NOVEL COMPOUND USEFUL FOR THE TREATMENT OF DEGENERATIVE AND INFLAMMATORY DISEASES

FIELD OF THE INVENTION

[0001] The present invention relates to a compound that is an inhibitor of JAK, a family of tyrosine kinases that are involved in inflammatory conditions, autoimmune diseases, proliferative diseases, transplantation rejection, diseases involving impairment of cartilage turnover, congenital cartilage malformations, and/or diseases associated with hypersecretion of IL6. The present invention also provides methods for the production of this compound, pharmaceutical compositions comprising this compound, and methods for the prevention and/or treatment of inflammatory conditions, autoimmune diseases, proliferative diseases, transplantation rejection, diseases involving impairment of cartilage turnover, congenital cartilage malformations, and/or diseases associated with hypersecretion of IL6 by administering a compound of the invention.

[0002] Janus kinases (JAKs) are cytoplasmic tyrosine kinases that transduce cytokine signaling from membrane receptors to STAT transcription factors. Four JAK family members are described, JAK1, JAK2, JAK3 and TYK2. Upon binding of the cytokine to its receptor, JAK family members auto- and/or transphosphorylate each other, followed by phosphorylation of STATs that then migrate to the nucleus to modulate transcription. JAK-STAT intracellular signal transduction serves the interferons, most interleukins, as well as a variety of cytokines and endocrine factors such as EPO, TPO, GH, OSM, LIF, CNTF, GM-CSF, and PRL (Vainchenker W. *et al.* (2008).

[0003] The combination of genetic models and small molecule JAK inhibitor research revealed the therapeutic potential of several JAKs. JAK3 is validated by mouse and human genetics as an immune-suppression target (O'Shea J. *et al.* (2004)). JAK3 inhibitors were successfully taken into clinical development, initially for organ transplant rejection but later also in other immuno-inflammatory indications such as rheumathoid arthritis (RA), psoriasis and Crohn's disease (http://clinicaltrials.gov/).

[0004] TYK2 is a potential target for immuno-inflammatory diseases, being validated by human genetics and mouse knock-out studies (Levy D. and Loomis C. (2007)).

[0005] JAK1 is a novel target in the immuno-inflammatory disease area. JAK1 heterodimerizes with the other JAKs to transduce cytokine-driven pro-inflammatory signaling. Therefore, inhibition of JAK1 and/or other JAKs is expected to be of therapeutic benefit for a range of inflammatory conditions as well as for other diseases driven by JAK-mediated signal transduction.

BACKGROUND OF THE INVENTION

[0006] The degeneration of cartilage is the hallmark of various diseases, among which rheumatoid arthritis and osteoarthritis are the most prominent. Rheumatoid arthritis (RA) is a chronic joint degenerative disease, characterized by inflammation and destruction of the joint structures. When the disease is unchecked, it leads to substantial disability and pain due to loss of joint functionality and even

premature death. The aim of an RA therapy, therefore, is not only to slow down the disease but to attain remission in order to stop the joint destruction. Besides the severity of the disease outcome, the high prevalence of RA (~ 0.8% of the adults are affected worldwide) means a high socio-economic impact. (For reviews on RA, we refer to Smolen and Steiner (2003); Lee and Weinblatt (2001); Choy and Panayi (2001); O'Dell (2004) and Firestein (2003)).

[0007] Osteoarthritis (also referred to as OA, or wear-and-tear arthritis) is the most common form of arthritis and is characterized by loss of articular cartilage, often associated with hypertrophy of the bone and pain. For an extensive review on osteoarthritis, we refer to Wieland *et al.*, (2005).

[0008] Osteoarthritis is difficult to treat. At present, no cure is available and treatment focuses on relieving pain and preventing the affected joint from becoming deformed. Common treatments include the use of non-steroidal anti-inflammatory drugs (NSAIDs). Although dietary supplements such as chondroitin and glucosamine sulphate have been advocated as safe and effective options for the treatment of osteoarthritis, a recent clinical trial revealed that both treatments did not reduce pain associated to osteoarthritis. (Clegg *et al.*, 2006). Taken together, no disease modifying osteoarthritic drugs are available.

[0009] Stimulation of the anabolic processes, blocking catabolic processes, or a combination of these two, may result in stabilization of the cartilage, and perhaps even reversion of the damage, and therefore prevent further progression of the disease. Various triggers may stimulate anabolic stimulation of chondrocytes. Insulin-like growth factor-I (IGF-I) is the predominant anabolic growth factor in synovial fluid and stimulates the synthesis of both proteoglycans and collagen. It has also been shown that members of the bone morphogenetic protein (BMP) family, notably BMP2, BMP4, BMP6, and BMP7, and members of the human transforming growth factor-ß (TGF-ß) family can induce chondrocyte anabolic stimulation (Chubinskaya and Kuettner, 2003). A compound has recently been identified that induces anabolic stimulation of chondrocytes (US 6,500,854; EP 1 391 211). However, most of these compounds show severe side effects and, consequently, there is a strong need for compounds that stimulate chondrocyte differentiation without these side effects.

[0010] Vandeghinste *et al.* (WO 2005/124342) discovered JAK1 as a target whose inhibition might have therapeutic relevance for several diseases including OA. JAK1 belongs to the Janus kinase (JAK) family of cytoplasmic tyrosine kinases, involved in cytokine receptor-mediated intracellular signal transduction. The JAK family consists of 4 members: JAK1, JAK2, JAK3 and TYK2. JAKs are recruited to cytokine receptors, upon binding of the cytokine, followed by heterodimerization of the cytokine receptor and a shared receptor subunit (common gamma-c chain, gp130). JAKs are then activated by auto- and/or transphosphorylation by another JAK, resulting in phosphorylation of the receptors and recruitment and phosphorylation of members of the signal transducer and activator of transcription (STATs). Phosphorylated STATs dimerize and translocate to the nucleus where they bind to enhancer regions of cytokine-responsive genes. Knockout of the JAK1 gene in mice demonstrated that JAK1 plays essential and nonredundant roles during development: JAK1-/- mice died within 24h

after birth and lymphocyte development was severely impaired. Moreover, JAK1 -/- cells were not, or less, reactive to cytokines that use class II cytokine receptors, cytokine receptors that use the gamma-c subunit for signaling and the family of cytokine receptors that use the gp130 subunit for signaling (Rodig *et al.*, 1998).

[0011] Various groups have implicated JAK-STAT signaling in chondrocyte biology. Li *et al.* (2001) showed that Oncostatin M induces MMP and TIMP3 gene expression in primary chondrocytes by activation of JAK/STAT and MAPK signaling pathways. Osaki *et al.* (2003) showed that interferon-gamma mediated inhibition of collagen II in chondrocytes involves JAK-STAT signaling. IL1-beta induces cartilage catabolism by reducing the expression of matrix components, and by inducing the expression of collagenases and inducible nitric oxide synthase (NOS2), which mediates the production of nitric oxide (NO). Otero *et al.*, (2005) showed that leptin and IL1-beta synergistically induced NO production or expression of NOS2 mRNA in chondrocytes, and that that was blocked by a JAK inhibitor. Legendre *et al.* (2003) showed that IL6/IL6Receptor induced downregulation of cartilage-specific matrix genes collagen II, aggrecan core and link protein in bovine articular chondrocytes, and that this was mediated by JAK/STAT signaling. Therefore, these observations suggest a role for JAK kinase activity in cartilage homeostasis and therapeutic opportunities for JAK kinase inhibitors.

[0012] JAK family members have been implicated in additional conditions including myeloproliferative disorders (O'Sullivan *et al*, 2007, Mol Immunol. 44(10):2497-506), where mutations in JAK2 have been identified. This indicates that inhibitors of JAK in particular JAK2 may also be of use in the treatment of myeloproliferative disorders. Additionally, the JAK family, in particular JAK1, JAK2 and JAK3, has been linked to cancers, in particular leukaemias *e.g.* acute myeloid leukaemia (O'Sullivan *et al*, 2007, Mol Immunol. 44(10):2497-506; Xiang *et al.*, 2008, "Identification of somatic *JAK1* mutations in patients with acute myeloid leukemia" Blood First Edition Paper, prepublished online December 26, 2007; DOI 10.1182/blood-2007-05-090308) and acute lymphoblastic leukemia (Mullighan *et al*, 2009) or solid tumours *e.g.* uterine leiomyosarcoma (Constantinescu *et al.*, 2007, Trends in Biochemical Sciences 33(3): 122-131), prostate cancer (Tam *et al.*, 2007, British Journal of Cancer, 97, 378 – 383). These results indicate that inhibitors of JAK, in particular of JAK1 and/or JAK2, may also have utility in the treatment of cancers (leukaemias and solid tumours e.g. uterine leiomyosarcoma, prostate cancer).

[0013] In addition, Castleman's disease, multiple myeloma, mesangial proliferative glomerulonephritis, psoriasis, and Kaposi's sarcoma are likely due to hypersecretion of the cytokine IL-6, whose biological effects are mediated by intracellular JAK-STAT signaling (Tetsuji Naka, Norihiro Nishimoto and Tadamitsu Kishimoto, Arthritis Res 2002, 4 (suppl 3):S233-S242). This result shows that inhibitor of JAK, may also find utility in the treatment of said diseases.

[0014] A link with autoimmune diseases has been established for JAK3 and Tyk2. Mutations in JAK3 but also in the upstream signaling components gamma-c receptor chain and IL7 receptor account in aggregate for ~70% of cases of human severe combined immunodeficiency ('OShea *et al.*, 2004).

Note that JAK1 cooperates with JAK3 in transducing signals from the gamma-c receptor chain. Tyk2 polymorphisms are seen in systemic lupus erythematosus (SLE) (O'Sullivan *et al*, 2007, Mol Immunol. 44(10):2497-506). Hence, targeting the JAK family may provide a therapeutic opportunity in the immuno-inflammation area.

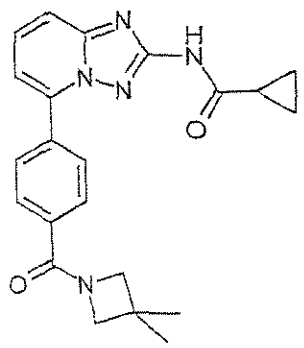
[0015] The current therapies are not satisfactory and therefore there remains a need to identify further compounds that may be of use in the treatment of inflammatory conditions, autoimmune diseases, proliferative diseases, transplantation rejection, diseases involving impairment of cartilage turnover, congenital cartilage malformations, and/or diseases associated with hypersecretion of IL6. The present invention therefore provides a compound, methods for its manufacture and pharmaceutical compositions comprising the compound of the invention together with a suitable pharmaceutical carrier. In particular, the compound exhibits high potency and selectivityfor JAK1 over other JAK family members in addition to 385 kinase and non-kinase targets. Additionally, data indicates that the compound has a good safety margin. Therefore it is concluded that the present invention offers a novel opportunity for treating JAK1 mediated diseases, in particular inflammatory diseases such as SLE (systemic lupus erythematosus) and RA.

[0016] The present invention also provides for the use of the compound of the invention in the preparation of a medicament for the treatment of these diseases and conditions.

SUMMARY OF THE INVENTION

[0017] The present invention is based on the discovery that inhibitors of JAK, in particular JAK1 are useful for the treatment of inflammatory conditions, autoimmune diseases, proliferative diseases, transplantation rejection, diseases involving impairment of cartilage turnover, congenital cartilage malformations, and/or diseases associated with hypersecretion of IL6. The present disclosure also provides methods for the production of these compounds, pharmaceutical compositions comprising these compounds and methods for treating inflammatory conditions, autoimmune diseases, proliferative diseases, transplantation rejection, diseases involving impairment of cartilage turnover, congenital cartilage malformations, and/or diseases associated with hypersecretion of IL6 by administering the compound of the invention.

[0018] Accordingly, in a first aspect of the invention, a compound of the invention is disclosed having a Formula I:



I

or a pharmaceutically acceptable salt thereof.

[0019] The compound of the invention is a novel inhibitor of JAK that exhibits a high potency and selectivity for JAK1 over other JAK family members and 385 kinase and non-kinase targets as well as showing a good safety margin. The use of a compound with this profile may result in advantages in the treatment of inflammatory diseases, in particular SLE and RA due to a lower incidence of off-target effects.

[0019a] In one aspect, there is provided a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a pharmaceutically effective amount of a compound, or pharmaceutically acceptable salt thereof, as defined above.

[0020] In one example, the present disclosure provides pharmaceutical compositions comprising the compound of the invention, and a pharmaceutical carrier, excipient or diluent. Moreover, the compound of the present disclosure useful in the pharmaceutical compositions and treatment methods disclosed herein, is pharmaceutically acceptable as prepared and used. In this example, the pharmaceutical composition may additionally comprise further active ingredients suitable for use in combination with the compound of the invention.

[0021] In another example, the present disclosure provides a method of treating a mammal susceptible to or afflicted with a condition from among those listed herein, and particularly, such condition as may be associated with aberrant JAK activity, for example inflammatory conditions, autoimmune diseases, proliferative diseases, transplantation rejection, diseases involving impairment of cartilage turnover, congenital cartilage malformations, and/or diseases associated with hypersecretion of IL6, which method comprises administering a therapeutically effective amount of the compound of the disclosure, or one or more of the pharmaceutical compositions herein described. In another example, this disclosure provides a method of treating a mammal susceptible to or afflicted with a condition as may be associated with aberrant JAK1 activity, in particular inflammatory conditions, proliferative diseases and diseases involving impairment of cartilage turnover.

[0021a] In one aspect, there is provided the use of a compound, or a pharmaceutically acceptable salt thereof, as defined above in the manufacture of a medicament.

[0021b] In another aspect, there is provided a compound, or pharmaceutically acceptable salt thereof, as defined above for use as a medicament.

[0021c] In another aspect, there is provided a compound, or pharmaceutically acceptable salt thereof, as defined above for use in the treatment, prevention or prophylaxis of inflammatory conditions, autoimmune diseases, proliferative diseases, transplantation rejection, diseases involving impairment of cartilage turnover, congenital cartilage malformations, and/or psoriasis.

[0021d] In another aspect, there is provided the use of a compound, or pharmaceutically acceptable salt thereof, as defined above in the preparation of a medicament for the treatment, prevention or prophylaxis of inflammatory conditions, autoimmune diseases, proliferative diseases, transplantation rejection, diseases involving impairment of cartilage turnover, congenital cartilage malformations, and/or psoriasis.

[0022] In another example, the present disclosure provides the compound of the invention for use in the treatment or prevention of a condition selected from those listed herein, particularly such conditions as may be associated with aberrant JAK activity such as inflammatory conditions, autoimmune diseases, proliferative diseases, transplantation rejection, diseases involving impairment of cartilage turnover, congenital cartilage malformations, and/or diseases associated with hypersecretion of IL6. In a specific example, the present disclosure provides the compound as described herein for use in the treatment or

prevention of conditions associated with aberrant JAK1 activity, in particular inflammatory conditions, proliferative diseases and diseases involving impairment of cartilage turnover.

[0023] In yet another example, the present disclosure provides a method for treating a mammal susceptible to or afflicted with a condition that is casually related to abnormal JAK activity as described herein, which method comprises administering an effective condition-treating or condition-preventing amount of one or more of the pharmaceutical compositions or the compound of the invention herein described. In a specific embodiment the abnormal JAK activity is abnormal JAK1 activity.

[0024] In another example, the present disclosure provides the compound as described herein for use in the treatment or prevention of a condition that is casually related to abnormal JAK activity. In a specific embodiment the abnormal JAK activity is abnormal JAK1 activity.

[0025] In additional examples, the present disclosure provides methods for synthesizing the compound as described herein, with representative synthetic protocols and pathways disclosed later on herein.

[0026] Accordingly, it is a principal object of this disclosure to provide a compound, which can modify the activity of JAK and thus prevent or treat any conditions that may be causally related thereto. In particular any conditions which may be causally related to the activity of JAK1.

[0027] It is further an object of the present disclosure to provide a compound that can treat or alleviate conditions or diseases or symptoms of same, such as inflammatory conditions, autoimmune diseases, proliferative diseases, transplantation rejection, diseases involving impairment of cartilage turnover, congenital cartilage malformations, and diseases associated with hypersecretion of IL6, that may be causally related to the activity of JAK. In particular disease or condition which may be causally related to the activity of JAK1.

[0028] Also disclosed are pharmaceutical compositions that may be used in the treatment or prevention of a variety of disease states, including the diseases associated with JAK activity such as inflammatory conditions, autoimmune diseases, proliferative diseases, transplantation rejection, diseases involving impairment of cartilage turnover, congenital cartilage malformations, and diseases associated with hypersecretion of IL6. In particular conditions associated with JAK1 activity.

[0029] Other objects and advantages will become apparent to those skilled in the art from a consideration of the ensuing detailed description.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

[0030] The following terms are intended to have the meanings presented therewith below and are useful in understanding the description and intended scope of the present invention.

[0031] When describing the invention, which may include compounds, pharmaceutical compositions containing such compounds and methods of using such compounds and compositions, the following terms, if present, have the following meanings unless otherwise indicated. It should also be understood

that when described herein any of the moieties defined forth below may be substituted with a variety of substituents, and that the respective definitions are intended to include such substituted moieties within their scope as set out below. Unless otherwise stated, the term "substituted" is to be defined as set out below. It should be further understood that the terms "groups" and "radicals" can be considered interchangeable when used herein.

[0032] The articles "a" and "an" may be used herein to refer to one or to more than one (i.e. at least one) of the grammatical objects of the article. By way of example "an analogue" means one analogue or more than one analogue.

[0033] 'Pharmaceutically acceptable' means approved or approvable by a regulatory agency of the Federal or a state government or the corresponding agency in countries other than the United States, or that is listed in the U.S. Pharmacopoeia or other generally recognized pharmacopoeia for use in animals, and more particularly, in humans.

[0034] 'Pharmaceutically acceptable salt' refers to a salt of the compound of the invention that is pharmaceutically acceptable and that possesses the desired pharmacological activity of the parent compound. In particular, such salts are non-toxic may be inorganic or organic acid addition salts and base addition salts. Specifically, such salts include: (1) acid addition salts, formed with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, and the like; or formed with organic acids such as acetic acid, propionic acid, hexanoic acid, cyclopentanepropionic acid, glycolic acid, pyruvic acid, lactic acid, malonic acid, succinic acid, malic acid, maleic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, 3-(4-hydroxybenzoyl) benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, 1,2-ethane-disulfonic acid, 2-hydroxyethanesulfonic acid, benzenesulfonic acid, 4-chlorobenzenesulfonic acid, 2-naphthalenesulfonic acid, 4-toluenesulfonic acid, camphorsulfonic acid, 4-methylbicyclo[2.2.2]-oct-2-ene-1-carboxylic acid, glucoheptonic acid, 3-phenylpropionic acid, trimethylacetic acid, tertiary butylacetic acid, lauryl sulfuric acid, gluconic acid, glutamic acid, hydroxynaphthoic acid, salicylic acid, stearic acid, muconic acid, and the like; or (2) salts formed when an acidic proton present in the parent compound either is replaced by a metal ion, e.g., an alkali metal ion, an alkaline earth ion, or an aluminum ion; or coordinates with an organic base such as ethanolamine, diethanolamine, triethanolamine, N-methylglucamine and the like. Salts further include, by way of example only, sodium, potassium, calcium, magnesium, ammonium, tetraalkylammonium, and the like; and when the compound contains a basic functionality, salts of non toxic organic or inorganic acids, such as hydrochloride, hydrobromide, tartrate, mesylate, acetate, maleate, oxalate and the like. The term "pharmaceutically acceptable cation" refers to an acceptable cationic counter-ion of an acidic functional group. Such cations are exemplified by sodium, potassium, calcium, magnesium, ammonium, tetraalkylammonium cations, and the like.

[0035] 'Pharmaceutically acceptable vehicle' refers to a diluent, adjuvant, excipient or carrier with which the compound of the invention is administered.

[0036] 'Prodrugs' refers to compounds, including derivatives of the compounds of the invention,which have cleavable groups and become by solvolysis or under physiological conditions the compounds of the invention which are pharmaceutically active in vivo. Such examples include, but are not limited to, choline ester derivatives and the like, N-alkylmorpholine esters and the like.

[0037] 'Solvate' refers to forms of the compound that are associated with a solvent, usually by a solvolysis reaction. This physical association includes hydrogen bonding. Conventional solvents include water, ethanol, acetic acid and the like. The compounds of the invention may be prepared e.g. in crystalline form and may be solvated or hydrated. Suitable solvates include pharmaceutically acceptable solvates, such as hydrates, and further include both stoichiometric solvates and non-stoichiometric solvates. In certain instances the solvate will be capable of isolation, for example when one or more solvent molecules are incorporated in the crystal lattice of the crystalline solid. 'Solvate' encompasses both solution-phase and isolable solvates. Representative solvates include hydrates, ethanolates and methanolates.

[0038] 'Subject' includes humans. The terms 'human', 'patient' and 'subject' are used interchangeably herein.

[0039] 'Therapeutically effective amount' means the amount of a compound that, when administered to a subject for treating a disease, is sufficient to effect such treatment for the disease. The "therapeutically effective amount" can vary depending on the compound, the disease and its severity, and the age, weight, etc., of the subject to be treated.

[0040] 'Preventing' or 'prevention' refers to a reduction in risk of acquiring or developing a disease or disorder (i.e., causing at least one of the clinical symptoms of the disease not to develop in a subject that may be exposed to a disease-causing agent, or predisposed to the disease in advance of disease onset.

[0041] The term 'prophylaxis' is related to 'prevention', and refers to a measure or procedure the purpose of which is to prevent, rather than to treat or cure a disease. Non-limiting examples of prophylactic measures may include the administration of vaccines; the administration of low molecular weight heparin to hospital patients at risk for thrombosis due, for example, to immobilization; and the administration of an anti-malarial agent such as chloroquine, in advance of a visit to a geographical region where malaria is endemic or the risk of contracting malaria is high.

[0042] 'Treating' or 'treatment' of any disease or disorder refers, in one embodiment, to ameliorating the disease or disorder (i.e., arresting the disease or reducing the manifestation, extent or severity of at least one of the clinical symptoms thereof). In another embodiment 'treating' or 'treatment' refers to ameliorating at least one physical parameter, which may not be discernible by the subject. In yet another embodiment, 'treating' or 'treatment' refers to modulating the disease or disorder, either physically, (e.g., stabilization of a discernible symptom), physiologically, (e.g., stabilization of a physical parameter), or both. In a further embodiment, "treating" or "treatment" relates to slowing the progression of the disease.

[0043] As used herein the term 'inflammatory condition(s)' refers to the group of conditions including, rheumatoid arthritis, osteoarthritis, juvenile idiopathic arthritis, psoriasis, allergic airway disease (e.g. asthma, rhinitis), inflammatory bowel diseases (e.g. Crohn's disease, colitis), endotoxin-driven disease states (e.g. complications after bypass surgery or chronic endotoxin states contributing to e.g. chronic cardiac failure), and related diseases involving cartilage, such as that of the joints. Partcicularly the term refers to rheumatoid arthritis, osteoarthritis, allergic airway disease (e.g. asthma) and inflammatory bowel diseases.

[0044] As used herein the term 'autoimmune disease(s)' refers to the group of diseases including obstructive airways disease, including conditions such as COPD, asthma (e.g intrinsic asthma, extrinsic asthma, dust asthma, infantily asthma) particularly chronic or inveterate asthma (for example late asthma and airway hyperreponsiveness), bronchitis, including bronchial asthma, systemic lupus erythematosus (SLE), multiple sclerosis, type I diabetes mellitus and complications associated therewith, atopic eczema (atopic dermatitis), contact dermatitis and further eczematous dermatitis, inflammatory bowel disease (e.g. Crohn's disease and ulcerative colitis), atherosclerosis and amyotrophic lateral sclerosis. Particularly the term refers to COPD, asthma, systemic lupus erythematosis, type I diabetes mellitus and inflammatory bowel disease.

[0045] As used herein the term 'proliferative disease(s)' refers to conditions such as cancer (e.g. uterine leiomyosarcoma or prostate cancer), myeloproliferative disorders (e.g. polycythemia vera, essential thrombocytosis and myelofibrosis), leukemia (e.g. acute myeloid leukaemia and acute lymphoblastic leukemia), multiple myeloma, psoriasis, restenosis, sclerodermitis or fibrosis. In particular the term refers to cancer, leukemia, multiple myeloma and psoriasis.

[0046] As used herein, the term 'cancer' refers to a malignant or benign growth of cells in skin or in body organs, for example but without limitation, breast, prostate, lung, kidney, pancreas, stomach or bowel. A cancer tends to infiltrate into adjacent tissue and spread (metastasise) to distant organs, for example to bone, liver, lung or the brain. As used herein the term cancer includes both metastatic tumour cell types, such as but not limited to, melanoma, lymphoma, leukaemia, fibrosarcoma, rhabdomyosarcoma, and mastocytoma and types of tissue carcinoma, such as but not limited to, colorectal cancer, prostate cancer, small cell lung cancer and non-small cell lung cancer, breast cancer, pancreatic cancer, bladder cancer, renal cancer, gastric cancer, glioblastoma, primary liver cancer, ovarian cancer, prostate cancer and uterine leiomyosarcoma.

[0047] As used herein the term 'leukemia' refers to neoplastic diseases of the blood and blood forming organs. Such diseases can cause bone marrow and immune system dysfunction, which renders the host highly susceptible to infection and bleeding. In particular the term leukemia refers to acute myeloid leukaemia (AML) and acute lymphoblastic leukemia (ALL).

[0048] As used herein the term 'transplantation rejection' refers to the acute or chronic rejection of cells, tissue or solid organ allo- or xenografts of e.g. pancreatic islets, stem cells, bone marrow, skin,

muscle, corneal tissue, neuronal tissue, heart, lung, combined heart-lung, kidney, liver, bowel, pancreas, trachea or oesophagus, or graft-versus-host diseases.

[0049] As used herein the term 'diseases involving impairment of cartilage turnover' includes conditions such as osteoarthritis, psoriatic arthritis, juvenile rheumatoid arthritis, gouty arthritis, septic or infectious arthritis, reactive arthritis, reflex sympathetic dystrophy, algodystrophy, Tietze syndrome or costal chondritis, fibromyalgia, osteochondritis, neurogenic or neuropathic arthritis, arthropathy, endemic forms of arthritis like osteoarthritis deformans endemica, Mseleni disease and Handigodu disease; degeneration resulting from fibromyalgia, systemic lupus erythematosus, scleroderma and ankylosing spondylitis.

[0050] As used herein the term 'congenital cartilage malformation(s)' includes conditions such as hereditary chondrolysis, chondrodysplasias and pseudochondrodysplasias, in particular, but without limitation, microtia, anotia, metaphyseal chondrodysplasia, and related disorders.

[0051] As used herein the term 'disease(s) associated with hypersecretion of IL6' includes conditions such as Castleman's disease, multiple myeloma, psoriasis, Kaposi's sarcoma and/or mesangial proliferative glomerulonephritis.

[0052] As used herein the term "JAK" relates to the family of Janus kinases (JAKs) which are cytoplasmic tyrosine kinases that transduce cytokine signaling from membrane receptors to STAT transcription factors. Four JAK family members are described, JAK1, JAK2, JAK3 and TYK2 and the term JAK may refer to all the JAK family members collectively or one or more of the JAK family members as the context indicates.

[0053] 'Compound(s) of the invention', and equivalent expressions, are meant to embrace the compound of the Formula as herein described, which expression includes the pharmaceutically acceptable salts, and the solvates, e.g., hydrates, and the solvates of the pharmaceutically acceptable salts where the context so permits. Similarly, reference to intermediates, whether or not they themselves are claimed, is meant to embrace their salts, and solvates, where the context so permits.

[0054] When ranges are referred to herein, for example but without limitation, C_1-C_8 alkyl, the citation of a range should be considered a representation of each member of said range.

[0055] Other derivatives of the compounds of this invention have activity in both their acid and acid derivative forms, but in the acid sensitive form often offers advantages of solubility, tissue compatibility, or delayed release in the mammalian organism (see, Bundgard, H., Design of Prodrugs, pp. 7-9, 21-24, Elsevier, Amsterdam 1985). Prodrugs include acid derivatives well know to practitioners of the art, such as, for example, esters prepared by reaction of the parent acid with a suitable alcohol, or amides prepared by reaction of the parent acid compound with a substituted or unsubstituted amine, or acid anhydrides, or mixed anhydrides. Simple aliphatic or aromatic esters, amides and anhydrides derived from acidic groups pendant on the compounds of this invention are particularly useful prodrugs. In some cases it is desirable to prepare double ester type prodrugs such as (acyloxy)alkyl esters or

((alkoxycarbonyl)oxy)alkylesters. Particular such prodrugs are the C_1 to C_8 alkyl, C_2-C_8 alkenyl, aryl, C_7-C_{12} substituted aryl, and C_7-C_{12} arylalkyl esters of the compounds of the invention.

[0056] As used herein, the term 'isotopic variant' refers to a compound that contains unnatural proportions of isotopes at one or more of the atoms that constitute such compound. For example, an 'isotopic variant' of a compound can contain one or more non-radioactive isotopes, such as for example, deuterium (^{2}H or D), carbon-13 (^{13}C), nitrogen-15 (^{15}N), or the like. It will be understood that, in a compound where such isotopic substitution is made, the following atoms, where present, may vary, so that for example, any hydrogen may be ^{2}H/D, any carbon may be ^{13}C, or any nitrogen may be ^{15}N, and that the presence and placement of such atoms may be determined within the skill of the art. Likewise, the invention may include the preparation of isotopic variants with radioisotopes, in the instance for example, where the resulting compounds may be used for drug and/or substrate tissue distribution studies. The radioactive isotopes tritium, i.e. ^{3}H, and carbon-14, i.e. ^{14}C, are particularly useful for this purpose in view of their ease of incorporation and ready means of detection. Further, compounds may be prepared that are substituted with positron emitting isotopes, such as ^{11}C, ^{18}F, ^{15}O and ^{13}N, and would be useful in Positron Emission Topography (PET) studies for examining substrate receptor occupancy.

[0057] All isotopic variants of the compound provided herein, radioactive or not, are intended to be encompassed within the scope of the invention.

[0058] Stereoisomers that are not mirror images of one another are termed 'diastereomers' and those that are non-superimposable mirror images of each other are termed 'enantiomers'. When a compound has an asymmetric center, for example, it is bonded to four different groups, a pair of enantiomers is possible. An enantiomer can be characterized by the absolute configuration of its asymmetric center and is described by the R- and S-sequencing rules of Cahn and Prelog, or by the manner in which the molecule rotates the plane of polarized light and designated as dextrorotatory or levorotatory (i.e., as (+) or (-)-isomers respectively). A chiral compound can exist as either individual enantiomer or as a mixture thereof. A mixture containing equal proportions of the enantiomers is called a 'racemic mixture'.

[0059] 'Tautomers' refer to compounds that are interchangeable forms of a particular compound structure, and that vary in the displacement of hydrogen atoms and electrons. Thus, two structures may be in equilibrium through the movement of π electrons and an atom (usually H). For example, enols and ketones are tautomers because they are rapidly interconverted by treatment with either acid or base. Another example of tautomerism is the aci- and nitro- forms of phenylnitromethane, that are likewise formed by treatment with acid or base.

[0060] Tautomeric forms may be relevant to the attainment of the optimal chemical reactivity and biological activity of a compound of interest.

[0061] The compound of the invention may possess one or more asymmetric centers; such compounds can therefore be produced as individual (R)- or (S)- stereoisomers or as mixtures thereof.

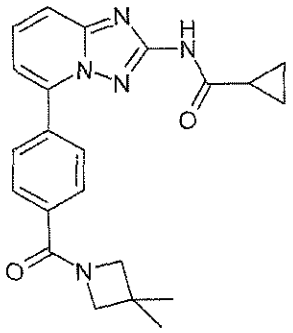
[0062] Unless indicated otherwise, the description or naming of a particular compound in the specification and claims is intended to include both individual enantiomers and mixtures, racemic or

otherwise, thereof. The methods for the determination of stereochemistry and the separation of stereoisomers are well-known in the art.

THE COMPOUND

[0063] The present invention is based on the discovery that inhibitors of JAK are useful for the treatment of inflammatory conditions, autoimmune diseases, proliferative diseases, transplantation rejection, diseases involving impairment of cartilage turnover, congenital cartilage malformations, and diseases associated with hypersecretion of IL6. The present invention also provides methods for the production of the compound, pharmaceutical compositions comprising the compound and methods for treating diseases involving cartilage degradation, bone and/or joint degradation and/or inflammation by administering the compound of the invention. The present compound is an inhibitor of members of the JAK family; specifically it inhibits the activity of of JAK1, JAK2, JAK3 and TYK2. In particular it inhibits the activity of JAK1.

[0064] Accordingly, in a first aspect of the invention, a compound of the invention is disclosed having a Formula I:



I

[0065] The compound of the invention is cyclopropanecarboxylic acid {5-[4-(3,3-dimethyl-azetidine-1-carbonyl)-phenyl]-[1,2,4]triazolo[1,5-a]pyridin-2-yl}-amide.

[0066] In one embodiment the compound of the invention is not an isotopic variant.

[0067] In one aspect the compound of the invention is present as the free base.

[0068] In one aspect the compound of the invention is a pharmaceutically acceptable salt.

[0069] In one aspect the compound of the invention is a solvate of the compound.

[0070] In one aspect the compound of the invention is a solvate of a pharmaceutically acceptable salt of the compound.

[0071] In certain aspects, the present invention provides prodrugs and derivatives of the compound according to the formula above. Prodrugs are derivatives of the compounds of the invention, which have metabolically cleavable groups and become by solvolysis or under physiological conditions the

compounds of the invention, which are pharmaceutically active, *in vivo*. Such examples include, but are not limited to, choline ester derivatives and the like, N-alkylmorpholine esters and the like.

[0072] Other derivatives of the compound of the invention have activity in both their acid and acid derivative forms, but the acid sensitive form often offers advantages of solubility, tissue compatibility, or delayed release in the mammalian organism (see, Bundgard, H., Design of Prodrugs, pp. 7-9, 21-24, Elsevier, Amsterdam 1985). Prodrugs include acid derivatives well know to practitioners of the art, such as, for example, esters prepared by reaction of the parent acid with a suitable alcohol, or amides prepared by reaction of the parent acid compound with a substituted or unsubstituted amine, or acid anhydrides, or mixed anhydrides. Simple aliphatic or aromatic esters, amides and anhydrides derived from acidic groups pendant on the compounds of this invention are preferred prodrugs. In some cases it is desirable to prepare double ester type prodrugs such as (acyloxy)alkyl esters or ((alkoxycarbonyl)oxy)alkylesters. Particularly useful are the C_1 to C_8 alkyl, C_2-C_8 alkenyl, aryl, C_7-C_{12} substituted aryl, and C_7-C_{12} arylalkyl esters of the compounds of the invention.

PHARMACEUTICAL COMPOSITIONS

[0073] When employed as a pharmaceutical, the compound of the invention is typically administered in the form of a pharmaceutical composition. Such compositions can be prepared in a manner well known in the pharmaceutical art and comprise at least one active compound. Generally, the compound of the invention is administered in a pharmaceutically effective amount. The amount of the compound actually administered will typically be determined by a physician, in the light of the relevant circumstances, including the condition to be treated, the chosen route of administration, the actual compound -administered, the age, weight, and response of the individual patient, the severity of the patient's symptoms, and the like.

[0074] The pharmaceutical compositions of the invention can be administered by a variety of routes including oral, rectal, transdermal, subcutaneous, intra-articular, intravenous, intramuscular, and intranasal. Depending on the intended route of delivery, the compound of this invention is preferably formulated as either injectable or oral compositions or as salves, as lotions or as patches all for transdermal administration.

[0075] The compositions for oral administration can take the form of bulk liquid solutions or suspensions, or bulk powders. More commonly, however, the compositions are presented in unit dosage forms to facilitate accurate dosing. The term "unit dosage forms" refers to physically discrete units suitable as unitary dosages for human subjects and other mammals, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect, in association with a suitable pharmaceutical excipient, vehicle or carrier. Typical unit dosage forms include prefilled, premeasured ampules or syringes of the liquid compositions or pills, tablets, capsules or the like in the case of solid compositions. In such compositions, the compound of the invention is usually a minor component (from about 0.1 to about 50% by weight or preferably from about 1 to about 40% by weight)

with the remainder being various vehicles or carriers and processing aids helpful for forming the desired dosing form.

[0076] Liquid forms suitable for oral administration may include a suitable aqueous or nonaqueous vehicle with buffers, suspending and dispensing agents, colorants, flavors and the like. Solid forms may include, for example, any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

[0077] Injectable compositions are typically based upon injectable sterile saline or phosphate-buffered saline or other injectable carriers known in the art. As before, the active compound in such compositions is typically a minor component, often being from about 0.05 to 10% by weight with the remainder being the injectable carrier and the like.

[0078] Transdermal compositions are typically formulated as a topical ointment or cream containing the active ingredient(s), generally in an amount ranging from about 0.01 to about 20% by weight, preferably from about 0.1 to about 20% by weight, preferably from about 0.1 to about 10% by weight, and more preferably from about 0.5 to about 15% by weight. When formulated as a ointment, the active ingredients will typically be combined with either a paraffinic or a water-miscible ointment base. Alternatively, the active ingredients may be formulated in a cream with, for example an oil-in-water cream base. Such transdermal formulations are well-known in the art and generally include additional ingredients to enhance the dermal penetration of stability of the active ingredients or the formulation. All such known transdermal formulations and ingredients are included within the scope of this invention.

[0079] The compound of the invention can also be administered by a transdermal device. Accordingly, transdermal administration can be accomplished using a patch either of the reservoir or porous membrane type, or of a solid matrix variety.

[0080] The above-described components for orally administrable, injectable or topically administrable compositions are merely representative. Other materials as well as processing techniques and the like are set forth in Part 8 of Remington's Pharmaceutical Sciences, 17th edition, 1985, Mack Publishing Company, Easton, Pennsylvania, which is incorporated herein by reference.

[0081] The compound of the invention can also be administered in sustained release forms or from sustained release drug delivery systems. A description of representative sustained release materials can be found in Remington's Pharmaceutical Sciences.

[0082] The following formulation examples illustrate representative pharmaceutical compositions that may be prepared in accordance with this invention. The present invention, however, is not limited to the following pharmaceutical compositions.

Formulation 1 - Tablets

[0083] The compound of the invention may be admixed as a dry powder with a dry gelatin binder in an approximate 1:2 weight ratio. A minor amount of magnesium stearate may be added as a lubricant. The mixture may be formed into 240-270 mg tablets (80-90 mg of active amide compound per tablet) in a tablet press.

Formulation 2 - Capsules

[0084] The compound of the invention may be admixed as a dry powder with a starch diluent in an approximate 1:1 weight ratio. The mixture may be filled into 250 mg capsules (125 mg of active amide compound per capsule).

Formulation 3 - Liquid

[0085] The compound of the invention (125 mg), may be admixed with sucrose (1.75 g) and xanthan gum (4 mg) and the resultant mixture may be blended, passed through a No. 10 mesh U.S. sieve, and then mixed with a previously made solution of microcrystalline cellulose and sodium carboxymethyl cellulose (11:89, 50 mg) in water. Sodium benzoate (10 mg), flavor, and color may be diluted with water and added with stirring. Sufficient water may then be added with stirring. Sufficient water may be then added to produce a total volume of 5 mL.

Formulation 4 - Tablets

[0086] The compound of the invention may be admixed as a dry powder with a dry gelatin binder in an approximate 1:2 weight ratio. A minor amount of magnesium stearate may be added as a lubricant. The mixture is formed into 450-900 mg tablets (150-300 mg of active amide compound) in a tablet press.

Formulation 5 - Injection

[0087] The compound of the invention may be dissolved or suspended in a buffered sterile saline injectable aqueous medium to a concentration of approximately 5 mg/mL.

Formulation 6 - Topical

[0088] Stearyl alcohol (250 g) and a white petrolatum (250 g) may be melted at about 75°C and then a mixture of the compound of the invention (50 g) methylparaben (0.25 g), propylparaben (0.15 g), sodium lauryl sulfate (10 g), and propylene glycol (120 g) dissolved in water (about 370 g) may be added and the resulting mixture may be stirred until it congeals.

METHODS OF TREATMENT

[0089] The compound of the invention may be used as a therapeutic agent for the treatment of conditions in mammals that are causally related or attributable to aberrant activity of JAK. In particular, conditions related to aberrant activity of JAK1. Accordingly, the compound and pharmaceutical compositions of the invention find use as therapeutics for preventing and/or treating inflammatory conditions, autoimmune diseases, proliferative diseases, transplantation rejection, diseases involving impairment of cartilage turnover, congenital cartilage malformations, and diseases associated with hypersecretion of IL6 in mammals including humans.

[0090] In additional method of treatment aspects, this invention provides methods of treating a mammal susceptible to or afflicted with an inflammatory condition. The methods comprise administering an effective condition-treating or condition-preventing amount of one or more of the pharmaceutical compositions or the compound of the invention herein described. In a specific embodiment, the inflammatory condition is selected from rheumatoid arthritis, osteoarthritis, allergic airway disease (e.g. asthma) and inflammatory bowel diseases.

[0091] In another aspect the present invention provides the compound of the invention for use in the treatment, prevention or prophylaxis of an inflammatory condition. In a specific embodiment, the inflammatory condition is selected from rheumatoid arthritis, osteoarthritis, allergic airway disease (e.g. asthma) and inflammatory bowel diseases.

[0092] In additional method of treatment aspects, this invention provides methods of treating a mammal susceptible to or afflicted with an autoimmune disease. The methods comprise administering an effective condition-treating or condition-preventing amount of one or more of the pharmaceutical compositions or the compound of the invention herein described. In a specific embodiment, the autoimmune disease is selected from COPD, asthma, systemic lupus erythematosis, type I diabetes mellitus and inflammatory bowel disease.

[0093] In another aspect the present invention provides the compound of the invention for use in the treatment, prevention or prophylaxis of an autoimmune disease. In a specific embodiment, the autoimmune disease is selected from COPD, asthma, systemic lupus erythematosis, type I diabetes mellitus and inflammatory bowel disease.

[0094] In further method of treatment aspects, this invention provides methods of treating a mammal susceptible to or afflicted with a proliferative disease, in particular cancer (e.g. solid tumors such as uterine leiomyosarcoma or prostate cancer), leukemia (e.g. AML or ALL), multiple myeloma and/or psoriasis. The methods comprise administering an effective condition-treating or condition-preventing amount of one or more of the pharmaceutical compositions or the compound of the invention herein described.

[0095] In another aspect the present invention provides the compound of the invention for use in the treatment, prevention or prophylaxis of a proliferative disease, in particular cancer (e.g. solid tumors such as uterine leiomyosarcoma or prostate cancer), leukemia (e.g. AML or ALL), multiple myeloma and/or psoriasis.

[0096] In further method of treatment aspects, this invention provides methods of treating a mammal susceptible to or afflicted with transplantation rejection. The methods comprise administering an effective condition-treating or condition-preventing amount of one or more of the pharmaceutical compositions or the compound of the invention herein described. In a specific embodiment, the invention provides methods of treating organ transplant rejection.

[0097] In another aspect the present invention provides the compound of the invention for use in the treatment, prevention or prophylaxis of transplantation rejection. In a specific embodiment, the invention provides methods of treating organ transplant rejection.

[0098] In a method of treatment aspect, this invention provides a method of treatment, prevention or prophylaxis in a mammal susceptible to or afflicted with diseases involving impairment of cartilage turnover. The methods comprise administering an effective condition-treating or condition-preventing amount of one or more of the pharmaceutical compositions or the compound of the invention herein described.

[0099] In another aspect the present invention provides the compound of the invention for use in the treatment, prevention or prophylaxis of diseases involving impairment of cartilage turnover.

[00100] The present invention also provides a method of treatment of congenital cartilage malformations. The methods comprise administering an effective condition-treating or condition-preventing amount of one or more of the pharmaceutical compositions or the compound of the invention herein described.

[00101] In another aspect the present invention provides the compound of the invention for use in the treatment, prevention or prophylaxis of congenital cartilage malformations.

[00102] In further method of treatment aspects, this invention provides methods of treating a mammal susceptible to or afflicted with diseases associated with hypersecretion of IL6, in particular Castleman's disease or mesangial proliferative glomerulonephritis. The methods comprise administering an effective condition-treating or condition-preventing amount of one or more of the pharmaceutical compositions or the compound of the invention herein described.

[00103] In another aspect the present invention provides the compound of the invention for use in the treatment, prevention or prophylaxis of diseases associated with hypersecretion of IL6, in particular Castleman's disease or mesangial proliferative glomerulonephritis.

[00104] As a further aspect of the invention there is provided the compound of the invention for use as a pharmaceutical especially in the treatment or prevention of the aforementioned conditions and diseases. Also provided herein is the use of the compound in the manufacture of a medicament for the treatment or prevention of one of the aforementioned conditions and diseases.

[00105] A particular regimen of the present method comprises the administration to a subject in suffering from an inflammatory condition, of an effective amount of the compound of the invention for a period of time sufficient to reduce the level of inflammation in the subject, and preferably terminate, the processes responsible for said inflammation. A special embodiment of the method comprises administering of an effective amount of the compound of the invention to a subject patient suffering from or susceptible to the development of rheumatoid arthritis, for a period of time sufficient to reduce or prevent, respectively, inflammation in the joints of said patient, and preferably terminate, the processes responsible for said inflammation.

[00106] A further particular regimen of the present method comprises the administration to a subject suffering from diseases involving impairment of cartilage turnover (e.g. osteoarthritis) of an effective amount of the compound of the invention for a period of time sufficient to reduce and preferably terminate, the self-perpetuating processes responsible for said degradation. A particular embodiment of the method comprises administering of an effective amount of the compound of the invention to a subject patient suffering from or susceptible to the development of osteoarthritis, for a period of time sufficient to reduce or prevent, respectively, cartilage degradation in the joints of said patient, and preferably terminate, the self-perpetuating processes responsible for said degradation. In a particular embodiment said compound may exhibit cartilage anabolic and/or anti-catabolic properties.

[00107] Injection dose levels range from about 0.1 mg/kg/hour to at least 10 mg/kg/hour, all for from about 1 to about 120 hours and especially 24 to 96 hours. A preloading bolus of from about 0.1 mg/kg to about 10 mg/kg or more may also be administered to achieve adequate steady state levels. The maximum total dose is not expected to exceed about 2 g/day for a 40 to 80 kg human patient.

[00108] For the prevention and/or treatment of long-term conditions, such as degenerative conditions, the regimen for treatment usually stretches over many months or years so oral dosing is preferred for patient convenience and tolerance. With oral dosing, one to five and especially two to four and typically three oral doses per day are representative regimens. Using these dosing patterns, each dose provides from about 0.01 to about 20 mg/kg of the compound of the invention, with particular doses each providing from about 0.1 to about 10 mg/kg and especially about 1 to about 5 mg/kg.

[00109] Transdermal doses are generally selected to provide similar or lower blood levels than are achieved using injection doses.

[00110] When used to prevent the onset of an inflammatory condition, the compound of the invention will be administered to a patient at risk for developing the condition, typically on the advice and under the supervision of a physician, at the dosage levels described above. Patients at risk for developing a particular condition generally include those that have a family history of the condition, or those who have been identified by genetic testing or screening to be particularly susceptible to developing the condition.

[00111] The compound of the invention can be administered as the sole active agent or it can be administered in combination with other therapeutic agents, including other compounds that demonstrate the same or a similar therapeutic activity, and that are determined to be safe and efficacious for such combined administration. In a specific embodiment, co-administration of two (or more) agents allows for significantly lower doses of each to be used, thereby reducing the side effects seen.

[00112] In one embodiment, the compound of the invention is co-administered with another therapeutic agent for the treatment and/or prevention of an inflammatory condition; particular agents include, but are not limited to, immunoregulatory agents e.g. azathioprine, corticosteroids (e.g. prednisolone or dexamethasone), cyclophosphamide, cyclosporin A, tacrolimus, Mycophenolate Mofetil, muromonab-CD3 (OKT3, e.g. Orthocolone®), ATG, aspirin, acetaminophen, ibuprofen, naproxen, and piroxicam.

[00113] In one embodiment, the compound of the invention is co-administered with another therapeutic agent for the treatment and/or prevention of arthritis (e.g. rheumatoid arthritis); particular agents include but are not limited to analgesics, non-steroidal anti-inflammatory drugs (NSAIDS), steroids, synthetic DMARDS (for example but without limitation methotrexate, leflunomide, sulfasalazine, auranofin, sodium aurothiomalate, penicillamine, chloroquine, hydroxychloroquine, azathioprine, and cyclosporin), and biological DMARDS (for example but without limitation Infliximab, Etanercept, Adalimumab, Rituximab, and Abatacept).

[00114] In one embodiment, the compound of the invention is co-administered with another therapeutic agent for the treatment and/or prevention of proliferative disorders; particular agents include but are not limited to: methotrexate, leukovorin, adriamycin, prenisone, bleomycin, cyclophosphamide, 5-fluorouracil, paclitaxel, docetaxel, vincristine, vinblastine, vinorelbine, doxorubicin, tamoxifen, toremifene, megestrol acetate, anastrozole, goserelin, anti-HER2 monoclonal antibody (e.g. Herceptin™), capecitabine, raloxifene hydrochloride, EGFR inhibitors (e.g. Iressa®, Tarceva™, Erbitux™), VEGF inhibitors (e.g. Avastin™), proteasome inhibitors (e.g. Velcade™), Glivec® and hsp90 inhibitors (e.g. 17-AAG). Additionally, a compound of the invention may be administered in combination with other therapies including, but not limited to, radiotherapy or surgery. In a specific embodiment the proliferative disorder is selected from cancer, myeloproliferative disease or leukaemia.

[00115] In one embodiment, the compound of the invention is co-administered with another therapeutic agent for the treatment and/or prevention of autoimmune diseases; particular agents include but are not limited to: glucocorticoids, cytostatic agents (e.g. purine analogs), alkylating agents, (e.g nitrogen mustards (cyclophosphamide), nitrosoureas, platinum compounds, and others), antimetabolites (e.g. methotrexate, azathioprine and mercaptopurine), cytotoxic antibiotics (e.g. dactinomycin anthracyclines, mitomycin C, bleomycin, and mithramycin), antibodies(e.g., anti-CD20, anti-CD25 or anti-CD3 (OTK3) monoclonal antibodies, Atgam® and Thymoglobuline®), cyclosporin, tacrolimus, rapamycin (sirolimus), interferons (e.g. IFN-β), TNF binding proteins (e.g. infliximab (Remicade™), etanercept (Enbrel™), or adalimumab (Humira™)), mycophenolate, Fingolimod, and Myriocin.

[00116] In one embodiment, the compound of the invention is co-administered with another therapeutic agent for the treatment and/or prevention of transplantation rejection; particular agents include but are not limited to: calcineurin inhibitors (e.g. cyclosporin or tacrolimus (FK506)), mTOR inhibitors (e.g. sirolimus, everolimus), anti-proliferatives (e.g. azathioprine, mycophenolic acid), corticosteroids (e.g. prednisolone, hydrocortisone), Antibodies (e.g. monoclonal anti-IL-2Rα receptor antibodies, basiliximab, daclizumab), polyclonal anti-T-cell antibodies (e.g. anti-thymocyte globulin (ATG), anti-lymphocyte globulin (ALG)).

[00117] In one embodiment, the compound of the invention is co-administered with another therapeutic agent for the treatment and/or prevention of asthma and/or rhinitis and/or COPD; particular agents include but are not limited to: beta$_2$-adrenoceptor agonists (e.g. salbutamol, levalbuterol, terbutaline and bitolterol), epinephrine (inhaled or tablets), anticholinergics (e.g. ipratropium bromide),

glucocorticoids (oral or inhaled) Long-acting β_2-agonists (e.g. salmeterol, formoterol, bambuterol, and sustained-release oral albuterol), combinations of inhaled steroids and long-acting bronchodilators (e.g. fluticasone/salmeterol, budesonide/formoterol), leukotriene antagonists and synthesis inhibitors (e.g. montelukast, zafirlukast and zileuton), inhibitors of mediator release (e.g. cromoglycate and ketotifen), biological regulators of IgE response (e.g. omalizumab), antihistamines (e.g. ceterizine, cinnarizine, fexofenadine), and vasoconstrictors (e.g. oxymethazoline, xylomethazoline, nafazoline and tramazoline).

[00118] Additionally, the compound of the invention may be administered in combination with emergency therapies for asthma and/or COPD, such therapies include oxygen or heliox administration, nebulized salbutamol or terbutaline (optionally combined with an anticholinergic (e.g. ipratropium), systemic steroids (oral or intravenous, e.g. prednisone, prednisolone, methylprednisolone, dexamethasone, or hydrocortisone), intravenous salbutamol, non-specific beta-agonists, injected or inhaled (e.g. epinephrine, isoetharine, isoproterenol, metaproterenol), anticholinergics (IV or nebulized, e.g. glycopyrrolate, atropine, ipratropium), methylxanthines (theophylline, aminophylline, bamiphylline), inhalation anesthetics that have a bronchodilatory effect (e.g. isoflurane, halothane, enflurane), ketamine, and intravenous magnesium sulfate.

[00119] In one embodiment, the compound of the invention is co-administered with another therapeutic agent for the treatment and/or prevention of irritable bowel disease (IBD); particular agents include but are not limited to: glucocorticoids (e.g. prednisone, budesonide) synthetic disease modifying, immunomodulatory agents (e.g. methotrexate, leflunomide, sulfasalazine, mesalazine, azathioprine, 6-mercaptopurine and ciclosporin) and biological disease modifying, immunomodulatory agents (infliximab, adalimumab, rituximab, and abatacept).

[00120] In one embodiment, the compound of the invention is co-administered with another therapeutic agent for the treatment and/or prevention of systemic lupus erythematosus; particular agents include but are not limited to: disease-modifying antirheumatic drugs (DMARDs) such as antimalarials (e.g. plaquenil, hydroxychloroquine), immunosuppressants (e.g. methotrexate and azathioprine), cyclophosphamide and mycophenolic acid; immunosuppressive drugs and analgesics, such as nonsteroidal anti-inflammatory drugs, opiates (e.g. dextropropoxyphene and co-codamol), opioids (e.g. hydrocodone, oxycodone, MS Contin, or methadone) and the fentanyl duragesic transdermal patch.

[00121] In one embodiment, the compound of the invention is co-administered with another therapeutic agent for the treatment and/or prevention of psoriasis; particular agents include but are not limited to: topical treatments such as bath solutions, moisturizers, medicated creams and ointments containing coal tar, dithranol (anthralin), corticosteroids like desoximetasone (Topicort™), fluocinonide, vitamin D_3 analogues (for example, calcipotriol), Argan oiland retinoids (etretinate, acitretin, tazarotene), systemic treatments such as methotrexate, cyclosporine, retinoids, tioguanine, hydroxyurea, sulfasalazine, mycophenolate mofetil, azathioprine, tacrolimus, fumaric acid esters or biologics such as Amevive™, Enbrel™, Humira™, Remicade™, Raptiva™ and ustekinumab (a IL-12 and IL-23 blocker). Additionally, a compound of the invention may be administered in combination with other therapies

including, but not limited to phototherapy, or photochemotherapy (e.g. psoralen and ultraviolet A phototherapy (PUVA)).

[00122] By co-administration is included any means of delivering two or more therapeutic- agents to the patient as part of the same treatment regime, as will be apparent to the skilled person. Whilst the two or more agents may be administered simultaneously in a single formulation this is not essential. The agents may be administered in different formulations and at different times.

GENERAL SYNTHETIC PROCEDURES

General

[00123] The compound of the invention can be prepared from readily available starting materials using the following general methods and procedures. It will be appreciated that where typical or preferred process conditions (i.e., reaction temperatures, times, mole ratios of reactants, solvents, pressures, etc.) are given, other process conditions can also be used unless otherwise stated. Optimum reaction conditions may vary with the particular reactants or solvent used, but such conditions can be determined by one skilled in the art by routine optimization procedures.

[00124] Additionally, as will be apparent to those skilled in the art, conventional protecting groups may be necessary to prevent certain functional groups from undergoing undesired reactions. The choice of a suitable protecting group for a particular functional group as well as suitable conditions for protection and deprotection are well known in the art. For example, numerous protecting groups, and their introduction and removal, are described in T. W. Greene and P. G. M. Wuts, *Protecting Groups in Organic Synthesis*, Second Edition, Wiley, New York, 1991, and references cited therein.

[00125] The following methods are presented with details as to the preparation of representative bicycloheteroaryls that have been listed hereinabove. The compound of the invention may be prepared from known or commercially available starting materials and reagents by one skilled in the art of organic synthesis.

[00126] All reagents were of commercial grade and were used as received without further purification, unless otherwise stated. Commercially available anhydrous solvents were used for reactions conducted under inert atmosphere. Reagent grade solvents were used in all other cases, unless otherwise specified. Column chromatography was performed on silica gel 60 (35-70 μm). Thin layer chromatography was carried out using pre-coated silica gel F-254 plates (thickness 0.25 mm). ^{1}H NMR spectra were recorded on a Bruker DPX 400 NMR spectrometer (400 MHz). Chemical shifts (δ) for ^{1}H NMR spectra are reported in parts per million (ppm) relative to tetramethylsilane (δ 0.00) or the appropriate residual solvent peak, i.e. $CHCl_3$ (δ 7.27), as internal reference. Multiplicities are given as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m) and broad (br). Coupling constants (J) are given in Hz. Electrospray MS spectra were obtained on a Micromass platform LC/MS spectrometer. Column Used for all LCMS analysis: Waters Acquity UPLC BEH C18 1.7μm, 2.1mm ID x 50mm L (Part No.186002350)).

Preparative HPLC: Waters XBridge Prep C18 5µm ODB 19mm ID x 100mm L (Part No.186002978). All the methods are using MeCN/H_2O gradients. H_2O contains either 0.1% TFA or 0.1% NH_3.

[00127] The following is a list of abbreviations used in the experimental section:

DCM	Dichloromethane
DiPEA	N,N-diisopropylethylamine
MeCN	Acetonitrile
BOC	tert-Butyloxy-carbonyl
MF	N,N-dimethylformamide
Cat.	Catalytic amount
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
NMR	Nuclear Magnetic Resonnance
DMSO	Dimethylsulfoxide
LC-MS	Liquid Chromatography- Mass Spectrometry
Ppm	part-per-million
Pd/C	Palladium on Charcoal 10%
PMB	Para-methoxy-benzyl
PyBOP	benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluoroborate
EtOAc	ethyl acetate
APCI	atmospheric pressure chemical ionization
Rt	retention time
s	singlet
br s	broad singlet
m	multiplet
min	minute
mL	milliliter
µL	microliter
g	gram
mg	milligram
$PdCl_2dppf$	[1,1'-Bis(diphenylphosphino)ferrocene] dichloropalladium(II)
TEA	Triethylamine
MMP	Matrix Metallo Proteinase
NHAC	Normal Human Articular Chondrocytes
shRNA	short hairpin RNA
RNA	Ribonucleic acid

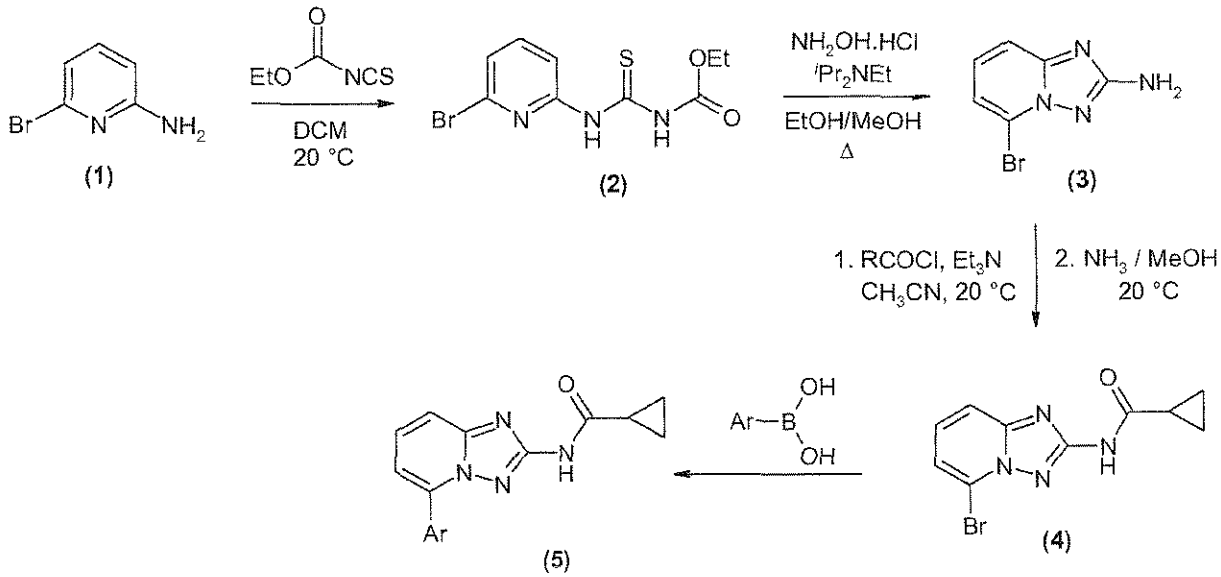
Ad-Si RNA	Adenoviral encoded siRNA
PBST	Phosphate buffered saline with Tween 3.2 mM Na2HPO4, 0.5 mM KH2PO4, 1.3 mM KCl, 135 mM NaCl, 0.05% Tween 20, pH 7.4
APMA	4-aminophenylmercuric acetate
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal bovine serum
hCAR	human cellular adenovirus receptor
3- *MOI*	*multiplicity of infection of 3*
dNTP	deoxyribonucleoside triphosphate
QPCR	quantitative polymerase chain reaction
cDNA	copy deoxyribonucleic acid
GAPDH	Glyceraldehyde phosphate dehydrogenase

Synthetic Preparation of the Compound of the Invention

[00128] The compound according to the invention can be produced according to the following scheme.

General Synthetic Method

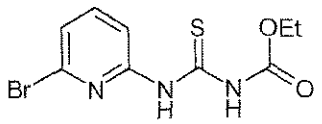
Scheme 1



wherein Ar represents phenyl-L1-heterocycloalkyl, where L1 is –CO- and the heterocycloalkyl group is *optionally substituted.*

General

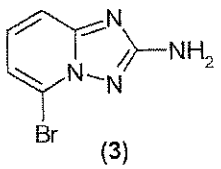
1.1.1 1-(6-Bromo-pyridin-2-yl)-3-carboethoxy-thiourea (2)



(2)

[00129] To a solution of 2-amino-6-bromopyridine (**1**) (253.8 g, 1.467 mol) in DCM (2.5 L) cooled to 5 °C is added ethoxycarbonyl isothiocyanate (173.0 mL, 1.467 mol) dropwise over 15 min. The reaction mixture is then allowed to warm to room temp. (20 °C) and stirred for 16 h. Evaporation *in vacuo* gives a solid which may be collected by filtration, thoroughly washed with petrol (3 ×600 mL) and air-dried to afford (**2**). The thiourea may be used as such for the next step without any purification. ^{1}H (400 MHz, $CDCl_3$) δ 12.03 (1H, br s, NH), 8.81 (1H, d, *J* 7.8 Hz, H-3), 8.15 (1H, br s, NH), 7.60 (1H, t, *J* 8.0 Hz, H-4), 7.32 (1H, dd, *J* 7.7 and 0.6 Hz, H-5), 4.31 (2H, q, *J* 7.1 Hz, CH_2), 1.35 (3H, t, *J* 7.1 Hz, CH_3).

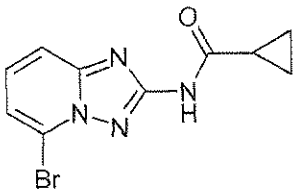
1.1.2 5-Bromo-[1,2,4]triazolo[1,5-a]pyridin-2-ylamine (3)



(3)

[00130] To a suspension of hydroxylamine hydrochloride (101.8 g, 1.465 mol) in EtOH/MeOH (1:1, 900 mL) is added *N,N*-diisopropylethylamine (145.3 mL, 0.879 mol) and the mixture is stirred at room temp. (20 °C) for 1 h. 1-(6-Bromo-pyridin-2-yl)-3-carboethoxy-thiourea (**2**) (89.0 g, 0.293 mol) is then added and the mixture slowly heated to reflux (Note: bleach scrubber is required to quench H_2S evolved). After 3 h at reflux, the mixture is allowed to cool and filtered to collect the precipitated solid. Further product is collected by evaporation *in vacuo* of the filtrate, addition of H_2O (250 mL) and filtration. The combined solids are washed successively with H_2O (250 mL), EtOH/MeOH (1:1, 250 mL) and Et_2O (250 mL) then dried *in vacuo* to afford the triazolopyridine derivative (**3**) as a solid. The compound may be used as such for the next step without any purification. ^{1}H (400 MHz, DMSO-d_6) δ 7.43-7.34 (2H, m, 2 × aromatic-H), 7.24 (1H, dd, *J* 6.8 and 1.8 Hz, aromatic-H), 6.30 (2H, br, NH_2); *m/z* 213/215 (1:1, $M+H^+$, 100%).

1.1.3 Cyclopropanecarboxylic acid (5-bromo-[1,2,4]triazolo[1,5-a]pyridin-2-yl)-amide (4):

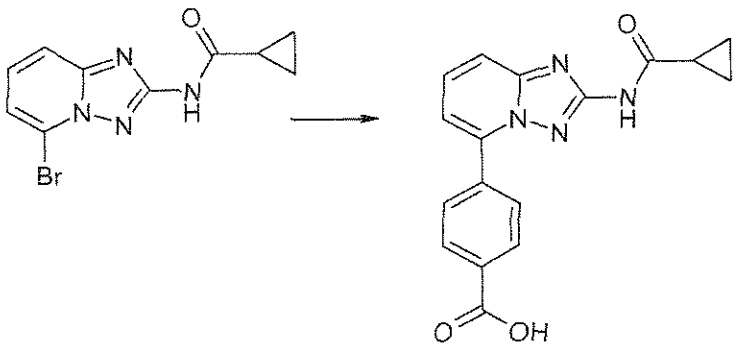


[00131] To a solution of the 2-amino-triazolopyridine **(3)** (7.10 g, 33.3 mmol) in dry CH_3CN (150 mL) at 5 °C is added Et_3N (11.6 mL, 83.3 mmol) followed by cyclopropanecarbonyl chloride (83.3 mmol). The reaction mixture is then allowed to warm to ambient temperature and stirred until all starting material **(3)** is consumed. If required, further Et_3N (4.64 mL, 33.3 mmol) and cyclopropanecarbonyl chloride (33.3 mmol) is added to ensure complete reaction. Following solvent evaporation *in vacuo* the resultant residue is treated with 7 N methanolic ammonia solution (50 mL) and stirred at ambient temp. (for 1-16 h) to hydrolyse any bis-acylated product. Product isolation is made by removal of volatiles *in vacuo* followed by trituration with Et_2O (50 mL). The solids are collected by filtration, washed with H_2O (2×50mL), acetone (50 mL) and Et_2O (50 mL), then dried *in vacuo* to give the required bromo intermediate **(4)**.

Synthetic procedure for the preparation of the compound of the invention

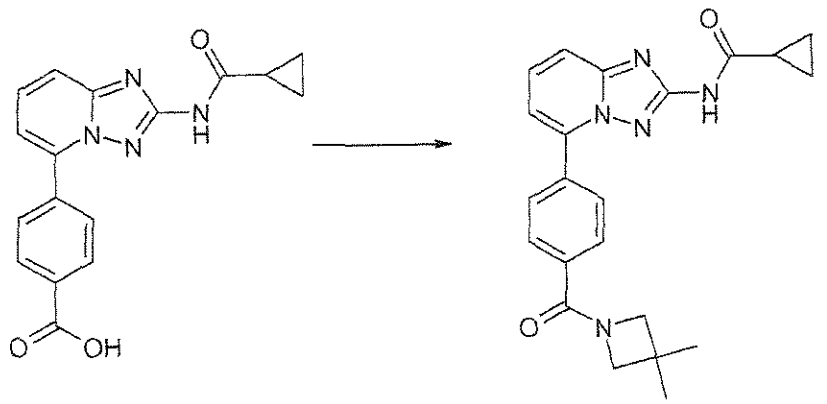
Compound 1

Step 1: *Suzuki coupling*



[00132] 4-Carboxyphenylboronic acid (3.5 g, 0.021 mol.) was added to a solution of cyclopropanecarboxylic acid (5-bromo-[1,2,4]triazolo[1,5-a]pyridin-2-yl)-amide (Intermediate 4, 5 g, 0.018 mol) in 1,4-dioxane/water (5:1). K_2CO_3 (5.0 g, 0.036 mol) and $PdCl_2dppf$ (5%) were added to the solution. The resulting mixture was then heated by traditional heating in an oil bath at 90°C for 16h. 1 M HCl solution was added and a precipitate was formed in the acidic solution. The precipitate was filtered, dried under vacuum to give the title compound used in the next step without further purification.

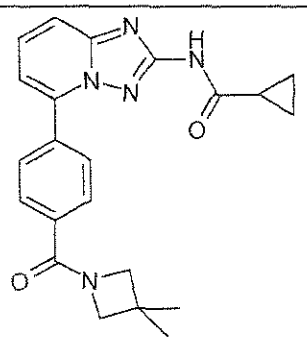
Step 2: Cyclopropanecarboxylic acid {5-[4-(3,3-dimethyl-azetidine-1-carbonyl)-phenyl]-[1,2,4]triazolo[1,5-a]pyridin-2-yl}-amide (Compound 1).



[00133] EDCI (3.59 g, 0.019 mol), HOBt (2.53 g, 0.019 mol) and DIPEA (4.48 mL) were added to a solution of 4-[2-(Cyclopropanecarbonyl-amino)-[1,2,4]triazolo[1,5-a]pyridin-5-yl]-benzoic acid (4 g, 0.012 mol) in DCM (150 mL) at room temperature. The resulting mixture was stirred for 10 min at room temperature. Dimethylazetidine hydrochloride salt (1.64 g, 0.013 mol) was added to the solution and the reaction is stirred for 16 hrs. Water was added to the reaction mixture. The organic layer was separated and washed with 2 N NaOH solution, 2N HCl solution and water. The organic phase was dried over $MgSO_4$, filtered and evaporated under vacuum. Purification by flash chromatography (eluant: 1:1 petrol/EtOAc to neat EtOAc) afforded cyclopropanecarboxylic acid {5-[4-(3,3-dimethyl-azetidine-1-carbonyl)-phenyl]-[1,2,4]triazolo[1,5-a]pyridin-2-yl}-amide.

[00134] The compound of the invention that has been or can be prepared according to the synthetic method described herein is listed in Table I below. The NMR spectral data of the compound of the invention is given in Table II.

[00135] **Table I: Mass spectral data of the Compound of the Invention**

Cpd #	Structures	Name	MW	MS Mes'd
1		Cyclopropanecarboxylic acid {5-[4-(3,3-dimethyl-azetidine-1-carbonyl)-phenyl]-[1,2,4]triazolo[1,5-a]pyridin-2-yl}-amide	389.46	390.0

[00136] **Table II: NMR Data of the Compound of the Invention**

Cpd #	NMR data (δ)
1	(^{1}H, DMSO-*d6*) 11.01 (1H, b, NH), 8.08 (2H, d, ArH), 7.79 (2H, d, ArH), 7.73 (1H, d, ArH), 7.72 (1H, s, ArH), 7.35 (1H, dd, ArH), 4.04 (2H, b, CH_2), 3.77 (2H, b, CH_2), 2.02 (1H, b, CH), 1.27 (6H, s, 2xCH_3), 0.81 (4H, m, 2xCH_2).

Biological Examples

Example 1 – *in vitro* assays

Example 1.1 JAK1 inhibition assay

[00137] Recombinant human JAK1 (catalytic domain, amino acids 866-1154; catalog number PV4774) was purchased from Invitrogen. 1 ng of JAK1 was incubated with 20 nM Ulight-JAK1(tyr1023) peptide (Perkin Elmer catalog number TRF0121) in kinase reaction buffer (25mM MOPS pH6.8, 0.016% Brij-35, 8.33mM MgCl2, 3.33mM DTT, 7μM ATP) with or without 4μL containing test compound or vehicle (DMSO, 1% final concentration), in a total volume of 20 μL, in a white 384 Luminotrac 200 plate (Greiner, catalog number 781075). After 60 min at room temperature, reactions were stopped by adding 20 μL/well of detection mixture (1 x detection buffer (Perkin Elmer, catalog number CR97-100C), 0.5nM Europium-anti-phosphotyrosine (PT66) (Perkin Elmer, catalog number AD0068), 10 mM EDTA). Readout is performed using the Envision with excitation at 320nm and measuring emission at 615 nm (Perkin Elmer). Kinase activity was calculated by subtracting relative fluorescence units (RFU) obtained in the presence of a positive control inhibitor (10 μM staurosporine) from RFU obtained in the presence of vehicle. The ability of a test compound to inhibit this activity was determined as:

[00138] Percentage inhibition = ((RFU determined for sample with test compound present – RFU determined for sample with positive control inhibitor) divided by (RFU determined in the presence of vehicle – RFU determined for sample with positive control inhibitor)) * 100.

[00139] Dose dilution series were prepared for the compounds enabling the testing of dose-response effects in the JAK1 assay and the calculation of the IC_{50} for the compound. Each compound is routinely tested at concentration of 20μM followed by a 1/5 serial dilution, 8 points (20μM - 4μM – 800nM – 160nM – 32nM – 6.4nM – 1.28nM – 0.26nM) in a final concentration of 1% DMSO. When potency of compound series increases, more dilutions are prepared and/or the top concentration are lowered (e.g. 5 μM, 1 μM). The data are expressed as the average IC_{50} from the assays ± standard error of the mean.

[00140] **TABLE III: JAK1 IC_{50} Values of Compound**

Cpd #	JAK1 (nM)
1	6.6 ± 0.7

Example 1.2 JAK1 Ki determination assay

[00141] For the determination of Ki, different amounts of inhibitor were mixed with the enzyme and the enzymatic reaction was followed as a function of ATP concentration. The Ki was determined by means of double reciprocal plotting of Km *vs* compound concentration (Lineweaver-Burk plot). JAK1 (Invitrogen, PV4774) was used at a final concentration of 500 ng/ml. The substrate was Poly(Glu,Tyr)sodium salt (4:1) , MW 20 000 - 50 000 (Sigma, P0275) The reaction was performed in 25mM Hepes pH 7.5; 0,01% Tween20, 10 mM $MgCl_2$ with varying concentrations of ATP and compound and stopped by addition of 150 mM phosphoric acid. Measurement of incorporated phosphate into the substrate polyGT was done by loading the samples on a filter plate (using a harvester, Perkin Elmer) and subsequent washing. Incorporated ^{33}P in polyGT is measured in a Topcount scintillation counter after addition of scintillation liquid to the filter plates (Perkin Elmer).

[00142] When Compound 1 was tested in this assay, a Ki value of 6.9 nM was measured.

[00143] Alternatively, for the determination of Ki, different amounts of inhibitor were mixed with the enzyme and the enzymatic reaction was followed as a function of ATP concentration. The Ki was determined by means of double reciprocal plotting of Km *vs* compound concentration (Lineweaver-Burk plot). 1 ng of JAK1 (Invitrogen, PV4774) was used in the assay. The substrate was 50nM Ulight-JAK-1 (Tyr1023) Peptide (Perkin Elmer, TRF0121) The reaction was performed in 25mM MOPS pH 6.8, 0.01%, 2 mM DTT, 5 mM $MgCl_2$ Brij-35 with varying concentrations of ATP and compound. Phosphorylated substrate was measured using an Eu-labeled anti-phosphotyrosine antibody PT66 (Perkin Elmer, AD0068). Readout was performed on the envision (Perkin Elmer) with excitation at 320 nm and emission followed at 615 nm and 665 nm.

[00144] When Compound 1 was tested in this assay, a Ki value of 5.6 nM was measured.

Example 1.3 JAK2 inhibition assay

[00145] Recombinant human JAK2 (catalytic domain, amino acids 866-1154; catalog number PV4210) was purchased from Invitrogen. 0.0125mU of JAK2 was incubated with 25 nM Ulight-JAK1(tyr1023) peptide (Perkin Elmer catalog number TRF0121) in kinase reaction buffer (41.66mM HEPES pH7.0, 0.016% Triton X-100, 12.5mM $MgCl_2$, 3.33mM DTT, 7.5µM ATP) with or without 4µL containing test compound or vehicle (DMSO, 1% final concentration), in a total volume of 20 µL, in a white 384 Luminotrac 200 plate (Greiner, catalog number 781075). After 60 min at room temperature, reactions were stopped by adding 20 µL/well of detection mixture (1xdetection buffer (Perkin Elmer, catalog number CR97-100C), 0.5nM Europium-anti-phosphotyrosine (PT66) (Perkin Elmer, catalog number AD0068), 10 mM EDTA). Readout is performed using the Envision with excitation at 320nm and measuring emission at 615 nm (Perkin Elmer). Kinase activity was calculated by subtracting relative fluorescence units (RFU) obtained in the presence of a positive control inhibitor (10 µM staurosporine) from RFU obtained in the presence of vehicle. The ability of a test compound to inhibit this activity was determined as:

[00146] Percentage inhibition = ((RFU determined for sample with test compound present – RFU determined for sample with positive control inhibitor) divided by (RFU determined in the presence of vehicle – RFU determined for sample with positive control inhibitor)) * 100.

[00147] Dose dilution series are prepared for compound enabling the testing of dose-response effects in the JAK2 assay and the calculation of the IC_{50} for the compound. Each compound is routinely tested at concentration of 20μM followed by a 1/5 serial dilution, 8 points (20μM - 4μM – 800nM – 160nM – 32nM – 6.4nM – 1.28nM – 0.26nM) in a final concentration of 1% DMSO. When potency of compound series increases, more dilutions are prepared and/or the top concentration are lowered (e.g. 5 μM, 1 μM). The data are expressed as the average IC_{50} from the assays ± standard error of the mean.

[00148] **TABLE IV: JAK2 IC_{50} Values of Compound**

Cpd #	JAK2 (nM)
1	67 ± 5

Example 1.4 JAK2 Ki determination assay

[00149] For the determination of Ki, different amounts of inhibitor were mixed with the enzyme and the enzymatic reaction was followed as a function of ATP concentration. The Ki was determined by means of double reciprocal plotting of Km *vs* compound concentration (Lineweaver-Burk plot). 0.025 mU of JAK2 (Invitrogen, PV4210) was used in the assay. The substrate was Poly(Glu,Tyr)sodium salt (4:1) , MW 20 000 - 50 000 (Sigma, P0275) The reaction was performed in 10 mM MOPS pH 7.5, 0.5 mM EDTA, 0.01% Brij-35, 1 mM DTT, 15 mM MgAc with varying concentrations of ATP and compound and stopped by addition of 150 mM phosphoric acid. Measurement of incorporated phosphate into the substrate polyGT was done by loading the samples on a filter plate (using a harvester, Perkin Elmer) and subsequent washing. Incorporated ^{33}P in polyGT is measured in a Topcount scintillation counter after addition of scintillation liquid to the filter plates (Perkin Elmer).

[00150] When Compound 1 was tested in this assay, a Ki value of 126 nM was measured.

[00151] Alternatively, for the determination of Ki, different amounts of inhibitor were mixed with the enzyme and the enzymatic reaction was followed as a function of ATP concentration. The Ki was determined by means of double reciprocal plotting of Km *vs* compound concentration (Lineweaver-Burk plot). 0.0125 mU of JAK2 (Invitrogen, PV4210) was used in the assay. The substrate was 50nM Ulight-JAK-1 (Tyr1023) Peptide (Perkin Elmer, TRF0121). The reaction was performed in 25 mM HEPES pH 7.0, 0.01% Triton X-100, 2 mM DTT, 7.5 mM $MgCl_2$ with varying concentrations of ATP and compound. Phosphorylated substrate was measured using an Eu-labeled anti-phosphotyrosine antibody PT66 (Perkin Elmer, AD0068). Readout was performed on the envision (Perkin Elmer) with excitation at 320 nm and emission followed at 615 nm and 665 nm.

[00152] When Compound 1 was tested in this assay, a Ki value of 35 nM was measured.

Example 1.5 JAK3 inhibition assay

[00153] Recombinant human JAK3 catalytic domain (amino acids 781-1124; catalog number PV3855) was purchased from Invitrogen. 0.025mU of JAK3 was incubated with 2.5 µg polyGT substrate (Sigma catalog number P0275) in kinase reaction buffer (25 mM Tris pH 7.5, 0.5 mM EGTA, 0.5 mM Na_3VO_4, 5 mM b-glycerolphosphate, 0.01% Triton X-100, 1 µM non-radioactive ATP, 0.25 µCi ^{33}P-gamma-ATP (GE Healthcare, catalog number AH9968) final concentrations) with or without 5µL containing test compound or vehicle (DMSO, 1% final concentration), in a total volume of 25 µL, in a polypropylene 96-well plate (Greiner, V-bottom). After 105 min at 30 °C, reactions were stopped by adding of 25 µL/well of 150 mM phosphoric acid. All of the terminated kinase reaction was transferred to prewashed (75 mM phosphoric acid) 96 well filter plates (Perkin Elmer catalog number 6005177) using a cell harvester (Perkin Elmer). Plates were washed 6 times with 300 µL per well of a 75 mM phosphoric acid solution and the bottom of the plates was sealed. 40 µL/well of Microscint-20 was added, the top of the plates was sealed and readout was performed using the Topcount (Perkin Elmer). Kinase activity was calculated by subtracting counts per minute (cpm) obtained in the presence of a positive control inhibitor (10 µM staurosporine) from cpm obtained in the presence of vehicle. The ability of a test compound to inhibit this activity was determined as:

Percentage inhibition = ((cpm determined for sample with test compound present – cpm determined for sample with positive control inhibitor) divided by (cpm determined in the presence of vehicle – cpm determined for sample with positive control inhibitor)) * 100.

[00154] Dose dilution series were prepared for the compound enabling the testing of dose-response effects in the JAK3 assay and the calculation of the IC_{50} for the compound. Each compound was routinely tested at concentration of 20µM followed by a 1/3 serial dilution, 8 points (20µM - 6.67µM - 2.22µM - 740nM - 247nM - 82nM - 27nM - 9nM) in a final concentration of 1% DMSO. When potency of the compound series increased, more dilutions were prepared and/or the top concentration was lowered (e.g. 5 µM, 1 µM). The data are expressed as the average IC_{50} from the assays ± standard error of the mean.

[00155] **TABLE V: JAK3 IC_{50} Values of Compound**

Cpd #	JAK3 (nM)
1	408 ± 62

Example 1.6 JAK3 Ki determination assay

[00156] For the determination of Ki, different amounts of inhibitor were mixed with the enzyme and the enzymatic reaction was followed as a function of ATP concentration. The Ki was determined by

means of double reciprocal plotting of Km *vs* compound concentration (Lineweaver-Burk plot). JAK3 (Carna Biosciences, 09CBS-0625B) was used at a final concentration of 10 ng/ml. The substrate was Poly(Glu,Tyr)sodium salt (4:1) , MW 20 000 - 50 000 (Sigma, P0275) The reaction was performed in 25mM Tris pH 7.5 , 0.01% Triton X-100 , 0.5mM EGTA, 2.5mM DTT, 0.5mM Na_3VO_4, 5mM b-glycerolphosphate, 10mM $MgCl_2$ with varying concentrations of ATP and compound and stopped by addition of 150 mM phosphoric acid. Measurement of incorporated phosphate into the substrate polyGT was done by loading the samples on a filter plate (using a harvester, Perkin Elmer) and subsequent washing. Incorporated ^{33}P in polyGT is measured in a Topcount scintillation counter after addition of scintillation liquid to the filter plates (Perkin Elmer)

[00157] When Compound 1 was tested in this assay, a Ki value of 188 nM was measured.

Example 1.7 TYK2 inhibition assay

[00158] Recombinant human TYK2 catalytic domain (amino acids 871-1187; catalog number 08-147) was purchased from Carna biosciences. 5 ng of TYK2 was incubated with 12.5 µg polyGT substrate (Sigma catalog number P0275) in kinase reaction buffer (25 mM Hepes pH 7.5, 100 mM NaCl, 0.2 mM Na_3VO_4, 0.1% NP-40, 0.1 µM non-radioactive ATP, 0.125 µCi ^{33}P-gamma-ATP (GE Healthcare, catalog number AH9968) final concentrations) with or without 5µL containing test compound or vehicle (DMSO, 1% final concentration), in a total volume of 25 µL, in a polypropylene 96-well plate (Greiner, V-bottom). After 90 min at 30 °C, reactions were stopped by adding of 25 µL/well of 150 mM phosphoric acid. All of the terminated kinase reaction was transferred to prewashed (75 mM phosphoric acid) 96 well filter plates (Perkin Elmer catalog number 6005177) using a cell harvester (Perkin Elmer). Plates were washed 6 times with 300 µL per well of a 75 mM phosphoric acid solution and the bottom of the plates was sealed. 40 µL/well of Microscint-20 was added, the top of the plates was sealed and readout was performed using the Topcount (Perkin Elmer). Kinase activity was calculated by subtracting counts per minute (cpm) obtained in the presence of a positive control inhibitor (10 µM staurosporine) from cpm obtained in the presence of vehicle. The ability of a test compound to inhibit this activity was determined as:

Percentage inhibition = ((cpm determined for sample with test compound present – cpm determined for sample with positive control inhibitor) divided by (cpm determined in the presence of vehicle – cpm determined for sample with positive control inhibitor)) * 100.

[00159] Dose dilution series were prepared for the compounds enabling the testing of dose-response effects in the TYK2 assay and the calculation of the IC_{50} for the compound. Each compound was routinely tested at concentration of 20µM followed by a 1/3 serial dilution, 8 points (20µM - 6.67µM - 2.22µM - 740nM - 247nM - 82nM - 27nM - 9nM) in a final concentration of 1% DMSO. When potency of compound series increased, more dilutions were prepared and/or the top concentration was lowered (e.g. 5 µM, 1 µM).

[00160] TABLE VI: TYK2 IC_{50} Values of Compound

Cpd #	TYK2 (nM)
1	219 ± 37

Example 2. Cellular assays

Example 2.1 JAK-STAT signalling assay:

[00161] HeLa cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat inactivated fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin. HeLa cells were used at 70 % confluence for transfection. 20,000 cells in 87 µL cell culture medium were transiently transfected with 40 ng pSTAT1(2)-luciferase reporter (Panomics), 8 ng of LacZ reporter as internal control reporter and 52 ng of pBSK using 0.32 µL Jet-PEI (Polyplus) as transfection reagent per well in 96-well plate format. After overnight incubation at 37°C, 10% CO_2, transfection medium was removed. 75 µL of DMEM + 1.5% heat inactivated fetal calf serum was added. 15 µL of compound at 6.7 x concentration was added for 60 min and then 10 µL of human OSM (Peprotech) at 33 ng/mL final concentration.

[00162] All compounds were tested in duplicate starting from 20 µM followed by a 1/3 serial dilution, 8 doses in total (20 µM – 6.6 µM – 2.2 µM – 740 nM – 250 nM – 82 nM – 27 nM – 9 nM) in a final concentration of 0.2% DMSO.

[00163] After overnight incubation at 37°C, 10% CO_2 cells were lysed in 100 µL lysis buffer/well (PBS, 0.9 mM $CaCl_2$, 0.5 mM MgCl2, 5% Trehalose, 0.025% Tergitol NP9, 0.15% BSA).

[00164] 40 µL of cell lysate was used to read β-galactosidase activity by adding 180 µL βGal solution (30µl ONPG 4mg/mL + 150 µL β-Galactosidase buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 1 mM $MgCl_2$)) for 20 min. The reaction was stopped by addition of 50 µL Na_2CO_3 1 M. Absorbance was read at 405 nm.

[00165] Luciferase activity was measured using 40 µL cell lysate plus 40 µl of Steadylite® as described by the manufacturer (Perkin Elmer), on the Envision (Perkin Elmer).

[00166] 10 µM of a pan-JAK inhibitor was used as a positive control (100% inhibition). As negative control 0.5% DMSO (0% inhibition) was used. The positive and negative controls were used to calculate z' and 'percent inhibition' (PIN) values.

[00167] Percentage inhibition = ((fluorescence determined in the presence of vehicle - fluorescence determined for sample with test compound present) divided by (fluorescence determined in the presence of vehicle – fluorescence determined for sample without trigger)) * 100.

[00168] PIN values were plotted for compounds tested in dose-response and EC_{50} values were derived.

[00169] TABLE VII: STAT signalling EC_{50} Values of Compound

Cpd #	EC_{50} (nM)
1	539 ± 130

Example 2.2 OSM/IL-1β signaling Assay

[00170] OSM and IL-1β were shown to synergistically upregulate MMP13 levels in the human chondrosarcoma cell line SW1353. The cells were seeded in 96 well plates at 15,000 cells/well in a volume of 120 µL DMEM (Invitrogen) containing 10% (v/v) FBS and 1% penicillin/streptomycin (InVitrogen) incubated at 37°C 5% CO_2. Cells were preincubated with 15 µL compound in M199 medium with 2% DMSO 1 hr before triggering with 15 µL OSM and IL-1β to reach 25 ng/mL OSM and 1 ng/mL IL-1β, and MMP13 levels were measured in conditioned medium 48 hours after triggering. MMP13 activity was measured using an antibody capture activity assay. For this purpose, 384 well plates (NUNC, 460518, MaxiSorb black) were coated with 35 µL of a 1.5 µg/mL anti-human MMP13 antibody (R&D Systems, MAB511) solution for 24 hours at 4°C. After washing the wells 2 times with PBS + 0.05% Tween, the remaining binding sites were blocked with 100 µL 5% non-fat dry milk (Santa Cruz, sc-2325, Blotto) in PBS for 24 hours at 4°C. Next, the wells were washed 2 times with PBS + 0.05% Tween and 35 µL of 1/10 dilution of culture supernatant containing MMP13 in 100-fold diluted blocking buffer was added and incubated for 4 hours at room temperature. Next the wells were washed twice with PBS + 0.05% Tween followed by MMP13 activation by addition of 35 µL of a 1.5 mM 4-Aminophenylmercuric acetate (APMA) (Sigma, A9563) solution and incubation at 37 °C for 1 hour. The wells were washed again with PBS + 0.05% Tween and 35 µL MMP13 substrate (Biomol, P-126, OmniMMP fluorogenic substrate) was added. After incubation for 24 hours at 37°C fluorescence of the converted substrate was measured in a Perkin Elmer Wallac EnVision 2102 Multilabel Reader (wavelength excitation: 320 nm, wavelength emission: 405 nm).

[00171] Percentage inhibition = ((fluorescence determined in the presence of vehicle - fluorescence determined for sample with test compound present) divided by (fluorescence determined in the presence of vehicle – fluorescence determined for sample without trigger)) * 100. The data are expressed as the average EC_{50} from the assays ± standard error of the mean.

[00172] **TABLE VIII: MMP13 EC_{50} Values of Compounds**

Cpd #	EC_{50} (nM)
1	4196 ± 2370

Example 2.3 PBL Proliferation assay

[00173] Human peripheral blood lymphocytes (PBL) are stimulated with IL-2 and proliferation measured using a BrdU incorporation assay. The PBL are first stimulated for 72 hrs with PHA to induce IL-2 receptor, fasted for 24 hrs to stop cell proliferation followed by IL-2 stimulation for another 72 hrs (including 24hr BrdU labeling). Cells are preincubated with test compounds 1 hr before IL-2 addition. Cells are cultured in RPMI 1640 containing 10% (v/v) FBS.

Example 2.4 Whole Blood Assays (WBA)

2.4.1 IFNα stimulation protocol

[00174] To predict the selectivity of the test compounds to inhibit JAK1- over JAK2-dependent signaling pathways *in vivo*, a physiologically relevant *in vitro* model was developed using human whole blood. In the WBA assay, blood drawn from human volunteers who gave informed consent, was treated *ex vivo* with compound (1h) and subsequently stimulated either for 30 minutes with interferon α (IFNα, *JAK1 dependent pathway*) or for 2 h with granulocyte macrophage-colony stimulating factor (GM-CSF, JAK2 dependent pathway).

2.4.1.1 Phospho – STAT1 Assay

[00175] For IFNα stimulation, increase in phosphorylation of Signal Transducers and Activators of Transcription 1 (pSTAT1) by IFNα in white blood cell extracts was measured using a pSTAT1 ELISA assay. Phosphorylation of Signal Transducer and Activator of Transcription 1 (STAT1) after interferon alpha (IFNα) triggering is a JAK1-mediated event. The Phospho-STAT1 Assay, which was used to measure Phospho-STAT1 levels in cellular extracts, was developed to assess the ability of a compound to inhibit JAK1-dependent signaling pathways.

[00176] Whole human blood, drawn from human volunteers who gave informed consent, was *ex vivo* treated with compound (1h) and subsequently stimulated for 30 minutes with IFNα. The increase in phosphorylation of STAT1 by IFNα in white blood cell extracts was measured using a phospho-STAT1 ELISA.

[00177] The ACK lysis buffer consisted of 0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA. The pH of the buffer was 7.3.

[00178] A 10x cell lysis buffer concentrate (part of the PathScan Phospho-STAT1 (Tyr701) sandwich ELISA kit from Cell Signaling) was diluted 10-fold in H_2O. Proteinase inhibitors were added to the buffer before use.

[00179] 20 µg IFNα is dissolved in 40 µL H_2O to obtain a 500 µg/mL stock solution. The stock solution was stored at -20°C.

[00180] A 3-fold dilution series of the compound was prepared in DMSO (highest concentration: 10 mM). Subsequently, the compound was further diluted in medium (dilution factor dependent on desired final compound concentration).

2.4.1.1.1 Incubation of blood with compound and stimulation with IFNα

[00181] Human blood was collected in heparinized tubes. The blood was divided in aliquots of 392 µL. Afterwards, 4 µL of compound dilution was added to each aliquot and the blood samples were incubated for 1 h at 37°C. The IFNα stock solution was diluted 1000-fold in RPMI medium to obtain a 500 ng/mL working solution. 4 µL of the 500 ng/mL work solution was added to the blood samples (final concentration IFNα: 5ng/ml). The samples were incubated at 37°C for 30 min.

2.4.1.1.2 Preparation of cell extracts

[00182] At the end of the stimulation period, 7.6 mL ACK buffer was added to the blood samples to lyse the red blood cells. The samples were mixed by inverting the tubes five times and the reaction was incubated on ice for 5 min. The lysis of the RBC should be evident during this incubation. The cells were pelleted by centrifugation at 300 g, 4°C for 7 min and the supernatant was removed. 10 mL 1x PBS was added to each tube and the cell pellet was resuspended. The samples were centrifuged again for 7 min at 300 g, 4°C. The supernatant was removed and the pellet resuspended in 500 µL of 1x PBS. Then, the cell suspension was transferred to a clean 1.5 mL microcentrifuge tube. The cells were pelleted by centrifugation at 700 g for 5 min at 4°C. The supernatant was removed and the pellet was dissolved in 150 µL cell lysis buffer. The samples were incubated on ice for 15 min. After that, the samples were stored at -80°C until further processing.

2.4.1.1.3 Measurement of STAT1 phosphorylation by ELISA

[00183] The Pathscan Phospho-STAT1 (Tyr701) Sandwich ELISA kit from Cell Signaling (Cat.n°: #7234) was used to determine Phospho-STAT1 levels.

[00184] The cellular extracts were thawed on ice. The tubes were centrifuged for 5 min at 16,000 g, 4°C and the cleared lysates were harvested. Meanwhile, the microwell strips from the kit were equilibrated to room temperature and wash buffer was prepared by diluting 20 x wash buffer in H_2O. Samples were diluted 2-fold in sample diluent and 100 µL was added to the microwell strips. The strips were incubated overnight at 4°C.

[00185] The following day, the wells were washed 3 times with wash buffer. 100 µL of the detection antibody was added to the wells. The strips were incubated at 37°C for 1 h. Then, the wells were washed 3 times with wash buffer again. 100 µL HRP-linked secondary antibody was added to each well and the samples were incubated at 37°C. After 30 min, the wells were washed 3 times again and 100 µL TMB substrate was added to all wells. When samples turned blue, 100 µL STOP solution was added to stop the reaction. Absorbance was measured at 450 nm.

2.4.1.2 IL-8 ELISA

[00186] For GM-CSF stimulation, increase in interleukin-8 (IL-8) levels in plasma was measured using an IL-8 ELISA assay. Granulocyte macrophage–colony stimulating factor (GM-CSF) - induced

interleukin 8 (IL-8) expression is a JAK2-mediated event. The IL-8 ELISA, which can be used to measure IL-8 levels in plasma samples, was developed to assess the ability of a compound to inhibit JAK2-dependent signaling pathways.

[00187] Whole human blood, drawn from human volunteers who gave informed consent, was *ex vivo* treated with compound (1h) and subsequently stimulated for 2 h with GM-CSF. The increase in IL-8 levels in plasma was measured using an IL-8 ELISA assay.

[00188] 10 µg GM-CSF was dissolved in 100 µL H_2O to obtain a 100 µg/mL stock solution. The stock solution was stored at -20°C.

[00189] A 3-fold dilution series of the test compound was prepared in DMSO (highest concentration: 10 mM). Subsequently, the compound was further diluted in medium (dilution factor dependent on desired final compound concentration).

2.4.1.2.1 Incubation of blood with compound and stimulation with GM-CSF

[00190] Human blood was collected in heparinized tubes. The blood was divided in aliquots of 245 µL. Afterwards, 2.5 µL test compound dilution was added to each aliquot and the blood samples were incubated for 1 h at 37°C. The GM-CSF stock solution was diluted 100-fold in RPMI medium to obtain a 1 µg/mL work solution. 2.5 µL of the 1 µg/mL work solution was added to the blood samples (final concentration GM-CSF: 10 ng/mL). The samples were incubated at 37°C for 2 h.

2.4.1.2.2 Preparation of plasma samples

[00191] The samples were centrifuged for 15 min at 1,000 g, 4°C. 100 µL of the plasma was harvested and stored at -80°C until further use.

2.4.1.2.3 Measurement of IL-8 levels by ELISA

[00192] The Human IL-8 Chemiluminescent Immunoassay kit from R&D Systems (Cat.n°: Q8000B) was used to determine IL-8 levels.

[00193] Wash buffer was prepared by diluting 10 x wash buffer in H_2O. Working glo reagent was prepared by adding 1 part Glo Reagent 1 to 2 parts Glo Reagent B 15 min to 4 h before use.

[00194] 100 µL assay diluent RD1-86 was added to each well. After that, 50 µL of sample (plasma) was added. The ELISA plate was incubated for 2 h at room temperature, 500 rpm. All wells were washed 4 times with wash buffer and 200 µL IL-8 conjugate was added to each well. After incubation for 3 h at room temperature, the wells were washed 4 times with wash buffer and 100 µL working glo reagent was added to each well. The ELISA plate was incubated for 5 min at room temperature (protected from light). Luminescence was measured (0.5 s/well read time).

2.4.1.3 Results

[00195] The pIC_{50} of Compound 1 for inhibiting the INFα induced increase of pSTAT1 levels was 6.32 ± 0.07 (SEM), while pIC_{50} for inhibition of GM-CSF induced increase of IL-8 was 4.87 ± 0.13 (SEM) (results obtained using 6 blood donors). This demonstrates that Compound 1 is 28-fold more selective for the JAK1 pathway versus the JAK2 pathway. Therefore, in a cellular setting it is clear that Compound 1 exhibits selectivity for JAK1 over JAK2. Compound 1 seems to exhibit a greater selectivity for JAK1 over JAK2 compared to known structurally related compounds, or other known JAK inhibitors.

2.4.1.3 Data curation treatment and Results

[00196] In order to further refine the results, inhibition of phosphoSTAT1 induction by IFNα in cell extracts or inhibition of IL-8 induction by GM-CSF in plasma were plotted against the compound concentration and IC_{50} values were derived using Graphpad software. Data were retained if R^2 was larger than 0.8 and the hill slope was smaller than 3 and only donors for which valid data were obtained for both assays were retained.

[00197] For example, when submitted to this additional data analysis protocol, the measured pIC_{50} of Compound 1 for inhibiting the INFα induced increase of pSTAT1 levels was 6.21 ± 0.09 (SEM), while pIC_{50} for inhibition of GM-CSF induced increase of IL-8 was 4.97 ± 0.14 (SEM) (results obtained using 6 blood donors). This confirms that Compound 1 is 17.6-fold more selective for the JAK1 pathway versus the JAK2 pathway. Therefore, in a cellular setting it is clear that Compound 1 exhibits selectivity for JAK1 over JAK2. Compound 1 seems to exhibit a greater selectivity for JAK1 over JAK2 compared to known structurally related compounds, or other known JAK inhibitors.

2.4.2 *IL-6 stimulation protocol*

[00198] A flow cytometry analysis was also performed to establish compound selectivity for JAK1 over JAK2, *ex vivo* using human whole blood. Therefore, blood was taken from human volunteers who gave informed consent. Blood was then equilibrated for 30 minutes at 37°C under gentle rocking, then aliquoted in Eppendorf tubes. Compound was added at different concentrations and incubated at 37°C for 30 minutes under gentle rocking and subsequently stimulated for 20 minutes at 37°C under gentle rocking with interleukin 6 (IL-6) for JAK1-dependent pathway stimulation or GM-CSF for JAK2-dependent pathway stimulation. Phospho-STAT1 and phospho-STAT5 were then evaluated using FACS analysis.

2.4.2.1 *Phospho–STAT1 Assays*

[00199] For IL-6-stimulated increase of Signal Transducers and Activators of Transcription 1 (pSTAT1) phosphorylation in white blood cell, human whole blood, drawn from human volunteers who gave informed consent, was *ex vivo* treated with the compound for 30 min and subsequently stimulated

for 20 minutes with IL-6. The increase in phosphorylation of STAT1 by IL-6 in lymphocytes was measured using anti phospho-STAT1 antibody by FACS.

[00200] The 5X Lyse/Fix buffer (BD PhosFlow, Cat. N°558049) was diluted 5-fold with distilled water and pre-warmed at 37°C. Remaining diluted Lyse/Fix buffer was discarded.

[00201] 10 µg rhIL-6 (R&D Systems, Cat N°206-IL) was dissolved in 1ml of PBS 0.1% BSA to obtain a 10µg/ml stock solution. The stock solution was stored aliquoted at -80°C.

[00202] A 3-fold dilution series of the compound was prepared in DMSO (10 mM stock solution). Control-treated samples received DMSO instead of compound. All samples were incubated with a 1% final DMSO concentration.

2.4.2.1.1 *Incubation of blood with compound and stimulation with IL-6*

[00203] Human blood was collected in heparinized tubes. The blood was divided in aliquots of 148.5µl. Then, 1.5 µl of compound dilution was added to each aliquot and the blood samples were incubated for 30 min at 37°C under gentle rocking. IL-6 stock solution (1.5µl) was added to the blood samples (final concentration 10ng/ml) and samples were incubated at 37°C for 20 min under gentle rocking.

2.4.2.1.2 *White blood cell preparation and CD4 labeling*

[00204] At the end of the stimulation period, 3ml of 1X pre-warmed Lyse/Fix buffer was immediately added to the blood samples, vortexed briefly and incubated for 15 min at 37°C in a water bath in order to lyse red blood cells and fix leukocytes, then frozen at -80°C until further use.

[00205] For the following steps, tubes were thawed at 37°C for approximately 20 minutes and centrifuged for 5 min at 400 g at 4°C. The cell pellet was washed with 3ml of cold 1X PBS, and after centrifugation the cell pellet was resuspended in 100µl of PBS containing 3% BSA. FITC-conjugated anti-CD4 antibody or control FITC-conjugated isotype antibody were added and incubated for 20 min at room temperature, in the dark.

2.4.2.1.3 *Cell permeabilization and labeling with anti Phospho-STAT1 antibody*

[00206] After washing cells with 1X PBS, the cell pellet was resuspended in 100µl of ice-cold 1X PBS and 900µl of ice-cold 100% methanol was added. Cells were then incubated at 4°C for 30 min for permeabilization.

[00207] Permeabilized cells were then washed with 1X PBS containing 3% BSA and finally resuspended in 80µl of 1X PBX containing 3% BSA.

[00208] 20µL of PE mouse anti-STAT1 (pY701) or PE mouse IgG2aκ isotype control antibody (BD Biosciences, Cat. N°612564 and 559319, respectively) were added and mixed, then incubated for 30 min at 4°C, in the dark.

[00209] Cells are then washed once with 1X PBS and analyzed on a FACSCanto II flow cytometer (BD Biosciences).

2.4.2.1.4 *Fluorescence analysis on FACSCanto II*

[00210] 50,000 total events were counted and Phospho-STAT1 positive cells were measured after gating on CD4+ cells, in the lymphocyte gate. Data were analyzed using the FACSDiva software and correspond to the percentage of inhibition of IL-6 stimulation calculated on the percentage of positive cells for phospho-STAT1 on CD4+ cells.

2.4.2.2 *Phospho–STAT5 Assay*

[00211] For GM-CSF-stimulated increase of Signal Transducers and Activators of Transcription 5 (pSTAT5) phosphorylation in white blood cell, human whole blood, drawn from human volunteers who gave informed consent, was *ex vivo* treated with compound for 30 min and subsequently stimulated for 20 minutes with GM-CSF. The increase in phosphorylation of STAT5 by GM-CSF in monocytes was measured using an anti phospho-STAT5 antibody by FACS.

[00212] The 5X Lyse/Fix buffer (BD PhosFlow, Cat. N°558049) was diluted 5-fold with distilled water and pre-warmed at 37°C. Remaining diluted Lyse/Fix buffer was discarded.

[00213] 10 µg rhGM-CSF (AbCys S.A., Cat N°P300-03) was dissolved in 100µl of PBS 0.1% BSA to obtain a 100µg/ml stock solution. The stock solution was stored aliquoted at -80°C.

[00214] A 3-fold dilution series of the compound was prepared in DMSO (10 mM stock solution). Control-treated samples received DMSO without any test compound. All samples were incubated with a 1% final DMSO concentration.

2.4.2.2.1 *Incubation of blood with compound and stimulation with GM-CSF*

[00215] Human blood was collected in heparinized tubes. The blood was divided in aliquots of 148.5µl. Afterwards, 1.5 µl of compound dilution was added to each aliquot and the blood samples were incubated for 30 min at 37°C under gentle rocking. GM-CSF stock solution (1.5µl) was added to the blood samples (final concentration 20pg/ml) and samples were incubated at 37°C for 20 min under gentle rocking.

2.4.2.2.2 *White blood cell preparation and CD14 labeling*

[00216] At the end of the stimulation period, 3ml of 1X pre-warmed Lyse/Fix buffer was immediately added to the blood samples, vortexed briefly and incubated for 15 min at 37°C in a water bath in order to lyse red blood cells and fix leukocytes, then frozen at -80°C until further use.

[00217] For the following steps, tubes were thawed at 37°C for approximately 20 minutes and centrifuged for 5 min at 400 g at 4°C. The cell pellet was washed with 3ml of cold 1X PBS, and after centrifugation the cell pellet was resuspended in 100µl of PBS containing 3% BSA. FITC mouse anti-

CD14 antibody (BD Biosciences, Cat. N°345784) or control FITC mouse IgG2bκ isotype antibody (BD Biosciences, Cat. N°555057) were added and incubated for 20 min at room temperature, in the dark.

2.4.2.2.3 *Cell permeabilization and labeling with anti phospho-STAT5 antibody*

[00218] After washing cells with 1X PBS, the cell pellet was resuspended in 100µl of ice-cold 1X PBS and 900µl of ice-cold 100% methanol was added. Cells were then incubated at 4°C for 30 min for permeabilization.

[00219] Permeabilized cells were then washed with 1X PBS containing 3% BSA and finally resuspended in 80µl of 1X PBS containing 3% BSA.

[00220] 20µL of PE mouse anti-STAT5 (pY694) or PE mouse IgG1κ isotype control antibody (BD Biosciences, Cat. N°612567and 554680, respectively) were added and mixed then incubated for 30 min at 4°C, in the dark.

[00221] Cells are then washed once with 1X PBS and analyzed on a FACSCanto II flow cytometer (BD Biosciences).

2.4.2.2.4 *Fluorescence analysis on FACSCanto II*

[00222] 50,000 total events were counted and Phospho-STAT5 positive cells were measured after gating on CD14+ cells. Data were analyzed using the FACSDiva software and correspond to the percentage of inhibition of GM-CSF stimulation calculated on the percentage of positive cells for phospho-STAT5 on CD14+ cells.

2.4.2.3 *Results*

[00223] When submitted to this protocol, the perrcentage of inhibition (PIN) obtained from the mean of 3 healthy volunteers was determined for the compound of the invention. For example, Compound 1 was tested and returned a pIC_{50} = 6.61 in the inhibition of STAT1 phosphorylation and a pIC_{50} = 5.37 in the inhibition of STAT5 phosphorylation

[00224] When comparing the effect of Compound 1 on STAT1 (JAK1-dependent pathway) and STAT5 (JAK2-dependent pathway), a 17.6-fold selectivity for JAK1 *vs.* JAK2 was measured.

Example 3. *In vivo* models

Example 3.1 CIA model

3.1.1 Materials

[00225] Completed Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) were purchased from Difco. Bovine collagen type II (CII), lipopolysaccharide (LPS), and Enbrel were obtained from Chondrex (Isle d'Abeau, France); Sigma (P4252, L'Isle d'Abeau, France), Whyett (25mg injectable syringe, France) Acros Organics (Palo Alto, CA), respectively. All other reagents used were of reagent grade and all solvents were of analytical grade.

3.1.2 Animals

[00226] Dark Agouti rats (male, 7-8 weeks old) are obtained from Harlan Laboratories (Maison-Alfort, France). DBA/1J mice (male, 7 weeks old) were obtained from Centre d'Elevage et de Reproduction JANVIER (CERJ) (Laval, France). Rats and mice were kept on a 12 hours light/dark cycle (0700 - 1900). The temperature was maintained at 22°C, and food and water were provided *ad libitum*.

3.1.3 Collagen induced arthritis (CIA)

[00227] One day before the experiment, CII solution (2 mg/mL) was prepared with 0.05 M acetic acid and stored at 4°C. Just before the immunization, equal volumes of adjuvant (IFA) and CII were mixed by a homogenizer in a pre-cooled glass bottle in an ice water bath. Extra adjuvant and prolonged homogenization might be required if an emulsion is not formed.

[00228] Mice: 0.1 mL of the emulsion was injected intradermally at the base of the tail of each mouse on day 1, a second booster intradermal injection (CII solution at 1 mg/mL in CFA 0.1 mL saline) was performed on day 21. This immunization method was modified from published methods (David D Brand Kary A Latham, & Edward F Rosloniec. Collagen-induced arthritis. Nature Methods 2 (5): 1269-1275, 2007).

[00229] Rat: 0.2 mL of the emulsion is injected intradermally at the base of the tail of each rat on day 1, a second booster intradermal injection (CII solution at 2 mg/mL in CFA 0.1 mL saline) is performed on day 9. This immunization method is modified from published methods (Sims NA *et al.*, (2004) Targeting osteoclasts with zoledronic acid prevents bone destruction in collagen-induced arthritis, Arthritis Rheum. 50 2338-2346; Jou *et al.*, 2005).

3.1.4 Study design

[00230] The therapeutic effects of the test compounds were tested in the rat or mouse CIA model. Animals were randomly divided into equal groups and each group contained 10 animals. All rats are immunized on day 1 and boosted on day 9. All mice were immunized on day 1 and boosted on day 21. Therapeutic dosing lasted from day 16 to day 30. The negative control group was treated with vehicle (MC 0,5%) and the positive control group with Enbrel (10 mg/kg, 3x week., s.c.). A compound of interest was typically tested at 3 doses, e.g. 3, 10, 30 mg/kg, p.o.

3.1.5 Clinical assessment of arthritis

[00231] Arthritis was scored according the method of Khachigian 2006, Lin *et al* 2007 and Nishida *et al.* 2004). The swelling of each of the four paws was ranked with the arthritic score as follows: 0-no symptoms; 1-mild, but definite redness and swelling of one type of joint such as the ankle or wrist, or apparent redness and swelling limited to individual digits, regardless of the number of affected digits; 2-moderate redness and swelling of two or more types of joints; 3-severe redness and swelling of the entire

paw including digits; 4-maximally inflamed limb with involvement of multiple joints (maximum cumulative clinical arthritis score 16 per animal) (Nishida *et al.*, 2004).

[00232] To permit the meta-analysis of multiple studies the clinical score values were normalised as follows:

[00233] *AUC of clinical score (AUC score)*: The area under the curve (AUC) from day 1 to day 14 was calculated for each individual rat. The AUC of each animal was divided by the average AUC obtained for the vehicle in the study from which the data on that animal was obtained and multiplied by 100 (i.e. the AUC was expressed as a percentage of the average vehicle AUC per study).

[00234] *Clinical score increase from day 1 to day 14 (End point score)*: The clinical score difference for each animal was divided by the average clinical score difference obtained for the vehicle in the study from which the data on that animal was obtained and multiplied by 100 (i.e. the difference was expressed as a percentage of the average clinical score difference for the vehicle per study).

3.1.6 Change in body weight (%) after onset of arthritis

[00235] Clinically, body weight loss is associated with arthritis (Shelton *et al.*, 2005; Argiles *et al.*, 1998; Rall, 2004; Walsmith *et al.*, 2004). Hence, changes in body weight after onset of arthritis could be used as a non-specific endpoint to evaluate the effect of therapeutics in the rat model. The change in body weight (%) after onset of arthritis was calculated as follows:

Mice: $$\frac{\text{Body Weight}_{(week6)} - \text{Body Weight}_{(week5)}}{\text{Body Weight}_{(week5)}} \times 100\%$$

Rats: $$\frac{\text{Body Weight}_{(week4)} - \text{Body Weight}_{(week3)}}{\text{Body Weight}_{(week3)}} \times 100\%$$

3.1.7 Larsen' score:

[00236] Post-mortem, a Larsen' score was derived for both hind paws of all rats by at least 2 scientists. The average of these scores was calculated to obtain one Larsen' score per rat. To allow an inter-study comparison of the Larsen' scores, the Larsen' score of each rat was divided by the average Larsen' score obtained for vehicle in the study to which that rat belongs and multiplied by 100 (i.e. expressing the Larsen' score as the percentage of average vehicle Larsen' score per study). The average Larsen' score of the different treatment groups was calculated and compared.

3.1.8 Radiology

[00237] X-ray photos were taken of the hind paws of each individual animal. A random blind identity number was assigned to each of the photos, and the severity of bone erosion was ranked by two independent scorers with the radiological Larsen's score system as follows: 0- normal with intact bony outlines and normal joint space; 1- slight abnormality with any one or two of the exterior metatarsal bones showing slight bone erosion; 2-definite early abnormality with any three to five of the exterior

metatarsal bones showing bone erosion; 3-medium destructive abnormality with all the exterior metatarsal bones as well as any one or two of the interior metatarsal bones showing definite bone erosions; 4-severe destructive abnormality with all the metatarsal bones showing definite bone erosion and at least one of the inner metatarsal joints completely eroded leaving some bony joint outlines partly preserved; 5-mutilating abnormality without bony outlines. This scoring system is a modification from Salvemini *et al.*, 2001; Bush *et al.*, 2002; Sims *et al.*, 2004; Jou *et al.*, 2005.

3.1.9 Histology

[00238] After radiological analysis, the hind paws of mice were fixed in 10% phosphate-buffered formalin (pH 7.4), decalcified with rapid bone decalcificant for fine histology (Laboratories Eurobio) and embedded in paraffin. To ensure extensive evaluation of the arthritic joints, at least four serial sections (5 µm thick) were cut and each series of sections were 100 µm in between. The sections were stained with hematoxylin and eosin (H&E). Histologic examinations for synovial inflammation and bone and cartilage damage were performed double blind. In each paw, four parameters were assessed using a four-point scale. The parameters were cell infiltration, pannus severity, cartilage erosion and bone erosion. Scoring was performed as follows: 1-normal, 2-mild, 3-moderate, 4-marked. These four scores were summed together and represented as an additional score, namely the 'RA total score'. To allow an inter-study comparison of the histology readouts, the overall histology score of each rat was divided by the average overall histology score obtained for vehicle in the study to which that rat belongs and multiplied by 100 (i.e. expressing the overall histology score as the percentage of average vehicle overall histology score per study). The average overall histology score of the different treatment groups was calculated and compared.

3.1.10 Micro-computed tomography (µCT) analysis of calcaneus (heel bone):

[00239] Bone degradation observed in RA occurs especially at the cortical bone and can be revealed by µCT analysis (Sims NA *et al.*, 2004; Oste L *et al.*, ECTC Montreal 2007). After scanning and 3D volume reconstruction of the calcaneus bone, bone degradation was measured as the number of discrete objects present per slide, isolated *in silico* perpendicular to the longitudinal axis of the bone. The more the bone that was degraded, the more discrete objects that were measured. 1000 slices, evenly distributed along the calcaneus (spaced by about 10.8 µm), are analyzed.

3.1.11 Results

[00240] Compound 1was tested in the mouse CIA study at 30 mg/kg andin the rat CIA study at 30, 10, 3, and 1 mg/kg. Compound 1 was efficacious in all readouts performed in the rat CIA study, with statistical significance in several of the readouts, in particular significant improvements were seen for: the AUC of the clinical score (from 10mg/kg), the endpoint clinical score (from 1 mg/kg), the Larsen' score (from 30 mg/kg) and paw swelling (from 1 mg/kg).

Example 3.2 Septic shock model

[00241] Injection of lipopolysaccharide (LPS) induces a rapid release of soluble tumour necrosis factor (TNF-alpha) into the periphery. This model is used to analyse prospective blockers of TNF release *in vivo*.

[00242] Six BALB/cJ female mice (20 g) per group are treated at the intended dosing once, po. Thirty minutes later, LPS (15 µg/kg; *E. Coli* serotype 0111:B4) is injected ip. Ninety minutes later, mice are euthanized and blood is collected. Circulating TNF alpha levels are determined using commercially available ELISA kits. Dexamethasone (5 µg/kg) is used as a reference anti-inflammatory compound. Selected compounds are tested at one or multiple doses, e.g. 3 and/or 10 and/or 30 mg/kg, po.

[00243] Compound 1 exhibited a statistically significant reduction in the TNF release (>50%) at 30mg/kg po.

Example 3.3 MAB model

[00244] The MAB model allows a rapid assessment of the modulation of an RA-like inflammatory response by therapeutics (Kachigian LM. Nature Protocols (2006) 2512-2516: Collagen antibody-induced arthritis). DBA/J mice are injected i.v. with a cocktail of mAbs directed against collagen II. One day later, compound treatment is initiated (vehicle: 10% (v/v) HPβCD). Three days later, mice receive an i.p. LPS injection (50 µg/mouse), resulting in a fast onset of inflammation. Compound treatment is continued until 10 days after the mAb injection. Inflammation is read by measuring paw swelling and recording the clinical score of each paw. The cumulative clinical arthritis score of four limbs is presented to show the severity of inflammation. A scoring system is applied to each limb using a scale of 0–4, with 4 being the most severe inflammation.

0 Symptom free

1 Mild, but definite redness and swelling of one type of joint such as the ankle or wrist, or apparent redness and swelling limited to individual digits, regardless of the number of affected digits

2 Moderate redness and swelling of two or more types of joints

3 Severe redness and swelling of the entire paw including digits

4 Maximally inflamed limb with involvement of multiple joints

Example 3.4 Oncology models

[00245] *In vivo* models to validate efficacy of small molecules towards JAK2-driven myleoproliferative diseases are described by Wernig *et al.* Cancer Cell 13, 311, 2008 and Geron *et al.* Cancer Cell 13, 321, 2008.

Example 3.5 Mouse IBD model

[00246] *In vitro* and *in vivo* models to validate efficacy of small molecules towards IBD are described by Wirtz *et al.* 2007.

Example 3.6 Mouse Asthma model

[00247] *In vitro* and *in vivo* models to validate efficacy of small molecules towards asthma are described by Nials *et al.*, 2008; Ip *et al.* 2006; Pernis *et al.*, 2002; Kudlacz *et al.*, 2008.

Example 4: Toxicity, DMPK and Safety Models

Example 4.1 Thermodynamic solubility

[00248] A solution of 1 mg/mL of the test compound is prepared in a 0.2M phosphate buffer pH7.4 or a 0.1M citrate buffer pH3.0 at room temperature in a glass vial.

[00249] The samples are rotated in a Rotator drive STR 4 (Stuart Scientific, Bibby) at speed 3.0 at room temperature for 24 hours.

[00250] After 24 hours, 800µL of the sample is transferred to an eppendorf tube and centrifuged 5 min at 14000rpm. 200 µL of the supernatant of the sample is then transferred to a MultiscreenR Solubility Plate (Millipore, MSSLBPC50) and the supernatant is filtered (10-12" Hg) with the aid of a vacuum manifold into a clean Greiner polypropylene V-bottom 96well plate (Cat no.651201). 5 µL of the filtrate is diluted into 95 µL (F20) of the same buffer used to incubate in the plate containing the standard curve (Greiner,Cat no.651201).

[00251] The standard curve for the compound is prepared freshly in DMSO starting from a 10mM DMSO stock solution diluted factor 2 in DMSO (5000µM) and then further diluted in DMSO up to 19.5µM. 3µL of the dilution series as from 5000µM is then transferred to a 97µL acetonitrile-buffer mixture (50/50). The final concentration range is 2.5 to 150 µM.

[00252] The plate is sealed with sealing mats (MA96RD-04S, www.kinesis.co.uk) and samples are measured at room temperature on LCMS (ZQ 1525 from Waters) under optimized conditions using Quanoptimize to determine the appropriate mass of the molecule.

[00253] The samples are analyzed on LCMS with a flow rate of 1mL/min. Solvent A is 15mM ammonia and solvent B is acetonitrile. The sample is run under positive ion spray on an XBridge C18 3.5µM (2.1 x 30mm) column, from Waters. The solvent gradient has a total run time of 2 minutes and ranges from 5% B to 95% B.

[00254] Peak areas are analyzed with the aid of Masslynx software package and peak areas of the samples are plotted against the standard curve to obtain the solubility of the compound.

[00255] Solubility values are reported in µM or µg/mL.

Example 4.2 Aqueous Solubility

[00256] Starting from a 10mM stock in DMSO, a serial dilution of the compound is prepared in DMSO. The dilution series is transferred to a 96 NUNC Maxisorb plate F-bottom (Cat no. 442404) and 0.2M phosphate buffer pH 7.4 or 0.1M citrate buffer pH3.0 at room temperature is added.

[00257] The final concentration ranged from 200µM to 2.5µM in 5 equal dilution steps. The final DMSO concentration did not exceed 2%. 200µM Pyrene is added to the corner points of each 96 well plate and serves as a reference point for calibration of Z-axis on the microscope.

[00258] The assay plates are sealed and incubated for 1 hour at 37°C while shaking at 230rpm. The plates are then scanned under a white light microscope, yielding individual pictures of the precipitate per concentration. The precipitate is analyzed and converted into a number which is plotted onto a graph. The first concentration at which the compound appears completely dissolved is the concentration reported, however the true concentration lies somewhere between this concentration and one dilution step higher.

[00259] Solubility values are reported in µg/mL

Example 4.3 Plasma Protein Binding (Equilibrium Dialysis)

[00260] A 10mM stock solution of the compound in DMSO is diluted with a factor 5 in DMSO. This solution is further diluted in freshly thawed human, rat, mouse or dog plasma (BioReclamation INC) with a final concentration of 10µM and final DMSO concentration of 0.5% (5.5µl in 1094.5µl plasma in a PP-Masterblock 96well (Greiner, Cat no. 780285))

[00261] A Pierce Red Device plate with inserts (ThermoScientific, Cat no. 89809) is prepared and filled with 750µL PBS in the buffer chamber and 500µL of the spiked plasma in the plasma chamber. The plate is incubated for 4 hours at 37°C while shaking at 230rpm. After incubation, 120µL of both chambers is transferred to 360µL acetonitrile in a 96-well round bottom, PP deep-well plates (Nunc, Cat no. 278743) and sealed with an aluminum foil lid. The samples are mixed and placed on ice for 30min. This plate is then centrifuged 30 min at 1200rcf at 4°C and the supernatant is transferred to a 96 v-bottom PP plate (Greiner, 651201) for analysis on LCMS.

[00262] The plate is sealed with sealing mats (MA96RD-04S) of www.kinesis.co.uk and samples are measured at room temperature on LCMS (ZQ 1525 from Waters) under optimized conditions using Quanoptimize to determine the appropriate mass of the molecule.

[00263] The samples are analyzed on LCMS with a flow rate of 1mL/min. Solvent A was 15mM ammonia and solvent B was acetonitrile. The sample was run under positive ion spray on an XBridge C18 3.5µM (2.1 x 30mm) column, from Waters. The solvent gradient has a total run time of 2 minutes and ranges from 5% B to 95% B.

[00264] Peak area from the compound in the buffer chamber and the plasma chamber are considered to be 100% compound. The percentage bound to plasma is derived from these results and was reported to the LIMS as percentage bound to plasma.

[00265] The solubility of the compound in the final test concentration in PBS is inspected by microscope to indicate whether precipitation is observed or not.

Example 4.4 Liability for QT prolongation

[00266] Potential for QT prolongation is assessed in the hERG patch clamp assay.

4.4.1 Conventional whole-cell patch-clamp

[00267] Whole-cell patch-clamp recordings are performed using an EPC10 amplifier controlled by Pulse v8.77 software (HEKA). Series resistance is typically less than 10 MΩ and compensated by greater than 60%, recordings are not leak subtracted. Electrodes are manufactured from GC150TF pipette glass (Harvard).

[00268] The external bathing solution contains: 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 5 mM Glucose, 10 mM HEPES, pH 7.4.

[00269] The internal patch pipette solution contains: 100mM Kgluconate, 20 mM KCl, 1mM $CaCl_2$, 1 mM $MgCl_2$, 5mM Na_2ATP, 2mM Glutathione, 11 mM EGTA, 10 mM HEPES, pH 7.2.

[00270] Drugs are perfused using a Biologic MEV-9/EVH-9 rapid perfusion system.

[00271] All recordings are performed on HEK293 cells stably expressing hERG channels. Cells are cultured on 12 mm round coverslips (German glass, Bellco) anchored in the recording chamber using two platinum rods (Goodfellow). hERG currents are evoked using an activating pulse to +40 mV for 1000 ms followed by a tail current pulse to –50 mV for 2000 ms, holding potential was -80 mV. Pulses are applied every 20s and all experiments are performed at room temperature.

4.4.2 Data Analysis

[00272] IC_{50} and IC_{20} values are calculated for each compound tested. The fold difference between the IC_{20} and the unbound C_{max} concentrations of the test compound obtained at relevant therapeutic doses as determined by results obtained from the rat CIA model is calculated.

[00273] For the concentration response curves, peak tail current amplitude is measured during the voltage step to -50 mV. Curve-fitting of concentration-response data is performed using the equation:

$$y = a + [(b - a)/ (1 + 10^{\wedge} ((\log c - x) d)]$$

[00274] where a is minimum response, b is maximum response and d is Hill slope, this equation can be used to calculate both IC_{50} (where y = 50 and c is the IC_{50} value) and IC_{20} (where y = 20 and c is the IC_{20} value). GraphPad® Prism® (Graphpad® Software Inc.) software was used for all curve fitting. A difference of 100 fold or greater indicates a low potential for QT prolongation.

Example 4.5 Microsomal stability

[00275] A 10mM stock solution of compound in DMSO was diluted 1000 fold in a 182 mM phosphate buffer pH7.4 in a 96 deep well plate (Greiner, Cat no.780285) and pre-incubated at 37°C.

[00276] 40µL of deionised water was added to a well of a polypropylene Matrix 2D barcode labelled storage tube (Thermo Scientific) and pre-incubated at 37°C.

[00277] A Glucose-6-phophate-dehydrogenase (G6PDH) working stock solution was prepared in 182mM phosphate buffer pH7.4 and placed on ice before use. A co-factor containing $MgCl_2$, glucose-6-phosphate and NADP+ was prepared in deionised water and placed on ice before use.

[00278] A final working solution containing liver microsomes (Xenotech) of a species of interest (human, mouse, rat, dog), previously described G6PDH and co-factors was prepared and this mix was incubated for no longer than 20 minutes at room temperature.

[00279] 30µL of the pre-heated compound dilution was added to 40µL of pre-heated water in the Matrix tubes and 30µL of the microsomal mix was added. Final reaction concentrations were 3µM compound, 1mg microsomes, 0.4U/mL GDPDH, 3.3mM $MgCl_2$, 3.3mM glucose-6-phosphate and 1.3mM NADP+.

[00280] To measure percentage remaining of compound at time zero MeOH or ACN was added (1:1) to the well before adding the microsomal mix. The plates were sealed with Matrix Sepra sealsTM (Matrix, Cat. No.4464) and shaken for a few seconds ensure complete mixing of all components.

[00281] The samples which were not stopped are incubated at 37°C, 300rpm and after 1 hour of incubation the reaction was stopped with MeOH or ACN (1:1).

[00282] After stopping the reaction the samples were mixed and placed on ice for 30min to precipitate the proteins. The plates were then centrifuged 30 min at 1200rcf at 4°C and the supernatant was transferred to a 96 v-bottom PP plate (Greiner, 651201) for analysis on LCMS.

[00283] These plates were sealed with sealing mats (MA96RD-04S) of www.kinesis.co.uk and samples were measured at room temperature on LCMS (ZQ 1525 from Waters) under optimized conditions using Quanoptimize to determine the appropriate mass of the parent molecule.

[00284] The samples were analyzed on LCMS with a flow rate of 1mL/min. Solvent A was 15mM ammonia and solvent B was methanol or acetonitrile, depending on the stop solution used. The samples were run under positive ion spray on an XBridge C18 3.5µM (2.1 x 30mm) column, from Waters. The solvent gradient had a total run time of 2 minutes and ranges from 5% B to 95% B.

[00285] Peak area from the parent compound at time 0 was considered to be 100% remaining. The percentage remaining after 1 hour incubation was calculated from time 0 and was calculated as the percentage remaining.The solubility of the compound in the final test concentration in buffer is inspected by microscope and results are reported.

[00286] The data on microsomal stability are expressed as a percentage of the total amount of compound remaining after 60 minutes.

[00287] **TABLE IX – Microsomal stability**

Compound #	Human	Rat

Compound #	Human	Rat
1	76-100 %	76-100%

Example 4.6 Caco2 Permeability

[00288] Bi-directional Caco-2 assays were performed as described below. Caco-2 cells were obtained from European Collection of Cell Cultures (ECACC, cat 86010202) and used after a 21 day cell culture in 24-well Transwell plates (Fisher TKT-545-020B).

[00289] $2x10^5$ cells/well were seeded in plating medium consisting of DMEM + GlutaMAXI + 1% NEAA + 10% FBS (FetalClone II) + 1% Pen/Strep. The medium was changed every 2 – 3 days.

[00290] Test and reference compounds (propranolol and rhodamine123 or vinblastine, all purchased from Sigma) were prepared in Hanks' Balanced Salt Solution containing 25 mM HEPES (pH7.4) and added to either the apical (125µL) or basolateral (600µL) chambers of the Transwell plate assembly at a concentration of 10 µM with a final DMSO concentration of 0.25%.

[00291] 50µM Lucifer Yellow (Sigma) was added to the donor buffer in all wells to assess integrity of the cell layers by monitoring Lucifer Yellow permeation. As Lucifer Yellow (LY) cannot freely permeate lipophilic barriers, a high degree of LY transport indicates poor integrity of the cell layer.

[00292] After a 1 hour incubation at 37°C while shaking at an orbital shaker at 150rpm, 70µL aliquots were taken from both apical (A) and basal (B) chambers and added to 100µLl 50:50 acetonitrile:water solution containing analytical internal standard (0.5µM carbamazepine) in a 96 well plate.

[00293] Lucifer yellow was measured with a Spectramax Gemini XS (Ex 426nm and Em 538nm) in a clean 96 well plate containing 150µL of liquid from basolateral and apical side.

[00294] Concentrations of compound in the samples were measured by high performance liquid-chromatography/mass spectroscopy (LC-MS/MS).

[00295] Apparent permeability (P_{app}) values were calculated from the relationship:

$P_{app} = [compound]_{acceptor\ final} \times V_{acceptor} / ([compound]_{donor\ initial} \times V_{donor}) / T_{inc} \times V_{donor} / surface\ area \times 60 \times 10^{-6}$ cm/s

V = chamber volume

T_{inc} = incubation time.

Surface area = $0.33cm^2$

[00296] The Efflux ratios, as an indication of active efflux from the apical cell surface, were calculated using the ratio of P_{app} B>A/ P_{app} A>B.

[00297] The following assay acceptance criteria were used:

Propranolol: P_{app} (A>B) value ≥ 20($\times 10^{-6}$ cm/s)

Rhodamine 123 or Vinblastine: P_{app} (A>B) value < 5 ($\times 10^{-6}$ cm/s) with Efflux ratio ≥5.

Lucifer yellow permeability: ≤100 nm/s

[00298] **TABLE X – Caco2 Efflux rate**

Compound #	Papp A>B (x10-6 cm/sec)	Efflux ratio
1	7.8 ± 0.8	6.1 ± 0.6

Example 4.7 Pharmacokinetic study

4.7.1 Pharmacokinetic study in rodents

Compounds are formulated in PEG200/physiological saline or PEG400/DMSO/physiological saline mixtures for the intravenous route and in 0.5% methylcellulose or 10-30% hydroxylpropyl-β-cyclodextrine pH3 or pH7.4 for the oral route. Test compounds are orally dosed as a single esophageal gavage at 5-10 mg/kg and intravenously dosed as a bolus via the caudal vein at 1 mg/kg. Each group consists of 3 rats. Blood samples are collected either via the jugular vein using cannulated rats or at the retro-orbital sinus with lithium heparin as anti-coagulant at the time points in the following range: 0.05 to 8 hours (intravenous route), and 0.25 to 6 or 24 hours (oral route). Whole blood samples are centrifuged at 5000 rpm for 10 min and the resulting plasma samples are stored at -20°C pending analysis.

4.7.2 Pharmacokinetic study in dogs

[00299] Compounds are formulated in PEG200/physiological saline mixtures for the intravenous route and in 0.5% methylcellulose or PEG400/ hydroxylpropyl-β-cyclodextrine mixtures acidified with citric acid to pH2-3 for the oral route. Test compounds are orally dosed via gavage at 5-30 mg/kg and intravenously dosed as a bolus or a 10 min infusion via the cephalic vein at 1 mg/kg. Each group consists of 3 male or female Beagle dogs. Blood samples are collected via the jugular vein with lithium heparin as anti-coagulant at time points between 0.083 to 24 hours postdose. Whole blood samples are centrifuged at 5000 rpm for 10 min and the resulting plasma samples are stored at -20°C pending analysis.

4.7.3 Quantification of compound levels in plasma

[00300] Plasma concentrations of each test compound are determined by an LC-MS/MS method in which the mass spectrometer is operated in positive electrospray mode.

4.7.4 Determination of pharmacokinetic parameters

[00301] Pharmacokinetic parameters are calculated using Winnonlin® (Pharsight®, United).

Example 4.8 7-Day rat toxicity study

[00302] A 7-day oral toxicity study with test compounds is performed in Sprague-Dawley male rats to assess their toxic potential and toxicokinetics, at daily doses of 100, 300 and 500 mg/kg/day, by gavage, at the constant dosage-volume of 5 mL/kg/day.

[00303] The test compounds are formulated in 30% (v/v) HPβCD in purified water. Each group includes 5 principal male rats as well as 3 satellite animals for toxicokinetics. A fourth group is given 30% (v/v) HPβCD in water only, at the same frequency, dosage volume and by the same route of administration, and acts as the vehicle control group.

[00304] The goal of the study is to determine the lowest dose that results in no adverse events being identified (no observable adverse effect level - NOAEL).

Example 4.9 Hepatocyte stability.

[00305] Models to evaluate metabolic clearance in hepatocyte are described by McGinnity *et al.* Drug Metabolism and Disposition **2008**, 32, *11*, 1247.

[00306] It will be appreciated by those skilled in the art that the foregoing descriptions are exemplary and explanatory in nature, and as indicated are intended to illustrate the invention and its preferred embodiments. Through routine experimentation, an artisan will recognise apparent modifications and variations that may be made without departing from the spirit of the invention. Thus, the invention is intended to be defined not by the above description, but by the following claims and their equivalents.

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[00308] All publications, including but not limited to patents and patent applications, cited in this specification are herein incorporated by reference as if each individual publication were specifically and individually indicated to be incorporated by reference herein as though fully set forth.

[00309] From the foregoing description, various modifications and changes in the compositions and methods of this invention will occur to those skilled in the art. All such modifications coming within the scope of the appended claims are intended to be included therein.

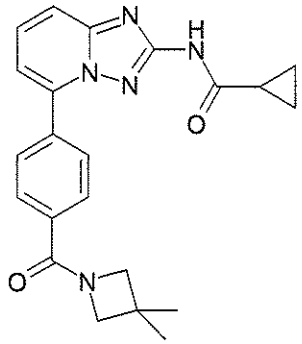
[00310] It should be understood that factors such as the differential cell penetration capacity of the various compounds can contribute to discrepancies between the activity of the compounds in the *in vitro* biochemical and cellular assays.

[00311] At least some of the chemical names of compounds of the invention as given and set forth in this application, may have been generated on an automated basis by use of a commercially available chemical naming software program, and have not been independently verified. Representative programs performing this function include the Lexichem naming tool sold by Open Eye Software, Inc. and the Autonom Software tool sold by MDL, Inc. In the instance where the indicated chemical name and the depicted structure differ, the depicted structure will control.

[00312] Chemical structures shown herein were prepared using either ChemDraw® or ISIS® /DRAW. Any open valency appearing on a carbon, oxygen or nitrogen atom in the structures herein indicates the presence of a hydrogen atom. Where a chiral center exists in a structure but no specific stereochemistry is shown for the chiral center, both enantiomers associated with the chiral structure are encompassed by the structure.

WHAT IS CLAIMED IS:

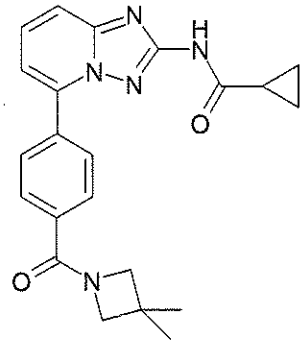
1. A compound according to Formula I:



I

or a pharmaceutically acceptable salt thereof.

2. A compound according to claim 1 which is



I

3. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a pharmaceutically effective amount of a compound, or pharmaceutically acceptable salt thereof, according to claims 1-2.

4. The pharmaceutical composition according to claim 3 comprising a further therapeutic agent.

5. The use of a compound, or a pharmaceutically acceptable salt thereof, according to claims 1-2 in the manufacture of a medicament.

6. A compound, or pharmaceutically acceptable salt thereof, according to claims 1-2 for use as a medicament.

7. A compound, or pharmaceutically acceptable salt thereof, according to claims 1-2 for use in the treatment, prevention or prophylaxis of inflammatory conditions, autoimmune diseases, proliferative diseases, transplantation rejection, diseases involving impairment of cartilage turnover, congenital cartilage malformations, and/or psoriasis.

8. The use of a compound, or pharmaceutically acceptable salt thereof, according to claim 1 in the preparation of a medicament for the treatment, prevention or prophylaxis of inflammatory conditions, autoimmune diseases, proliferative diseases, transplantation rejection, diseases involving impairment of cartilage

turnover, congenital cartilage malformations, and/or psoriasis.

9. A use according to claim 8, wherein the inflammatory condition is rheumatoid arthritis.

10. A use according to claim 8, wherein the condition or disease involves inflammation.

11. The compound to claim 6, wherein the compound according to claims 1-2 is to be administered in combination with a further therapeutic agent.

12. The use according to claim 8, wherein the compound according to claims 1-2 is to be administered in combination with a further therapeutic agent.

13. The pharmaceutical composition according to claim 4,wherein the further therapeutic agent is an agent for the treatment, prevention or prophylaxis of inflammatory conditions, autoimmune diseases, proliferative diseases, transplantation rejection, diseases involving impairment of cartilage turnover, congenital cartilage malformations, and/or psoriasis.

14. The use according to claim 12, wherein the further therapeutic agent is an agent for the treatment, prevention or prophylaxis of inflammatory conditions, autoimmune diseases, proliferative diseases, transplantation rejection, diseases involving impairment of cartilage turnover, congenital cartilage malformations, psoriasis

NOVEL COMPOUND USEFUL FOR THE TREATMENT OF DEGENERATIVE AND INFLAMMATORY DISEASES

Abstract

A compound is disclosed that has a formula represented by the following: (I). This compound may be prepared as a pharmaceutical composition, and may be used for the prevention and treatment of a variety of conditions in mammals including humans, including by way of non-limiting example, inflammatory conditions, autoimmune diseases, proliferative diseases, transplantation rejection, diseases involving impairment of cartilage turnover, congenital cartilage malformations, and/or diseases associated with hypersecretion of IL6.

Figure I.