodies are described herein.

Thermo HCS Arrayscan Algorithm Creation and Analysis

Plate-based cell assays were imaged using the Thermo HCS Arrayscan (Thermo Scientific) and quantified by algorithm generation. Templates were used to generate algorithms using HCS Studio software and included the *Nuclear Translocation* algorithm template for p53 translocation analysis and *Target Activation* algorithm template for DAPI nuclear count and the EdU proliferation assay.

Data Analysis and Statistics

Method of statistical analysis and corresponding *p* values are stated within individual figures other than for transcriptional profiling time course and dose response studies which are described below.

Transcriptional profiling time course and dose response studies: Statistical analysis was performed using Wilcoxon signed-rank test using SAS software for the transcriptional profiling time course study, with each statistical comparison occurring between vehicle and DMF-treated per time point. For the transcriptional profiling dose response study, one-way analysis of variance (ANOVA) was performed with Dunnett's multiple comparison test. Significance is indicated in **Tables 15** and **16** as either $p < .01$ (dark gray) or $p < .05$ (light gray).

Example 8: Delayed-Release Dimethyl Fumarate Pharmacodynamic Responses are Tissue-Specific and Nrf2-Dependent

RATIONALE

A potential mechanism to treat neurodegenerative disease is to utilize and enhance the activity of existing cellular defense mechanisms to overcome the degenerative and toxic effects of pathogenic stimuli. Elucidating how these defensive signaling pathways regulate cellular protection at the molecular level can contribute to the development of therapeutic approaches for combating neurodegenerative disease. The major cellular defense system activated during periods of oxidative and electrophilic stress is the Nrf2 pathway, which regulates the expression of genes that are pro-survival in nature and enable cells to better mitigate potentially toxic stimuli. DMF is believed to mediate its effect, at least in part, via the Nrf2 pathway; however, the exact mechanism of action is unknown. Therefore, evaluating the detailed pharmacodynamic responses to DMF treatment would provide important insight into the molecular nature of the DMF mechanism of action. We measured transcriptional changes in wild type and Nrf2 knockout mice treated with DMF to compare the pharmacodynamic responses throughout the central nervous system (CNS) and periphery in order to understand how changes in target gene expression may confer DMF functional activity, and also to determine the necessity for Nrf2 in these processes.

RESULTS

DMF treatment leads to increased levels of MMF in all tissues of interest

DMF is known to be rapidly hydrolyzed to its bioactive metabolite, MMF, within minutes following oral intake (Werdenberg et al., 2003). To confirm that DMF was present at tissue sites analyzed for gene expression, MMF concentrations were measured in these tissues as an indicator of compound exposure. Following a single dose of 100mg/kg DMF in wild type C57BL/6 mice, MMF exposure was detected in all six tissues of interest; liver, spleen, kidney, jejunum, brain and plasma (**FIGURE 37**). The highest level of MMF exposure was seen within plasma samples and the lowest levels were found to be in the liver. In the remaining tissues, comparable exposure levels were seen between the peripheral organs and the brain. These findings suggest that DMF administration can result in effects within the CNS and that the metabolite of MMF can cross the blood brain barrier to contribute to DMF-mediated mechanisms.

DMF-induces differential gene expression across tissue types

To determine the effects of a single 100 mg/kg DMF dose on transcription regulation, a detailed gene transcriptional profiling study was conducted that measured the expression of 48 genes (**Table 15**) across eight time points using the Fluidigm BioMark™ HD System. This system maintains the sensitivity of classical real time PCR analysis while utilizing nanofluidic technology to collect larger data sets (upwards of 9,000 data points) in a single run. After receiving a single dose of DMF or vehicle, cohorts of animals were harvested at 2, 4, 6, 8, 12.5, 16.5, 23 or 37.5 hours. DMF and vehicle treated animals were harvested in parallel at each time point to eliminate the effects of diurnal variation, as all DMF samples were compared directly to time-matched vehicle controls to determine fold-changes in target gene expression. Data collected using this system identified time-dependent transcriptional changes following DMF treatment within peripheral (liver, spleen, kidney, jejunum and whole blood) and CNS (cerebellum, hippocampus, striatum and cortex) tissues. DMF-dependent transcription differed between the genes regulated within these tissue types as well as the onset and duration of the response (**FIGURES 38-41; Table 16**).

DMF-dependent transcription differed between the specific genes regulated within tissue types, with each tissue exhibiting a distinct profile of the 48 genes selected for analysis. In **FIGURES 38** and **39**, identified DMF-regulated genes in peripheral tissues were graphed to show the differential expression of these genes in various tissues types against VEGFA, a non-

regulated baseline control gene. Identified genes included ARB18, GCLC, GDF15, NQO1, SRXN1, SQSTM1, TXNRD1, OSGIN1 and RBP4. Within peripheral tissues, less regulation in these genes was seen to occur in the liver and spleen compared to the kidney and jejunum, with the largest number of genes being regulated in the kidney and the largest magnitude of response occurring within the jejunum (**FIGURE 38**). Classical Nrf2-regulated genes such as AKRB18, GCLC, NQO1, SRXN1 and TXNRD1 showed fairly universal expression across the four analyzed peripheral tissues compared to more localized expression in specific peripheral regions for GDF15, SQSTM1, RBP4 and OSGIN1 (**FIGURES 38 and 39**).

Gene regulation following DMF treatment in CNS tissues was defined by a smaller subset of gene changes compared to the periphery, with gene transcription only affecting levels of NQO1, OSGIN1 and BDNF (**FIGURES 40 and 41**). Furthermore, as was seen in the periphery, the expression of these genes was differentially regulated in the four analyzed brain regions. NQO1 levels only changed slightly (1.5 to 2-fold) within brain tissues, with the exception of the striatum where no expression of NQO1 could be detected at the selected time points. The largest and most universal transcriptional changes within the brain were seen with OSGIN gene regulation, which expressed changes up to 4-5-fold above vehicle values in the hippocampus and cortex. OSGIN changes could also be detected within the cerebellum and striatum albeit at lower expression levels. In contrast to OSGIN and NQO1 in the brain, BDNF expression was unique to the striatum following DMF treatment.

Transcriptional changes following DMF treatment also resulted in distinct patterns in the onset and duration of the response. Within peripheral tissues, gene regulation peaked between 4 and 8 hours post-dose with the exception of OSGIN1 and GDF15 in the kidney, which exhibited an earlier peak regulation of around 2 hours (**FIGURE 38**). For the few genes regulated within CNS tissues, gene expression also tended to peak at an earlier time point between 2 and 6 hours following DMF dosing (**FIGURES 40 and 41**). For most regulated genes in most tissues, overall expression gradually decreased to baseline between 12 and 24 hours; however, when expressed, NQO1 and AKR1B8 tended to have more prolonged response in tissues. This can be seen in the small change in NQO1 within the CNS, as well as the more pronounced regulation of NQO1 and AKR1B8 within the kidney and jejunum. Although CNS regulation of NQO1 was slight, the prolonged expression tended to last out to 16.5 hours. In the kidney and jejunum prolonged expression continued past 24 hours. A unique time course following DMF could also be seen for

BDNF expression in the striatum and RBP4 in the spleen, with peak regulation of these genes occurring at 4 hours post-dose and then dropping below normal levels before returning to baseline at around 16 hours.

Table 15. *Gene selections for Fluidigm real-time PCR.*

GO TERM	GO ID	Gene Name	Gene Symbol
Autophagy	GO:0006914	ATG2 autophagy related 2 homolog A	ATG2A
		sequestosome 1 (p62)	SQSTM1
Cell cycle	GO:0007049	cyclin-dependent kinase inhibitor 1A (P21)	CDKN1A
		growth differentiation factor 15	GDF15
Cell differentiation	GO:0030154	vesicular endothelial growth factor	VEGFA
Cellular matrix organization	GO:0030198	matrix metalloproteinase 11 (stromelysin 3)	MMP11
Cell migration	GO:0016477	snail homolog 1	SNAI1
Cholesterol homeostasis		ATP-binding cassette, sub-family A (ABC1), member 1	ABCA1
Developmental growth	GO:0048589	stimulated by retinoic acid gene 6 homolog (mouse)	STRA6
Dopamine receptor signaling pathway	GO:0007212	neuron-specific protein family member 2	NSG2
Glutamine biosynthesis process	GO:0005642	glutamate-ammonia ligase	GLUL
L-glutamate transport	GO:0015813	solute carrier family 1, member 2 (EAAT2)	SLC1A2
Metabolic process	GO:0008152	glutathione S-transferase alpha 2	GSTA2
Myelination	GO:0042552	early growth response 2	EGR2

Negative regulation of apoptotic process	GO:0043065	DnaJ (Hsp40) homolog, subfamily A, member 3	DNAJA3
		apoptosis antagonizing transcription factor	AATF
		nerve growth factor- γ	NGFG
Negative regulation of cell growth	GO:0008083	Oxidative stress induced growth inhibitor 1	OSGIN1
		nitric oxide synthase 1	NOS1
		sirtuin 1	SIRT1
Neuron development	GO:0048666	nuclear receptor subfamily 2, group F, member 6	NR2F6
Neuron fate commitment	GO:0048663	oligodendrocyte transcription factor 1	OLIG1
Nucleoside diphosphate metabolic process	GO:0009132	nudix (nucleoside diphosphate linked moiety X)	NUDT7
Oxidation-reduction process	GO:0055114	aldo-keto reductase family 1, member B8	AKR1B8
		copper chaperone for superoxide dismutase	CCS
		oxidoreductase NAD-binding domain containing 1	OXNAD1
Positive regulation of excitatory postsynaptic membrane potential	GO:2000463	neuropilin (NRP) and tolloid (TLL)-like 2	NETO2
Regulation of cell growth	GO:0001558	insulin-like growth factor binding protein	IGFBP
Regulation of synaptic plasticity	GO:0048167	brain-derived neurotrophic factor	BDNF
Response to oxidative stress	GO:0006979	amyotrophic lateral sclerosis 2 (juvenile)	ALS2
		apolipoprotein E	APOE
		GRB2-associated binding protein 1	GAB1

		glutamate-cysteine ligase, catalytic subunit	GCLC
		glutamate-cysteine ligase, modifier subunit	GCLM
		NAD(P)H dehydrogenase, quinone 1	NQO1
		peroxiredoxin 1	PRDX1
		prion protein	PRNP
		prostaglandin-endoperoxide synthase 2	PTGS2
		superoxide dismutase	SOD1
		sulfiredoxin 1	SRXN1
		thioredoxin reductase 1	TXNRD1
		glial fibrillary acidic protein	GFAP
Retinol metabolic process	GO:0042574	retinol binding protein 4, plasma	RBP4
Signal transduction	GO:0007165	NF-kappa-B inhibitor, beta	NFKBIB
Tissue regeneration	GO:0042246	ninjurin 1	NINJ1

Table16. *Transcriptional profiling time course statistical analysis.*

Tissue	Gene	P-value 2 hrs	P-value 4 hrs	P-value 6 hrs	P-value 8 hrs	P-value 12.5 hrs	P-value 16.5 hrs	P-value 23 hrs	P-value 37 hrs
Liver	AKR1B8	0.51	0.008*	0.0011*	0.013	0.010	0.99	0.15	0.88
	GCLC	0.0007*	0.0005*	0.0016*	0.029	0.021	0.027	0.0056*	0.74
	NQO1	0.88	0.15	0.0081*	0.0042*	0.0015*	0.14	0.0059*	0.013
	OSGIN1	0.0048*	0.21	0.19	0.79	0.022	0.13	0.27	0.30

	SRXN1	0.17	< 0.0001*	< 0.0001*	0.0005*	0.0007*	0.36	0.21	0.021
Spleen	NQO1	0.14	0.98	0.064	0.0046*	0.046	0.038	0.99	0.93
	OSGIN1	0.078	0.0012*	< 0.0001*	0.0039*	0.29	0.44	0.31	0.76
	SQSTM1	0.44	0.032	0.041	0.0035*	0.38	0.70	0.35	0.010
	SRXN1	0.050	0.030	0.017	0.0008*	0.011	0.029	0.22	0.54
	TXNRD1	0.0087*	< 0.0001*	< 0.0001*	< 0.0001*	0.0091*	0.98	0.57	0.67
Kidney	AKR1B8	0.82	0.082	0.0061*	0.0075*	0.0012*	0.028	0.31	0.86
	GCLC	0.081	0.0003*	0.0001*	0.0026*	0.019	0.098	0.14	0.028
	GDF15	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	0.0001*	0.0041*	0.038
	NQO1	0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	0.18
	OSGIN1	< 0.0001*	< 0.0001*	0.066	0.0056*	0.015	0.50	0.025	0.088
	SQSTM1	0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	0.0047*	0.19	0.84	0.45
	SRXN1	0.0009*	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	0.0052*	0.16	0.026
	TXNRD1	0.0002*	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	0.0030*	0.28	.002*
Jejun.	AKR1B8	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	0.22
	GCLC	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	0.0001*	0.0007*	0.060
	NQO1	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	.003*
	OSGIN1	.0002*	< 0.0001*	0.011	0.017	0.13	0.012	0.56	0.22
	SRXN1	0.0029*	0.0003*	0.0002*	0.0011*	0.0051*	0.037	0.28	0.31
	TXNRD1	0.0009*	< 0.0001*	< 0.0001*	0.0017*	0.0019*	0.0039*	0.056	0.68
Cortex	OSGIN1	0.0019*	0.0020*	0.097	0.16	0.036	0.36	0.37	0.36
Cereb.	OSGIN1	< 0.0001*	< 0.0001*	0.0033*	0.0048*	0.99	0.36	0.028	0.12
Hippo.	NQO1	0.28	0.51	0.0035*	0.26	0.30	0.35	0.79	0.65
	OSGIN1	0.0005*	0.012	0.0044*	0.052	0.55	0.50	0.37	0.61

Jejun.=jejunum; Cereb.=cerebellum; Hipp.=hippocampus. * represent of $p < .01$ based on Wilcoxon signed-rank test.

The magnitude of DMF-induced gene expression is dose-dependent

Dose-dependent changes in DMF-induced gene expression were also measured against the gene panel (**Table 15**) analyzed in the abovementioned time course using the Fluidigm BioMark™ HD System. This study aimed to capture DMF-dependent transcriptional changes with increasing concentrations of DMF that ranged from 50 to 600mg/kg. DMF concentrations were administered at a constant time point of 7 hours that was selected based on optimal gene regulation across tissues identified in the previous time course. Data from this study shows the magnitude of DMF transcriptional response to be dose-dependent for all genes in all tissues tested except for the BDNF response in striatal tissue (**FIGURE 42-45; Table 17**). In contrast to all other regulated genes in the periphery and CNS which showed dose-dependent expression of genes previously identified in the time course, BDNF expression in the striatum increased at lower doses of 50 and 100 mg/kg but returned to baseline at concentrations above 100 mg/kg.

In the presence of higher DMF concentrations, genes not identified in the time course study were found to be induced. The most obvious example of this is the expression of CDKN1A at concentrations above 100mg/kg within the liver, kidney and all CNS tissues (**FIGURES 42-45**). Interestingly, the liver, which did not express large transcriptional changes in the time course study, amplified CDKN1A over 40-fold at the highest dose following DMF treatment (**FIGURE 43B**). Furthermore, AKR1B8, which had the most profound expression in the jejunum in the time course study, was surpassed in expression by GSTA2 at concentrations above 200mg/kg (**FIGURE 42B**). Other genes that increased in expression at higher doses with DMF included NETO, IGFBP3 and NINJ1 in the spleen and GSTA2 in the jejunum. Reductions in gene expression were also seen at higher doses of DMF including NUDT7 in the liver and OLIG1 in the kidney (**FIGURES 42A and 43B**).

In the CNS, the cortex, cerebellum and hippocampus portrayed similar gene regulation patterns for CDKN1A, OSGIN1 and NQO1 following higher concentrations of DMF, with the largest magnitude of expression seen in OSGIN1 (**FIGURES 44 and 45A**). In contrast to the

latter tissues and as seen in the time course data, the striatum continued to show differential regulation of genes in the presence of DMF compared to other CNS brain regions (**FIGURE 45B**). As mentioned above, BDNF regulation was only present in the striatum and only at low doses; however, there were also reductions in GAB1 and EGR2 seen within the striatum with increasing concentrations of DMF that reduced the expression of these in half. Furthermore, unlike neighboring brain regions, the striatum did not show dose-dependent increases in NQO1.

Table 17. *Transcriptional profiling dose response statistical analysis.*

Tissue	Gene	P-value	P-value	P-value	P-value	P-value
		50mpk	100mpk	200mpk	400mpk	600mpk
Liver	AKR1B8	0.2078	0.0180*	0.0006#	< 0.0001#	< 0.0001#
	CDKN1A	> 0.9999	0.9999	0.9958	0.1512	0.0019#
	GCLC	0.3035	0.1414	0.1571	0.0007#	< 0.0001#
	NQO1	0.1285	< 0.0001#	0.0028#	< 0.0001#	< 0.0001#
	NUDT7	0.6611	0.8941	0.3174	0.0006#	0.0001#
	SQSTM1	0.7532	0.3684	0.1855	0.0041#	0.0026#
	SRXN1	0.8096	0.0288*	0.0502	0.0149*	0.0006#
Spleen	GDF15	0.9999	0.9957	0.9179	0.0373*	0.0006#
	GLUL	0.9946	0.5730	0.4302	0.0019#	< 0.0001#
	NINJ1	0.6906	0.0910	0.0240*	< 0.0001#	< 0.0001#
	NQO1	0.9579	0.5392	0.3811	0.0632	0.0088#
	OSGIN1	0.9673	0.8205	0.0392*	< 0.0001#	< 0.0001#
	SQSTM1	0.9997	0.2289	0.3902	0.0081#	< 0.0001#
	SRXN1	0.9922	0.4780	0.5188	0.0456*	0.0022#
	TXNRD1	0.9997	0.1512	0.2418	0.0005#	< 0.0001#

Kidney	GCLC	0.5500	0.0357*	0.1326	< 0.0001#	0.0002#
	GCLM	0.0159*	0.0004#	0.0003#	< 0.0001#	< 0.0001#
	GDF15	0.9164	0.2127	0.1689	0.0002#	< 0.0001#
	GSTA2	0.7974	0.0005#	0.0260*	0.0040#	< 0.0001#
	NQO1	0.0183*	< 0.0001#	< 0.0001#	< 0.0001#	< 0.0001#
	OSGIN1	0.7770	0.5161	0.0548	< 0.0001#	< 0.0001#
	PRDX1	0.1131	0.0086#	0.0307*	0.0012#	0.0036#
	PRNP	0.2796	0.1519	0.1220	0.0046#	0.0410*
	SQSTM1	0.1257	0.0044#	0.0008#	< 0.0001#	< 0.0001#
	SRXN1	0.8774	0.1240	0.1268	< 0.0001#	< 0.0001#
	TXNRD1	0.0692	< 0.0001#	< 0.0001#	< 0.0001#	< 0.0001#
Jejunum	AKR1B8	< .0001#	< 0.0001#	< 0.0001#	< 0.0001#	< 0.0001#
	CDKN1A	0.9997	0.9813	0.5640	0.0921	0.0104*
	GCLC	0.8189	0.0265*	0.0142*	< 0.0001#	< 0.0001#
	GCLM	0.3128	0.0194*	0.0005#	< 0.0001#	< 0.0001#
	GDF15	0.9054	0.8633	0.9174	0.2186	0.0036#
	GSTA2	0.9806	0.3975	0.1193	< 0.0001#	0.0037#
	NQO1	0.0186*	0.0002#	< 0.0001#	< 0.0001#	< 0.0001#
	OSGIN1	0.5395	0.3733	0.0596	< 0.0001#	0.0003#
	SRXN1	0.4836	0.1905	0.0461*	0.0047#	0.0007#
	TXNRD1	0.0717	0.0050#	0.0015#	< 0.0001#	< 0.0001#
Cortex	CDKN1A	0.9867	0.9984	0.9786	0.0358*	0.0003#
	GCLC	0.3499	0.5443	0.4165	0.0060#	0.0002#
	IGFBP3	0.9981	0.8788	0.9918	0.0358*	0.0006#

	NQO1	0.9914	0.0036#	0.0008#	< 0.0001#	< 0.0001#
	OSGIN1	0.9949	0.9763	0.1509	0.0062#	0.0321*
Cerebellum	CDKN1A	0.9999	0.9938	0.4639	0.0002#	< 0.0001#
	GAB1	0.8488	0.8932	0.6950	0.0346*	0.0099#
	NQO1	0.4832	0.0133*	0.1517	< 0.0001#	< 0.0001#
	OSGIN1	0.6673	0.4070	0.1447	0.1406	0.0292*
Hippocampus	CDKN1A	> 0.9999	0.9915	0.7042	0.0002#	< 0.0001#
	GAB1	0.9997	0.6539	0.7792	0.0890	0.0322*
	GCLC	0.4169	0.0009#	0.2017	< 0.0001#	0.0013#
	NQO1	0.3692	0.0241	0.2579	0.0091#	0.0021#
	OSGIN1	0.9936	0.8693	0.6327	0.2702	0.0146*
Striatum	BDNF	0.4307	0.0062#	0.8007	0.9594	0.3467
	CDKN1A	0.9999	0.9982	0.7790	0.0051#	< 0.0001#
	EGR2	0.9999	0.1539	0.5928	0.0008#	0.0016#
	GAB1	0.9025	0.9934	0.8852	0.0431*	0.0011#
Whole Blood	GCLC	0.9998	0.7210	0.0478*	0.0010#	0.0041#
	NINJ1	0.7168	0.9965	0.1031	0.0236*	0.0006#
	NGFG	> 0.9999	0.9719	0.5310	0.0098#	0.0016#
	NQO1	0.9986	0.9877	0.5893	0.6577	0.0434*
	SRXN1	0.8727	0.8770	0.0374*	0.0350*	0.0038#
	TXNRD1	0.3182	0.4550	0.1012	0.0248*	0.0260*

*= $p < .05$ and #= $p < .01$ based on one-way analysis of variance (ANOVA) performed with Dunnett's multiple comparison test

DMF transcriptional regulation in whole blood

Gene regulation following DMF treatment in the time course and dose response paradigms was also measured in whole blood samples for each animal analyzed. Whole blood data tended to be more variable than results detected in peripheral and CNS tissues; however, 2-fold increases in GFAP, PRDX1, OSGIN1 and NQO1 could be detected after a single 100 mg/kg dose of DMF (**FIGURE 46A**). These gene expression increases peaked at 2 hours for OSGIN1, between 4 and 6 hours for GFAP and PRDX1, and 8 hours for NQO1. Differential gene regulation in whole blood could also be detected with higher concentrations of DMF (**FIGURE 46B**) in a dose-dependent manner at a single 7 hour time point. Increased expression of GCLC and NINJ1 were detected at concentrations ≥ 200 mg/kg and decreased expression of NFKBIB and NSG2 were also identified at higher concentrations of DMF.

DMF-induced gene expression is Nrf2-dependent

To determine if the DMF-induced transcriptional changes identified in the aforementioned time course and dose response studies were Nrf2-dependent, we measured the regulation of 5 of these identified genes in an Nrf2 knockout mouse. The panel of five genes included NQO1, AKR1B8, GDF15, OSGIN1 and BDNF, and were measured for transcriptional regulation at 4, 8 and 16 hours post 100mg/kg DMF. This small gene panel was selected to specifically measure genes that were of particular interest and/or genes that are not generally identified as classical Nrf2-regulated genes. In the absence of Nrf2, our results indicate a loss of NQO1, AKR1B8, GDF15 and OSGIN1 expression following dosing with a single concentration of DMF in the periphery and the CNS (**FIGURES 47 and 48**). Furthermore, the baseline expression of these genes in the knockout animals was decreased below endogenous expression levels of wild type controls in tissues where these genes could be induced by DMF.

Of these five analyzed genes BDNF was the only target that did not result in a complete loss of DMF-induced signal (**FIGURE 48B**). Although variable, loss of Nrf2 resulted in increased levels of BDNF expression at baseline, and in the presence of DMF these increases were gradually returned to the level of wild type values over time.

DMF transcriptional regulation translates to protein expression

To confirm that transcriptional regulation of Nrf2 target genes translate to protein following DMF administration, western blot analysis was conducted to analyze protein levels of genes identified to be regulated following transcriptional analysis. Protein levels in tissue extracts from Nrf2^{-/-} and wild type mice 6 hours post 100mg/kg DMF administration were analyzed for expression changes of NQO1, TXNRD1, GCLC and SQSTM1 in peripheral tissues (**FIGURE 49**) and NQO1 and BDNF in CNS tissues (**FIGURE 50**).

Protein analysis in peripheral tissue extracts identified increased expression of selected proteins following DMF treatment in kidney (**FIGURE 49A**) and jejunum (**FIGURE 49B**). In the absence of Nrf2, these protein changes were either decreased or lost following DMF administration. In CNS tissues, basal levels of BDNF in the striatum (**FIGURE 50A**) and NQO1 in the cortex (**FIGURE 50B**) were not altered in the absence of Nrf2; however, although variable, a trend of increased protein expression in the presence of DMF could be identified.

Discussion

Recent studies have identified inflammation and generation of excessive oxidative stress as contributing factors in a number of neurodegenerative disorders. Reactive oxygen species (ROS), a natural byproduct of cellular metabolism, exists in all aerobic cells in a physiological balance with neutralizing antioxidants (Andersen et al., 2004). However, when this balance is disrupted and ROS production surpasses antioxidant capacity, toxic free radicals can damage cells leading to oxidative stress (OS) (Orient et al., 2007). Inflammatory processes are also linked to the generation of excessive oxidative stress; the release of significant ROS via a “metabolic burst” mechanism from activated microglia and infiltrating macrophages in the CNS. Disturbances in the normal redox state, either through dysregulation of normal homeostatic processes or from aberrant inflammatory activity in the CNS, are believed to be a common pathogenic mechanism in aging as well as in neurodegenerative diseases such as Parkinson’s disease (Jenner, 2007), Alzheimer’s disease (Emerit et al., 2004), amyotrophic lateral sclerosis (Carrì et al., 2003) and multiple sclerosis (Offen et al., 2004). Previous studies have suggested that targeting the Nrf2 pathway could be a promising therapeutic target for these diseases since this pathway is a ubiquitously expressed defense system for combatting against inflammatory, oxidative and electrophilic stress.

A functional Nrf2 pathway has been shown to be required for DMF protection against toxic oxidative stress *in vitro* (Scannevin et al, 2012), and for mediating DMF efficacy *in vivo* in the EAE model (Linker et al, 2011). The primary aim of our current study was to further characterize DMF pharmacodynamic responses throughout the CNS and periphery, and also to understand the requirement for Nrf2 in mediating these responses. These experiments provide further understanding of the specific genes, onset of expression, duration and relative magnitude of DMF pharmacodynamic responses that occur across many different tissues. These data will help inform the sequence of events involved with DMF-mediated anti-inflammatory effects and cellular protection.

Our results suggest that DMF-induces distinct gene changes within peripheral and CNS tissues, with differentiation in the onset and duration of the response observed between genes and tissue type. These data also support earlier studies demonstrating that BG-12 activates transcription of Nrf2-dependent genes in the CNS and periphery. Although many classical Nrf2-dependent genes, such as NQO1, AKR1B8, GCLC, SRXN1 and TRXND1, were found to be regulated across many tissue types, many gene expression changes specific to certain tissues were also identified, including RPB4 in the spleen and BDNF in the striatum (**FIGURES 39A and 40B**).

The observations of tissue-specific gene expression suggest additional levels of transcriptional regulation that are specific to individual mRNA and particular cells types. The data also suggest that the dosage of DMF and corresponding relative DMF/MMF exposure may potentially effect transcriptional regulation of specific genes in different tissue types, based on the observation that increased concentrations of DMF can induce unique gene regulation in tissues that were not originally detected at lower doses. In addition to distinct gene changes, most transcriptional changes tended to be transient, with expression levels peaking between 2 and 8 hours post-dose (**FIGURES 38-41**). As there was differentiation in the persistence of individual mRNA, even those induced to a similar peak magnitude (**FIGURE 38B**. NQO1 vs GCLC), this suggests that specific mRNA stability may be governed by multiple pathways. Notably, some of these responses persisted for an extended period of time before returning to baseline (**FIGURE 38A and B**; NQO1, AKR1B8).

A goal of the study was to characterize CNS gene regulation following DMF treatment to a greater detail. The presented data suggests that the bioactive metabolite of DMF, MMF, can cross the blood-brain barrier (**FIGURE 37**) and induce differential gene expression in various brain regions. Although the regulation of genes in the CNS did not appear as robust in magnitude or in overall number of genes as within the periphery, small but significant changes in the classical Nrf2 target gene NQO1 as well as novel expression of OSGIN1 throughout all brain regions were identified (**FIGURES 40** and **41**). Although one study identifies OSGIN1 as a candidate Nrf2 target, this analysis supports in an in vivo model that Osgin1 expression is mediated via Nrf2 (Chorley et al, 2012). Current literature on OSGIN1 suggests that various splice-variants of this gene interact with p53 to mediate cell death and survival within the cell (Hu et al, 2012).

BDNF has also been shown to be regulated in the brain by DMF, specifically within the striatum; however, results from Nrf2^{-/-} studies suggest that BDNF is not Nrf2-dependent. Moreover, basal expression of BDNF may increase in the absence of Nrf2, and DMF may reduce expression levels back to baseline.

The transcriptional profiling data reported herein suggests differential gene regulation across tissue types following treatment with DMF, which gives insight into the unique Nrf2-dependent pathways stimulated, or repressed, within distinct tissues and biofluids.

Example 9: OSGIN1 contributes to the Cytoprotective Properties of Monomethyl Fumarate (MMF)

RATIONALE

Previous studies using DMF and its bioactive metabolite, MMF, show Nrf2 stabilization and increased antioxidant protein expression following treatment of human astrocyte and oligodendrocyte cultures (Nguyen et al., 2009, Scannevin et al., 2012). Furthermore, MMF treatment in these cultures systems, as well as in primary mixed neuronal cultures, can protect against oxidative injury induced by hydrogen peroxide. This neuronal protection is also evident in the ability of DMF/MMF to improve clinical score and reduce neuroinflammation in the rat

EAE model of MS (Linker et al., 2011). There is strong evidence that DMF/MMF increase cellular levels of Nrf2 resulting in antioxidant gene upregulation. Understanding the specific sequence of events involved with Nrf2-mediated cellular protection is important for delineating the role fumarates play in these pathways. Following the identification of OSGIN1 as an Nrf2 transcriptional target that is upregulated in the brain following oral administration of DMF, the importance of OSGIN1 in DMF-mediated cytoprotection was investigated.

RESULTS

MMF induces Nrf2 target genes and is protective against oxidative insult

Based on current literature that suggests Nrf2 regulation in the CNS is predominantly via astrocytes, primary human astrocytes were measured for Nrf2 target gene regulation following treatment with MMF (Vargas et al., 2009, Miao et al., 2011). As previously mentioned, DMF is rapidly metabolized to MMF upon oral administration and only MMF exposure is known be detected in the CNS (**FIGURE 37**; Werdenberg et al, 2003); therefore, to parallel *in vivo* all *in vitro* cultures were treated with MMF. To confirm that OSGIN1 is endogenously expressed in human astrocytes and confirm OSGIN1 induction with MMF treatment, OSGIN1 and five classical Nrf2 targets were analyzed via q-PCR. Following 24 hour treatment with MMF, q-PCR analysis identified OSGIN1 to be dose-dependently induced to a greater extent than other classical Nrf2 target genes such as NQO1, TXNRD1, SRXN1, GCLC and HMOX1 in the presence of MMF (**FIGURE 51**). These results support *in vivo* studies that OSGIN1 is a MMF-regulated transcriptional target and that MMF regulates Nrf2 target genes.

Since the Nrf2 pathway is considered the major cellular response pathway to oxidative and electrophilic stress, the effect of MMF on cellular viability in the presence of oxidative insult was evaluated. Previous studies investigating the cytoprotective effects of MMF have shown this compound to protect human astrocytes from the oxidative insult hydrogen peroxide (H₂O₂) (Scannevin et al, 2012). To find whether MMF can protect cells from oxidative damage, human astrocytes were treated with a titration of MMF for 24 hours followed by oxidative challenge with H₂O₂ (**FIGURE 52**). To measure viability of cells following experimentation, LIVE/DEAD analysis was performed. Addition of 300μM H₂O₂ to DMSO-treated cells resulted in approximately 85% reduction in LIVE fluorescence intensity measured by calcein-AM

incorporation into cells. In the presence of MMF, astrocytes challenged with H₂O₂ were protected against the toxic effects of this insult resulting in only a 50% reduction in cell loss (**FIGURE 52D**). Also, images were acquired from samples analyzed in the LIVE/DEAD assay (**FIGURE 52A-C**). H₂O₂ challenge alone resulted in a dramatic loss of cell number as well as an increase in dying cells (**FIGURE 52B**). In comparison, pre-treatment with MMF was able to preserve cellular viability of astrocytes and reduce cell death (**FIGURE 52C**). These data support previous studies that MMF is cytoprotective against the effects of oxidative stress *in vitro*. Furthermore, the increases in OSGIN1 (**FIGURE 52B**) following MMF treatment correlate with increased cellular survival against H₂O₂, suggesting this gene may play a mechanistic role in this paradigm.

Loss of Nrf2 abolishes MMF cytoprotection and depletes OSGIN1

Following confirmation of MMF-mediated cytoprotection against oxidative insult, the importance of Nrf2 in this protection was assessed. Human astrocytes were transfected with siRNA targeted against Nrf2 to reduce mRNA and protein levels of this gene. Nrf2 siRNA transfection resulted in an approximate 80% reduction in Nrf2 mRNA levels and almost a complete loss of Nrf2 protein expression (**FIGURE 53B**). In the presence of MMF, no significant regulation of Nrf2 transcript was observed in either control or Nrf2 siRNA transfected astrocytes (**FIGURE 53A**). Since Nrf2 is constitutively expressed and degraded under normal homeostatic conditions by the protein Keap1 and inhibition of this interaction is believed to regulate Nrf2 protein expression (see section 2.3 of Chapter I; Itoh et al., 1997), no effect on Nrf2 mRNA induction is expected. MMF is believed to interact with the cysteine residues on Keap1 to inhibit the degradation of Nrf2 and allow for Nrf2 accumulation (Linker et al., 2011). Protein accumulation of Nrf2 following MMF treatment was observed with control siRNA and these accumulations were lost in cells transfected with Nrf2 siRNA (**FIGURE 53B**). These findings support previous findings that MMF regulates Nrf2 expression on a protein level to activate this pathway (Scannevin et al., 2012).

To determine if the loss of Nrf2 expression in human astrocytes correlates with the reduction in OSGIN1 expression seen in *Nrf2*^{-/-} mice, q-PCR analysis was conducted to measure OSGIN1 transcript levels following Nrf2 knockdown (**FIGURE 54**). Loss of Nrf2

reduced total OSGIN1 expression in astrocytes by greater than 60% and significantly reduced the ability of MMF to induce OSGIN1 transcript levels. This supports *in vivo* Nrf2 knockout studies where induction of OSGIN1 was found to be strongly regulated by Nrf2 and Nrf2 was necessary for MMF-mediated regulation of OSGIN1. Although other Nrf2 transcriptional targets have been identified to be mediated by MMF *in vitro* (Scannevin et al., 2012), OSGIN1 is the most significantly regulated Nrf2 target shown to be mediated in the presence of MMF, suggesting that this gene is an important target of Nrf2.

In order to determine if activation of the Nrf2 pathway contributes to the cytoprotective properties of MMF, human astrocytes were transfected with Nrf2 siRNA and treated with MMF for 24 hours followed by oxidative challenge with H₂O₂. In the absence of Nrf2, MMF-mediated cytoprotection of human astrocytes was completely abolished, as can be seen in both LIVE/DEAD acquired images and viable nuclear DAPI counts (**FIGURE 55**). Nrf2-deficient astrocytes also demonstrated increased sensitivity to oxidative insult as seen in the reduced viability of DMSO-treated cells lacking Nrf2 compared to control transfected cells. These results support previous findings that activation of the Nrf2 pathway is important for MMF-mediated protection against oxidative damage in astrocytes.

Loss of OSGIN1 reduces MMF cytoprotection in the presence of oxidative insult

OSGIN1 was first identified as an Nrf2 target in *in vitro* studies where the loss of Nrf2 inhibited the ability of the oxidation product, Ox-PAPC, to induce OSGIN1 (Li et al, 2006). Although these studies were confirmed in 2014 by Yan et al, further understanding of the role of OSGIN1 as an Nrf2 target has not been thoroughly investigated (Yan et al, 2014). The *in vivo* findings presented herein as well as the inability of MMF to induce OSGIN1 in the absence of Nrf2, suggest that OSGIN1 may be involved in mediating some of the protective effects of activating the Nrf2 pathway. To investigate the possibility of a role for OSGIN1 in MMF-mediated cytoprotection, human astrocytes were transfected with OSGIN1 siRNA to diminish OSGIN1 levels and the effect on oxidative challenge was studied as previously described with Nrf2 siRNA knockdown.

siRNA knockdown of OSGIN1 resulted in greater than 70% loss of OSGIN1 transcript levels compared to scrambled siRNA control (**FIGURE 56A**). In the presence of MMF,

OSGIN1 induction was also significantly diminished following reduction in basal levels of OSGIN1 (**FIGURE 56A**). Furthermore OSGIN1 knockdown did not affect the level of Nrf2 transcripts, supporting findings described herein that OSGIN1 is a downstream target of Nrf2 (**FIGURE 27B**).

To determine if OSGIN1 induction contributes to MMF-mediated cytoprotection, astrocytes transfected with OSGIN1 siRNA were treated with a titration of MMF for 24 hours followed by oxidative challenge with H₂O₂. Knockdown of OSGIN1 resulted in a reduced ability of astrocytes to be protected against H₂O₂ following MMF treatment. This can be visualized in the increased number of viable cells indicated by LIVE-positive staining and reductions in overall DEAD-positive cells in LIVE/DEAD acquired images (**FIGURE 57A**). These findings were further quantified by fluorescence measurement of LIVE-positive cells and viable nuclear DAPI count (**FIGURE 57B** and **C**). In comparison to Nrf2 siRNA transfection (**FIGURE 55**), OSGIN1 knockdown did not completely abolish MMF-mediated cytoprotection. Although this could be a result of incomplete loss of OSGIN1 in this cellular system, it may be more likely that other transcriptional targets of Nrf2 also contribute to the protection inferred by MMF.

OSGIN1 siRNA transfection in the presence of H₂O₂ alone tended to be slightly more resistant to oxidative damage compared to scrambled siRNA control astrocytes (**FIGURE 57**). Although there was not a significant difference observed in total cell count between OSGIN1 and control siRNA transfected astrocytes, this could still be due to increased proliferation in the absence of OSGIN1. OSGIN1 has previously been identified to regulate cell cycle and loss of OSGIN1 has been shown to induce proliferation of cancer cell lines (Ong et al., 2004; Huynh et al. 2001, Wang et al., 2005).

Identification of OSGIN1 isoform induction in the presence of MMF

Generation of OSGIN1 isoform specific antibodies: OSGIN1 undergoes alternative splicing to yield various isoforms, three of which have been investigated by researchers (Ong et al., 2004). These include variants encoding for 52kDa and 61kDa ORF's, as well as a third isoform encoding a 38kDa ORF that has been researched but is not accepted as a likely variant. Research investigating the importance of these variants suggests that individual regulation of OSGIN1 isoforms may yield divergent biological functions (Hu et al., 2012). Therefore,

following identification of OSGIN1 as a downstream target of Nrf2 that contributes to MMF cytoprotection, investigation into the regulation of OSGIN1 isoforms in the presence of MMF was pursued. To deduce whether OSGIN1 variants are differentially regulated by MMF, three isoform-specific antibodies were generated using affinity purification conducted by New England Peptide (NEP) that recognize the following three human OSGIN1 variants; OSGIN1-38kDa, OSGIN1-52kDa and OSGIN1-61kDa. Based on the splicing characteristics of these isoforms, only the OSGIN1-61kDa antibody should be unique, with the OSGIN1-52kDa antibody recognizing both the 52kDa and 61kDa forms and the OSGIN1-38kDa antibody recognizing all three variants (section 1.2.1 of Chapter II; **FIGURE 36**).

To determine the specificity of these antibodies, peptide competition studies were conducted. In these studies human astrocyte cell lysates were probed with three different conditions; antibody alone (NP), antibody pre-incubated with antibody specific peptide (P) and antibody pre-incubated with non-specific control peptide (CP) (**FIGURE 58**). Peptides generated against the different isoforms were used as control peptides (example: OSGIN1-52kDa peptide was a control for OSGIN1-61kDa antibody optimization). OSGIN1-52kDa and OSGIN1-61kDa antibodies detected specific immunoreactive bands that correlated with the appropriate predicted size of their amino acid sequence (**FIGURE 58**). Both of these bands were lost when the antibody was pre-incubated with an epitope specific peptide but not with a control peptide. Interestingly, pre-incubation of the OSGIN1-61kDa antibody with the control peptide (in this case the OSGIN1-52kDa peptide) resulted in depletion of a lower immunoreactive band (**FIGURE 53B**). This could be a result of OSGIN1-52kDa antibody contamination in the OSGIN1-61kDa antibody pool, particularly since this band runs at a similar size to the 52kDa OSGIN1 protein. No immunoreactive bands were identified in the presence of the OSGIN1-38kDa antibody.

Following optimization of the OSGIN1-52kDa and OSGIN1-61kDa antibodies, siRNA knockdown of OSGIN1 in human astrocytes was conducted to determine if immunoreactive bands associated with these antibodies were depleted in the absence of OSGIN1. Loss of OSGIN1 correlated with depletion of immunoreactive bands identified during optimization, with almost complete loss of the OSGIN1-61kDa immunoreactive band (**FIGURE 59**). Abundance of the OSGIN1-52kDa immunoreactive band seemed to be higher than that of OSGIN1-61kDa;

however, whether this difference was due to antibody specificity is unclear. However, the modest reduction observed with the OSGIN1-52kDa antibody following OSGIN1 knockdown may be indicative of incomplete knockdown and suggest that this isoform is more abundant.

Evidence for induction of OSGIN1-61kDa isoform in the presence of MMF

To determine if OSGIN1 isoforms are differentially regulated in the presence of MMF, human astrocytes were transfected with either control or OSGIN1 specific siRNA followed by treatment with MMF for 24 hours. OSGIN1-52kDa and OSGIN1-61kDa antibodies were probed against cell lysates via western blot (**FIGURE 60**). Although a similar reduction in immunoreactivity was observed with the OSGIN1-52kDa antibody similar to previous findings, no induction was seen in the presence of MMF (**FIGURE 60A**). In contrast, control siRNA transfected astrocytes probed with the OSGIN1-61kDa antibody detected an increase in immunoreactivity in the presence of 30uM MMF which was lost in the absence of OSGIN1 (**FIGURE 60B**). These findings suggest that addition of MMF to astrocytes specifically induces expression of the 61kDa encoding OSGIN1 ORF.

Overexpression of the 61kDa encoding OSGIN1 isoform has been shown to be less toxic to tumorigenic cell lines, suggesting this specific variant may function independently of apoptotic induction and thus may be regulated differentially. Since OSGIN1 is an Nrf2-regulated target gene and loss of Nrf2 reduces OSGIN1 transcript expression, the regulation of the identified OSGIN1-61kDa reactive band was examined following Nrf2 depletion. Human astrocytes were transfected with control or Nrf2 specific siRNA and cell lysates were probed with the OSGIN1-61kDa antibody. Nrf2 knockdown resulted in depletion of OSGIN1-61kDa immunoreactivity which correlated with OSGIN1 knockdown (**FIGURE 60C**), supporting the regulation of this isoform in an Nrf2-dependent manner.

Further examination of both the OSGIN1-52kDa and OSGIN1-61kDa generated antibodies was also conducted via immunocytochemistry. Human astrocytes treated with MMF for 24 hours were fixed and probed with either the OSGIN1-52kDa or OSGIN1-61kDa antibody. Stained cells were imaged on an automated Thermo HCS Arrayscan technology and immunoreactive puncta were quantified using HCS Studio algorithm software. Total fluorescent spot count indicated a significant increase in immunoreactive puncta probed with the OSGIN1-

61kDa antibody compared to no change seen following identification with OSGIN1-52kDa immunoreactive puncta (**FIGURE 61**). Furthermore, the total fluorescent spot count was observably higher in the 52kDa group compare to the 61kDa, providing further evidence that the 61kDa OSGIN1 isoform is less abundant than the 52kDa form.

Alteration in the 5'UTR of OSGIN1 transcripts identified by RACE

Although MMF regulation of the OSGIN1-61kDa isoform was identified via protein analysis, confirmation of this specific transcript was unable to be confirmed via q-PCR based on the location of the primer/probe set within the overlapping region of the OSGIN1 isoforms. Therefore, Northern Blot analysis was attempted to isolate the transcript associated with the identified protein product. RNA probes were generated against the probe sequence used for OSGIN1 q-PCR and probed against human astrocyte purified RNA treated with MMF. Rapid amplification of cDNA ends (RACE) was performed. 3' RACE was performed using RNA extracted from human astrocytes treated with a titration of MMF and probes designed based on the primer/probe sequences used for OSGIN1 q-PCR. 3' RACE resulted in a 1.3kb sequence that was confirmed to match the 3' end of OSGIN1 following DNA sequencing (**FIGURE 62A**). The identified 3' RACE product correlated with an increase in total 3' sequence abundance in the presence of MMF, confirming upregulation of this sequence following MMF treatment (**FIGURE 62A**).

Following confirmation of the 3' RACE product by sequencing, primers were generated within the identified 3' sequenced region for 5'RACE analysis. Similar to 3' RACE, RNA extracted from MMF-treated astrocytes treated was used as a template. 5' RACE analysis identified a .6kb sequence that was found to match the 5' end of the OSGIN1-52kDa encoding region following DNA sequencing (**FIGURE 57B**). Interestingly, the identified 5'RACE product was depleted in a dose-dependent manner in the presence of MMF, suggesting that the 5' end of OSGIN1 in the presence of MMF is differentially regulated.

Further DNA sequencing analysis of the .6kb 5' RACE product identified two identical transcripts that differed only in two nucleotide substitutions within the 5' region of OSGIN1 (**FIGURE 62B**). Alterations in the 5'UTR of OSGIN1 have been previously identified to regulate the protein expression of OSGIN1 (Ong 2007); therefore to determine if MMF

differentially regulated these two transcripts, Taqman primer/probe sets specific for each transcript were generated and analyzed against human astrocytes treated with and without MMF. q-PCR analysis detected a significant MMF-dependent alteration in expression of these two transcripts suggesting that they are not simply allele variants (**FIGURE 63**). Furthermore, MMF induced expression of the “ALT” transcript variant which substitutes a C for an A at position 59 and a G to an A at position 62 of the OSGIN1 sequence (NM_182981.2), which results in a potential ATG start site and a premature stop site (**FIGURE 62B**). These findings suggest that alterations in the 5' region of OSGIN1 may be regulated in the presence of MMF.

p53 is downstream of OSGIN1 and contributes to OSGIN1-mediated cytoprotection

Current literature investigating the role of OSGIN1 describes this gene as a mediator of cell cycle and apoptosis that is believed to be regulated via p53 (Hu et al., 2012; Yao et al., 2008). p53 has been shown to bind to the promoter of OSGIN1 to regulate its transcription as well as interact with OSGIN1 in the cytoplasmic space to induce apoptosis (Hu et al., 2012). To determine if OSGIN1 is transcriptionally regulated by p53, human astrocytes were transfected with siRNA targeted against p53 or OSGIN1 and transcript regulation analyzed. p53 knockdown resulted in an approximate 80% loss of p53 transcript expression (**FIGURE 64A**); however, this reduction in p53 did not affect basal transcriptional levels of OSGIN1 (**FIGURE 64C**). Furthermore, siRNA knockdown of OSGIN1 had no effect on p53 transcription (**FIGURE 64A**). These findings suggest that p53 is not a transcriptional regulator of OSGIN1 in this cell model.

Following 24 hour treatment with MMF, p53 transcript levels were shown to be significantly increased in human astrocytes transfected with scrambled (control) as well as Nrf2 siRNA (**FIGURE 64A**). This is of particular interest since p53 has been identified within the literature to contribute to Nrf2-mediated transcriptional control and co-regulate the expression of proteins involved in protection against oxidative stress (Toledano et al., 2009); therefore, the effect of p53 knockdown on Nrf2 transcription was also analyzed in human astrocytes. Loss of p53 was found to have no effect on transcriptional regulation of Nrf2 in astrocytes (**FIGURE 64B**); however, since Nrf2 regulation is mediated at the protein level and p53 is also believed to be regulated in a similar manner as Nrf2, protein induction of p53 was investigated (Wakabayashi et al., 2010). In the presence of MMF, control transfected astrocytes exhibited a

visible induction of p53 protein that was abolished in p53 knockdown and diminished in astrocytes transfected with OSGIN1 and Nrf2 siRNA (**FIGURE 65A** and **C**). Furthermore, knockdown of p53 did not alter the MMF-mediated accumulation of Nrf2. These findings suggest that p53 is induced downstream of Nrf2 and OSGIN1 in the presence of MMF. Since OSGIN1 has already been identified to be downstream of Nrf2 (**FIGURE 54**), regulation of p53 is most likely regulated via an OSGIN1-induced pathway at a protein level.

As mentioned above, p53 protein levels are tightly regulated within cells similar to Nrf2. In resting conditions, p53 protein is maintained at low levels by proteasomal degradation and activation of p53 expression can reduce ROS levels by inducing the expression of anti-oxidative stress proteins (Vurusaner et al., 2012; Levine et al., 2006). Since MMF is known to induce Nrf2 accumulation and translocation into the nucleus, the effect of MMF on p53 nuclear translocation was investigated. Human astrocytes were treated with a titration of MMF for 24 hours followed by fixation and detection with p53 antibody. Plates were imaged using the Thermo HCS Arrayscan and quantification of p53 localization in the nucleus and cytoplasm was accomplished by algorithm creation using HCS Studio software. Addition of MMF to human astrocytes resulted in a significant translocation of p53 protein from the nucleus which correlated with reduced p53 protein levels in the cytoplasm (**FIGURE 66**). These results were also supported using a p53 nuclear TransAM ELISA (**FIGURE 67**).

Following identification of p53 nuclear translocation with MMF treatment, the importance of p53 in MMF-mediated cytoprotection was evaluated. Human astrocytes were transfected with p53-specific or control siRNA and treated with MMF for 24 hours followed by oxidative challenge with H₂O₂. MMF regulated protection was reduced in this model in the absence of p53 to a similar extent as observed in OSGIN1 knockdown cultures (**FIGURES 68** and **57**). These results suggest that the nuclear translocation of p53 may contribute to the cytoprotective properties of MMF.

MMF time course of OSGIN1, p53, Nrf2 and NQO1.

In order to confirm the transcript and protein regulation patterns of these genes following MMF treatment, time course analysis was conducted. Human astrocytes were treated with MMF for 3, 6, 9, 12, 24 or 36 hours followed by RNA and protein collection. Transcript expression of

OSGIN1 and the classical Nrf2 target, NQO1, were analyzed. Following addition of MMF, OSGIN1 was found to be induced early, peaking at 6 hours and remaining elevated at 36 hours post treatment (**FIGURE 69A**). This early, sharp response parallels *in vivo* studies measuring the OSGIN1 time course in mouse brain (**FIGURE 40**). In comparison, NQO1 transcript levels elevated in a more gradual manner, peaking at around 24 hours and remaining elevated in a more consistent pattern (**FIGURE 69B**). Peak induction of OSGIN1 was almost four fold higher compared to NQO1 induction following MMF treatment.

Since Nrf2 and p53 have only be shown to be predominantly regulated at the protein level, they were measured for protein expression along with NQO1 and OSGIN1 following MMF treatment (**FIGURE 70**). Nrf2 accumulated first following MMF addition to human astrocytes beginning at the three hour post-dose and returning to near baseline levels by 36 hours. Interestingly, even though OSGIN1 transcript levels increased first, NQO1 protein levels increased before OSGIN1 with OSGIN1 protein accumulation not occurring to almost 24 hours. This suggests that alterations of OSGIN1 may occur at either the transcript or protein level to eventually lead to protein accumulation. p53 protein levels accumulated last between 24 and 36 hours, supporting that p53 protein regulation occurs downstream of Nrf2 and OSGIN1. Together these findings suggest that Nrf2 is activated first in the presence of MMF following by OSGIN1 accumulation and eventually p53.

MMF inhibits cell proliferation: preliminary studies.

Although OSGIN1 has been shown to contribute to MMF-induced cytoprotection in an Nrf2-dependent and potentially p53-dependent manner, the exact mechanisms associated with the protective characteristics of OSGIN1 are unclear.

MMF reduces cell proliferation independent of apoptosis

Regulation of cell cycle is considered to be major a cellular pathway controlled by OSGIN1 expression (Liu et al., 2014; Huynh et al., 2001; Ong et al., 2004); however, whether OSGIN1 contributes to cell cycle regulation in astrocytes is unknown. To investigate a role for OSGIN1 in cell cycle regulation, human astrocytes were transfected with scrambled (control) or OSGIN1 siRNA followed by 24 hour treatment with MMF. Astrocytes were pulse labeled with

5-ethynyl-2'-deoxy-uridine (EdU) to label dividing cells and analyzed using the Thermo HCS Arrayscan imaging and algorithm creation technology. Treatment with MMF in control transfected cells resulted in a significant, dose-dependent reduction in total proliferating cells (**FIGURE 71**). In the absence of OSGIN1, this effect on proliferation in the presence of MMF was significantly reduced (**FIGURE 71**). To confirm that reductions in proliferation in the presence of MMF were independent of apoptosis, transfected astrocytes treated with MMF were analyzed for apoptosis using the TiterTACS™ apoptotic assay. Knockdown of OSGIN1 or p53 did not significantly increase apoptosis compared to control knockdown (**FIGURE 72**). These findings suggest that OSGIN1 may regulate cell proliferation. Interestingly, loss of p53 significantly induced apoptosis in the presence of MMF.

A potential role for PADI4

As previously mentioned, p53 is cited in the literature to regulate OSGIN1 expression; however, the results discussed in this chapter suggest that OSGIN1 may instead regulate p53 nuclear translocation (Hu et al., 2012). Another protein cited to regulate OSGIN1 expression is peptidyl arginine deiminase type IV (PADI4), which has been shown to negatively regulate OSGIN1 expression (Yao et al., 2008). Furthermore, PADI4 has been shown to regulate gene transcription by regulating the deimination of arginines on histones and antagonizing arginine methylation and may contribute to cell cycle control (Tanikawa et al., 2009; Chang et al., 2011). To determine if PADI4 is transcriptionally regulated following MMF treatment, PADI4 was measured in the previous time course analysis described in section 2.6 of this chapter. PADI4 was demonstrated to be regulated transcriptionally following MMF treatment in a similar manner as NQO1 (**FIGURE 69B**), with peak expression occurring at 24 hours and a more gradual induction of expression compared to OSGIN1 (**FIGURE 69A**). Since these findings demonstrated PADI4 to be regulated after OSGIN1 induction, PADI4 transcript levels were measured in PADI4, p53 and Nrf2 siRNA knockdown in human astrocytes to determine where PADI4 is targeted. q-PCR analysis of PADI4 levels following siRNA transfection with PADI4, OSGIN1 and p53 resulted in significant reductions in PADI4 expression compared to control samples (**FIGURE 74A**). In contrast, Nrf2 knockdown had no effect on PADI4 transcript expression. Furthermore, MMF induction of PADI4 was abolished in the absence of OSGIN1

(FIGURE 74B). These findings suggest PADI4 is regulated downstream of p53 since p53 has previously been identified to be regulated downstream of Nrf2 and OSGIN1.

As mentioned above PADI4 has been described in the literature to negatively regulate transcription of OSGIN1 (Yao et al., 2008); however, in the absence of PADI4, no differences in OSGIN1 transcriptional expression were observed (FIGURE 75A). Furthermore, p53 transcript levels were also not altered in the absence of PADI4 (FIGURE 75B). Overall, the studies outlined in this section suggest that PADI4 is regulated independently of Nrf2 induction but is also a downstream target of OSGIN1 and p53.

Discussion

Endogenous regulation of cellular defense systems such as the Nrf2 pathway have become of particular interest to the field of neurodegeneration. A unifying aspect of nervous system disease is the accumulation of ROS leading to an overall state of oxidative stress. Activation of Nrf2 by small-molecule compounds such as DMF has shown to increase antioxidant defense genes leading to cytoprotection in various models of neurodegenerative disease (Linker 2011); however, the exact mechanisms underlying Nrf2-mediated cytoprotection are unclear. In Chapter III, transcriptional profiling studies were conducted to evaluate gene regulation following oral dosing with DMF in an attempt to identify specific targets of this compound in the CNS. These studies identified the Nrf2 transcriptional target OSGIN1 to be significantly upregulated in the CNS *in vivo*. Current literature describing the biological functions of OSGIN1, describe this gene to be a major mediator of cell apoptosis under the control of the tumor suppressor protein, p53 (Yao et al., 2008; Hu et al., 2012). However, the *in vitro* findings in human astrocytes discussed herein suggest OSGIN1 is strongly Nrf2-regulated and contributes to the cytoprotective properties of the bioactive metabolite of DMF, MMF, against oxidative insult.

Regulation of OSGIN1 via an Nrf2-dependent mechanism in contrast to p53 transcriptional control raised the question of whether splice variants of OSGIN1 contribute to alternate biological functions of this gene. The predominant literature associated with OSGIN1 function has been conducted in tumorigenic cell lines and have identified the shorter OSGIN1 isoforms, OSGIN1-52kDa and the less accepted OSGIN1-38kDa, to be strong inducers of

apoptosis under the control of p53 (Hu et al., 2012; Yao et al., 2008). In contrast, the longer OSGIN1-61kDa isoform is shown to be less toxic to cells and was also the major variant identified to be regulated in astrocytes in the presence of MMF in an Nrf2-dependent manner (Hu et al., 2012). The specific, cytoprotective regulation of OSGIN1-61kDa under the transcriptional control of Nrf2 supports a mechanism for OSGIN1 independent of the recognized function of this gene in tumor cell lines. These findings suggest that functional domains within OSGIN1 variants may result in differing biological effects regulated under specific cellular conditions. This correlates with findings that OSGIN1 has been identified to contribute to a diverse set of cellular functions other than apoptosis, including anti-inflammatory actions, regulation of cell cycle and protection against oxidative stress (Li et al., 2007; Li et al., 2010; Oguri et al., 2010). OSGIN1 may have a role in anti-inflammatory effects based on the increased expression of TNF- α following knockdown of OSGIN1 in astrocytes (**FIGURE 76**). This correlates with previous findings that identify OSGIN1 to reduce oxidative stress and inflammation in cells challenged with Ox-PAPC (Romanoski et al., 2011; Li et al., 2007; Hammad et al., 2009).

Although the specific transcript encoding the identified OSGIN1-61kDa protein product was not detected in RACE analysis, a distinct reduction in expression of the 5'-end of the MMF-induced transcript was identified. DNA sequencing determined the MMF-induced transcript to encode for the 52kDa OSGIN1 isoform; however, this could potentially be a result of GC enrichment in the 5' region of the known 61kDa encoding sequence or be a result of low abundance of this transcript, resulting in an inability to fully sequence the long form of OSGIN1. In general, these findings suggest that alterations in the 5' region do occur in the presence of MMF. This is further supported by identification of two nucleotide substitutions in the 5' end of the identified transcript that were induced following MMF treatment in astrocytes. This is interesting since these nucleotide substitutions resulted in a potential AUG start site encoding a small ORF. Although the Kozak region preceding this new start site was not incredibly strong, there has been evidence in the literature suggesting that upstream AUG sites encoding uORFs in 5'UTR regions can decrease the frequency of AUG starts sites to initiate transcription in the main open reading frame (Morris and Geballe, 2000). Furthermore, the generation of OSGIN1 small encoding ORFs in the 5'UTR, have been previously identified to negatively control

OSGIN1 protein translation (Ong et al., 2007). Therefore, the regulation of specific OSGIN1 transcripts by Nrf2 could potentially result in selection of the 61kDa protein over the 52kDa form through downregulation of OSGIN1-52kDa protein expression independent of transcript regulation.

p53 was not shown to regulate OSGIN1 expression in human astrocytes. Instead, studies with MMF in these cell types identified p53 to be a downstream protein target of Nrf2-regulated OSGIN1. This was exemplified by the inability of p53 to accumulate and translocate to the nucleus in the absence of OSGIN1, suggesting this molecule may activate p53-mediated transcriptional control. Previous studies have suggested that p53 and Nrf2 work together to regulate gene expression in the presence of oxidative stress and p53 itself has also been shown to be protective against oxidative damage (Toledano et al. 2009; Chen et al., 2012). Thus, the activation of OSGIN1 by Nrf2 may be a potential mechanism through which Nrf2 regulates p53 during periods of oxidative stress. Since OSGIN1 is activated before classical Nrf2 targets, it is possible that the role of OSGIN1 is to induce p53 translocation to the nucleus where it can interact with Nrf2 to regulate other Nrf2 target genes. Previous data have already identified the ability of p53 and OSGIN1 to interact in the cytoplasm, but there may also be a role for OSGIN1 in inhibiting degradation of p53 similar to Nrf2.

The translocation of p53 by OSGIN1 may also induce or suppress gene regulation independent of Nrf2. Evidence for this includes the regulation of PADI4 identified in astrocytes to be regulated dependently by p53 but independently of Nrf2. In the preliminary studies described herein, PADI4 was identified to also be transcriptionally regulated by OSGIN1, suggesting that OSGIN1-mediated translocation of p53 induces PADI4 expression. Interestingly, similar to p53, the regulation of PADI4 in association with OSGIN1 contrasts with current literature that suggests PADI4 to be a negative regulator of OSGIN1 (Yao et al., 2008). As discussed above, these alternate functions of OSGIN1 in MMF-treated astrocytes may be due to the expression of the OSGIN1-61kDa isoform or potentially be due to cell type-specific characteristics of OSGIN1. PADI4 has been shown to regulate DNA methylation by citrullination of histone residues to regulate DNA transcription and a role for p53 in this process has been described (Tanikawa et al., 2009). Therefore, the role of PADI4 in our model may be to inhibit DNA transcription in a p53-dependent manner to reduce cell proliferation. This process is

independent of apoptosis based on the findings that MMF administration does not induce apoptosis but does reduce cell proliferation. Various research studies have identified inhibition of cell cycle entry to be a protective mechanism within cells, allowing conservation of energy and limiting the probability of damaged DNA to be replicated (Price et al., 2003; Giovanni et al., 2005).

Overall, these results indicate a role for the Nrf2 transcriptional target OSGIN1 in MMF-mediated cytoprotection in the presence of oxidative stress. Furthermore, these results lay the groundwork for a potential mechanism of OSGIN1-dependent protection (**FIGURE 77**). This mechanism involves activation of Nrf2 by MMF through inhibition of Keap1 degradation, resulting in the translocation of Nrf2 to the nucleus. Once inside the nucleus, Nrf2 regulates the transcription of various genes including OSGIN1, which is one of the first Nrf2 transcribed targets. Translation of the OSGIN1-61kDa protein then results in the accumulation and subsequent translocation of p53 to the nucleus, leading to the induction of PADI4 regulation and potentially inhibition of cell proliferation. Whether p53 also regulates other genes independently or in collaboration with Nrf2 is unknown. Furthermore, inhibiting cell cycle may be a protective mechanism. Without wishing to be bound by any particular theory, this inhibition could involve proliferative inhibition of immune cells and a subsequent inflammatory response since loss of OSGIN1 also increased TNF- α levels (**FIGURE 76**).

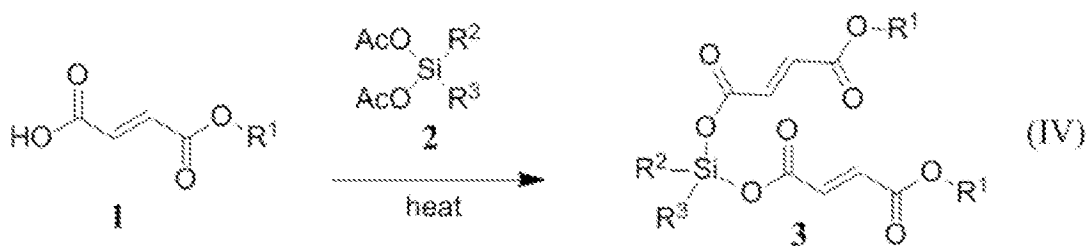
Taken together, these data suggest that Nrf2-mediated cytoprotection consists of a complicated network of pathways that together function to protect the cell during periods of stress.

Example 10: Preparation of compounds (III)-(VI)

The compounds of Formulae (III)-(VI) may be prepared using methods known to those skilled in the art, or the methods disclosed in the present invention.

Specifically, the compounds of this invention of Formula IV may be prepared by the exemplary reaction in Scheme 1.

Scheme 1

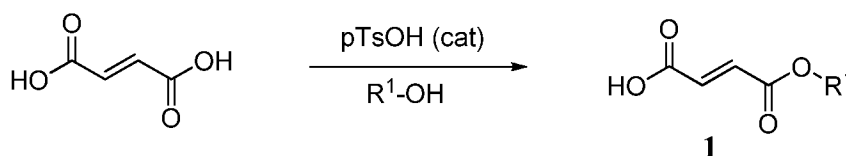


wherein R^1 , R^2 , and R^3 are each defined above for Formula IV.

Reaction of fumaric acid ester **1** with silane diacetate intermediate **2** in a refluxing organic solvent such as diethyl ether, toluene, or hexane to give the desired siloxane **3**.

Some of the fumaric acid esters **1** are commercially available. Fumaric acid ester **1** can also be prepared, for example, using synthetic methods known by one of ordinary skill in the art. For example, fumaric acid can be converted by reacting alcohol (R^1-OH) with a catalytic amount of p-toluene sulfonic acid at room temperature for a few hours to overnight as shown in Scheme 2.

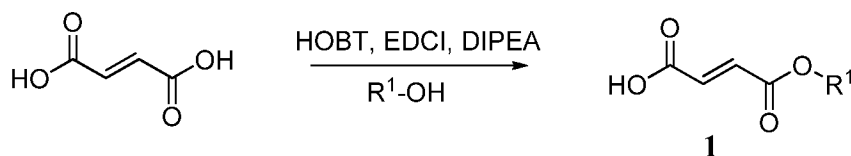
Scheme 2



wherein R^1 is defined above for Formula III.

Alternatively, fumaric acid ester **1** can be prepared by reacting alcohol (R^1-OH) under the coupling conditions of hydroxybenzotriazole (HOBt), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI), and diisopropyl amine (DIPEA) as shown in Scheme 3.

Scheme 3



wherein R^1 is defined above for Formula III.

Some of the silanes that can be used in the present invention are commercially available. Commercially available silyl halides include trimethylsilyl chloride, dichloromethylphenylsilane, dimethyldichlorosilane, methyltrichlorosilane, (4-aminobutyl)diethoxymethylsilane, trichloro(chloromethyl)silane, trichloro(dichlorophenyl)silane, trichloroethylsilane, trichlorophenylsilane, and trimethylchlorosilane. Commercial sources for silyl halides include Sigma Aldrich and Acros Organics.

Silanes used in the present invention can be prepared, for example, using synthetic methods known by one of ordinary skill in the art. For example, trichlorosilane may be prepared by the exemplary reaction in Scheme 4.

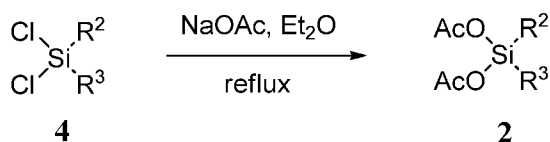
Scheme 4



The silylation of styrene derivatives catalyzed by palladium is described in Zhang, F. and Fan, Q.-H., *Organic & Biomolecular Chemistry* 7:4470-4474 (2009) and in Bell, J.R., *et al.*, *Tetrahedron* 65:9368-9372 (2009).

Diacetate intermediate **2** may be prepared by treatment of dichlorosubstituted silicon compound **4** with sodium acetate in diethyl ether under reflux as shown in Scheme 5.

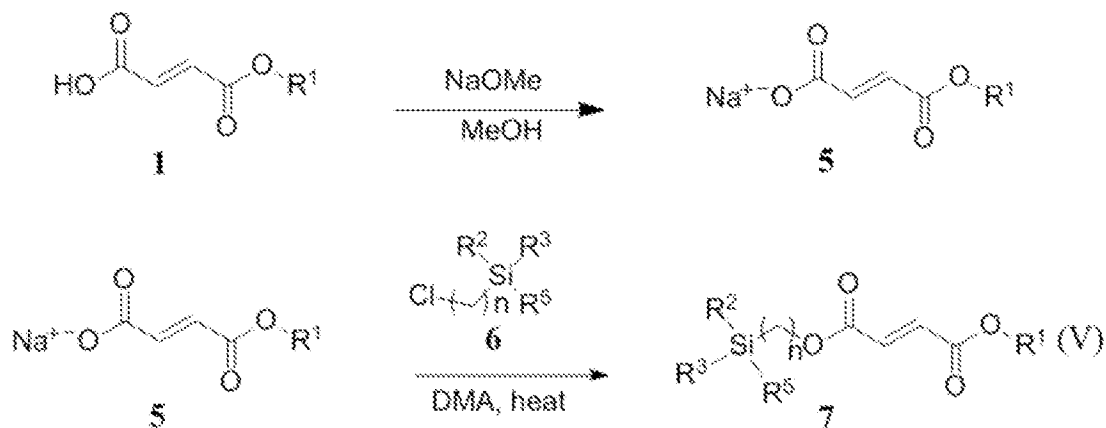
Scheme 5



wherein R^2 and R^3 are each defined above for Formula IV.

Specifically, the compounds of this invention of Formula V may be prepared by the exemplary reaction in Scheme 6.

Scheme 6

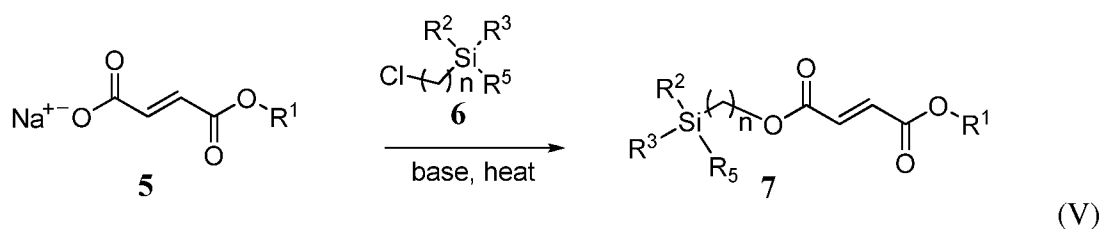


wherein R^1 , R^2 , R^3 , and R^5 are as defined above for Formula V.

Fumaric acid ester **1** can be converted to the sodium salt **5** using, for example, sodium methoxide in methanol at room temperature. Removal of the solvent would afford sodium salt **5**. Treatment of the sodium salt **5** with silane **6** in an organic solvent such as dimethylformamide under reflux would generate ester **7**. The synthesis of structurally related (trimethoxysilyl)-methyl esters is described in Voronkov, M.G., *et al.*, *Zhurnal Obshchei Khimii* 52:2052-2055 (1982).

Alternatively, the compounds of this invention of Formula V may be prepared by the exemplary reaction in Scheme 7.

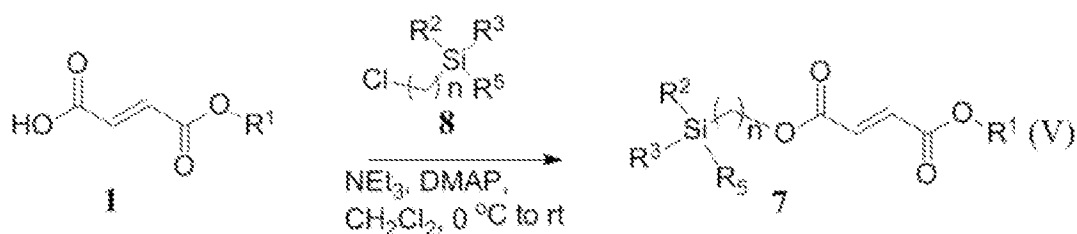
Scheme 7



wherein R^1 , R^4 , R^5 , R^6 , and n are as defined above for Formula V.

Treatment of the sodium salt **5** with silane **6** in an organic solvent such as dimethylformamide under heating with or without an acid scavenger would generate ester **7**.

Scheme 8

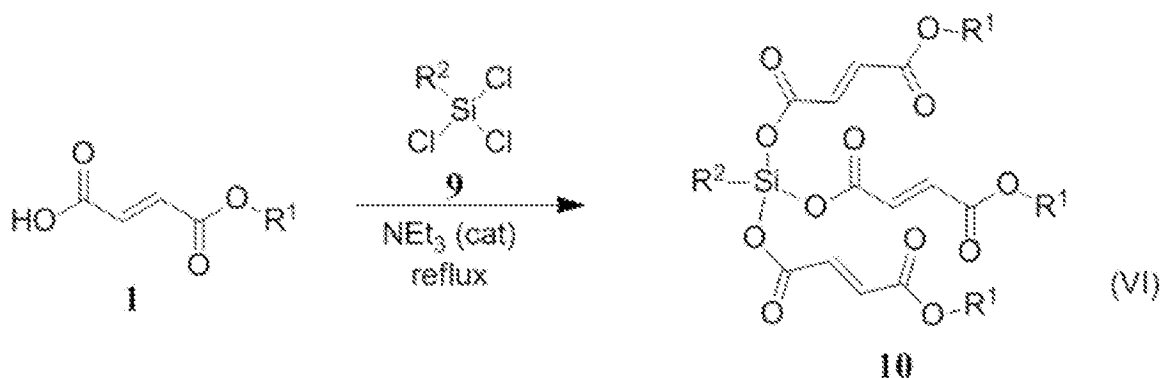


wherein R^1 , R^4 , R^5 , R^6 , and n are as defined above for Formula V.

Reaction of fumaric acid ester **1** with tri-substituted silane alcohol **8** in methylene chloride with mild base such as triethyl amine and 4-*N,N*-dimethyl amino pyridine (DMAP) at room temperature generates fumarate **7**. See Coelho, P.J., *et al.*, *Eur. J. Org. Chem.* 3039-3046 (2000).

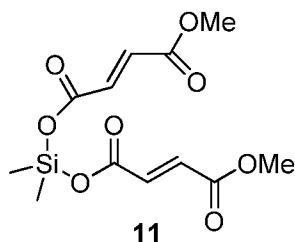
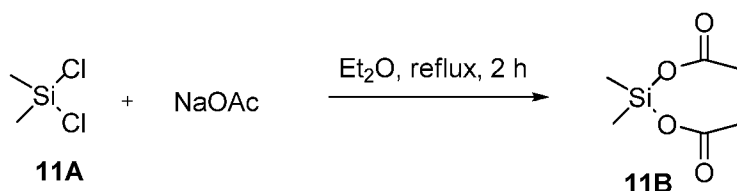
Specifically, the compounds of this invention of Formula VI can be prepared by the exemplary reaction in Scheme 9.

Scheme 9

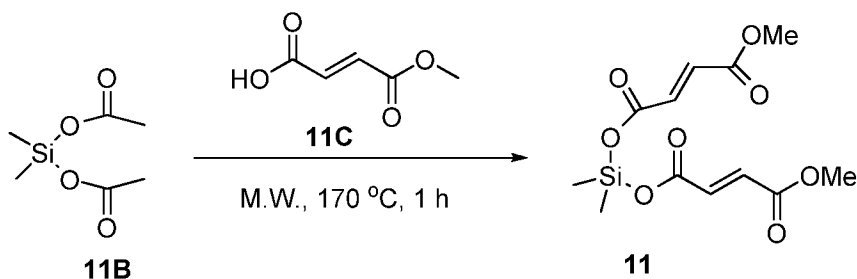


wherein R^1 and R^2 are as defined above for Formula VI.

Reaction of fumaric acid **1** with trichlorosilane **9** in a refluxing organic solvent such as hexane or toluene using a catalytic amount of a base such as triethylamine generates the trifumarate silane **10**. The reaction of acetic and methacrylic acids with 1-silyladamantanes is described in Fedotov, N.S., *et al.*, *Zhurnal Obshchei Khimii* 52:1837-1842 (1982).

Example 11: Synthesis of (*E*)-*O,O'*-(dimethylsilanediyl)dimethyl difumarate (Compound 11)**Step 1: Preparation of dimethylsilanediyl diacetate 11B**

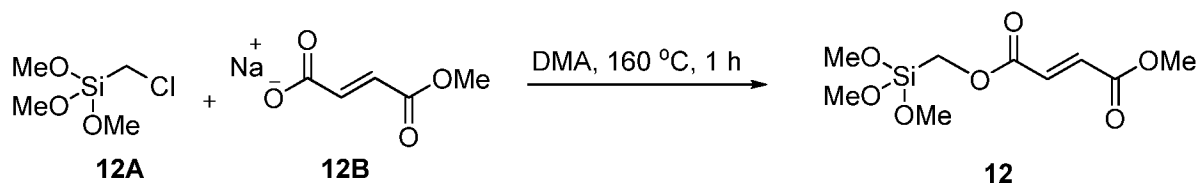
To a slurry of sodium acetate (8.2 g, 100 mmol, 2.0 equiv.) in anhydrous diethyl ether (40 mL) was slowly added a solution of dimethyldichloro silane **11A** (6.45 g, 50 mmol, 1.0 equiv.) in anhydrous diethyl ether (10 mL). After addition was completed, the mixture was heated at reflux for 2 hours, and then filtered under N₂. The filtrate was concentrated under vacuum at 40 °C to give diacetate **11B** as a colorless oil (6.1 g, 70%). ¹H NMR (400 MHz, CDCl₃) δ ppm: 2.08 (s, 6H), 0.48 (s, 6H).

Step 2: Preparation of (*E*)-*O,O'*-(dimethylsilanediyl)dimethyl difumarate 11

A mixture of **11B** (2.0 mL, 12 mmol, 1.5 equiv.) and **11C** (1.04 g, 8.0 mmol, 1.0 equiv.) in a sealed tube was heated at 170 °C with stirring under microwave condition for 1 hour. After

cooling to 50 °C, the mixture was transferred to a round bottom flask and the excess silica reactant **11B** was removed under vacuum at 100 °C to afford compound **11** as brown oil (1.47 g, 60%). ¹H NMR (400 MHz, CDCl₃) δ ppm: 6.82-6.80 (m, 4H), 3.79 (s, 6H), 0.57 (s, 6H).

Example 12: Synthesis of methyl ((trimethoxysilyl)methyl) fumarate (Compound 12)

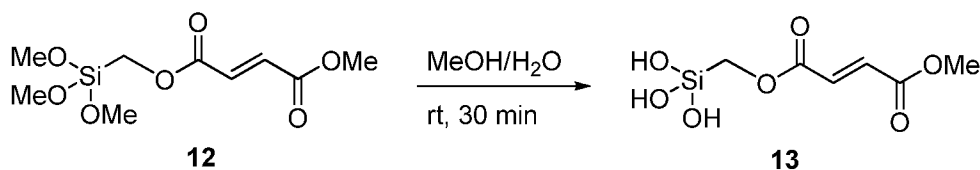


To a stirred solution of monomethyl fumarate (3.5 g, 27 mmol, 1.0 equiv.) in anhydrous THF (35 mL) at room temperature was added sodium hydride (1.08 g, 27 mmol, 1.0 equiv.) in small portions. After addition, the mixture was heated to reflux for 3 hours, and then cooled to room temperature. The solid was collected by filtration and washed twice with diethyl ether, and further dried *in vacuo* to give 3.8 g of **12B** (93%).

To a suspension of **12B** (760 mg, 5.0 mmol, 1.0 equiv.) in dry DMA (5 mL) at 100 °C under nitrogen was added a solution of **12A** (1.03 g, 6.0 mmol, 1.2 equiv.) in dry DMA (1 mL) dropwise. The resulting mixture was heated to 160 °C and stirred for 1 hour, and then cooled to room temperature. The solid was filtered, and the filtrate was evaporated under reduced pressure to give the titled compound **12**, 513 mg (37%), as a red viscous liquid.

¹H NMR (400 MHz, CDCl₃) δ ppm: 6.90-6.86 (m, 2H), 3.97 (s, 2H), 3.82 (s, 3H), 3.62 (s, 9H).

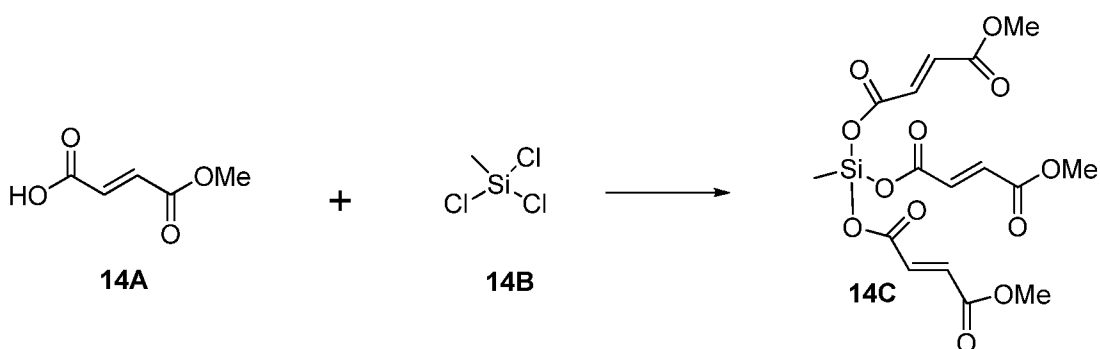
Example 13: Synthesis of methyl ((trihydroxysilyl)methyl) fumarate (Compound 13)



To a solution of **12** (1.0 g, 3.8 mmol, 1.0 equiv., prepared in Example 2) in MeOH (10 mL) at room temperature was added water (341 mg, 19.0 mmol, 5.0 equiv.) dropwise. After addition, the mixture was stirred at room temperature for 30 minutes, with white solids precipitated out. The solids were collected through filtration, washed with methanol three times, and dried at 60 °C *in vacuo*, to provide the titled compound **13**, 500 mg (59%), as a white solid.

¹H NMR (400 MHz, DMSO-*d*₆) δ ppm: 6.79-6.74 (m, 2H), 3.91-3.58 (m, 6H), 3.18-3.15 (m, 2H).

Example 14: Synthesis of trimethyl (methylsilanetriyl) trifumarate (Compound 14)



Following the procedure described in Scheme 9, monomethyl fumarate **14A** would react with trichloromethane-silane **14B** in refluxing toluene or hexanes with a catalytic amount of triethylamine to provide (2'E, 2''E)-trimethyl O,O',O''-(methylsilanetriyl) trifumarate **14C**.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed.

What is claimed is:

1. A method of evaluating, monitoring or stratifying a subject, *e.g.*, a subject having or at risk for MS, comprising:
 - a) acquiring a value for the expression of the *OSGIN1* gene for a sample from the subject;
 - b) responsive to said value,
 - i) classifying said subject, *e.g.*, classifying said subject as in need of treatment with DMF, MMF, or a prodrug of MMF; or
 - ii) selecting said subject for treatment with DMF, MMF, or a prodrug of MMF, or with a treatment other than DMF, MMF, or a prodrug of MMF,provided that the method comprises directly acquiring the value, or directly acquiring a sample from which the value is acquired.
2. DMF, MMF, or a prodrug of MMF for use in a method of treating a subject having or at risk for MS, wherein the method comprises:
 - a) acquiring a value for the expression of the *OSGIN1* gene;
 - b) responsive to said value,
 - i) administering DMF or MMF, or a prodrug of MMF, to said subject.
3. The method of claim 1, comprising classifying said subject as in need of treatment with DMF or MMF.
4. The method of claim 1 or 3, comprising selecting said subject for treatment with DMF, MMF, or a prodrug of MMF, or with a treatment other than DMF, MMF, or a prodrug of MMF.
5. The method of any one of claims 1 and 3-4, or DMF, MMF, or the prodrug for use according to claim 2, wherein acquiring a value for the expression of the *OSGIN1* gene comprises hybridization of a probe specific for *OSGIN1* with an *OSGIN1* mRNA or an *OSGIN1* cDNA.

6. The method of any one of claims 1 and 3-5, or DMF, MMF, or the prodrug for use according to claim 2 or 5, wherein acquiring a value for the expression of the *OSGIN1* gene comprises amplifying, *e.g.*, with PCR amplification, an *OSGIN1* mRNA or an *OSGIN1* cDNA.

7. The method of any one of claims 1 and 3-6, or DMF, MMF, or the prodrug for use according to any one of claims 2, 5 and 6, wherein the value for expression of the gene comprises a value for a transcriptional parameter, *e.g.*, the level of an mRNA encoded by the gene.

8. The method of any one of claims 1 and 3-7, or DMF, MMF, or the prodrug for use according to any one of claims 2 and 5-7, wherein acquiring a value for the expression of the *OSGIN1* gene comprises normalizing a value for an *OSGIN1* mRNA or an *OSGIN1* cDNA with the level of a second gene, *e.g.*, gene the expression of which is not affected by MS.

9. The method of any one of claims 1 and 3-5, or DMF, MMF, or the prodrug for use according to claim 2 or 5, wherein the value for expression of the gene comprises a value for a translational parameter, *e.g.*, the level of a protein encoded by the gene, *e.g.*, by an antibody based measurement.

10. The method of any one of claims 1 and 3-9, or DMF, MMF, or the prodrug for use according to any one of claims 2 and 5-9, wherein the subject, *e.g.*, a human subject, has MS.

11. The method of any one of claims 1 and 3-10, or DMF, MMF, or the prodrug for use according to any one of claims 2 and 5-10, wherein the subject with MS has a relapsing form of MS.

12. The method of any one of claims 1 and 3-11, or DMF, MMF, or the prodrug for use according to any one of claims 2 and 5-11, wherein the subject has been administered DMF, MMF, or a prodrug of MMF, *e.g.*, prior to, or at the time of, acquiring the value.

13. The method of any one of claims 1 and 3-12, or DMF, MMF, or the prodrug for use according to any one of claims 2 and 5-12, wherein a tissue of the subject, *e.g.*, the peripheral blood or a tissue of CNS, comprises, greater than background levels, *e.g.*, therapeutic levels, of DMF, MMF, or a prodrug of MMF, or a combination thereof, *e.g.*, prior to, or at the time of, acquiring the value.

14. The method of any one of claims 1 and 3-13, or DMF, MMF, or the prodrug for use according to any one of claims 2 and 5-13, wherein the value for expression of the gene is for cerebral spinal fluid or blood, *e.g.*, whole blood.

15. The method of any one of claims 1, 3-4 and 9-14, or DMF, MMF, or the prodrug for use according to any one of claims 2 and 9-14, wherein the value for expression of the gene comprises a value for a translational parameter, *e.g.*, the level of a protein encoded by the gene, in cerebral spinal fluid or blood, *e.g.*, whole blood.

16. The method of any one of claims 1 and 3-15, or DMF, MMF, or the prodrug for use according to any one of claims 2 and 5-15, wherein the value for expression of the gene is for a blood sample, or a blood derived sample, *e.g.*, serum, or an NK-cell containing fraction, from the subject.

17. The method of any one of claims 1 and 3-16, or DMF, MMF, or the prodrug for use according to any one of claims 2 and 5-16, wherein the sample is blood, and comprises, greater than background levels, *e.g.*, therapeutic levels, of DMF, MMF, or a prodrug of MMF, or a combination thereof.

18. A method of evaluating, or monitoring, an MS treatment, *e.g.*, an MS treatment with DMF, MMF, or a prodrug of MMF, in a subject having MS, or at risk for developing MS, said method comprising:

acquiring from a sample obtained from said subject a value for the expression of the *OSGIN1* gene, wherein the subject has been administered an MS treatment, *e.g.*, an MS treatment with DMF, MMF, or a prodrug of MMF,

wherein a change in the gene expression is indicative of a differential response to DMF, MMF, or a prodrug of MMF.

19. The method of claim 18, further comprising, responsive to said value, selecting and/or altering one or more of: the course of the MS treatment, the dosing of the MS treatment, the schedule or time course of the MS treatment, or administration of a treatment other than DMF, MMF, or a prodrug of MMF.

20. DMF, MMF, or a prodrug of MMF for use in a method of treating a subject having MS, or at risk for developing MS, wherein the method comprises:

administering DMF, MMF, or the prodrug to the subject; and

acquiring from said subject a value for the expression of the *OSGIN1* gene,

wherein a change in the gene expression is indicative of a differential response to DMF, MMF, or a prodrug of MMF.

21. DMF, MMF, or the prodrug for use according to claim 20, wherein the method further comprises, responsive to said value, treating, selecting and/or altering one or more of: the course of the MS treatment, the dosing of the MS treatment, the schedule or time course of the MS treatment, or administration of a treatment other than DMF, MMF, or a prodrug of MMF.

22. DMF, MMF, or a prodrug of MMF for use in a method of treating a subject having, or at risk of having, MS, wherein the subject has been identified for treatment with DMF, MMF, or a prodrug of MMF, on the basis of a value for the expression of a the *OSGIN1* gene.

23. DMF, MMF, or the prodrug for use according to claim 22, wherein the subject, *e.g.*, a human subject, has MS.

24. DMF, MMF, or the prodrug for use according to claim 22 or 23, wherein the subject with MS has a relapsing form of MS.

25. DMF, MMF, or the prodrug for use according to any one of claims 22-24, wherein the subject has been administered DMF, MMF, or a prodrug of MMF, *e.g.*, prior to, or at the time of, acquiring the value.

26. DMF, MMF, or the prodrug for use according to any one of claims 22-25, wherein a tissue of the subject, *e.g.*, the peripheral blood, comprises, greater than background levels, *e.g.*, therapeutic levels, of DMF, MMF, or a prodrug of MMF, or a combination thereof, *e.g.*, prior to, or at the time of, acquiring the value.

27. DMF, MMF, or the prodrug for use according to any one of claims 22-26, wherein the value for expression of the gene comprises a value for a transcriptional parameter, *e.g.*, the level of an mRNA encoded by the gene.

28. DMF, MMF, or the prodrug for use according to any one of claims 22-26, wherein the value for expression of the gene comprises a value for a translational parameter, *e.g.*, the level of a protein encoded by the gene.

29. DMF, MMF, or the prodrug for use according to any one of claims 22-28, wherein the value for expression of the gene is for cerebral spinal fluid or blood, *e.g.*, whole blood.

30. DMF, MMF, or the prodrug for use according to any one of claims 22-29, wherein the value for expression of the gene comprises a value for a translational parameter, *e.g.*, the level of a protein encoded by the gene, in cerebral spinal fluid or blood, *e.g.*, whole blood.

31. DMF, MMF, or the prodrug for use according to any one of claims 22-30, wherein the value for expression of the gene is for a blood sample, or a blood derived sample, *e.g.*, serum, or an NK-cell containing fraction, from the subject.

32. DMF, MMF, or the prodrug for use according to any one of claims 22-31, wherein the sample is blood, and comprises, greater than background levels, *e.g.*, therapeutic levels, of DMF, MMF, or a prodrug of MMF, or a combination thereof.

33. A device comprising:

one, or a plurality of, probes, each probe being specific for a product, *e.g.*, a translational product or transcriptional product, of the *OSGIN1* gene,
wherein the device includes less than 10, 25, 50, 100, 200, 250, 300, or 500 probes specific for products, *e.g.*, a translational product or transcriptional product, of a gene other than *OSGIN1*.

34. The device of claim 33, further comprising, a sample from a tissue of a subject, *e.g.*, the peripheral blood, which comprises greater than background levels, *e.g.*, therapeutic levels, of DMF, MMF, or a prodrug of MMF, or a combination thereof.

35. A method of using a device described herein comprising:

providing a device of claim 33;

contacting the device with the sample from a tissue of a subject, *e.g.*, the peripheral blood, *e.g.*, a tissue which comprises greater than background levels, *e.g.*, therapeutic levels, of DMF, MMF, or a prodrug of MMF, or a combination thereof,
thereby using the device.

36. A reaction mixture comprising:

a sample from a tissue of a subject, *e.g.*, the peripheral blood, *e.g.*, tissue which comprises greater than background levels, *e.g.*, therapeutic levels, of DMF, MMF, or a prodrug of MMF, or a combination thereof;

one, or a plurality of, probes each probe being specific for a product, *e.g.*, a translational product or transcriptional product, of the *OSGIN1* gene,
wherein the reaction mixture includes less than 10, 25, 50, 100, 200, 250, 300, or 500 probes specific for products, *e.g.*, a translational product or transcriptional product, of genes other than *OSGIN1*.

37. A method of making a reaction mixture comprising:

providing a sample from a tissue of a subject, *e.g.*, the peripheral blood, *e.g.*, tissue which comprises greater than background levels, *e.g.*, therapeutic levels, of DMF, MMF, or a prodrug of MMF, or a combination thereof;

contacting the sample with one or a plurality of probes specific for *OSGIN1*, wherein the reaction mixture includes less than 10, 25, 50, 100, 200, 250, 300, or 500 probes specific for products, *e.g.*, a translational product or transcriptional product, of genes other than *OSGIN1*,

thereby making a reaction mixture.

38. An *OSGIN1* antagonist for use in modulating an immune response in a subject, wherein the antagonist is selected from the group consisting of an anti-*OSGIN1* antibody or *OSGIN1*-binding fragment thereof, siRNA, shRNA, antisense RNA, miRNA, and combinations thereof.

39. The *OSGIN1* antagonist for use according to claim 38, wherein level and/or activity of TNF- α is increased by antagonizing expression of *OSGIN1* in the subject.

40. An *OSGIN* agonist for use in modulating an immune response in a subject, wherein the agonist is selected from the group consisting of an expression vector encoding *OSGIN1* (*e.g.*, retroviral, lentiviral, among other expression vectors), mRNA encoding *OSGIN1*, *OSGIN1* translation product, and combinations thereof.

41. The *OSGIN* agonist for use according to claim 40, wherein level and/or activity of TNF- α is decreased by agonizing expression of *OSGIN1* in the subject.

MMF Exposures 30 min Post Oral Dosing DMF vs MMF (100 mg/kg)

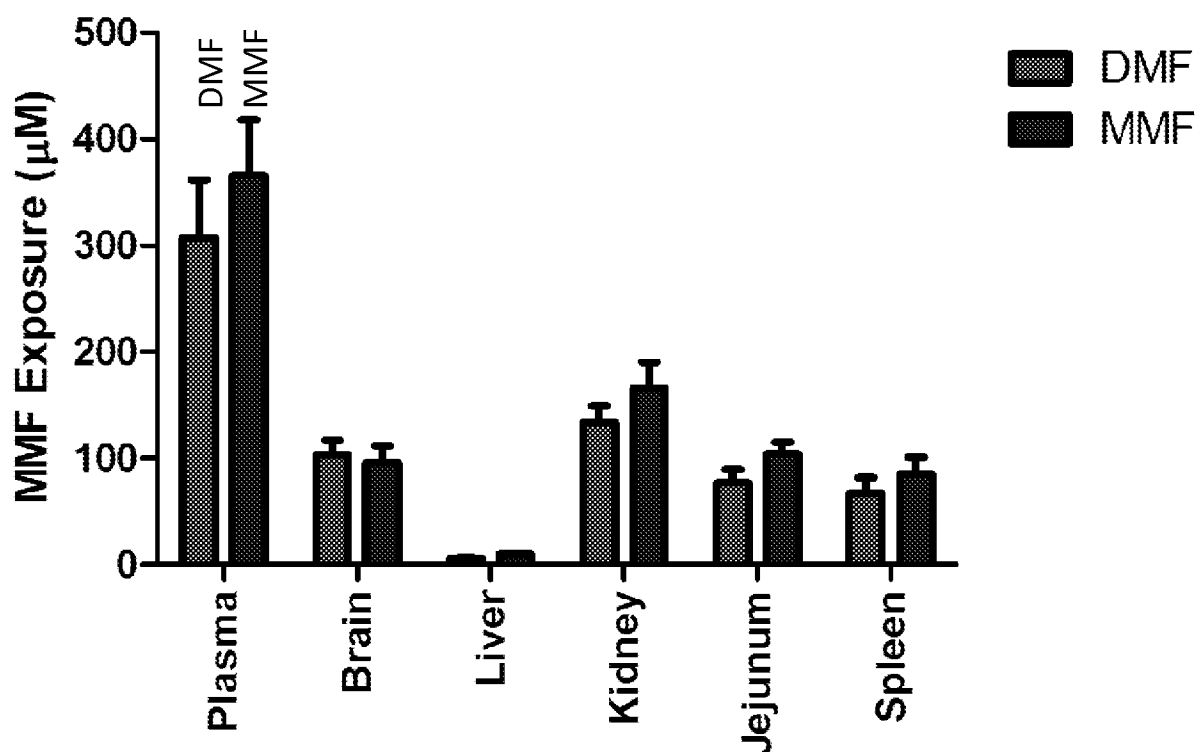
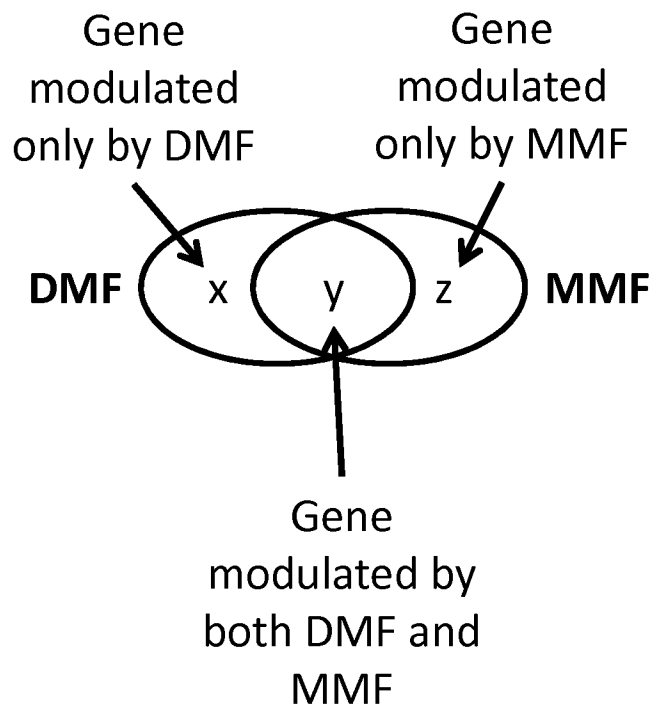


FIGURE 1

Overall Summary:

Venn Diagrams comparing differentially Expressed Genes (DEGs) in each tissue



* Data presented as an aggregation of the three time points for each tissue.

FIGURE 2A

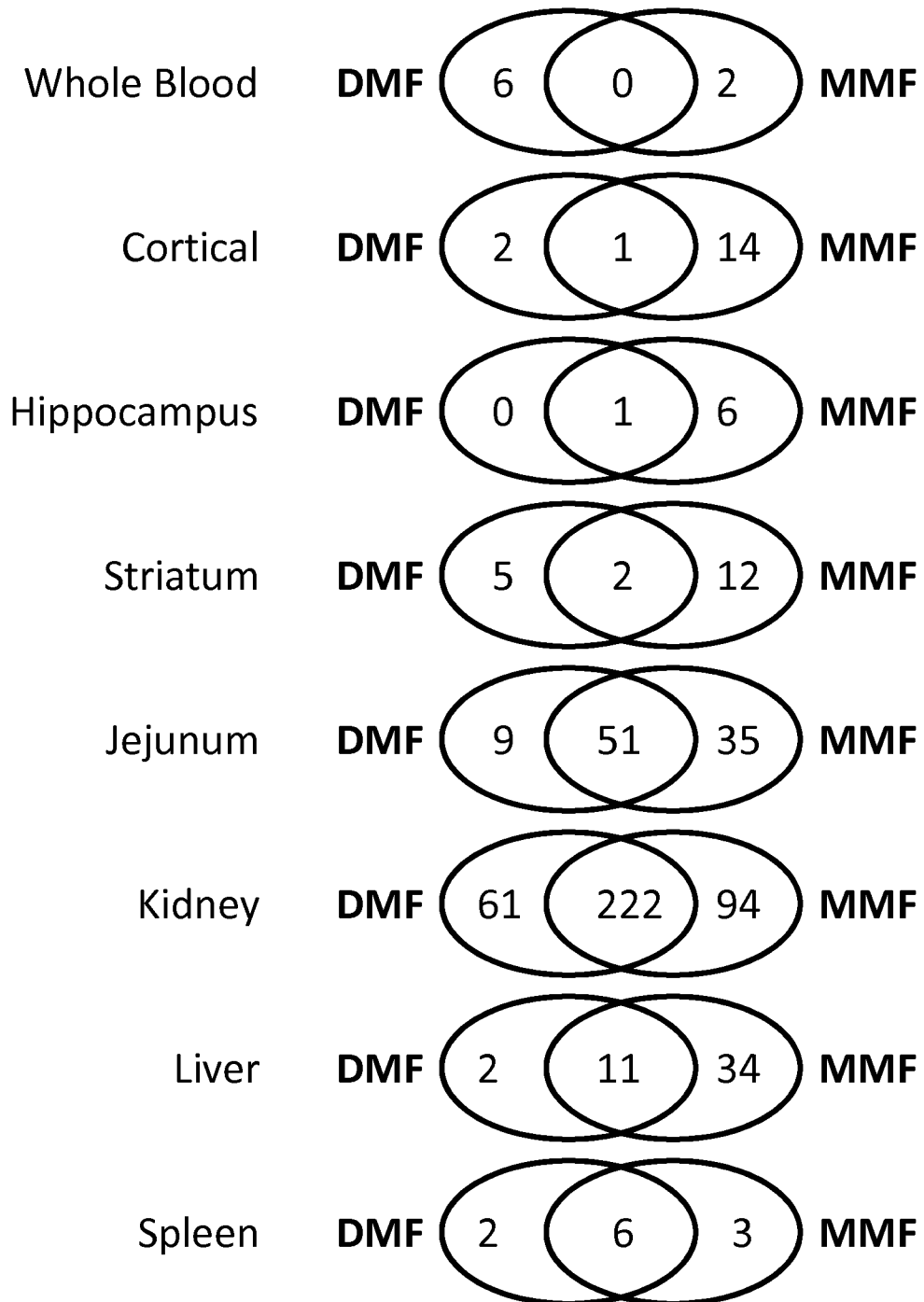


FIGURE 2B

Whole Blood DEGs

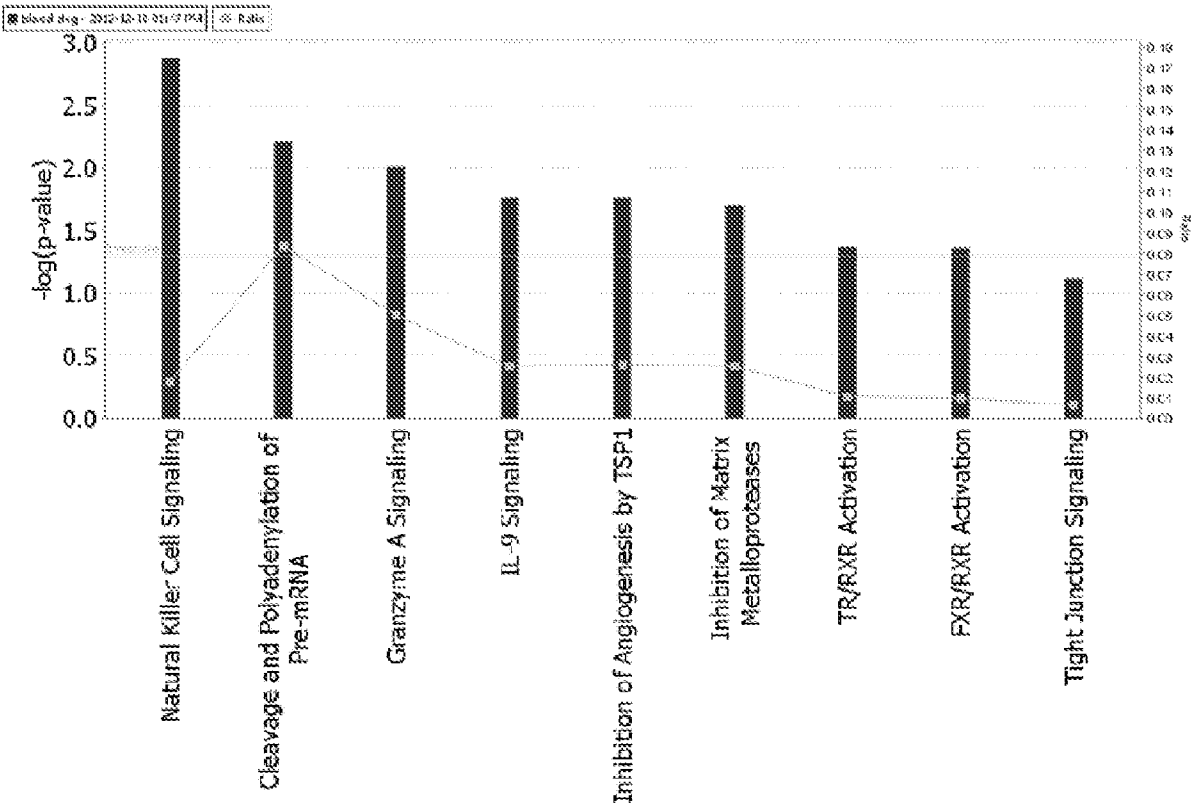
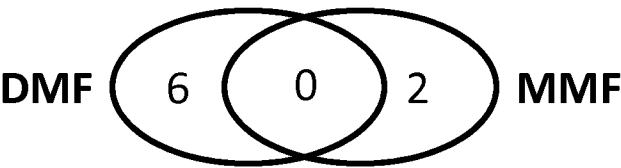
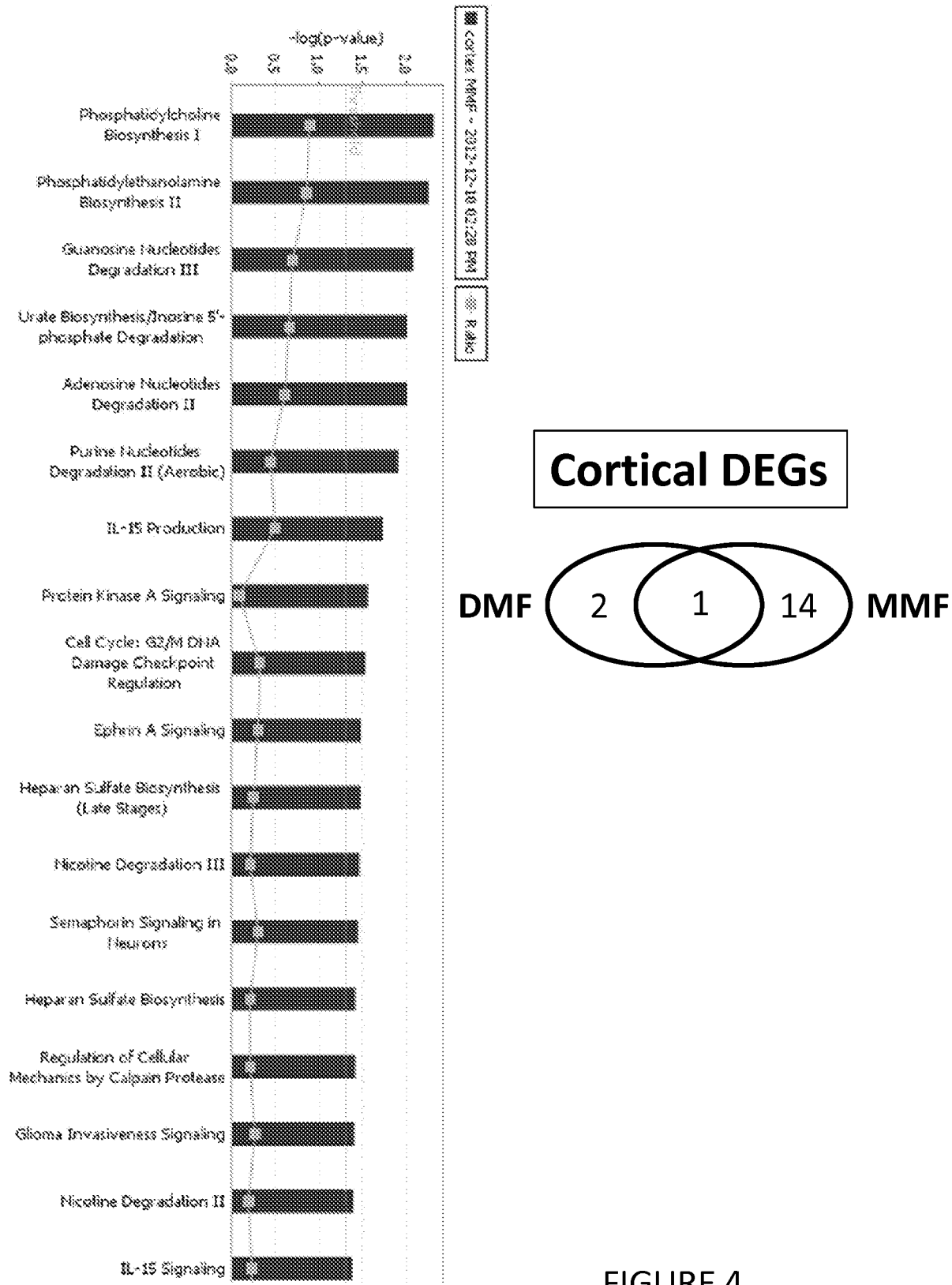


FIGURE 3



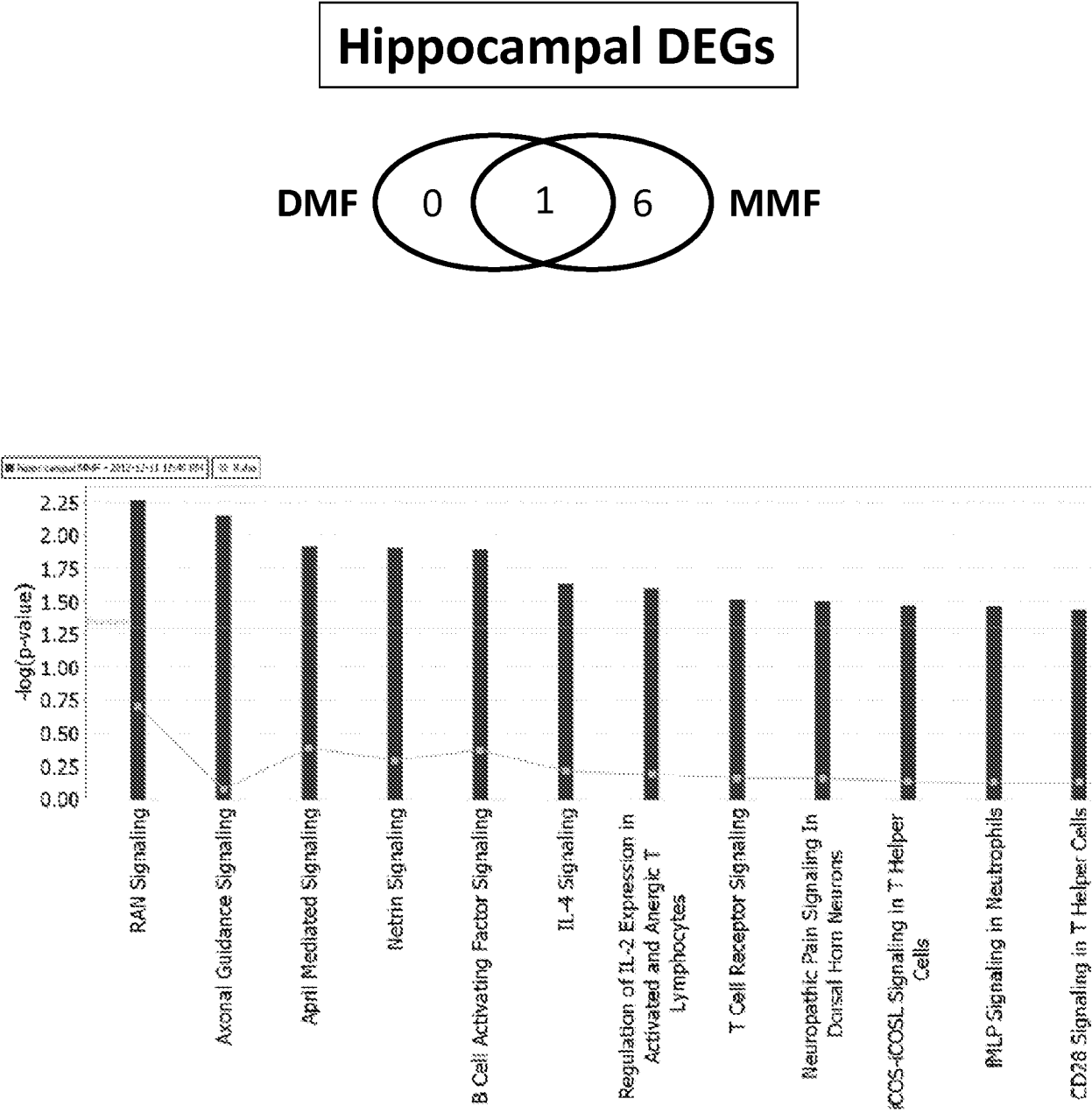


FIGURE 5

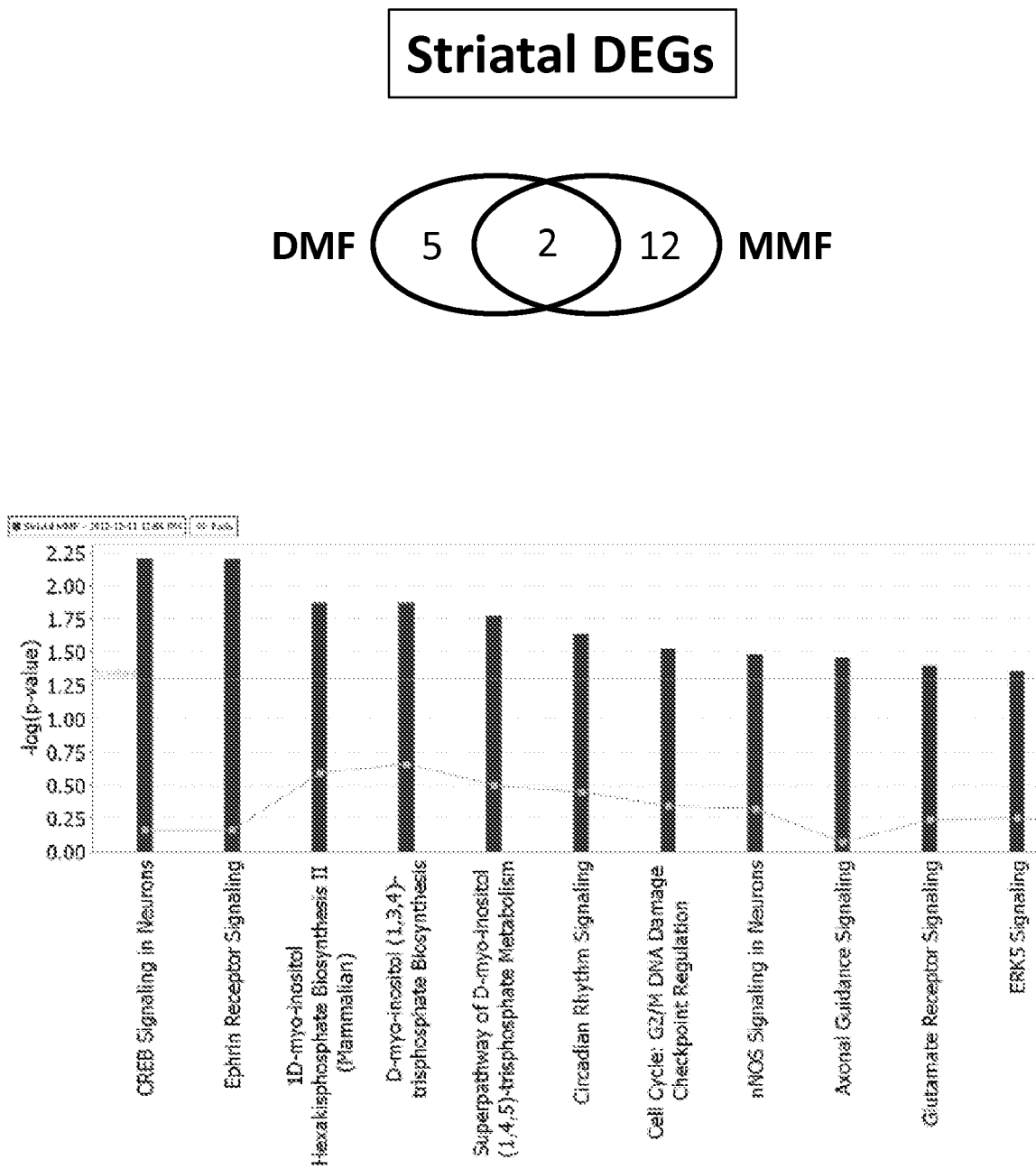
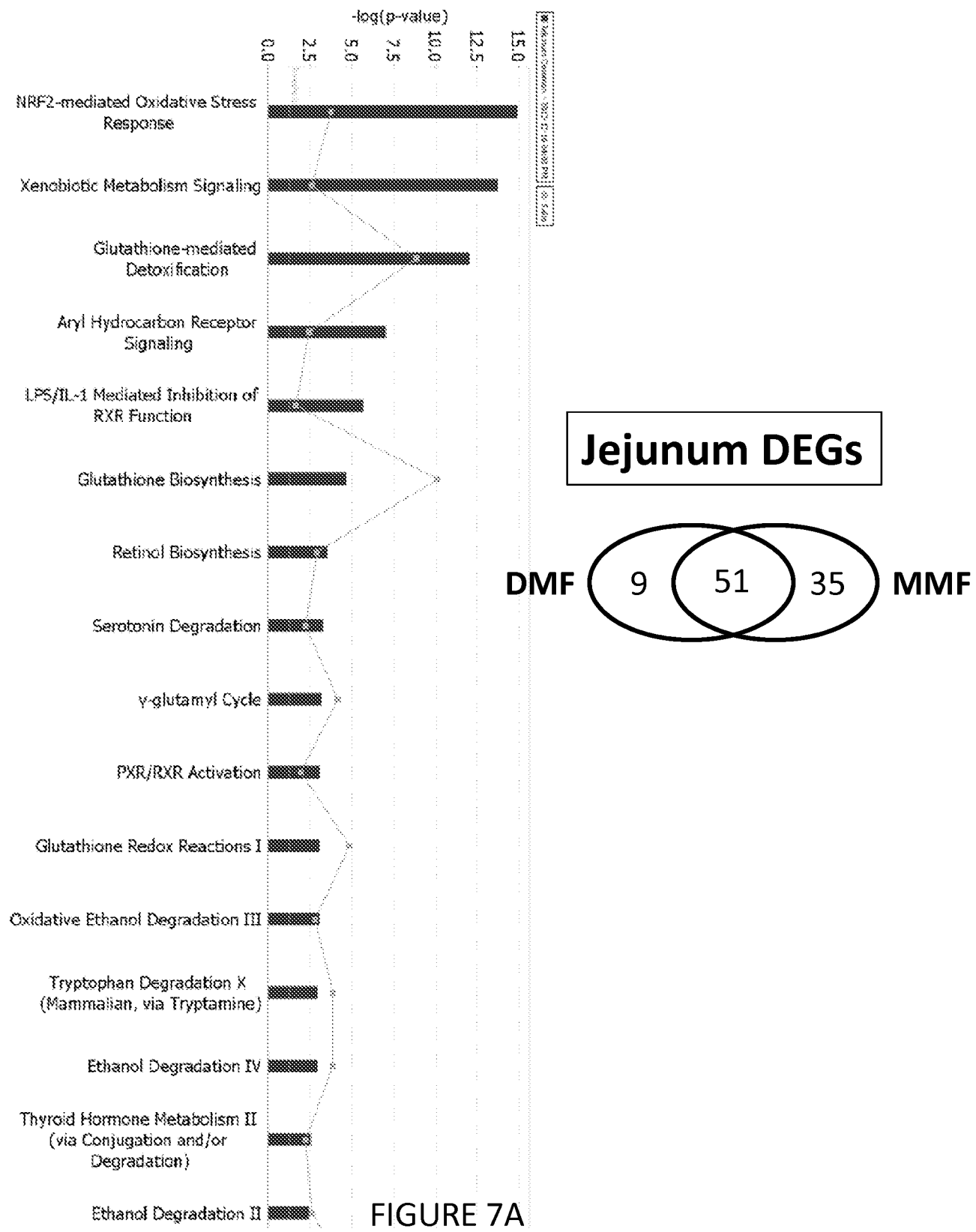


FIGURE 6



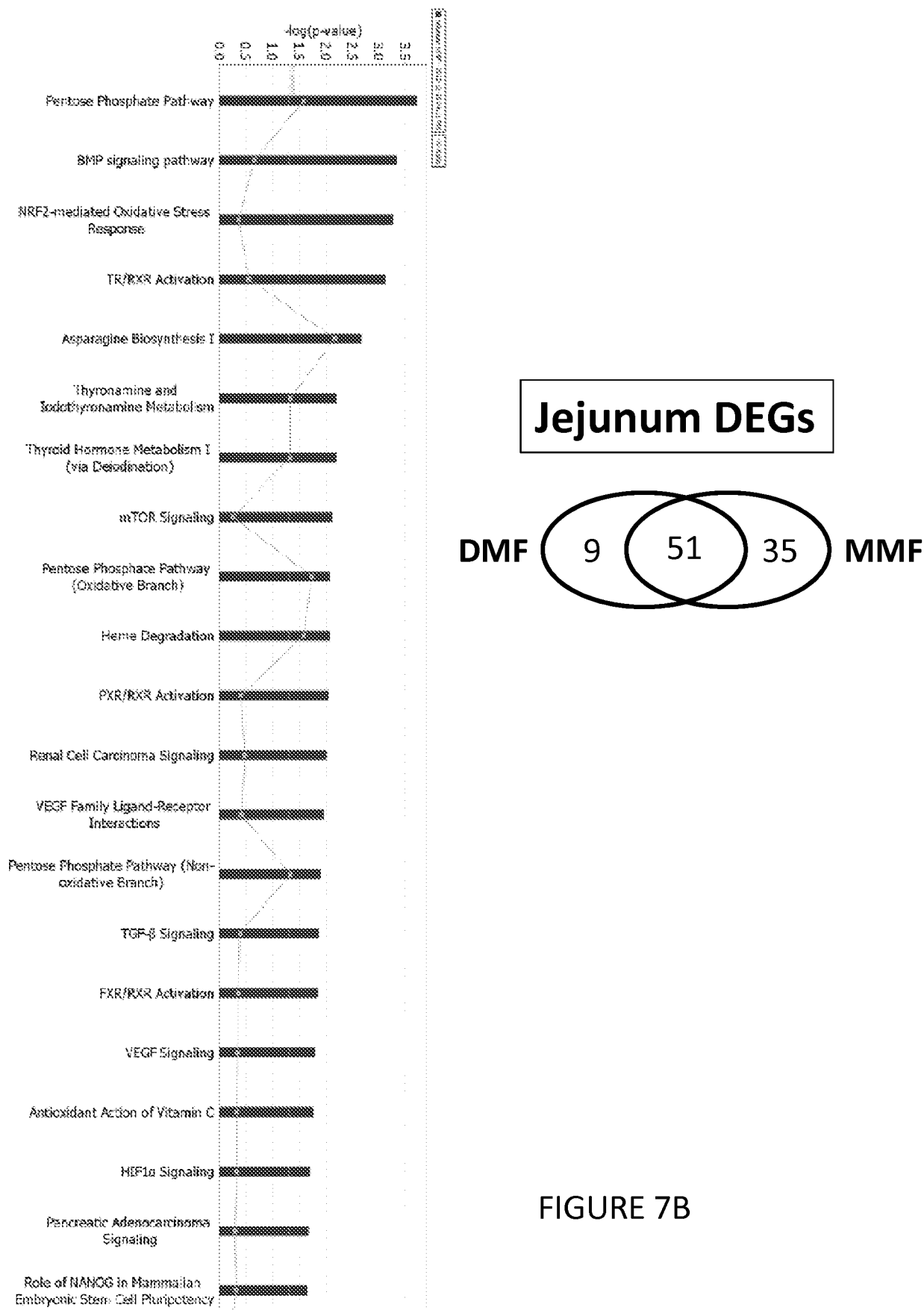
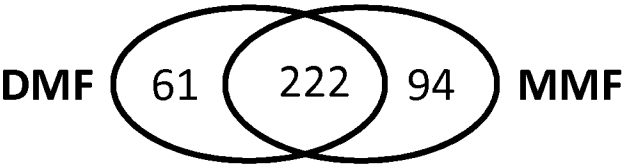


FIGURE 7B

Kidney DEGs



A

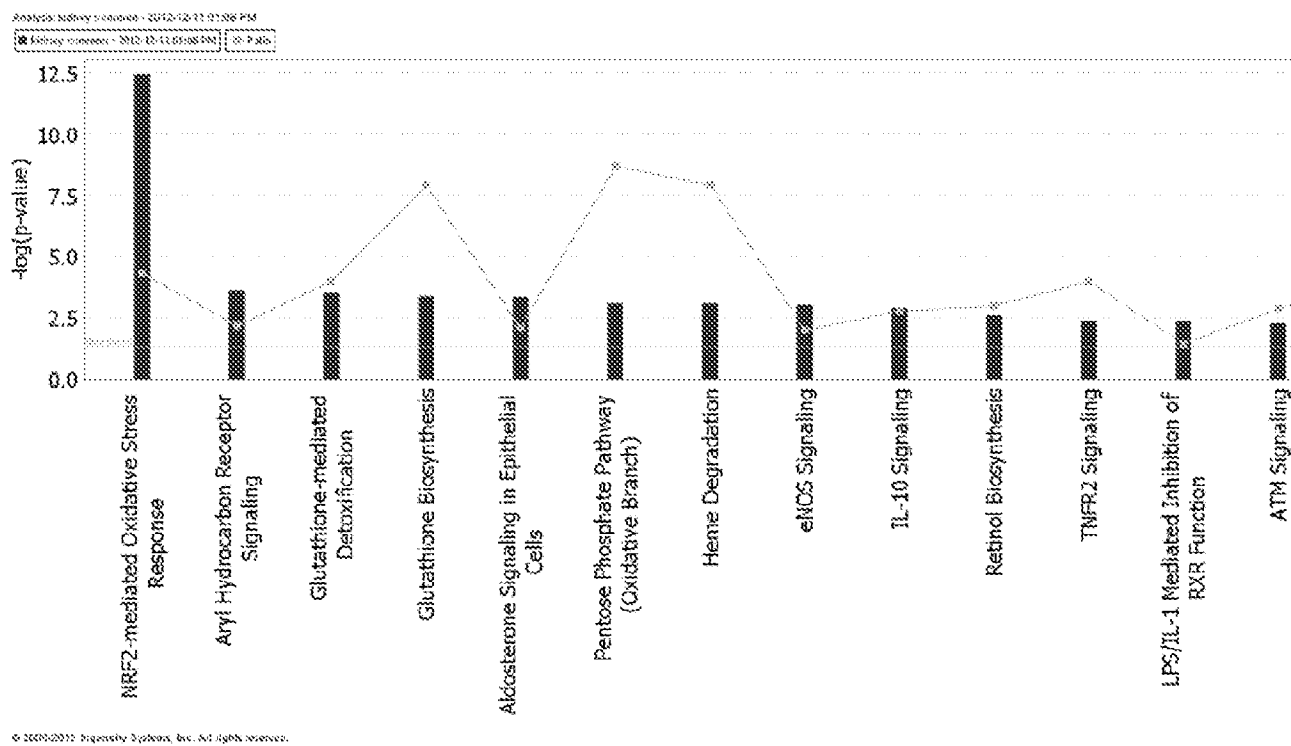


FIGURE 8

B

Supplementary Fig. 8-2015-12-11 09:15 PM
[Downloaded from 202.12.10.11 PM] [0.5.6.3]

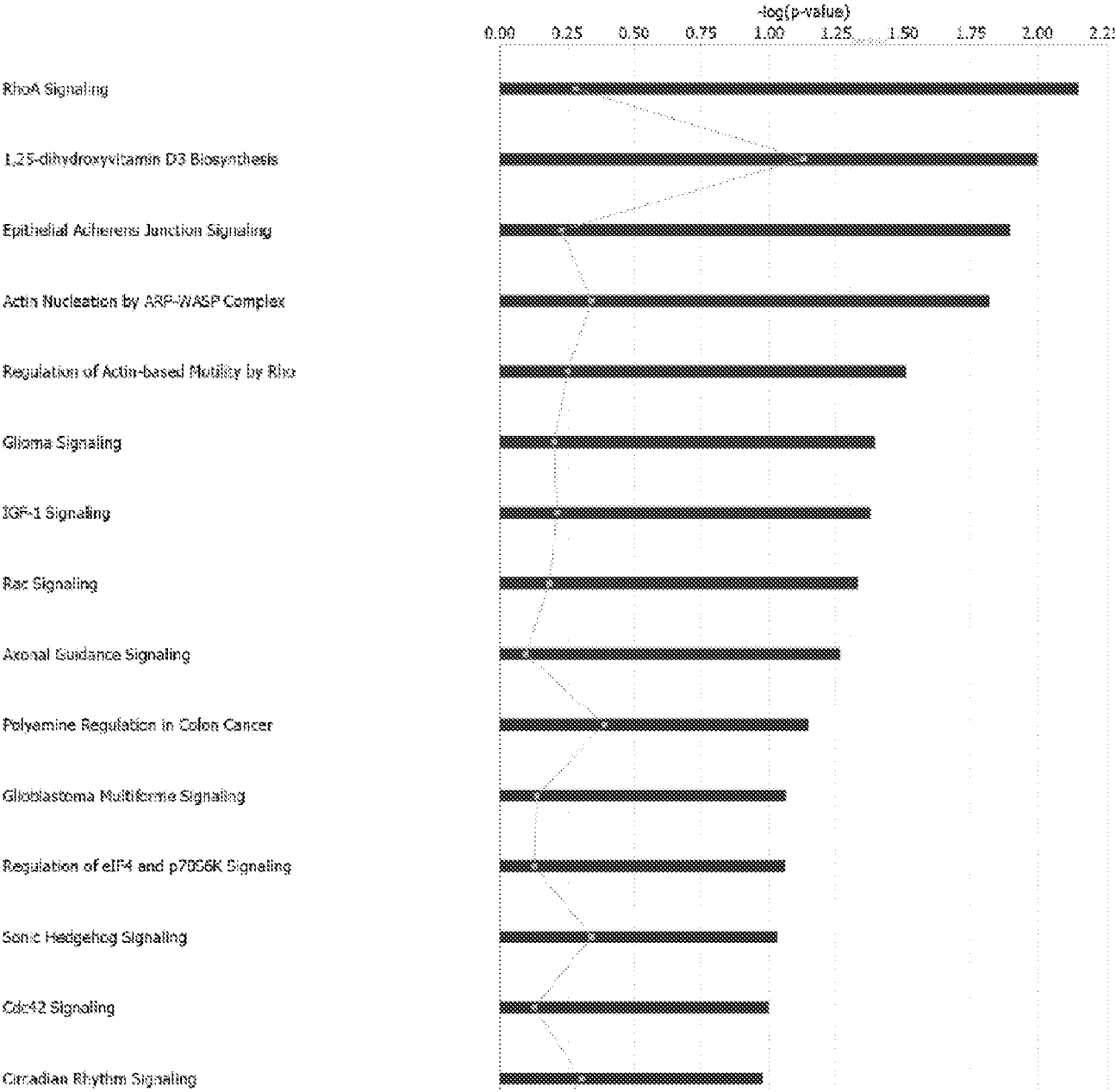


FIGURE 8 CONT'D

C

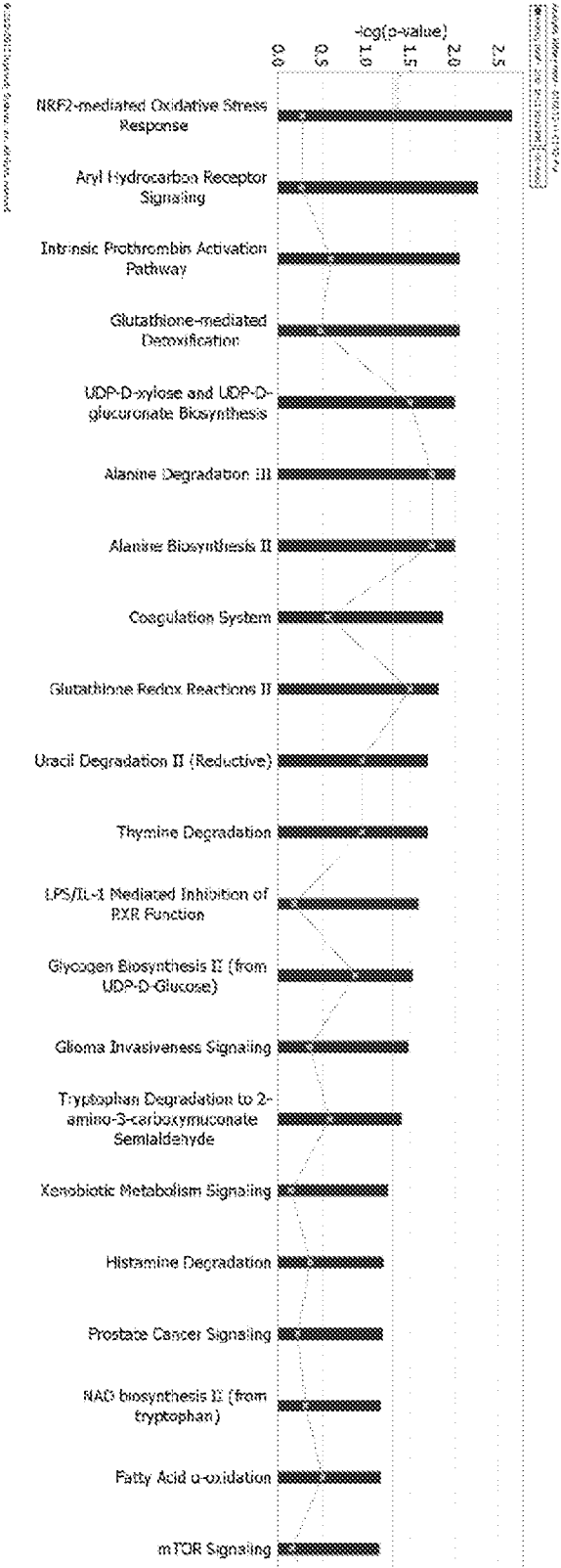
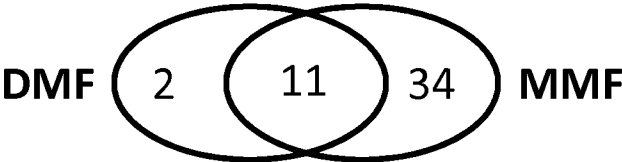


FIGURE 8 CONT'D

Liver DEGs



bioRxiv preprint doi: <https://doi.org/10.1101/011111>; this version posted March 1, 2015. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

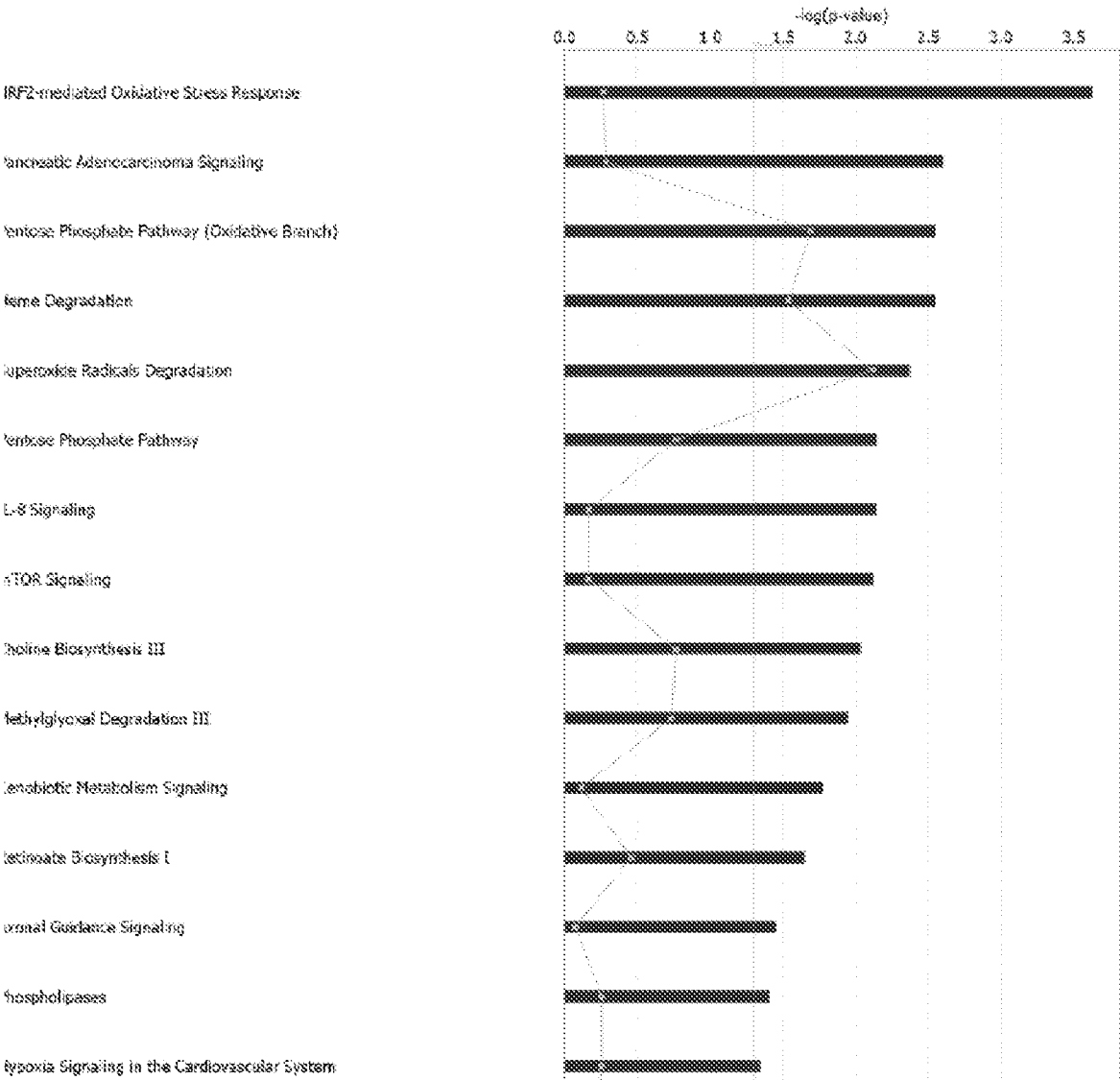
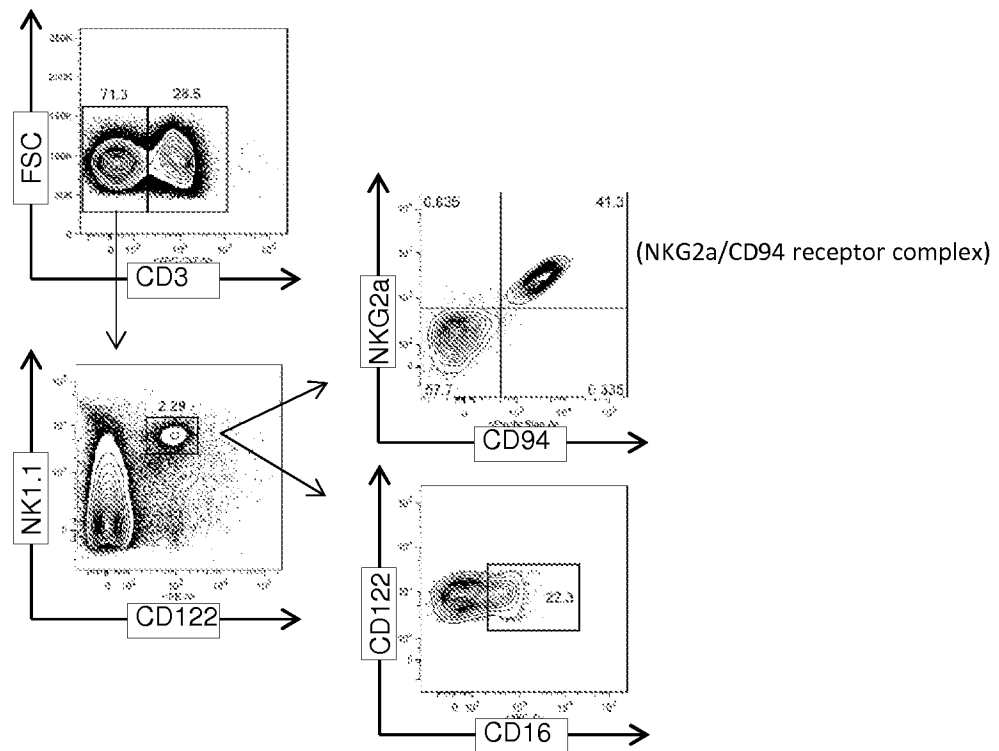


FIGURE 9

Gating strategy:



Protein expression quantified by mean fluorescent intensity (MFI):

- NK1.1 (*klrb1b*)
- Nkg2d (*klrk1*)
- NKp46 (*ncr1*)
- Nkg2a (*klrc1*)
- CD94 (*klrd1*)

FIGURE 10

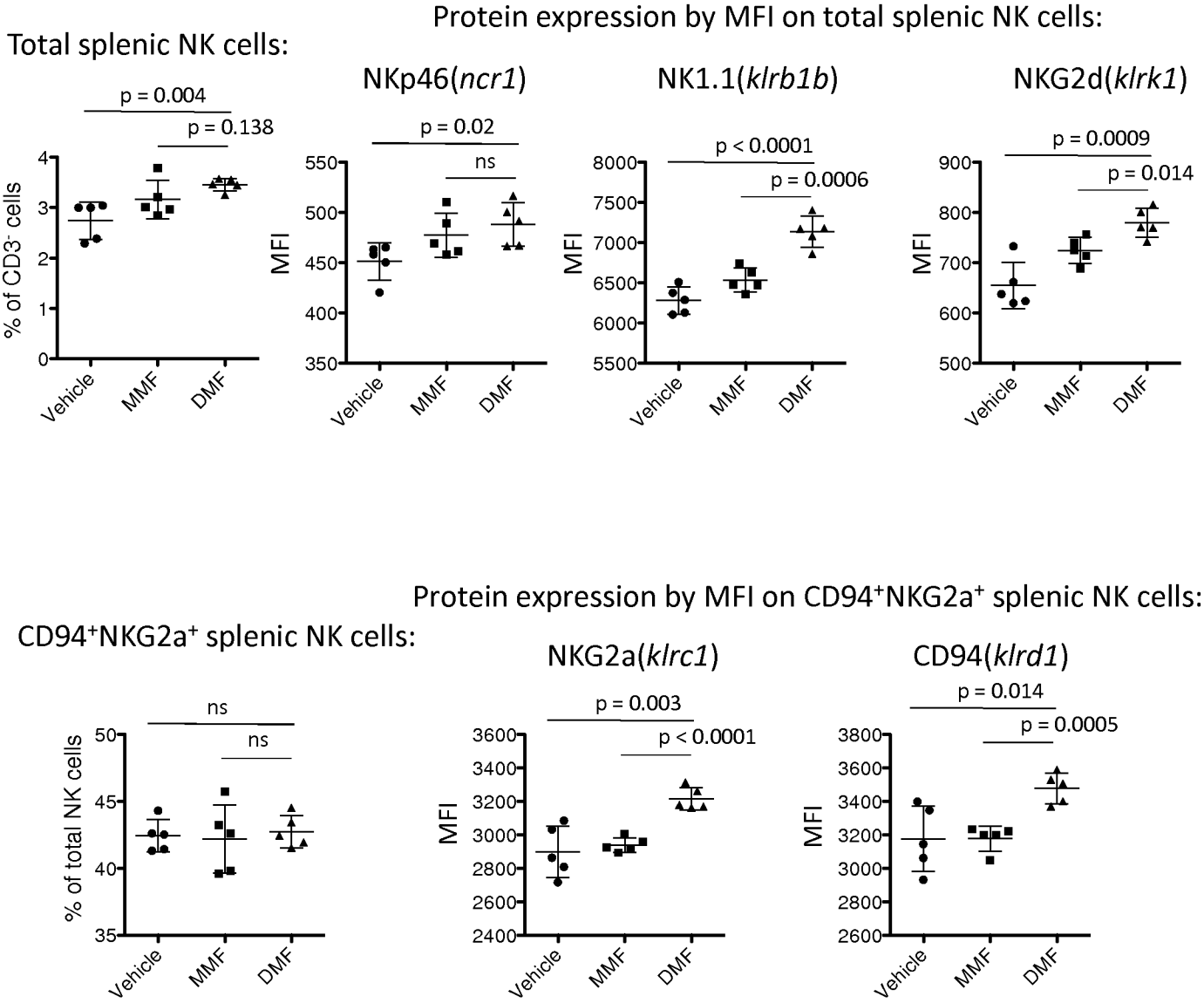


FIGURE 11

	T cell/Treg	titration		NK cell	titration		B cell	titration		Myeloid cell	titration	
A488	CD25	1uL		NKG2a	1uL		IgM	1uL		CD169	0.2uL	
PcP-Cy5.5	CD4	1uL		NKp46	1uL		B220	1uL		Ly6C	1uL	
PE	Foxp3	1uL		CD122	1uL		CD138	0.1uL		CCR2	5uL	
PE-CF594	NKG2D	1uL		NKG2d	1uL		X			CD11c	1uL	
PE-Cy7	CD62L	0.5uL		NK1.1	0.5uL		Fas	0.125uL		CD11b	0.5uL	
APC	CD122	1uL		CD16/32	1uL		IgG1	1uL		F4/80	1uL	
A700	X			X			CD38	0.33uL		CD86	1uL	
APC-H7	CD3	1uL		CD3	1uL		X			X		
V450	CD44	1uL		CD94	1uL		CD19	1uL		CD3, CD19, Ly6G, NK1.1		1uL each
V500	CD8	1uL		CD4	1uL		X			MHC II	1uL	
Regulatory T cells				NK cells			Germinal Center response			Monocytes - resident and inflammatory		
CD4 naïve, Tcm, Tem				Activating/Inhibitory receptors			B cells			Macrophages		
CD8 naïve, Tcm, Tem				Fc-receptors						Dendritic Cells		
Total NK cells												

FIGURE 12

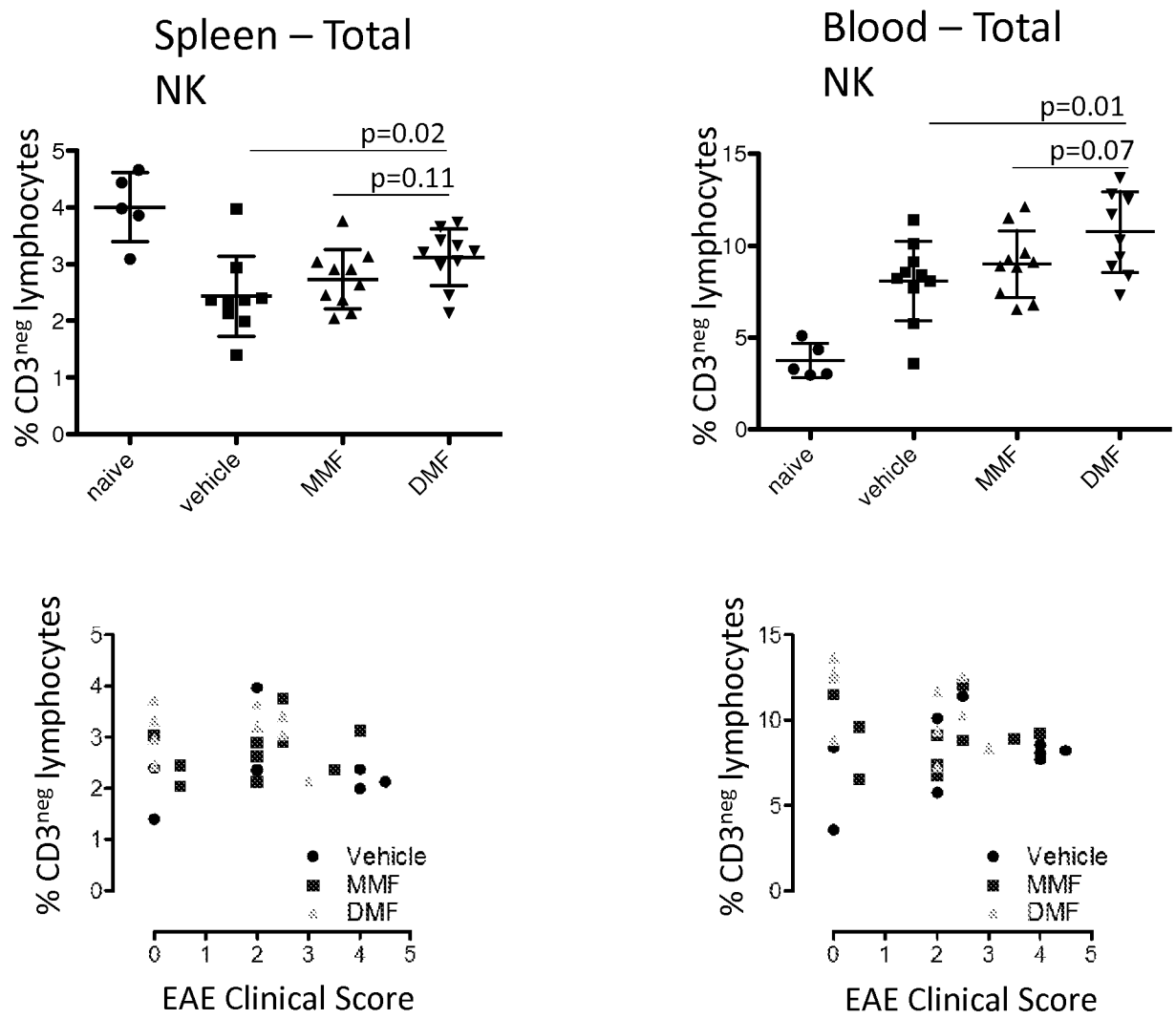


FIGURE 13

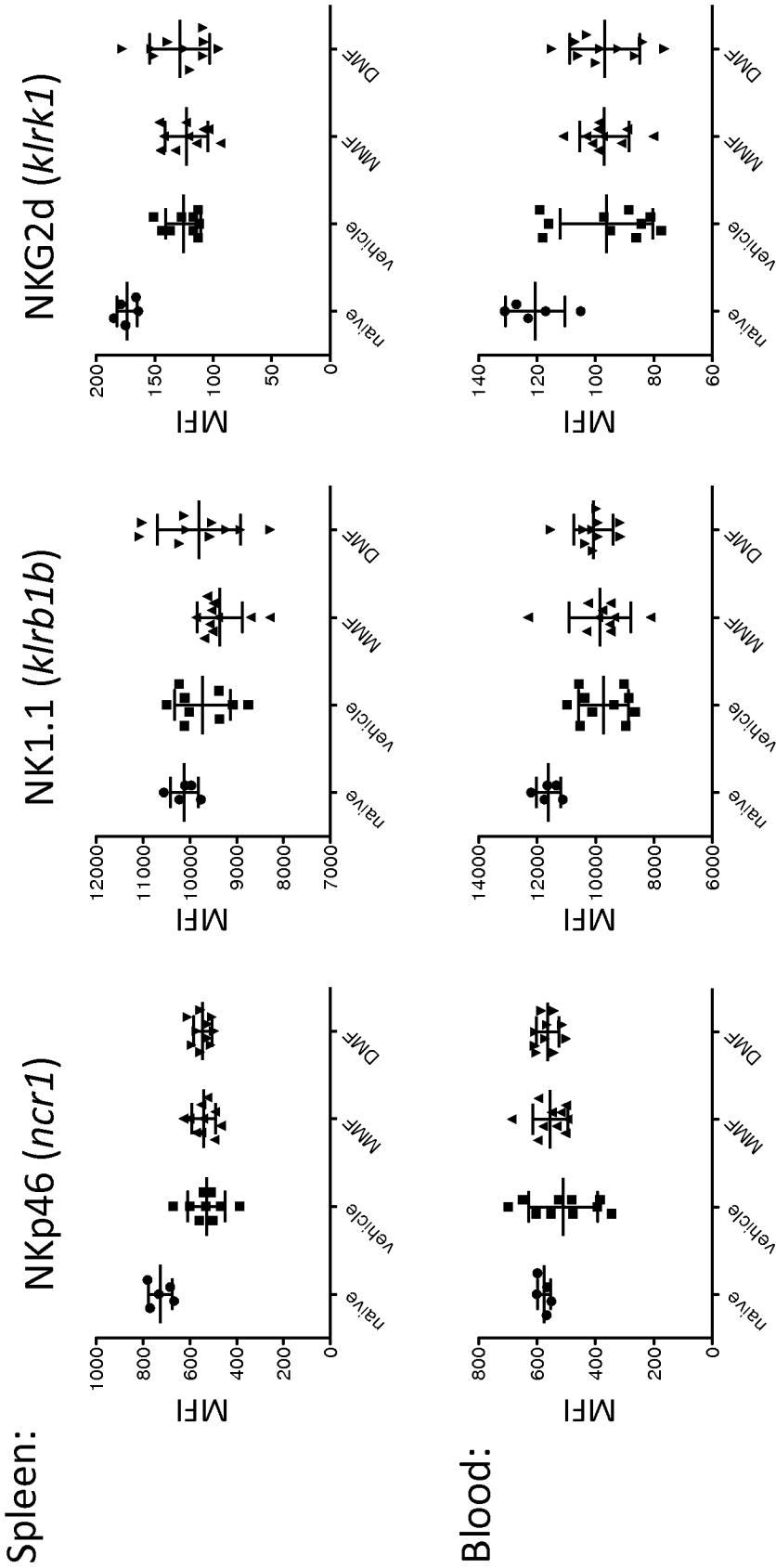


FIGURE 14

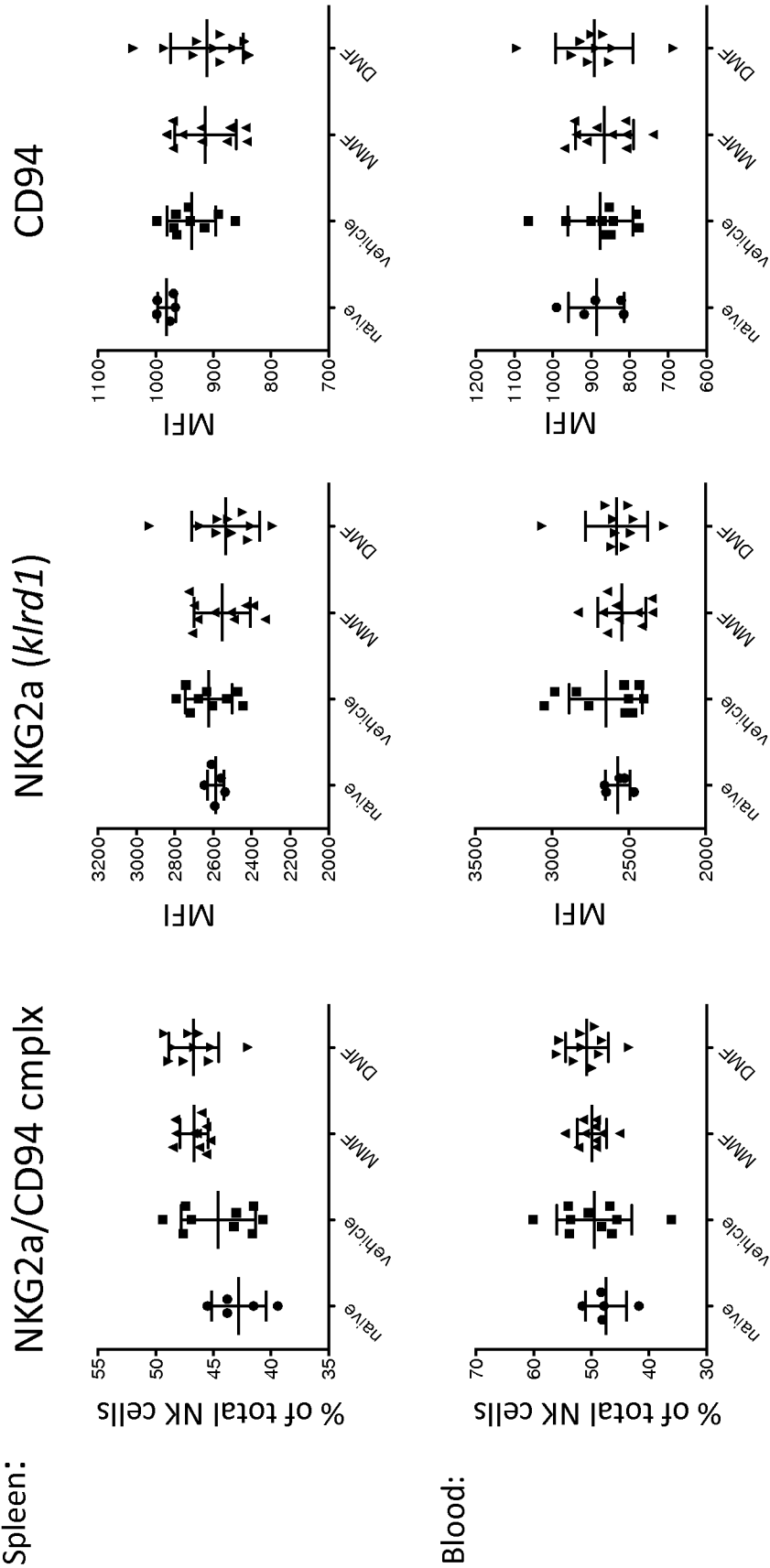


FIGURE 15

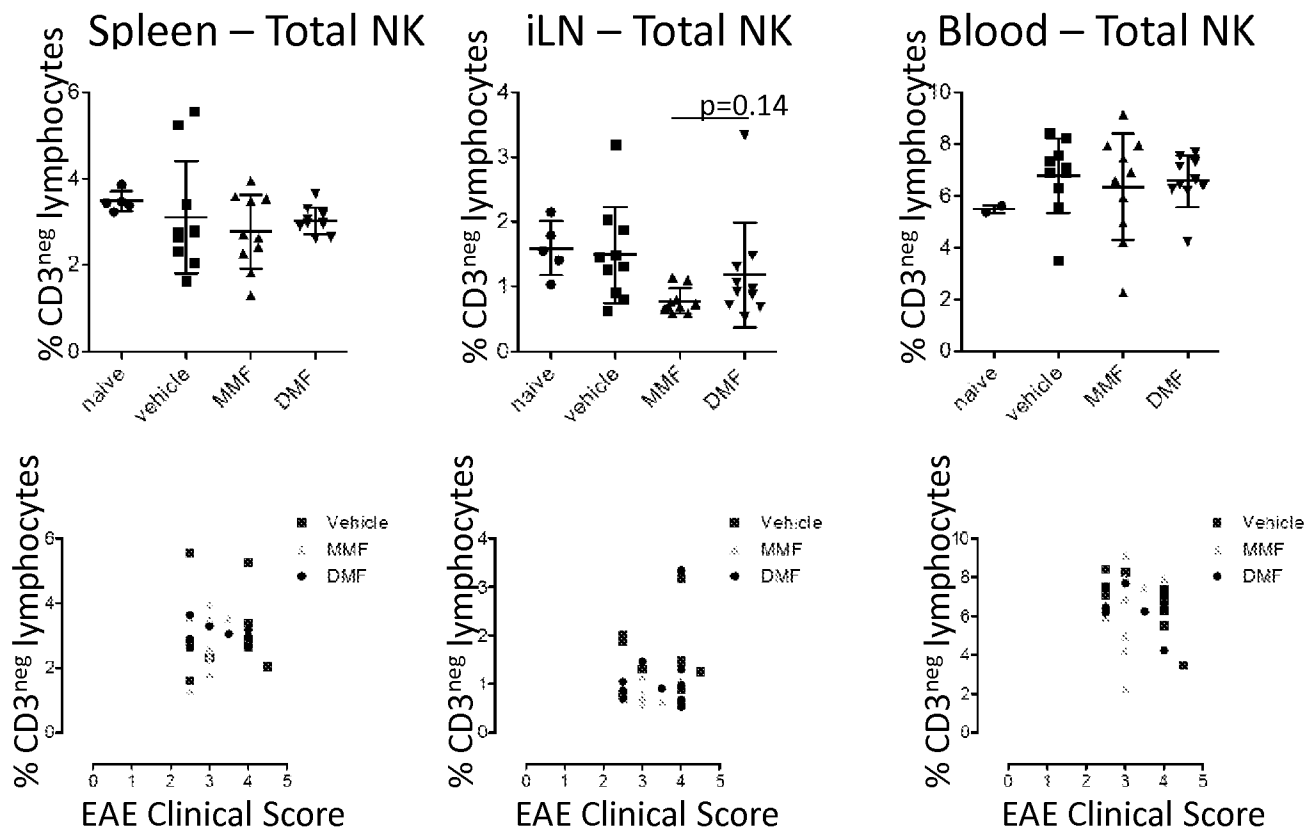


FIGURE 16

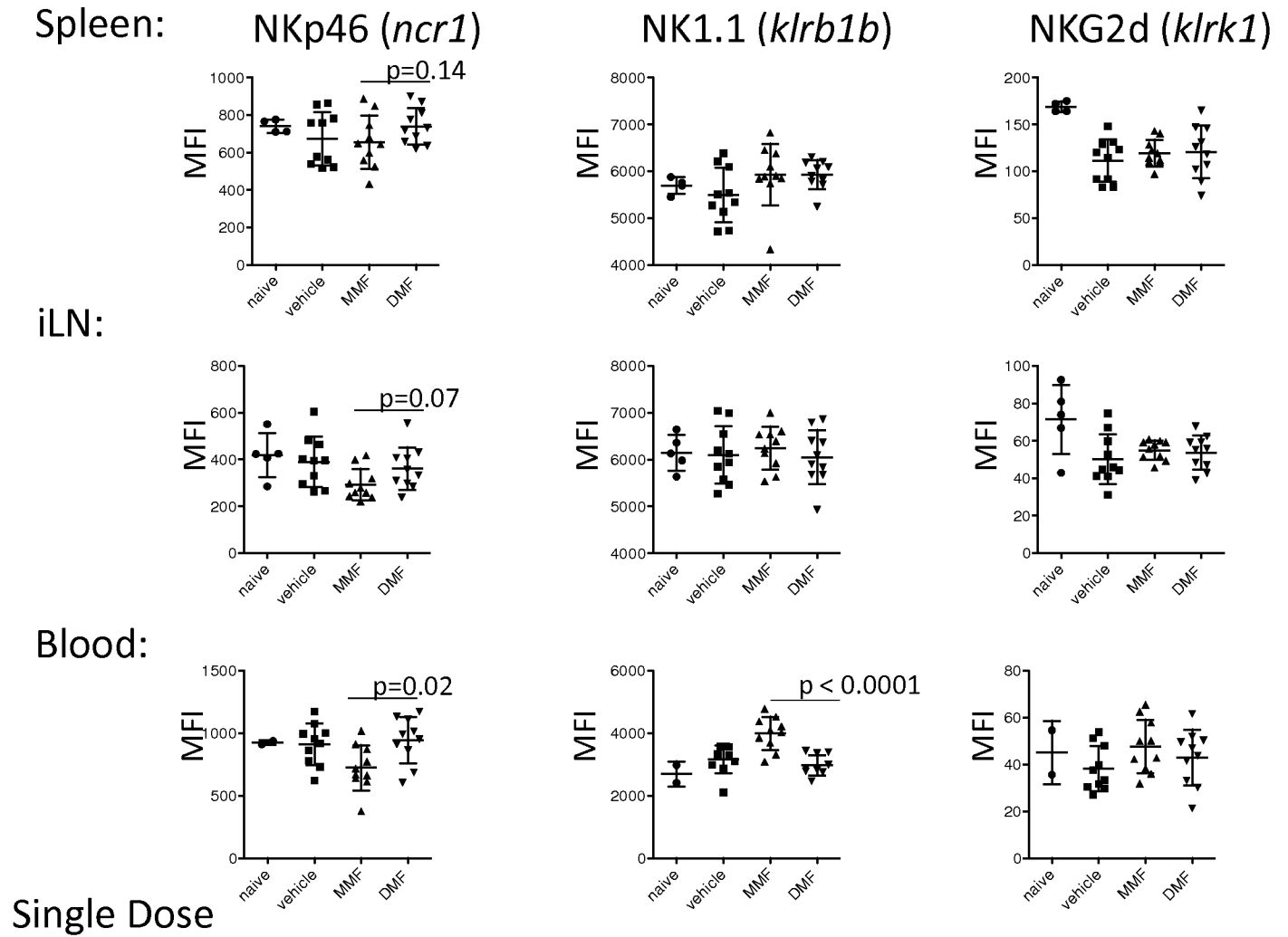


FIGURE 17

Spleen: NKG2a/CD94 cmplx

NKG2a (*kldr1*)

CD94

iLN:

Blood:

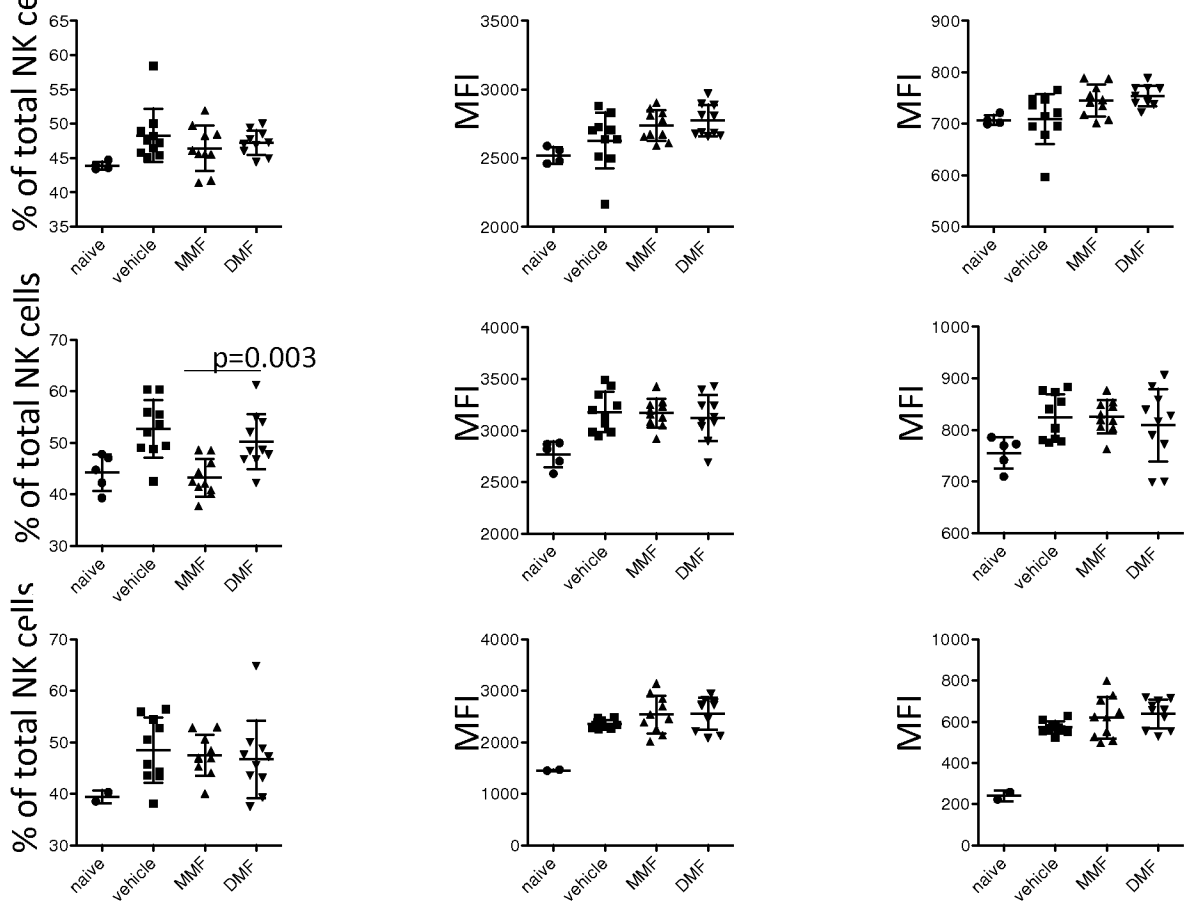


FIGURE 18

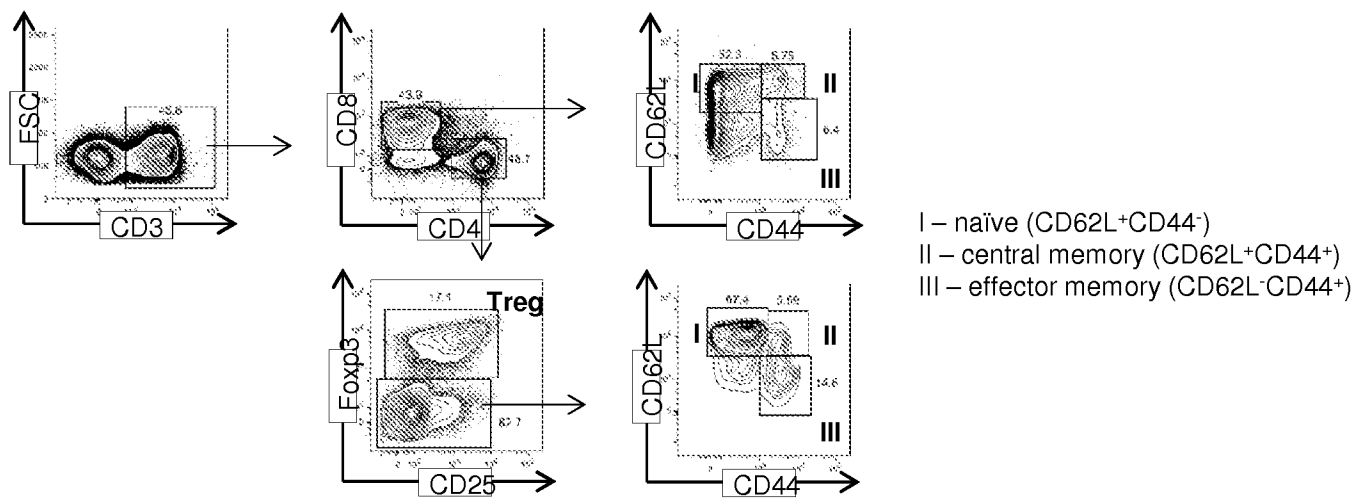


FIGURE 19

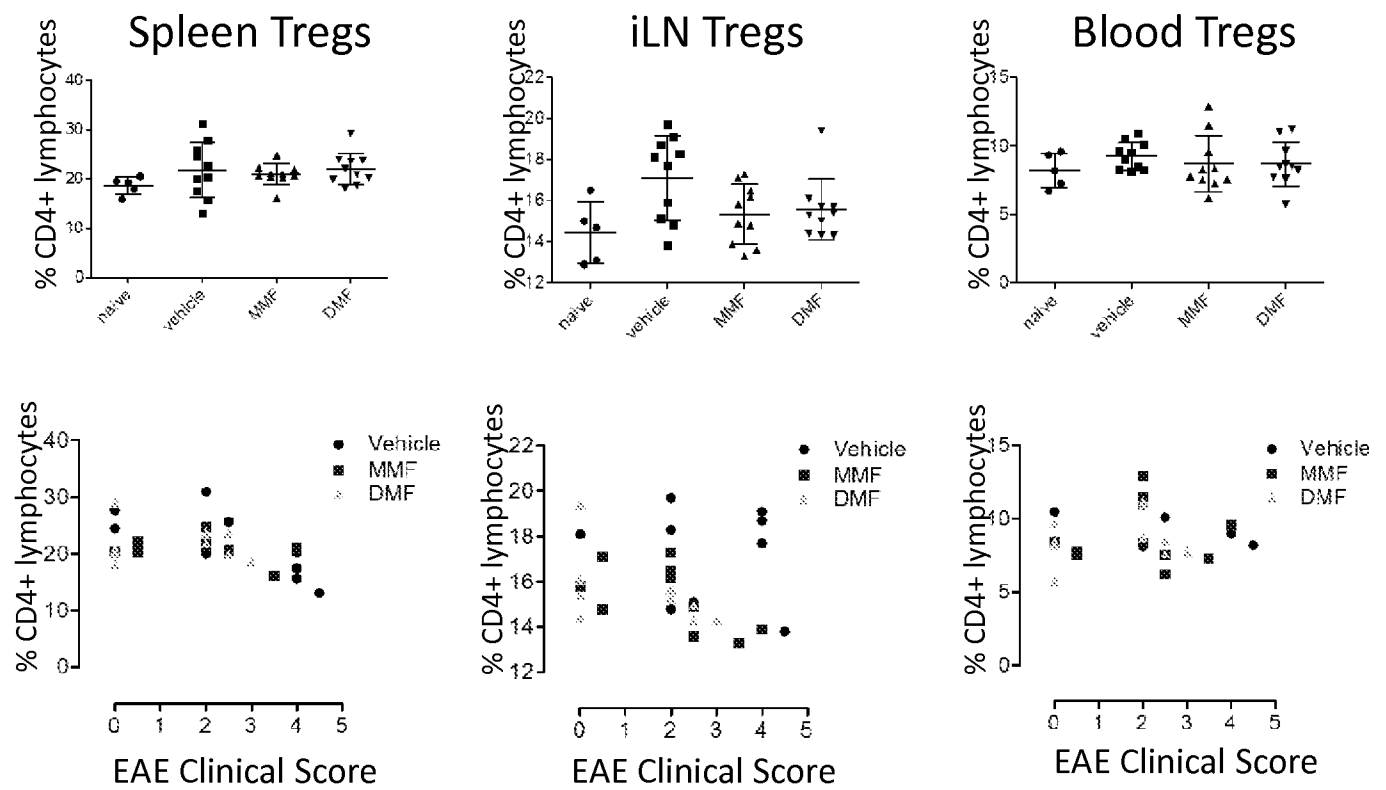


FIGURE 20

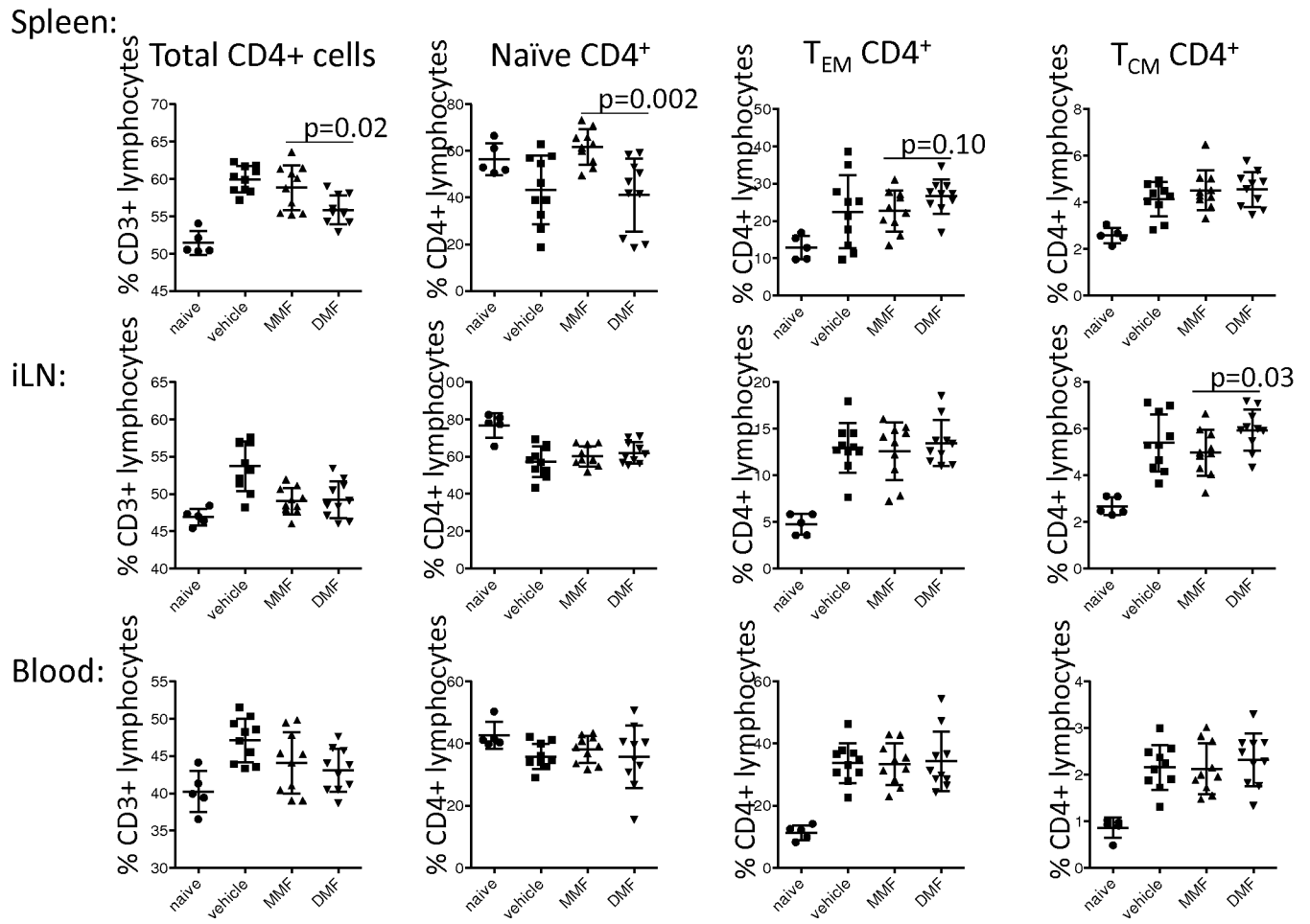


FIGURE 21

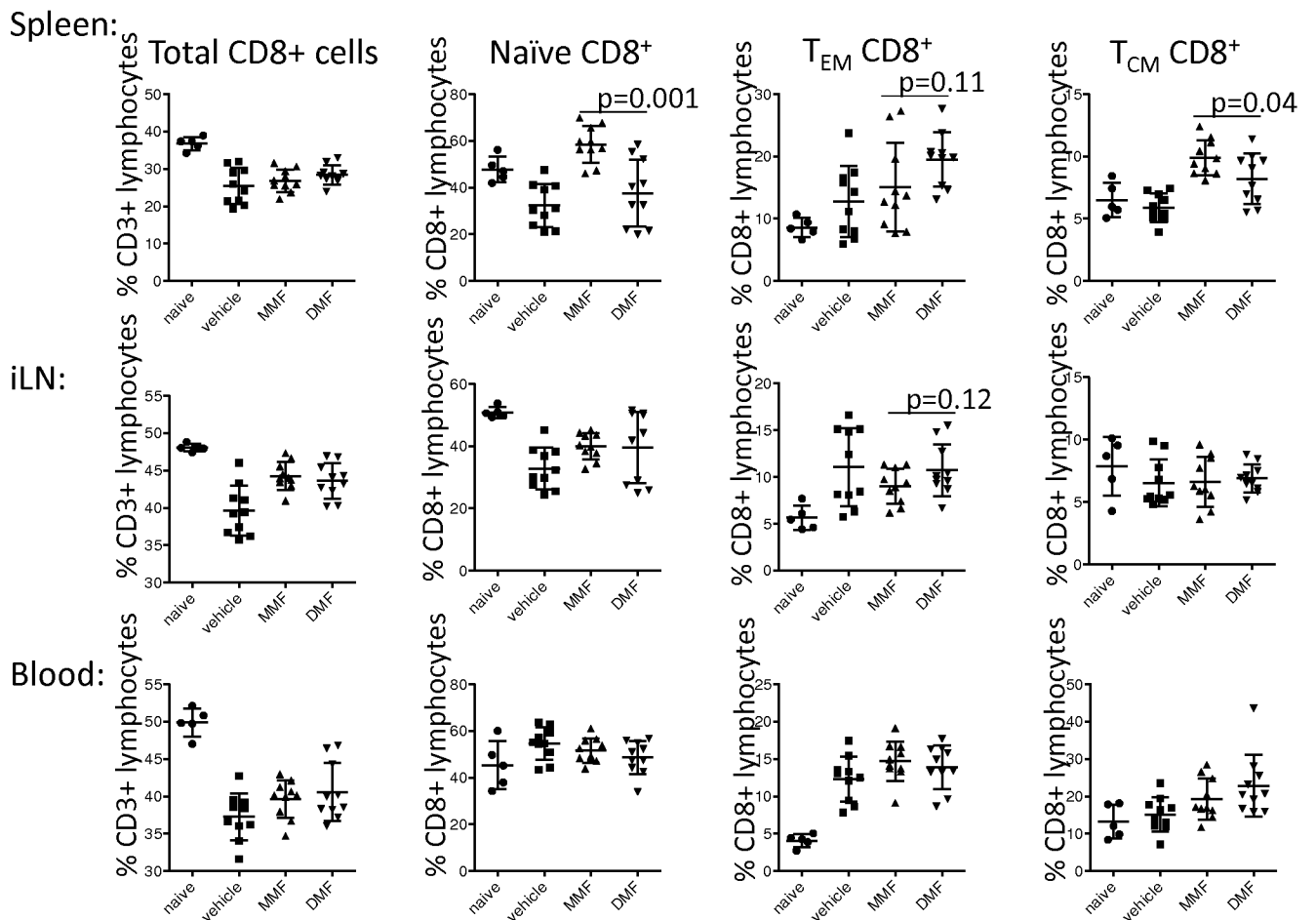


FIGURE 22

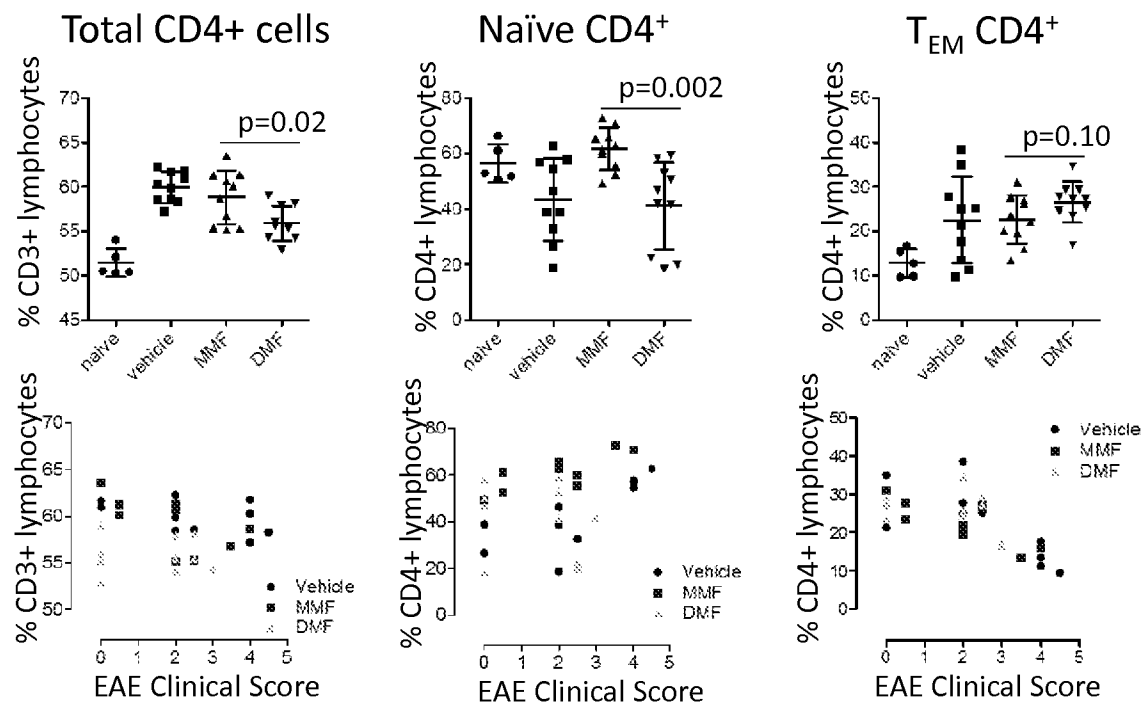


FIGURE 23

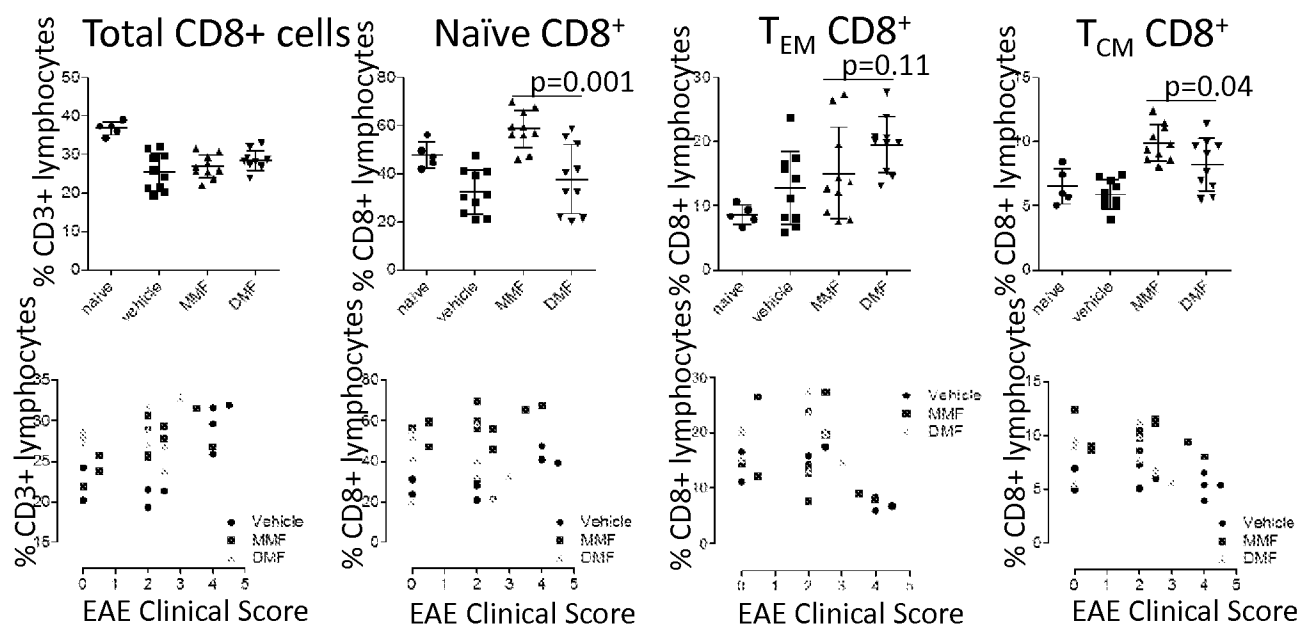


FIGURE 24

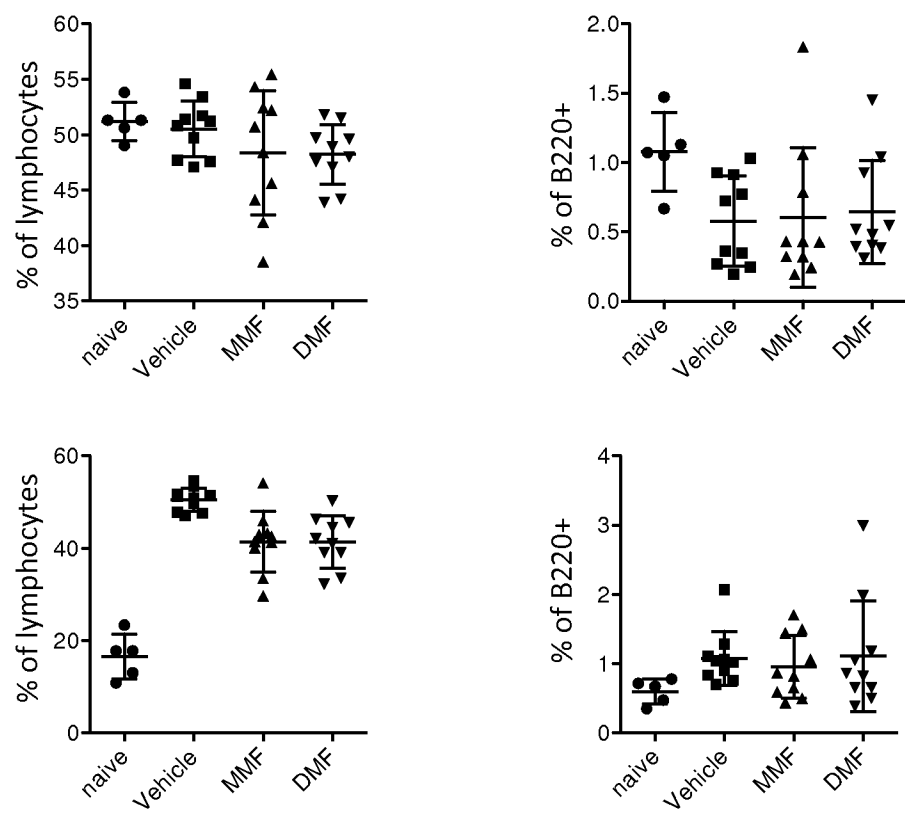
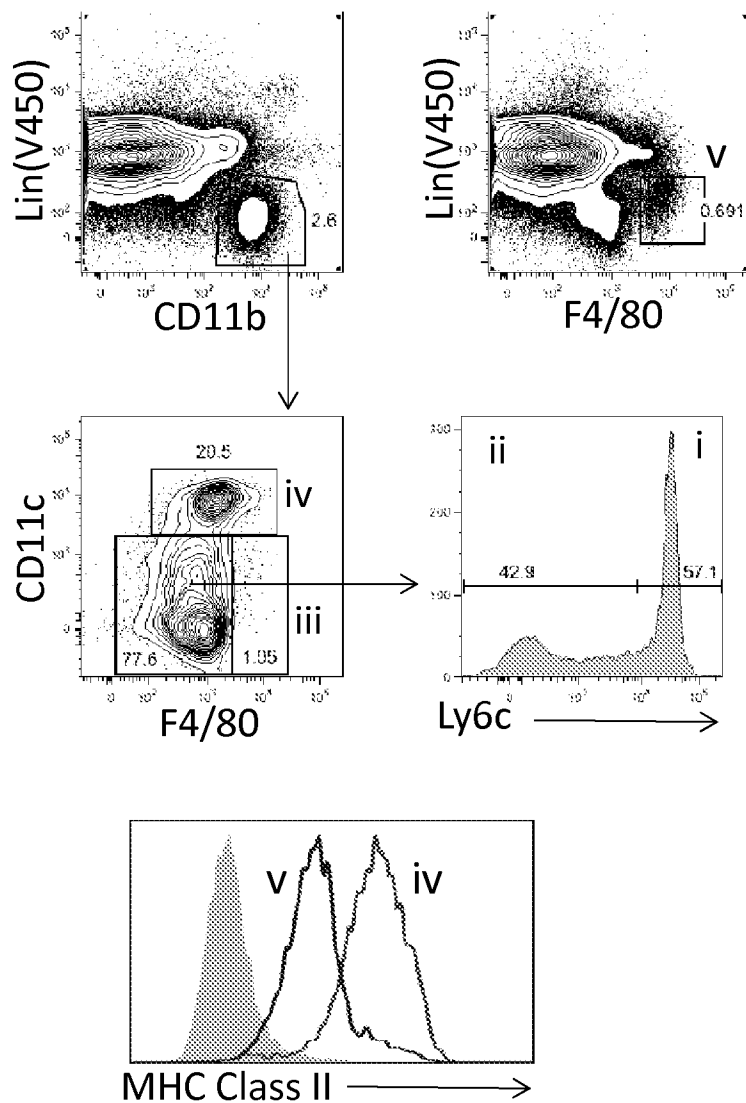


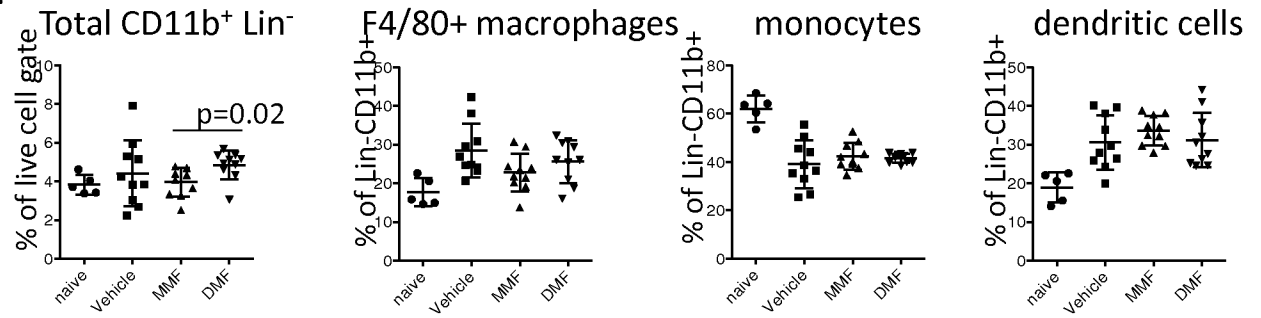
FIGURE 25



- i – Ly-6C^{high} (inflammatory) monocytes
- ii – Ly-6C^{low} (resident) monocytes
- iii – CD11b⁺F4/80⁺ macrophages
- iv – CD11b⁺CD11c⁺ DCs
- v – CD11b⁻F4/80⁺ macrophages

FIGURE 26

Spleen:



iLN:

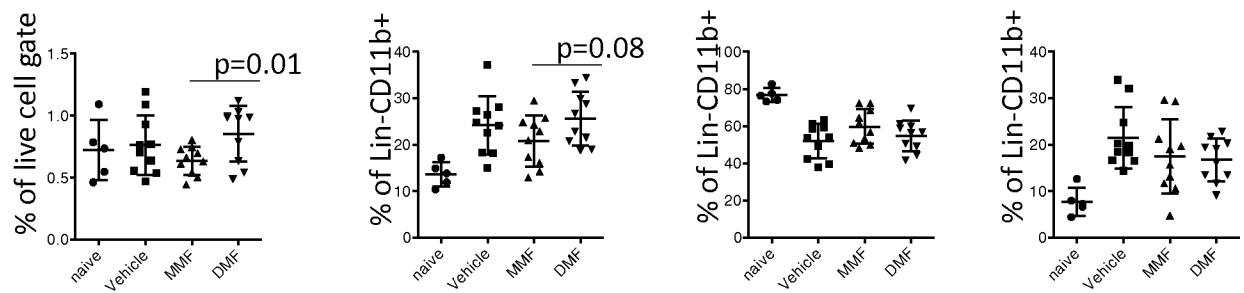


FIGURE 27

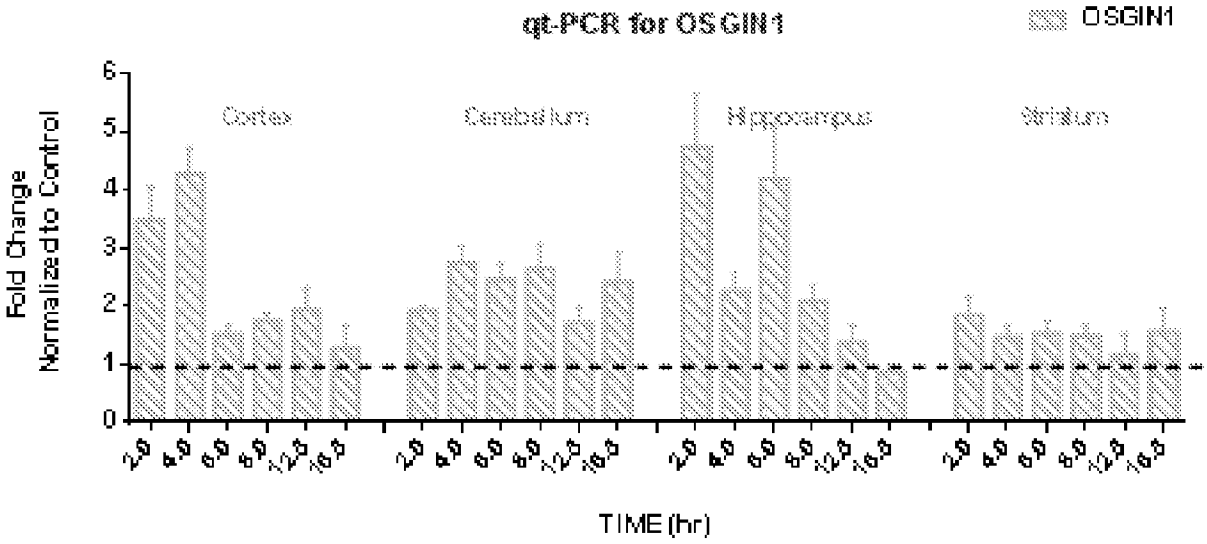


FIGURE 28

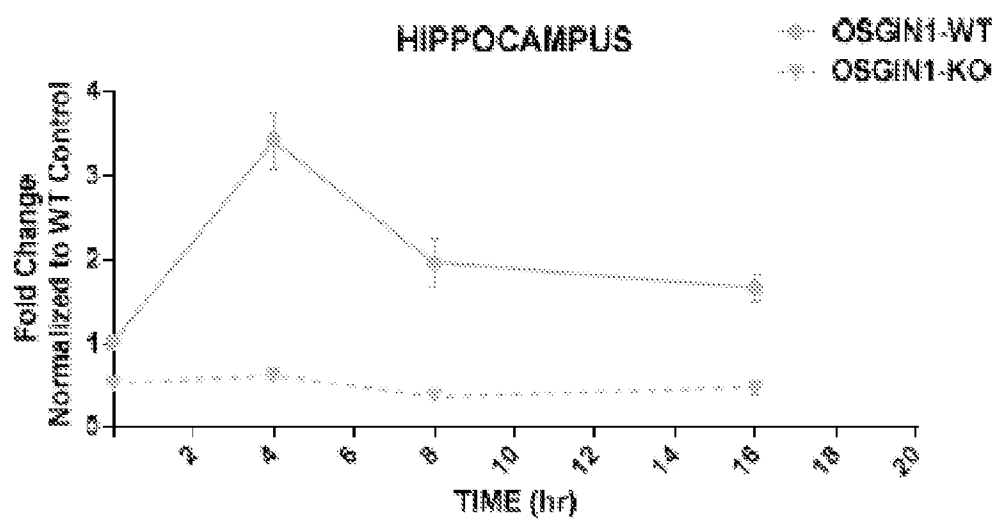


FIGURE 29A

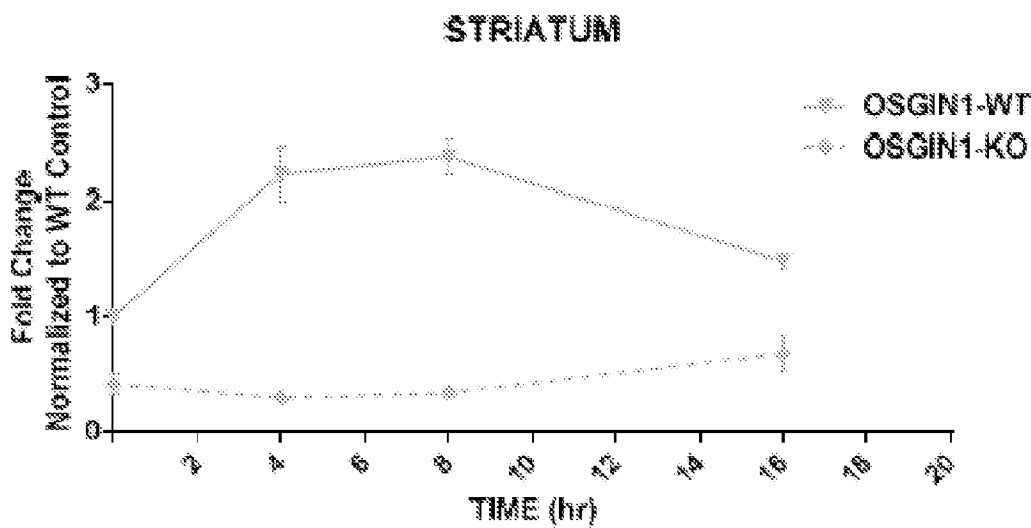


FIGURE 29B

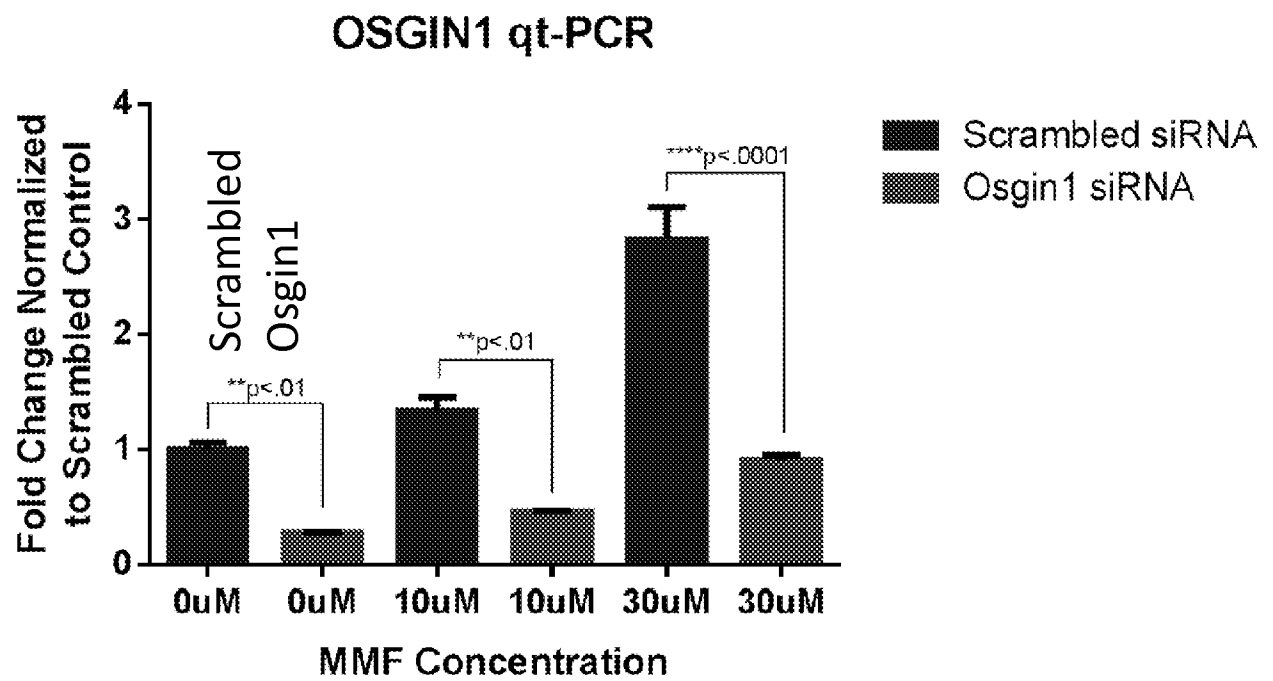


FIGURE 30

siRNA Knockdown in Human Astrocytes
Treated with MMF and 200uM H2O2:
Calcein AM Positive Cells

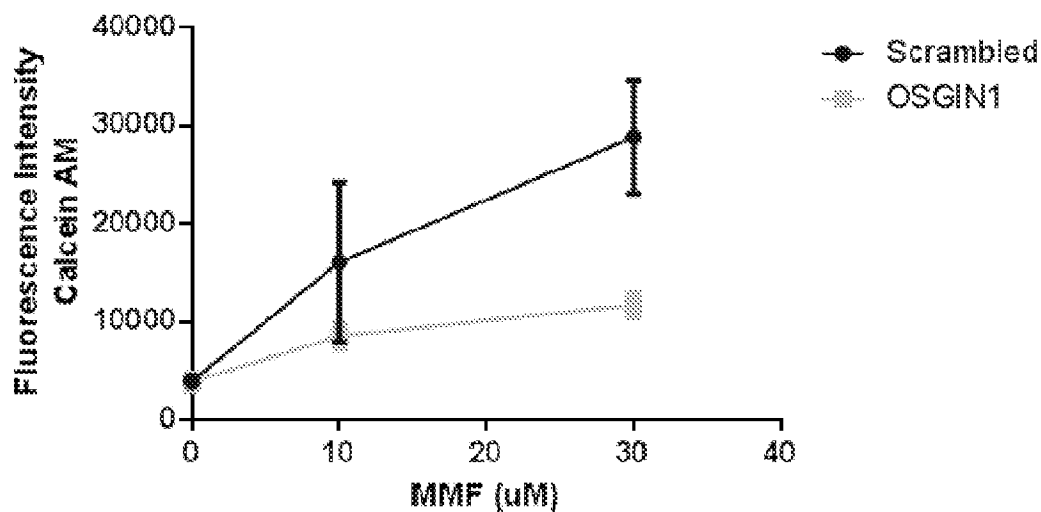


FIGURE 31A

siRNA Knockdown in Human Astrocytes
Treated with MMF and 200uM H2O2:
Viable Nuclear Count

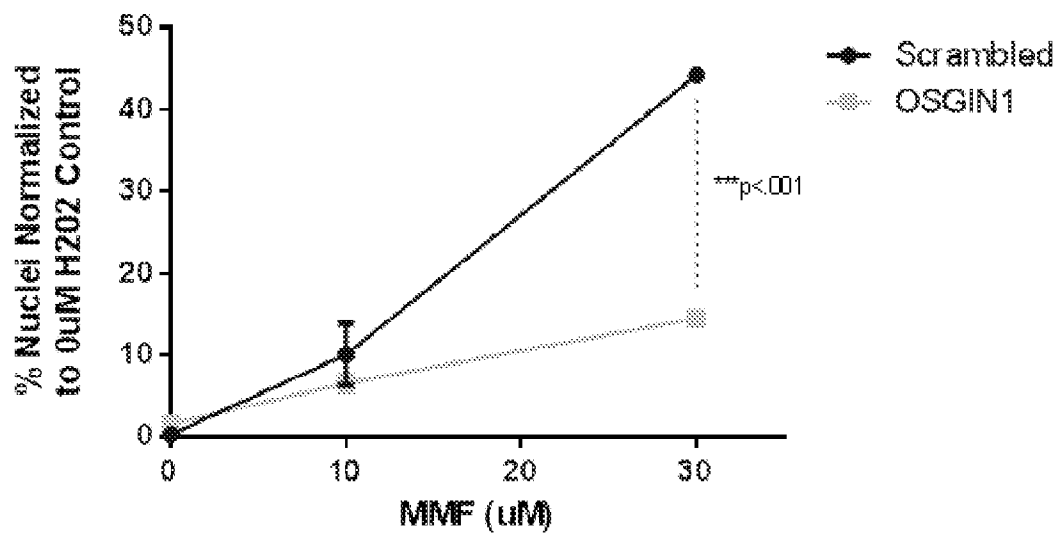


FIGURE 31B

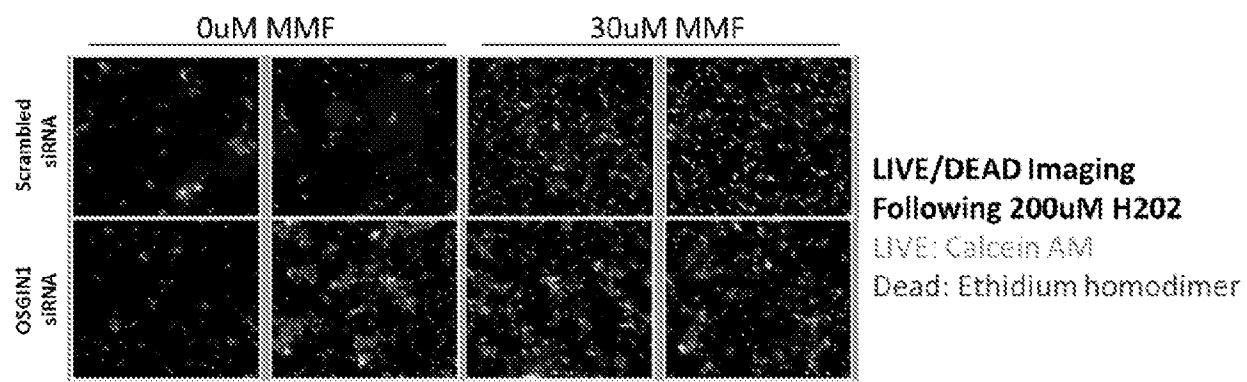


FIGURE 31C

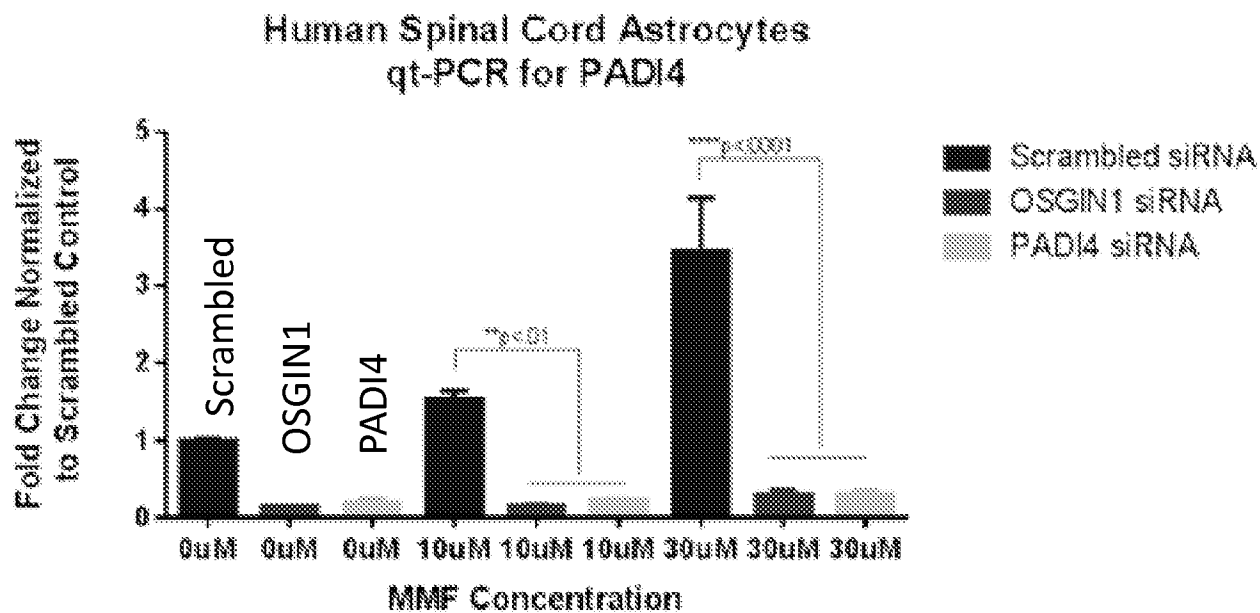


FIGURE 32A

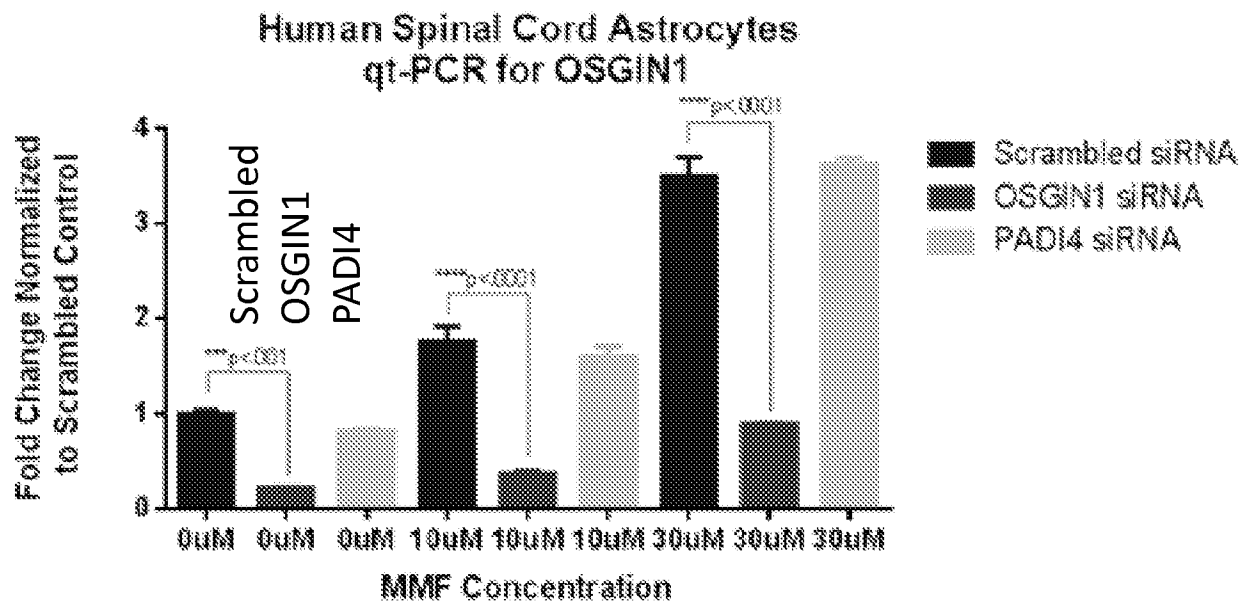


FIGURE 32B

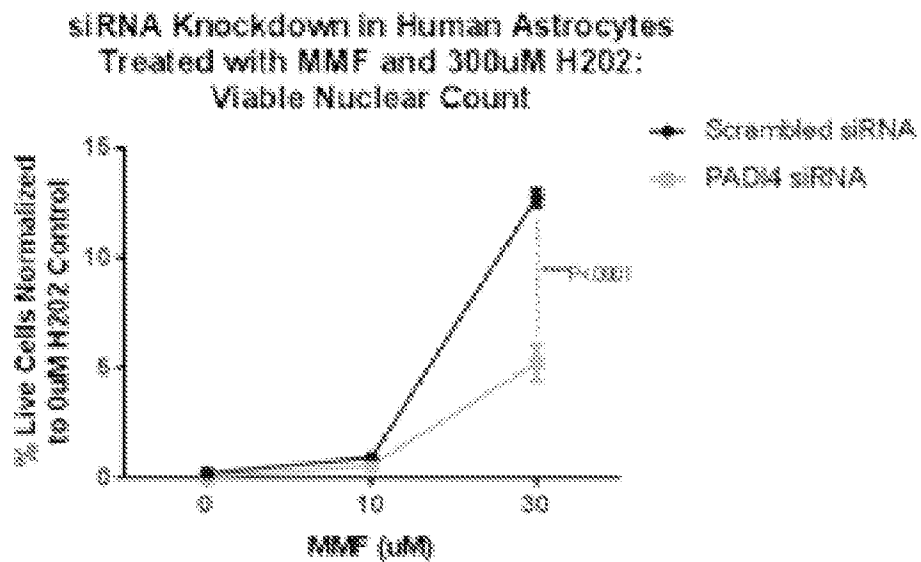


FIGURE 33A

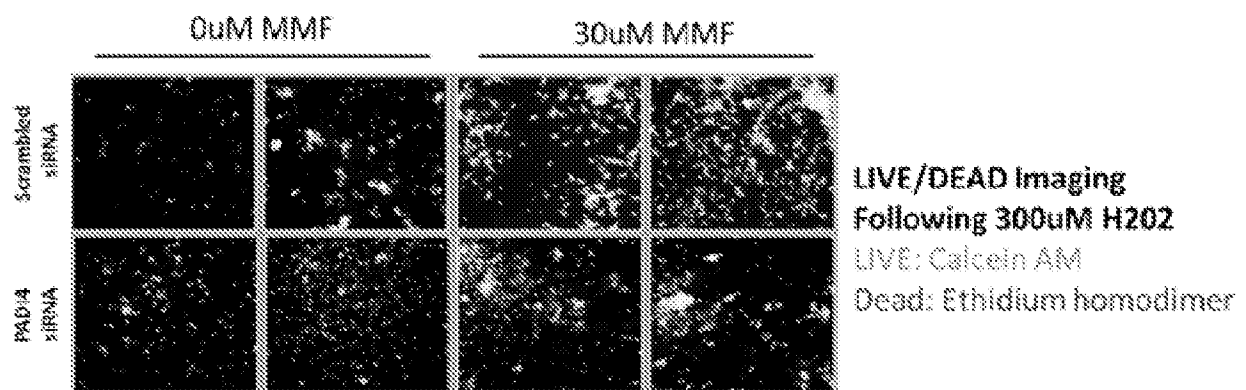


FIGURE 33B

**siRNA Knockdown in Human Astrocytes
Treated with MMF and 300uM H2O2:
Viable Nuclear Count**

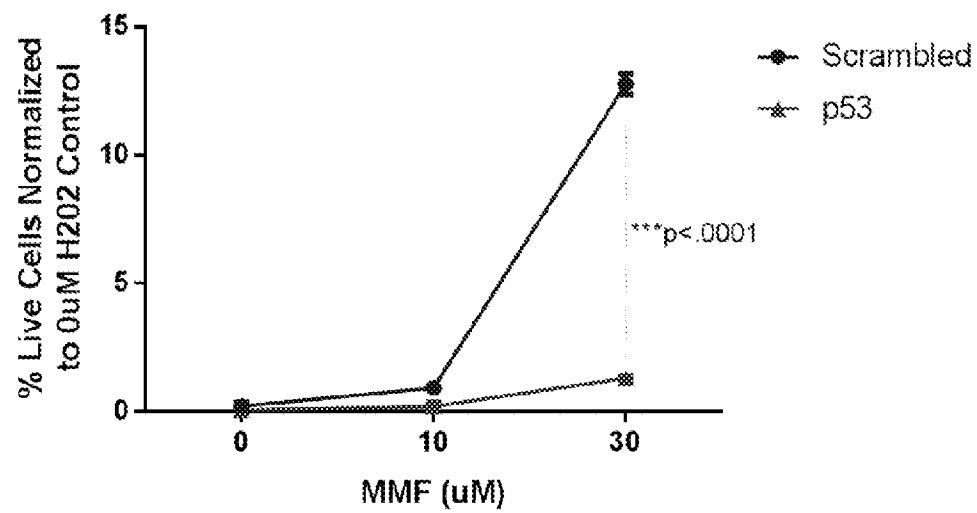


FIGURE 34

GO TERM	GO ID	Gene Name	Gene Symbol
Autophagy	GO:0006914	ATG2 autophagy related 2 homolog A sequestosome 1 (p62)	ATG2A SQSTM1
Cell cycle	GO:0007049	cyclin-dependent kinase inhibitor 1A (P21) growth differentiation factor 15	CDKN1A GDF15
Cell differentiation	GO:0030154	vesicular endothelial growth factor	VEGFA
Cellular matrix organization	GO:0030198	matrix metalloproteinase 11 (stromelysin 3)	MMP11
Cell migration	GO:0016477	snail homolog 1	SNAIL
Cholesterol homeostasis		ATP-binding cassette, sub-family A (ABC1), member 1	ABCA1
Developmental growth	GO:0048589	stimulated by retinoic acid gene 6 homolog (mouse)	SIRA6
Dopamine receptor signaling pathway	GO:0007212	neuron-specific protein family member 2	NSG2
Glutamine biosynthesis process	GO:0005642	glutamate-ammonia ligase	GLUL
L-glutamate transport	GO:0015813	solute carrier family 1, member 2 (EAAT2)	SLC1A2
Metabolic process	GO:0008152	glutathione S-transferase alpha 2	GSTA2
Myelination	GO:0042552	early growth response 2	EGR2
Negative regulation of apoptotic process	GO:0043065	DnaJ (Hsp40) homolog, subfamily A, member 3 apoptosis antagonizing transcription factor nerve growth factor-γ	DNAJA3 AATF NGFG
Negative regulation of cell growth	GO:0008083	Oxidative stress induced growth inhibitor 1 nitric oxide synthase 1 sirtuin 1	OSGIN1 NOS1 SIRT1
Neuron development	GO:0048666	nuclear receptor subfamily 2, group F, member 6	NR2F6
Neuron fate commitment	GO:0048663	oligodendrocyte transcription factor 1	OLIG1
Nucleoside diphosphate metabolic process	GO:0009132	nudix (nucleoside diphosphate linked moiety X)	NUDT7
Oxidation-reduction process	GO:0055114	aldo-keto reductase family 1, member B8 copper chaperone for superoxide dismutase oxidoreductase NAD-binding domain containing 1	AKR1B8 CCS OXNAD1
Positive regulation of excitatory postsynaptic membrane potential	GO:2000463	neuroligin (NRP) and toll-like (TL)-like 2	NETO2
Regulation of cell growth	GO:0001558	insulin-like growth factor binding protein	IGFBP
Regulation of synaptic plasticity	GO:0048167	brain-derived neurotrophic factor	BDNF
Response to oxidative stress	GO:0006979	amyotrophic lateral sclerosis 2 (juvenile) apolipoprotein E GRB2-associated binding protein 1 glutamate-cysteine ligase, catalytic subunit glutamate-cysteine ligase, modifier subunit NAD(P)H dehydrogenase, quinone 1 peroxiredoxin 1 prion protein prostaglandin-endoperoxide synthase 2 superoxide dismutase sulfiredoxin 1 thioredoxin reductase 1 glial fibrillary acidic protein	ALS2 APOE GAB1 GCLC GCLM NQO1 PRDX1 PRNP PTGS2 SOD1 SRXN1 TXNRD1 GFAP
Retinol metabolic process	GO:0042574	retinol binding protein 4, plasma	RBP4
Signal transduction	GO:0007165	NF-κappa-B inhibitor, beta	NFKBIB
Tissue regeneration	GO:0042246	ninjurin 1	NINJ1

FIGURE 35

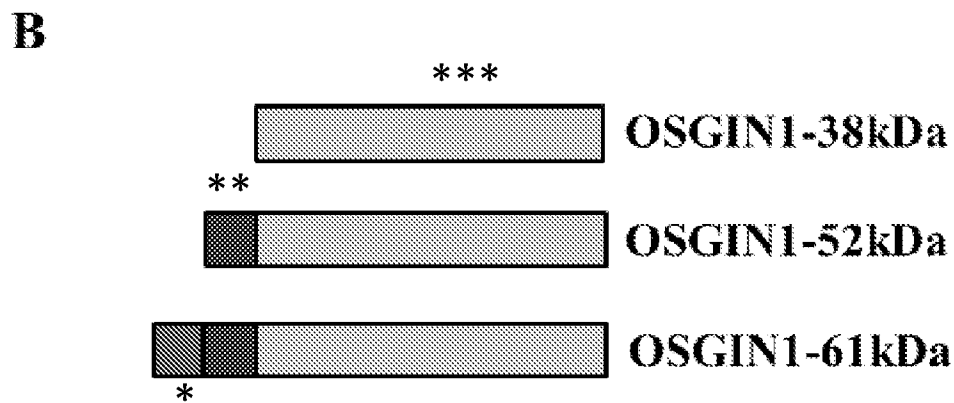
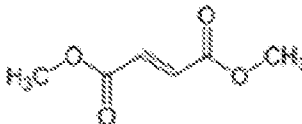
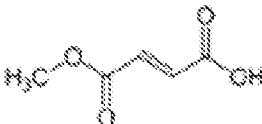


FIGURE 36

A

Compound Name	Dimethyl Fumarate	Monomethyl Fumarate
Abbreviation	DMF	MMF
Structure		
Molecular Weight	144.13	130.1
Molecular Formula	C ₆ H ₈ O ₄	C ₅ H ₆ O ₄

B

MMF Exposures 30 min Post Oral Dosing
DMF 100 mg/kg in Mice

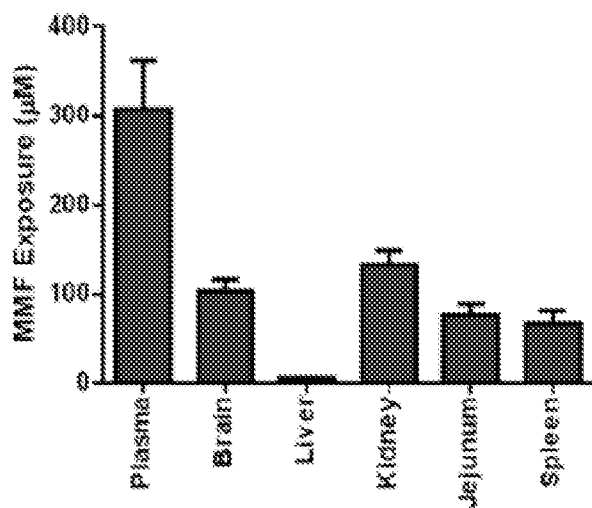


FIGURE 37

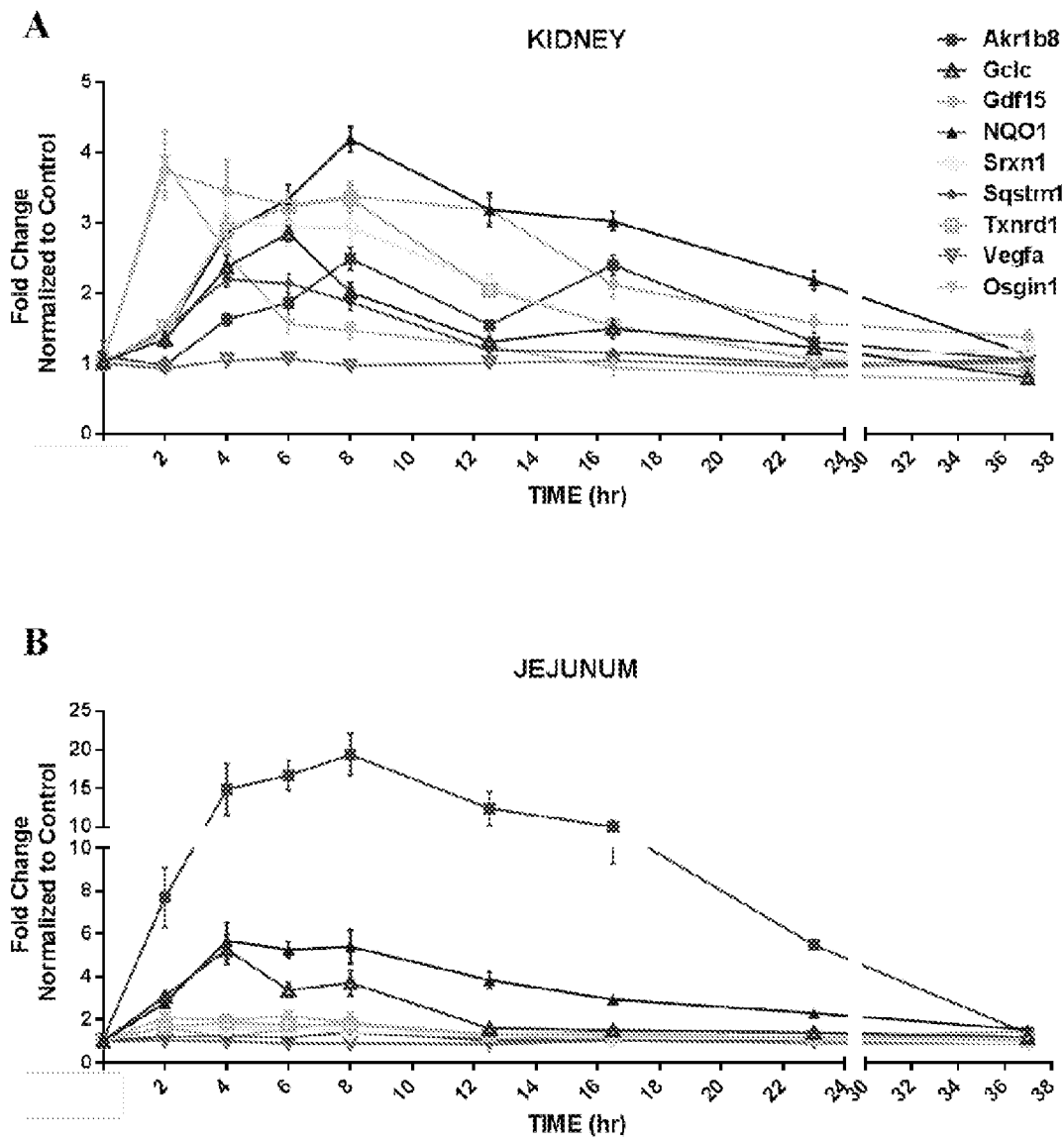


FIGURE 38

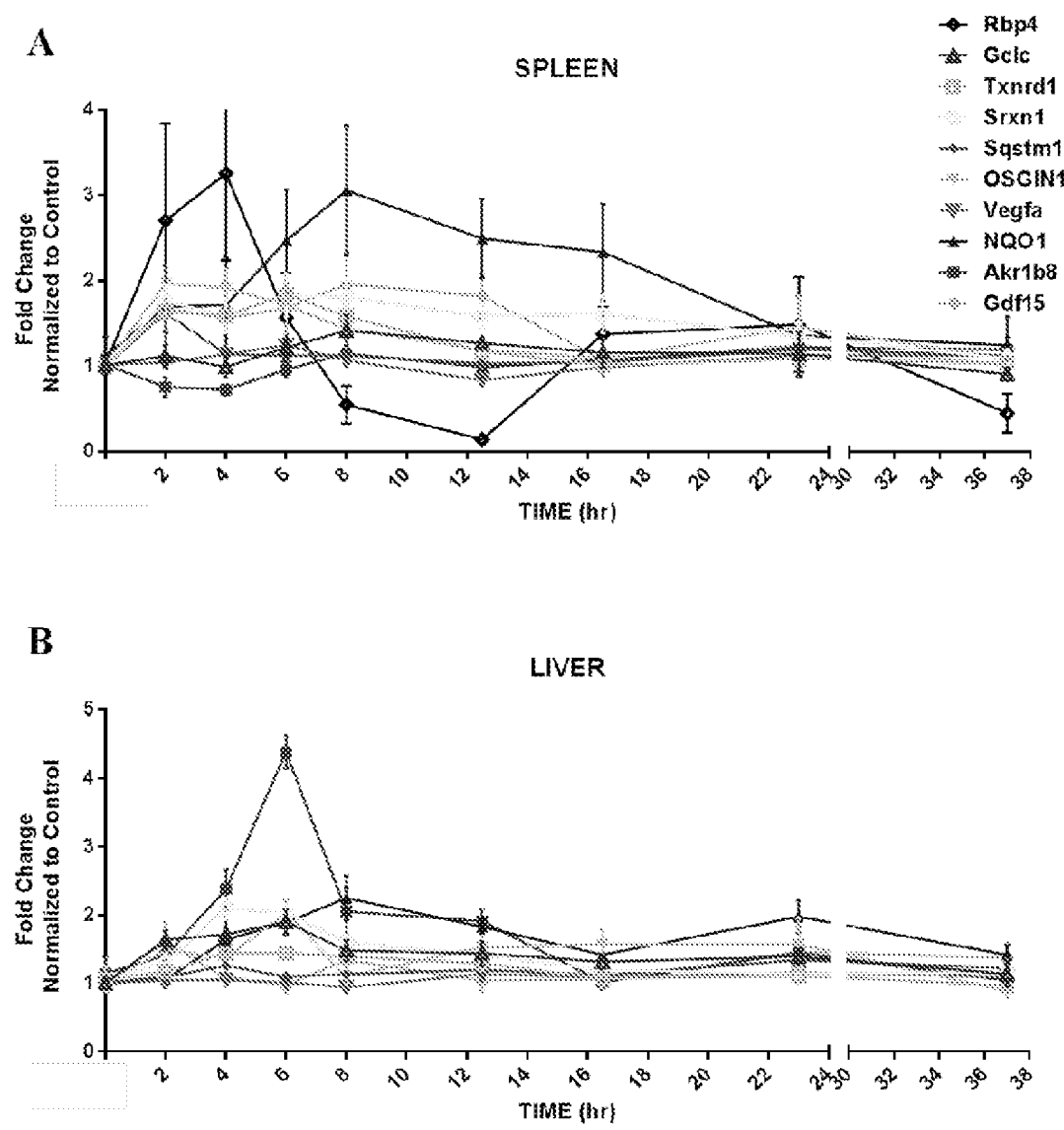


FIGURE 39

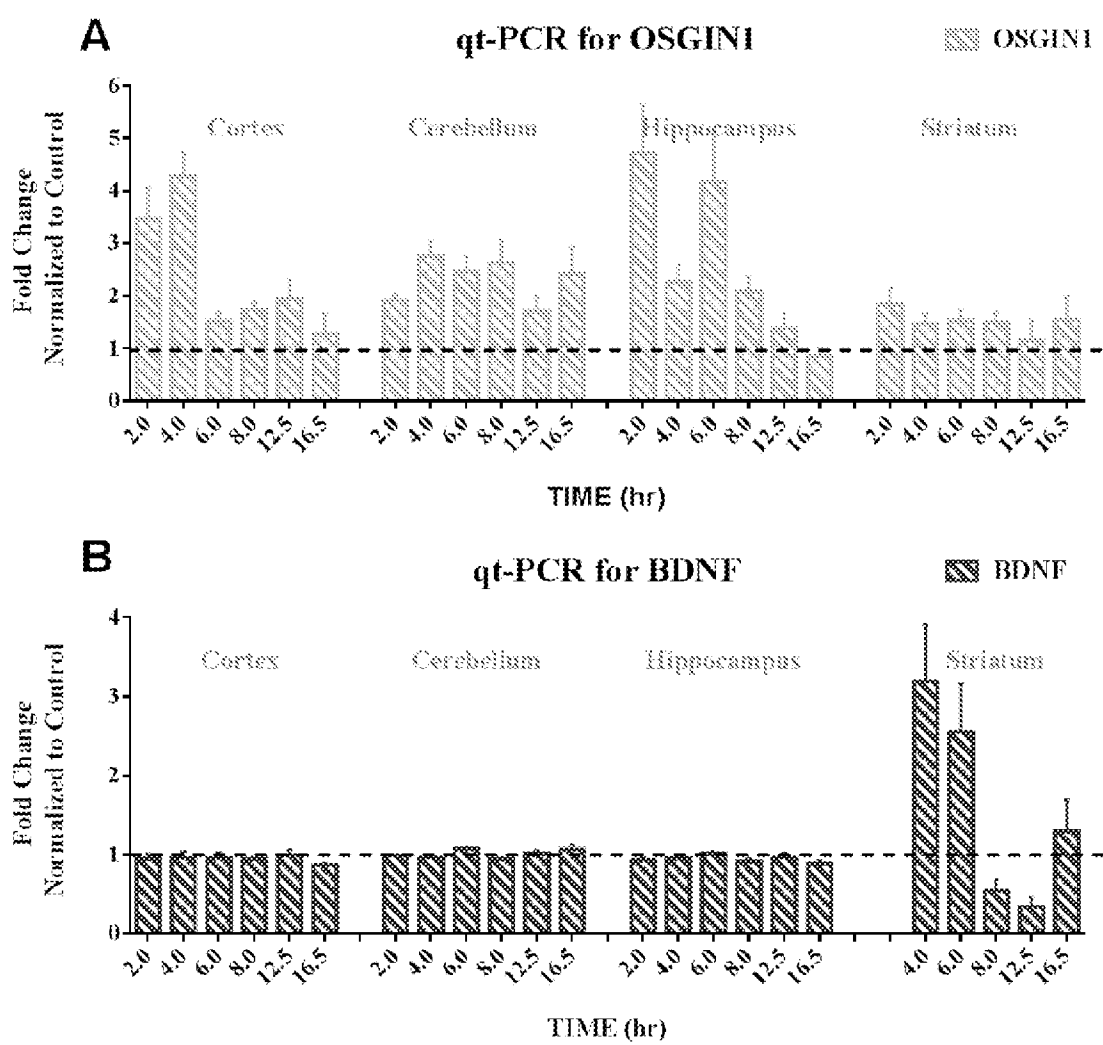


FIGURE 40

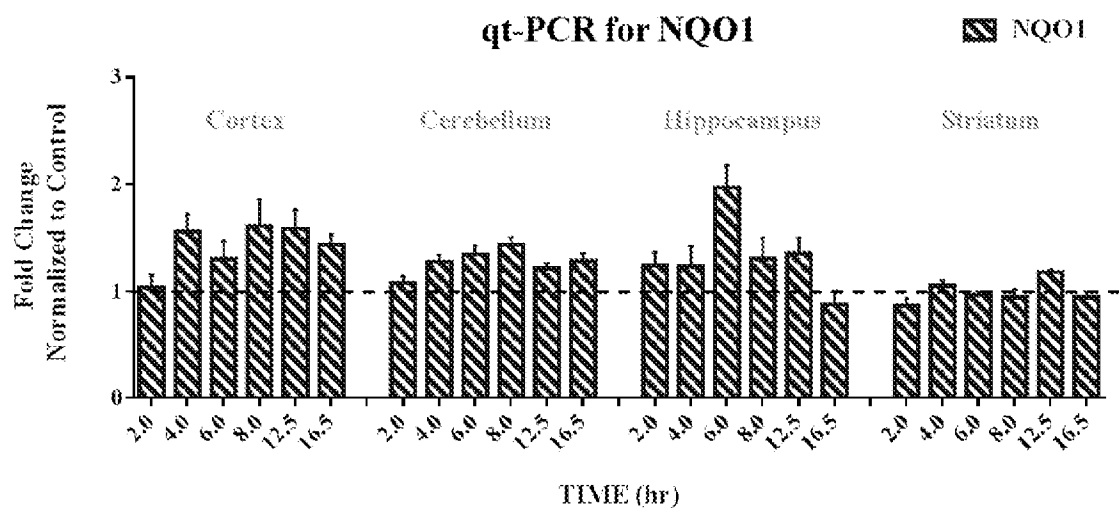


FIGURE 41

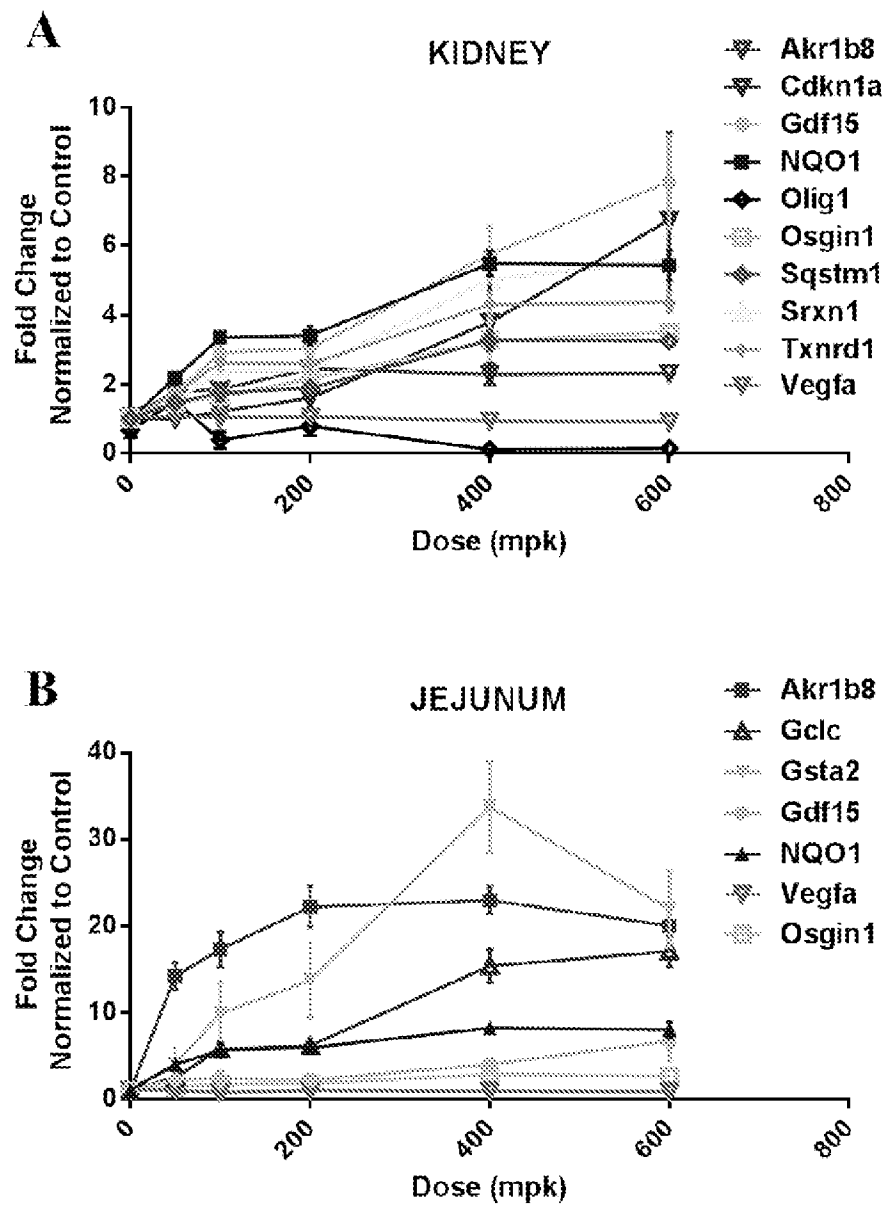


FIGURE 42

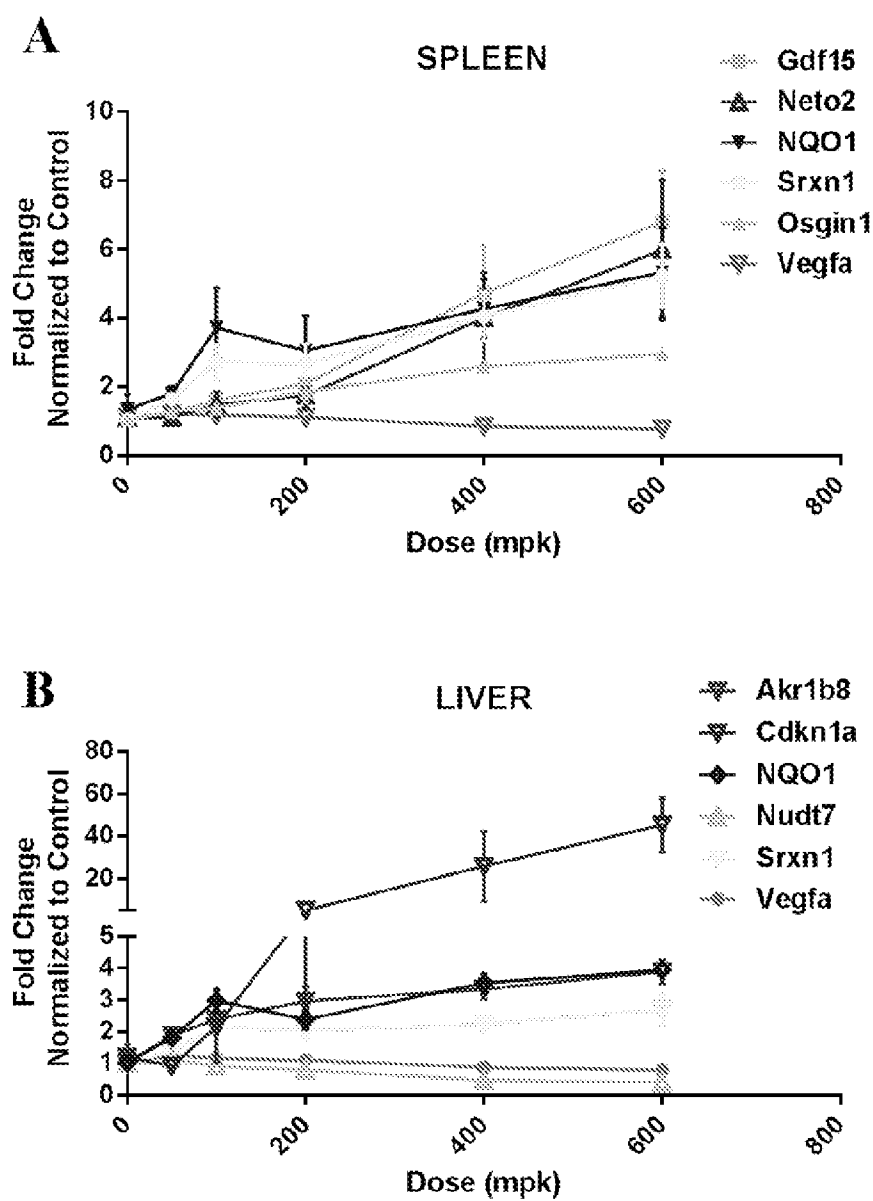


FIGURE 43

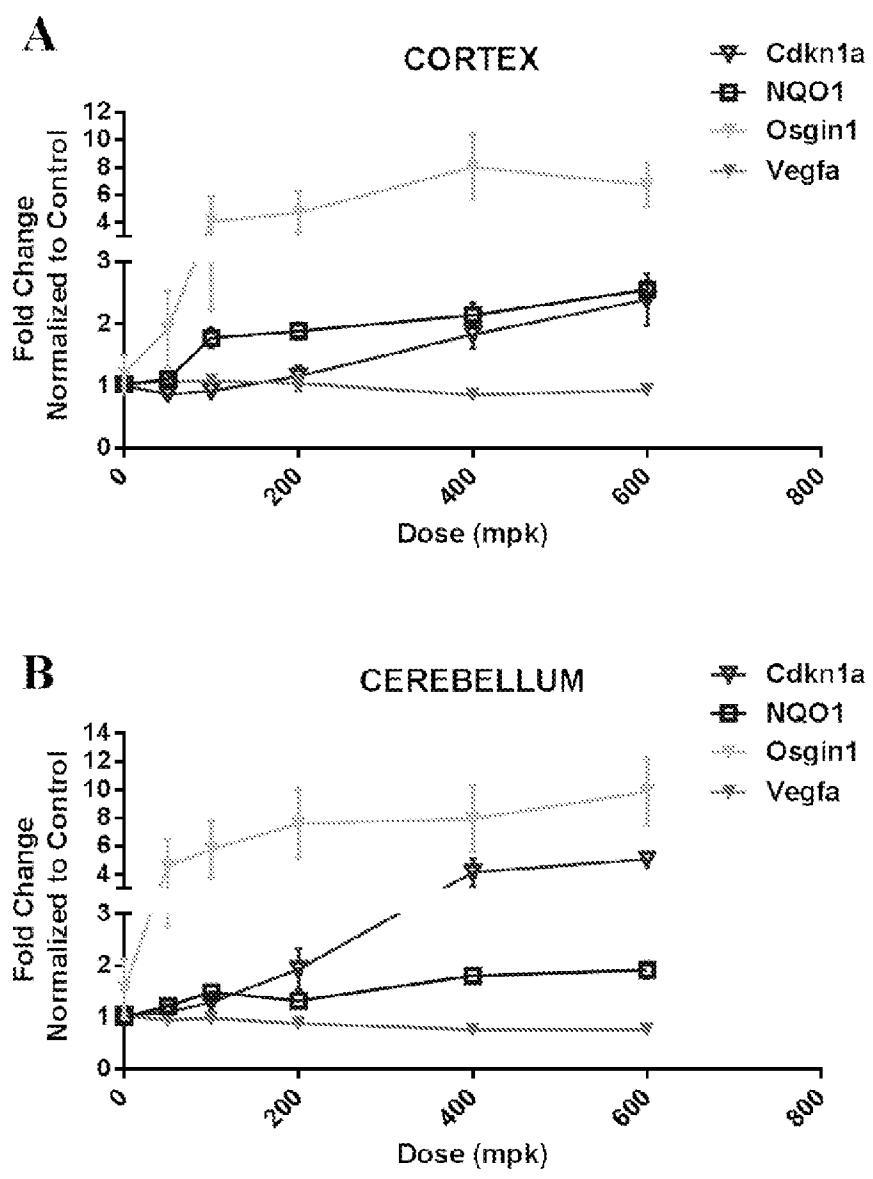


FIGURE 44

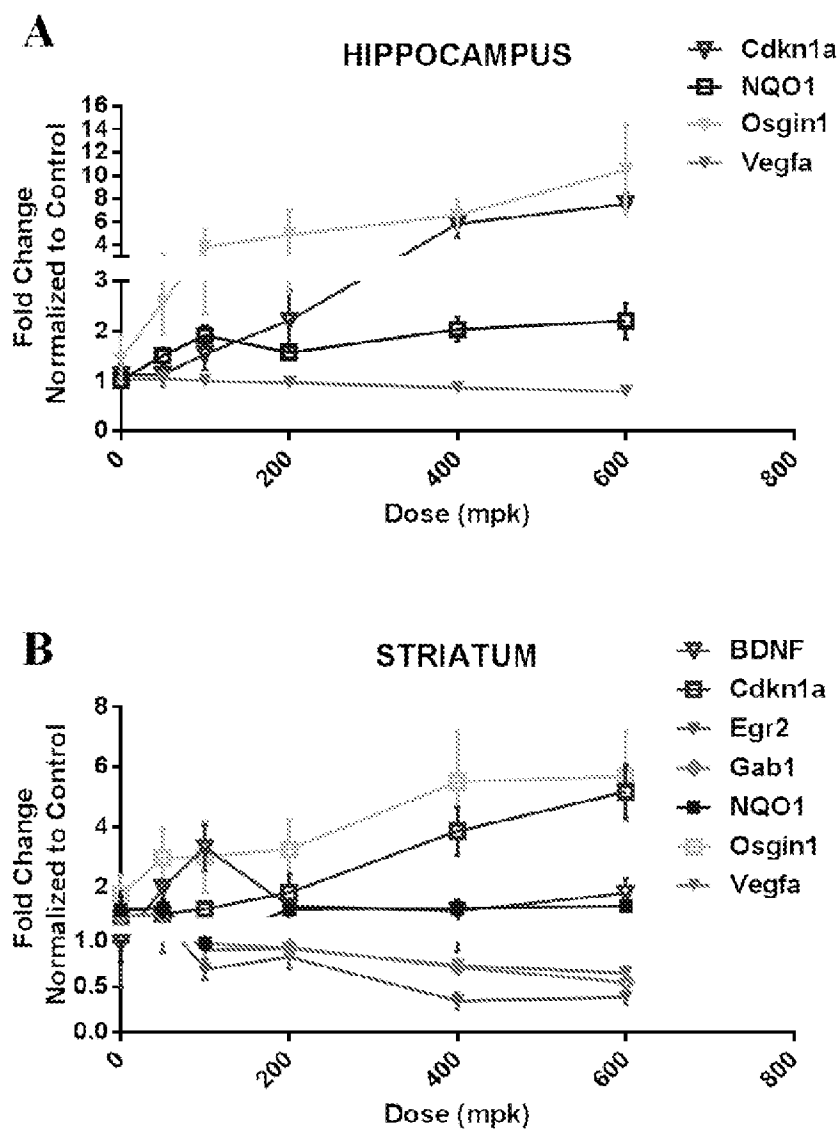


FIGURE 45

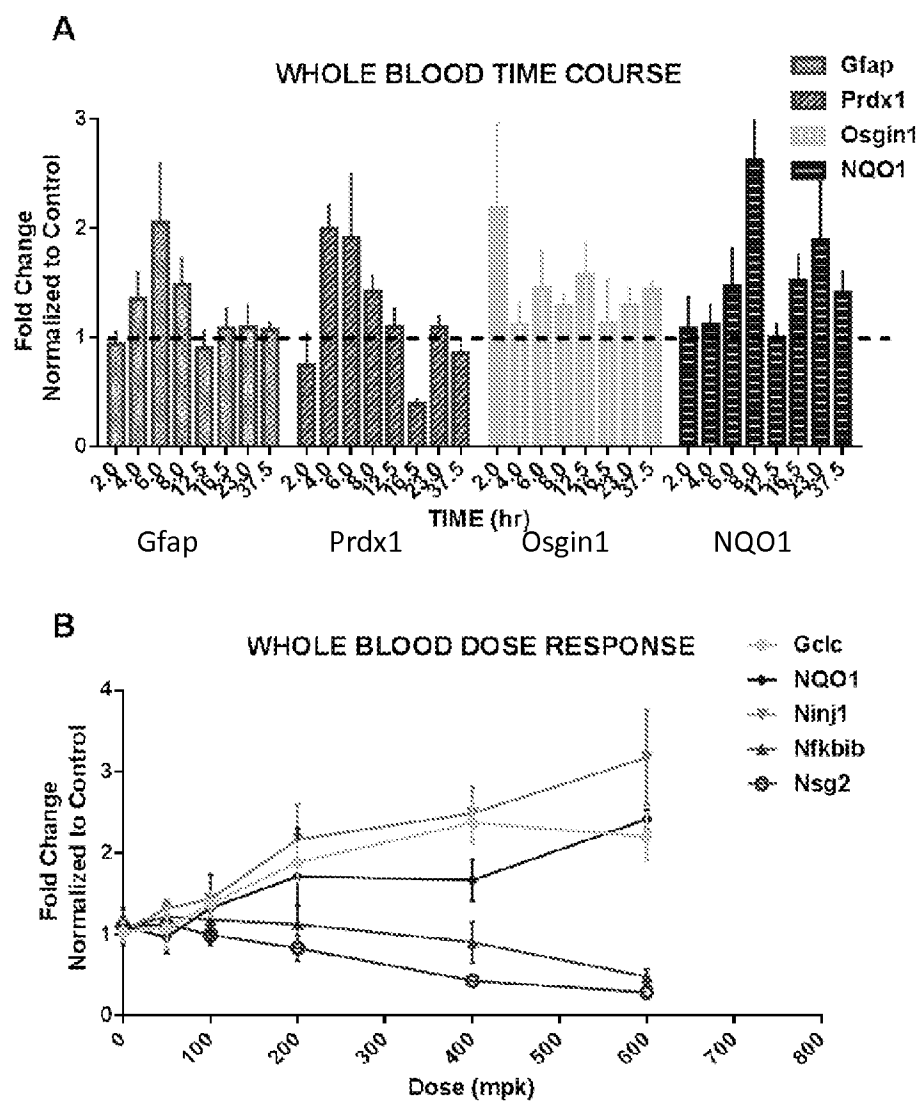


FIGURE 46

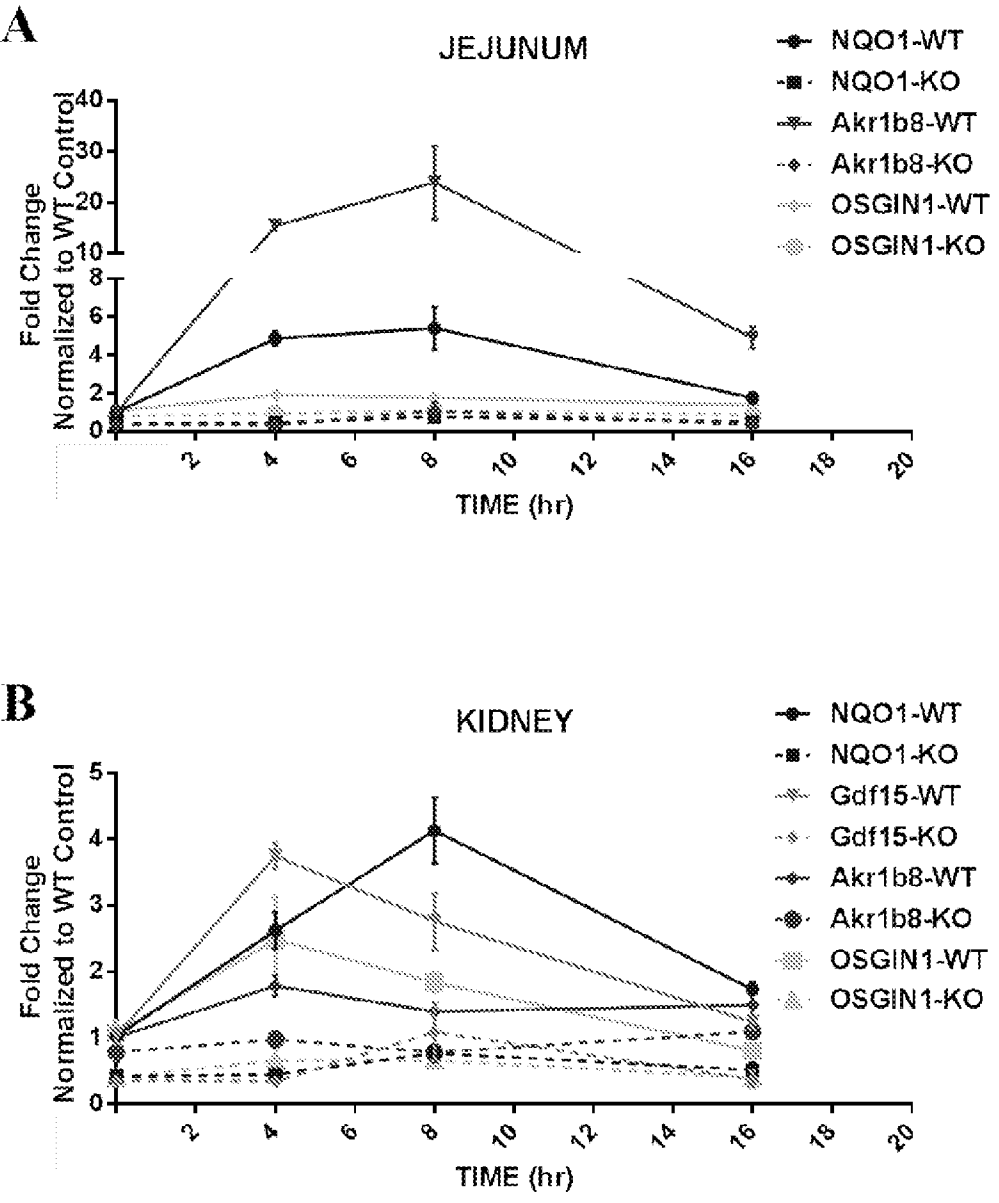


FIGURE 47

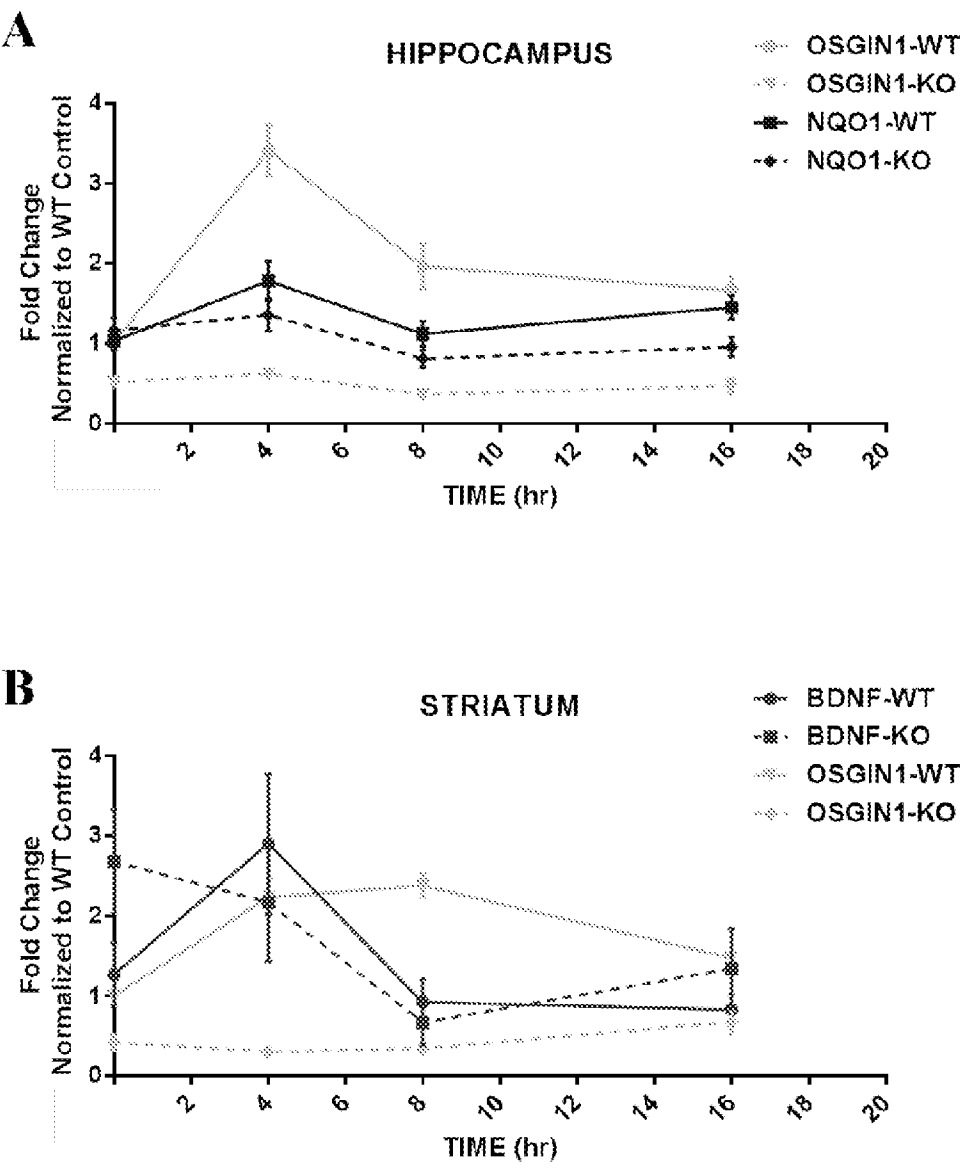


FIGURE 48

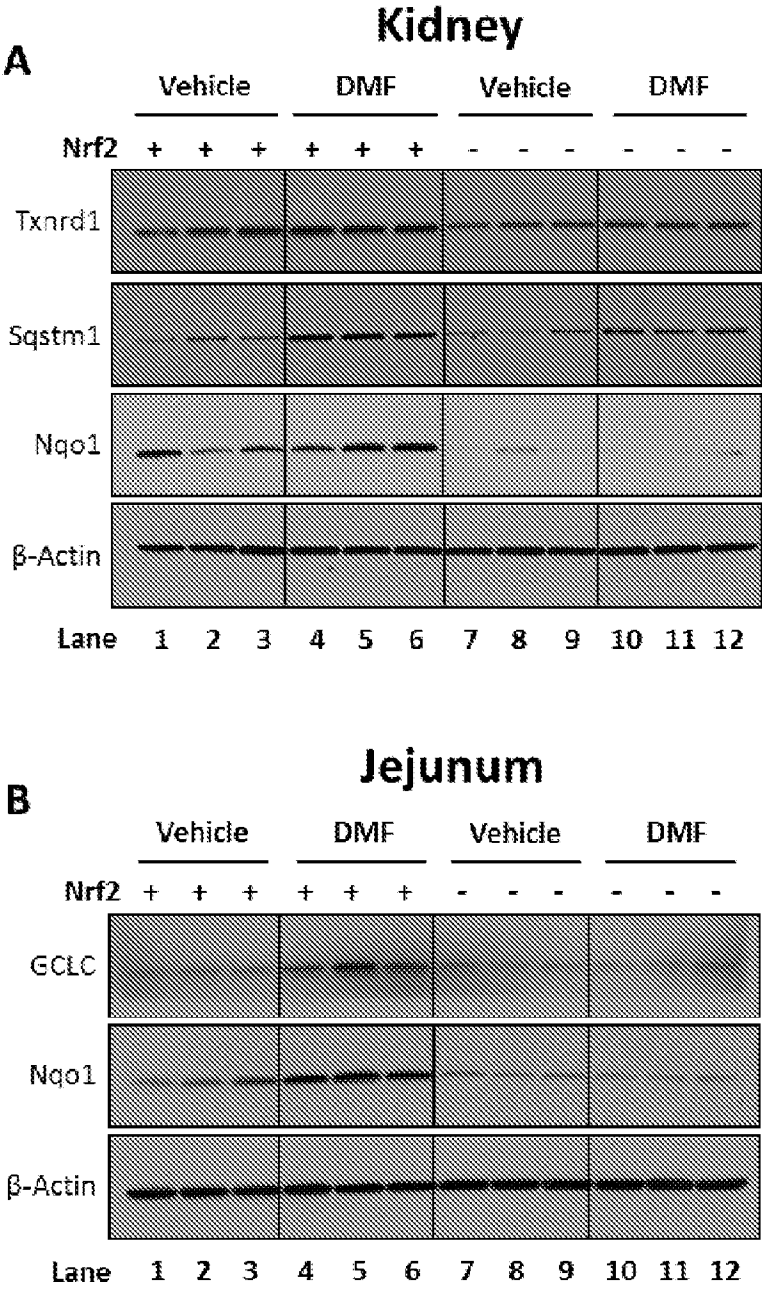


FIGURE 49

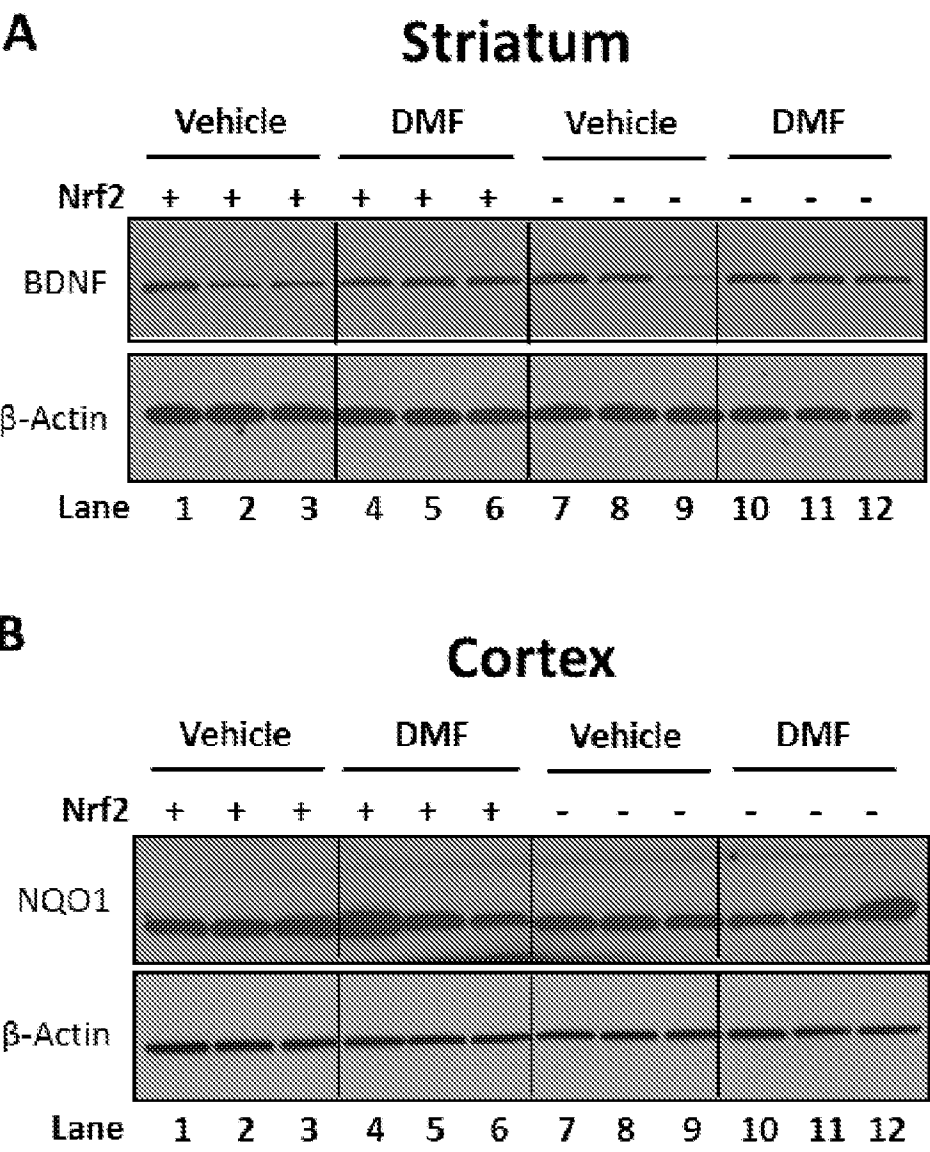
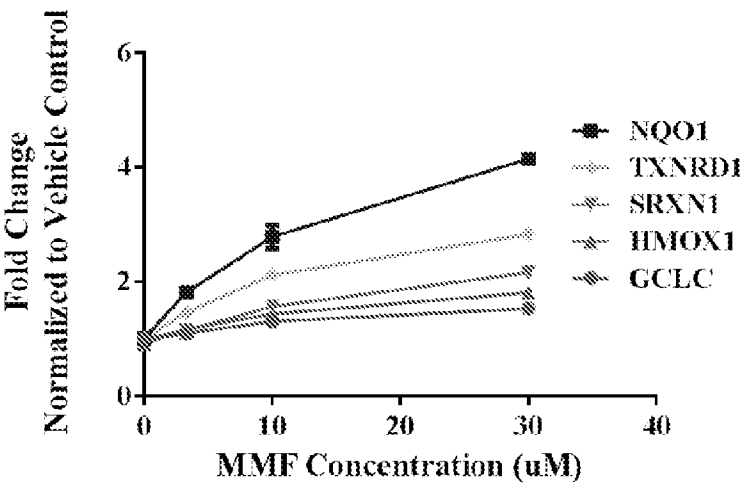


FIGURE 50

A

q-PCR for Classical Nrf2 Target Genes



B

q-PCR for OSGIN1

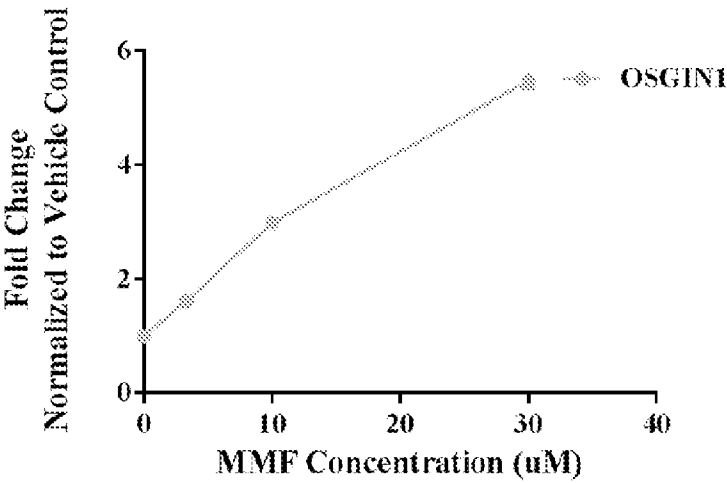


FIGURE 51

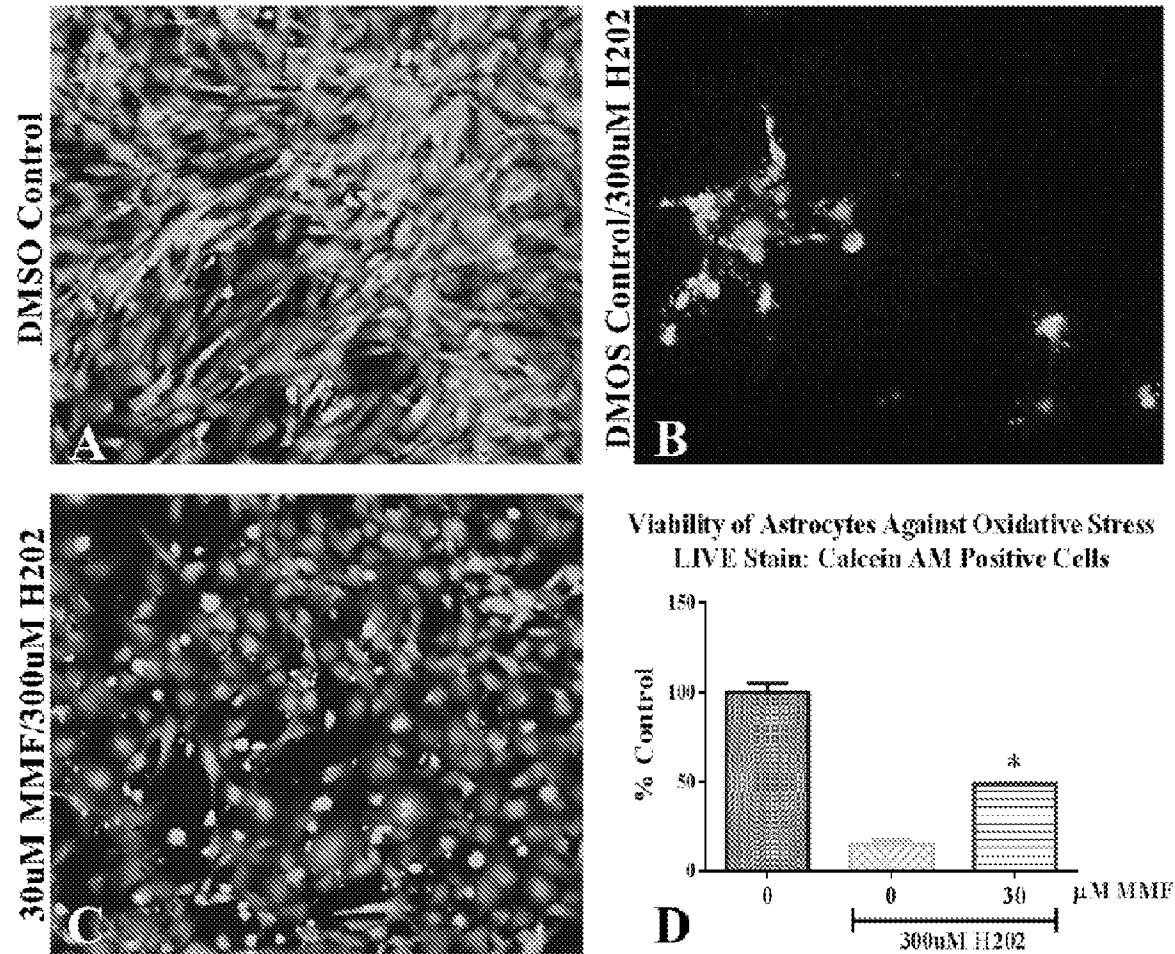


FIGURE 52

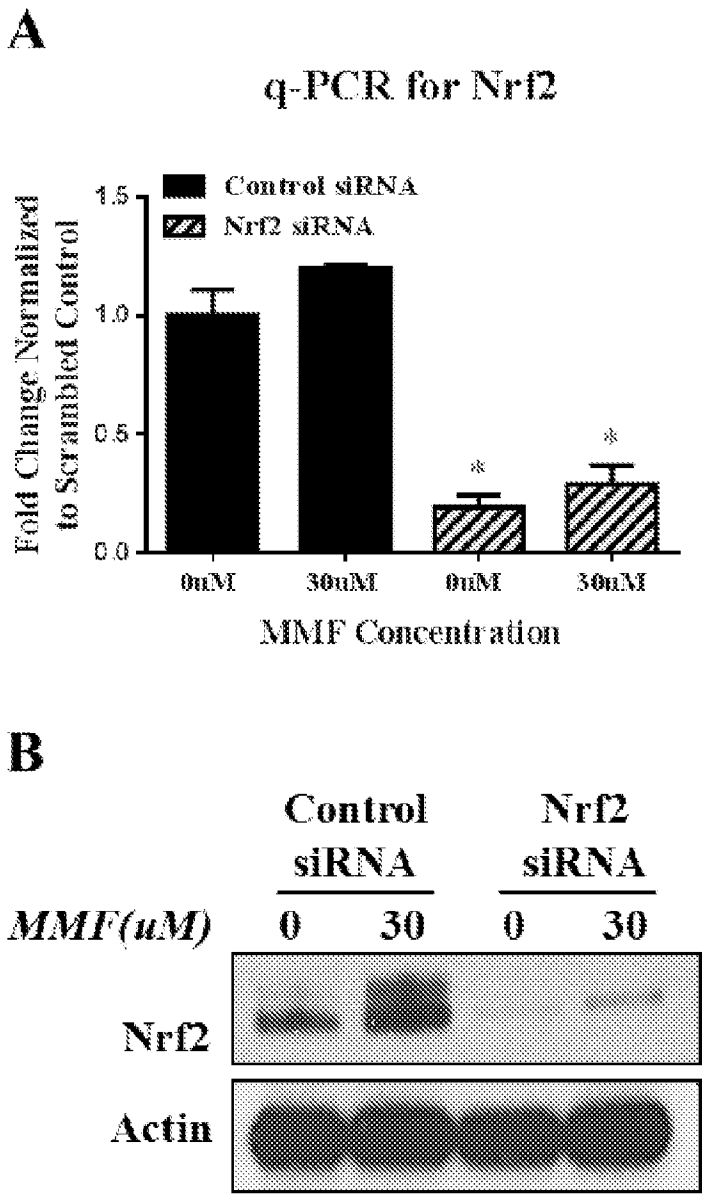


FIGURE 53

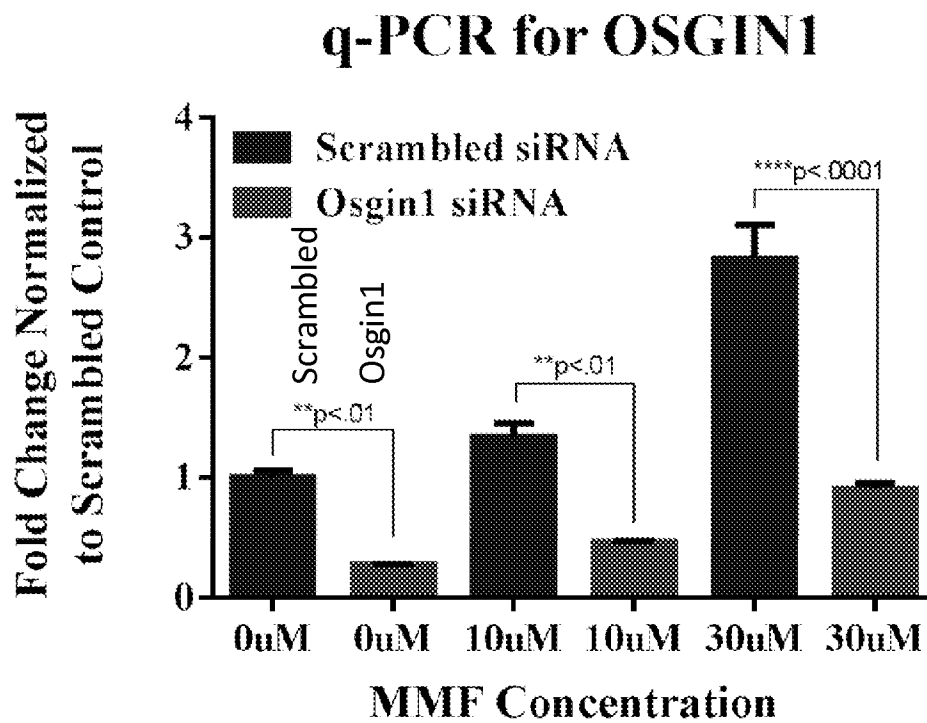


FIGURE 54

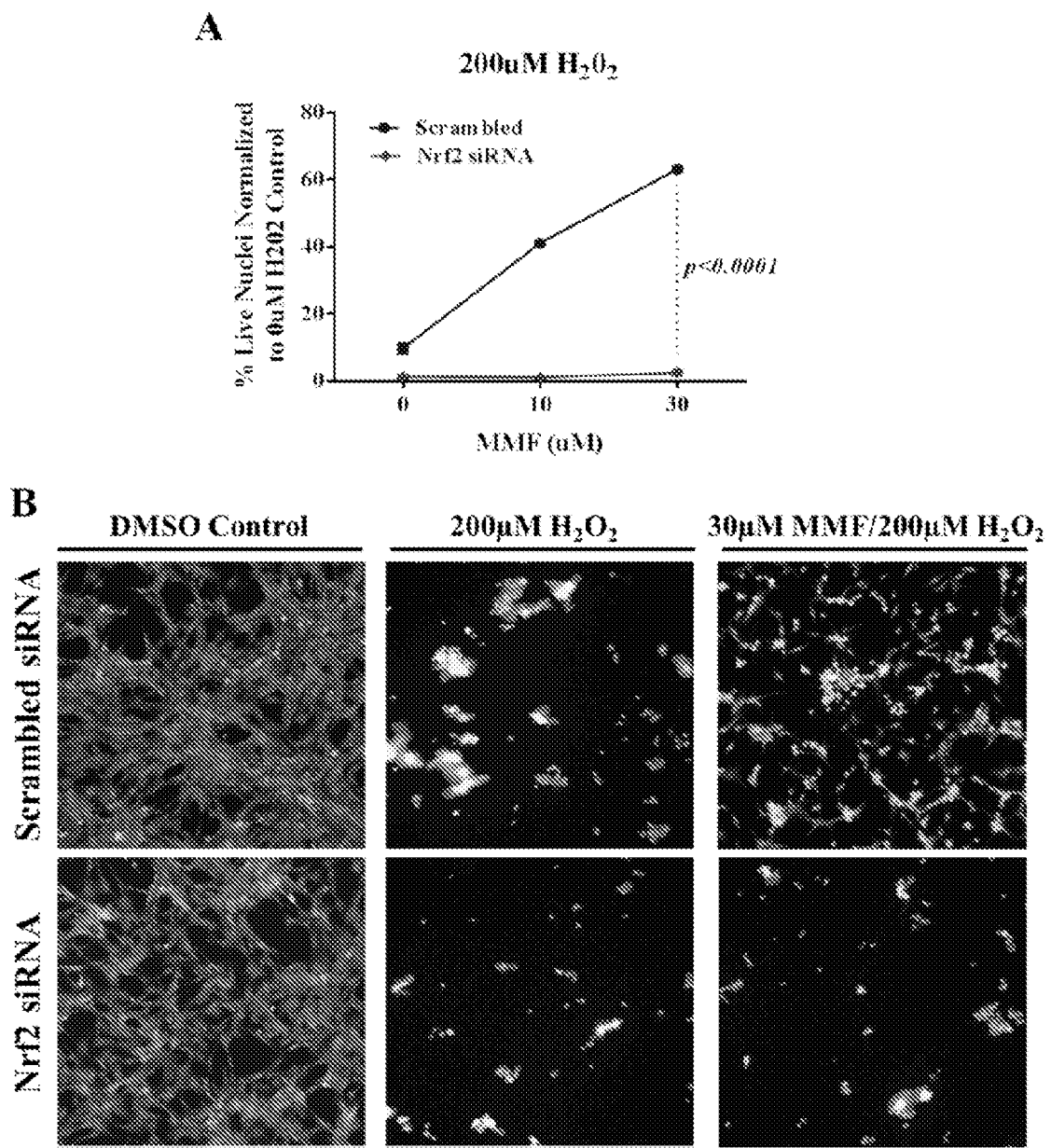


FIGURE 55

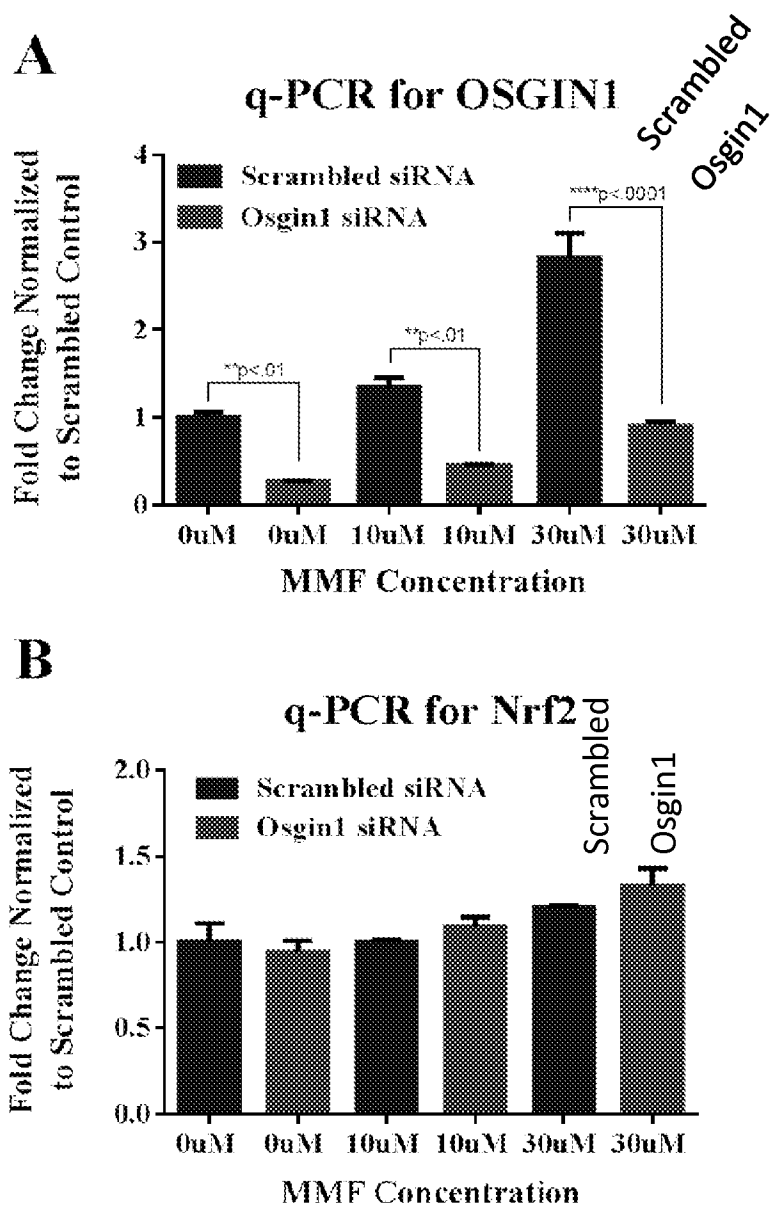


FIGURE 56

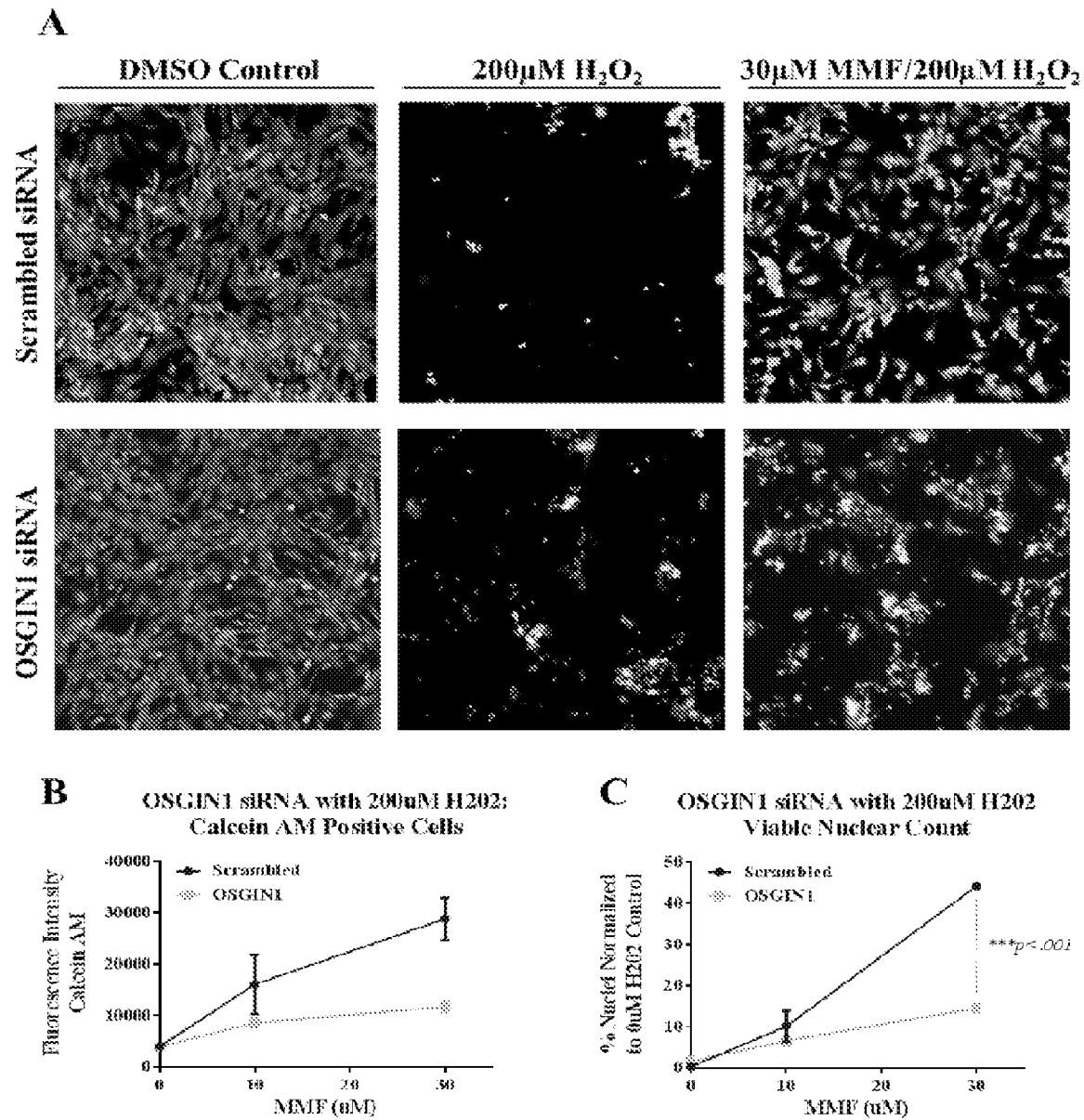


FIGURE 57

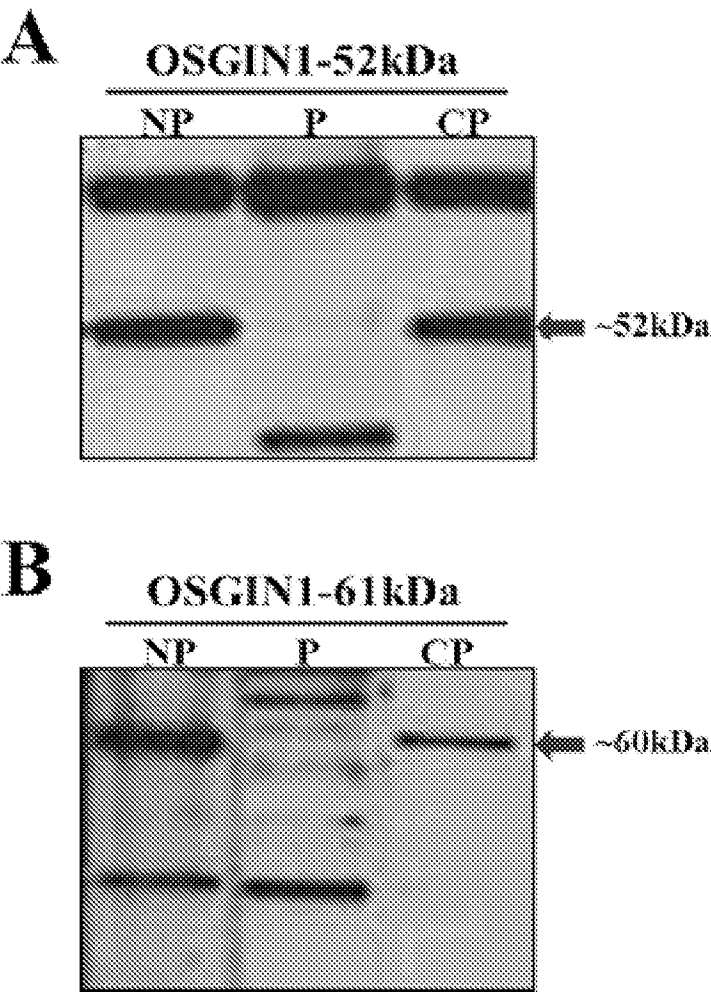


FIGURE 58

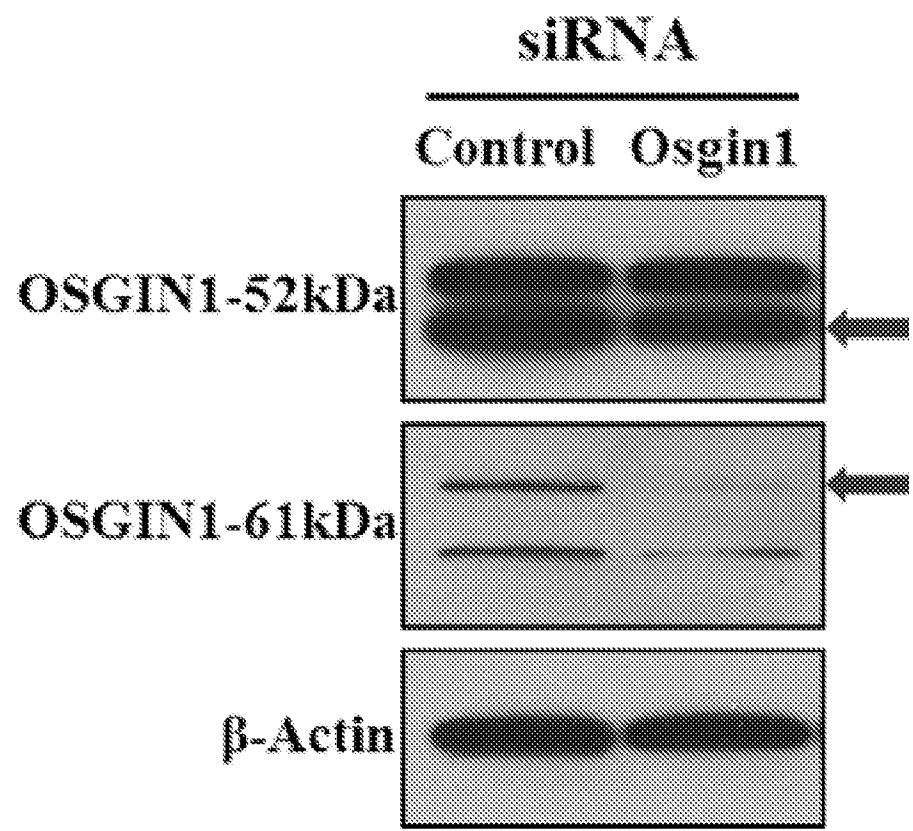


FIGURE 59

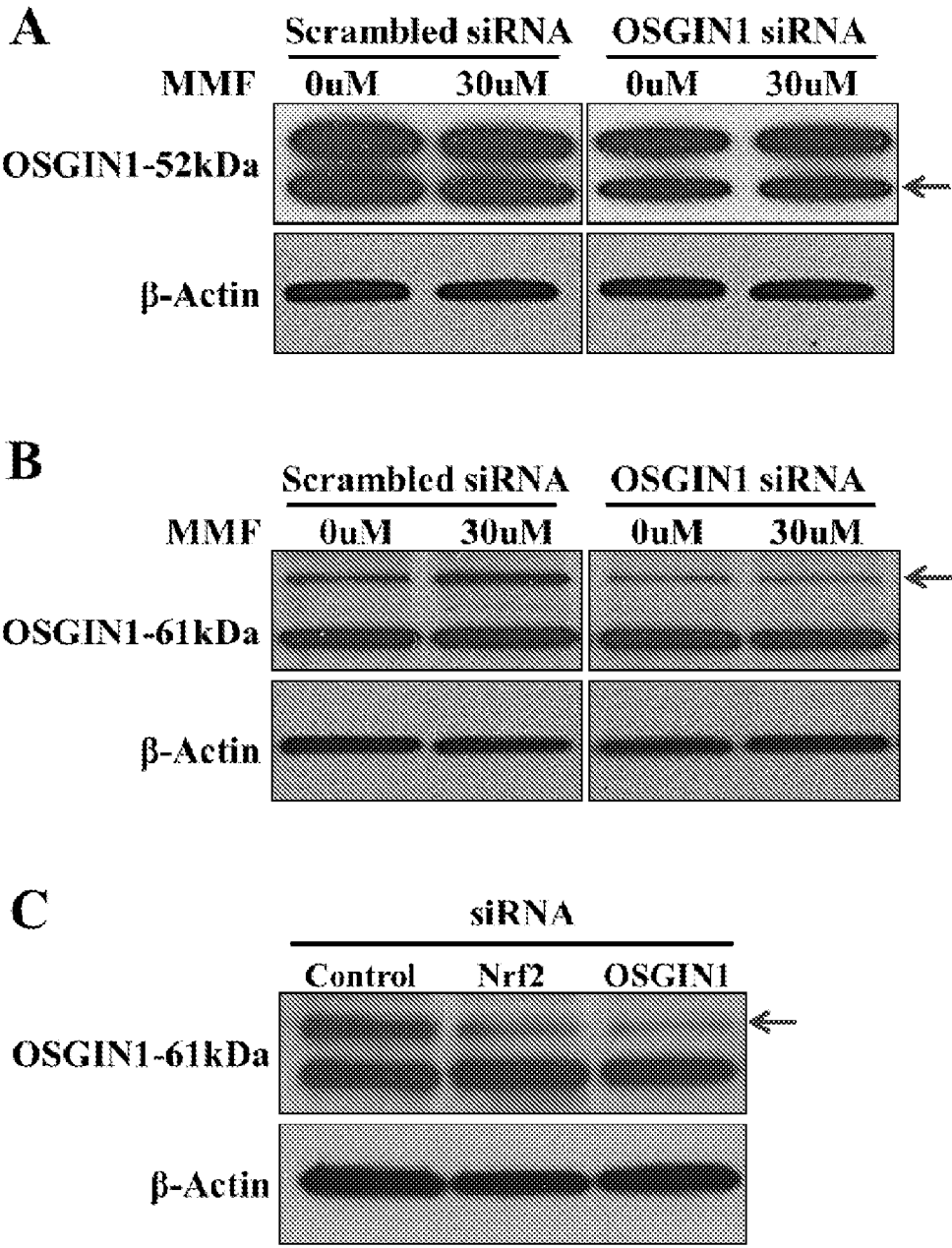


FIGURE 60

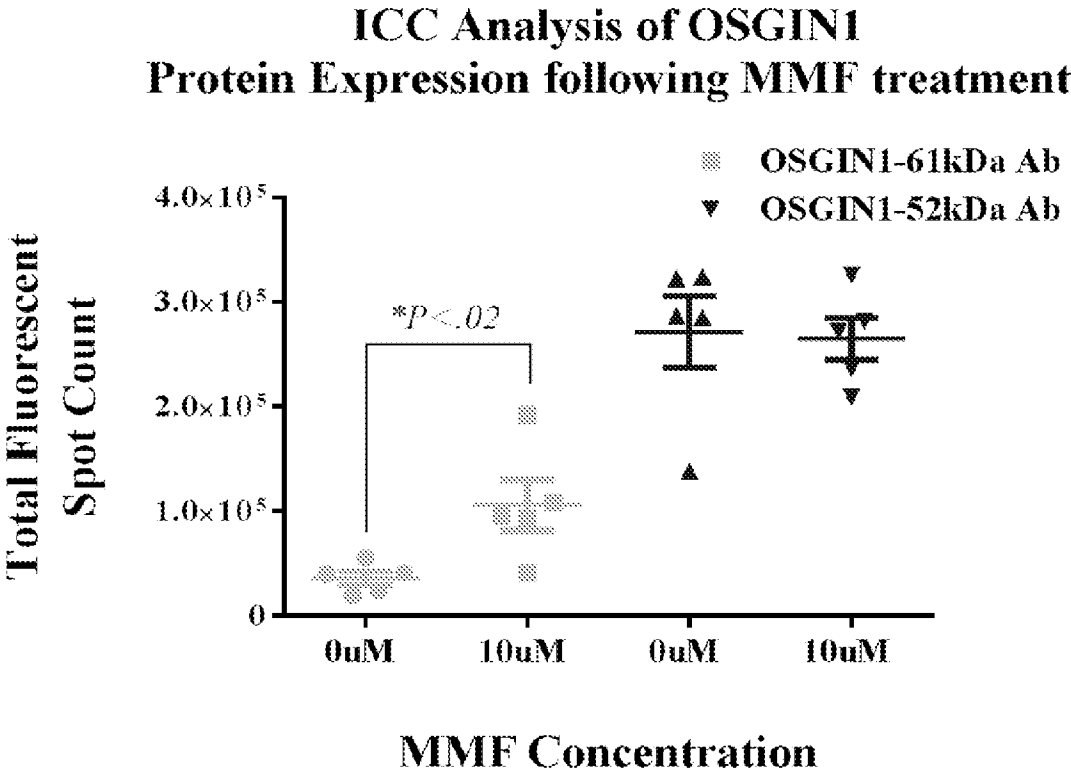


FIGURE 61

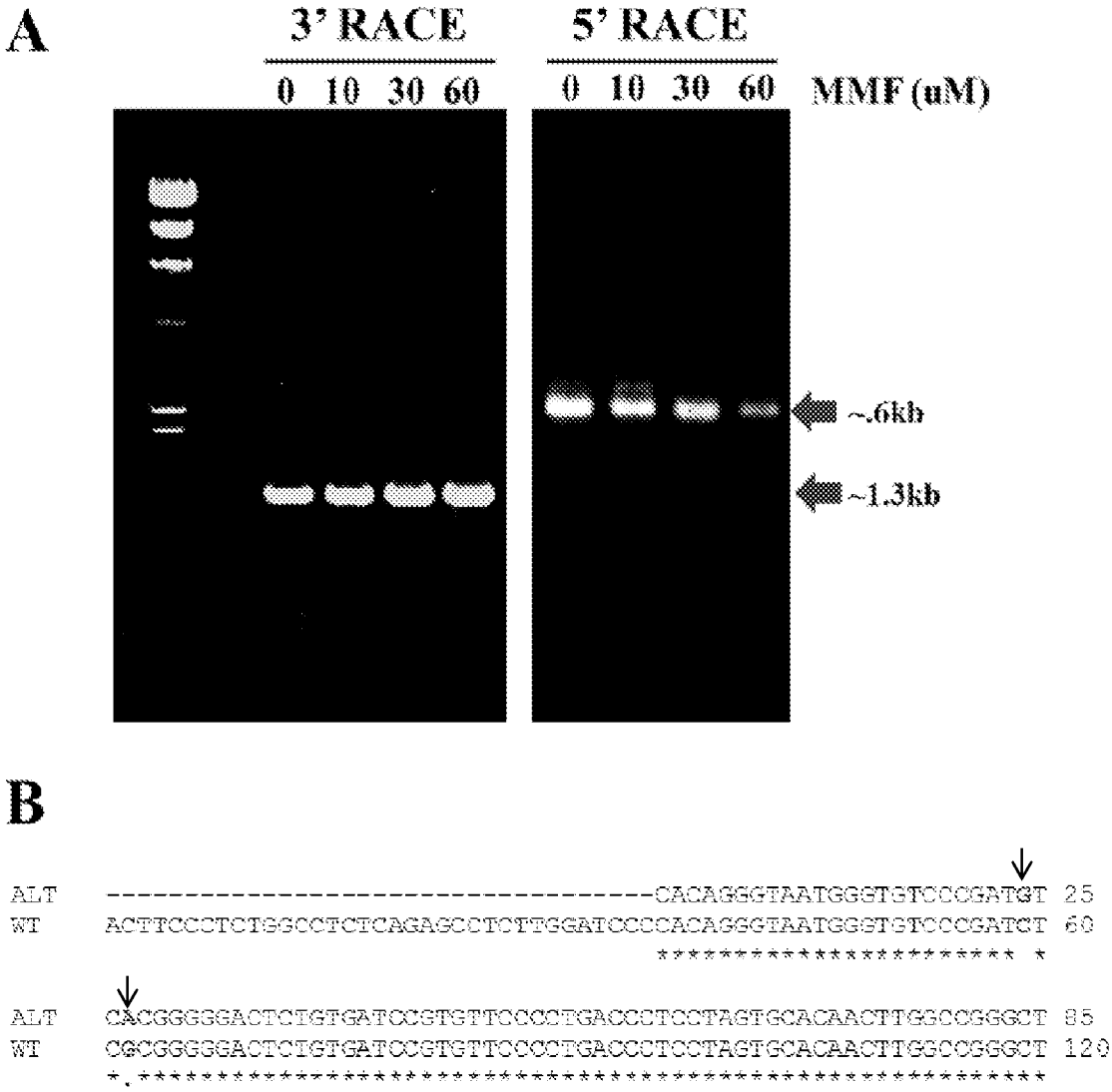


FIGURE 62

q-PCR of OSGIN1 5'UTR Transcript Variants in MMF-treated Human Astrocytes

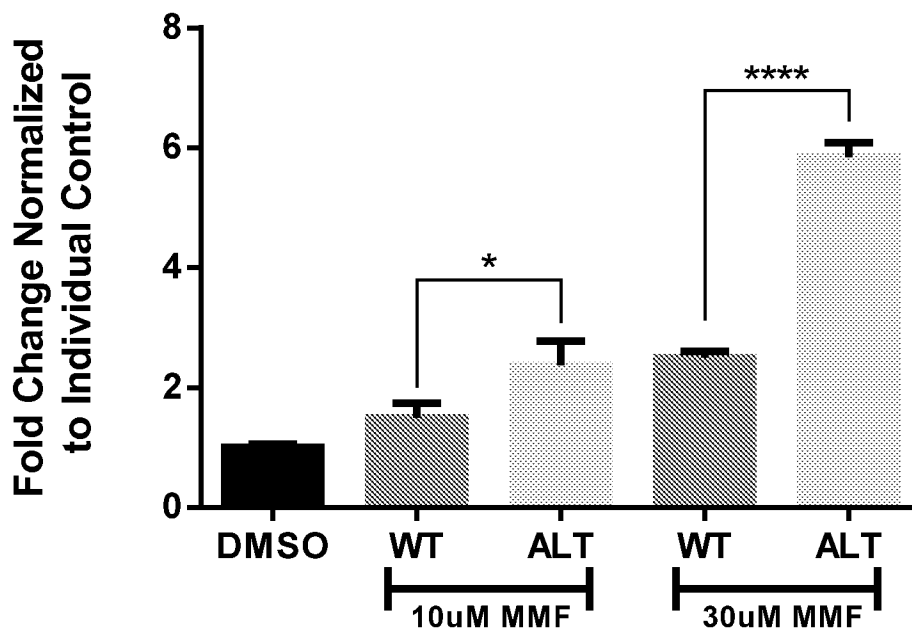


FIGURE 63

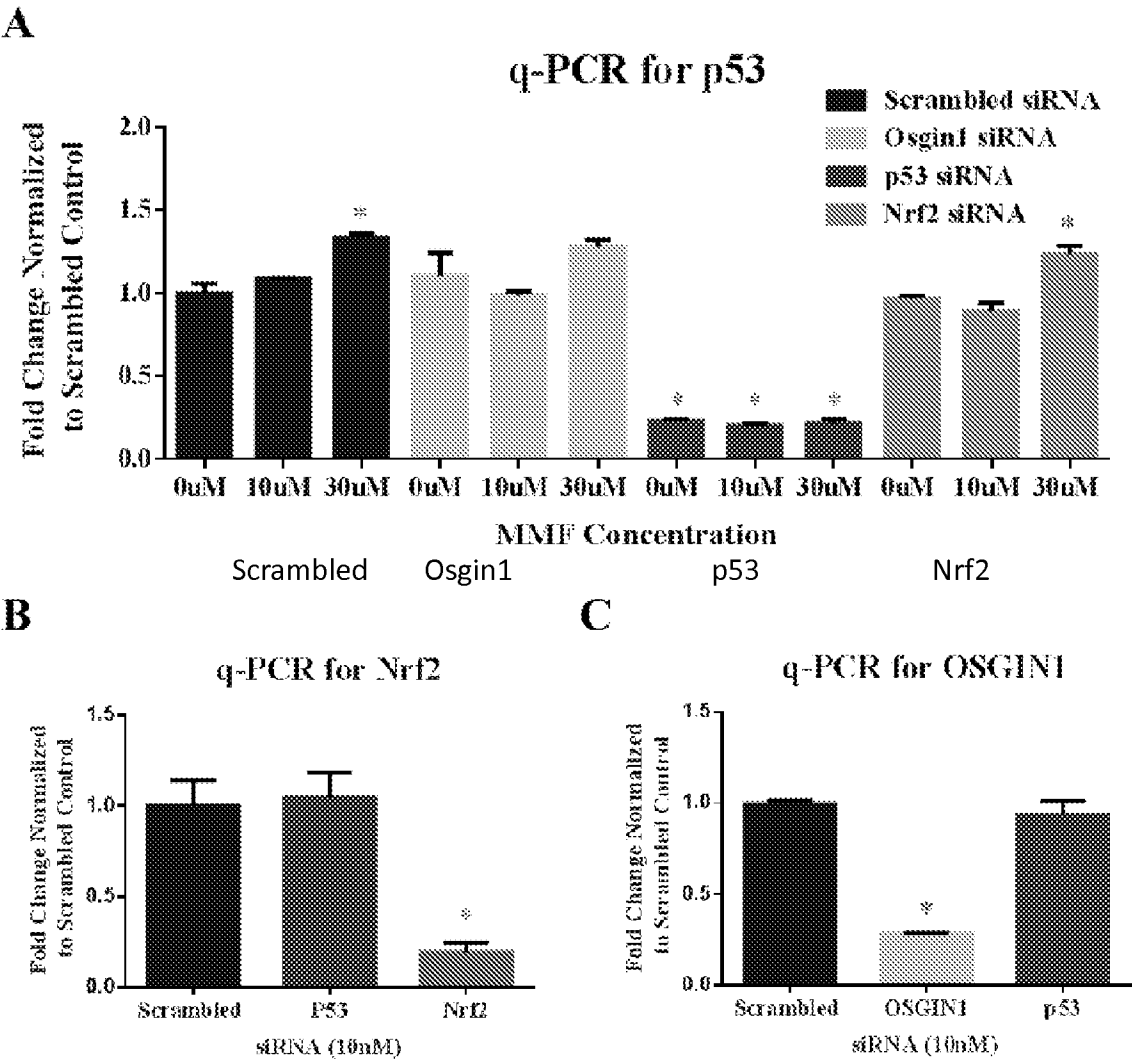


FIGURE 64

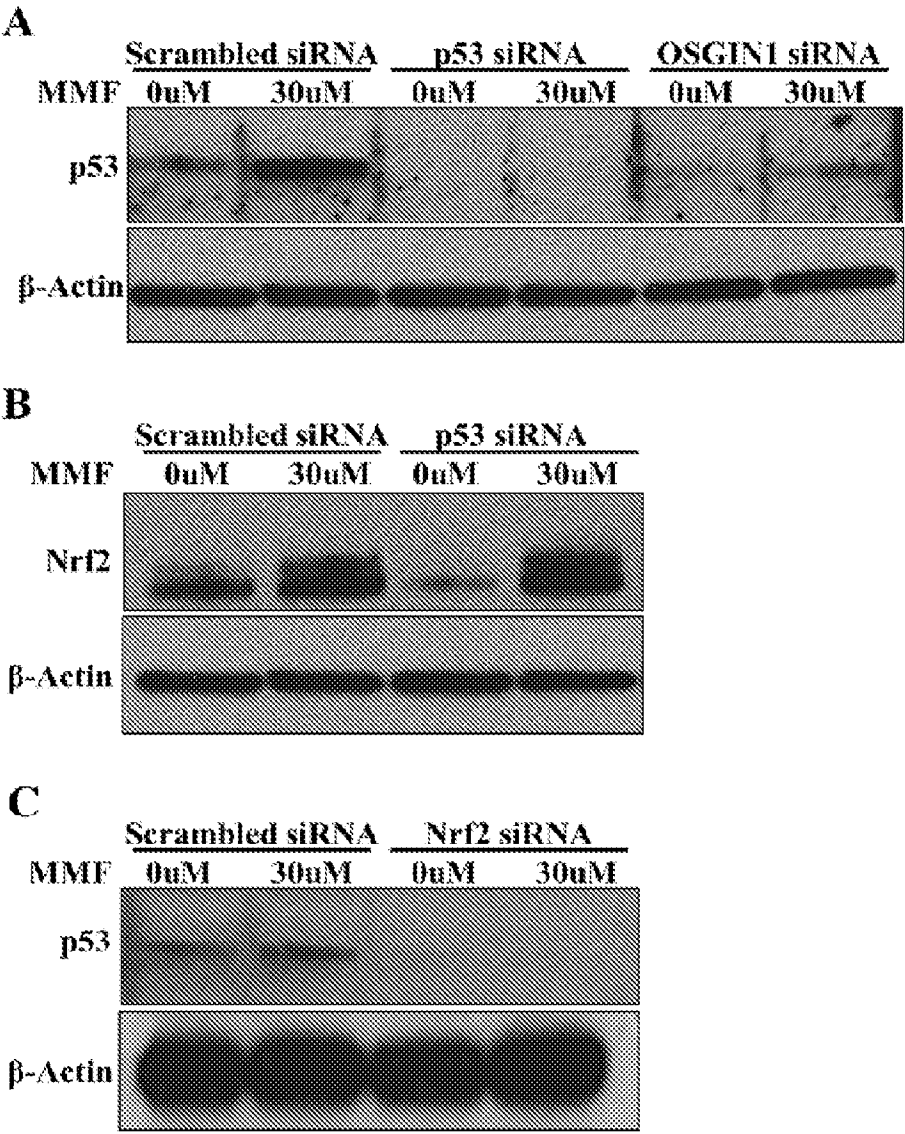


FIGURE 65

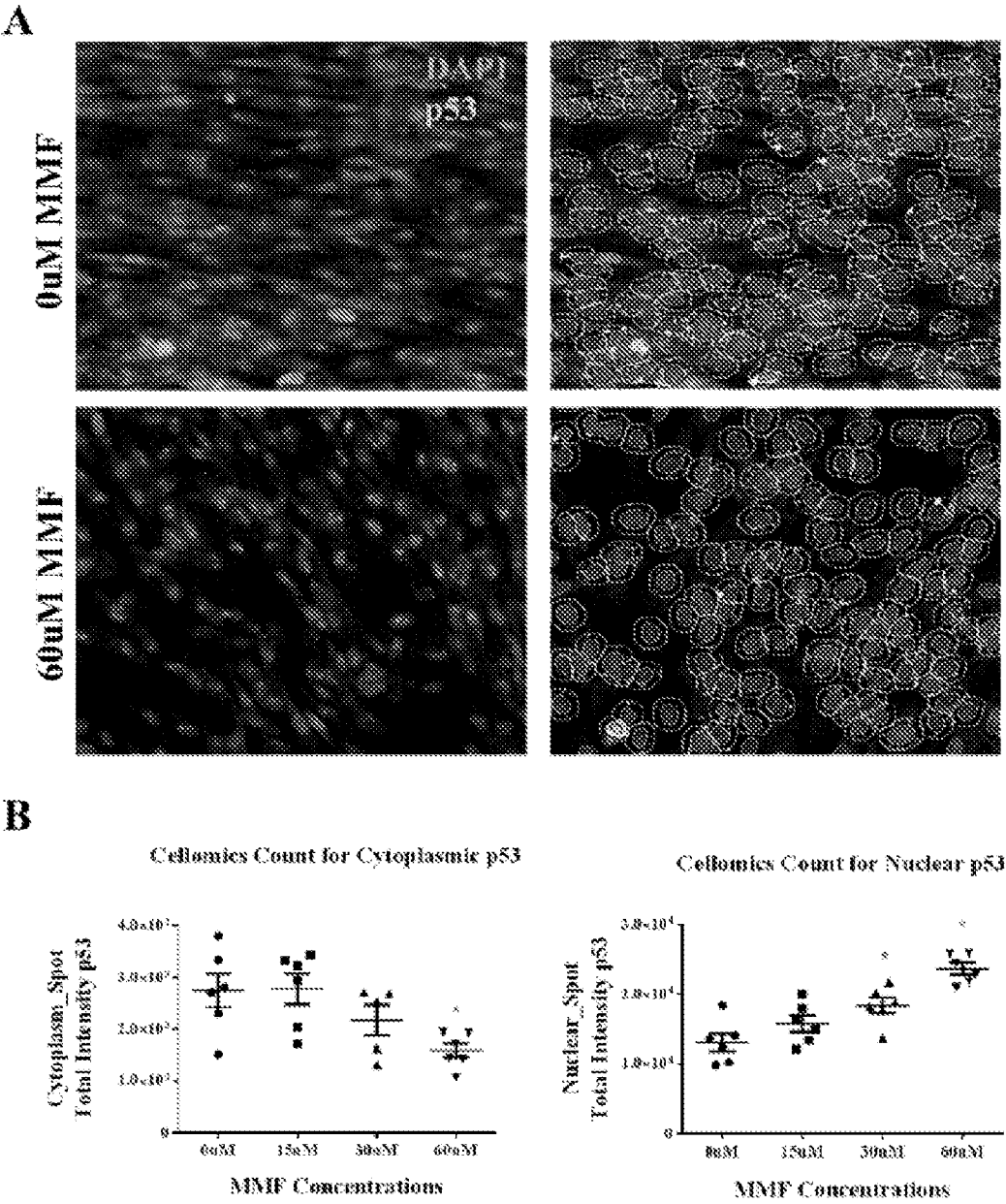


FIGURE 66

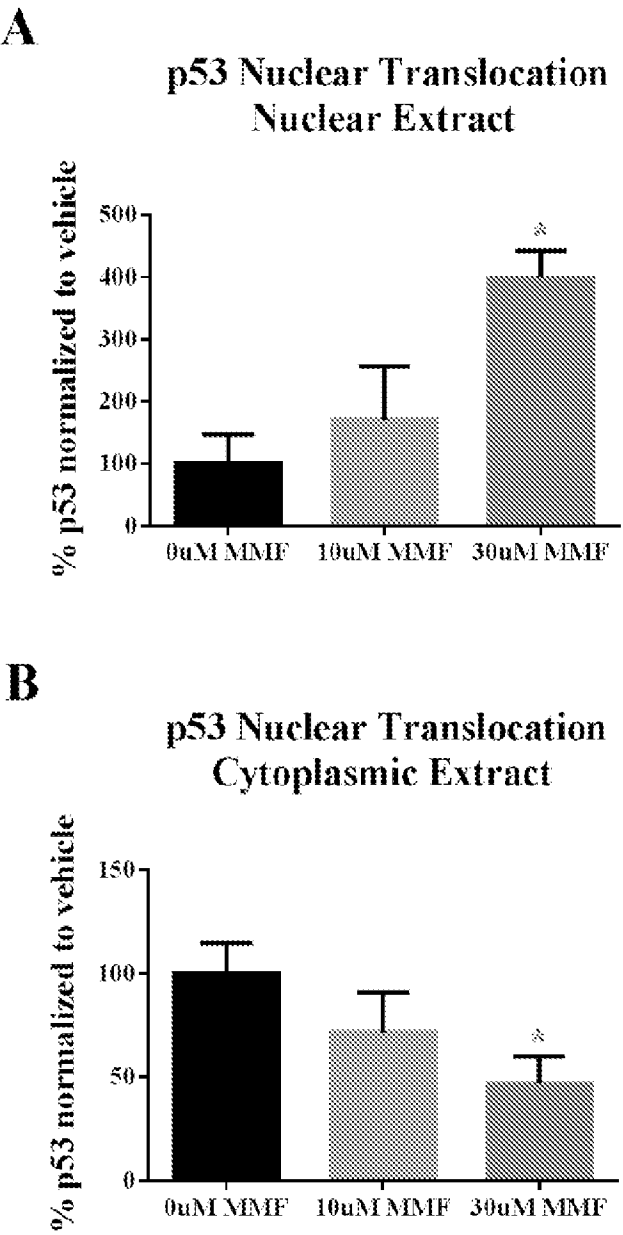


FIGURE 67

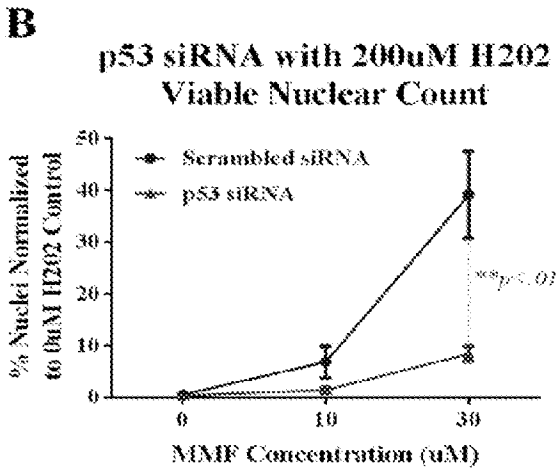
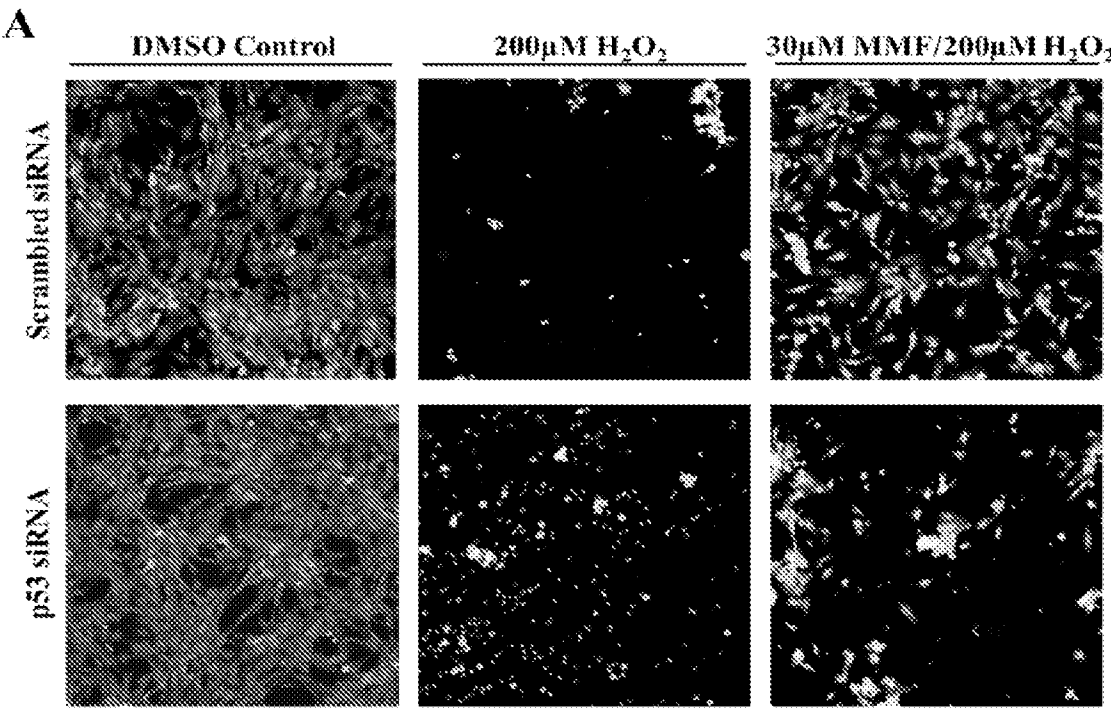
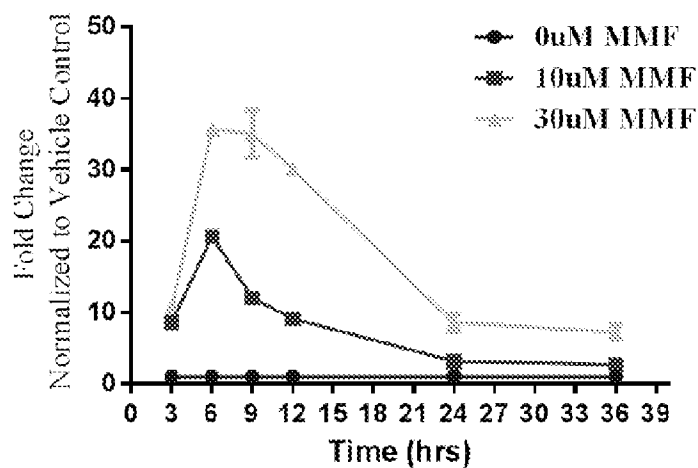


FIGURE 68

A

Human Astrocytes Treated with MMF over Time
qt-PCR for OSGIN1

**B**

Human Astrocytes Treated with MMF over Time
qt-PCR for NQO1

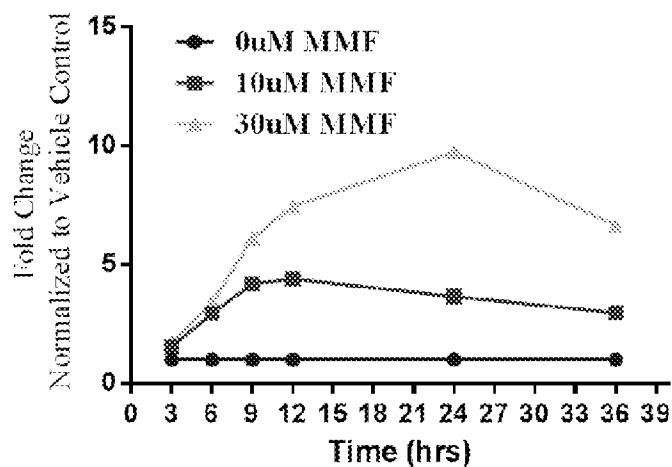


FIGURE 69

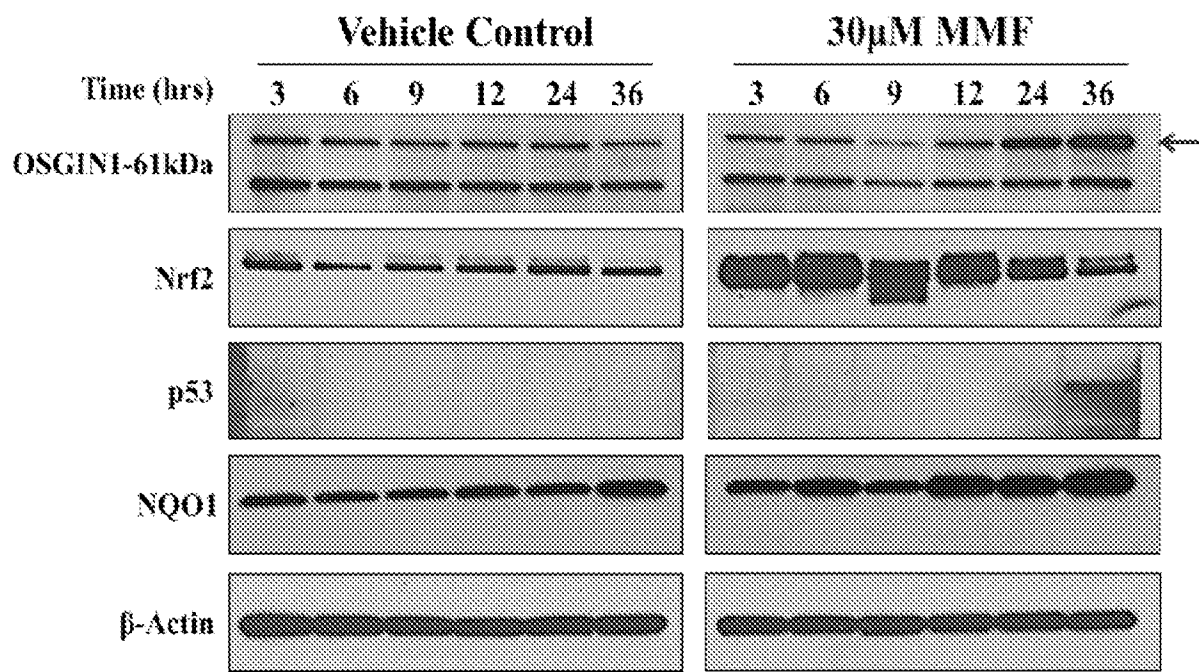


FIGURE 70

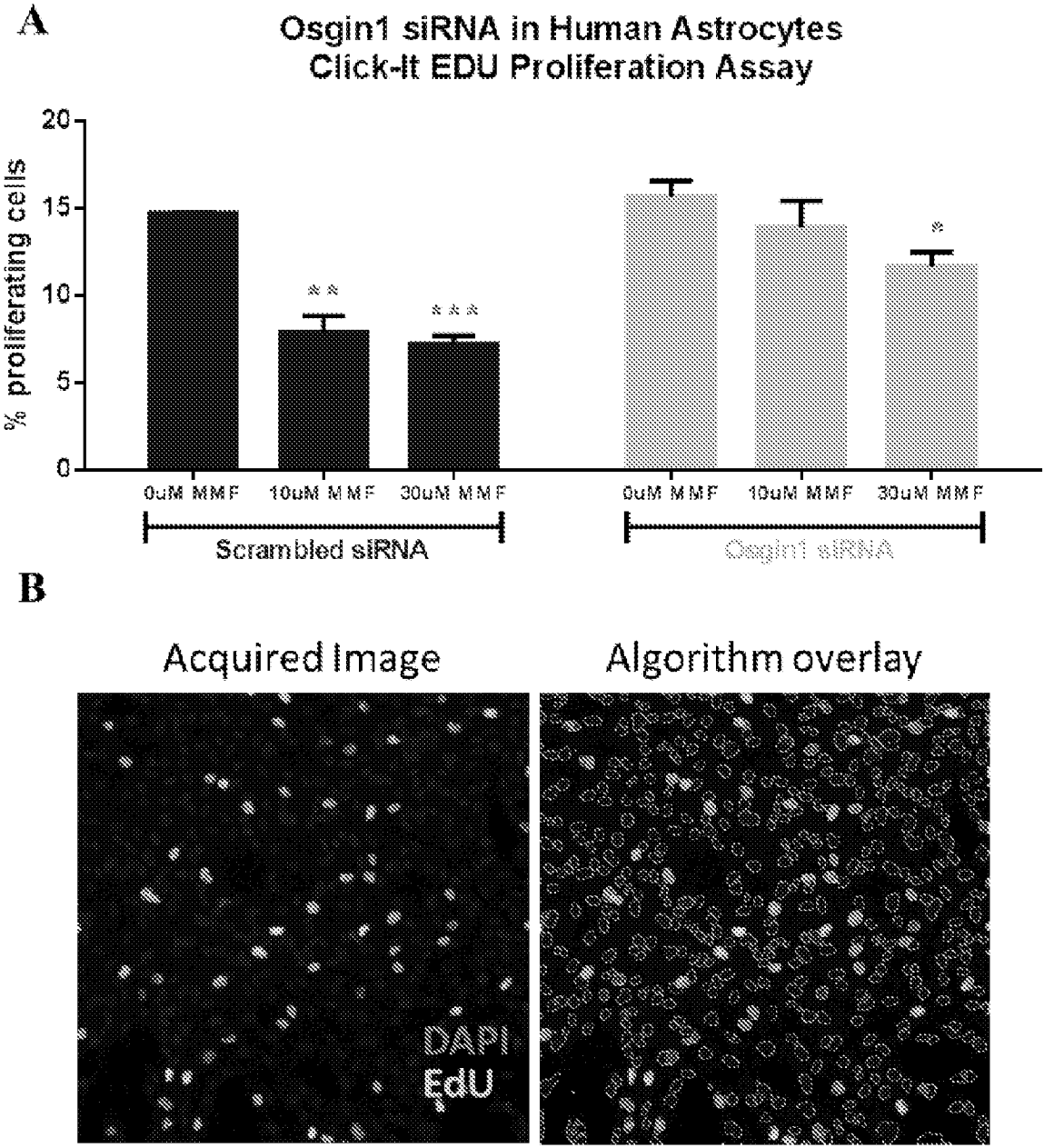


FIGURE 71

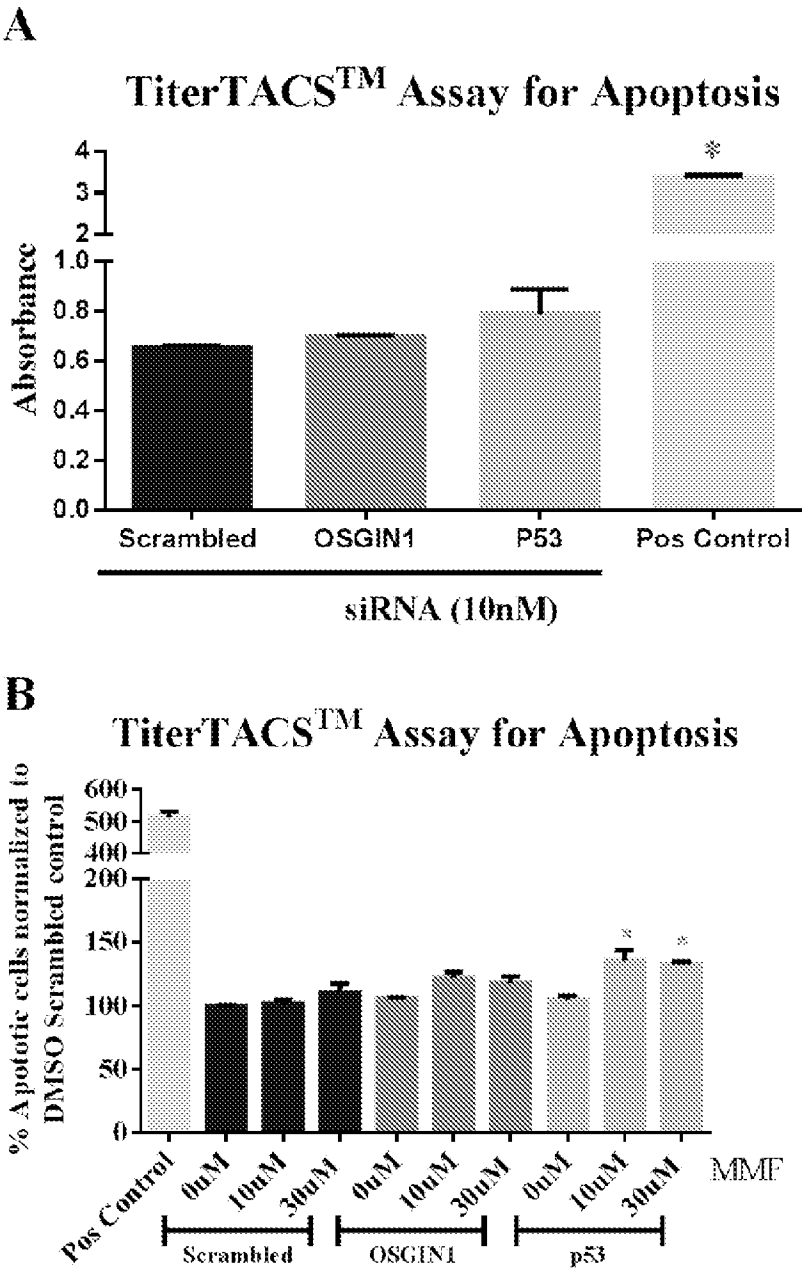


FIGURE 72

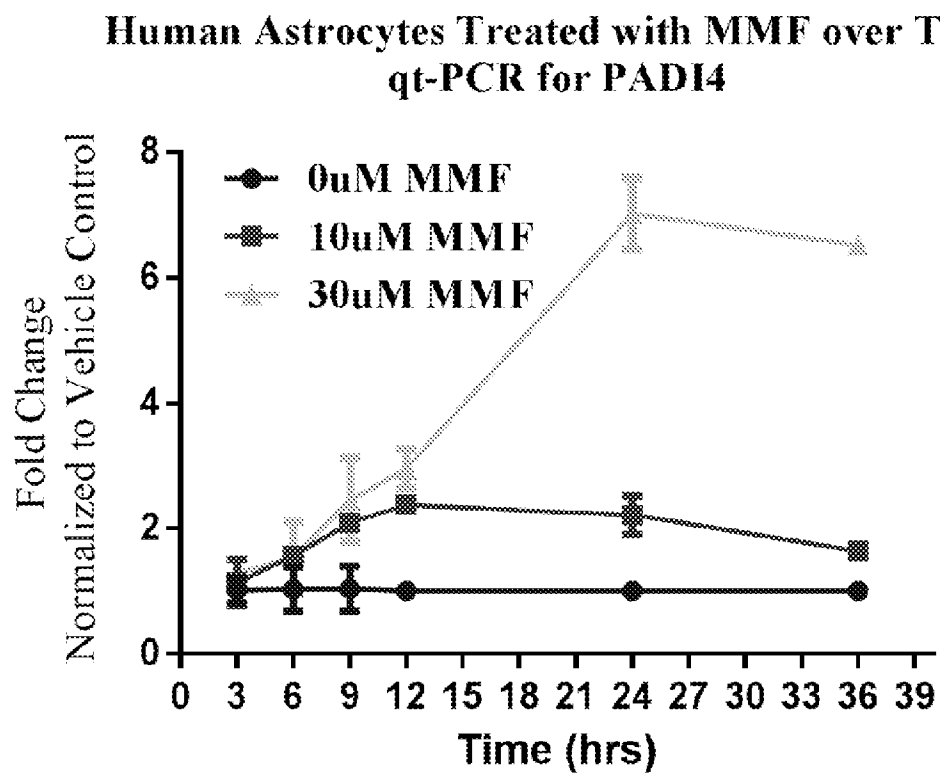


FIGURE 73

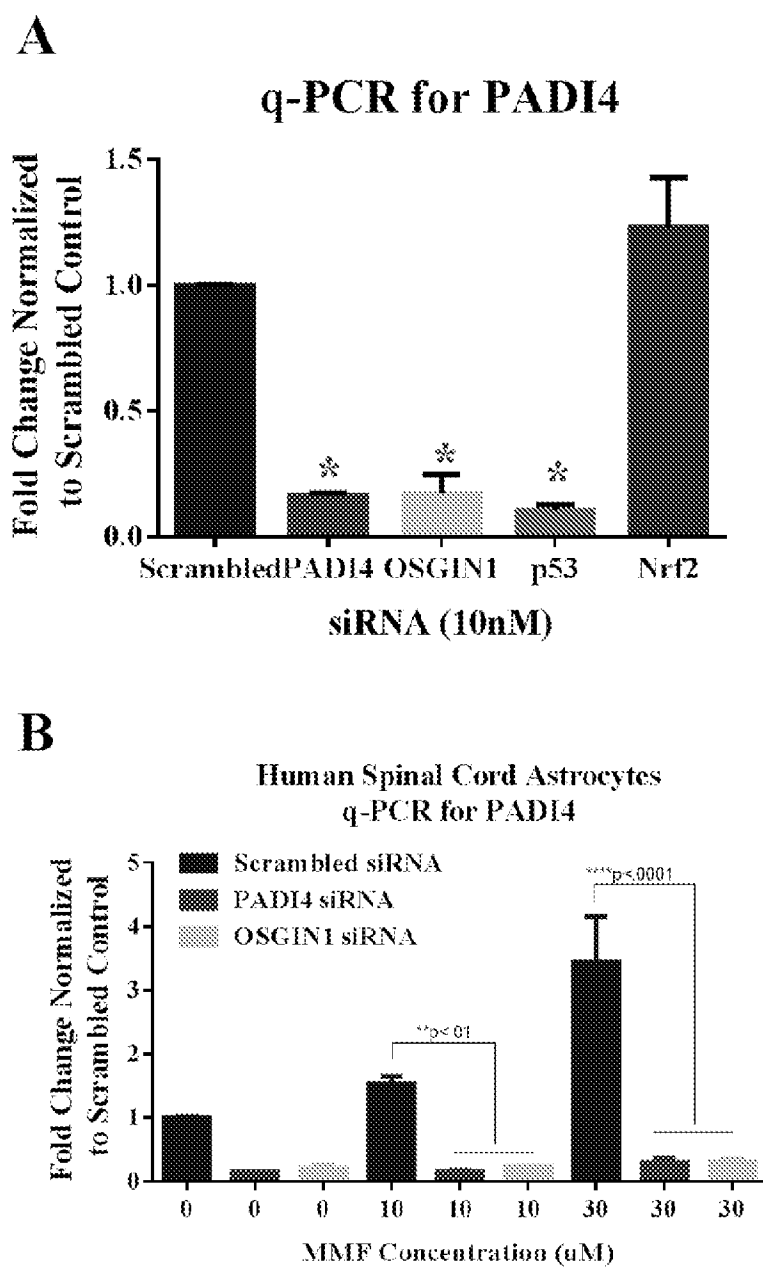


FIGURE 74

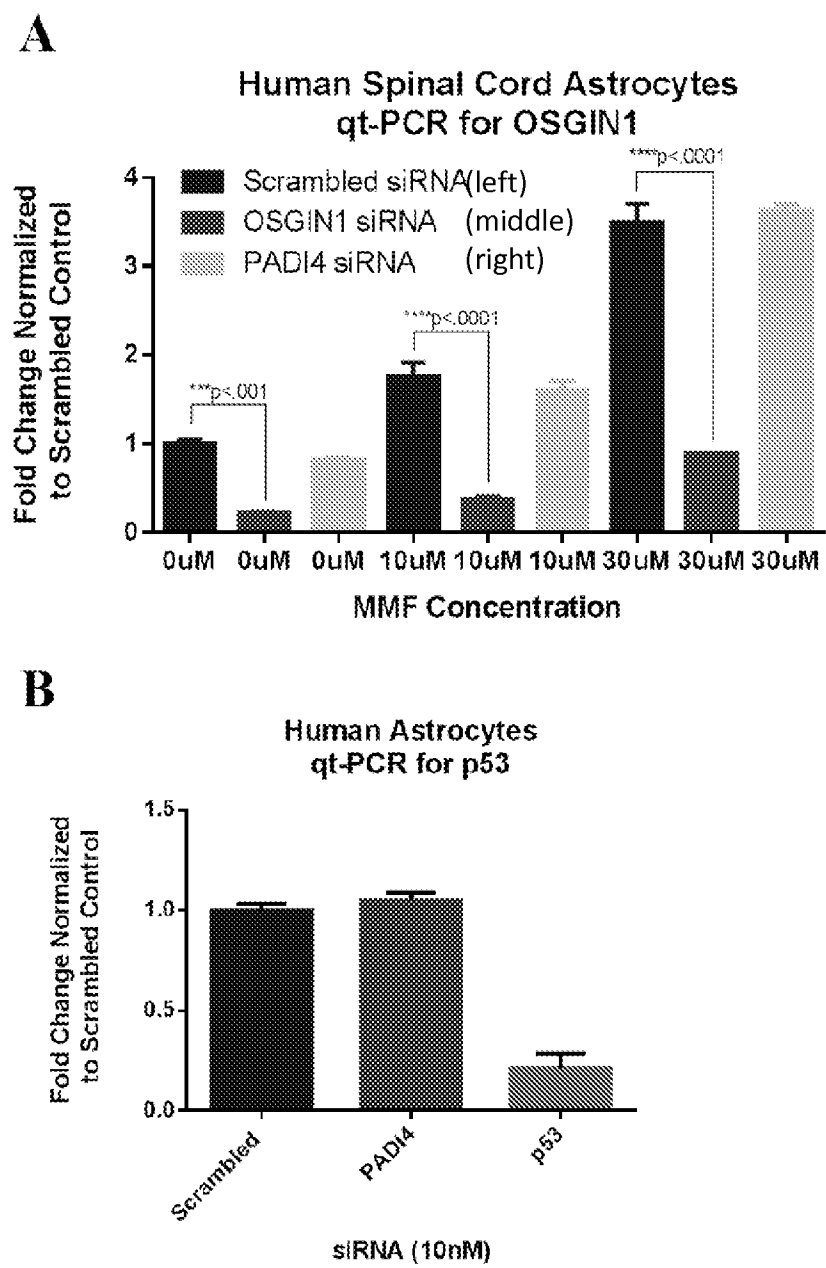


FIGURE 75

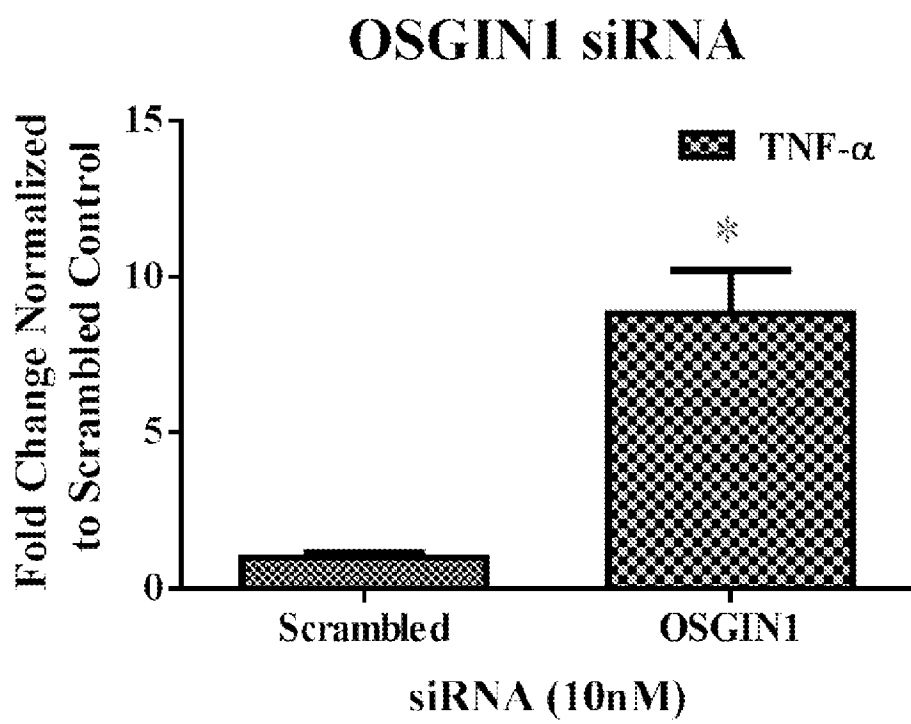


FIGURE 76

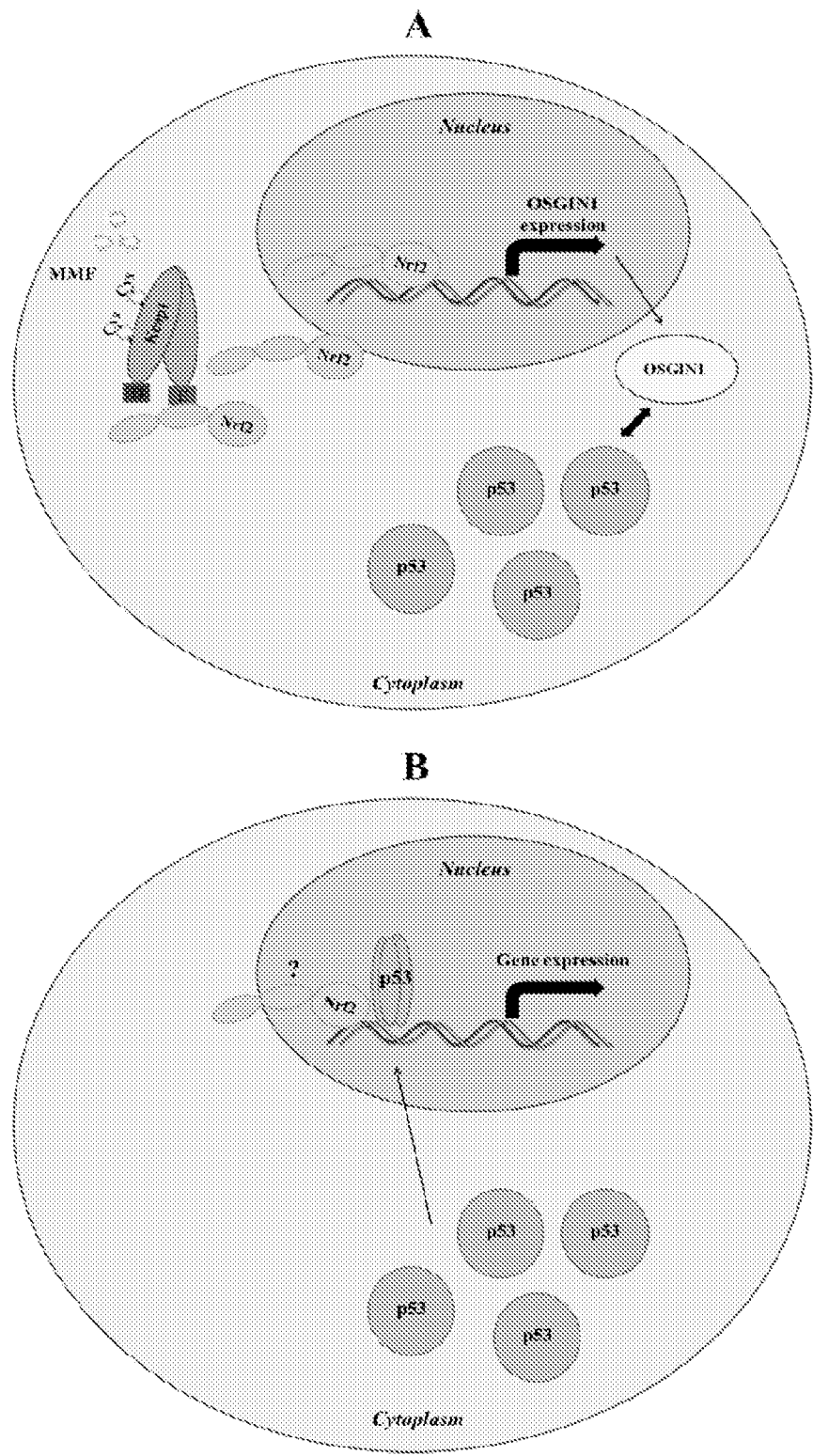


FIGURE 77

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2015/029993

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	C. E. ROMANOSKI ET AL: "Network for Activation of Human Endothelial Cells by Oxidized Phospholipids: A Critical Role of Heme Oxygenase 1", CIRCULATION RESEARCH, vol. 109, no. 5, 7 July 2011 (2011-07-07), pages e27-e41, XP055202393, ISSN: 0009-7330, DOI: 10.1161/CIRCRESAHA.111.241869	38,39
A	page e29, column 1, paragraph 3	1-37,40,41
X	----- US 2003/166519 A1 (HUYNH THE HUNG [SG]) 4 September 2003 (2003-09-04)	40,41
A	the whole document	1-39
X	----- Europeans Medicines Agency;United Kingdom An agency of the European Union: "Assessment Report: Tecfidera", 26 November 2013 (2013-11-26), XP055202838, Retrieved from the Internet: URL:http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Public_assessment_report/human/002601/WC500162070.pdf [retrieved on 2015-07-16]	1-32
A	page 111 - page 115; figure 23 page 120 - page 121 page 124	34-41
A	----- BROOKS M HYBERTSON ET AL: "Oxidative stress in health and disease: The therapeutic potential of Nrf2 activation", MOLECULAR ASPECTS OF MEDICINE, PERGAMON PRESS, OXFORD, GB, vol. 32, no. 4, 11 October 2011 (2011-10-11), pages 234-246, XP028119045, ISSN: 0098-2997, DOI: 10.1016/J.MAM.2011.10.006 [retrieved on 2011-10-15] abstract page 237, paragraph 3 page 240 - page 242; figure 1 page 243, paragraph 8 ----- -/--	1-41

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2015/029993

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BRENNAN M S ET AL: "BG-12 (Dimethyl Fumarate) pharmacodynamic responses have distinct temporal and dose-dependent profiles and are Nrf2-dependent", ABSTRACTS OF THE ANNUAL MEETING OF THE SOCIETY FOR NEUROSCIENCE, SOCIETY FOR NEUROSCIENCE, WASHINGTON, DC, US, vol. 43, 13 November 2013 (2013-11-13), XP009185292, ISSN: 0190-5295 the whole document -----	1-41
A	US 2012/108562 A1 (CHEN GONG [US] ET AL) 3 May 2012 (2012-05-03) paragraph [0119]; figure 15; example 16 -----	1-41
A	WO 2009/067812 A1 (UNIV MONTREAL [CA]; SEKALY RAFICK-PIERRE [CA]; HADDAD ELIAS [CA]; WALK) 4 June 2009 (2009-06-04) the whole document -----	1-41

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2015/029993

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2003166519	A1	04-09-2003	NONE
US 2012108562	A1	03-05-2012	CN 103189063 A 03-07-2013
		US 2012108562	A1 03-05-2012
		US 2014288059	A1 25-09-2014
		WO 2012061390	A2 10-05-2012
WO 2009067812	A1	04-06-2009	AU 2008329530 A1 04-06-2009
		CA 2742926	A1 04-06-2009
		EP 2225397	A1 08-09-2010
		US 2011008777	A1 13-01-2011
		WO 2009067812	A1 04-06-2009