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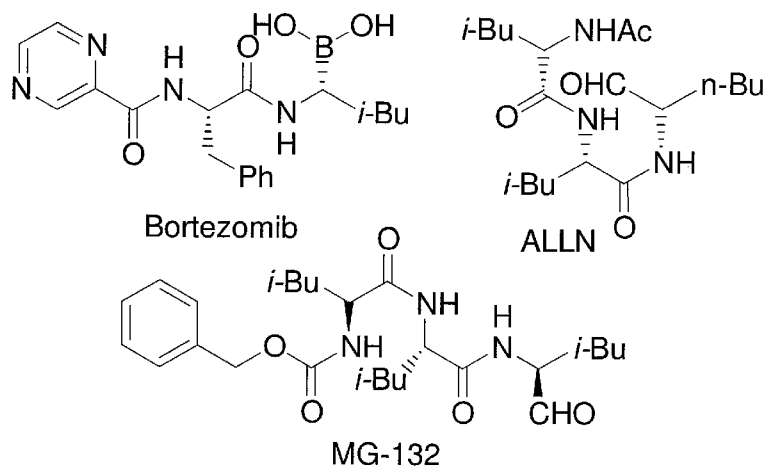
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(54) Title: NON-COVALENT INHIBITION OF THE 26S PROTEASOME AND USES THEREOF

Figure 1



(57) Abstract: The present inventions relate to compositions and methods for treating inflammatory diseases and cancer by administering proteasome inhibitors. In particular, the present inventions provide a new class of orally available non-covalent proteasome inhibitors capable of reducing NF-kappaB for mediating cytokine production in vivo. Further, the use of a small molecular weight inhibitor of the 26S proteasome via a non-covalent type inhibition is contemplated for use as a means to treat NF-kB mediated diseases, including but not limited to multiple myeloma and rheumatoid arthritis.

NON-COVALENT INHIBITION OF THE 26S PROTEASOME AND USES THEREOF

FIELD OF THE INVENTION

5 The present inventions relate to compositions and methods for treating inflammatory diseases and cancer by administering proteasome inhibitors. In particular, the present inventions provide a new class of orally available non-covalent proteasome inhibitors capable of reducing NF-kappaB for mediating cytokine production in vivo. Further, the use of a small molecular weight inhibitor of the 26S
10 proteasome via a non-covalent type inhibition is contemplated for use as a means to treat NF-κB mediated diseases, including but not limited to multiple myeloma and rheumatoid arthritis.

BACKGROUND OF THE INVENTION

15 The clinical benefit of proteasome inhibitors was demonstrated by the use of bortezomib in multiple myeloma and mantle cell lymphoma (in addition to multiple clinical trials with patients with advanced solid tumors, breast cancer, pancreatic cancer, colon cancer and non small lung cell cancer).

 However, problems remain with host toxicity (neuropathy) and development
20 of host resistance.

 Thus more effective treatments are necessary for safer and more effective treatment of cancer such as multiple myeloma.

SUMMARY OF THE INVENTION

25 The present inventions relate to compositions and methods for treating inflammatory diseases and cancer by administering proteasome inhibitors, including inhibitors that bind reversibly and inhibitors that bind irreversibly. In particular, the present inventions provide a new class of orally available non-covalent proteasome inhibitors capable of reducing NF-kappaB for mediating cytokine production in vivo.
30 Further, the use of a small molecular weight inhibitor of the 26S proteasome via a non-covalent type inhibition is contemplated for use as a means to treat NF-κB mediated diseases, including but not limited to multiple myeloma and rheumatoid arthritis.

While not limited to any particular mechanism, the present inventions are directed to the treatment of inflammatory and autoimmune diseases as well as cancer by administering proteasome inhibitors that, it is believed, interfere with the activation of NF-kappaB (NF- κ B) via the ubiquitin-proteasome pathway. The use of a small molecular weight inhibitor of the 26S proteasome via a non-covalent type inhibition is described herein. Non-covalent inhibition of the 26S proteasome is contemplated for use as a means to treat NF- κ B mediated diseases, including but not limited to multiple myeloma and rheumatoid arthritis. The described and related compounds are orally available showed potent inhibition of NF- κ B mediated inflammatory responses in cell culture, stimulated human blood and in vivo. Some compounds bind at a place different than the substrate binding site and are therefore classic non-competitive inhibitors.

The inventions provide a pharmaceutical composition comprising a small molecule non-covalent inhibitor of the 26S proteasome. In one embodiment, said inhibitor inhibits the 20S catalytic core of the 26S proteasome. In one embodiment, said inhibitor is selected from the group consisting of imidizolines 1-9 (see Figure 8). In one embodiment, said compound is a therapeutically effective amount for reducing a symptom of a disease selected from the group consisting of cancer and inflammatory disease. In one embodiment, said cancer is multiple myeloma. In one embodiment, said inflammatory disease is rheumatoid arthritis.

The inventions provide a method, comprising, a) providing, i) a pharmaceutical composition comprising a small molecule non-covalent inhibitor of the catalytic region of a 26S proteasome and ii) a patient, wherein said patient is in need of treatment and b) administering said pharmaceutical composition to said patient as a therapeutically effective amount. In one embodiment, said inhibitor inhibits the 20S catalytic core of the 26S proteasome. In one embodiment, said small molecule is selected from the group consisting of imidizolines 1-9 (see Figure 8). In one embodiment, said patient shows a symptom selected from the group consisting of a cancer and an inflammatory disease. In one embodiment, said cancer is multiple myeloma. In one embodiment, said inflammatory disease is rheumatoid arthritis.

While non-covalent inhibition has been emphasized herein, the present invention also contemplates, in one embodiment, covalent inhibitors (e.g. TCH-062) that bind irreversibly to the 20S proteasome.

DEFINITIONS

The use of the article "a" or "an" is intended to include one or more.

The use of terms defined in the singular are intended to include those terms defined in the plural and vice versa.

5 The term "proteasome" refers to a large protein complex inside eukaryote cells and archaea, and some bacteria.

 The term "26S proteasome" refers to a common form of a proteasome also referred to as an "ATP-dependant proteolytic complex" that is responsible for ubiquitin-dependent protein degradation. A 26S proteasome is about 2000 kilodaltons
10 (kDa) in molecular mass and contains one 20S core particle complex. Additionally, this complex may further comprise two 19S regulatory subunits (caps). An alternative form of regulatory subunit called the 11S particle can associate with the core in essentially the same manner as the 19S particle.

 The term "20S proteasome" or "20S core particle" refers to a complex that
15 provides the catalytic core of the 26S proteasome. The number and diversity of subunits contained in the 20S core particle depends on the organism.

 The term "inflammatory" is used to refer to pertaining, characterized by, causing, resulting from, or becoming affected by inflammation. An inflammation is a fundamental pathologic process consisting of a dynamic complex of cytologic and
20 chemical reactions that occur in the affected blood vessels and adjacent tissues in response to an injury or abnormal stimulation caused by a physical, chemical, or biologic agent; these reactions include the local reactions and resulting morphologic changes, the destruction or removal of the injurious material, and the responses that lead to repair and healing.

25 An "inflammatory disease" refers to a disease caused by or resulting from or resulting in inflammation.

 The term "anti-inflammatory" is used to refer to an effect or compound which has an effect of preventing, inhibiting, alleviating or decreasing inflammation or components of an inflammatory reaction, either completely or partially.

30 The terms "anticancer agent" and "anticancer drug," as used herein, refer to any therapeutic agents (e.g., chemotherapeutic compounds and/or molecular therapeutic compounds), radiation therapies, or surgical interventions, used in the treatment of hyperproliferative diseases such as cancer (e.g., in mammals).

The term "therapeutically effective amount," as used herein, refers to that amount of the therapeutic agent sufficient to result in amelioration of one or more symptoms of a disorder, or prevent advancement of a disorder, or cause regression of the disorder. For example, with respect to the treatment of cancer, a therapeutically effective amount preferably refers to the amount of a therapeutic agent that decreases the rate of tumor growth, decreases tumor mass, decreases the number of metastases, increases time to tumor progression, or increases survival time by at least 5%, preferably at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 100%.

The terms "prevent," "preventing," and "prevention," as used herein, refer to a decrease in the occurrence of pathological cells (e.g., hyperproliferative or neoplastic cells) in an animal. The prevention may be complete, e.g., the total absence of pathological cells in a subject. The prevention may also be partial, such that the occurrence of pathological cells in a subject is less than that which would have occurred without the present invention.

As used herein, the term "patient" refers to a human or animal. As used herein, the term "patient" and "subject" are used interchangeably.

As used herein, the term "administering" refers to any means of treating a patient with a composition of the present invention. In a preferred embodiment, administration is orally.

As used herein, the term "sample" is used in its broadest sense. In one sense, it is meant to include a specimen or culture obtained from any source, as well as biological and environmental samples. Biological samples may be obtained from an animal, including a human; a particular biological sample may be a fluid (for example, blood, plasma and serum), a solid (for example, stool), or a tissue; other biological samples may be obtained from other biological sources, such as food, and may be a liquid food (for example, milk), or a solid food (for example, vegetables). Environmental samples include environmental material such as surface matter, soil, water, crystals, and industrial samples. These examples are not to be construed as limiting the sample types applicable to the present invention.

DESCRIPTION OF THE FIGURES

Figure 1 shows exemplary examples of covalent proteasome inhibitors.

Figure 2 shows exemplary examples of non-covalent proteasome inhibitors.

Figure 3A shows exemplary Inhibition of the chymotrypsin-like activity by TCH-018, Figure 3B shows exemplary inhibition of the post-glutamyl peptidase-like activity, Figure 3C shows exemplary inhibition of the Tryptsin-like activity.

Figure 4 shows an exemplary Western blot of total nuclear p65: Lane 1: untreated; lane 2: 1% DMSO, lane 3: imidazoline **1** (20 mM), lane 4: TNF-a, lane 5: TNF-a and MG-132 (10 mM), lane 6: TNF-a and imidazoline **1** (20 mM), lane 7: TNF-a and imidazoline **1** (10 mM), lane 8: TNF-a and imidazoline **1** (1 mM), lane 9: TNF-a and imidazoline **1** (0.1 mM).

Figure 5 shows an exemplary Western blot of total I κ B in THP-1 cells. Lane 1: untreated; lane 2: 1% DMSO, lane 3: **1** (20 mM), lane 4: TNF-a, lane 5: TNF-a and MG-132 (10 mM), lane 6: TNF-a and **1** (20 mM).

Figure 6 shows an exemplary Imidazoline **1** induces an accumulation of modified I κ Ba. Lane 1) No treatment, 2) 10 ng/mL TNFa, 3) 50 mM parthenolide with 10 ng/mL TNFa, 4) 10 mM MG132 with 10 ng/mL TNFa, 5-7) 20, 10 and 1 mM imidazoline **1** respectively with TNFa, 8-10) 20, 10 and 1 mM imidazoline **1** respectively with 10 mM MG132 and TNFa.

Figure 7 shows an exemplary Ubiquitinylation of I κ Ba. Lane 1) untreated; lane 2) TNF-a only; lane; 3) 10 mM MG132 with TNF-a; lane 4) 50 mM parthenolide with TNF-a; lanes 5 - 7) 20 mM, 10 mM, 1 mM imidazoline **1**.

Figure 8 shows an exemplary A) dose response activity of imidazoline **1** in inhibition of luciferase production in HeLa-NF- κ B-luc cells. Cells were unstimulated/stimulated with 25 ng/mL TNF-a in the absence or presence of imidazoline **1** in 1% DMSO. Fold-induction (%) of the Luciferase activity, normalized for all samples to TNF-a stimulation, is shown. All data is an average of two independent experiments (error bars included in Figure). B) Structures of imidazolines 1-9.

Figure 9 shows an exemplary inhibition of IL-6. Human blood (1:10 RPMI) was unstimulated/stimulated with 200 U/mL IL-1b in the absence or presence of imidazoline **1** in 1% DMSO. Fold-induction of the IL-6 levels of stimulated (calculated from unstimulated) cells was determined. Data are an average of two

independent experiments (error bars included in Figure). For the treatment of Multiple Myeloma

Figure 10 shows an exemplary inhibition of TNF- α . Human blood (1:10 RPMI) was unstimulated/stimulated with 200 U/mL IL-1 β in the absence or presence of imidazoline **1** in 1% DMSO. Fold-induction of the TNF- α levels of stimulated (calculated from unstimulated) cells was determined. A contemplated use is for Rheumatoid Arthritis.

Figure 11 shows exemplary cytotoxicity of imidazoline **1**. Human blood was exposed to imidazoline **1** for 24 hours at stated concentrations and the lymphocytes were evaluated for survival.

Figure 12 shows an exemplary correlation of proteasome activity with NF- κ B mediated gene transcription and reduction of IL-6 levels in blood.

Figure 13 shows an exemplary induction of cell death in MM cells by imidazoline **1**. RPMI-8226 cells were treated with various concentrations of imidazoline **1** in duplicate and incubated for 24 hours. Percent survival was extrapolated based on mitochondrial activity as determined by MTS assay.

Figure 14 shows an exemplary LPS challenge in BALB/C mice with and without TCH-018.

Figure 15 shows exemplary schematics of activation pathways associated with MM and RA and exemplary treatment compounds.

Figure 16 is a plot showing inhibition with TCH-018 using three substrates.

Figure 17a is a plot showing inhibition with TCH-018 is non-competitive.

Figure 17b is a plot showing TCH-018 does not bind in the catalytic domains.

Figure 18 is a plot showing the addition of TCH-018 to the sulfone resulted in additive activities for CT-inhibition.

Figure 19 is a plot showing the binding of TCH-018 is reversible.

Figure 20 is a plot showing TCH-062 binds irreversibly bind to the 20S proteasome.

Figure 21 shows the structure of TCH-062.

DESCRIPTION OF THE INVENTION

The present inventions relate to compositions and methods for treating inflammatory diseases and cancer by administering proteasome inhibitors. In particular, the present inventions provide a new class of orally available non-covalent

proteasome inhibitors capable of reducing NF-kappaB for mediating cytokine production in vivo. Further, the use of a small molecular weight inhibitor of the 26S proteasome (e.g. via inhibition of the 20S catalytic core of the 26S proteasome) via a non-covalent type inhibition is contemplated for use as a means to treat NF-κB mediated diseases, including but not limited to multiple myeloma and rheumatoid arthritis.

Host toxicity of covalent proteasome inhibitors has precluded them as clinically relevant anti-inflammatory agents. The imidazolines described in this disclosure differ from current proteasome inhibitors, it is believed, by their mechanism of proteasome inhibition. These agents have demonstrated in vivo efficacy and no apparent in vivo toxicity, making them clinically relevant for anticancer (for example multiple myeloma) as well as anti-inflammatory (rheumatoid arthritis) treatments.

As described herein a new class of orally available non-covalent proteasome inhibitors capable of reducing NF-kappaB mediate cytokine production in vivo are provided. The compounds inhibit a clinically validated target protein (for example, IL-6) via a novel mechanism of action. This may have significant advantages related to patient toxicity and drug-resistance.

A clinical benefit of proteasome inhibitors was demonstrated by the use of bortezomib in multiple myeloma and mantle cell lymphoma (in addition to multiple clinical trials with patients with advanced solid tumors, breast cancer, pancreatic cancer, colon cancer and non small lung cell cancer). However, host toxicity of covalent proteasome inhibitors has precluded them as clinically relevant anti-inflammatory agents.

The imidazolines described in this disclosure and in U.S. Patents including U.S. patent No. 6,878,735 B2 and U.S. applications related to U.S. Patent No. 6,878,735 B2, herein incorporated by reference in their entirety, differ from current proteasome inhibitors by their mechanism of proteasome inhibition and thus are contemplated to overcome either host toxicity or other adverse clinical outcomes in order to provide significant benefits to patients. These agents have demonstrated in vivo efficacy and no apparent in vivo toxicity, making them clinically relevant for anticancer (for example multiple myeloma) as well as anti-inflammatory (rheumatoid arthritis) treatments.

The mammalian nuclear transcription factor NF- κ B is responsible for the transcription of multiple cytokines, including the pro-inflammatory cytokines tumor necrosis factor alpha (TNF-R) and interleukin 6 (IL-6). Elevated levels of pro-inflammatory cytokines play an important role in the pathogenesis of inflammatory disorders such as rheumatoid arthritis (RA). Inhibition of the pro-inflammatory transcription factor NF- κ B has therefore been identified as a possible therapeutic treatment for RA. We describe herein the synthesis and biological activity of a series of imidazoline-based scaffolds as potent inhibitors of NF- κ B mediated gene transcription in cell culture as well as inhibitors of TNF-R and IL-6 production in interleukin 1 beta (IL-1B) stimulated human blood (Kahlon, et al., Nuclear Factor-KB Mediated Inhibition of Cytokine Production by Imidazoline Scaffolds, J. Med. Chem., Article ASAP • DOI: 10.1021/jm8013162 • Publication Date (Web): 16 February 2009, herein incorporated by reference in its entirety).

The 20S proteasome. The 20S proteasome is the catalytic core of the 26S proteasome. This threonine protease exhibits three distinct peptidase-like hydrolytic activities: Chymotrypsin-like, Trypsin-like and Post-glutamyl-like.

Covalent proteasome inhibitors: Since the ATP-dependent degradation of many regulatory proteins is required to regulate multiple key intracellular regulatory cascades, inhibition of the proteasome has been identified as a means to treat inflammatory diseases and cancer.¹ Inhibition of the 20S proteasome has been validated in the clinic approval of the proteasome inhibitor, bortezomib, for the treatment of multiple myeloma. One rationale is that the proteins that regulate cell cycle and apoptotic events have short half-lives, thus will be more affected by modulation of the proteasome. Tumor types relying on NF- κ B mediated cytokes, such as IL-6 and TNF- α , should be particularly sensitive to proteasome inhibition, while normal cells would remain unresponsive.

Nearly all proteasome inhibitors reported, inhibit the chymotrypsin-like activity of the 20S proteasome via the formation of a covalent adduct to the N-terminal threonine on the active site of the beta-subunit. Representative examples among the known covalent proteasome inhibitors are: peptide aldehydes (MG-132, ALLN), peptide vinyl sulfones (Ac-YLLN-vs), peptide boronates (bortezomib), peptide epoxyketones (epoxomicin) and beta-lactones (lactacystine, salinosporamide A).¹ Although many of these agents are very potent, inherent drawbacks include a lack of target specificity and the highly reactive and often unstable nature of the

agent. Applications of these covalently-linked inhibitors in vivo often induce apoptosis and cell death. By far the most successful clinical example of inhibitors of the 20S proteasome is bortezomib (also known as velcade and formally PS-341, Figure 1). Bortezomib represents the first selective and reversible covalent proteasome inhibitor to demonstrate both significant preclinical activity in several tumor models and significant efficacy in patients with refractory or relapsed MM.¹⁴ Bortezomib is a covalent proteasome inhibitor and thus exerts significant cell death as well as toxicity via the modulation of multiple signaling pathways.

Non-covalent proteasome inhibitors: It can be reasoned that the toxicity of a reversible and time-limited proteasome inhibitor may be reduced.^{16, 17} Unfortunately, examples of non-covalent inhibitors are scarce, with TMC-95 being one of the most cited examples.¹⁸ The natural product and cyclic peptide, TMC-95, is structurally unrelated to the aforementioned covalent inhibitors and binds to all three proteolytically active binding sites in the beta-subunits of the 20S proteasome. The binding of TMC-95 in the proteasome sites is similar to the vinyl sulfone inhibitors, albeit non-covalently to the N-terminal threonine. The further preclinical development of TMC-95 is hampered by its complex total synthesis.²⁰⁻²⁷ Novartis has also reported some non-covalent-peptide based 20S proteasome inhibitors with selectivity towards the chymotrypsin-like active site, but not further cellular or in vivo studies were reported.^{17, 28, 29} To the best of our knowledge, non-covalent small molecule inhibitors have not yet been reported in the literature.

NF-kB-MEDIATED DISEASES, Relationship between proteasome inhibition and NF-kB inhibition.

Upon the covalent addition of poly-ubiquitin chains (E1-E3 ubiquitin conjugation pathway), proteins are recognized by the multifunctional 26S proteasome, transferred to the 20S proteolytic core particle and degraded by proteolysis. This degradation pathway is critical for the turnover of proteins essential to cell proliferation, cell differentiation and inflammation. The involvement of this proteasomal degradation process is intimately linked to the regulation of NF-kB activation and its control over apoptosis and inflammation.

The mammalian transcription factor NF-kappaB (NF-kB) is an ubiquitous transcription factor responsible for the regulation of more than 150 genes impacting virtually every aspect of cellular adaptation including responses to stress,

inflammatory stimuli, activation of immune cell function, cellular proliferation, programmed cell death (apoptosis), and oncogenesis. In normal non-stimulated cells, NF- κ B is typically sequestered in the cytoplasm by NF- κ B's inhibitory protein, I κ B. Upon stimulation by an appropriate extracellular signal, such as pro-inflammatory cytokines TNF- α and IL-1 β , or DNA damaging agents, the NF- κ B pathway becomes
5 activated.^{40, 48-51} During this process I κ B undergoes a phosphorylated-driven polyubiquitination and proteasomal degradation by the 26S proteasome, resulting in the release of NF- κ B.⁴⁷ Liberation of NF- κ B allows for its rapid translocation into the nucleus.⁵² Following its nuclear translocation, NF- κ B binds to DNA and initiates the
10 transcription of a host of pro-inflammatory signaling genes or survival genes. Subsequently, deregulation of the NF- κ B pathway has been directly implicated in the pathogenesis of inflammatory diseases such as rheumatoid arthritis (RA), inflammatory bowel disease, helicobacter pylori-associated gastritis, atherosclerosis, multiple sclerosis, asthma as well as cancer.

15

REPRESENTATIVE THERAPEUTIC APPLICATIONS:

Multiple Myeloma: In multiple myeloma cells, NF- κ B is constitutively activated (Figure 3) and the DNA damaging drugs melphalan and doxorubicin (clinically used to treat MM) have been found to induce even higher levels of NF- κ B activation.⁶⁵

Current therapeutic treatments of MM: Multiple myeloma (MM) is typically characterized as a type of cancer of plasma cells in the bone marrow. MM is a malignant disorder of differentiated B-cells (plasma cells) and remains incurable,^{3, 5, 66-72} with a median survival rate of just 33 months.⁷³ Due to the intrinsic resistance of MM cells to classical chemotherapeutics, only few treatment options slow its progression. Traditional MM therapies include the DNA alkylating agents melphalan and doxorubicin in combination with vincristine and/or dexamethasone.⁷² In the last decade, the US Food and Drug Administration has approved three new drugs for the treatment of MM:^{74, 75} the proteasome inhibitor bortezomib,^{2, 76} the anti-angiogenic and immunomodulator thalidomide and its analogue lenalidomide.^{77, 78} Bortezomib-based regimens have since moved to the forefront as front-line therapies.^{5, 79, 80} The various treatment options/regimes are typically determined a IL-6 based staging method⁸¹⁻⁸³ and will be indicative of the patient's eligibility for autologous stem cell transplantation. In the last few years, the combination of either thalidomide/dexamethasone, bortezomib-based regimes and/or lenalidomide/dexamethasone combinations have emerged as the most recommended strategies in newly diagnosed patients.⁷² Standard DNA alkylating agent therapies still include melphalan and/or doxorubicin with prednisone, thalidomide or bortezomib and are typically used for patients that have a more advanced stage of the disease and are not transplant candidates.^{72, 84} In addition to the limited survival-times, these treatment options have multiple complications and toxicities related to them.^{85, 86}

Rheumatoid Arthritis: Given the critical role of NF- κ B-mediated expression of cytokines, this transcription factor has been actively pursued as a therapeutic target for these types of inflammatory disorders.^{39, 87}

It is well documented that NF- κ B stimulates the expression of multiple genes responsible for many aspects of inflammatory responses and the pathogenesis of inflammatory diseases.^{88, 89} Cytokines (IL-1, IL-2, IL-6 and TNF- α) expressed by NF-

kB induce the amplification of inflammatory signals. Of these cytokines, IL-6⁹⁰⁻⁹² and TNF- α ⁹³⁻⁹⁷ have been identified as key targets in rheumatoid arthritis (RA) and other inflammatory disorders. Pharmacologic intervention in RA was improved drastically with the advent of biologicals that specifically target IL-6⁹¹ or TNF- α ⁹⁸⁻¹⁰⁰

- 5 Unfortunately, these and alternative treatment options for RA are limited, suffer from high costs and involve undesirable methods of administration. Furthermore, these therapies lack data pertaining to their long-term safety, tolerability and sustained efficacy.⁹⁹ In addition, variability in responses to these anti-inflammatory drugs is found due to the complex network of alternative cytokine-mediated pathways.¹⁰¹
- 10 Inhibition of pro-inflammatory transcription factors, such as NF-kB, may therefore represent a better alternative to modulate the complex cytokine network that induces inflammatory response.⁸⁸ In addition, inhibition of NF-kB mediated gene transcription by a small molecule would represent an attractive therapeutic alternative to the current clinical options, which still primarily include anti TNF- α mAbs and anti IL-6 receptor
- 15 antibodies.^{89, 102}

Current therapeutic treatment of rheumatoid arthritis (RA): RA is a highly variable and difficult disease to control, can severely deform joints and, in severe cases, shorten a patient's life. No cure for this disease is presently available. The

20 only small molecule therapy for disease modification in rheumatoid arthritis (RA), methotrexate, is effective in only approximately 20% of RA patients and its use is limited by toxicity issues at higher exposures.¹⁰³⁻¹⁰⁵ The most effective disease modifying approaches currently in use for RA rely on protein therapeutic agents that interfere with signaling by the potent proinflammatory cytokines, TNF- α (Infliximab,

25 Adalimumab, Etanercept,) and IL-1B (Anakinra), or an anti-CD20 monoclonal antibody (Rituximab) that depletes B-cells. These biologics are expensive therapeutics, require parenteral administration, and have variable responses in patients.

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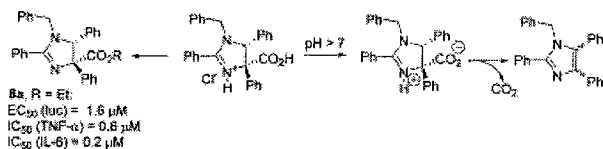
Article

**Nuclear Factor- κ B Mediated Inhibition of
Cytokine Production by Imidazoline Scaffolds**

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Nuclear Factor- κ B Mediated Inhibition of Cytokine Production by Imidazoline Scaffolds

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The mammalian nuclear transcription factor NF- κ B is responsible for the transcription of multiple cytokines, including the pro-inflammatory cytokines tumor necrosis factor alpha (TNF- α) and interleukin 6 (IL-6). Elevated levels of pro-inflammatory cytokines play an important role in the pathogenesis of inflammatory disorders such as rheumatoid arthritis (RA). Inhibition of the pro-inflammatory transcription factor NF- κ B has therefore been identified as a possible therapeutic treatment for RA. We describe herein the synthesis and biological activity of a series of imidazoline-based scaffolds as potent inhibitors of NF- κ B mediated gene transcription in cell culture as well as inhibitors of TNF- α and IL-6 production in interleukin 1 beta (IL-1 β) stimulated human blood.

Introduction

Rheumatoid arthritis (RA¹) is a common and often debilitating human autoimmune disease that involves chronic inflammation of joints, tissues, and organs.¹ Compared to tissues in a normal joint, RA joint tissues have increased cell densities with an abundance of neutrophils, macrophages, and T lymphocytes. Analysis of the synovial fluid from patients with RA has revealed that this tissue is rich in primarily two cytokines: tumor necrosis factor alpha (TNF- α) and interleukin 1 beta (IL-1 β).^{2–4} Elevated production of both TNF- α and IL-1 β have been found in primary fibroblast-like synoviocytes from patients with rheumatoid arthritis (RA) as well as osteoarthritis (OA). Furthermore, cultured rheumatoid synovial cells continuously expressed IL-1 and TNF mRNA, indicating that these cytokines play important roles in the pathology of rheumatoid arthritis.⁵ It is well-documented that TNF- α and IL-1 β also act as pivotal signaling agents for the production of many additional cytokines, such as IL-6,^{6–8} via the activation of the mammalian nuclear transcription factor, NF- κ B.^{9–12} IL-6 is found in abundance in RA synovium, and due to its role in B cell and hematocytic cell growth, differentiation, and proliferation of synovial fibroblasts, IL-6's overproduction contributes to many RA symptoms.^{13–15} The activation of this pro-inflammatory cytokine signaling cascade induces the release of matrix metalloproteinases (MMPs), which lead to the destruction of bone and cartilage, resulting in permanent tissue damage.¹⁶

TNF- α and IL-1 β are considered classical (or canonical) activators of the NF- κ B pathway.^{17–20} NF- κ B is formed from various members of the Rel family of transcription factors (NF- κ B1(p50), NF- κ B2(p52), c-Rel, RelA(p65), and RelB) as either a homodimer or heterodimer, where the RelA(p65)/p50 het-

erodimer is the most abundant.^{4,7} Each NF- κ B dimer has unique transcriptional activity, as transcriptional activators (RelA(p65)/p50, p50/c-Rel, RelA(p65)/RelA(p65), and p65/c-Rel) and as repressors (p50/p50 and p52/p52).¹⁸ NF- κ B is sequestered in the cytoplasm by the I κ Bs in normal nonstimulated cells.²¹ Upon cellular stimulation, I κ B is phosphorylated and subsequently polyubiquitinated and proteolytically degraded by the 26S proteasome.^{21–24} These events unmask the nuclear localization signal (NLS) of NF- κ B, allowing it to translocate into the nucleus.²⁵ Following its nuclear translocation, NF- κ B binds to DNA and induces gene transcription of a wide variety of genes, including those responsible for proinflammatory responses. The levels of NF- κ B-DNA binding are consequently much greater in tissues from RA patients compared to control patients.^{26,27,28} Hence, regulation of the NF- κ B mediated expression of cytokines has been actively pursued as a means of therapeutic intervention in these types of inflammatory disorders.^{27,28}

Pharmacologic intervention in RA was improved drastically with the advent of biologicals that target TNF- α , providing a proof-of-principle for therapeutic intervention in RA by targeting TNF- α .^{29–31} Unfortunately, these and alternative treatment options for RA are limited and suffer from high costs, undesirable methods of administration, and lack of data on long-term safety, tolerability, and sustained efficacy. In addition, variability in responses to these anti-inflammatory drugs is found due to the complex network of alternative cytokine-mediated pathways.³² Inhibition of pro-inflammatory transcription factors, such as NF- κ B, may therefore represent a better alternative to modulate the complex cytokine network that induces inflammatory responses.³³ Consequently, inhibition of NF- κ B by a small molecule would represent an attractive therapeutic alternative to the current clinical options.^{12,34}

We previously described the synthesis and biological activity of a series of imidazolines as potent small molecule inhibitors of NF- κ B transcription, with imidazoline **1** being the most effective in cell culture.^{35,36} The imidazolines are formed via trimethylsilyl chloride mediated 1,3-dipolar cycloaddition of oxazol-5-(4*H*)-ones and imines (Scheme 1).^{37,38} Even though the parent molecule **1** displayed excellent activity in cell culture, additional studies found that the imidazoline scaffold **1** was fairly unstable upon standing and easily converted to a zwitterionic form **2** (Scheme 2). X-ray crystal structures of **1** and **2**

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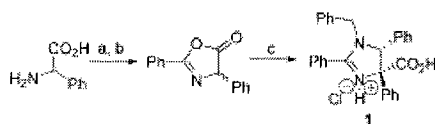
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^{||} Abbreviations: RA, rheumatoid arthritis; TNF- α , tumor necrosis factor α ; IL-1 β , interleukin-1 β ; OA, osteoarthritis; IL-6, interleukin-6; NLS, nuclear localization signal; TFAA, trifluoroacetic anhydride; TMS, trimethylsilyl; EDCI, 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide; DMAP, 4-dimethylaminopyridine.

Scheme 1. Synthesis of the *trans*-Imidazolines³⁹

³⁹ (a) Benzoyl chloride, 1 N NaOH (aq), overnight; (b) ITAA, CH₂Cl₂, 2 h, room temperature, 77% overall; (c) TMSCHN₂, imine, CH₂Cl₂, reflux, overnight, 68%.

are provided in the Supporting Information). Zwitterion **2** is readily decarboxylated to form the ylide **3**,³⁹ which provided a mixture of three products: the *cis*-dihydroimidazoline **4**, the *trans*-dihydroimidazoline **5**, and the fully aromatized imidazole **6** (Scheme 2). Furthermore, the *cis*- and *trans*-dihydroimidazolines (**4** and **5**) isomerized upon standing and dehydrogenated to the imidazole **6** at room temperature when exposed to polar solvents such as DMSO. This *cis/trans* isomerization and oxidation of the decarboxylated form of diaryl-substituted imidazolines is well-documented,^{40–42} and thus this zwitterion induced decarboxylation needed to be addressed to pursue its further clinical potential. The instability of imidazoline **1** results in undesirable pharmacological properties and could be the reason for the inconsistent activity seen when imidazoline **1** was tested in cell culture and whole blood (Table 1). Fortunately, chemical manipulation and derivatization of the carboxyl moiety readily restores stability and potency. In this paper, we describe the synthesis of derivatives of imidazoline **1** aimed to prevent the aforementioned degradation and the new compounds were evaluated for their stability and inhibition of NF- κ B mediated TNF- α and IL-6 production. Biological evaluation indicated that three analogues substantially inhibited NF- κ B mediated transcription in cell culture and inhibited TNF- α and IL-6 production in IL-1 β stimulated human blood at low micromolar concentrations.

Synthesis and Biological Evaluation

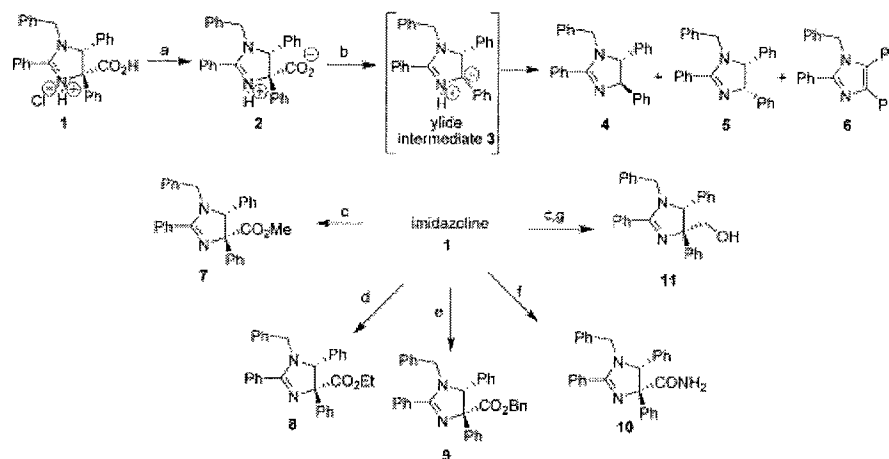
Synthesis of Imidazoline Derivatives. We envisioned that the replacement of the carboxylic acid in **1** with esters or a less acidic alcohol or amide functionalities would avoid the formation of the zwitterion. The imidazoline scaffold was prepared via our previously reported protocol using a silicon mediated 1,3-dipolar cycloaddition (Scheme 1).^{37,38} The resulting imidazoline·HCl salt **1** was treated with TMSCHN₂ to yield the methyl ester **7** in excellent yield (Scheme 2). The ethyl ester **8** was prepared via the acid chloride. To form the benzyl ester **9**, the imidazoline **1** was coupled with benzyl alcohol, EDCI, and DMAP. To determine the importance of the ester moiety, imidazoline **1** was converted to the amide **10** using similar coupling conditions and (NH₄)₂CO₃. Finally, the ester was reduced to the alcohol **11** with lithium aluminum hydride to study the significance of the carbonyl moiety for activity (Scheme 2).

Inhibition of NF- κ B-Mediated Gene Transcription. The ability of the imidazolines **1**, **2** and **4–11** to modulate NF- κ B-mediated gene transcription was evaluated using a luciferase reporter assay in human cervical epithelial (HeLa) cells with a stably transfected NF- κ B-luc gene. These cells maintain, through hygromycin selection, a chromosomal integration of a luciferase reporter construct regulated by multiple copies of the NF- κ B response element. The cells were activated with TNF- α (25 ng/mL) in the presence or absence of imidazolines **1**, **2**, **4–11**. Because activation of the NF- κ B pathway requires proteasomal degradation of I κ B, a classic proteasome inhibitor **12** (N-CBZ-Leu-Leu-leucinal, also known as MG-132, Figure 1B)⁴³ was

used as positive control and DMSO (vehicle) served as the negative control. Luciferase production was evaluated after 8 h, and all samples were normalized to the TNF- α activation control. Treatment of HeLa/NF- κ B-luc cells with imidazolines without any TNF- α activation did not induce any significant amount of luciferase activity at 20 μ M, indicating that the imidazolines alone did not stimulate the NF- κ B pathway. Pretreatment of the cells with the imidazolines followed by TNF- α stimulation resulted in a dose-dependent decrease in luciferase production for several of the analogues of **1**, **7–9**, whereas the zwitterion **2** was devoid of activity (Table 1). While the decarboxylated products **4** and **5** displayed significantly reduced activity, the imidazoline ester **8** was the most active alternative to compound **1** with an EC₅₀ value in HeLa cells of 2.5 μ M (Figure 1A). The imidazole **6**, amide **10**, and alcohol **11** were significantly less active, with EC₅₀ values at or greater than 20 μ M. To verify that the results for luciferase production in this integrated cell line were due to inhibition of NF- κ B and not to cell death, HeLa cells were transiently transfected with 6 $\times$ κ B driven reporter gene pNF- κ B-luc (Stratagene) and the internal control plasmid pRL-TK (Stratagene), which provides low levels of *Renilla* luciferase expression. After activation with TNF- α in the presence or absence of imidazoline **8**, luciferase production was assayed and the data was normalized to the *Renilla* luminescence (see Supporting Information). The two luciferase assays corresponded well with one another, confirming the results from the luciferase assay using the stable HeLa/NF- κ B-luc cell line. These results indicate that the ester moiety represented a comparable alternative to imidazoline **1** for NF- κ B inhibition.

Inhibition of TNF- α and IL-6 Production by Imidazolines in Human Whole Blood. The results from cell culture prompted the analysis of these imidazolines for their anti-inflammatory potential in whole blood. The imidazolines were subsequently evaluated for their ability to inhibit a NF- κ B mediated cytokine response in IL-1 β stimulated human blood. Human blood samples were pretreated with imidazolines **4–11** for 2 h followed by IL-1 β stimulation. Plasma was harvested 22 h after stimulation and IL-1 β induced TNF- α production was measured using a human TNF- α ELISA (R&D Systems) assay. The circulating TNF- α levels in IL-1 β stimulated blood samples were significantly higher than in unstimulated or the vehicle treated blood. Pretreatment of the blood for 2 h with the imidazolines **4–11**, followed by IL-1 β stimulation, resulted in a strong dose-dependent inhibition of TNF- α production, as compared to the vehicle treated control (Figure 1C). The amide **10** and the alcohol **11** were inactive (Table 1). Therefore, imidazolines **7–9** were effective in decreasing TNF- α levels, presumably via inhibition of NF- κ B mediated gene transcription.

To evaluate the effect of inhibiting TNF- α production on other downstream pro-inflammatory cytokines, such as IL-6, the same plasma samples were subjected to an IL-6 ELISA (R&D Systems). As seen with the TNF- α response, IL-1 β stimulation caused a robust increase in IL-6 production when compared to the unstimulated control or the vehicle-only treated blood samples. Pretreatment of the blood for 2 h with the imidazolines **4**, **5**, **7–11**, followed by IL-1 β stimulation, resulted in a strong dose-dependent inhibition of IL-6 production, as compared to the vehicle treated control (Figure 1D). Consistent with the TNF- α data as well as the NF- κ B mediated luciferase activity, compounds **10** and **11** were significantly less active. The reduction of IL-6 levels clearly indicates that inhibition of NF- κ B activation causes a decline in the production of additional pro-inflammatory cytokines. Even though the decarboxylated

Scheme 2. Synthesis of Imidazolines Derivatives^a

^a (a) NaHCO₃ (aq); (b) DMSO, rt or THF, reflux; (c) TMSOTf, benzene:MeOH (9:1), 0 °C; (d) (1) (COCl)₂, CH₂Cl₂, 0 °C; (2) EtOH; (e) EDCl, DMAP, benzyl alcohol, CH₂Cl₂; (f) EDCl, HOBT, DIPEA, (NH₄)₂CO₃, THF; (g) LiAlH₄, THF.

Table 1

compd	HeLa NF- κ B-luc EC ₅₀ ^a , μ M	whole blood TNF- α IC ₅₀ ^b , μ M	whole blood IL-6 IC ₅₀ ^c , μ M
1	0.95 ^d	>20	>20
4	4.6	NT ^e	2.5
5	11.0	NT ^e	2.4
6	>20	NT ^e	NT ^e
7	7.5	2.8	3.0
8	2.5	1.2	0.8
8a (<i>R,R</i>)	1.6	0.6	0.2
8b (<i>S,S</i>)	2.9	0.7	0.6
9	3.5	3.2	5.9
10	>20	>20	>20
11	>20	11.9	>20

^a EC₅₀ values for inhibition of luciferase production in pNF- κ B-luc HeLa cells following TNF- α activation. ^b IC₅₀ values for inhibition of TNF- α production in human whole blood following IL-1 β stimulation. ^c IC₅₀ values for inhibition of IL-6 production in human whole blood following IL-1 β stimulation. EC₅₀ and IC₅₀ values were calculated from logarithmic curves (see Supporting Information for complete list of log values and standard errors). ^d Reference 35. ^e NT: Not tested.

compounds (4 and 5) inhibited IL-6 production in the low micromolar range, their ability to isomerize and aromatize to the imidazole 6 made them unattractive as potential drug candidates.

From this data, compound 8 was determined to be the superior compound of this series based on the activities in all three assays. Considering the difference between the types of assays, the activity of the compounds in cell culture corresponded very well with the activity in IL-1 β stimulated whole blood for both TNF- α and IL-6 inhibition (Table 1). This data indicates that imidazoline 8 is a very effective inhibitor of NF- κ B mediated gene transcription, resulting in a decrease in the levels of the pro-inflammatory cytokines TNF- α and IL-6 in IL-1 β stimulated human blood. To demonstrate the pharmacological relevance of these imidazolines, human blood samples were first stimulated with IL-1 β and then treated with imidazoline 8 at various doses (see Supporting Information). The IC₅₀ value for post-treatment was comparable to that for pretreatment, suggesting the imidazolines inhibit inflammatory response after it has been initiated.

Cytotoxicity of Imidazoline 8. To exclude the possibility of decreased cytokine production due to an increase of imida-

zoline mediated cell death, both the HeLa cells and white blood cells were evaluated for cell death at various concentrations of imidazoline 8. An LDH release assay showed that incubation of HeLa/NF- κ B-luc cells with up to 10 μ M imidazoline 8 for 8 h did not induce any significant amount of cell death (see Supporting Information). FACS analysis of these samples confirmed the cells were healthy (see Supporting Information). To confirm the cell viability data in the whole blood experiments, human white blood cell counts also indicated no significant cell cytotoxicity at 10 μ M or less imidazoline 8 (see Supporting Information). Therefore, the inhibition of luciferase production and inhibition of TNF- α and IL-6 production by imidazoline 8 was not due to a decrease in cell number but rather due to inhibition of NF- κ B transcription.

Stability of Imidazoline 8. Sterically accessible carboxylic esters are often susceptible to hydrolysis by blood and cellular esterases, a conversion that could present a potential problem due to the reoccurrence of the zwitterionic form 2 and hence lead to instability of the imidazoline scaffold. The ethyl ester derivative (compound 8) was therefore incubated in whole human blood for 24 h and evaluated for hydrolysis. Compound 8 was found to be stable to blood esterolytic activity for up to 24 h at 37 °C as determined by LC/MS analysis of compound-spiked human blood. LC/MS analysis indicated no detection of cleavage products over this 24 h incubation period (see Supporting Information). These results indicated that imidazoline 8 is the active compound in the assays and not a pro-drug for imidazoline 1.

Evaluation of Enantiomers. To optimize the anti-inflammatory efficacy of our scaffold, each enantiomer (8a-*R,R*) and 8b-*S,S*) of the racemic compound 8 was evaluated for its biological activity. Previously, we found that both enantiomers of compound 1 were active in cell culture, however the (*R,R*) enantiomer of imidazoline 1 was approximately 5-fold more potent than the (*S,S*) enantiomer.³⁶ To evaluate the two enantiomers of compound 8, the enantiomers were readily resolved by chiral HPLC (see Supporting Information). This procedure gives excellent resolution (~5 min between enantiomers), and both enantiomers were isolated and evaluated for their activities. Similar to the imidazoline 1, both enantiomers showed strong inhibition of NF- κ B mediated gene transcription.

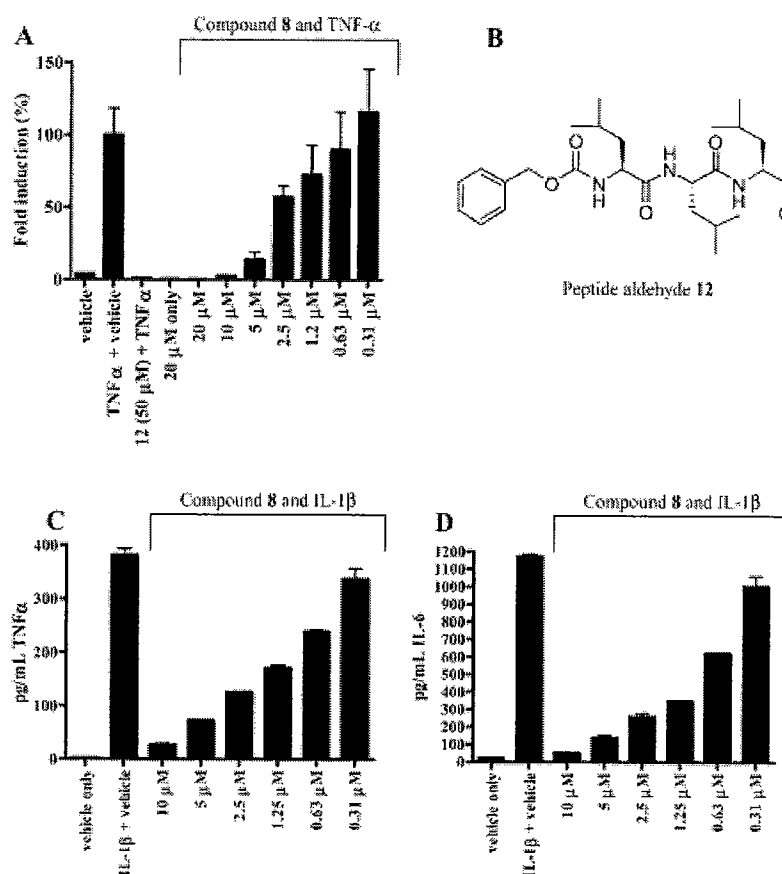


Figure 1. Dose-response activity of imidazoline **8** in (A) inhibition of luciferase production in HeLa-NF- κ B-luc cells, (C) inhibition of TNF- α production in IL-1 β stimulated human blood, and (D) inhibition of IL-6 production in IL-1 β stimulated human blood. (A) Cells were unstimulated/stimulated with 25 ng/mL TNF- α in the absence or presence of imidazoline **8** in 1% DMSO. Fold-induction (%) of the luciferase activity, normalized for all samples to TNF- α stimulation, is shown. (B) Structure of peptide aldehyde **12**. (C) Human blood (1:10 RPMI) were unstimulated/stimulated with 200 U/mL IL-1 β in the absence or presence of imidazoline **8** in 1% DMSO. Fold-induction of the TNF- α levels of stimulated (calculated from unstimulated) cells was determined. (D) Human blood (1:10 RPMI) were unstimulated/stimulated with 200 U/mL IL-1 β in the absence or presence of imidazoline **8** in 1% DMSO. Fold-induction of the IL-6 levels of stimulated (calculated from unstimulated) cells was determined. All experiments were run in duplicate.

with the (*R,R*) enantiomer **8a** ($EC_{50} = 1.6 \mu\text{M}$) somewhat more potent than the (*S,S*) enantiomer **8b** ($EC_{50} = 2.9 \mu\text{M}$) (Table 1). The enantiomers were also tested for their ability to inhibit cytokine production in whole human blood. Once more, the (*R,R*) enantiomer **8a** was found to be slightly more potent than the (*S,S*) enantiomer **8b**. The IC_{50} values for TNF- α and IL-6 inhibition for the (*R,R*) enantiomer **8a** were 0.6 and 0.2 μM , respectively. The IC_{50} values for the (*S,S*) enantiomer were in the same range, albeit slightly less potent. These results indicate that both enantiomers of **8** are able to inhibit cytokine production at the low micromolar level, with the (*R,R*) enantiomer to be slightly superior.

Conclusion

The NF- κ B inhibitor imidazoline **1** was previously found to be a potent inhibitor of NF- κ B mediated gene transcription in cell culture. The carboxylic acid functionality of this class of compounds was found to be responsible for subsequent decomposition (Scheme 1). Herein, we described the synthesis and biological activity of structurally modified imidazolines, incapable of forming a zwitterionic species. The ethyl ester

derivative **8** was found to be a potent inhibitor of NF- κ B mediated gene transcription in HeLa cells with low micromolar EC_{50} values and stable against degradation and hydrolysis by esterases. The cellular activity of the imidazolines translated well in whole human blood assays, resulting in nanomolar IC_{50} values for TNF- α and IL-6 production after IL-1 β mediated NF- κ B stimulation. Furthermore, evaluation of isolated enantiomers revealed the (*R,R*) enantiomer of imidazoline **8** to be slightly more potent than the (*S,S*) enantiomer at inhibiting NF- κ B mediated gene transcription and cytokine production. The further clinical potential of these stable noncytotoxic small molecule NF- κ B inhibitors as potential anti-inflammatory agents is currently under investigation in our laboratories.

Experimental Section

General Information. All commercial reagents were purchased from commercial suppliers and used without further purification. All solvents were reagent grade. Peptide aldehyde **12** was obtained from Calbiochem (San Diego, CA). TNF- α was purchased from Invitrogen (Carlsbad, CA), and IL-1 β was obtained from Roche Applied Sciences (Indianapolis, IN). THF was freshly distilled from

sodium/benzophenone under nitrogen. CH_2Cl_2 was dispensed from a delivery system, which passes the solvents through a column packed with dry neutral alumina. Melting points were obtained using an electrothermal capillary melting point apparatus and are uncorrected. Column chromatography was carried out on silica gel 60 (230–400 mesh) supplied by EM Science. Yields refer to chromatographically and spectroscopically pure compounds unless otherwise stated. Infrared spectra were recorded on a Nicolet IR/42 spectrometer. Proton and carbon NMR spectra were recorded on a Varian Unity Plus-500 spectrometer. High-resolution 70 eV EI mass spectra and FAB mass spectra were obtained at the RTSF Mass Spectrometry Facility of the Michigan State University, using a JEOL AX-505H and a JEOL HX-110 double focusing mass spectrometer (JEOL USA, Peabody, MA), respectively. Combustion analysis was performed on a PerkinElmer 2400 Series II CHNS/O analyzer. All final compounds possess purity $>95\%$ as determined by combustion analysis, high-resolution mass spectrometry, or HPLC.

DL-(4R,5R)-1-Benzyl-2,4,5-triphenyl-4,5-dihydro-1H-imidazole-4-carboxylic Acid Hydrochloride (1). Melting point 152–155 °C. ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 4.18 (d, 1H, $J = 16.0$ Hz), 4.86 (d, 1H, $J = 16.0$ Hz), 5.59 (s, 1H), 6.71 (d, 2H, $J = 7.5$ Hz), 7.10 (t, 2H, $J = 7.7$ Hz), 7.19 (t, 2H, $J = 7.5$ Hz), 7.45–7.59 (m, 7H), 7.72–7.77 (m, 4H), 7.84 (t, 1H, $J = 7.2$ Hz), 7.97 (d, 2H, $J = 7.5$ Hz), 12.86 (br s, 1H), 13.99 (br s, 1H). ^{13}C NMR (125 MHz, $\text{DMSO}-d_6$) δ 48.7, 73.8, 75.9, 121.4, 127.2, 128.3, 128.7, 128.9, 129.0, 129.1, 129.4, 129.5, 129.9, 132.8, 133.2, 134.3, 139.2, 165.7, 167.6.

DL-(4R,5R)-1-Benzyl-2,4,5-triphenyl-4,5-dihydro-1H-imidazole-3-ium-4-carboxylate (2). DL-(4R,5R)-1-benzyl-2,4,5-triphenyl-4,5-dihydro-1H-imidazole-4-carboxylic acid hydrochloride (1) was dissolved in dichloromethane. Then it was washed 2 $\times$ saturated NaHCO_3 solution. The organic layer was separated, dried over MgSO_4 , and concentrated in vacuo to yield the zwitterion 2 as an off-white solid; mp 119–121 °C. ^1H NMR (500 MHz, CDCl_3) δ 3.78 (d, 1H, $J = 16.0$ Hz), 4.60 (d, 1H, $J = 16.0$ Hz), 4.91 (s, 1H), 6.60 (d, 2H, $J = 7.5$ Hz), 7.06 (t, 2H, $J = 7.5$ Hz), 7.15 (t, 1H, $J = 7.5$ Hz), 7.26–7.33 (m, 5H), 7.38–7.41 (m, 5H), 7.49 (t, 1H, $J = 7.5$ Hz), 7.54 (d, 2H, $J = 7.5$ Hz), 7.82 (d, 2H, $J = 7.0$ Hz). ^{13}C NMR (125 MHz, $\text{DMSO}-d_6$) δ 48.7, 75.8, 79.3, 123.3, 125.9, 127.0, 127.7, 128.2, 128.5 (2 peaks), 129.1, 129.2, 129.3, 129.3, 129.6, 133.2, 134.0, 136.2, 143.3, 165.1, 168.4.

DL-(4R,5R)-1-Benzyl-2,4,5-triphenyl-4,5-dihydro-1H-imidazole (4). The synthesis of compound 4 has been previously reported.⁴⁴ The title compound was isolated in small amounts via a decarboxylation of imidazoline 1. A solution of imidazoline 1 (0.7 g, 1.62 mmol) in 100 mL of THF was refluxed for 5 h, resulting in complete decomposition of starting material. The solution was then concentrated in vacuo, resulting in a crude mixture of imidazolines 4 and 5 and imidazole 6 in a 2:1:1 ratio, respectively. The crude residue was then purified via flash column chromatography using silica gel (1:9 methanol:dichloromethane as eluant), isolating the product as a white solid (112 mg, 18%-unoptimized); mp 78–80 °C. ^1H NMR (500 MHz, CDCl_3) δ 3.99 (d, 1H, $J = 16$ Hz), 4.41 (d, 1H, $J = 8.5$ Hz), 4.77 (d, 1H, $J = 15.5$ Hz), 5.07 (d, 1H, $J = 8.5$ Hz), 7.00–7.01 (m, 2H), 7.18–7.23 (m, 2H), 7.24–7.30 (m, 4H), 7.31–7.33 (m, 2H), 7.34–7.39 (m, 3H), 7.42–7.45 (m, 2H), 7.53–7.55 (m, 3H), 7.88–7.90 (m, 2H). ^{13}C NMR (125 MHz, CDCl_3) δ 49.6, 72.6, 77.9, 126.7, 127.0, 127.1, 127.4, 127.7, 127.9, 128.3, 128.4, 128.6, 128.6, 128.8, 130.1, 131.3, 136.4, 141.8, 143.9, 165.9. IR (neat): 3063 cm^{-1} , 3030 cm^{-1} , 1595 cm^{-1} . HRMS (FAB): m/z calculated for $[\text{C}_{26}\text{H}_{20}\text{N}_2 + \text{H}]^+$, 389.2018; found, 389.2020.

Because of the difficulty in separation and to verify the structures of compounds 5 and 6, each compound was prepared individually via an alternative route.

DL-(4R,5S)-1-Benzyl-2,4,5-triphenyl-4,5-dihydro-1H-imidazole (5). A solution of DL-(4R,5S)-2,4,5-triphenyl-4,5-dihydro-1H-imidazole⁴⁵ (0.3 g, 1.01 mmol) and benzyl bromide (0.2 g, 1.06 mmol) in 20 mL of anhydrous benzene was treated with triethyl amine (0.2 g, 2.02 mmol). The solution was refluxed for 15 h and

then washed with saturated NaHCO_3 solution (2 $\times$ 20 mL) and brine solution (1 $\times$ 20 mL). The solution was then dried over Na_2SO_4 and concentrated in vacuo. The resulting crude residue was purified via flash column chromatography using silica gel (1:9 methanol:dichloromethane as eluant), affording the product as a yellow oil (77 mg, 20%). ^1H NMR (500 MHz, CDCl_3) δ 3.85 (d, 1H, $J = 15.5$ Hz), 4.77 (d, 1H, $J = 16$ Hz), 4.92 (d, 1H, $J = 11.5$ Hz), 5.55 (d, 1H, $J = 11$ Hz), 6.90–6.96 (m, 5H), 6.97–7.00 (m, 4H), 7.02–7.09 (m, 3H), 7.25–7.29 (m, 3H), 7.50–7.53 (m, 3H), 7.81–7.83 (m, 2H). ^{13}C NMR (125 MHz, CDCl_3) δ 49.0, 68.4, 72.9, 126.2, 127.1, 127.3, 127.5, 127.8, 127.9, 127.9, 128.1, 128.5, 128.6, 128.7, 130.2, 131.2, 136.6, 136.8, 139.3, 167.1. IR (neat): 3030 cm^{-1} , 2924 cm^{-1} , 1595 cm^{-1} . HRMS (FAB): m/z calculated for $[\text{C}_{26}\text{H}_{20}\text{N}_2 + \text{H}]^+$, 389.2018; found, 389.2017.

1-Benzyl-2,4,5-triphenyl-1H-imidazole (6). To a flame-dried 100 mL round-bottom flask was added 2,4,5-triphenylimidazole (2 g, 6.76 mmol) and anhydrous THF (60 mL). Sodium hydride (432 mg, 10.81 mmol) was then added and the mixture stirred for 5 min. Then benzyl chloride (1.24 mL, 10.81 mmol) was added dropwise and the mixture refluxed overnight under a nitrogen atmosphere. The solid precipitate was filtered off and the THF was concentrated down. The crude residue was put into solution using ethyl acetate (50 mL) and was washed with brine solution (1 $\times$ 30 mL). The organics were dried using anhydrous sodium sulfate and concentrated. The crude residue was purified by flash column chromatography using silica gel (8:2 hexane:ethyl acetate as eluant) affording the product as a white powder (1.33 g, 51%); mp 159–161 °C. ^1H NMR (500 MHz, CDCl_3) δ 5.1 (s, 2H), 6.78–6.81 (m, 2H), 7.1–7.23 (m, 8H), 7.28–7.4 (m, 6H), 7.57–7.6 (m, 2H), 7.64–7.67 (m, 2H). ^{13}C NMR (125 MHz, CDCl_3) δ 48.2, 125.9, 126.2, 126.7, 127.2, 128.0, 128.51, 128.52, 128.55, 128.72, 128.81, 129.0, 130.0, 130.9, 131.0, 134.4, 137.5, 138.0, 148.0. IR (NaCl): 3065 cm^{-1} , 3032 cm^{-1} , 1956 cm^{-1} , 1889 cm^{-1} , 1812 cm^{-1} , 1762 cm^{-1} . HRMS (ESI): m/z calculated for $[\text{C}_{26}\text{H}_{20}\text{N}_2 + \text{H}]^+$, 387.1861; found, 387.1873.

DL-(4R,5R)-Methyl 1-Benzyl-4,5-dihydro-2,4,5-triphenyl-1H-imidazole-4-carboxylate (7). A flame-dried flask under nitrogen was charged with DL-(4R,5S)-1-benzyl-4,5-dihydro-2,4,5-triphenyl-1H-imidazole-4-carboxylic acid (1) (0.50 g, 1.07 mmol) in 9:1 benzene:methanol (50 mL) and cooled to 0 °C. After 5 min, trimethylsilyldiazomethane (1.1 mL, 2.13 mmol) was added dropwise and then the reaction mixture was allowed to stir at 0 °C for 5 h. The reaction mixture was concentrated to minimal residue and purified by running through a small silica plug to give the product as a cream-colored solid (0.24 g, 30%); mp 114–116 °C. ^1H NMR (500 MHz, CDCl_3) δ 3.18 (s, 3H), 3.84 (d, 1H, $J = 16.0$ Hz), 4.63 (d, 1H, $J = 16.0$ Hz), 4.91 (s, 1H), 6.73 (d, 2H, $J = 7.5$ Hz), 7.05 (t, 2H, $J = 7.5$ Hz), 7.10 (t, 1H, $J = 7.5$ Hz), 7.25–7.28 (m, 1H), 7.31–7.34 (m, 3H), 7.37 (d, 4H, $J = 4.5$ Hz), 7.47 (d, 1H, $J = 1.5$ Hz), 7.48 (d, 1H, $J = 2.0$ Hz), 7.73 (m, 2H), 7.77 (d, 1H, $J = 3.5$ Hz), 7.78 (d, 1H, $J = 1.5$ Hz). ^{13}C NMR (125 MHz, CDCl_3) δ 48.7, 51.8, 73.9, 83.1, 126.8, 127.2, 127.4, 127.5, 128.0, 128.1, 128.3, 128.4, 128.5, 128.6, 128.9, 130.4, 130.5, 136.6, 137.9, 144.0, 165.6, 171.4. IR (NaCl): 3061 cm^{-1} , 3031 cm^{-1} , 2948 cm^{-1} , 1734 cm^{-1} , 1595 cm^{-1} . HRMS (ESI): m/z calculated for $[\text{C}_{26}\text{H}_{22}\text{N}_2\text{O}_2 + \text{H}]^+$, 447.2070; found, 447.2070.

DL-(4R,5R)-Ethyl 1-Benzyl-4,5-dihydro-2,4,5-triphenyl-1H-imidazole-4-carboxylate (8). Into a flame-dried flask under nitrogen was placed DL-(4R,5S)-1-benzyl-4,5-dihydro-2,4,5-triphenyl-1H-imidazole-4-carboxylic acid (1) (20.0 g, 46.5 mmol) and dichloromethane (300 mL). After cooling the reaction mixture to 0 °C, oxalyl chloride (11.7 mL, 138.8 mmol) was added over 5 min, followed by DMF (10 mL/1 mL of dichloromethane). The reaction mixture was allowed to stir at 0 °C for 2 h, after which the solvent was removed on the rotary evaporator. The residue was placed on a vacuum line for 1 h. The flask was then placed under nitrogen and cooled to 0 °C and ethanol (400 mL) was added. After stirring for an addition 2.5 h, the ethanol was removed on the rotary evaporator and dichloromethane (300 mL) was added. The organic solution was washed with saturated NaHCO_3 solution (1 $\times$ 100 mL) and H_2O (1 $\times$ 100 mL), dried over MgSO_4 , filtered, and the

solvent was removed under reduced pressure to give a yellow oil. The product was purified using flash column chromatography on silica gel (1:1 hexane:ethyl acetate as eluant) to give a white solid (16.1 g, 76%); mp 86–88 °C. ¹H NMR (500 MHz, CDCl₃) δ 0.83 (t, 3H, *J* = 7.2 Hz), 3.63 (qq, 1H, *J* = 7.2 Hz), 3.73 (qq, 1H, *J* = 7.2 Hz), 3.86 (td, 1H, *J* = 16.0 Hz), 4.64 (d, 1H, *J* = 15.5 Hz), 4.96 (s, 1H), 6.78 (d, 2H, *J* = 7.0 Hz), 7.09 (t, 2H, *J* = 7.2 Hz), 7.14 (t, 1H, *J* = 7.2 Hz), 7.28–7.32 (m, 1H), 7.34–7.38 (m, 3H), 7.39–7.45 (m, 4H), 7.49–7.51 (m, 3H), 7.79–7.81 (m, 4H). ¹³C NMR (125 MHz, CDCl₃) δ 13.4, 48.6, 60.9, 73.7, 82.8, 126.7, 127.1, 127.2, 127.3, 127.9, 128.1, 128.2, 128.3, 128.4, 128.5, 128.8, 130.2, 130.6, 136.6, 137.9, 144.1, 165.3, 170.7. IR (NaCl): 3061 cm⁻¹, 3031 cm⁻¹, 2981 cm⁻¹, 1732 cm⁻¹, 1597 cm⁻¹. HRMS (ESI): *m/z* calculated for [C₂₅H₂₃N₃O₂ + H]⁺, 461.2229; found, 461.2225.

***m*-(4*R*,5*R*)-Benzyl 1-Benzyl-4,5-dihydro-2,4,5-triphenyl-1*H*-imidazole-4-carboxylate (9).** To a stirred solution of DL-(4*R*,5*S*)-1-benzyl-4,5-dihydro-2,4,5-triphenyl-1*H*-imidazole-4-carboxylic acid (1) (200 mg, 0.43 mmol) in dichloromethane was added 1-ethyl-(3-dimethylaminopropyl) carbodiimide (120 mg, 0.64 mmol). After 5 min was added 4-(*N,N*-dimethylamino)pyridine (52 mg, 0.43 mmol). After stirring for an addition 10 min, benzyl alcohol (92 mg, 0.85 mmol) was added. The reaction mixture was stirred at room temperature overnight. The reaction mixture was washed with 10% HCl solution (1 × 5 mL), saturated NaHCO₃ solution (1 × 5 mL), H₂O (1 × 5 mL), and brine solution (1 × 5 mL). The product was extracted using dichloromethane, dried over MgSO₄, filtered, and the solvent was removed under reduced pressure. The product was purified using column chromatography on silica gel (9:1 dichloromethane:ethyl acetate as eluant) to give a light-yellow oil (120 mg, 54%). ¹H NMR (500 MHz, CDCl₃) δ 3.79 (d, 1H, *J* = 15.5 Hz), 4.50 (d, 1H, *J* = 12.5 Hz), 4.61 (d, 1H, *J* = 15.5 Hz), 4.76 (d, 1H, *J* = 12.5 Hz), 4.92 (s, 1H), 6.74 (d, 2H, *J* = 7.5 Hz), 6.95 (dd, 2H, *J* = 7.5, 2.5 Hz), 7.04–7.08 (m, 2H), 7.10–7.13 (m, 1H), 7.17–7.21 (m, 3H), 7.27–7.29 (m, 4H), 7.30–7.34 (m, 4H), 7.47–7.49 (m, 3H), 7.74–7.77 (m, 4H). ¹³C NMR (125 MHz, CDCl₃) δ 48.6, 66.6, 73.7, 82.7, 126.8, 127.1, 127.4, 127.5, 127.7, 127.9, 128.0 (2 peaks), 128.4 (2 peaks), 128.5, 128.6, 128.8, 130.4, 135.4, 136.3, 137.3, 143.5, 165.6, 170.5. IR (NaCl): 3063 cm⁻¹, 3032 cm⁻¹, 2924 cm⁻¹, 1740 cm⁻¹, 1593 cm⁻¹. HRMS (ESI): *m/z* calculated for [C₃₀H₂₅N₃O₂ + H]⁺, 523.2356; found, 523.2350.

***m*-(4*R*,5*R*)-1-Benzyl-4,5-dihydro-2,4,5-triphenyl-1*H*-imidazole-4-carboxamide (10).** To a stirred solution of DL-(4*R*,5*S*)-1-benzyl-4,5-dihydro-2,4,5-triphenyl-1*H*-imidazole-4-carboxylic acid (1) (100 mg, 0.21 mmol), *N*-hydroxybenzotriazole (32 mg, 0.23 mmol), and 1-ethyl-(3-dimethylaminopropyl) carbodiimide (45 mg, 0.23 mmol) in anhydrous THF (2 mL) was added *N,N*-diisopropylethylamine (30 mg, 0.23 mmol). The reaction mixture was stirred at ambient temperature for 10 min. Then ammonium carbonate (50 mg, 0.64 mmol) was added in one portion and the resulting suspension was stirred at ambient temperature overnight. The reaction mixture was concentrated to minimal residue. The residue was treated with 1:1 saturated NaHCO₃ solution:H₂O (2 mL) and stirring was continued for 2 h. The mixture was placed into a separatory funnel, and the product was extracted using ethyl acetate, dried over MgSO₄, and the solvent was removed under reduced pressure. The product was purified using column chromatography on silica gel (3:7 hexane:ethyl acetate as eluant) to give a light-yellow solid (65 mg, 71%); mp 65–68 °C. ¹H NMR (500 MHz, CDCl₃) δ 3.80 (d, 1H, *J* = 16.5 Hz), 4.56 (d, 1H, *J* = 16.5 Hz), 4.86 (s, 1H), 5.15 (br s, 1H), 6.71 (d, 2H, *J* = 7.5 Hz), 7.05 (t, 2H, *J* = 7.5 Hz), 7.11 (t, 1H, *J* = 7.2 Hz), 7.26–7.28 (m, 1H), 7.30–7.36 (m, 7H), 7.50–7.54 (m, 3H), 7.71–7.73 (m, 2H), 7.79 (d, 2H, *J* = 7.5 Hz). ¹³C NMR (125 MHz, CDCl₃) δ 48.4, 74.3, 81.6, 126.3, 126.9, 127.3, 127.4, 128.1, 128.3, 128.4, 128.5, 128.8, 130.6, 136.7, 137.8, 144.8, 165.4, 173.4. IR (NaCl): 3442 cm⁻¹, 3063 cm⁻¹, 3031 cm⁻¹, 2926 cm⁻¹, 1688 cm⁻¹, 1591 cm⁻¹. HRMS (FAB): *m/z* calculated for [C₂₆H₂₃N₃O + H]⁺, 432.2076; found 432.2073.

***m*-(4*R*,5*R*)-1-Benzyl-4,5-dihydro-2,4,5-triphenyl-1*H*-imidazol-4-yl)methanol (11).** In a flame-dried flask a suspension of lithium aluminum hydride (7 mg, 0.18 mmol) in anhydrous THF (8 mL) was cooled to 0 °C. Then a solution of DL-(4*R*,5*S*)-methyl 1-benzyl-

4,5-dihydro-2,4,5-triphenyl-1*H*-imidazole-4-carboxylate (7) (50 mg, 0.11 mmol) in THF (3 mL) was added and the reaction mixture was stirred under nitrogen and warmed to room temperature. The reaction mixture was quenched using saturated NH₄Cl solution (3 mL). The mixture was allowed to stir for 10 min and then placed in a separatory funnel. The product was extracted using ethyl acetate (20 mL). The aqueous layer was washed with fresh ethyl acetate (5 mL). The combined organic layers were washed with brine solution, dried over MgSO₄, filtered, and the solvent was removed under reduced pressure. The crude material was purified via column chromatography on silica gel (100% ethyl acetate as eluant) to yield a white solid (29 mg, 62%); mp 138–141 °C. ¹H NMR (500 MHz, CDCl₃) δ 2.78 (br s, 1H), 3.47–3.50 (m, 1H), 3.53–3.56 (m, 1H), 3.87 (d, 1H, *J* = 15.5 Hz), 4.59 (s, 1H), 4.72 (d, 1H, *J* = 15.5 Hz), 6.72 (d, 2H, *J* = 6.5 Hz), 7.05–7.10 (m, 3H), 7.21–7.22 (m, 1H), 7.32 (t, 2H, *J* = 7.5 Hz), 7.35–7.41 (m, 6H), 7.54 (t, 3H, *J* = 3.5 Hz), 7.84–7.85 (m, 2H). ¹³C NMR (125 MHz, CDCl₃) δ 49.0, 67.0, 73.8, 76.9, 125.8, 126.8, 127.5, 127.6, 128.1, 128.4, 128.4, 128.5, 128.8, 128.8, 130.5, 130.7, 135.7, 135.9, 146.3, 166.5. IR (NaCl): 3183 cm⁻¹ (broad), 3061 cm⁻¹, 3029 cm⁻¹, 2924 cm⁻¹, 1597 cm⁻¹. HRMS (ESI): *m/z* calculated for [C₂₆H₂₃N₃O + H]⁺, 419.2123; found 419.2125.

Biological Methods. Cell Culture. The human cell line HeLa-NF-κB-luc was purchased from Panomics Inc. (Fremont, CA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose, 3.7 g/L bicarbonate, and supplemented with 5% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate, 0.2 mM L-glutamine, and 100 μg/mL hygromycin B. The human cell line HeLa was purchased from ATCC. The cells were maintained in DMEM media containing 4.5 g/L glucose, 3.7 g/L bicarbonate, and supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 mg/mL streptomycin, 1 mM sodium pyruvate, and 0.2 μM L-glutamine. Cells were cultured at 37 °C, 5% CO₂ atmosphere, 97% relative humidity and were routinely passaged by trypsin-EDTA treatment to maintain a cell density between 2 × 10⁶ to 5 × 10⁶.

Viability Assays. Human white blood cells were counted manually using a BD Unopette reservoir and a hemacytometer.

NF-κB-luc Reporter Assay. HeLa NF-κB-luc cells (5.0 × 10⁵ cells/mL) were seeded into a 96-well white opaque plate using DMEM medium supplemented with 5% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate, 0.2 mM L-glutamine, and 100 μg/mL hygromycin B. After 24 h, the cell culture medium was replaced with DMEM medium supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin. Cell cultures were pretreated with vehicle (1% DMSO), 50 μM peptide aldehyde 12 (positive control), or imidazole (final concentrations were 20, 10, 5, 1, 0.5, 0.1, 0.05 μM) for 30 min at 37 °C in 5% CO₂. TNF-α was added to a final concentration of 25 ng/mL, and the samples were further incubated for 8 h at 37 °C in 5% CO₂. Cells were assayed for firefly luciferase production using the Steady-Glo luciferase reporter assay (Promega, Madison, WI) according to manufacturer's protocol. The luminescence of each well was measured using a Veritas microplate luminometer. All reported data are the average of two independent experiments unless otherwise indicated. The data was analyzed using GraphPad Prism 4.0. The data was normalized to TNF-α activation, and the EC₅₀ values were calculated using the equation for the sigmoidal curve for variable slope.

NF-κB-luc/Renilla-luc Dual Reporter Assay. HeLa cells (3.5 × 10⁵ cells/mL) were seeded into a 6-well plate using DMEM media supplemented with 10% fetal bovine serum and no antibiotics. Transient transfections were performed using lipofectamine 2000. Briefly, 0.8 μg of plasmid DNA was combined with 1.5 μL lipofectamine 2000. HeLa cells were transiently cotransfected with internal control pRL-TK. A constitutively activated plasmid pEC-MEKK served as positive control and pCIS-CK was the negative control. The mixture was incubated at room temperature for 20 min and mixed with cells in Opti-MEM 1 medium. Cells were transfected for 5–6 h at 37 °C in 5% CO₂. Cells were allowed to

grow in complete growth medium for 24 h in 5% CO₂. Cell cultures were pretreated with vehicle (1% DMSO), 50 μ M peptide aldehyde **12** (positive control), or imidazole **8** (final concentrations were 20, 10, 5, 2.5, 1.25, 0.625, 0.312 μ M) for 30 min at 37 °C in 5% CO₂. TNF- α was added to a final concentration of 25 ng/mL, and the samples were further incubated for 8 h at 37 °C in 5% CO₂. Cells were assayed for both firefly luciferase production as well as *Renilla* luciferase (coreporter) using the Dual-Glo reporter assay according to the manufacturer's protocol. The results were read on Veritas microplate luminometer as relative light units. Values for the luciferase activity were normalized to the *Renilla* luciferase and analyzed using GraphPad Prism 4.0 (see Supporting Information).

Human Whole Blood IL-1 β Challenge. After obtaining the appropriate approval for deidentified human cell lines, human whole blood was obtained through the Jasper Research Clinic, Kalamazoo, MI, from a single healthy, fasted human volunteer and was collected in glass citrated endotoxin-free tubes by venipuncture. Only samples with a white blood count falling within the normal range (4800–10800 white blood cells per liter) were used. To support the viability of white blood cells, blood was diluted 1:10 in RPMI-1640 media supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin. Aliquots of diluted blood (1 mL) were preincubated with vehicle (0.1% DMSO, final concentration) or imidazole **8** (final concentrations were 10, 3, 1, 0.3, and 0.1 μ M) for 2 h at 37 °C, 5% CO₂. IL-1 β was added to a final concentration of 200 U/mL, and the samples were further incubated for 18 h at 37 °C, 5% CO₂. At the end of the incubation period, the blood samples were centrifuged at 3000 RPM for 5 min. The plasma was removed, snap frozen, and stored at –80 °C. TNF- α and IL-6 levels were determined by ELISA (R&D Systems, Inc.).

CytoTox-ONE Homogeneous Membrane Integrity Assay. HeLa NF- κ B-luc cells (3.8 $\times$ 10⁵ cells/mL) were seeded into a 96-well black opaque plate using DMEM media supplemented with 2% fetal bovine serum, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 100 μ g/mL hygromycin B. Cells were treated with either vehicle (1% DMSO) or imidazole **8** (final concentrations were 40, 20, 10, 5, 2, 0.5, 0.2 μ M) for 8 h at 37 °C, 5% CO₂. Media-only wells served as negative control to determine background fluorescence. Untreated cells with 1% DMSO served as vehicle control. The plate and CytoTox-ONE assay reagent (Promega, Madison, WI) were equilibrated to room temperature. To determine the maximum LDH release, 2 μ L of lysis solution (9% w/v Triton X-100 in water) was added to high control wells. Background LDH release from tissue culture cells was considered as low (media) control. An equal volume of CytoTox-ONE assay reagent was subsequently added to each well. The contents of the plate were gently mixed for 30 s and allowed to incubate at room temperature for 10 min. The fluorescence of each well was recorded on SpectraMax M5⁺ with an excitation wavelength of 560 nm and an emission wavelength of 590 nm. All reported data are the average of four independent experiments unless otherwise indicated. The data was normalized to maximum LDH release (see Supporting Information).

FACS Analysis. Cells were treated with either vehicle (1% DMSO) or imidazole **8** (final concentrations were 40, 20, 10, 5, 2, 0.5, 0.2 μ M) for 8 h at 37 °C, 5% CO₂. For the analysis of cell cycle parameters, the adherent cell cultures were trypsinized and resuspended in 1 mL of 1 $\times$ PBS with 50% FBS. Then the cells were fixed with 70% (v/v) ice-cold ethanol overnight. After centrifugation of the cells, the pellet was washed two times with 5 mL of ice-cold 1 $\times$ PBS containing 10% FBS. Then the cell pellet was resuspended in staining solution (PBS containing 50 μ g propidium iodide, 10 μ L 0.1 M EDTA, 14 units RNaseA). The DNA content was analyzed by flow cytometry using a FACS Vantage SE analyzer with ModFit LT software (see Supporting Information).

LC/MS Assay for Stability of Compound **8 in Whole Blood.** Plasma aliquots (50 μ L) from blood incubated with imidazole **7** for different incubation times up to 24 h were added to 200 μ L of ice-cold HPLC-grade acetonitrile in a 1.5 mL microcentrifuge tube,

After centrifugation (5000g, 15 min; 4 °C), 100 μ L of each supernatant was transferred to an autosampler vial containing 100 μ L of 0.15% aqueous formic acid. These solutions were analyzed by LC/MS on a Waters LCT Premier mass spectrometer equipped with Shimadzu SIL-5000 autosampler and LC-20AD pumps. Analyses were performed using a Waters Atlantis dC18 column (2.1 mm $\times$ 50 mm, 3 μ m particles) using a flow rate of 0.3 mL/min. The gradient consisted of 10 mM aqueous ammonium acetate (solvent A) and acetonitrile (solvent B), starting at 90% A with a linear gradient to 100% B at 20 min. Volumes of 10 μ L were injected and analyzed using positive mode electrospray ionization. Extracted ion chromatograms were generated for the protonated compound **8** (*m/z* 461.2) and the hydrolyzed product (compound **1**, *m/z* 433.2). Retention times and response factors were obtained through analysis of authentic standards of compounds **1** and **8**.

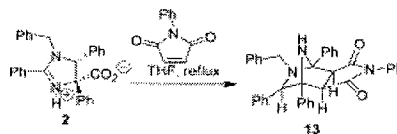
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Supporting Information Available: Experimental data for reporter assays, ELISA IL-6, ELISA TNF- α , FACS, and cell lysis experiments. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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- (39) The proposed ylide **3** (Scheme 2) was trapped with the dipolarophile *N*-phenylimaleimide. Compound **2** was treated with *N*-phenylimaleimide in THF and refluxed for 3 h. The crude material was purified by flash column chromatography using silica gel with 2:8 ethyl acetate:hexane to 3:5:6:5 ethyl acetate:hexane as eluent. The exocyclic product **13** was isolated in 24% yield (unoptimized conditions).



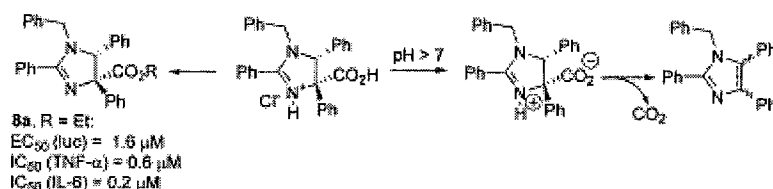
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EXPERIMENTAL

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the experimental disclosure which follows, the following abbreviations apply: M (molar); mM (millimolar); μ M (micromolar); nM (nanomolar); mol (moles); mmol (millimole); μ mol (micromole); nmol (nanomole); gm (gram); mg (milligram); μ g (microgram); pg (picogram); L (liter); ml (milliliter); μ l (microliter); cm (centimeter); mm (millimeter); μ m (micrometer); nm (nanometer); $^{\circ}$ C. (degrees Centigrade or Celsius), s (second), h and hr (hour), and wk (week), and



Example I

This Example demonstrates that trans-substituted imidazolines represented by TCH-018 show potent inhibition of the 20S proteasome. TCH-018 inhibited the chymotrypsin-like Figure 3A and post-glutamyl peptidase –like Figure 3B domains of the 20S proteasome. No significant activity was seen in the trypsin-like domain (Figure 3C).

Example II

This Example demonstrates that the proteasome inhibitor TCH-018 blocks nuclear translocation of the p65 subunit of NF- κ B after activation of the NF- κ B pathway. Thus inhibition of the proteasome should inhibit the activation of the pro-survival and pro-inflammatory nuclear transcription factor kappa B (NF- κ B). Inhibition of this transcription by a small molecule proteasome inhibitor, blocked the nuclear translocation and subsequent gene transcription of pro-survival genes and pro-inflammatory genes.

Inhibition of p65 nuclear translocation by imidazoline 1 (TCH-013, i.e. the racemic form of TCH-018): In order to evaluate mechanism for NF- κ B mediated

transcription of IL-6 production, the effects of imidazoline 1 (TCH-018) on the nuclear translocation of the p65 subunit of NF- κ B was investigated. In order to monitor NF- κ B's nuclear translocation, THP-1 cells were activated with TNF- α (to induce the nuclear translocation) in the presence and absence of a range of imidazoline 1 concentrations and nuclear extracts were investigated for p65 levels. Nuclear extracts were isolated and analyzed by Western blot using a p65 total (Santa Cruz) antibody. As shown in Figure 4, imidazoline 1 does not induce the activation of NF- κ B (lane 3), whereas TNF- α induces the nuclear translocation of p65 (lane 4). The commercial proteasome inhibitor MG-132 blocks TNF- α -induced nuclear translocation (lane 5). Similar to MG-132, imidazoline 1 inhibits the TNF- α -induced nuclear translocation of p65 in a dose response manner (lanes 6-9). This data indicates that imidazoline 1 inhibits the nuclear translocation of NF- κ B in THP-1 cells after TNF- α activation.

Example III

This Example demonstrates accumulation of I κ B by imidazoline 1. The effect of imidazoline TCH-013 on the modification and subsequent degradation of I κ B was investigated by Western blot analysis using an I κ B polyclonal antibody (Santa Cruz). In control lanes 1-3, I κ B remains un-modified and is accumulated in the cytoplasm. Upon treatment with TNF- α 10 ng/mL for 30 minutes, I κ B becomes phosphorylated, ubiquitinated and degraded by the proteasome (Figure 5). The presence of a weak band in Lane 3 indicates that most of I κ B has been degraded. Addition of the proteasome inhibitor MG-132 (lane 5) indicates accumulation of I κ B (i.e. prevention of its degradation). A second band is visible in lane 5, which is the phosphorylated form of I κ B that is recognized by the antibody. Figure 5 indicates that imidazoline 1 accumulated the unmodified form of I κ B (Figure 5, lane 6).

Example IV

The following examples demonstrate exemplary embodiments of the present inventions.

This Example demonstrates accumulation of I κ B isoforms: The effects of imidazoline 1 (TCH-013) on I κ B modification were investigated in more detail using Western blot analysis. HeLa cells were pretreated with test agents for two hours and

then challenged with 10 ng/mL TNF- α for 30 minutes. Whole cell extracts were prepared in cell lysis buffer containing complete protease inhibitor cocktail. 50 μ g of each sample was heated in an equal volume of SDS-PAGE sample buffer containing reducing agent (DTT), resolved by SDS-PAGE, and transferred to a PVDF
5 membrane. The membrane was blocked in TBS-T (containing 5% nonfat dry milk. IkB- α was detected using polyclonal antibody IkB- α (SC371). Bound antibodies were detected with enhanced chemiluminescence. Figure 6, indicates that upon treatment with TNF- α IkB quickly degrades, resulting in a decrease in the total amount of IkB (lane 2) compared to the untreated control (lane 1). Addition of
10 the kinase inhibitor, parthenolide, prior to TNF- α activation (lane 3) prevents the phosphorylation and thus degradation of IkB. The proteasome inhibitor MG132, also prevented the degradation of IkB following TNF- α activation, resulting in the presence of the phospho-IkB and multiple ubiquitinated forms of IkB (lane 4). Comparison of lanes 3 and 4 shown that the IKK kinase inhibitor parthenolide
15 prevents the formation of phospho-IkB. However, in the presence of imidazole 1, IkB appears to be stabilized, and multiple sumoylated and mono-ubiquitinated forms are predominant (lanes 5- 7), which are not apparent with parthenolide (lane 3). By combining MG132 with imidazole 1, followed by TNF- α activation, a similar profile remains, although the poly-ubiquitinated IkB and the phosphorylated IkB
20 forms appear to be intensified (lanes 8-10). The absence of a phospho-IkB product in lanes 2 (TNF only) and 5-7 (TNF & imidazole 1) could indicate that IkB does proceed through the phosphorylation step, but gets blocked in its next post-translational modification by the imidazole.

This Example demonstrates accumulation of ubiquitinated IkB.

25 Ubiquitination of IkB was confirmed by performing a complementary experiment in which IkB α was immunoprecipitated and the ubiquitinated species was detected by western blot analysis. HeLa cells were transfected with pMT123, a plasmid encoding an epitope tagged (HA) ubiquitin. pMT123 was received as a kind gift from Professor Dirk Bohmann, University of Rochester. Cells were transfected with
30 pMT123 using Lipofectamine 2000. Twenty-four hours later, transfected cells were pretreated with test agents for two hours and then challenged with 10 ng/mL TNF- α (Sigma) for 90 minutes. The extended time (90 minutes vs 30 minutes (see preliminary data section 2.4 and 2.5) was used to investigate the fate of IkB, following ample IkB activation. Whole cell extracts were prepared in cell lysis buffer

(20 mM Tris-HCL [pH 7.5], 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton) containing complete protease inhibitor cocktail. For immunoprecipitation, the whole cell extract was incubated overnight at 4°C with a specific antibody for IκBα (sc-371) covalently bound to protein G agarose. Samples were heated in an equal volume of SDS-PAGE sample buffer containing reducing agent (DTT), resolved by SDS-PAGE, and transferred to a PVDF membrane. The membranes were blocked in TBS-T (50 mM Tris [pH 8.0], 150 mM NaCl, 0.05% Tween 20) containing 5% nonfat dry milk. Ubiquitinated species of IκBα were detected using polyclonal antibody to HA conjugated to HRP (sc-8017). Bound antibodies were detected with enhanced chemiluminescence. Figure 7 indicates that imidazoline **1** clearly induces accumulation of polyubiquitinated IκBα's (lanes 5-7) as compared to the TNF-α control (lane 2), consistent with the profile of proteasome inhibitors such as MG-132.

This Example demonstrates inhibition of NF-κB mediated gene transcription. In order to test the ability of the imidazolines to inhibit NF-κB-mediated gene transcription, we evaluated the compounds using a luciferase reporter assay. Human cervical epithelial HeLa cells with stably transfected NF-κB-luc gene were purchased from Panomics (Freemont, CA.). These cells maintain, through hygromycin selection, a chromosomal integration of a luciferase reporter construct regulated by multiple copies of the NF-κB response element. The cells were activated with TNF-α (25 ng/mL) in the presence or absence of the imidazolines. DMSO and TNF-α were used as negative and positive controls respectively and luciferase production was evaluated after 8h using the Steady-Glo luciferase assay system. All samples were normalized to the TNF-α activated control. Treatment of HeLa/ NF-κB-luc cells with the imidazolines without any TNF-α activation did not induce a significant amount of luciferase activity, suggesting that the compounds had no effect on NF-κB activation. Pre-treatment of the cells with the imidazolines followed by TNF-α stimulation resulted in a dose dependent decrease in luciferase production (Figure 8). A representative dose-response of imidazoline **1** is shown in Figure 8. Compounds **1-9** were evaluated and their respective IC₅₀ values are listed in Table 1. (error margins shown in Table 2). Limited structure activity relationship of the carboxylic acid moiety identified imidazoline ester **1** as a potential viable alternative to **2a**.

Table 1. IC₅₀ values for inhibition of luciferase production in pNF-κB-luc HeLa cells following TNF-α(α) activation. Data are an average of 2 independent experiments.

Compound	NF- κ B-luc (IC ₅₀)
1	1.2 μ M
2a	0.95 μ M
2b	>20 μ M
3	11 μ M
4	5 μ M
5	>20 μ M
6	7.5 μ M
7	3.5 μ M
8	~20 μ M
9	>20 μ M

This Example demonstrates inhibition of NF- κ B mediated IL-6 production in human blood. In order to evaluate the potential of these compounds to inhibit NF- κ B mediated gene transcription beyond cell culture, human blood samples were pretreated with imidazoline **1** followed by IL-1 β to stimulate NF- κ B mediated gene transcription of IL-6. IL-1 β was chosen as stimulus, because second to IL-6, this mediator has been implicated as part of the primary mechanism for bone destruction in patients with multiple myeloma. In addition, IL-1 β is known to be a potent inducer of IL-6 in multiple myeloma. IL-6 was chosen as an end-point read-out, because this NF- κ B-regulated cytokine plays a key role in the pathogenesis of multiple myeloma.

After obtaining the appropriate approval for de-identified human cell lines, human whole blood was obtained through the Jasper Research Clinic, Kalamazoo, MI, from a single healthy, fasted human volunteer and was collected in glass citrated tubes by venipuncture. Only samples with a white blood count falling within the normal range (4,800-10,800 white blood cells per liter) were used. To support the viability of white blood cells, blood was diluted 1:10 in RPMI-1640 media. Aliquots of diluted blood (1 mL) were pre-incubated with vehicle (0.1% DMSO, final concentration) or imidazoline (at various concentrations) for 2 hours at 37° C, 5% CO₂. IL-1 β was added to a final concentration of 200 U/mL and the samples were further incubated for 22 hours at 37° C, 5% CO₂. At the end of the incubation period, the blood samples were centrifuged at 3000 RPM for 5 minutes. The plasma was removed, snap frozen and stored at -80° C. IL-6 levels were determined by ELISA. As clearly indicated in Figure 11 the circulating cytokine levels in IL-1 β stimulated samples were significantly higher (>1000 fold) than in unstimulated or the vehicle treated blood. IL-6 levels significantly reduced in the presence of the imidazoline in a clear dose response (Figure 9). The IC₅₀ of the racemic compound **1** was determined

to be 0.8 M for IL-6 inhibition. In addition to imidazoline **1**, compounds **6-9** were also evaluated for activity (Table 2).⁶⁸

The inventors observed that the imidazoline **1**, when added post-treatment (add imidazoline 1 hour *after* IL-1B activation of NF-kB pathway), instead of pre-treatment (2 hours prior to IL-1B treatment), was still able of significantly reducing IL-6 production (IC₅₀ 2.5 M).

This Example inhibition of NF-κB mediated TNF-α production in human blood: NF-κB regulates multiple cytokines, including IL-2, IL-6, IL-8 and TNF-α. In order to exclude the potential of imidazoline **1** to inhibit IL-6 production via an NF-kB independent mechanism, the same human blood samples were evaluated inhibition of another NF-kB regulated cytokine, TNF-α. It should be noted that TNF-α is also a cytokine directly related to the pathogenesis of MM and strong stimulus for IL-6 production in MM. The levels of TNF-α production were measured using the same blood samples as described above and similarly a strong dose-response inhibition of TNF-α was found following IL-1B stimulation. Plasma was harvested 22 hours after stimulation and IL-1B induced TNF-α production was measured using a human TNF-α ELISA (R&D Systems) assay. The circulating TNF-α levels in IL-1B stimulated blood samples were significantly higher (~400 fold) than in unstimulated or the vehicle treated blood (Figure 10). Pretreatment of the blood for 2 hours with the imidazoline **1**, followed by IL-1B stimulation resulted in a strong dose-dependent inhibition of TNF-α production, as compared to the vehicle control (Figure 10). The IC₅₀ of compound **1** (TCH-013, racemate) was determined to be 1.2 μM for TNF-α inhibition. In addition, **6-9** were also evaluated for inhibition of TNF-α production (Table 2).

This Example Cell shows cytotoxicity effects. In order to exclude the possibility of decreased cytokine production due to an increase of imidazoline mediated cell death, both the HeLa cells and the white blood cells were evaluated for cell death at various concentrations of imidazoline **1**. An LDH release assay showed that incubation of HeLa/NF-kB-luc cells with up to 10 microM imidazoline **1** for 8 hours did not induce any significant amount of cell death (data not shown).⁶⁸ FACS analysis of these samples confirmed the cells were healthy (data not shown).⁶⁸ In addition, to evaluate the toxicity of imidazoline **1** in human blood, de-identified human whole blood was obtained from the Jasper Clinic (Kalamazoo, Michigan) and diluted 1:1 in PBS. Lymphocytes were isolated from whole blood by layering over

Lymphoprep solution. The samples were centrifuged at 800 X g for 30 minutes in a swinging bucket rotor at room temperature with the break turned off. The lymphocyte band was transferred to a fresh tube and diluted with RPMI-1640 containing 10% fetal bovine serum until there were 1×10^6 cells/mL. The lymphocytes were aliquoted into culture dishes and treated with 20, 10, 3, and 1 μ M compound **1** in quadruplicate. The cells were incubated at 37°C, 5% CO₂ for twenty-four hours. Cells were diluted 1:1 in trypan blue solution and live cells were counted. No significant cell cytotoxicity could be detected at 10 μ M or lower (Figure 11, white blood cell count after 24 hours at various concentration of imidazoline **1**). Therefore, the inhibition of luciferase production and inhibition of IL-6 and TNF- α production was not due to a decrease in cell number of the HeLa cells or white blood cells, respectively.

Whole blood cytokine inhibition *versus* cellular reporter assay. The correlation between the activity seen in HeLa cells (NF- κ B-luc reporter assay) and in whole blood (IL-6 and TNF- α , ELISA assay) was highly consistent and reproducible (Table 2). As mentioned above, compounds **1** (and enantiomers **1a** and **1b**), **3-9** were evaluated for inhibition of IL-6 and TNF- α production and the data is summarized in Table 2. Comparison of the IC₅₀ values from all assays indicates that the *R,R* enantiomers of **1** (**1b**) is the most potent compound thus far (IL-6: IC₅₀ 0.2 μ M). To illustrate the error margin from experiment to experiment most accurately, error margins are listed as the standard error of the log EC₅₀'s.

Table 2. ^aLog EC₅₀ values for inhibition of luciferase production in pNF-κB-luc HeLa cells following TNF-α activation. ^bStandard error of log EC₅₀ values for inhibition of luciferase production in pNF-κB-luc HeLa cells following TNF-α activation. ^cEC₅₀ values calculated from the log EC₅₀ values for inhibition of luciferase production in pNF-κB-luc HeLa cells following TNF-α activation. ^dLog IC₅₀ values for inhibition of TNF-α production in human blood following IL-1β stimulation. ^eStandard error of log IC₅₀ values for inhibition of TNF-α production in human blood following IL-1β stimulation. ^fIC₅₀ values calculated from the log IC₅₀ values for inhibition of TNF-α production in human blood following IL-1β stimulation. ^gLog IC₅₀ values for inhibition of IL-6 production in human blood following IL-1β stimulation. ^hStandard error of log IC₅₀ values for inhibition of IL-6 production in human blood following IL-1β stimulation. ⁱIC₅₀ values calculated from the log IC₅₀ values for inhibition of IL-6 production in human blood following IL-1β stimulation.

15 This Example demonstrates Inhibition of the chymotrypsin-like activity of the 20S proteasome is consistent with the inhibition of NF-κB mediated gene

Compound	HeLa NF-κB-luc Log EC ₅₀ ^a	HeLa NF-κB-luc Std. Error log EC ₅₀ ^b	HeLa NF-κB-luc EC ₅₀ ^c	Whole Blood TNF-α Log IC ₅₀ ^d	Whole Blood TNF-α Std. Error Log IC ₅₀ ^e	Whole Blood TNF-α IC ₅₀ ^f	Whole Blood IL-6 Log IC ₅₀ ^g	Whole Blood IL-6 Std. Error Log IC ₅₀ ^h	Whole Blood IL-6 IC ₅₀ ⁱ
1	0.40	0.04	2.5 μM	0.07	0.04	1.2 μM	-0.10	0.05	0.8 μM
1b	0.21	0.10	1.6 μM	-0.18	0.07	0.6 μM	-0.64	0.09	0.2 μM
1a	0.47	0.11	2.9 μM	-0.13	0.05	0.7 μM	-0.23	0.05	0.6 μM
3	1.04	0.03	11.0 μM	-	-	-	0.38	0.16	2.4 μM
4	0.66	0.07	4.6 μM	-	-	-	0.39	0.03	2.5 μM
5	1.44	1.27	> 20 μM	-	-	-	-	-	-
6	0.87	0.01	7.5 μM	0.45	0.08	2.8 μM	0.48	0.11	3.0 μM
7	0.54	0.11	3.5 μM	0.51	0.13	3.2 μM	0.77	0.33	5.9 μM
8	1.55	0.06	~ 20 μM	1.67	0.27	> 20 μM	1.67	0.55	> 20 μM
9	-2.1e+7	1.8e+014	> 20 μM	1.07	0.15	11.9 μM	1.29	12432	~ 20 μM

transcription. The in vitro activity of the chymotrypsin-like activity of the 20S proteasome was evaluated for several active and inactive imidazolines. The activity correlated well with the inhibition of NF-κB mediated gene transcription of (Hela-luc reporter assay) as well as with the activity of the compounds in reducing cytokine production in human blood (Figure 12).

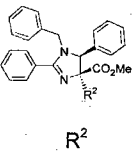
This Example demonstrates effeciacy for treatment of Multiple Myeloma:

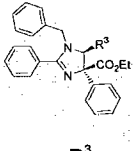
Given the critical role NF-κB-mediated IL-6 production plays in the growth and

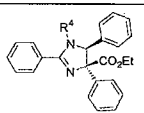
parthenogenesis of multiple myeloma, we investigated the use of imidazoline **1** in somewhat more detail in a MM cell line (RPMI-8226). Multiple myeloma is a key target disease for NF- κ B as well as IL-6 inhibitors, since nearly all multiple myeloma cells (including RPMI-8226 cells) contains constitutively active NF- κ B. Similar to bortezomib (or velcade), imidazoline **1** was found to be effective as a single agent in MM cells. The CC_{50} of imidazoline **1** in the RPMI-8226 MM cell lines was 4.0 microM (Figure 13), which is comparable to the clinically significant doxorubicin (CC_{50} 1.2 microM) but better than melphalan (CC_{50} 30.6 microM) in this particular cell line. Importantly, unlike other MM drugs no significant cytotoxicity towards white blood cells was observed at effective concentrations (<20 microM).

This Example demonstrates Structure activity relationship. A series of imidazolines were prepared and investigated for their ability to reduce NF- κ B activity (in cell culture, HeLa-luc reporter assay) and cytokine production (in human blood, ELISA assay). The IC_{50} values of the compounds are listed in Table 3 below:

Table 3. ^aLog EC_{50} values for inhibition of luciferase production in pNF- κ B-luc HeLa cells following TNF- α activation. ^bStandard error of log EC_{50} values for inhibition of luciferase production in pNF- κ B-luc HeLa cells following TNF- α activation. ^c EC_{50} values calculated from the log EC_{50} values for inhibition of luciferase production in pNF- κ B-luc HeLa cells following TNF- α activation. ^dLog IC_{50} values for inhibition of IL-6 production in human whole blood following IL-1 β stimulation. ^eStandard error of log IC_{50} values for inhibition of IL-6 production in human whole blood following IL-1 β stimulation. ^f IC_{50} values calculated from the log IC_{50} values for inhibition of IL-6 production in human whole blood following IL-1 β stimulation.

Compound	 R ²	HeLa NF-κB-luc Log EC ₅₀ ^f	HeLa NF-κB-luc Std. Error log EC ₅₀ ^g	HeLa NF-κB-luc EC ₅₀ ^c	Whole Blood IL-6 Log IC ₅₀ ^d	Whole Blood IL-6 Std. Error Log IC ₅₀ ^d	Whole Blood IL-6 IC ₅₀ ⁱ
10	Ph	0.87	0.01	7.5 μM	0.48	0.11	3.0 μM
11	Me	-9.6x10 ⁶	2.4x10 ¹³	>20 μM	0.47	0.05	3.0 μM
12	ⁱ Pr	1.37	0.09	~20 μM	0.85	0.03	7.1 μM
13	methyl-indol	1.11	0.05	13.0 μM	1.21	0.20	16.1 μM
14	Bn	0.72	0.04	5.3 μM	0.61	0.07	4.1 μM

Compound	 R ³	HeLa NF-κB-luc Log EC ₅₀ ^a	HeLa NF-κB-luc Std. Error log EC ₅₀ ^b	HeLa NF-κB-luc EC ₅₀ ^c	Whole Blood IL-6 Log IC ₅₀ ^g	Whole Blood IL-6 Std. Error Log IC ₅₀ ^h	Whole Blood IL-6 IC ₅₀ ⁱ
15	4-pyr	-1.6x10 ⁷	6.4x10 ¹³	>20 μM	0.97	0.10	9.4 μM
16	4- Ph-NO ₂	0.53	0.09	3.4 μM	-0.27	0.03	0.5 μM
17	4- Ph-NH ₂	1.02	0.04	10.4 μM	-0.34	0.13	0.5 μM
18	4- Ph-OMe	0.56	0.05	3.6 μM	0.11	0.05	1.3 μM
19	4- Ph-F	0.69	0.02	5.0 μM	0.02	0.05	1.1 μM
20	4- Ph-Cl	0.19	0.12	1.5 μM	-0.48	0.06	0.3 μM
21	4- Ph-CF ₃	0.13	0.09	1.4 μM	-0.07	0.03	0.8 μM
22	2-Furan	0.88	0.02	7.6 μM	0.28	0.02	1.9 μM

Compound	 R ⁴	HeLa	HeLa	HeLa	Whole Blood	Whole Blood	Whole Blood
		NF-κB-luc	NF-κB-luc	NF-κB-luc	IL-6	IL-6	IL-6
		Log EC ₅₀ ^a	Std. Error lo EC ₅₀ ^b	EC ₅₀ ^c	Log IC ₅₀ ^g	Std. Error Log IC ₅₀ ^h	IC ₅₀ ⁱ
23	H	2.06	1.03	>20 μM	1.54	0.14	>20 μM
24	Ac	2.22	1.82	>20 μM	-	-	>20 μM
25	Bz	1.40	0.04	~20 μM	0.82	0.06	6.6 μM
26	Tos	1.56	0.46	~20 μM	0.82	0.14	6.6 μM
27	4-Bn-OMe	0.69	0.06	4.6 μM	.014	0.03	1.4 μM
28	4-Bn-Me	0.23	0.10	1.7 μM	0.66	0.05	4.6 μM
29	4- Ph-Br	0.44	0.05	2.7 μM	-0.30	0.02	0.5 μM
30	4- Ph-Cl	0.62	0.05	4.2 μM	0.20	0.12	1.6 μM
31	4-Ph-F	0.71	0.03	5.2 μM	0.08	0.02	1.2 μM
32	4-Ph-CF ₃	0.82	0.05	5.5 μM	0.17	0.03	1.5 μM
33	2-Furan	0.74	0.05	4.2 μM	0.11	0.02	1.3 μM

This Example demonstrates In vivo activity. The activity of the imidazolines was evaluated in BALB/C mice using a standard LPS challenge. In the control group, mice were injected ip with 1.0 mg/Kg or 0.1 mg/Kg LPS in saline solution to initiate a massive inflammatory response. In the treatment groups, mice were given 200 mg/Kg TCH-018 in vegetable oil by oral lavage, 1 hour prior to LPS injection. After the LPS injection, blood was analyzed for TNF-α levels. Figure 14 indicates that LPS induced high levels of TNF-α after 2 hours (2,000 pg/mL TNF-α) in female mice treated with 1.0 mg/Kg LPS. In the mice pretreated with TCH-018 this level was significantly reduced (400 pg/mL). Similar results were obtained with male mice treated with 0.1 mg/Kg LPS. See, Figure 14. LPS challenge in BALB/C mice with and without TCH-018.

This Example shows TCH-018 inhibits the 20S proteasome: The ability of TCH-018 to inhibit the 20S proteasome was determined in vitro using purified human 20S proteasome and the following fluorogenic peptides as substrates: Suc-LLVY-AMC (substrate for CT-L activity), Boc-LRR-AMC (substrate for T-L activity) and Z-LLE-AMC (substrate for PGPH activity). The rates of hydrolysis were monitored by fluorescence increase and the initial linear portion of the curves were used to calculate the IC₅₀ values, resulting in inhibition of the CT-L, (IC₅₀ 0.95 uM) and PGPH (also referred to a caspase-like) (IC₅₀ 1.6 uM) activities of the 20S catalytic core. The T-L activity was not inhibited (IC₅₀ >10 uM, **Figure 16**). An apparent slight activation of the trypsin-activity (~10%) was seen at the highest concentration of TCH-018 (10 uM), not uncommon to proteasome inhibition.

This Example shows TCH-018 inhibits via non-competitive binding: A classical non-competitive inhibitor has no effect on substrate binding (i.e. the inhibitor and substrate bind independently at different sites). A non-competitive inhibitor decreases the V_{max}, but has no effect on the K_m value. Kinetic analysis of CT-L activity indicate that when substrate (Suc-LLVY-AMC) concentration were increased and measurements were taken at five different concentrations of TCH-018 or vehicle, the V_{Max} of the CT-L activity diminished with the increasing concentration of substrate and the K_M of the substrate remained constant (**Figure 17a**). The collected data followed the model of non-competitive inhibition: calculated maximum velocities, V_{max}, were 0.018, 0.243, 0.722, 0.855, 0.822 and 0.991 FU/sec released by 1 nM protein over thirty minutes at 37° C. Over a concentration range from 10 uM - 0.6 uM of TCH-018 respectively, the Michaelis constant, K_M, remained constant at 54 uM for all concentrations. This data indicates that the substrate concentration has no affect on the activity of the inhibitor. The mechanism for inhibition of PGPH-like (or caspase-like) activity was found to be un-competitive. This is a classic representation of a non-competitive type of inhibition. *However*, it should be noted that non-competitive kinetics could be mis-interpreted if the agent binds irreversibly in the catalytic domains

This Example shows TCH-018 does not bind in the catalytic domains. To eliminate (or confirm) irreversible binding of TCH-018 in the catalytic domains, we incubated the 20S proteasome with the biotinylated sulfone (**Figure 17b**, Ada-

Lys(biotinyl)-(Ahx)3-(Leu)3-vinyl sulfone) to label the three core catalytic sites following the work of Overkleeft and Ploegh. The affinity label (1 μ M) is incubated for 1 h with purified human 20S proteasome (200 ng) where it binds covalently and irreversibly to the three catalytic sites (**Figure 1**, β 1, β 2 and β 5). The complex was
5 denatured, exposed to gel electrophoresis (12%), transferred to a PVDF membrane, where after blocking with 3% gelatin in TBS, the covalent biotin-protein adducts are visualized using avidin-HRP. Panel b illustrates that the biotin-labeled sulfone bind irreversibly to the three domains, β 1, β 2 and β 5 (lane 1). Pretreatment of the 20S proteasome of 1 h with different concentrations of TCH-018, prior to the biotinylated
10 sulfone, does not interfere with the ability of the probe to bind (lanes 3-7), whereas the competitive inhibitor MG-132 or Velcade (i.e. bortezomib) blocks access to the β 1 and β 5 sites as reported previously.⁷⁰ This data eliminates the possibility that TCH-018 binds irreversibly to any of the three catalytic domains. Thus, indicating that TCH-018 inhibits the proteolytic activity at the β 1 (PGP-like) and β 5 (CT-like) sites
15 via a non-competitive mechanism at a site other than the catalytic sites.

Consistent with this hypothesis, the addition of TCH-018 to the sulfone, resulted in additive activities for CT-inhibition (**Figure 18**). This follows the same trend as the combination studies with the competitive binding agent, bortezomib, as described in C3.2. Therefore, (1) **Figure 16** illustrates the inhibition of proteolytic
20 activity of the 20S proteasome by TCH-018. (2) The kinetics of binding in **Figure 17a**, indicates a non-competitive mechanism, and (3) **Figure 17b** indicates that binding of TCH-018 to the proteasome does not occur at any of the three catalytic sites. We recognize that individually these data do not fully constitute the case for allosteric inhibition. However, taken together these data strongly suggest that TCH-
25 018 inhibits the 20S proteasome by binding a site other than the catalytic sites resulting in inhibition of its catalytic activity (typically/often referred to as negative allosteric inhibition).

This Example shows TCH-018 does not inhibit other proteases: Inhibition was not limited to human proteasome tested, but TCH-018 was equally active with the human
30 20S immunoproteasome and was also effective toward 20S proteasome isolated from *S. cerevisiae* (IC₅₀ 2.8 μ M). However, when TCH-018 was evaluated for its modulation of other proteases including calpain, caspase and cathepsin B, the compound did not exhibit any detectable inhibition (data not shown). This data

indicates that TCH-018 is not a general protease inhibitor, but has primarily (or selectively) targets the 20S proteasome.

This Example shows TCH-018 binds reversibly. In order to investigate whether the

5 TCH-018 binding is reversible or irreversible, we evaluated plot of $V_{\max}$ versus amount of enzyme added to distinguish between a reversible and irreversible non-competitive inhibitor. Irreversible inhibition can be distinguished from reversible binding by plotting $V_{\max}$ vs. $[E]$. If the compound binding is reversible, the curve will have a smaller slope than the control curve and go through the origin. An irreversible
10 inhibitor's curve will have the same slope but intersect on the horizontal axis at the position equivalent to the amount of enzyme that is irreversibly inactivated by the inhibitor. As illustrate in **Figure 19**, TCH-018 (2.5 μ M) and control both intersect the plot origin, indicative of classical non-competitive reversible binding. This data indicates that TCH-018 behaves as a classical non-competitive reversible inhibitor.

15 **This Example shows that TCH-062 binds irreversibly.** TCH-062 was found to irreversibly bind to the 20S proteasome and as evaluated by the plot of $V_{\max}$ versus amount of enzyme added (Figure 20).

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in the relevant fields are intended to be within the scope of the following claims.

10

CLAIMS:

1. A pharmaceutical composition comprising a small molecule non-covalent inhibitor of the 26S proteasome.

5

2. The composition of Claim 1, wherein said inhibitor inhibits the 20S catalytic core of the 26S proteasome.

10

3. The composition of claim 1, wherein the small molecule inhibitor is selected from the group consisting of imidizolines 1-9 as set forth in Figure 8.

4. The composition of claim 1, wherein the compound is a therapeutically effective amount for reducing a symptom of a disease selected from the group consisting of cancer and inflammatory disease.

15

5. The composition of claim 1, wherein the cancer is multiple myeloma.

6. The composition of claim 1, wherein the inflammatory disease is rheumatoid arthritis.

20

7. A method, comprising,

a) providing,

i) a pharmaceutical composition comprising a small molecule non-covalent inhibitor of the catalytic region of a 26S proteasome and

25

ii) a patient, wherein said patient is in need of treatment and

b) administering said pharmaceutical composition to said patient as a therapeutically effective amount

30

8. The method of Claim 7, wherein said inhibitor inhibits the 20S catalytic core of the 26S proteasome.

9. A method, comprising,

a) providing,

- iii) a pharmaceutical composition comprising a small molecule non-competitive inhibitor of the 26S proteasome and
- iv) a patient, wherein said patient is in need of treatment; and
- b) administering said pharmaceutical composition to said patient.

5

10. The method of claim 9, wherein the small molecule is selected from the group consisting of imidizolines 1-9 as set forth in Figure 8.

11. The method of claim 9, wherein the patient shows a symptom selected from
10 the group consisting of a cancer and an inflammatory disease.

12. The method of claim 11, wherein the cancer is multiple myeloma.

13. The method of claim 11, wherein the inflammatory disease is rheumatoid
15 arthritis.

Figure 1

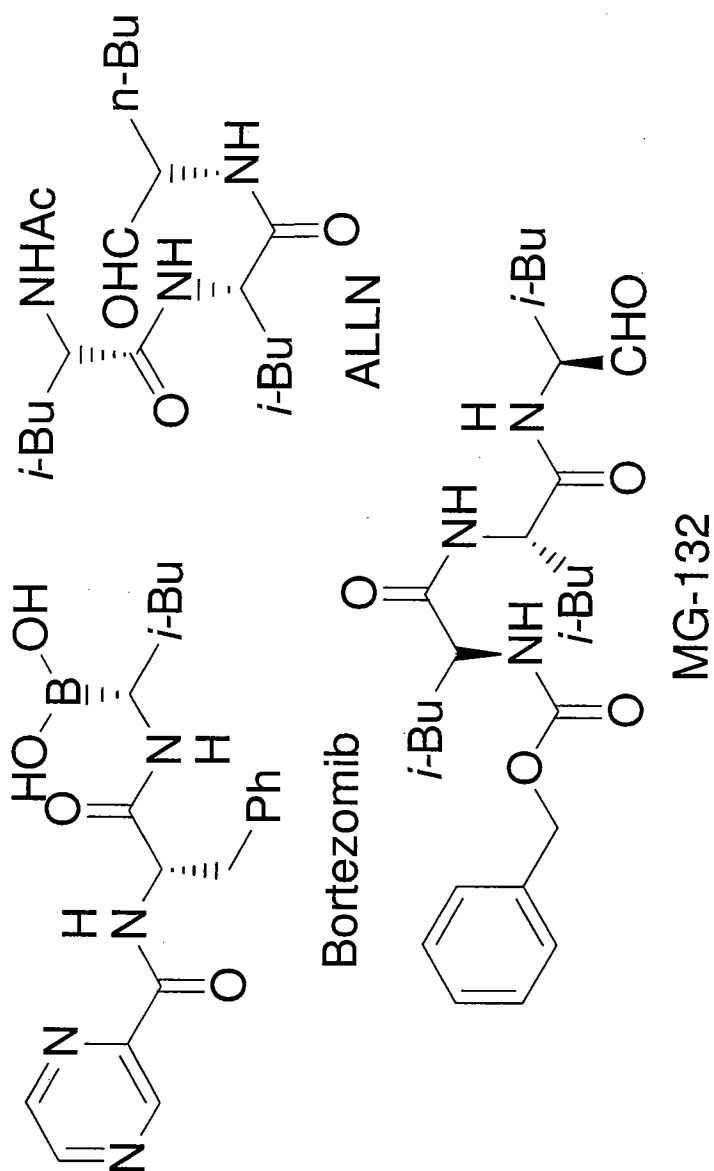


Figure 2

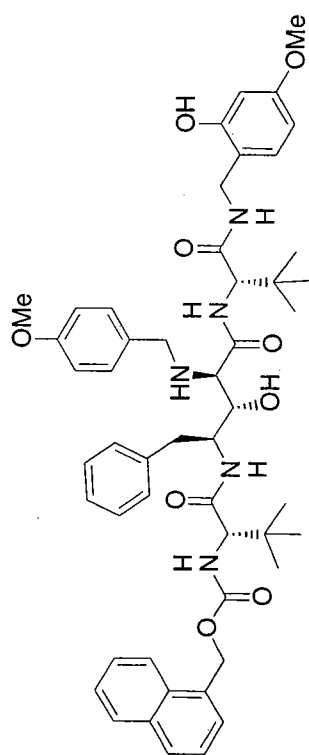
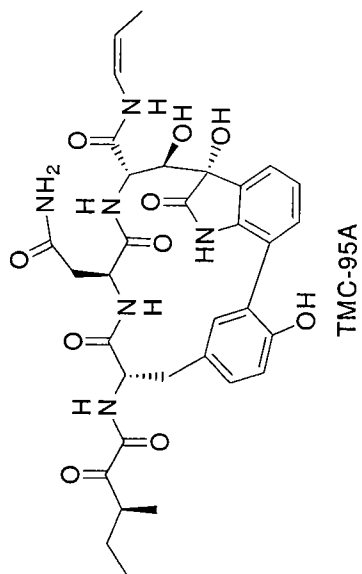
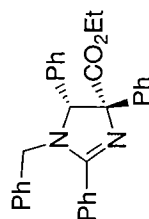
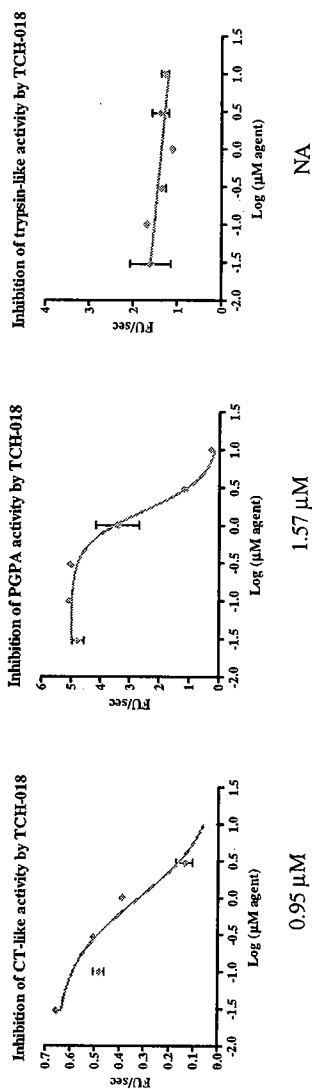
Novartis (BMCL 2001, 11, 1317) CT-only IC₅₀ 0.1 μM

Figure 3

TCH-018



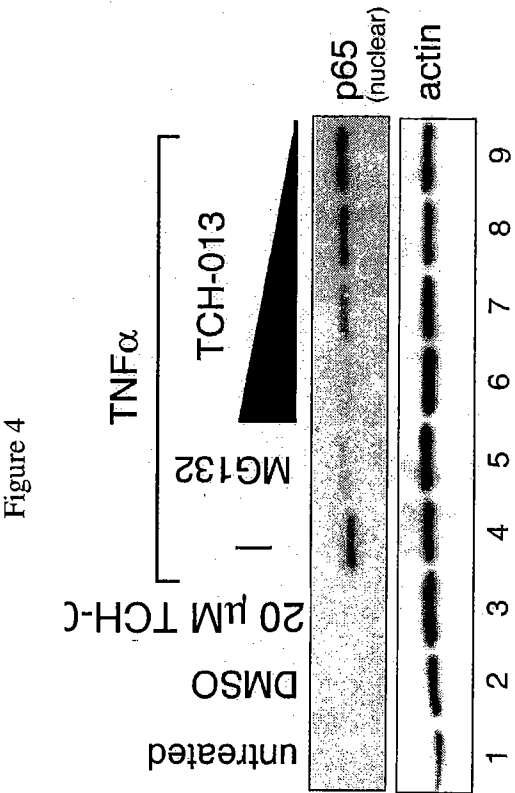


Figure 5

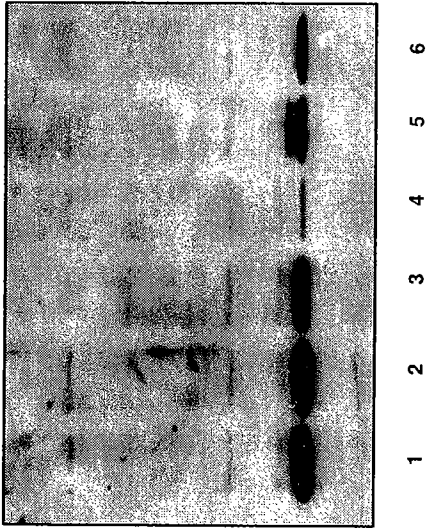


Figure 6

1 2 3 4 5 6 7 8 9 10

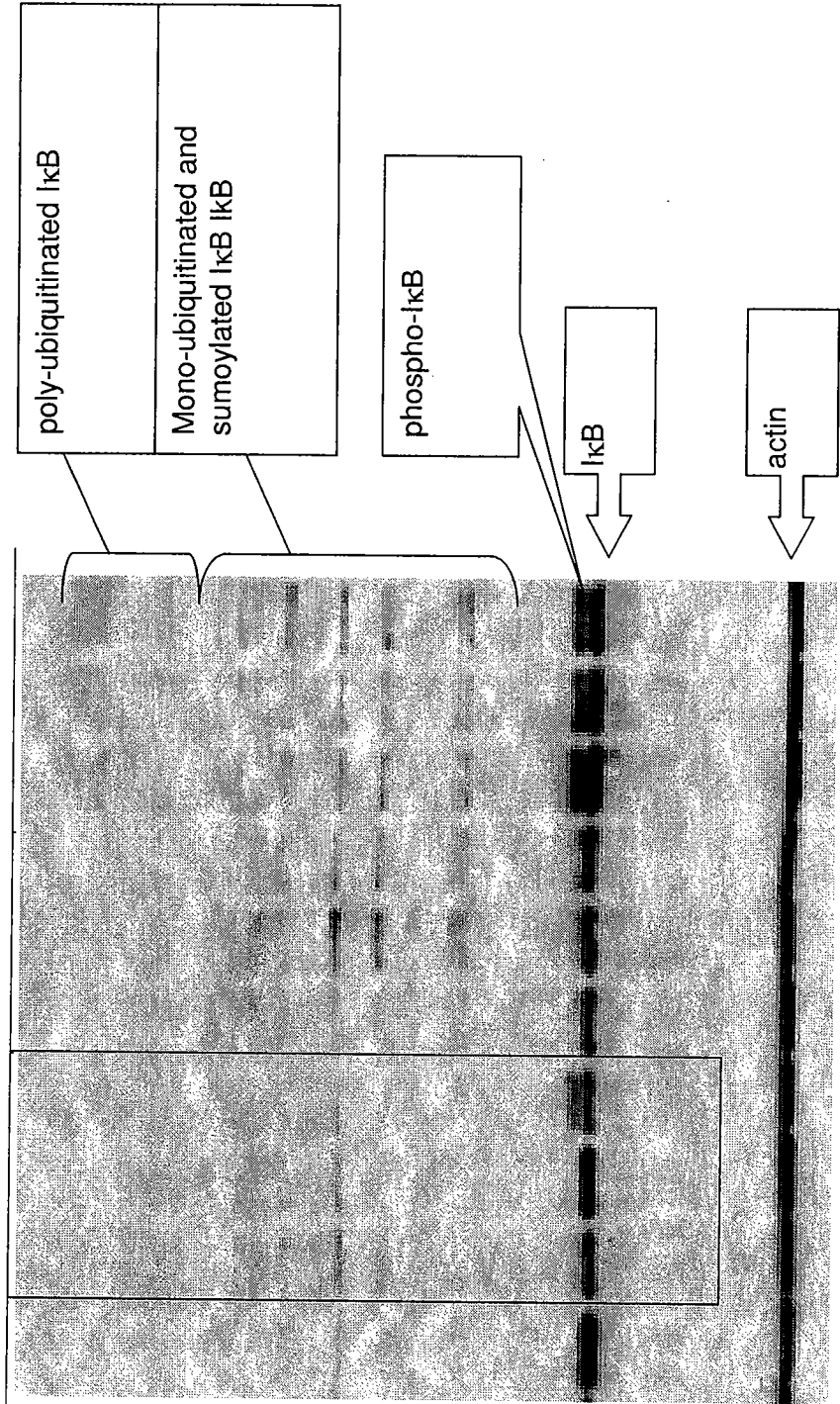


Figure 7

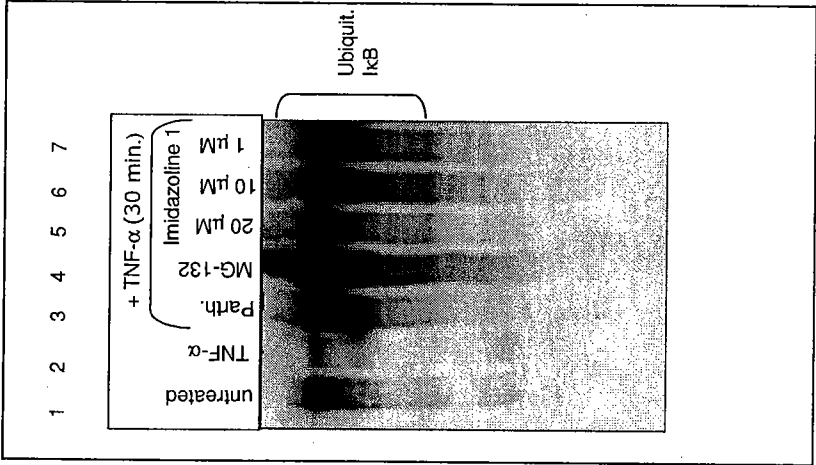
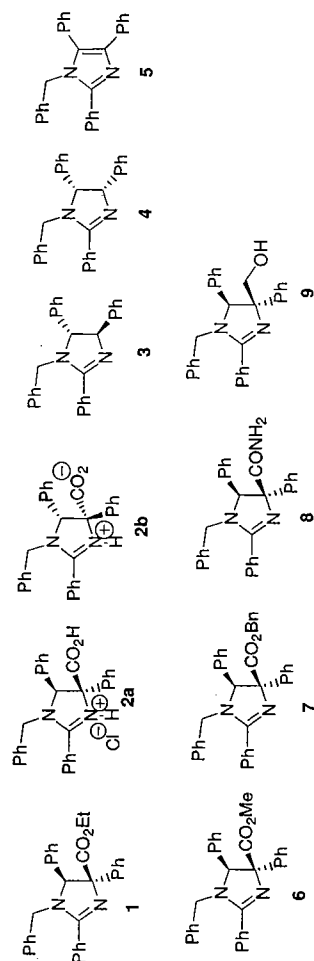
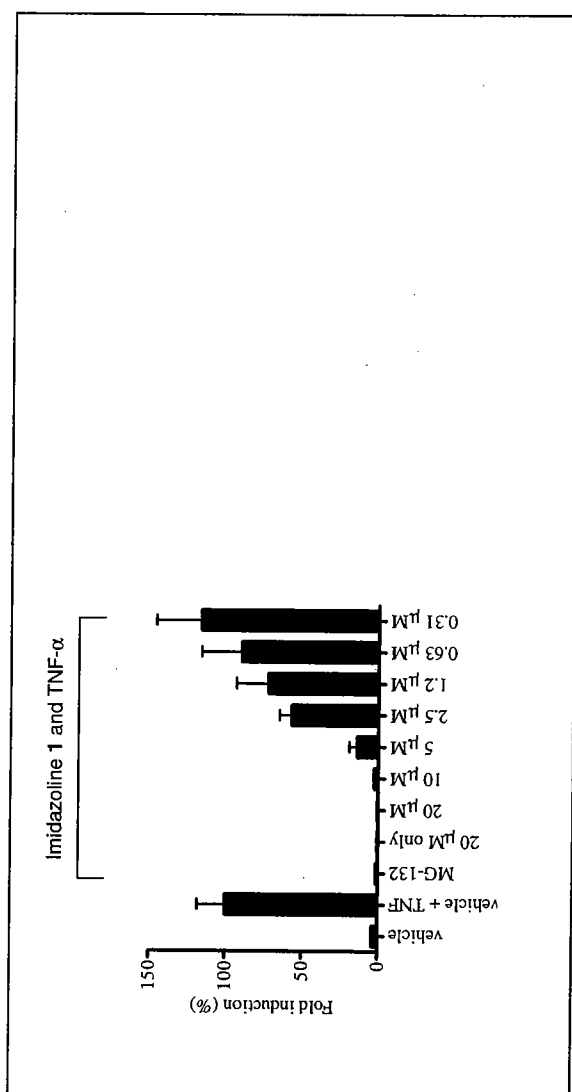


Figure 8



Structures of imidazolines 1-9

Figure 9

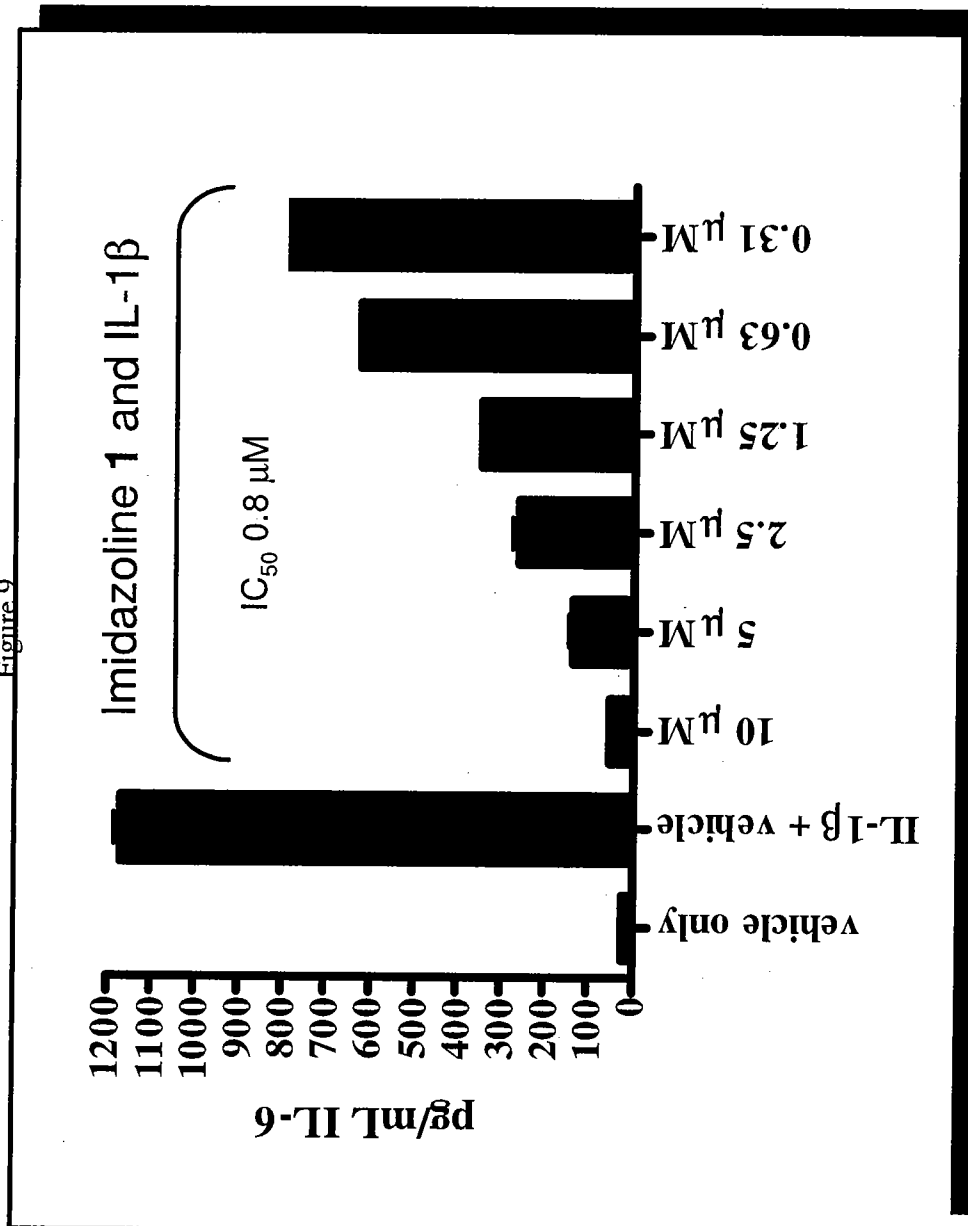


Figure 10

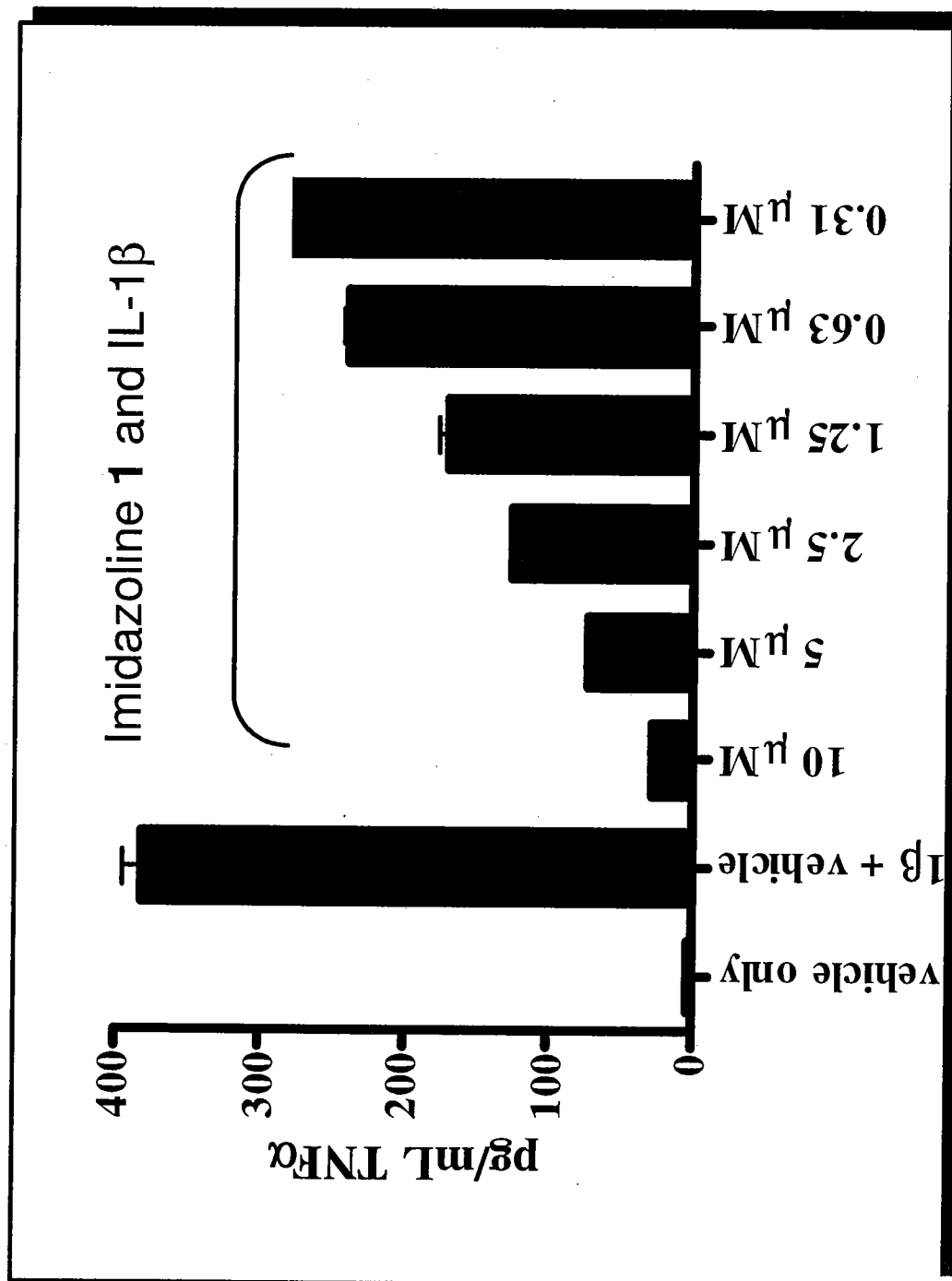


Figure 11

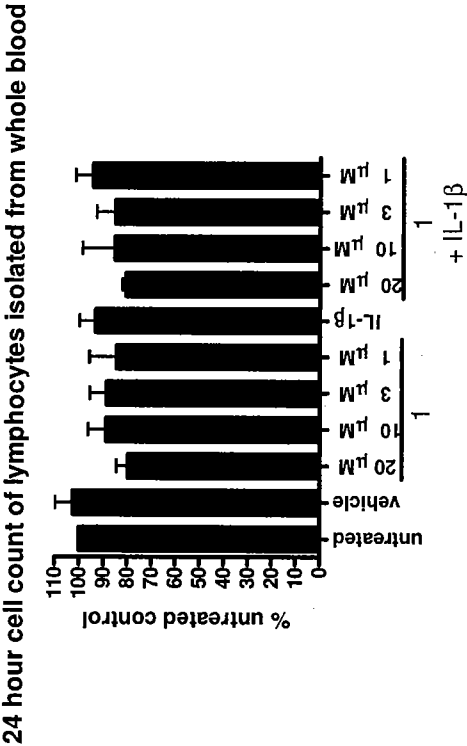


Figure 12

Entry	structure	20S proteasome CT only (μM)	NF- κB -luc Hela cells (μM)	IL-6 Human blood (μM)
TCH-018		0.95-1.7	1.6	0.2
TCH-047		>10	>20	>20
TCH-015		>10	>20	Not tested
TCH-34		>10	>20	>20
TCH-063		1.6	1.9	0.3
TCH-072		1.4	2.6	Not tested
TCH-088		1.9	6.0	Not tested
TCH-023 Exception:		2.4	>20	>20

Figure 13

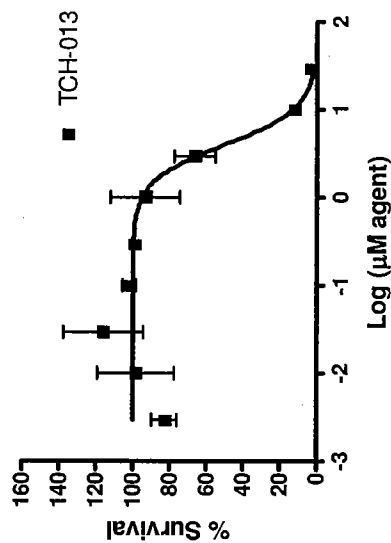


Figure 14

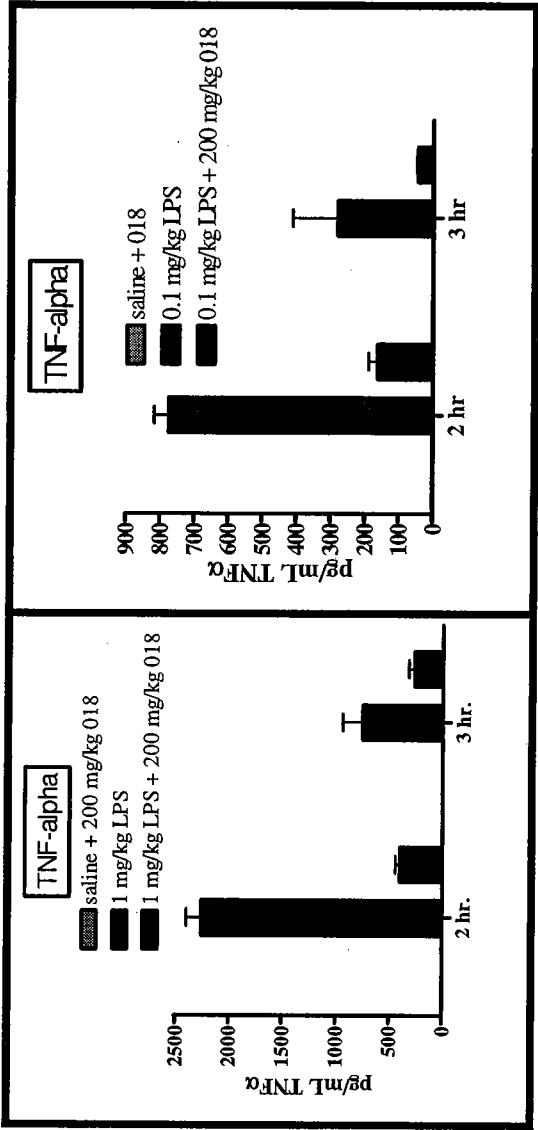
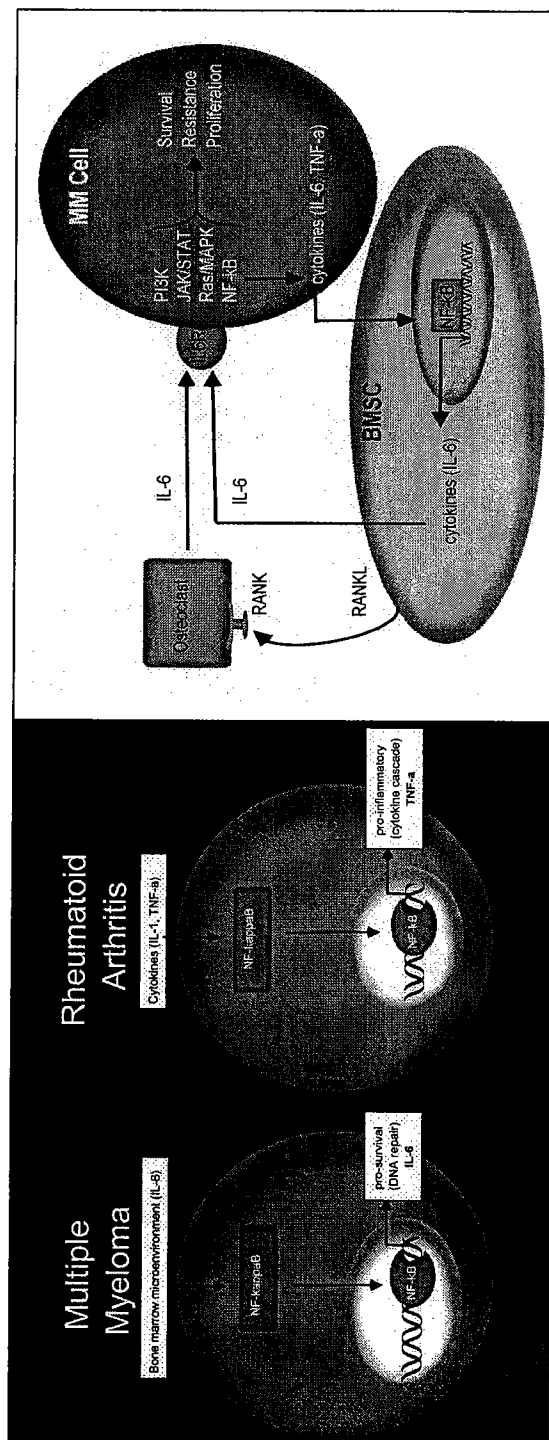
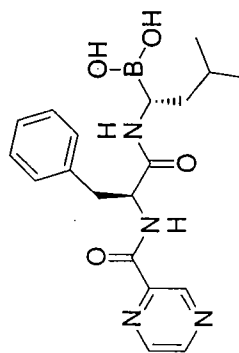


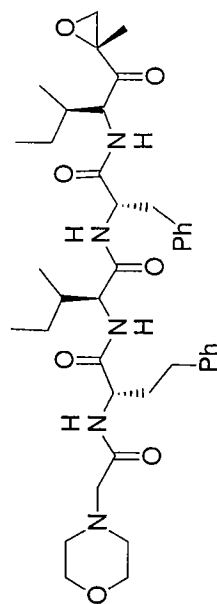
Figure 15



Clinical biomarker for disease progression is **IL-6** level in blood.

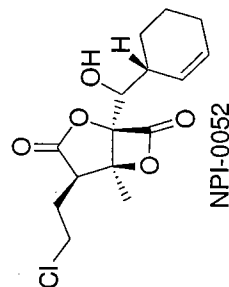


Bortezomib for relapsing MM (iv)
Problems: Host toxicity
(neuropathy) and resistance



PR-171

Carfilzomib (PR-171):
Phase II MM
Phase II solid tumors
Phase I in lymphoma



NPI-0052

Salinosporamide
(NPI-0052 (iv)):
in Phase 1 trial for MM, NSCL,
pancreatic, melanoma and lymphoma)

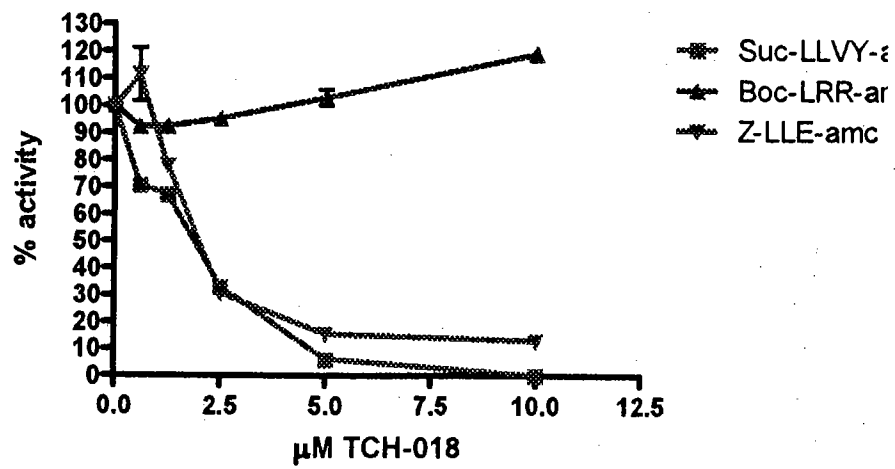


Fig. 16

Fig. 18

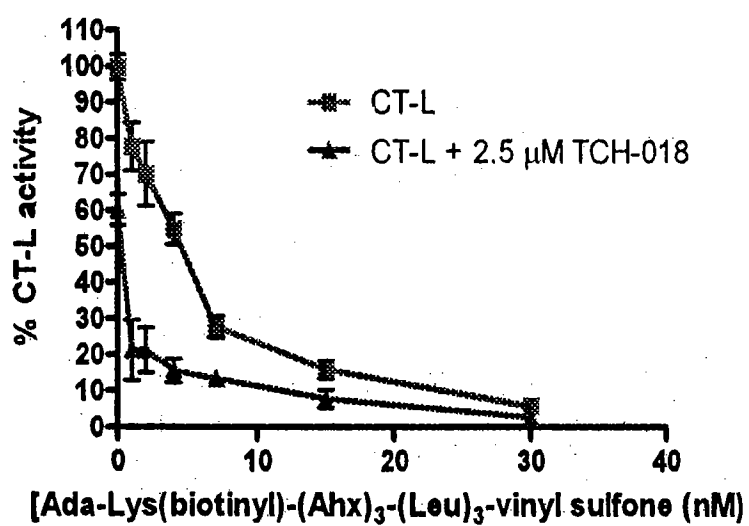


Figure 18. Ada-Lys(biotinyl)-(Ahx)₃-(Leu)₃-vinyl sulfone does not prevent inhibition by TCH-018.

Fig. 19

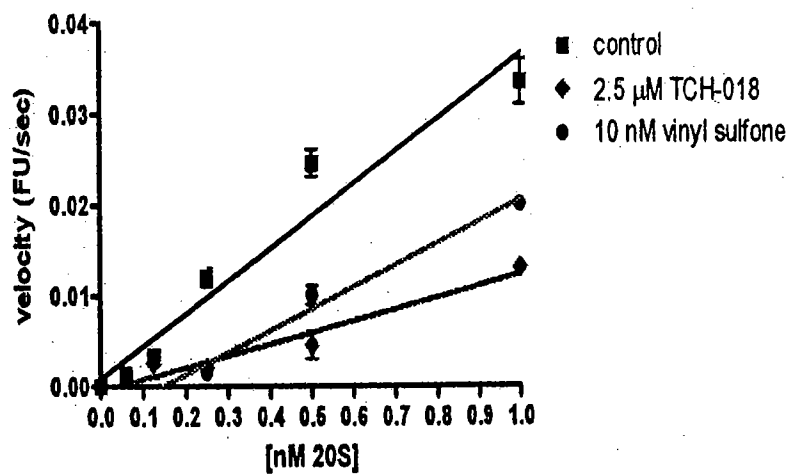


Figure 19. Vmax measured in the presence of TCH-018 (2.5 μM) with varying enzyme concentrations ([E]). Interception of the origin by both control and TCH-018 is indicative of classical non-competitive reversible inhibition.

Fig. 20

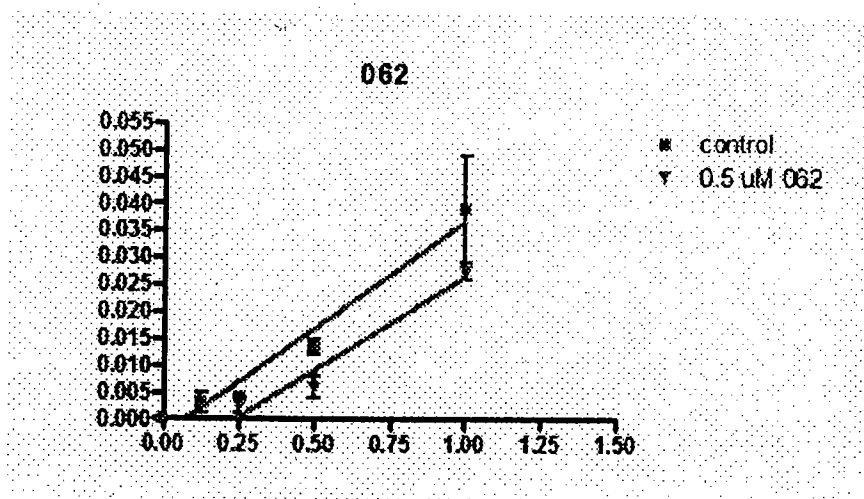


Figure 20. Vmax measured in the presence of TCH-062 (0.5 mM) with varying enzyme concentrations indicating an irreversible (likely covalent) binding.

Fig. 21

