(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
29 December 2016 (29.12.2016)

WIPO | PCT

(10) International Publication Number
WO 2016/207204 A1

(51) International Patent Classification:
A61K 31/16 (2006.01) *A61P 1/02* (2006.01)
A61K 31/4015 (2006.01) *A61P 19/02* (2006.01)
A61K 45/06 (2006.01) *A61P 19/10* (2006.01)
A61P 3/04 (2006.01) *A61P 15/16* (2006.01)

(21) International Application Number:
PCT/EP2016/064388

(22) International Filing Date:
22 June 2016 (22.06.2016)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
15173322.7 23 June 2015 (23.06.2015) EP

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(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).

Declarations under Rule 4.17:

— *as to the identity of the inventor (Rule 4.17(i))*

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

Published:

— *with international search report (Art. 21(3))*

WO 2016/207204 A1

(54) Title: N-ALKYLAMIDES FOR TREATMENT OF BROMODOMAIN PROTEIN RELATED DISEASES

(57) Abstract: The invention relates to compounds in particular N,N-dimethyl acetamide and 1-vinyl-2- pyrrolidone, for use in a method for prevention or treatment of a disease that can be modulated or ameliorated by the inhibition of bromodomain containing proteins and enhancement of bone morphogenetic protein receptor signalling. In particular these diseases comprise weight gain, obesity, osteoporosis, osteoarthritis, periodontitis and/or peri- implantitis.

N-Alkylamides for treatment of bromodomain protein related diseases

Field of the invention

The present invention relates to the use of N,N-dimethyl acetamide and 1-vinyl-2-pyrrolidone for use prevention and treatment of obesity and bone loss, particularly in osteoporosis, osteoarthritis, periodontitis and peri-implantitis and for use as a male contraceptive.

Background of the invention

Acetylation of histones is an epigenetic mechanism that has been linked to an opening of the chromatin architecture and an increase in transcriptional activation. ε-N-acetylation of lysine residues is primarily recognized by bromodomains first identified in the brahma gene from Drosophila melanogaster. Acetylation marks act as scaffolds for the assembly of macromolecular complexes that alter chromatin accessibility to transcription factors and allow the recruitment and activation of RNA polymerases. Until today, 61 bromodomains have been recognized in the human genome, which are present in 46 diverse nuclear and cytoplasmic proteins.

The bromodomain is an evolutionarily conserved, ~110 amino acid motif comprised of four left-handed, antiparallel α-helices. The medical potency of bromodomain inhibitors was first demonstrated by the ischemin/MS120, a weak inhibitor of human p300/CBP associated factor bromodomain, the action of which prevents apoptosis in ischemic cardiomyocytes. High affinity nano-molar range inhibitors were finally developed by the Structural Genomic Consortium and at GlaxoSmithKline. Their efficiency was demonstrated in several medical applications. In an induced periodontitis model in rats, JQ1 proved to suppress inflammation by a reduction of the inflammatory cytokine release and bone destruction by the inhibition of osteoclast maturation. Another potential area for high affinity bromodomain inhibitor application is as male contraceptive by blocking the bromodomains of BrdT, a spermatocyte and spermatid specific member of the BET family.

Bone regeneration by the deposition of newly formed bone tissue is performed mainly by osteoblasts under the influence of bone morphogenetic proteins and is required for the healing of bone defects and fractures, to compensate for bone loss and for normal bone turnover. Bone loss can be caused by cancer, inflammation, bone metastases, sepsis, rheumatoid arthritis, periodontitis, peri-implantitis, osteoporosis, osteoarthritis or other bone degrading disorders. Known bromodomain inhibitors such as JQ1 cannot be used for the treatment of diseases connected to bone degradation, fracture etc, because these

substances do not only inhibit osteoclast formation but also osteoblast differentiation, whereas the latter is an important mechanism in bone formation.

The problem underlying the present invention is to provide means for the treatment of a disease that can be modulated or ameliorated by the inhibition of bromodomain containing proteins and/or enhancement of bone morphogenetic protein receptor signalling. This problem is solved by the subject-matter of the independent claims.

Terms and definitions

In the context of the present specification, the term *bromodomain* is used in its meaning known in the art of molecular biology and biochemistry; it refers to an extensive family of evolutionarily conserved protein modules originally found in proteins associated with chromatin. Bromodomains are protein modules that function as acetyl-lysine binding domains (Zeng et al. FEBS Letters, 513 (2002) 124-128). They recognize monoacetylated lysine residues most commonly, but not exclusively found on the N-terminal tails of histones.

In the context of the present specification, the term *bromodomain inhibitor* is used in its meaning known in the art of molecular biology and biochemistry; it refers to a compound that inhibits the binding of a bromodomain with its cognate acetylated lysine or lysines of one or more proteins. In particular, said cognate acetylated lysines are among the residues of one or more histones.

In the context of the present specification, the term *bromodomain and extraterminal (BET) family of proteins* is used in its meaning known in the art of molecular biology and biochemistry; it refers to readers of the epigenetic code that couple acetylation of lysine residues on histones to changes in chromatin structure and gene expression. The BET family includes the four known proteins Brd2 (P25440), Brd3 (Q15059), Brd4 (O60885), and BrdT (Q58F21). All BET proteins are widely expressed across diverse tissues, with the exception of BrdT, whose expression is restricted to the testes. Every BET protein contains tandem bromodomains in the N-terminal region that specifically bind acetylated lysine residues in histones H3 and H4. Once bound to histones, BET proteins recruit protein complexes that modulate gene transcription either directly, such as transcriptional activators or repressors, or indirectly such as chromatin remodeling complexes. BET proteins have been identified as important mediators of altered gene expression profiles found in numerous diseases including cancer, diabetes, obesity, atherosclerosis, cardiovascular and renal disorders and viral infection (Muller, S., et al. , Expert Rev. Mol. Med., 13: e29 (2011)).

In the context of the present specification, the term *bone morphogenetic protein (BMP)* is used in its meaning known in the art of molecular biology and biochemistry; it refers to a group of multi-functional growth factors and cytokines that belong to the transforming growth

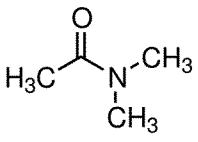
factor beta superfamily. BMPs constitute a group of pivotal morphogenetic signals, orchestrating tissue architecture throughout the body. BMPs play a role in differentiation, proliferation, growth inhibition, and arrest of maturation of a wide variety of cells depending on the cellular microenvironment and the interactions with other regulatory factors. In this specification BMP refers to any one of the family of growth factors or fragments thereof with the ability to induce the formation of bone and/or cartilage. It is not intended that BMP refer to any particular protein sequence, provided that the molecule has sufficient structural homology to any one of the known BMPs described below and retains some functional ability to promote bone growth, cartilage growth, or osteoblast differentiation. BMP-2 is known to induce bone and cartilage formation and play a role in osteoblast differentiation. BMP-3 is known to induce bone formation. BMP-4 is known to regulate the formation of teeth, limbs and bone from the mesoderm and play a role in fracture repair. BMP-5 is known to function in cartilage development. BMP-6 is known to play a role in joint integrity and bone formation/repair. BMP-7 and BMP-9 are known to play a role in osteoblast differentiation. BMP-1 is a known metalloprotease that acts on procollagen I, II, and III and is involved in cartilage development.

In the context of the present specification, the term *bone morphogenetic protein receptor* is used in its meaning known in the art of molecular biology and biochemistry; it refers to receptors for BMPs that are complexes of two different types of membrane-bound serine/threonine kinases: type I BMP receptors and type II receptors. After ligand binding, the type II receptor phosphorylates the type I receptor. The activated type I receptor then phosphorylates members of downstream signalling pathways such as p38 or Smad proteins.

Protein identifiers provided in this specification are identifiers of the Uniprot database (www.uniprot.org) if not indicated otherwise.

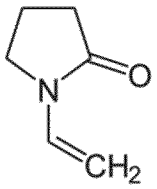
Summary of the invention

The surprising finding was made that N,N-dimethyl acetamide (DMA, CAS No. 127-19-5; formula I)



(I)

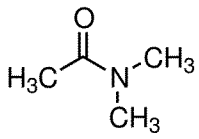
and 1-vinyl-2-pyrrolidone (NVP, CAS No. 88-12-0; formula II)


(II)

Inhibit binding of bromodomains to their cognate targets. Furthermore they enhance the maturation of osteoblasts by a bromodomain independent effect via bone morphogenetic protein related pathways, and as a result augment bone regeneration. In this context it was discovered that they inhibit osteoclast differentiation and the formation of multinucleated osteoclasts needed for bone destruction not only via bromodomain binding but also by the augmentation of Bone morphogenetic protein (BMP) receptor signalling.

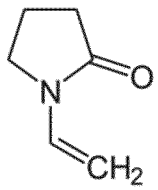
According to a first aspect of the invention, a compound for use in a method for prevention or treatment of a disease selected from pathological weight gain, obesity, osteoporosis, osteoarthritis, periodontitis and peri-implantitis is provided. The compound is selected from the group comprising:

a) N,N-dimethyl acetamide (I) (DMA, CAS No. 127-19-5)


(I),

and

b) 1-vinyl-2-pyrrolidone (II) (NVP, CAS No. 88-12-0)


(II).

In certain embodiments, the disease is selected from (pathological or disease-causing) weight gain, obesity, osteoporosis, osteoarthritis, periodontitis and/or peri-implantitis.

In certain embodiments, the bromodomain containing protein that is inhibited to achieve prevention or treatment of the disease is selected from Brd2, Brd3, Brd4 or BrdT.

According to a second aspect of the invention, a bromodomain inhibitor for use in prevention or treatment of weight gain, obesity, osteoporosis, osteoarthritis, periodontitis and/or peri-implantitis is provided. The compound is selected from N,N-dimethyl acetamide (DMA, CAS No. 127-19-5) or 1-vinyl-2-pyrrolidone (NVP, CAS No. 88-12-0).

According to a third aspect of the invention, a method for the inhibition of bromodomain containing proteins and/or enhancement of bone morphogenetic protein receptor signalling is provided. The method comprises the steps of administering N,N-dimethyl acetamide (DMA, CAS No. 127-19-5) and/or 1-vinyl-2-pyrrolidone (NVP, CAS No. 88-12-0) to a mammal.

In certain embodiments, the bromodomain containing protein is selected from Brd2, Brd3, Brd4 or BrdT.

In certain embodiments, the inhibition of BrdT inhibits fertility in a male mammal.

According to a fourth aspect of the invention, a pharmaceutical composition comprising a compound selected from N,N-dimethyl acetamide (DMA, CAS No. 127-19-5) and/or 1-vinyl-2-pyrrolidone (NVP, CAS No. 88-12-0) is provided. The composition is provided for use in prevention or treatment of pathological weight gain, obesity, osteoporosis, osteoarthritis, periodontitis and/or peri-implantitis.

According to a fifth aspect of the invention, a contraceptive for a male mammal is provided. The contraceptive comprises a compound selected from N,N-dimethyl acetamide (DMA, CAS No. 127-19-5) or 1-vinyl-2-pyrrolidone (NVP, CAS No. 88-12-0).

According to a sixth aspect of the invention, the use of N,N-dimethyl acetamide (DMA, CAS No. 127-19-5) or 1-vinyl-2-pyrrolidone (NVP, CAS No. 88-12-0) in manufacture of a medicament is provided. The medicament is provided for use in prevention or treatment of a disease that can be modulated or ameliorated by the inhibition of bromodomain containing proteins and/or enhancement of bone morphogenetic protein receptor signalling.

In certain embodiments the disease is selected from weight gain, obesity, osteoporosis, osteoarthritis, periodontitis and/or peri-implantitis.

Wherever alternatives for single separable features such as, for example, an isotype protein inhibitor or activator or medical indication are laid out herein as “embodiments”, it is to be understood that such alternatives may be combined freely to form discrete embodiments of the invention disclosed herein.

Specific description of the invention

The present invention is based on *in vitro* and *in vivo* studies, which reveal that N,N-dimethyl acetamide and 1-vinyl-2-pyrrolidone are bromodomain inhibitors and enhancers of bone morphogenetic protein receptor signaling.

In an *in vitro* assay, wherein acetyl-residues as analogues for an epigenetic mark of acetylation were assessed for binding to single or tandem bromodomains of BET-proteins, Brd2, Brd3, Brd4 and BrdT, the inventors surprisingly found that N,N-dimethyl acetamide and

1-vinyl-2-pyrrolidone inhibit this binding (Fig.1). This discovery revealed that N,N-dimethyl acetamide and 1-vinyl-2-pyrrolidone are bromodomain inhibitors and therefore are useful for male contraception via BrdT inhibition, prevention and treatment of bone loss via Brd4 inhibition, prevention and treatment of obesity via Brd2 inhibition and inflammation, osteoarthritis, periodontitis and peri-implantitis via Brd2 and Brd4 inhibition.

Beyond the bromodomain inhibiting activity and in contrast to the high affinity bromodomain inhibitor JQ1 both N,N-dimethyl acetamide (Fig. 2) and 1-vinyl-2-pyrrolidone (Fig. 3) are able to enhance BMP-2 activity and overcome the inhibitory effect on osteoblast differentiation and maturation normally seen with bromodomain inhibitors. Osteoblasts are the cells that deposit new bone and regenerate bone defects. In studies on the effect of N,N-dimethyl acetamide on model cell lines for mesenchymal progenitor cells (C2C12) and preosteoblasts (MC3T3), it was surprisingly found that alkaline phosphatase activity, which is an early marker for bone formation and maturation of preosteoblasts to osteoblast, was increased in the presence of N,N-dimethyl acetamide (Fig 2). The same behavior was also found for 1-vinyl-2-pyrrolidone (Fig. 3). A closer inspection of this result revealed that N,N-dimethyl acetamide enhances the signaling of the receptors for bone morphogenetic proteins (BMP) for Smad and p38 phosphorylation (Fig 4). Since BMPs are central for bone formation, regeneration and maturation of osteoblasts N,N-dimethyl acetamide and 1-vinyl-2-pyrrolidone enhance bone formation and bone regeneration most likely by increasing the signaling of the BMP-receptor via an enhanced phosphorylation of Smads and p38 which triggers the transcription of factors leading the cell towards the osteoblastic lineage.

In summary, N,N-dimethyl acetamide and 1-vinyl-2-pyrrolidone enhance the maturation of preosteoblasts to osteoblasts and the kinase activity of the BMP-receptor for Smad and p38 and therefore the formation and regeneration of bone.

The counterparts of the bone depositing osteoblasts are the bone degrading osteoclasts. When the effect of N,N-dimethyl acetamide on osteoclasts or a model cell line for osteoclasts (RANKL-stimulated RAW264.7 cells) was examined, it was surprisingly found that N,N-dimethyl acetamide is able to inhibit osteoclastogenesis, the differentiation to osteoclasts, which is needed for bone degradation.

RAW264.7 cells were chosen because they are a good model system to study osteoclastogenesis. The tartrate-resistant acid phosphatase (TRAP) is needed to generate the acidic milieu to dissolve the calcium-phosphates of bone. The examination of the effect of N,N-dimethyl acetamide on TRAP activity induced by RANKL (Fig. 5) revealed that TRAP activity was significantly reduced in the cells treated with RANKL and N,N-dimethyl acetamide, compared to the cells treated with RANKL alone. Moreover, N,N-dimethyl acetamide inhibited the formation of multinucleated osteoclasts (Fig.5) and dramatically

reduced the number of nuclei per osteoclast suggesting that N,N-dimethyl acetamide could modulate also the fusion process (Fig. 5).

In summary, N,N-dimethyl acetamide inhibits osteoclast differentiation and maturation and therefore the ability to degrade bone.

As an *in vivo* model to assess the inhibition of bone destruction by N,N-dimethyl acetamide, oestrogen depletion by ovariectomy in rats was chosen. Surprisingly, weekly administration of N,N-dimethyl acetamide reduced bone destruction of the trabecular bone as assessed by microcomputer tomography (Fig. 6) and histology (Fig.7) of the femur heads.

Another cause for bone destruction is inflammation as manifested in osteoarthritis but above all in peri-implantitis and periodontitis. RAW 264.7 macrophages were stimulated by lipopolysaccharides (LPS) and used as an *in vitro* model to study inflammatory processes. The surprising result of these studies was that 1-vinyl-2-pyrrolidone reduced LPS-induced nitric oxide (NO) production, a mediator for inflammation, to a similar extent than achieved by the high affinity bromodomain inhibitor JQ1 (Fig.8).

In essence, 1-vinyl-2-pyrrolidone as other bromodomain inhibitors reduces inflammation by decreasing NO production in macrophages.

Another outcome of the ovariectomy experiments was that N,N-dimethyl acetamide is able to reduce weight gain normally occurring during estrogen depletion (Fig.9). This reduced weight gain is due to an inhibition of adipocyte differentiation and maturation. In the presence of N,N-dimethyl acetamide, pre-adipocytes stimulated with differentiation medium inducing adipogenesis show a significant reduction in fat deposition monitored by oil red staining (Fig.10).

The invention is further illustrated by the following examples and figures, from which further embodiments and advantages can be drawn. These examples are meant to illustrate the invention but not to limit its scope.

Short description of the figures

Fig. 1 shows binding assays (AlphaScreening Assays; Perkin Elmer) of recombinant bromodomains of the BET-family members Brd2 (A), Brd3 (B), Brd4 (C) and BrdT (D) and bromodomain ligands in the presence of N,N-dimethyl acetamide (DMA) and 1-vinyl-2-pyrrolidone (NVP). DMA and NVP cause a reduction of the acetyl-binding activity of the bromodomain. This was observed with recombinant BET proteins with either one (BD1 or BD2) or both bromodomains (BD1BD2). For comparison binding data of the already known bromodomain inhibitors N-Methyl-2-pyrrolidone (NMP) and JQ1 are shown. The concentration in the test was set to 10 mmol/l for DMA, NVP and NMP

and 1 µmol/l for JQ1 for Brd2, Brd3 and Brd4. For BrdT JQ1 concentration was 10 µmol/l. (E) Determination of millimolar half-maximum inhibitory concentration (IC50) for DMA on BRD2. (F) Determination of millimolar half-maximum inhibitory concentration (IC50) for DMA on BRD4 binding activity.

Fig. 2 shows measurement of ALP (alkaline phosphatase) activity as a marker of osteoblastic differentiation in C2C12 (left panel) and MC3T3 cells (right panel). C2C12 cells treated with rhBMP-2 and increasing amounts of N,N-dimethyl acetamide (DMA) exhibited a up to 2 times higher ALP activity than cells treated with rhBMP-2 alone. Similarly, the pre-osteoblastic cell line MC3T3 (right panel) responds to increasing amounts of DMA with an up to 3 fold increased ALP activity, with and without rhBMP-2 supplementation. The upper smaller graphs show an enlargement of the no growth factor (No GF) data from the lower graphs.

Fig. 3 shows measurement of alkaline phosphatase (ALP) activity as a marker of osteoblastic differentiation in C2C12 cells. C2C12 cells treated with rhBMP-2 and increasing amounts of 1-vinyl-2-pyrrolidone (NVP) exhibited a 2 fold higher ALP activity than cells treated with rhBMP-2 alone. In contrast, the high affinity bromodomain inhibitor JQ1 inhibits osteoblast differentiation and maturation.

Fig. 4 shows the phosphorylation of the BMP receptor-specific Smad proteins (pSmad 1/5/8) and p38 (pp38), which is also involved in BMP signalling, in response to stimulation with rhBMP-2 and increasing concentrations of DMA. Phosphorylation levels of Smad proteins 1/5/8 and p38 are increased with rising concentrations of DMA. As control substance 5 mM NMP was used.

Fig. 5 shows the effects of N,N-dimethyl acetamide (DMA) on receptor activator of nuclear factor kappa-B ligand (RANKL)-induced osteoclastogenesis in RAW264.7 cells. The microscopic images show that, RAW264.7 cells differentiate in the presence of RANKL into mature tartrate-resistant acid phosphatase (TRAP) positive multinucleated osteoclasts (MNCs). DMA reduced the formation and number of TRAP positive MNCs in a concentration-dependent manner. The occurrence of MNCs is quantified in the lower left graph. TRAP activity (lower right graph) was measured in response to treatment with RANKL and DMA. DMA reduced TRAP activity determined as optical density at 405 nm (OD405)n a concentration dependent manner. NMP: N-Methyl-2-pyrrolidone.

Fig. 6 shows reduced bone destruction in an osteoporosis model after treatment with N,N-dimethyl acetamide (DMA). Female rats underwent either bilateral laparotomy (Sham Veh, Ntotal=10) or bilateral ovariectomy (OVX, Ntotal=20). One week after recovering from surgery, the OVX rats were divided into 2 groups: OVX with vehicle (OVX Veh, Ntotal=10) and OVX with DMA (OVX DMA, 1/3 of LD50 = 92µl/100g/week, equals an overall concentration of 10.5 mM, Ntotal=10). Weekly treatment via intraperitoneal injection was initiated 1 week after OVX and lasted for 15 weeks. In rat femur bones the cortical structure (A) remains but the trabecular structure (B) is reduced by oestrogen depletion induced osteoporosis. This effect can be prevented by weekly administration of DMA.

Fig. 7 shows (A) serum levels of estradiol after ovariectomy. The results of the Sham group were compared with OVX Veh group, and the results of the OVX DMA group were compared with the results of OVX Veh. Data are mean ± s.d.. Two independent experiments were performed with 3 animals each (n=6). (B) Changes of serum levels of osteocalcin after ovariectomy (C) Bone parameters were analyzed by micro-CT after 15 weeks of DMA treatment in OVX rats. Graphs represented bone volume (BV/TV), trabecular separation (Tb. Sp), trabecular number (Tb. N), and trabecular thickness (Tb. Th). Data are mean ± s.d. (n = 6 per group). (D) histological images of femur bones from the experiment described in figure 6 and example 4. The trabecular structure deteriorates by oestrogen depletion induced osteoporosis (OVX Veh) as compared to sham operated animals (Sham Veh). Disturbances of trabecular structure in response to oestrogen depletion can be reduced by weekly administration of N,N-dimethyl acetamide (DMA) (OVX DMA).

Fig. 8 shows the effect of 1-vinyl-2-pyrrolidone (NVP) and N,N-dimethyl acetamide (DMA) on LPS-induced inflammation in RAW264.7 cells. RAW264.7 cells were stimulated with LPS (1µg/ml) for 48 h in the absence or presence of NVP (1, 5 and 10 mM) or DMA and nitric oxide (NO) levels were measured. NVP and DMA decrease LPS-induced NO production in a concentration dependent manner. A similar result was achieved by using the high affinity bromodomain inhibitor JQ1. (C) RAW264.7 cells were treated with LPS (1 µg/ml) in the absence or presence of DMA, total RNA (6 h after stimulation) or whole cell lysates (48 h after stimulation) were isolated. iNOS mRNA levels were determined by real-time PCR and normalized to GAPDH level and expressed as ΔΔCT. Whole cell lysates were subjected to Western blot analysis with antibody against iNOS. Data are expressed as mean ± s.d. (n = 6). (D) Effect

of DMA on LPS-induced pro-inflammatory cytokines. RAW264.7 cells were treated with LPS (1 µg/ml) in the absence or presence of DMA, and total RNA was isolated 6 h after treatment. IL-1α, IL-β, TNF-α, and IL-6 mRNA levels were determined by real-time PCR and normalized to GAPDH level Results are expressed as ΔΔCT. Data are expressed as mean ± s.d. (n = 6).

Fig. 9 shows body weight development in ovariectomized (OVX) and sham operated (Sham) rats as described in figure 6 and example 4 in response to weekly treatment with N,N-dimethyl acetamide (DMA) as compared to vehicle only (Veh). OVX causes an increased weight gain as compared to sham operated rats. DMA treatment does reduce this effect of OVX and results in a reduced weight gain compared to vehicle treated OVX rats.

Fig. 10 shows the effect of N,N-dimethyl acetamide (DMA) on fat accumulation in adipocytes. Adipogenic differentiation was induced in 3T3-L1 cells by stimulation with Methyl iso-butyl xanthine (500 µM), Dexamethasone (0.25 µM) and Insulin (10 µg/ml) (MDI). Accumulation of lipids in adipocytes was determined via Oil red staining. Treatment with MDI induces the maturation of adipocytes resulting in increased fat accumulation. Simultaneous stimulation with DMA (5 mM) abolishes the increase observed in MDI stimulated pre-adipocytes.

Fig. 11 shows the effect of N,N-dimethyl acetamide (DMA) and 1-vinyl-2-pyrrolidone (NVP) in a bone regeneration model. Six Rabbits were used and four defects of 6 mm in diameter were generated in the calvarial bone. One defect per animal was left untreated, one was covered by a membrane of poly-lactide-co-glycolide (PLGA), one was treated with a PLGA membrane loaded with 10 weight % DMA and the other one with a membrane loaded with 15 weight % NVP. Bone regeneration was measured in histological sections from the middle section of the defects 4 weeks after implantation. Bone regeneration was significantly increased when the membranes were either loaded with N,N-dimethyl acetamide (DMA) or 1-vinyl-2-pyrrolidone (NVP). Therefore both substances accelerate and increase bone regeneration and bone formation.

Examples

The invention is illustrated by the following examples.

For statistical analysis, unpaired Students T-test was implemented by a commercially available software package (SSPE, Chicago, Il). All values are represented as mean ± standard error of the mean.

Example 1: N,N-dimethyl acetamide and 1-vinyl-2-pyrrolidone are bromodomain inhibitors.

AlphaScreening Assays were performed using recombinant bromodomains and bromodomain ligands or recombinant BET bromodomains and BET Ligand from BPS Bioscience (San Diego, USA). The AlphaScreening signal from the assay is correlated with the amount of bromodomain/BET ligand binding to the bromodomain. AlphaScreening signal was measured using EnSpire Alpha 2390 Multilabel reader (Perkin Elmer).

Binding experiments were performed in duplicate. AlphaScreening data were analyzed using the computer software, Graphpad Prism. In the absence of the compound, the AlphaScreening signal (At) in each data set was defined as 100% activity. In the absence of the bromodomain/BET Ligand, the AlphaScreening signal (Ab) in each data set was defined as 0% activity. The percent activity in the presence of each compound was calculated according to the following equation: % activity = [(A- Ab)/(At - Ab)]×100, where A= AlphaScreening signal in the presence of the compound, Ab = AlphaScreening signal in the absence of the bromodomain/BET Ligand, and At = AlphaScreening signal in the absence of the compound. The percent inhibition was calculated according to the following equation: % inhibition = 100 - % activity. The results of these experiments as illustrated in figure 1 show that both N,N-dimethyl acetamide and 1-vinyl-2-pyrrolidone are able to inhibit the binding of a natural bromodomain ligand to bromodomains of the BET-protein family (Brd2, Brd3, Brd4, BrdT).

DMA inhibits the binding activity of BRD2 and BRD4 with an IC50 value of 11 mM and 6 mM, respectively (Fig 1E, F).

Example 2: N,N-dimethyl acetamide and 1-vinyl-2-pyrrolidone enhances maturation of osteoblasts and N,N-dimethyl acetamide BMP signaling in C2C12 cells.

C2C12 cells are pluripotent myogenic cells able to transdifferentiate into a variety of tissues such as bone and adipose tissue. Therefore, they are often used as a model for mesenchymal stem cells. Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum and antibiotics (100U/mL penicillin G and 100mg/mL streptomycin). Cells were trypsinized and passaged every 2 to 3 days, and cultures were

never allowed to become confluent. All cells were grown at 37°C in humidified air mixed with 5% carbon dioxide.

ALP (alkaline phosphatase) activity was measured as a marker of osteoblastic differentiation in C2C12 cells. Cells were seeded at a density of $5x10^4$ cells/cm2 in 6- or 24-well plates (n=3 per group). Treatment with N,N-dimethyl acetamide or 1-vinyl-2-pyrrolidone and BMP-2 diluted in the tissue culture medium started 5 h after seeding and incubation was continued for 6 more days. After 6 days of incubation, medium was removed, and cells were washed with PBS and then scraped in 0.56 M 2-amino-2-methyl-1-propanol, pH 10.5. The pellets were then homogenized for 10 s. After centrifugation, the supernatant was collected and tested for ALP activity using p-nitrophenyl phosphate (Sigma-Aldrich) as a substrate. The protein content of the lysates was determined using Bradford protein assay reagent (Bio-Rad Laboratories; Hercules, CA). Experiments were performed independently in triplicate.

As shown in figure 2, C2C12 cells treated with rhBMP-2 and increasing amounts of N,N-dimethyl acetamide exhibited an up to 2 times higher ALP activity than cells treated with rhBMP-2 alone. Therefore, N,N-dimethyl acetamide enhances maturation of preosteoblasts to osteoblasts in a concentration dependent manner. The same applies to the pre-osteoblastic cell line MC3T3, since increasing amounts of N,N-dimethyl acetamide increase ALP activity with and without rhBMP-2 supplementation, due to the fact that MC3T3 cells produce autologous BMPs.

As shown in figure 3, C2C12 cells treated with rhBMP-2 and increasing amounts of 1-vinyl-2-pyrrolidone exhibited an up to 2 fold higher ALP activity than cells treated with rhBMP-2 alone. Therefore, 1-vinyl-2-pyrrolidone enhances maturation of preosteoblasts to osteoblasts in a concentration dependent way.

To explore the mechanisms by which N,N-dimethyl acetamide enhances BMP-2 activity, the effect of N,N-dimethyl acetamide on the kinase activity of the intracellular portion of BMPR I in preosteoblastic cells was examined. 15 min after application of 5 mM of N,N-dimethyl acetamide and 0.5 mg/mL of BMP-2, the phosphorylation of BMPR-specific Smad proteins was induced (Fig. 4). In addition to Smad phosphorylation, BMP also signals via p38 phosphorylation, which can only be studied at low serum concentrations. When the cells were treated under low serum conditions with 5mM of N,N-dimethyl acetamide and 0.5 mg/mL of BMP-2, Smad and p38 phosphorylation increased after 1 h, suggesting a longer-lasting effect of N,N-dimethyl acetamide on BMP signaling.

Example 3: N,N-dimethyl acetamide suppresses RANKL-induced osteoclastogenesis in RAW264.7 cells.

To show the effects of N,N-dimethyl acetamide on osteoclastogenesis, RAW 264.7 cells were treated with the receptor activator of nuclear factor kappa-B ligand (RANKL) (25 ng/ml). RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and antibiotics (100 U/ml penicillin G and 100 mg/ml streptomycin). The cultures were never allowed to become confluent. Incubations were performed at 37°C in 5% CO_2 in humidified air. TRAP activity is an early marker for maturation of preosteoclasts to osteoclasts. The effect of N,N-dimethyl acetamide on TRAP activity induced by RANKL was examined, and the results are shown in figure 5. For determination of TRAP activity, RAW 264.7 cells were plated into a 12-well culture dish (Corning, NY) with different concentrations of N,N-dimethyl acetamide in the presence of 25 ng/ml of RANKL. The medium and factors were replaced every 2 days. After 6 days of culture, the medium was removed, and the cell monolayer was gently washed twice with PBS. The cells were then lysed with 200 µl of 0.1% Triton X-100. TRAP activity in cell lysate was determined using TRAP solution (sodium acetate 0.1 M pH 5.8, ascorbic acid 1 mM, KCl 0.15 M, disodium tartrate 10 mM and p-nitrophenyl phosphate 10 mM). An aliquot of cell lysate was added to TRAP solution and was incubated for 30 minutes at 37°C. The reaction was stopped with NaOH 0.3 N, and the absorbance was measured at 410 nm using a micro-plate reader (BioTek). Results, normalized to protein content, are expressed in percentage of the activity obtained in RANKL stimulated cells. Data are expressed as mean S.D. (n=3). The data show that TRAP activity was significantly reduced in the cells treated with RANKL and N,N-dimethyl acetamide compared to cells treated with RANKL alone (Fig. 5). Therefore, N,N-dimethyl acetamide can inhibit the maturation of preosteoclasts to osteoclasts. To further illustrate these findings, RAW264.7 cells were seeded on a 24-well culture plate, treated with RANKL alone or with different concentration of N,N-dimethyl acetamide as indicated in figure 5. After 6 days of incubation, cells were washed with PBS, fixed, and stained for TRAP after differentiation into osteoclasts. TRAP staining of the cells was performed using a leukocyte acid phosphatase kit (Sigma-Aldrich). Cultured cells were fixed with formaldehyde for 5 min at room temperature, washed with PBS and air-dried. After TRAP-staining, an inspection of the plate by phase-contrast microscopy revealed that the number of multinucleated cells was reduced by N,N-dimethyl acetamide in a concentration dependent manner (Fig. 5). In the absence of N,N-dimethyl acetamide RAW264.7 cells differentiate into mature TRAP-positive multinucleated osteoclasts (MNCs), while N,N-dimethyl acetamide (1, 2.5 and 5 mmol/L) reduced the formation and numbers of TRAP-positive MNCs in a concentration-dependent manner (Fig. 5). Therefore, N,N-dimethyl acetamide inhibits the maturation of preosteoclasts to bone resorbing osteoclasts. Moreover,

N,N-dimethyl acetamide dramatically reduced the number of nuclei per osteoclast suggesting that N,N-dimethyl acetamide could modulate the fusion process (Fig. 5). In the presence of N,N-dimethyl acetamide (5 mM), the MNCs number per well induced by RANKL (25 ng/ml) was reduced approximately by 50%, whereas 5 mmol/L NMP totally abolished the formation of MNCs. The results of this example show, that exposure of osteoclast precursor cells to N,N-dimethyl acetamide inhibit their RANKL induced maturation to osteoclasts.

Example 4: In an oestrogen deficiency rat model N,N-dimethyl acetamide inhibits bone destruction

Osteoporosis is a major problem in our aging society and induced by reduction of estrogen in elderly women. It is associated with bone destruction and an increased fracture risk and weight gain. An established *in vivo* model for osteoporosis is the ovariectomized (OVX) rat.

Healthy female Sprague-Dawley (SD) rats (wt. 230 ± 10 g) were obtained and acclimatized to laboratory environment for 2 week before the experiment. In three independent experiments, a total of 30 animals were used. The acclimatized rats underwent either bilateral laparotomy (Sham Veh, Ntotal=10) or bilateral ovariectomy (OVX, Ntotal=20). One week after recovering from surgery, the OVX rats were divided into 2 groups: OVX with vehicle (OVX Veh, Ntotal=10) and OVX with DMA (OVX DMA, 1/3 of LD50 = 92µl/100g/week, equals an overall concentration of 10.5 mM, Ntotal=10). Treatment via intraperitoneal injection was initiated 1 week after OVX and lasted for 15 weeks. The body mass of each rat was monitored weekly, and the administered dose was adjusted accordingly.

The estradiol level in the OVX Veh group was significantly lower than in Sham Veh group (Fig. 7A). DMA was not able to rescue estradiol levels and therefore acts independent of estradiol levels. Analysis of the bone turnover marker osteocalcin revealed an increase in the OVX vehicle group compared with those in Sham vehicle group which was prevented when the OVX group was treated with DMA (Fig. 7B).

15 weeks after surgery, the femurs were dissected and the adherent tissue removed before placing the samples in 70 % ethanol and later used for bone mineral density (BMD) measurement and trabecular microarchitecture analysis. For Microcomputed Tomography (µCT) Analysis, the rat femur samples were measured with a cone-beam microCT (µCT 100, SCANCO MEDICAL AG, Brüttisellen, Switzerland). The reference point was used to define the region of interests (ROI), and the bone was automatically segmented, based on its gray scale value in the CT slices. 200 slices were evaluated for every sample (volume of interests: VOI). The three-dimensional images were reconstructed with the purpose of visualization and display. After analyzing the VOI, morphometric bone parameters, including bone volume over total volume (BV/TV) were obtained. The VOI analysis was performed blindly by the

same operator. The results reveal that compared to sham operated controls (Sham/PBS) with a BV/TV of 15.96, OVX/PBS reduced BV/TV to 6.43 and OVX with administration of DMA (OVX/DMA) inhibited bone degradation and yielded a value of 9.49, which was significantly higher than BV/TV of OVX/PBS (Fig. 7C). Furthermore additional morphometric bone parameters such as trabecular number (Tb.N), trabecular spacing (Tb.Sp) and trabecular thickness were significantly improved by DMA treatment as compared to OVX vehicle. Therefore, the degradation of the trabecular structure is reduced upon treatment with N,N-dimethyl acetamide. This is further supported by histologies as illustrated in figure 7D.

Example 5: 1-vinyl-2-pyrrolidone and N,N-dimethyl acetamide suppress LPS-induced production of NO in RAW264.7 cells as marker for macrophages.

Macrophages play a crucial role in both the specific and non-specific immune responses. Activation by inflammatory stimuli produces a variety of inflammatory mediators such as nitric oxide (NO). To show the effect of 1-vinyl-2-pyrrolidone on the suppression of inflammatory mediators like NO, RAW264.7 macrophages were used as model system. The RAW264.7 macrophage cell line was obtained from ATCC and cultured in DMEM supplemented with 10% FBS and antibiotics (100 units/ml penicillin G and 100 mg/ml streptomycin). The cultures were never allowed to become confluent. Incubations were performed at 37 °C in 5% CO_2 in humidified air.

To study the effect of 1-vinyl-2-pyrrolidone and N,N-dimethyl acetamide on NO production, RAW264.7 cells were stimulated with LPS (1µg/ml) in the absence or presence of 1-vinyl-2-pyrrolidone or N,N-dimethyl acetamide, and the accumulation of NO was measured using Griess reagent. For this purpose, cells were plated in 24-well plates and then incubated with LPS in the absence or presence of 1-vinyl-2-pyrrolidone (1, 5 and 10 mM) for 48 h. Nitrite levels in culture media were determined using the Nitrite/Nitrate colorimetric assay kit (Sigma) according to the manufacturer's instruction. After 10 min incubated at room temperature, absorbance at 540 nm was measured in a microplate reader (Synergy HT, BioTek). Fresh culture medium was used as the blank in all experiments. The amount of nitrite in the samples was quantified using the serial dilution standard curve from sodium nitrite. The results as shown in figure 8 reveal that in RAW264.7 macrophages the production of NO was increased by LPS stimulation and that treatment with 1-vinyl-2-pyrrolidone or N,N-dimethyl acetamide suppressed NO production concentration-dependently.

Since nuclear factor-κB (NF-κB) is involved in the transcriptional regulation of cytokines and targeted by bromodomain inhibitors, it was examined whether DMA could suppress the nuclear translocation of p65 which is associated with the activation of NFκB. The cellular location changes of NF-κB/p65 after LPS stimulation were examined with or without DMA pre-treatment. In unstimulated cells NF-κB/p65 was located in the cytoplasm. In LPS-treated

cells NF-κB/p65 translocated into the nucleus and pre-treatment with DMA partially suppressed the LPS-induced p65 translocation, suggesting that DMA inhibits the LPS-induced NF-κB activation. Macrophages are a major source of many cytokines involved in immune response, hematopoiesis, and other homeostatic processes. mRNA expression of IL-1α, IL-1β, IL-6 and TNF-α were markedly up-regulated after LPS stimulation, however, significantly down-regulated by DMA pretreatment (Fig. 8D).

Thus, 1-vinyl-2-pyrrolidone and N,N-dimethyl acetamide reduces the production and release of NO from LPS-stimulated RAW264.7 macrophages and is able to ameliorate inflammation as such and inflammation induced bone destruction.

Example 6: N,N-dimethyl acetamide reduces weight gain, obesity in ovariectomized rats and differentiation and maturation of adipocytes.

During the time course of the ovariectomy experiment, as described earlier, it became evident that upon treatment with N,N-dimethyl acetamide body weight gain of these animals was significantly reduced. To further unravel this effect, pre-adipocytes (3T3-L1 cells) were maintained in low glucose DMEM (Gibco) and 10% fetal calf serum. On day 3 adipogenesis was induced by addition of MDI which contains **M**ethyl iso-butyl xanthine (500 µM), **D**examethasone (0.25 µM) and **I**nsulin (10 µg/ml). Adipogenesis was quantified by oil red staining determined by absorbance at 500 nm. As shown in figure 10, 5 mM N,N-dimethyl acetamide administered with MDI inhibited the maturation of pre-adipocytes to adipocytes.

Example 7: N,N-dimethyl acetamide and 1-vinyl-2-pyrrolidone enhance bone regeneration in vivo

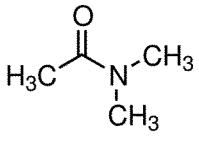
To investigate the effect of N,N-dimethyl acetamide and 1-vinyl-2-pyrrolidone on bone formation and bone regeneration a guided bone regeneration model was employed and N,N-dimethyl acetamide and 1-vinyl-2-pyrrolidone were applied via a biodegradable membrane. Significantly more bone formed in this non-critical size defects when the membrane were loaded with N,N-dimethyl acetamide or 1-vinyl-2-pyrrolidone, compared to membrane alone (Fig. 11). Therefore N,N-dimethyl acetamide and 1-vinyl-2-pyrrolidone enhance bone regeneration and repair of bony defects.

Bone regeneration was tested in a guided bone regeneration model at the calvarial bone of 6 rabbits (female, 26 week old, New Zealand white rabbit) as described in Karfeld-Sulzer et al., Journal of tissue engineering and regenerative medicine, 2014. DMA loading of the membrane was performed by vapor deposition to yield 10% weight increase. Sample size was determined by power analysis.

Claims

1. A compound for use in a method for prevention or treatment of a disease selected from pathological weight gain, obesity, osteoporosis, osteoarthritis, periodontitis and peri-implantitis, wherein said compound is selected from the group comprising:

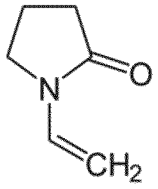
   a) N,N-dimethyl acetamide (I)

   (I),

   and

   b) 1-vinyl-2-pyrrolidone (II)

   (II).

2. A bromodomain inhibitor for use in prevention or treatment of pathological weight gain, obesity, osteoporosis, osteoarthritis, periodontitis and/or peri-implantitis selected from N,N-dimethyl acetamide or 1-vinyl-2-pyrrolidone.

3. A pharmaceutical composition comprising a compound selected from N,N-dimethyl acetamide and/or 1-vinyl-2-pyrrolidone for use in prevention or treatment of pathological weight gain, obesity, osteoporosis, osteoarthritis, periodontitis and/or peri-implantitis.

4. A contraceptive for a male mammal comprising a compound selected from N,N-dimethyl acetamide or 1-vinyl-2-pyrrolidone.

Fig. 1 A + B

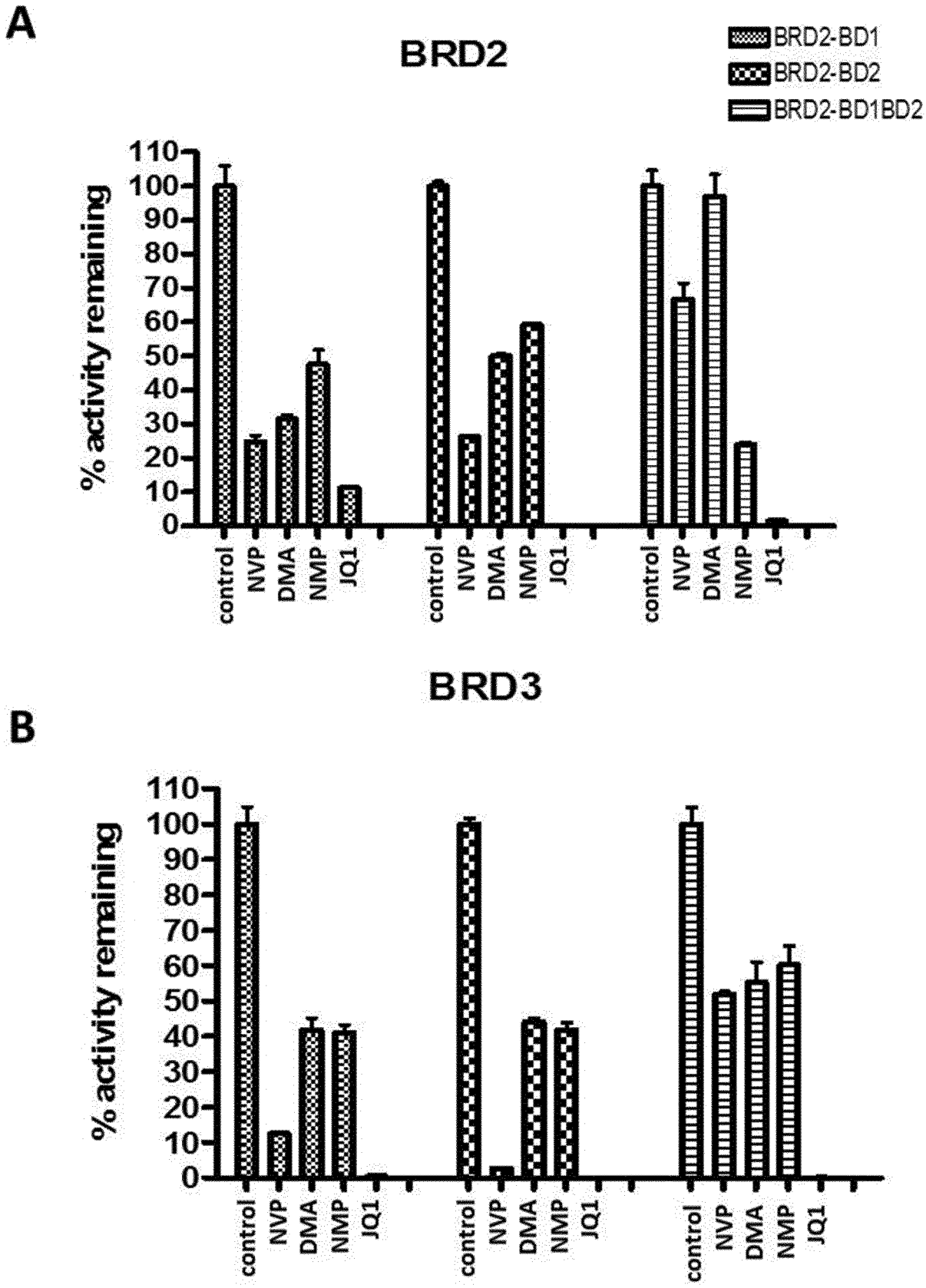

A
BRD2
BRD2-BD1
BRD2-BD2
BRD2-BD1BD2
% activity remaining
110
100
90
80
70
60
50
40
30
20
10
0
control
NVP
DMA
NMP
JQ1
B
BRD3
% activity remaining
110
100
90
80
70
60
50
40
30
20
10
0
control
NVP
DMA
NMP
JQ1

Fig. 1 C + D.

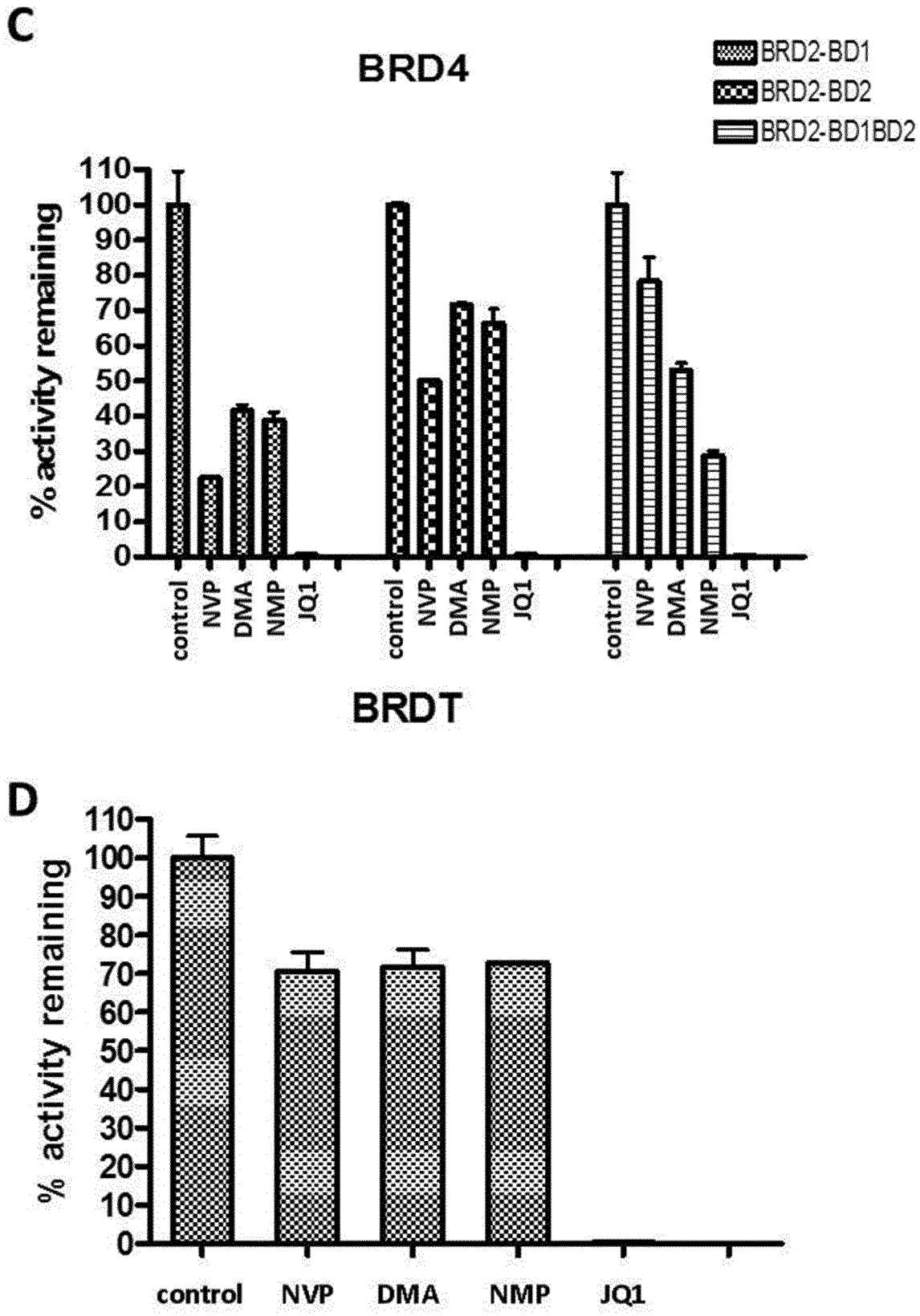

C
BRD4
BRD2-BD1
BRD2-BD2
BRD2-BD1BD2
% activity remaining
110
100
90
80
70
60
50
40
30
20
10
0
control
NVP
DMA
NMP
JQ1
BRDT
D
% activity remaining
control
NVP
DMA
NMP
JQ1

Fig. 1 E + F

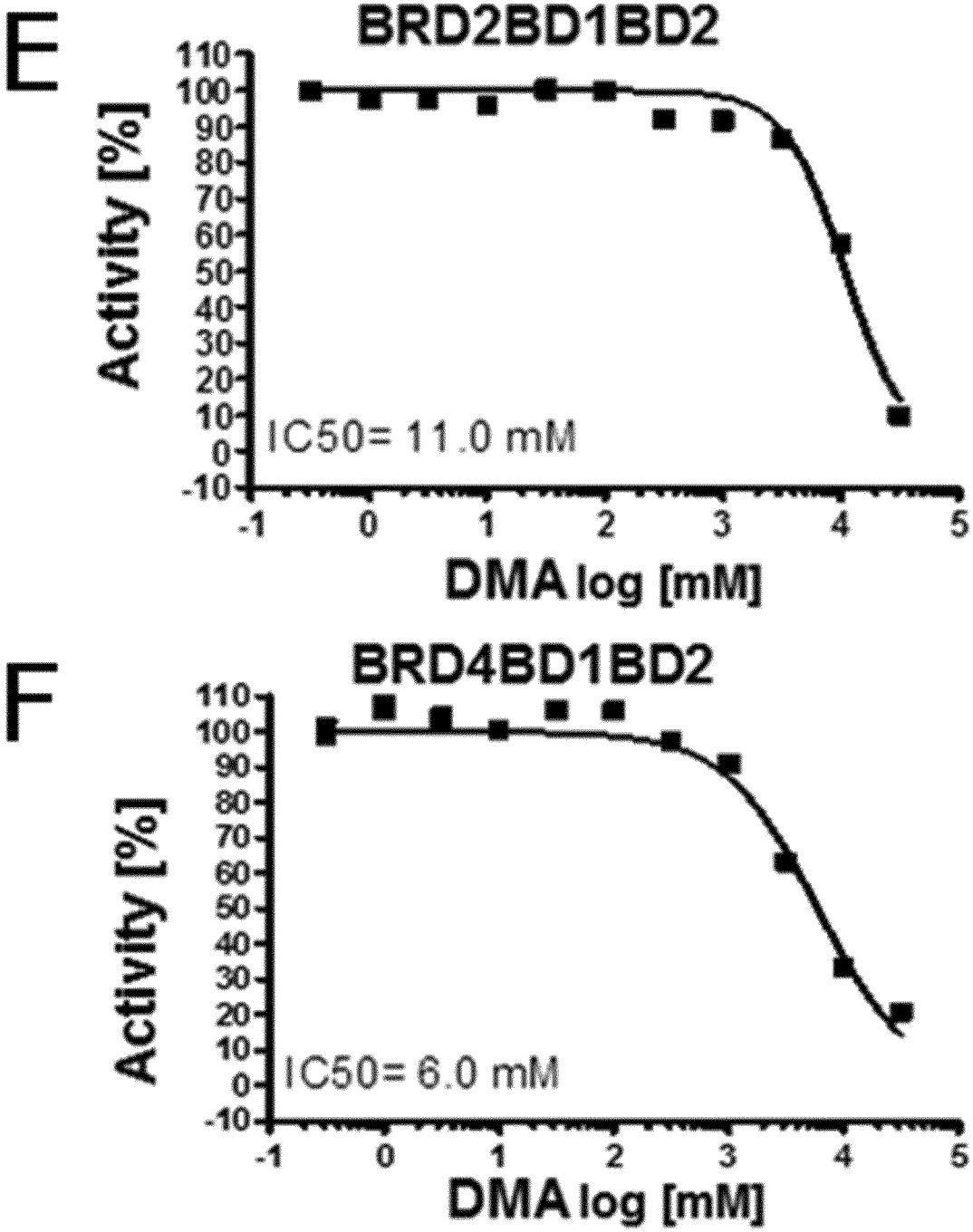

E
BRD2BD1BD2
Activity [%]
110
100
90
80
70
60
50
40
30
20
10
0
-10
IC50= 11.0 mM
-1
0
1
2
3
4
5
DMA log [mM]
F
BRD4BD1BD2
Activity [%]
IC50= 6.0 mM
DMA log [mM]

Fig. 2

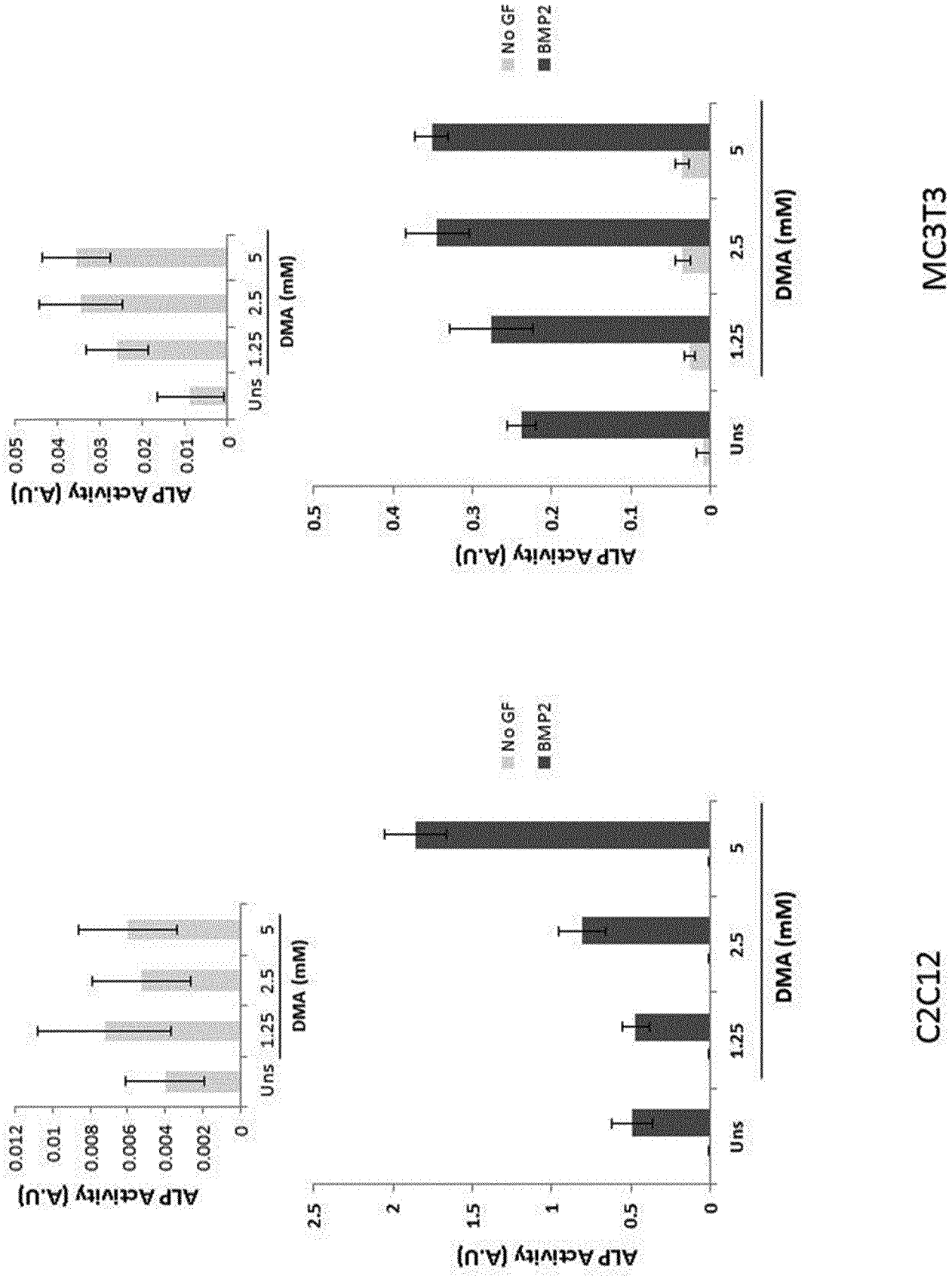

MC3T3
C2C12
No GF
BMP2
ALP Activity (A.U)
DMA (mM)
Uns
1.25
2.5
5
0
0.01
0.02
0.03
0.04
0.05
0.1
0.2
0.3
0.4
0.5
0.002
0.004
0.006
0.008
0.012
0.5
1
1.5
2
2.5

Fig. 3

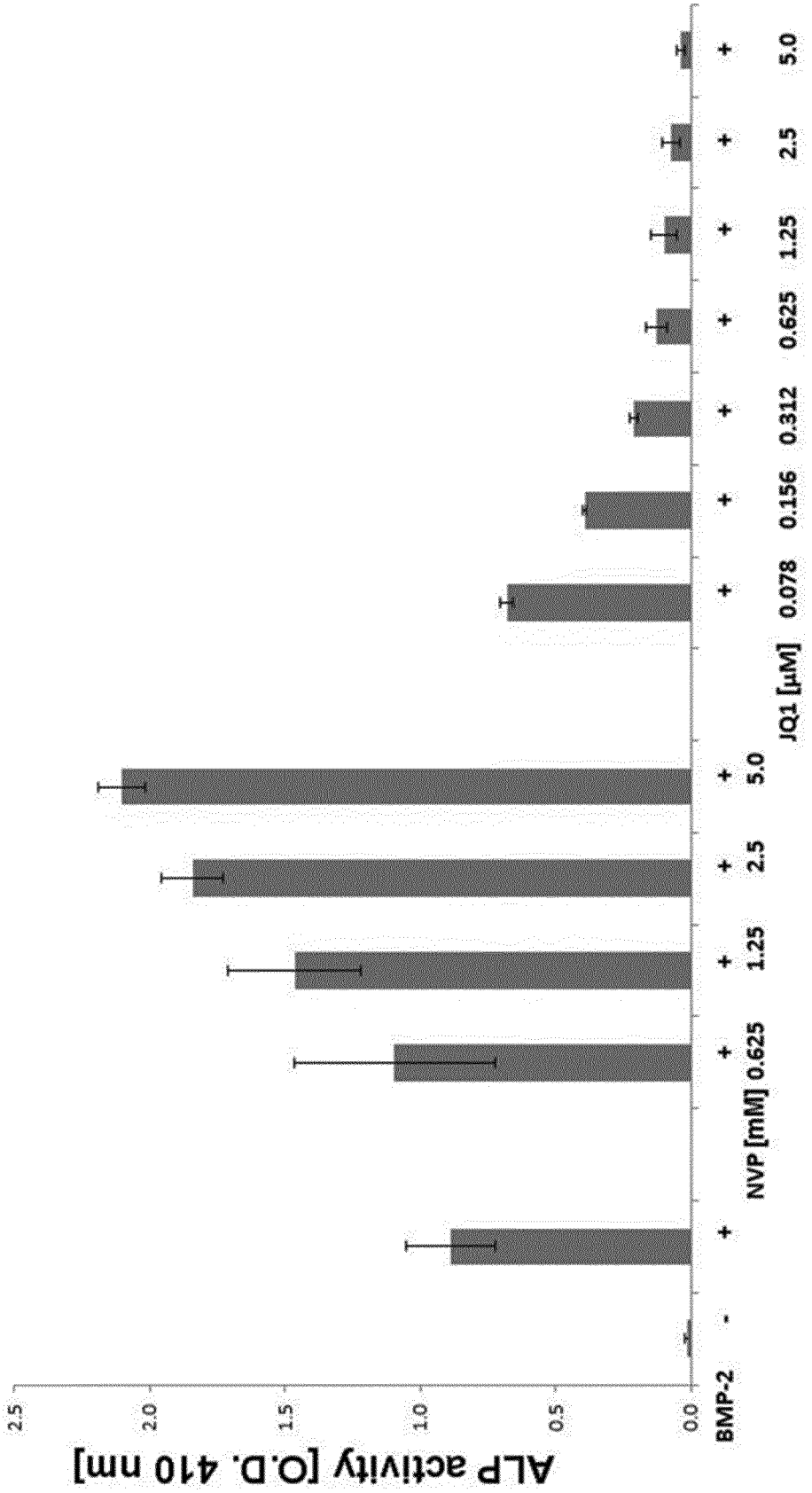

ALP activity [O.D. 410 nm]
2.5
2.0
1.5
1.0
0.5
0.0
BMP-2
-
+
+
+
+
+
+
+
+
+
+
NVP [mM]
0.625
1.25
2.5
5.0
JQ1 [µM]
0.078
0.156
0.312
0.625
1.25
2.5
5.0

Fig. 4

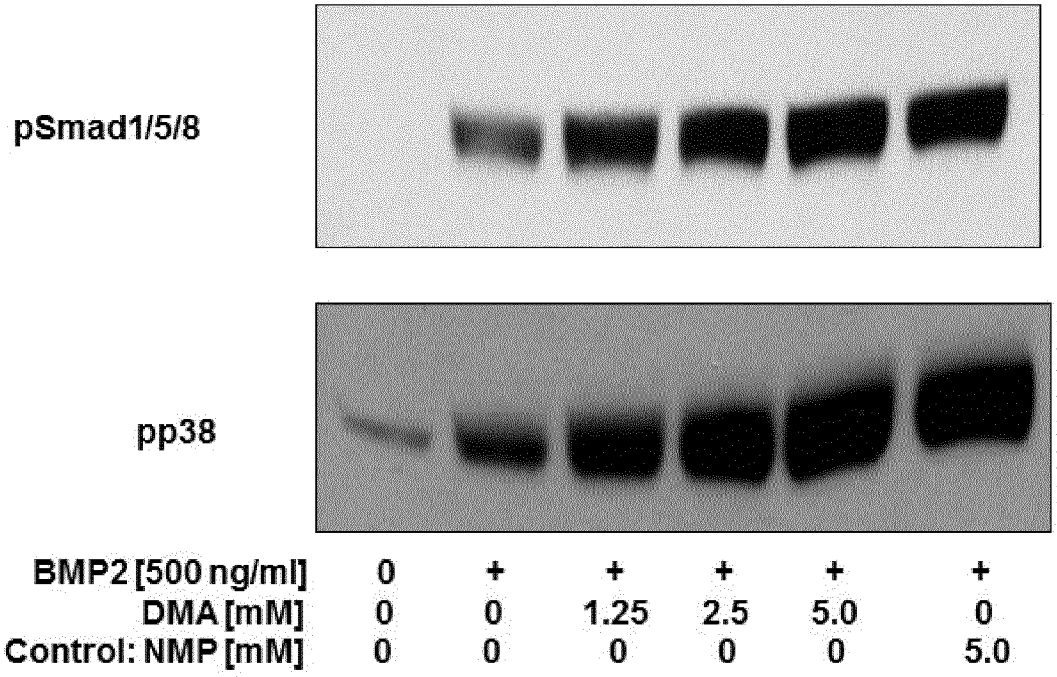

pSmad1/5/8
pp38
BMP2 [500 ng/ml] 0 + + + + +
DMA [mM] 0 0 1.25 2.5 5.0 0
Control: NMP [mM] 0 0 0 0 0 5.0

Fig. 5

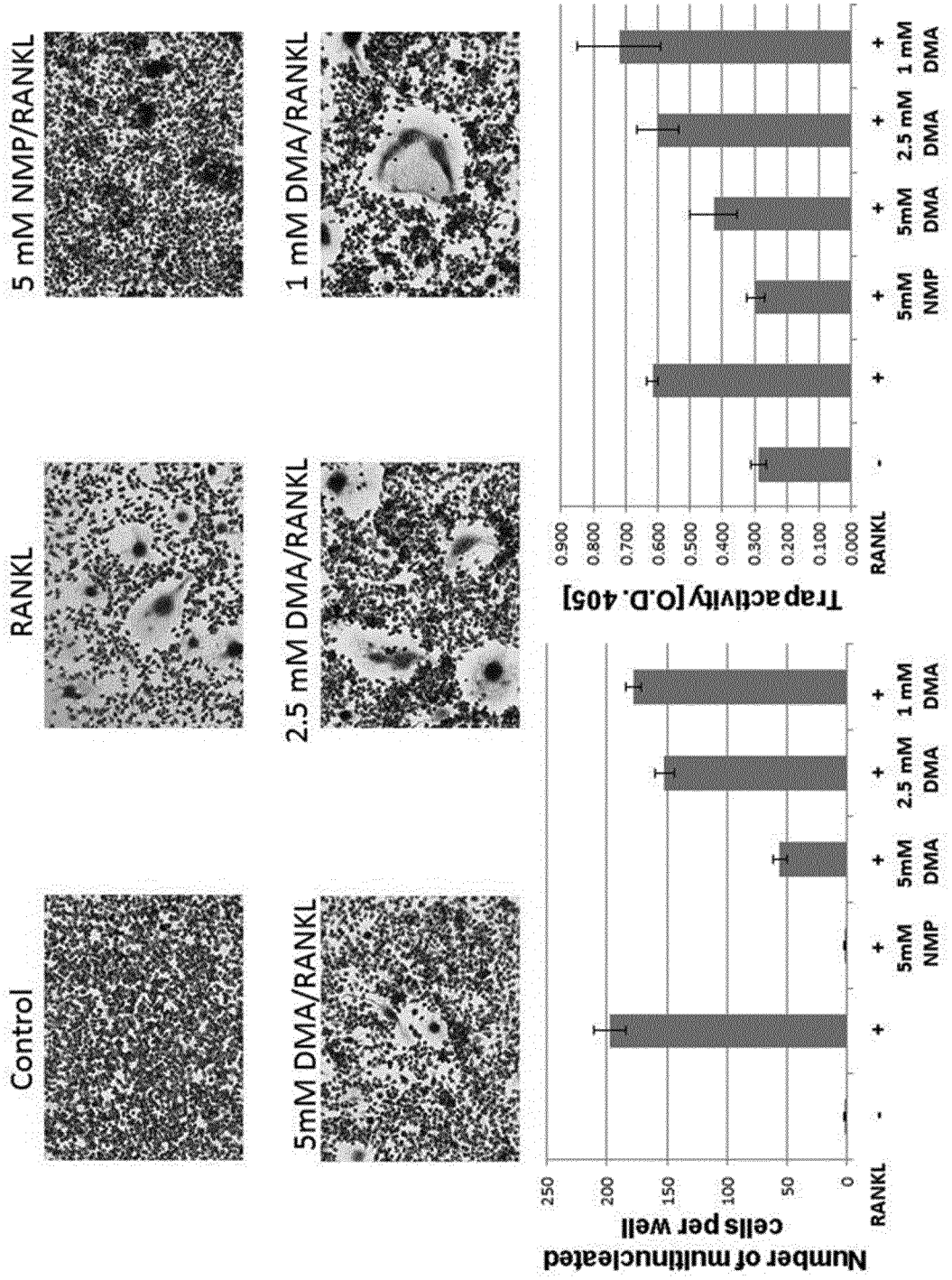

Control
RANKL
5mM DMA/RANKL
2.5 mM DMA/RANKL
5 mM NMP/RANKL
1 mM DMA/RANKL
Number of multinucleated cells per well
250
200
150
100
50
0
RANKL - + + + + +
5mM NMP + + + +
5mM DMA + + + +
2.5 mM DMA + + + +
1 mM DMA + + + +
Trap activity [O.D. 405]
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
RANKL - + + + + +
5mM NMP + + + +
5mM DMA + + + +
2.5 mM DMA + + + +
1 mM DMA + + + +

Fig. 6

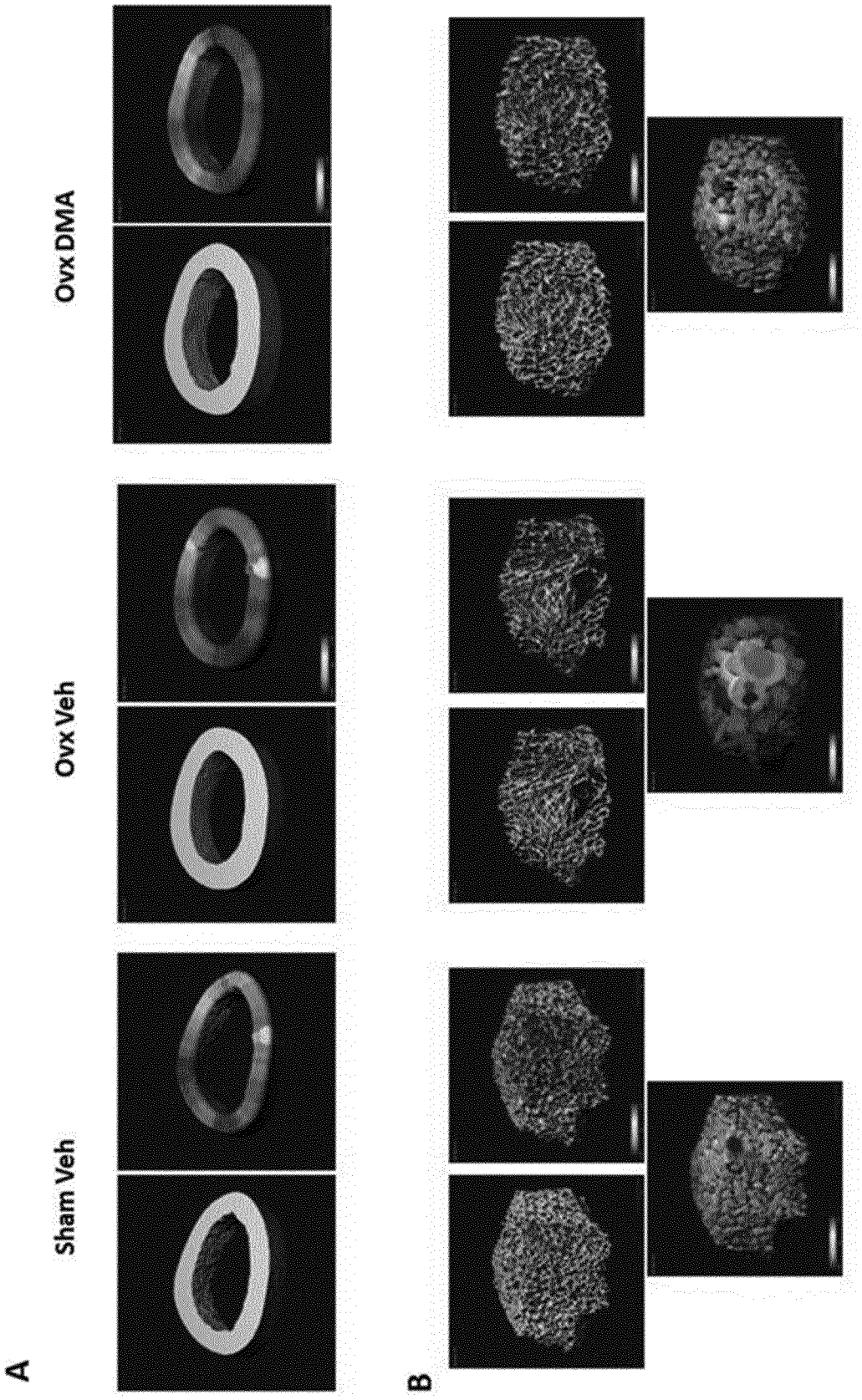


Fig. 7 A + B + C

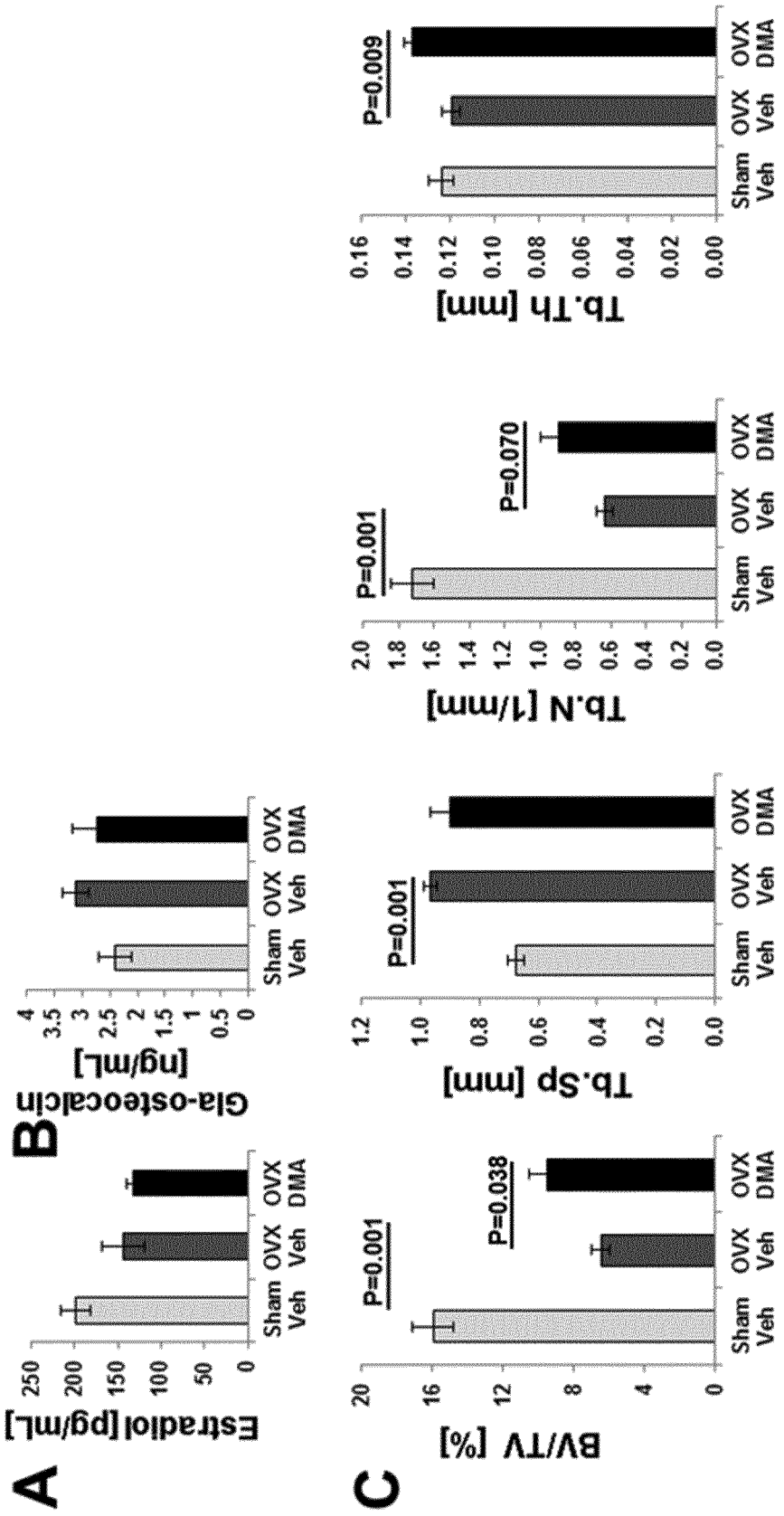

A
Estradiol [pg/mL]
250
200
150
100
50
0
Sham Veh
OVX Veh
OVX DMA
B
Gla-osteocalcin [ng/mL]
4
3.5
3
2.5
2
1.5
1
0.5
0
C
BV/TV [%]
20
16
12
8
4
0
P=0.001
P=0.038
Tb.Sp [mm]
1.2
1.0
0.8
0.6
0.4
0.2
0.0
P=0.001
P=0.070
Tb.N [1/mm]
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
P=0.001
P=0.009
Tb.Th [mm]
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00

Fig. 7 D

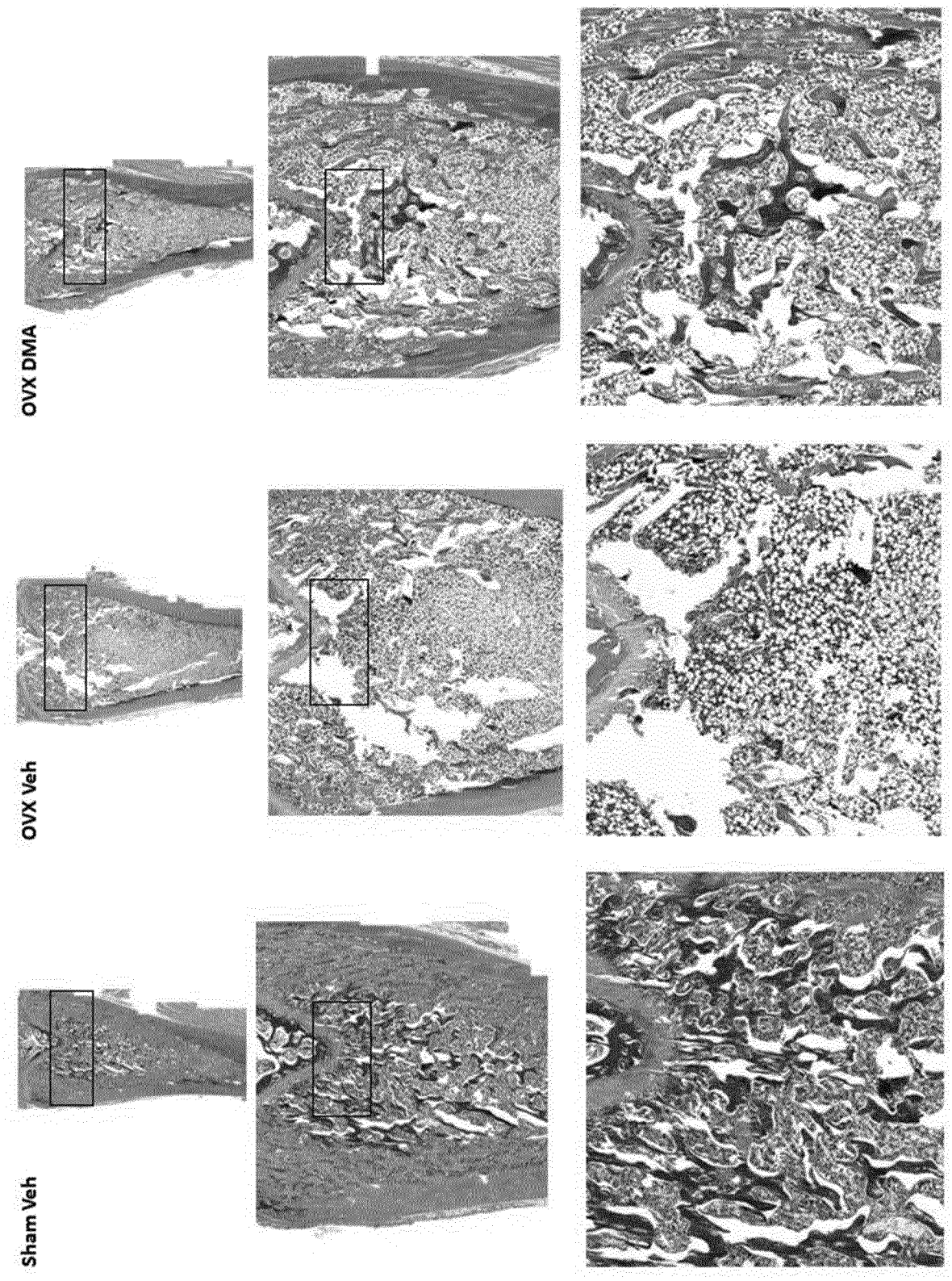


Fig. 8

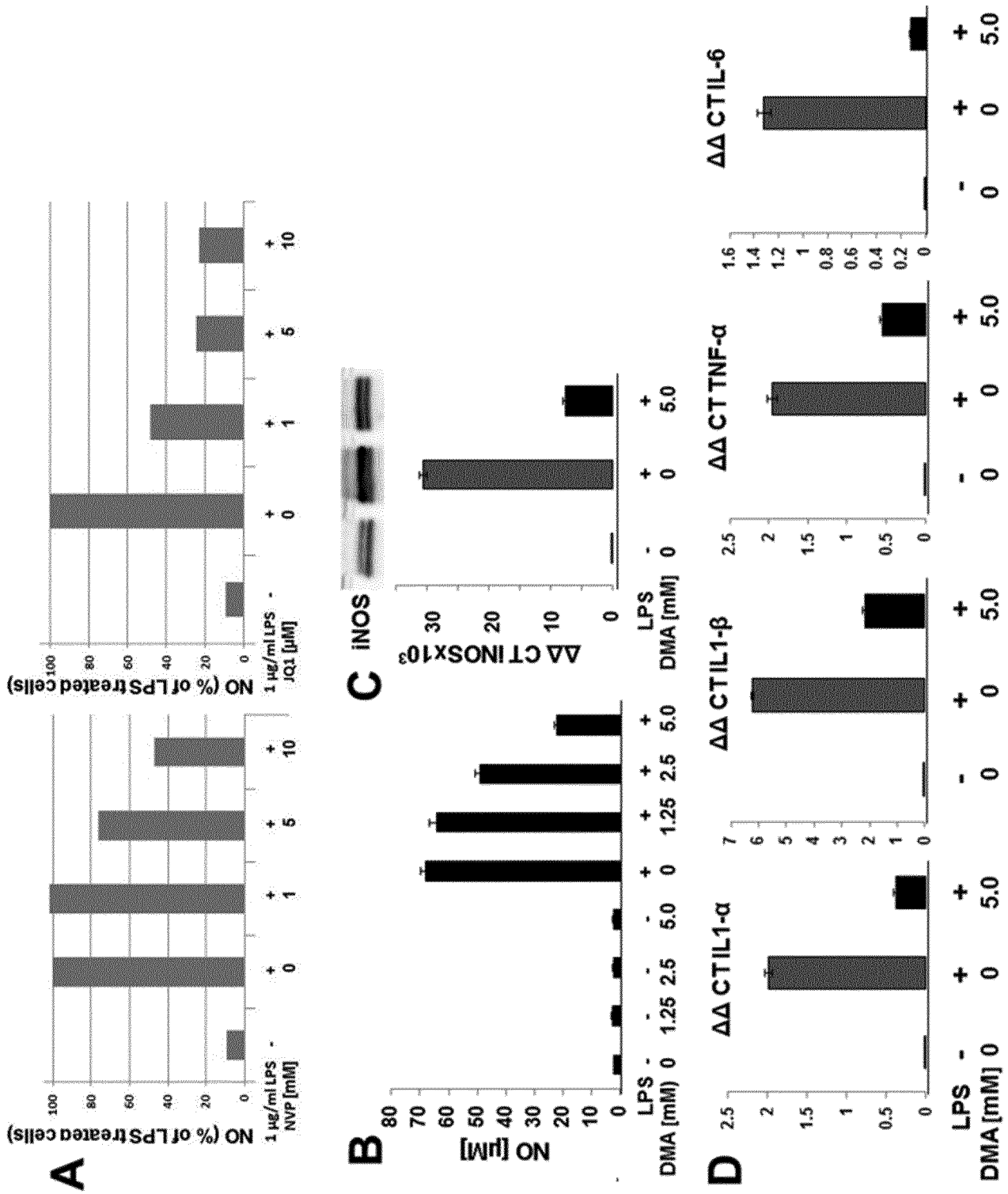

A
NO (% of LPS treated cells)
1 µg/ml LPS
NVP [mM]
JQ1 [µM]
B
NO [µM]
LPS
DMA [mM]
C
iNOS
ΔΔ CT INOSx10³
D
ΔΔ CT IL1-α
ΔΔ CT IL1-β
ΔΔ CT TNF-α
ΔΔ CT IL-6

Fig. 9

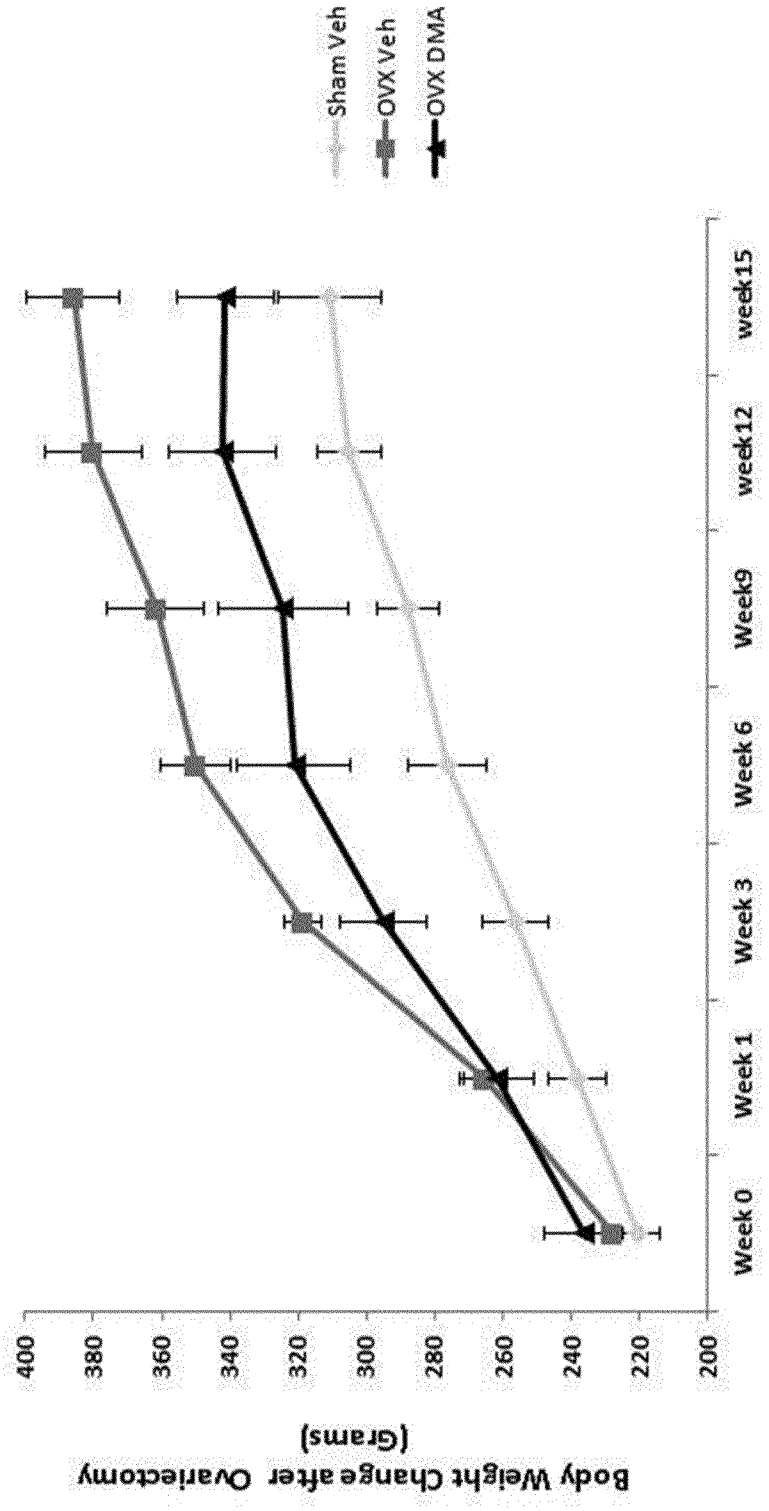

Body Weight Change after Ovariectomy
(Grams)
400
380
360
340
320
300
280
260
240
220
200
Week 0
Week 1
Week 3
Week 6
Week9
week12
week15
Sham Veh
OVX Veh
OVX DMA

Fig. 10

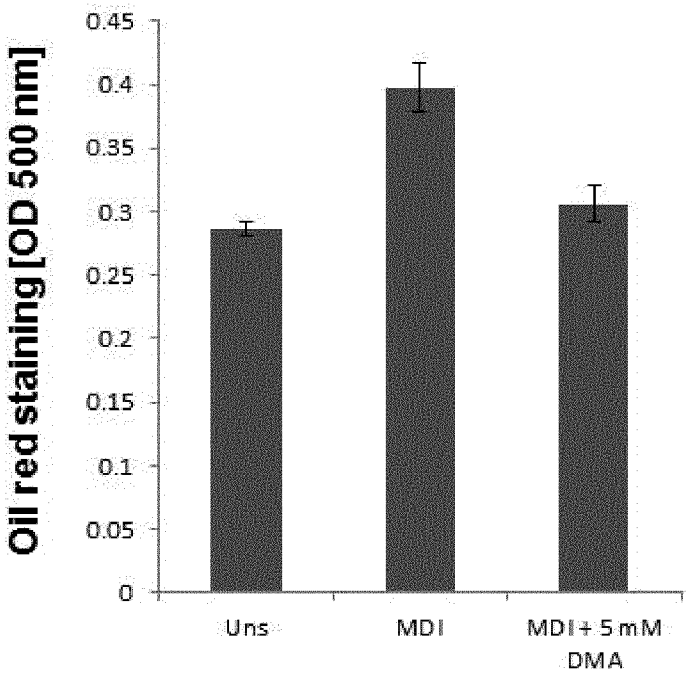
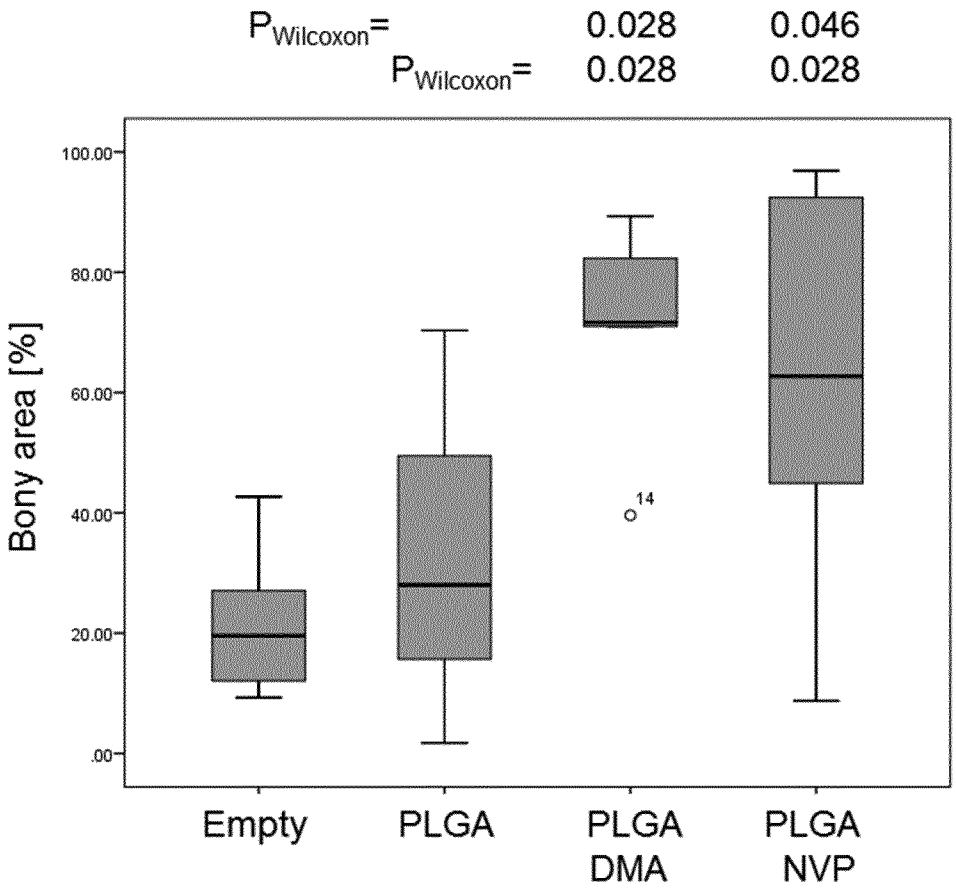

Oil red staining [OD 500 nm]
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Uns
MDI
MDI + 5 mM DMA

Fig. 11


$P_{Wilcoxon}$= 0.028 0.046
$P_{Wilcoxon}$= 0.028 0.028
Bony area [%]
100.00
80.00
60.00
40.00
20.00
.00
14
Empty
PLGA
PLGA DMA
PLGA NVP

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2016/064388

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/064388

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/16 A61K31/4015 A61K45/06 A61P3/04 A61P1/02
A61P19/02 A61P19/10 A61P15/16
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2014/005272 A1 (REZNIK SANDRA EVE [US]) 2 January 2014 (2014-01-02) abstract; claims 1,6,13, -----	1-4
X	WO 2008/099190 A2 (INION LTD [GB]; CHOPRA BIKRAMJIT [GB]; MCKENZIE GRAHAME [GB]; HARDWICK) 21 August 2008 (2008-08-21) page 18, last paragraph; claims 1, 2 -----	1-4

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 July 2016	26/07/2016

Name and mailing address of the ISA/	Authorized officer
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2016/064388

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2014005272 A1	02-01-2014	NONE	
WO 2008099190 A2	21-08-2008	GB 2446653 A	20-08-2008
		WO 2008099190 A2	21-08-2008

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows: