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(71) Applicants: **INSERM (INSTITUT NATIONAL DE LA SANTÉ ET DE LA RECHERCHE MÉDICALE)** [FR/FR]; 101, rue de Tolbiac, 75013 Paris (FR). **UNIVERSITÉ DE VERSAILLES SAINT-QUENTIN-EN-YVELINES** [FR/FR]; 55 avenue de Paris, 78000 Versailles (FR). **ASSISTANCE PUBLIQUE-HÔPITAUX DE PARIS (APHP)** [FR/FR]; 3, avenue Victoria, 75004 Paris (FR).

(72) Inventors: **BONAY, Marcel**; UFR Sciences de la Santé Simone Veil - U1179 2 avenue de la Source de la Bièvre, 78180 Montigny-le-bretonneux (FR). **DERAMAUDT, Thérèse**; Ufr Sciences De La Sante Simone Veil - U1179 2 Av De La Source De La Bievre, 78180 Montigny-le-bretonneux (FR).

(74) Agent: **COLLIN, Matthieu**; 7 rue Watt, 75013 Paris (FR).

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(54) Title: NRF2 ACTIVATORS FOR THE TREATMENT OF MYCOBACTERIAL INFECTIONS

(57) Abstract: The present invention relates to methods and pharmaceutical compositions for the treatment of mycobacterial infections. In particular, the present invention relates to a method of treating a mycobacterial infection in a subject in need thereof comprising administering to the subject a therapeutically effective amount of an Nrf2 activator.

NRF2 ACTIVATORS FOR THE TREATMENT OF MYCOBACTERIAL INFECTIONS

FIELD OF THE INVENTION:

The present invention relates to methods and pharmaceutical compositions for the treatment of mycobacterial infections.

BACKGROUND OF THE INVENTION:

Mycobacteria included in the group of rapid growing mycobacteria (RGM) are increasingly becoming a public health concern worldwide. RGM have proven to be a great challenge to eradicate since these organisms present a high tolerance to antibiotics, which greatly limits treatments, and are highly prevalent in immunosuppressed patients. *Mycobacterium abscessus* (Mabs), a nontuberculous mycobacterium of the RGM group, is an opportunistic pathogen found in the environment. *M. abscessus* is an emerging pathogen that has been increasingly involved in patients with cystic fibrosis and in immunosuppressed patients (1-3), and more generally in exacerbation of lung infections. Association of *M. abscessus* with patients suffering from a pre-existing condition and at risk of developing chronic airway infections, makes for a poor clinical outcome. Alike *M. tuberculosis*, *M. abscessus* survives in phagocytic cells and uses the host immune cells as a reservoir for proliferation by blocking the maturation of phagosomes into phagolysosomes. Recent in vitro studies on THP-1-derived macrophages showed that *M. abscessus* appears to thrive in oxidative environment. The bacterial growth is enhanced in oxidative condition such as presence of oxygen free radicals, while its growth is inhibited in the presence of oxidant scavengers such as MnTE-2-PyP and N-acetyl-L-cysteine (4). MnTE-2-PyP is able to diminish *M. abscessus* load in infected macrophages by inducing the fusion of mycobacteria-containing phagosomes with lysosomes into phagolysosomes, thus promoting cell survival (5). This imbalance between oxidants and antioxidants in the infected host cell activates the antioxidant signaling pathway controlled by the transcriptional factor Nuclear factor E2-related factor 2 (Nrf2). Nrf2 is a key regulator in adaptive responses to oxidative stress by inducing the transcription of antioxidant and cytoprotective genes (6). In normal physiological conditions, NRF2 is sequestered in the cytoplasm by its negative regulator Kelch-like ECH via the Keap1-Cullin-3 based E3 ligase complex (7). Upon oxidative stress, infection or chemical stimulation, Nrf2 is released from Keap-1, and translocates to the nucleus, where it

heterodimerizes with transcription factors including Maf, c-Jun, c-Fos, and members of the AP-1 family (8). The cofactor complex binds specifically to the antioxidant responsive element (ARE) sequences found in a wide range of antioxidant genes coding for antioxidant enzymes such as NADPH quinone oxidoreductase-1, epoxide hydrolase-1, HO-1, UDP-glucuronyl transferase, glutathione-S-transferases(9, 10). Previous studies have shown that *M. tuberculosis* escapes mycobactericidal killing in infected host cells by eliciting necrosis cell death (11). Activation of this energy-independent cell death by *M. tuberculosis* allows the release of mycobacteria and subsequent infection of neighboring phagocytes. An important defense mechanism utilized by the innate immune system is the triggering of the programmed cell death, also known as cell apoptosis, to reduce the viability of pathogens. Cell apoptosis is an energy-dependent process and presents bactericidal properties. Apoptotic bodies issued from infected apoptotic macrophages maintain plasma membrane integrity and as such antigen presentation, which facilitate T-cell response and induce direct mycobacterial killing by uninfected neighboring macrophages (12).

SUMMARY OF THE INVENTION:

The present invention relates to methods and pharmaceutical compositions for the treatment of mycobacterial infections. In particular, the present invention is defined by the claims.

DETAILED DESCRIPTION OF THE INVENTION:

Mycobacterium abscessus (Mabs), a non-tuberculous mycobacterium, is an emerging and rapidly growing opportunistic pathogen that is frequently found in patients with cystic fibrosis and in immunosuppressed patients. Its high tolerance to antibiotics is of great concern for public health and new strategies to enhance bactericidal effects against Mabs are highly expected. In the present disclosure, the inventors showed that human THP-1-derived macrophages infected with *M. abscessus* presented an increase in ROS production and cell necrosis. In addition, *M. abscessus* infection triggered activation of the Nuclear factor E2-related factor 2 (Nrf2) signaling pathway, and the induction of HO-1 and NQO1 expression levels. Interestingly, pretreatment of macrophages with sulforaphane (SFN), an activator of the antioxidant key regulator Nrf2, followed by *M. abscessus* infection significantly decreased mycobacterial burden. We demonstrated that this reduction in mycobacterial growth was due to an activation in cell apoptosis in SFN pretreated and *M. abscessus* infected macrophages. Pretreatment with specific MAPK inhibitors, PD98059, SP600125, and SB203580 to ERK,

JNK, and p38 respectively, failed to inhibit induction of Nrf2 expression, suggesting Nrf2 signaling pathway was upstream of MAPK signaling. Activation of cell apoptosis was caspase 3/7-independent but p38 MAPK-dependent. Moreover, p38 MAPK induction was abolished in macrophages transfected with Nrf2 siRNA. In addition, p38 inhibitor abolished Nrf2-dependent apoptosis in infected macrophages. Taken together, our results indicate that modulation of the Nrf2 signaling using Nrf2 activators may help potentiate the actual drug therapies used to treat mycobacterial infection.

Accordingly, one object of the present invention relates to a method of treating a mycobacterial infection in a subject in need thereof comprising administering to the subject a therapeutically effective amount of an Nrf2 activator.

As used herein, the term "subject" refers to a human or another mammal (e.g., mouse, rat, rabbit, hamster, dog, cat, cattle, swine, sheep, horse or primate). In some embodiments, the subject is a human being. The term “subject” does not denote a particular age, and thus encompass adults, children and newborns.

In some embodiments, the subject is immunocompromised. An immunocompromised subject is a subject who is incapable of developing or unlikely to develop a robust immune response, usually as a result of disease, malnutrition, or immunosuppressive therapy. An immunocompromised immune system is an immune system that is functioning below normal. Immunocompromised subjects are more susceptible to mycobacterial infections. Those who can be considered to be immunocompromised include, but are not limited to, subjects with AIDS (or HIV positive), subjects with severe combined immune deficiency (SCID), diabetics, subjects who have had transplants and who are taking immunosuppressives, and those who are receiving chemotherapy for cancer. Immunocompromised individuals also includes subjects with most forms of cancer (other than skin cancer), sickle cell anemia, cystic fibrosis, those who do not have a spleen, subjects with end stage kidney disease (dialysis), and those who have been taking corticosteroids on a frequent basis by pill or injection within the last year. Subjects with severe liver, lung, heart disease, or neurological and muscular disabilities also may be immunocompromised.

As used herein, the term "treatment" or "treat" refer to both prophylactic or preventive treatment as well as curative or disease modifying treatment, including treatment of subjects

at risk of contracting the disease or suspected to have contracted the disease as well as subjects who are ill or have been diagnosed as suffering from a disease or medical condition, and includes suppression of clinical relapse. The treatment may be administered to a subject having a medical disorder or who ultimately may acquire the disorder, in order to prevent, cure, delay the onset of, reduce the severity of, or ameliorate one or more symptoms of a disorder or recurring disorder, or in order to prolong the survival of a subject beyond that expected in the absence of such treatment. By "therapeutic regimen" is meant the pattern of treatment of an illness, e.g., the pattern of dosing used during therapy. A therapeutic regimen may include an induction regimen and a maintenance regimen. The phrase "induction regimen" or "induction period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the initial treatment of a disease. The general goal of an induction regimen is to provide a high level of drug to a subject during the initial period of a treatment regimen. An induction regimen may employ (in part or in whole) a "loading regimen", which may include administering a greater dose of the drug than a physician would employ during a maintenance regimen, administering a drug more frequently than a physician would administer the drug during a maintenance regimen, or both. The phrase "maintenance regimen" or "maintenance period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the maintenance of a subject during treatment of an illness, e.g., to keep the subject in remission for long periods of time (months or years). A maintenance regimen may employ continuous therapy (e.g., administering a drug at a regular intervals, e.g., weekly, monthly, yearly, etc.) or intermittent therapy (e.g., interrupted treatment, intermittent treatment, treatment at relapse, or treatment upon achievement of a particular predetermined criteria [e.g., disease manifestation, etc.]).

As used herein, the term "mycobacterial infection" has its general meaning in the art and refers to an infection caused or mediated by mycobacteria. The term "mycobacterial infection" is associated with mycobacterial infections caused by *Mycobacterium tuberculosis* or a nontuberculosis mycobacterium and in particular an infection caused or mediated by a resistant or highly virulent strain of a mycobacterium. Examples of mycobacteria other than *Mycobacterium tuberculosis*, include *Mycobacterium avium*, *Mycobacterium kansasii, Mycobacteriumfortuitum, Mycobacterium chelonae, Mycobacterium leprae, Mycobacterium avium subspecies paratuberculosis, Mycobacterium intracellular, Mycobacterium scrofulaceum, Mycobacterium xenopi, Mycobacterium abscessus, Mycobacterium marinum,* and *Mycobacterium ulcerans*. Diagnosis of mycobacterial infection is commonly achieved

using a skin test, which e.g. involves intradermal exposure to tuberculin PPD (protein-purified derivative); wherein a measurable induration at the injection site by 48-72 hours after injection indicates exposure to mycobacterial antigens. Alternatively, blood tests may be used to confirm or rule out latent or active tuberculosis. These tests measure how the immune system reacts to tuberculosis bacteria by interferon γ release assays. Quantiferon*-TB Gold in -tube test and T-Spot.TB test* are examples of TB blood tests. Diagnosis of mycobacterial infection can also be achieved using one or more additional methods known in the art including, but not limited to, body fluid (sputum, gastric washings, laryngeal swab, bronchoalveolar lavage, bronchial washings) smears and cultures for acid-fast bacilli, and polymerase chain reaction or gene probe tests for detecting the mycobacteria. In some embodiments, the mycobacterial infection may be inactive reactivated, or active. In some embodiments, the mycobacterial infection is caused by a multi-drug resistant strain.

The terms "Nrf2 activator" and "Nuclear factor (erythroid-derived 2)-like 2 activator" as used herein relate to chemical compounds or elements that increase the activity of Nrf2. The term "activity of Nrf2" as used herein relates to the activity of Nrf2 and in particular activation in cell apoptosis. Nrf2 activators are typically classified based on their chemical structures: Diphenols, Michael reaction acceptors, isothiocyanates, thiocarbamates, trivalent arsenicals, 1,2-dithiole-3-thiones, hydroperoxides, vicinal dimercaptans, heavy metals, and polyenes.

In some embodiments, the Nrf2 activator of the present invention is selected from the group consisting of Chalcone derivatives as disclosed in J. Med. Chem., 2011, 54 (12), pp 4147-4159, such as 2-trifluoromethyl-2′ -methoxychalcone, auranofin, ebselen, 1,2-naphthoquinone, cynnamic aldehyde, caffeic acid and its esters, curcumin, reservatrol, artesunate, tert-butylhydroquinone, and -quinone, (tBHQ, tBQ), vitamins K1, K2 and K3, preferably menadione, fumaric acid esters, i.e. fumaric acid mono- and/or diester which is preferably selected from the group of monoalkyl hydrogen fumarate and dialkyl fumarate, such as monomethyl hydrogen fumarate, dimethyl fumarate, monoethyl hydrogen fumarate, and diethyl fumarate, 2-cyclopentenones, ethacrynic acid and its alkyl esters, bardoxolone methyl (methyl 2-cyano-3,12-dioxooleana-1,9(11)dien-28-oate) (CDDO-Me, RTA 402), ethyl 2-cyano-3,12-dioxooleana-1,9(11)dien-28-oate, 2-cyano-3,12-dioxooleana-1,9(11)dien-28-oic acid (CDDO), 1 [2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im), (2-cyano-N-methyl-3,12-dioxooleana-1,9(11)-dien-28 amide (CDDO-methyl amide, CDDO-

MA), isothiocyanate such as sulforaphane, 1,2-dithiole-3-thione such as oltipraz, 3,5-di-tert-butyl-4-hydroxytoluene, 3-hydroxycoumarin, 4-hydroxynonenal, 4-oxononenal, malondialdehyde, (E)-2-hexenal, capsaicin, allicin, allylisothiocyanate, 6-methylthiohexyl isothiocyanate, 7-methylthioheptyl isothiocyanate, sulforaphane, 8-methylthiooctyl isothiocyanate, corticosteroids, such as dexamethasone, 8-iso prostaglandin A2, alkyl pyruvate, such as methyl and ethyl pyruvate, diethyl or dimethyl oxaloproprionate, 2-acetamidoacrylate, methyl or ethyl-2-acetamidoacrylate, hypoestoxide, parthenolide, eriodictyol, 4-Hydroxy-2-nonenal, 4-oxo-2nonenal, geranial, zerumbone, aurone, isoliquiritigenin, xanthohumol, [10]-Shogaol, eugenol, 1′ -acetoxychavicol acetate, allyl isothiocyanate, benzyl isothiocyanate, phenethyl isothiocyanate, 4-(Methylthio)-3-butenyl isothiocyanate and 6-Methylsulfinylhexyl isothiocyanate, ferulic acid and its esters, such as ferulic acid ethyl ester, and ferulic acid methyl ester, sofalcone, 4-methyl daphnetin, imperatorin, auraptene, poncimarin, bis[2-hydroxybenzylidene]acetones, alicylcurcuminoid, 4-bromo flavone, β-naphthoflavone, sappanone A, aurones and its corresponding indole derivatives such as benzylidene-indolin-2-ones, perillaldehyde, quercetin, fisetin, koparin, genistein, tanshinone HA, BHA, BHT, PMX-290, AL-1, avicin D, gedunin, fisetin, andrographolide, tricyclic bis(cyano enone) TBE-31 [(±)-(4bS,8aR,10aS)-10a-ethynyl-4-b,8,8-trimethyl-3,7-dioxo-3,4-b,7,8,8a,9,10,10a-octahydrophenanthrene-2,6-dicarbonitrile], MCE-1, MCE5, TP-225, ADT as referred to in in Medicinal Research Reviews, 32, No. 4, 687-726, 2012, and the respective quinone or hydroquinone forms of the aforementioned quinone and hydroquinone derivatives and stereoisomers, tautomers or pharmacologically active derivatives of the aforementioned agents.

In some embodiments, the Nrf2 activator of the present invention is selected from the group consisting of fumaric acid derivatives (Joshi and Strebel, WO 2002/055063, US 2006/0205659, and U.S. Pat. No. 7,157,423 (amide compounds and protein-fumarate conjugates); Joshi et al., WO 2002/055066 and Joshi and Strebel, U.S. Pat. No. 6,355,676 (mono and dialkyl esters); Joshi and Strebel, WO 2003/087174 (carbocyclic and oxacarbocylic compounds); Joshi et al., WO 2006/122652 (thiosuccinates); Joshi et al., US 2008/0233185 (dialkyl and diaryl esters) and salts (Nilsson et al., US 2008/0004344) Controlled release pharmaceutical compositions comprising fumaric acid esters are also disclosed by Nilsson and Willer, WO 2007/042034. Prodrugs are described by Nielsen and Bundgaard, J Pharm Sci 1988, 77(4), 285-298 and in WO2010/022177.

Additional examples of Nrf2 activators can be found in US2011/0250300, US 2004/0002463, US 20130172391, US20140275205, WO2014100728 the disclosures of each of which are hereby incorporated by reference herein.

By a "therapeutically effective amount" is meant a sufficient amount of the Nrf2 activator of the present invention for treating or reducing the symptoms at reasonable benefit/risk ratio applicable to any medical treatment. It will be understood that the total daily usage of the compounds and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular subject will depend upon a variety of factors including the disorder being treated and the severity of the disorder; activity of the specific compound employed; the specific composition employed, the age, body weight, general health, sex and diet of the subject; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination with the Nrf2 activator of the present inventions; and like factors well known in the medical arts. For example, it is well within the skill of the art to start doses of the compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. However, the daily dosage of the products may be varied over a wide range from 0.01 to 1,000 mg per adult per day. Typically, the compositions contain 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, 50.0, 100, 250 and 500 mg of the Nrf2 activator of the present invention for the symptomatic adjustment of the dosage to the subject to be treated. A medicament typically contains from about 0.01 mg to about 500 mg of the Nrf2 activator of the present invention, typically from 1 mg to about 100 mg of the Nrf2 activator of the present invention. An effective amount of the drug is ordinarily supplied at a dosage level from 0.0002 mg/kg to about 20 mg/kg of body weight per day, especially from about 0.001 mg/kg to 7 mg/kg of body weight per day.

Typically the Nrf2 activator of the present invention of the present invention is combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form pharmaceutical compositions. The term "Pharmaceutically" or "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent,

encapsulating material or formulation auxiliary of any type. The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetables oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin. In the pharmaceutical compositions of the present invention, the Nrf2 activator of the present inventions of the invention can be administered in a unit administration form, as a mixture with conventional pharmaceutical supports. Suitable unit administration forms comprise oral-route forms such as tablets, gel capsules, powders, granules and oral suspensions or solutions, sublingual and buccal administration forms, aerosols, implants, subcutaneous, transdermal, topical, intraperitoneal, intramuscular, intravenous, subdermal, transdermal, intrathecal and intranasal administration forms and rectal administration forms.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:

Figure 1: (A) *Mabs* induces Nrf2 protein expression level in THP-1-derived macrophages. Protein expression levels were normalized to β-actin in the total protein extracts, and to Lamin A/C in the nuclear extracts. **(B)** RT-qPCR: Nrf2 signaling pathway is activated by *M. abscessus* and SFN. The Nrf2 targets HO-1, and NQO1 mRNA expression levels were normalized to the housekeeping gene ubiquitin. Data shown are the means ± SEM of three independent experiments done in triplicates. **(C)** Protein expression levels of HO-1 and NQO1 were detected by immunoblotting. Densitometric quantification of protein signals were normalized to β-actin.

Figure 2: SFN showed a decrease in mycobacterial burden in infected macrophages. THP-1-derived macrophages were pretreated with DMSO or 10 μM SFN 3 hours prior to infection with *M. abscessus* for 3 hours. The unincorporated mycobacteria were incubated in culture medium containing 250 μg/ml amikacin for 1 hour before thorough washes. The cells were then incubated in medium with 50 μg/ml amikacin for the indicated time period (0, 1, 3, 7 days). Intracellular mycobacteria were released by cell lysing in ice-cold water, serially diluted, and seeded on agar plates. Colony-forming units were counted 5 days after incubation at 37°C. The graph represents the means ± SEM of 2 independent experiments done in triplicates. * $p<0.03$, ** $p<0.01$.

Figure 3: Apoptosis induction by SFN in *M. abscessus* infected macrophages. THP-1-derived macrophages pretreated with SFN or DMSO for 3 hours were infected with *M. abscessus* at the MOI of 10:1 for 24 h or 48 h. **(A)** The percentage of apoptotic cells was determined after the indicated post infection times using Annexin V-FITC labeling. # $p<0.03$ compared to SFN treated cells. **(B)** Colocalization of *Mabs*-mCherry in annexin V-FITC positive cells. Data represent the means ± SEM of 3 independent experiments. * $p<0.04$ compared to *Mabs*-mCherry infected cells.

Figure 4: Nrf2 signaling pathway regulates MAPK cascade. **(A)** THP-1 derived macrophages were pretreated with MAPK specific inhibitors PD98059, SB203580, and SP600125 of ERK, p38, and JNK respectively, 2 hours prior to SFN stimulation and/or *Mabs* infection. Cells were lyzed 24 hours after infection and protein lysates were analyzed by western blot. **(B)** THP-1 derived macrophages were transfected with scrambled or Nrf2 siRNA 24 hours prior to SFN stimulation and/or *M. abscessus* infection. Cells were incubated for an additional 24 hours after infection before lysis and western blots were performed using Nrf2 antibody and GAPDH antibody, as an internal control. **(C)** Immunoblots were performed on total protein lysates using specific phosphorylated antibodies against ERK, JNK, and p38.

Figure 5: (A) Annexin V-FITC assay in THP-1 derived macrophages pretreated with MAPK inhibitors SB203580, SP600125, and PD98059. Infected cells were collected 24 hours post infection. Data were from two independent experiments of n=10 000 events that were imaged and analyzed using MARKII imaging cytometer. * $p<0.03$ compared to SFN treated

cells. **(B)** THP-1 derived macrophages were pretreated with MAPK inhibitors prior to SFN pretreatment and *Mabs* infection. Mycobacteria were collected 48 hours after infection by lysing cells in ice-cold water, serially diluted, and seeded on agar plates. CFUs were obtained 5 days after incubation at 37°C. * $p<0.03$ compared to scramble transfected cells.

EXAMPLE:

Materials and methods

Antibodies and reagents

Sulforaphane (SFN), phorbol 12-myristate 13-acetate (PMA), and amikacin were purchased from Sigma-Aldrich. RPMI 1640, penicillin/streptomycin, HEPES, sodium pyruvate, fetal bovine serum (FBS), fluorescein-5-isothiocyanate, LysoTracker Red DND-99, and pre-designed Nrf2 siRNA (Ambion), Middlebrook 7H9 broth and Oleic acid/bovine albumin/dextrose/catalase enrichment (Difco, Becton Dickinson) were purchased from Fisher Scientific (Illkirch, France). HiPerfect transfection reagent and scrambled siRNA were purchased from Qiagen (Courtaboeuf, France). DC protein assay kit was purchased from BioRad (Marne-la-Coquette, France). Complete protease inhibitor cocktail tablets were purchased from Roche. ECL prime detection reagent was purchased from Amersham (GE Healthcare). 2′, 7′- dichlorodihydrofluorescein diacetate (Calbiochem), anti-phospho ERK1/2, anti-MAPK were purchased from Millipore. MAPK inhibitors PD98059, SB203580, SP600125, annexin-V FITC apoptosis detection kit, anti-lamin A/C, anti-β actin, and anti-HO-1 antibodies were purchased from Abcam. Anti-Nrf2 antibody was purchased from Santa Cruz. Anti-phospho p38, anti-p38, anti-phospho JNK, and anti-JNK were purchased from BD biosciences. EurobioGreen qPCR mix and FAM-FLICA Caspase 3/7 detection kit (ImmunoChemistry Technologies) were purchased from Eurobio. Fluoromount-G (Southern Biotech) was purchased from Clinisciences. HRP-conjugated anti-mouse or rabbit IgG were purchased from Jackson ImmunoResearch laboratories.

Cell culture

The human THP-1 monocytic cell line was maintained in RPMI 1640 medium with Glutamax supplemented with 10 % heat inactivated FBS, 1 mM sodium pyruvate, 10 mM HEPES, 1 % penicillin-streptomycin, and 0.05 mM β-mercaptoethanol in a humidified atmosphere at 37 °C and 5 % CO_2. Cells were maintained at a density between 2.5 x 10^5

cells/ml and 1 x 10^6 cells/ml. Terminal differentiation of THP-1 to macrophages was obtained by rinsing the cells twice with PBS prior to treatment with 10 µM PMA for 48 hours. Depending on the indicated conditions, THP-1-derived macrophages were pretreated with 10 µM sulforaphane or DMSO for 3 hours before mycobacterial infection. When indicated, cells were pretreated with MAPK inhibitor (PD98059, SB203580, or SP600125) 1 hour prior to SFN pretreatment and/or *M. abscessus* infection.

Mycobacterial strain

M. abscessus strain used for this study displayed a smooth morphology. *Mabs* expressing mCherry or GFP fluorochrome was generated by transforming *Mabs* with mCherry or GFP expressed pMV261-kanamycin plasmid. *Mabs*-mCherry and *Mabs*-GFP were cultured aerobically at 37 °C in Middlebrook 7H9 broth supplemented with 0.05 % Tween 80, 10 % (v/v) oleic acid/albumin/ dextrose/catalase enrichment, and supplemented with the appropriate antibiotics (250 µg/ml kanamycin and 1 mg/ml hygromycin respectively). Prior to infection, bacteria were washed twice in PBS and single bacilli were obtained by passing the bacteria suspension sequentially through a 25-G needle and a 29-G insulin syringe 10 times each. The number of bacteria was determined by counting the fluorescent bacteria in a Malassez counting chamber using an epifluorescence microscope.

In vitro cell infection and intracellular growth measurements

Forty-eight hours after seeding THP-1 in 24-well plates at 5 x 10^5 cells/well, and inducing differentiation to macrophages with PMA, the cells were infected with *Mabs*-mCherry at the MOI of 10:1 (bacilli to THP-1). After 3 hours infection at 37 °C and 5 % CO_2, unincorporated bacilli were eliminated by thoroughly washing twice with PBS. Infected and control cells were then treated for 1 hour with 250 µg/ml amikacin to eliminate the remaining unattached bacilli, washed with PBS, and cultured for up to 7 days in culture cell medium supplemented with 50 µg/ml amikacin. The colony-forming-unit (CFU) counts were determined at day 0, 1, 3, and 7 after infection. The intracellular bacilli were collected by lysing cells with ice-cold distilled water and plating 10-fold serial dilutions on Luria Bertani agar plates. The number of bacilli was determined by counting individual colonies after 5-7 days of growth at 37 °C.

Phagosomal acidification assay

Mabs-mCherry was surface labeled with the pH sensitive fluorescein-5-isothiocyanate (FITC). THP-1 seeded in 24-well plate at 5 x 10^5 cells/well were infected with doubly labeled *Mabs* at the MOI of 10:1 for 20 minutes at 4 °C. After washing with PBS, and supplementing the cells with PBS-1 % FBS, fluorescence signal intensities were detected using the Fluoroskan Ascent FL spectrophotometer (Fisher scientific). FITC and mCherry signal intensities were acquired every 5 minutes for 1 hour then every 10 minutes for 2 hours at 34 °C by sequential excitation at 485 nm and 544 nm, respectively. For each experiment, a standard pH curve was determined by correlating the fluorescence intensities to standardized pH buffers.

Cell transfection

To knockdown Nrf2, THP-1-derived macrophages were transfected 24 hours prior to pretreatment or infection with a pre-designed Nrf2 siRNA (siNrf2), or a universal control RNAi (scramble), using HiPerfect transfection reagent following the manufacturer's recommended protocol.

Subcellular fractionation and immunoblotting

For immunoblot analysis, THP-1-derived macrophages were rinsed with cold PBS then lyzed with cold RIPA buffer (150 mM NaCl, 1 % Triton X-100, 0.5 % sodium deoxycholate, 0.1 % SDS, 50 mM Tris-HCl, pH 7.5, supplemented with Complete protease inhibitor cocktail mixture). Protein concentrations were determined using DC protein assay kit. Twenty-µg of total proteins were resolved by SDS-PAGE (4-20 % gradient gels) and transferred to polyvinylidene difluoride membrane. Membrane blocking was performed in 5 % BSA/TBST (10 mMTris-HCl, pH 7.4, 150 mM NaCl, and 0.1 % Tween 20) for 1 hour prior to incubation with primary antibodies. The corresponding horseradish peroxidase-conjugated secondary antibodies were used at dilution 1/20 000. Immunoreactivity was visualized using the ECL prime detection reagent and detected using the QuantityOne software (ChemiDoc XRS, BioRad). Immunoblots shown are representative of 3 independent experiments.

Total RNA isolation, reverse transcription, and quantitative real-time PCR

Total RNA was isolated from treated cells using the RNeasy Mini kit (Qiagen). RNA concentration and purity were determined using the GE NanoVue spectrophotometer (GE Healthcare). RNA was reverse transcribed into cDNA using Superscript III First strand

synthesis kit (Life Technologies) with an oligo(dT) primer, according to the manufacturer's instructions. cDNA was analyzed using real time qPCR, with each sample done in triplicate. qPCR was performed using the CFX96 thermocycler (BioRad) and EurobioGreen qPCR mix. The specific oligonucleotides for Nrf2, HO-1, and NQO1, were designed using Primer-BLAST (NCBI website), and purchased from Eurogentec. Gene expression levels were calculated as a ratio to the expression of the reference gene ubiquitin C (UBC). Data were analyzed on the BioRad CFX manager 3.1 using the ΔΔCt method.

Apoptosis and necrosis assays, activated Caspase 3/7 assay, and ROS production assay

Apoptosis assay was performed according to the manufacturer's procedure. Briefly, THP-1 cells cultured in 6-well plate at 1 x 10^6 cells/well were pretreated with SFN or DMSO for 3 hours prior to mycobacterial infection. At the indicated times, cells were trypsinized, washed once with PBS, resuspended in Annexin binding buffer 1X, and stained with Annexin V-FITC for 5 minutes in the dark and at room temperature. After 1 PBS wash, the cell pellet was suspended in 100 µl Annexin binding buffer 1X. Quantification of necrotic THP-1-derived macrophages was performed by incubating the infected and non-infected cells with 2 µg/ml propidium iodide (PI).

For assessing Caspase 3/7 activity in THP-1 cells, the non-cytotoxic Fluorescent Labeled Inhibitors of Caspases (FLICA) probe was used. The FLICA probe, composed of the irreversible caspase inhibitor DEVD- fluoromethyl ketone fused to a carboxyfluorescein, binds specifically and covalently to activated Caspase 3/7 enzymes. Following the manufacturer's protocol, cells were incubated 1 hour at room temperature and in the dark. After 2 washes, the cells were resuspended in wash buffer 1X.

The generation of ROS in THP-1-derived macrophages was monitored using the cell-permeable fluorogenic probe, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA). Treated cells tested for apoptosis, necrosis, ROS generation and activated caspases 3/7 production were analyzed by imaging flow cytometry (MARK II, Merck-Millipore) as described below.

Imaging flow cytometry

Apoptotic/necrotic/infected cells, H_2DCFDA-labeled cells, and activated Caspase 3/7 FLICA-labeled cells were quantified using ImageStream® Mark II (Amnis, Merck-Millipore) imaging flow cytometer, which allows simultaneous imaging and analysis of cells. Depending

on the assay, data from 5,000 to 10,000 events were acquired at 40X magnification and using 488, 658 and 785 nm lasers. Compensation settings were adjusted on single-color controls for each fluorochrome and analyses were performed using the IDEAS® 5.0 data analysis software (Amnis). The brightfield images were used to verify cell integrity. A brightfield area versus brightfield aspect ratio scatterplot was used to gate on single cells and eliminate cell aggregates. The single cells were then plotted on an Annexin V bright detail intensity versus brightfield area to gate on the Annexin V^+ cells. The Annexin V^+ cells were then plotted on an M.absessus-mCherry bright detail intensity versus *Mabs*-mCherry area to gate on the Annexin V^+ *Mabs*$^+$ cells. The same procedure was applied to the PI, FLICA and H_2DCFDA labeling.

Lysosomal labeling and indirect immunofluorescence imaging

THP-1-derived macrophages were seeded at 2.5 x 10^5 cells per well on coverslips in 24-well plates and were incubated in PMA-containing medium. After 48 hour incubation, cells were transfected using Hiperfect transfection reagent (Qiagen) and scrambled or siNrf2 24 hours prior to pretreatment with MAPK inhibitor 4 hours before infection. SFN or DMSO pretreatment was done 3 hours prior to infection with *Mabs*-GFP for 3 hours. Labeling with LysoTracker Red DND-99 (Life Technologies) was done according to the manufacturer's instructions. Cells were rinsed with PBS and fixed with 4 % paraformaldehyde at room temperature for 30 minutes. After 1 rinse with PBS, coverslips were mounted on slides using Fluoromount-G. Cells were observed using a confocal microscope (Leica TCS SPE). Images were treated and analyzed using ImageJ software.

Statistical analyses

Results are presented as means ± SEM of 3 independent experiments done in triplicates. Imaging flow cytometry results presented are means ± SEM of 2 to 3 independent experiments of 5 000-10 000 events. Statistical comparisons were performed using two-tailed Student's t test and differences were considered to be significant at a value of $p < 0.05$.

Results

Activation of the Nrf2 dependent antioxidant pathway by SFN and/or M. abscessus

To determine the effect of *Mabs* infection on the antioxidant pathway, we sought to define the expression level of Nrf2 in macrophages derived from phorbol 12-myristate 13-acetate (PMA)-differentiated human THP-1 cells. Macrophages were pretreated 3 hours with

sulforaphane (SFN) prior to infection with *Mabs*. *Mabs* infection induced Nrf2 protein expression level 24 hours post infection 2.8-fold higher as compared with that of DMSO (Figure 1A). Nrf2 expression level in macrophages treated with SFN was increased more than 3-fold compared to DMSO treated cells. Interestingly, *Mabs* infection in SFN treated macrophages strongly increased Nrf2 protein level to 10.5-fold. Nrf2 activation is confirmed by analyzing nuclear proteins extracted from SFN pretreated and/or *Mabs* infected macrophages. Immunoblots revealed that Nrf2 is translocated to the nucleus in SFN pretreated macrophages (4.7-fold increase) and confirmed the strong increase in Nrf2 previously seen in total protein extracts in SFN pretreated/*Mabs* infected cells (6.2-fold increase). Interestingly, infection with *Mabs* alone augmented Nrf2 in the total protein extracts but is not reflected in the nuclear Nrf2.

Quantitative PCR of two downstream targets of Nrf2 showed a rapid and significant increase in mRNA levels of heme oxygenase-1 (HO-1) and NAD(P)H dehydrogenase, quinone 1 (NQO1) with the persistence of the mRNA levels 10 hours after SFN treatment alone or SFN pretreatment/*M. abscessus* infection of THP-1 derived macrophages (Figure 1B). Infection with *Mabs* alone led to a weak induction of HO-1 mRNA or an induction of NQO1 mRNA that decreased 6 hours after *Mabs* infection. Immunoblots using total protein extracts of macrophages lyzed 24 hours post infection confirmed the significant 37.4-fold increase in HO-1 protein level in SFN pretreated/*Mabs* infected cells (Figure 1C) comparable to the pattern observed with total Nrf2 extracts. It is noteworthy that NQO1 protein levels showed a comparable pattern with the one seen in nuclear Nrf2 immunoblot.

Sulforaphane treated cells showed decreased mycobacterial proliferation

Since *M. abscessus* has been previously shown to proliferate more favorably in oxidative stress conditions[5], we sought to determine the effect of Nrf2 activation on the bactericidal activity of macrophages against *M. abscessus*. THP-1-derived macrophages were infected with *Mabs* for 3 hours at a multiplicity of infection of 10 bacilli for 1 cell, thoroughly washed and incubated in amikacin and SFN supplemented medium. After the indicated post infection times, macrophages were lyzed and live internalized mycobacteria were quantified using the colony counting method (CFU). The results showed an increase in *Mabs* viability and proliferation over the 7-day observation period in DMSO pretreated cells (Figure 2). Interestingly, SFN pretreated macrophages infected with *Mabs* yielded a 2-fold decrease in CFU at day 3 post infection and a 3-fold decrease at day 7 post infection compared to DMSO

treated cells. These data suggest that SFN may have an inhibitory effect on mycobacterial proliferation in macrophages.

SFN-induced mycobacterial growth decrease is independent from phagosomal acidification

Numerous pathogens including *M. tuberculosis* have developed complex mechanisms in order to survive and proliferate in host phagocytes by interfering with the phagosomal maturation process thus blocking the fusion of phagosomes with lysosomes and the generation of phagolysosomes. This impediment prevents exposition of the ingested bacteria to reactive oxygen metabolites, lysosomal hydrolases and general acidification of the phagolysosome to pH below 5.0, allowing the use of macrophages as proliferation reservoir. To determine whether *Mabs* or/and SFN has an effect on phagosomal maturation, phagosome acidification assay was used. *Mabs* expressing mCherry fluorochrome were surface labeled with the pH sensitive FITC and were used to infect THP-1 derived macrophages. FITC, which emission intensities are pH dependent, was used to determine phagosomal acidification, while mCherry, a pH-independent fluorochrome, was used as an internal indicator of the number of bacteria (data not shown). Our results showed that *Mabs* efficiently inhibited the maturation of phagosomes (pH acidification in DMSO treated macrophages was 6.27 ± 0.07). Interestingly, reduction in mycobacterial burden in SFN treated macrophages (Figure 2) was not due to a decrease in phagosomal pH since the phagosomes of SFN treated macrophages remained stable at pH = 6.17 ± 0.14 (data not shown).

Using the ROS-sensitive indicator, H_2DCFDA, we analyzed the intracellular oxidative stress level in *Mabs* infected macrophages pretreated with SFN or DMSO (data not shown). ROS production was significantly augmented by *Mabs* infection at 2 hours post infection and in SFN pretreated/ *Mabs* infected macrophages compared to DMSO treated cells. Macrophages pretreated with SFN alone did not show a significant modulation of the ROS-sensitive indicator compared to DMSO treated cells. These data suggest that infection with *Mabs* increases ROS production with no effect of SFN pretreatment on the latter.

Mabs infection induces cell necrosis in THP-1 derived macrophages

Mabs expressing mCherry were used to infect for 3 hours THP-1 derived macrophages that were pretreated with SFN or DMSO. Analysis by imaging flow cytometry on 24 hour- and 48 hour- post-infected cells showed firstly, an increase in the number of bacteria infected cells at 48 hours post infection compared to that of 24 hours post infection cells, and

secondly, that SFN pretreatment had no significant effect on *Mabs* phagocytosis by THP-1-derived macrophages (data not shown).

Unlike the well-studied *M. tuberculosis*, very little is known about the effect of *Mabs* infection and cell death. To establish the effect of *M. abscessus* infection on cell necrosis, THP-1-derived macrophages were infected with *Mabs*-mCherry for 3 hours and incubated for 24 and 48 hours. Cells were then stained with propidium iodide and analysis was performed by imaging flow cytometry. At 24 hours post infection, SFN alone did not significantly induce cell necrosis, nor did *M. abscessus* infection alone or the combination of both. Interestingly, *M. abscessus* dependent cell necrosis was raised from 2.26 ± 0.17 % at 24 hours post infection to 5.66 ± 0.6 % at 48 hours after infection (data not shown). Similar results were obtained in SFN pretreated and *Mabs* infected THP-1, suggesting that SFN pretreatment did not modulated *M. abscessus* induced cell necrosis.

Mabs infection induces apoptosis in SFN treated THP-1-derived macrophages

Since SFN did not affect phagosomal maturation nor cell necrosis, we hypothesized that SFN might reduce mycobacterial burden through cell apoptosis. Indeed, one of the mechanisms phagocytes utilize to increase mycobactericidal activity is for the infected macrophages to trigger apoptosis, generating apoptotic bodies that will induce killing by non-infected bystander macrophages[13]. In order to test the apoptotic response of macrophages following mycobacterial infection, THP-1-derived macrophages were infected with *Mabs* and left incubated for 24 hours and 48 hours. Early cell apoptosis was detected using an Annexin V-FITC probe and quantified by imaging flow cytometry (data not shown).

As can be seen, no significant difference in cell apoptosis between *Mabs* infected macrophages at 24 or 48 hours and DMSO or SFN alone treated cells (Figure 3A). Interestingly, macrophages that were pretreated with SFN and subsequently infected with *Mabs* showed a strong increase in cell apoptosis at 24 and 48 hours post infection (Figure 3A). Quantification of apoptotic cells that colocalized with mycobacteria showed a significant increase in SFN pretreated cells compared to *Mabs* infected cells (Figure 3B), thus suggesting a role of SFN in inducing cell apoptosis in macrophages infected by *Mabs*.

Mabs infection in SFN treated cells induces cell apoptosis in a caspases 3/7 independent pathway

Since activation of the caspase cascade is an essential process in cell apoptosis[14], we sought to determine whether the cell apoptosis observed in SFN treated and *Mabs* infected

macrophages was caspase-dependent. THP-1 cells treated with SFN were infected with *Mabs*, and activated caspases 3/7 were assessed using a non-cytotoxic fluorescent inhibitor of caspases probe (FLICA) which binds covalently to active caspases 3 and 7 and analyzed by imaging flow cytometry (data not shown). At 24 hours post infection, no significant activation of caspases 3/7 was seen in SFN pretreated, *Mabs* infected, and SFN pretreated-*Mabs* infected macrophages compared to DMSO treated cells (data not shown). However, cells incubated 48 hours after infection showed a significant increase in caspases 3/7 activation in *Mabs* infected cells compared to DMSO treated cells and in SFN pretreated-*Mabs* infected macrophages compared to SFN pretreated cells. Treatment with SFN did not modify caspases 3/7 activity in presence or absence of *Mabs*. Colocalization of caspases 3/7 positive cells and *Mabs*-mCherry positive cells confirmed that SFN does not activate caspases 3/7 pathway in infected cells compared to untreated cells (data not shown).

Nrf2 regulates the MAPK signaling pathway

MAPK signaling pathway is known to play an important role in the regulation of cell death decisions, which prompted us to hypothesize that MAPK cascade may be involved in the caspase-independent apoptosis process observed in SFN treated and *Mabs* infected cells. The three well-characterized MAPK subfamilies ERK, JNK, and p38 are involved in the pro- and anti-apoptotic pathways[15]. PD98059, SB203580, and SP600125, are specific inhibitors of ERK, p38, and JNK respectively, and were used to pretreat the macrophages prior to SFN pretreatment and/or *Mabs* infection. Inhibitory effect of PD98059, SB203580, and SP600125 were verified by immunoblotting using phosphorylated antibodies to ERK, p38, and JNK (data not shown). Interestingly, immunoblots showed that increased expression of Nrf2 in SFN pretreatment and *Mabs* infected macrophages was not inhibited by the specific MAPK inhibitors suggesting that induction of Nrf2 expression is upstream from ERK, p38, and JNK pathways (Figure 4A).

To determine whether Nrf2 is implicated in the activation of ERK, p38, and JNK pathways, macrophages were transfected with siRNA designed to specifically silence Nrf2 expression levels 24 hours prior to SFN and/or *Mabs* treatment. Western blotting of protein lysates extracted 24 hours after infection showed a significant decrease in Nrf2 protein level although the abolition was not complete (Figure 4B).

In macrophages transfected with scrambled siRNA, SFN increased phosphorylation of ERK, and p38 in SFN pretreated macrophages and SFN pretreated/*Mabs* infected macrophages (Figure 4C). Phosphorylation of JNK was observed only in SFN

pretreated/*Mabs* macrophages. Infection with *Mabs* activated p38 with no significant effect on ERK and JNK. Interestingly, in macrophages transfected with siRNA targeting Nrf2, phosphorylation of ERK and JNK were higher in SFN treated cells, *Mabs* infected cells and SFN pretreated/*Mabs* cells compared to that seen in scrambled siRNA transfected macrophages. These results suggest that SFN-induced Nrf2 has an inhibitory effect on ERK and JNK signaling pathways. Conversely, p38 phosphorylation is reduced to the basal level detected in DMSO treated macrophages. Thus, Nrf2 controls the activation of the p38 pathway in our cell model (Figure 4C).

p38 signaling pathway in the SFN/Mabs induced apoptosis

To determine whether p38 signaling pathway played a central role in the increase in cell apoptosis induced by SFN and *Mabs* infection, THP-1 derived macrophages were pretreated with MAPK inhibitors of p38, JNK, and ERK, prior to pretreatment with SFN and *Mabs* infection, and annexin V-FITC labeling were performed. A significant decrease in apoptosis in SB203580 and SP600125 pretreated macrophages compared to SFN pretreated cells, while no significant difference was observed in PD98059 pretreated cells (Figure 5A).

Moreover, THP-1 derived macrophages were pretreated with the MAPK inhibitors, SFN, and infected with *Mabs*. After 48 hours, live mycobacteria were collected and seeded on LB-agar plates. The results showed a 2-fold increase in mycobacterial load in SB203580 pretreated cells, and a significant decrease in PD98059 pretreated cells (Figure 5B). Cells pretreated with SP600125 showed an increase in the mycobacterial burden that was not significant. The results validate further the implication of the pro-apoptotic p38 signaling pathway activated by Nrf2, and to a lesser extent JNK signaling pathway, in SFN/*Mabs* induced apoptosis.

Discussion

Mycobacterium abscessus (*Mabs*) is able to cause skin, bone and soft tissue infections and has been increasingly involved in exacerbations of lung infections and pulmonary diseases[16]. Currently, its high resistance to antibiotics greatly limits patient treatment which may account for the likelihood of developing chronic airway infections and increase risk of fatal outcome. Thus, development of new anti-mycobacterial drugs and treatment that may potentiate the actual drug therapies is urgently needed. SFN is a well-known activator of Nrf2 and has been shown to have several beneficial effects[17] including an antibacterial effect on *H.*

pylori[18,19]. We used SFN as a pretreatment in our *in vitro* model of macrophage infection by *M. abscessus*. In this study, we describe a new mechanism by which SFN can inhibit bacterial proliferation. The important finding in this study is that SFN triggers a caspase-independent cell apoptosis in infected macrophages that requires activation of Nrf2 and p38 signaling pathways.

The role of oxidative stress in pathogen infection and propagation remains partially understood. Upon infection, microorganisms are detected, enveloped, and then phagocytosed by inflammatory cells from the innate immune defense system. These cells produce highly unstable and free radicals like ROS, comprising metabolites from partially reduced oxygen (superoxide anion, hydrogen peroxide, and hydroxyl radical), that will inflict irreversible damage to DNA, proteins, and lipids. This oxidative burst is crucial in pathogen clearance, but it appears that microorganisms, such as some mycobacteria, may survive and even preferentially thrive in an oxidative environment. *M. abscessus*, like *M. tuberculosis*, uses the host immune cells as a reservoir for proliferation and its growth is even enhanced in presence of oxygen free radicals[5,20]. Here, we showed that although infection with *M. abscessus* induced ROS production in THP-1 derived macrophages, *M. abscessus* was able to prevent phagosomal pH acidification and thus proliferate intracellularly. Moreover, infection with *M. abscessus* led to an increase in cell necrosis with a negligible amount of cell apoptosis.

The consequence of this oxidative burst is an imbalance in oxidants/antioxidants which activates triggering a cascade of cytoprotective and antioxidant defense mechanisms to maintain a redox homeostasis. This antioxidant cascade is controlled by Nrf2, the major transcriptional activator of ARE-mediated phase II enzymes. Although not fully understood, the Nrf2 signaling pathway seems to play an important role, either beneficial or detrimental, in microbial infections[21]. Our results showed that infection of THP-1 derived macrophages with *M. abscessus* activates the antioxidant signaling pathway regulated by Nrf2. *M. abscessus* infection induced Nrf2 expression level and its translocation into the nucleus. Even though this activation is lower than that seen with SFN, it is still able to induce expression of HO-1 and NQO1, two downstream targets of Nrf2 (Figure 1). Recently, Abdalla et al. have described that infection of THP-1 induced macrophages by *M. abscessus* induced HO-1 expression and contributed to *M. abscessus* growth and survival in phagosomes of macrophages[22].

Interestingly, *M. abscessus* infected macrophages showed a significant decrease in mycobacterial growth 7 days post infection in cells pretreated with SFN compared to untreated cells suggesting that activation of Nrf2 signaling pathway by SFN promote

mycobacterial growth inhibition. We showed that SFN had neither an effect in the early stage of pH acidification or on phagosomal maturation, nor on the phagocytosis mechanism since the amount of internalized *M. abscessus* was similar to infected macrophages. Interestingly, SFN showed no effect on either cell necrosis or cell apoptosis when macrophages were treated with SFN alone, but showed a significantly strong increase in cell apoptosis in SFN pretreated macrophages that were subsequently infected with *M. abscessus*. This apoptotic mechanism triggered by SFN in infected macrophages is in contradiction with the well-known protective effect of Nrf2 signaling pathway that promotes the survival of normal and cancerous cells[23-25].

One efficient mechanism utilized by the innate immunity to fight mycobacterial infection is to undergo apoptosis. This mechanism has been reported to directly kill intracellular bacteria and apoptotic bodies enhance bacterial phagocytosis by activated and uninfected neighboring macrophages[12]. One mechanism used by *M. tuberculosis* is to inhibit the apoptotic pathway to prevent the programmed death of infected macrophages and thus enhance its intracellular survival[26,27]. Here, Nrf2 and HO-1 protein expression levels were strongly induced in SFN pretreated and *M. abscessus* infected macrophages and this observation correlates with an increase in cell apoptosis in these cells. Macrophages pretreated with SFN alone or infected with *M. abscessus* alone did not have any apoptotic effect. We demonstrated that cell apoptosis induced by SFN pretreatment in infected macrophages was caspase-independent and p38 MAPK dependent (data not shown). It is likely that, as for *M. tuberculosis*, *M. abscessus* is also able to inhibit the typical apoptosis pathway in the infected macrophages causing the cells to induce the caspase-independent apoptosis that we observe in our study.

Various caspase-independent cell death have been recently described in the literature. The cell death mechanistic pathway triggered by SFN in infected macrophages strongly resemble the defense mechanism activated by the innate immunity following viral infection and previously described as necroptosis[28,29]. This alternate cell death pathway is triggered in phagocytes to help eliminate phagocytosed viruses that are able to block the classical apoptosis pathway. We speculate that SFN may be able to help *M. abscessus* infected macrophages overcome the blockade in cell apoptosis and eliminate mycobacteria by triggering a mechanism similar to necroptosis. One other caspase 3-independent mechanism that has been recently implicated in *M. tuberculosis* infected macrophages is the inflammation related pyropotosis[30].

In conclusion, the present study showed that activation of the Nrf2 signaling pathway by SFN can reduce *M. abscessus* proliferation in macrophages by inducing a caspase-independent cell apoptosis. To our knowledge, we describe for the first time the anti-bactericidal properties of sulforaphane against mycobacteria with the involvement of Nrf2 and p38 signaling pathways in the mechanism involved in inhibition of bacterial proliferation. These findings indicate that modulation of the Nrf2 signaling using Nrf2 activators may potentiate the actual multi-drug therapies used to treat patients diagnosed with *M. abscessus* infection.

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Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

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CLAIMS:

1. A method of treating a mycobacterial infection in a subject in need thereof comprising administering to the subject a therapeutically effective amount of an Nrf2 activator.

2. The method of claim 1 wherein the subject is immunocompromised.

3. The method of claim 1 wherein the subject is selected from the group consisting of subjects with AIDS (or HIV positive), subjects with severe combined immune deficiency (SCID), diabetics, subjects who have had transplants and who are taking immunosuppressives, subjects who do not have a spleen, subjects with end stage kidney disease (dialysis), and subject who have been taking corticosteroids.

4. The method of claim 1 wherein the subject suffers from a disease selected from the group consisting of sickle cell anemia, cystic fibrosis, severe liver, lung, heart disease, and neurological and muscular disabilities.

5. The method of claim 1 wherein the mycobacterial infection is caused or mediated by a mycobacterium selected from the group consisting of *Mycobacterium tuberculosis, include Mycobacterium avium, Mycobacterium kansasii, Mycobacteriumfortuitum, Mycobacterium chelonae, Mycobacterium leprae, Mycobacterium avium subspecies paratuberculosis, Mycobacterium intracellular, Mycobacterium scrofulaceum, Mycobacterium xenopi, Mycobacterium abscessus, Mycobacterium marinum*, and *Mycobacterium ulcerans*.

6. The method of claim 1 wherein the mycobacterial infection is caused or mediated by a resistant or highly virulent strain of a mycobacterium.

7. The method of claim 1 wherein the Nrf2 activator is sulforaphane.

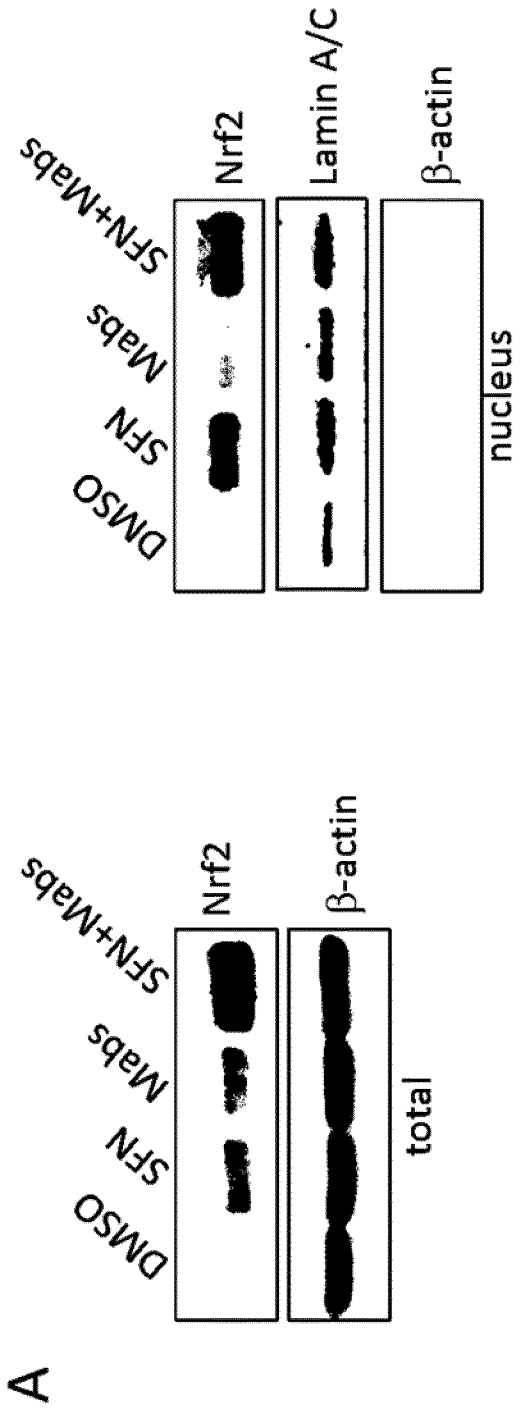
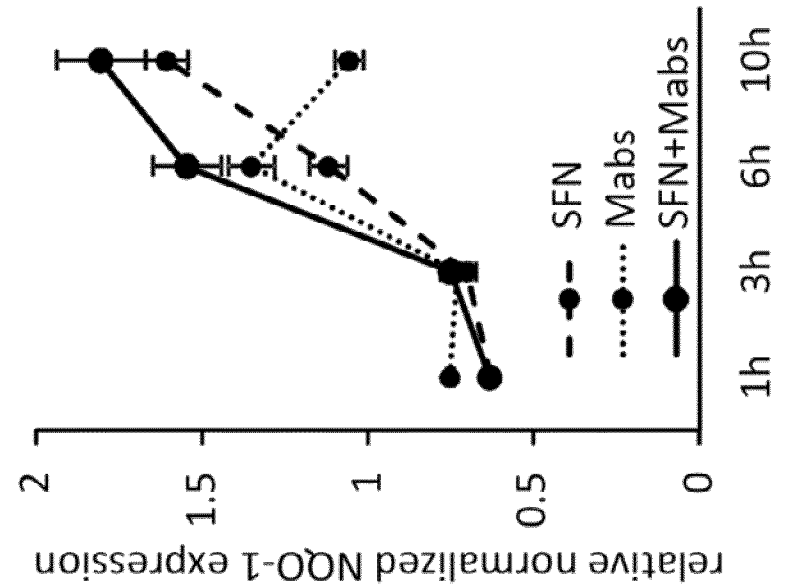

Nrf2
Lamin A/C
β-actin
SFN+Mabs
Mabs
SFN
DMSO
nucleus
Nrf2
β-actin
SFN+Mabs
Mabs
SFN
DMSO
total
A

Figure 1A


relative normalized NQO-1 expression
2
1.5
1
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1h
3h
6h
10h
SFN
Mabs
SFN+Mabs

B

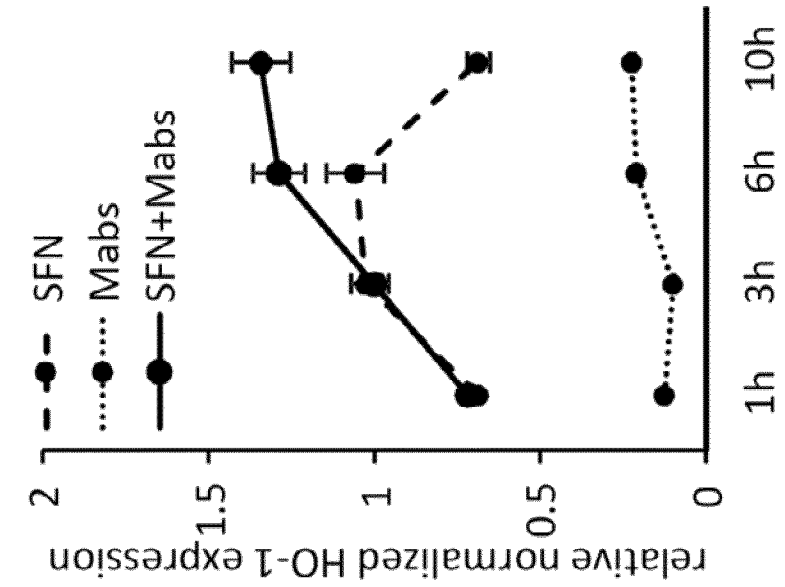

relative normalized HO-1 expression
2
1.5
1
0.5
0
1h
3h
6h
10h
SFN
Mabs
SFN+Mabs

Figure 1B

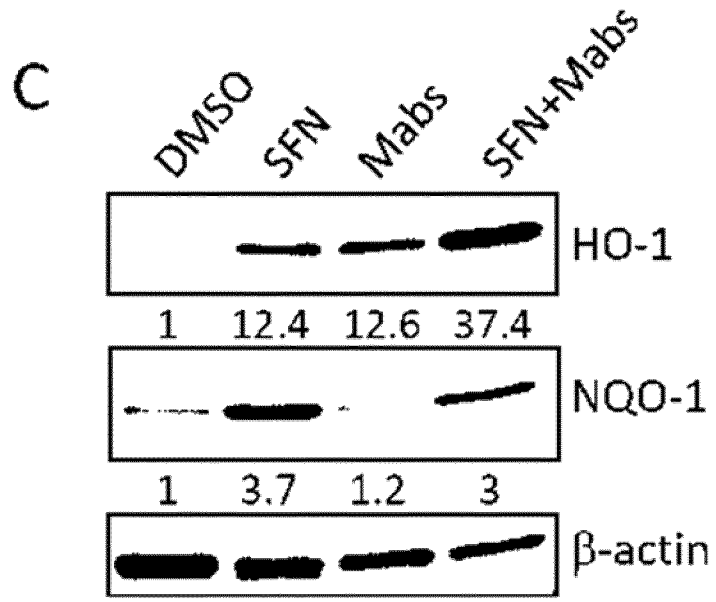


Figure 1C

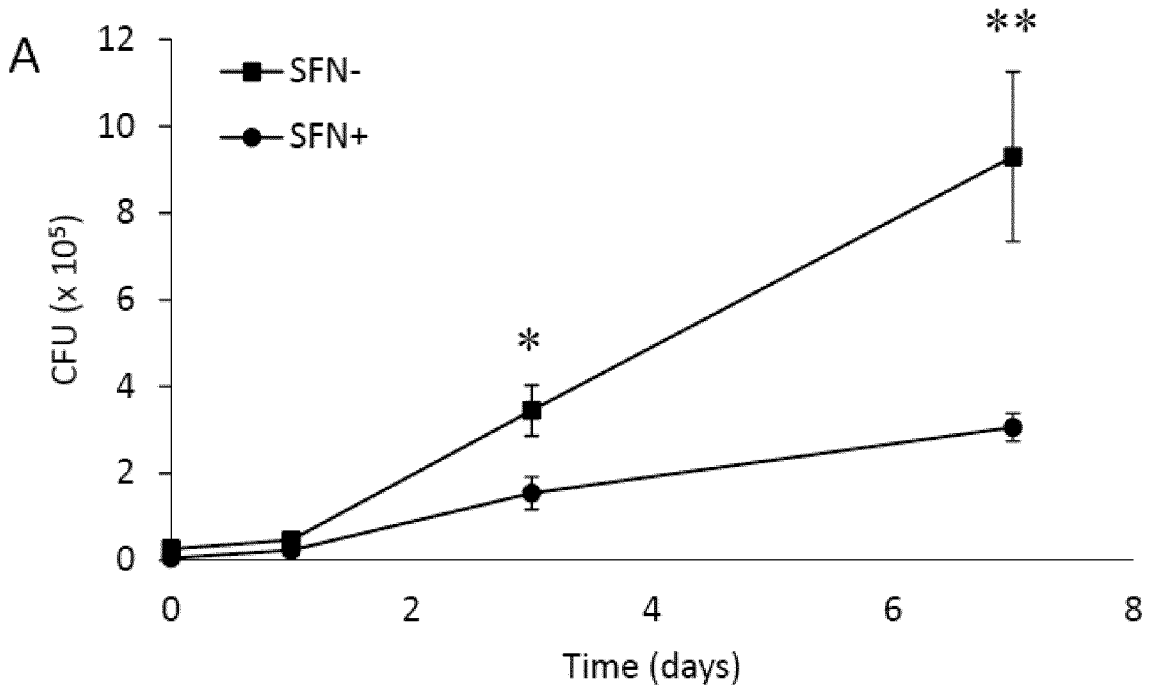


Figure 2

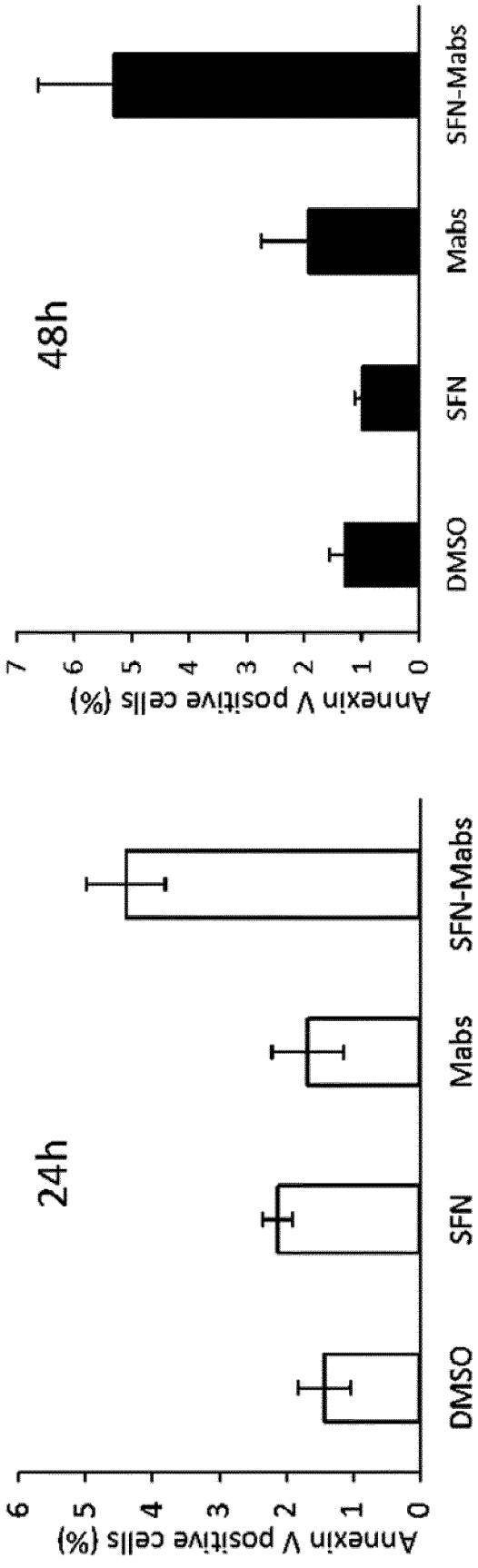

48h
24h
Annexin V positive cells (%)
0
1
2
3
4
5
6
7
DMSO
SFN
Mabs
SFN-Mabs

Figure 3A

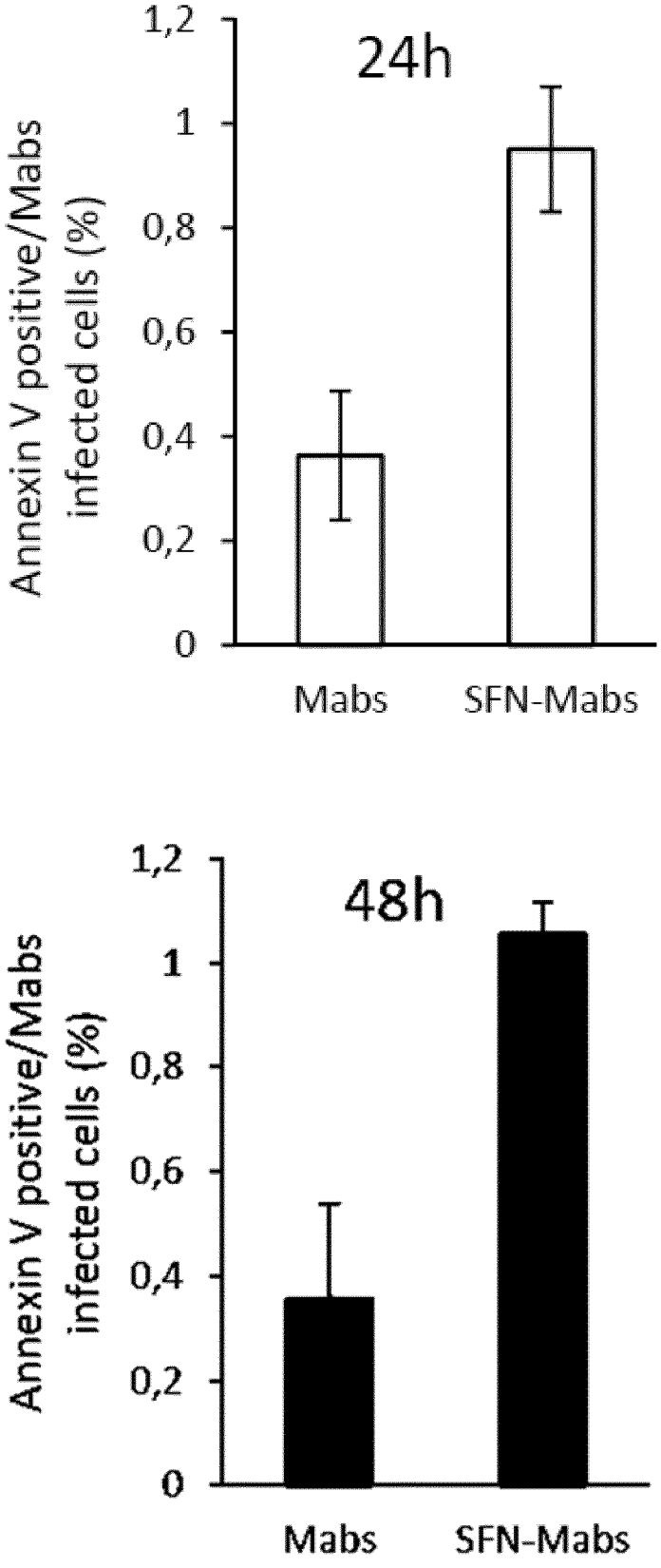

24h
Annexin V positive/Mabs infected cells (%)
1,2
1
0,8
0,6
0,4
0,2
0
Mabs
SFN-Mabs
48h
Annexin V positive/Mabs infected cells (%)
1,2
1
0,8
0,6
0,4
0,2
0
Mabs
SFN-Mabs

Figure 3B

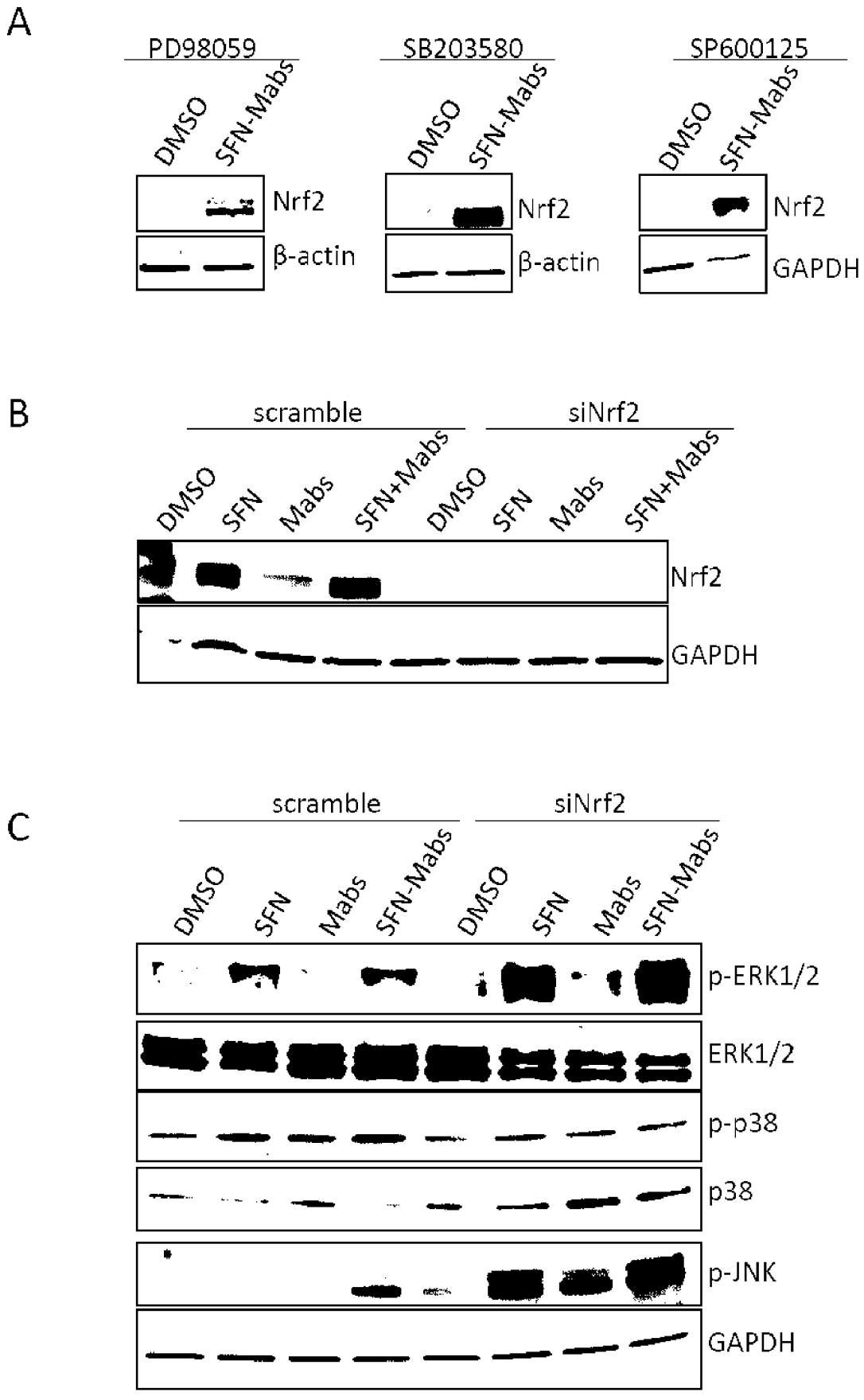
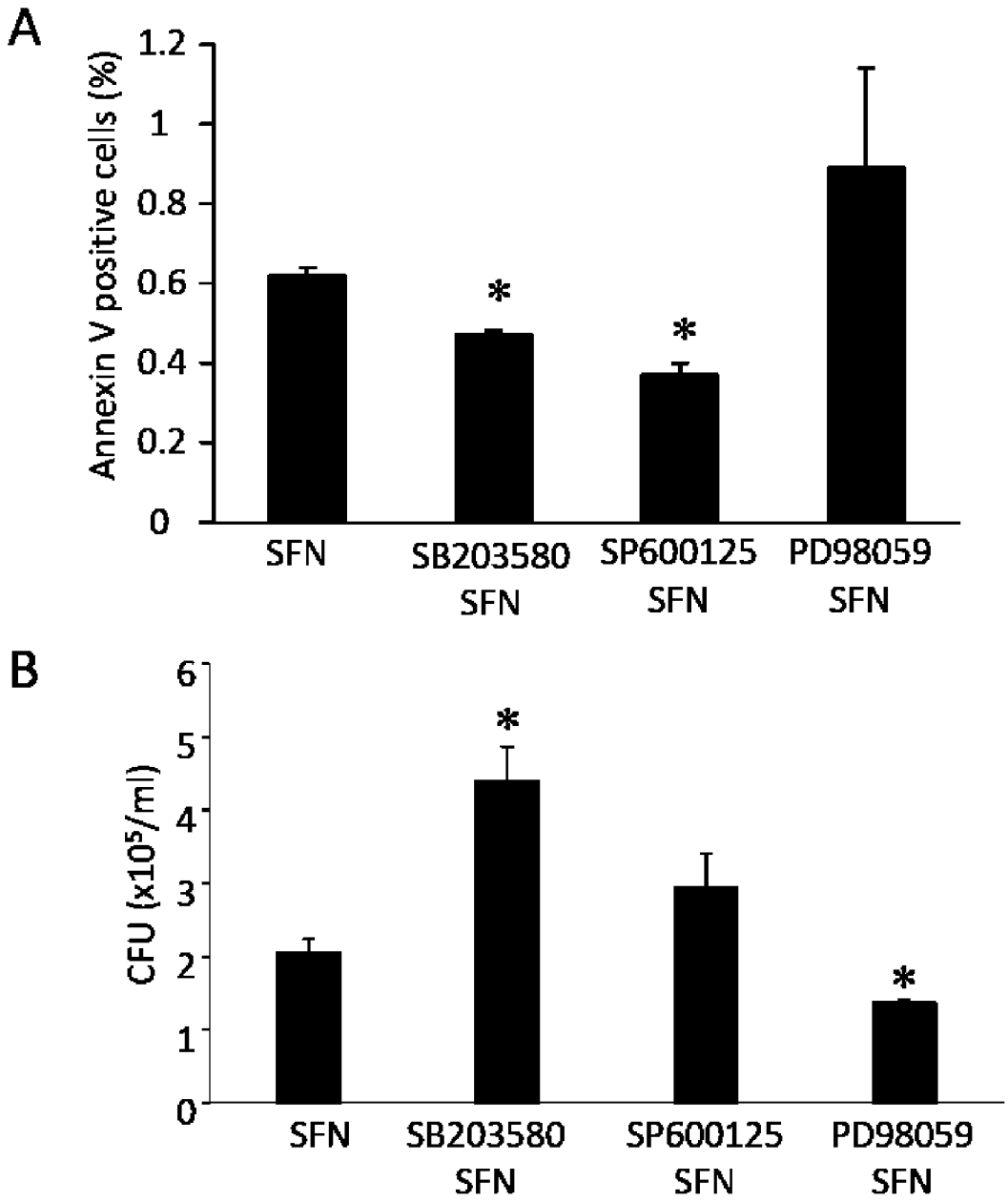

A
PD98059
SB203580
SP600125
DMSO
SFN-Mabs
Nrf2
β-actin
GAPDH
B
scramble
siNrf2
DMSO
SFN
Mabs
SFN+Mabs
Nrf2
GAPDH
C
scramble
siNrf2
DMSO
SFN
Mabs
SFN-Mabs
p-ERK1/2
ERK1/2
p-p38
p38
p-JNK
GAPDH

Figure 4



Figures 5A and 5B

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/067482

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/26 A61P31/04 A61P31/06 A61K31/00
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

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, 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	KR 2014 0013792 A (PUSAN NAT UNIV IND COOP FOUND [KR]) 5 February 2014 (2014-02-05) the whole document -----	1-7
X	WO 2014/008341 A2 (NUTRAMAX LAB INC [US]) 9 January 2014 (2014-01-09) paragraph [0016] page 22, paragraph 67 -----	1-7
X	WO 01/21164 A2 (ADVANCED LIFE SCIENCES INC [US]; LIN YUH MEEJ [US]) 29 March 2001 (2001-03-29) page 8, lines 9-16 page 14, line 31 - page 20, line 2 -----	1-7
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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"&" 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 October 2016	26/10/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Strack, Eberhard

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/067482

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2016/067482

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