Characterization of LY2228820 dimesylate, a potent and selective inhibitor of p38 MAPK with anti-tumor activity

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Abbreviations: MAPK = Mitogen-Activated Kinase, p38 = p38 MAPK, TME = tumor microenvironment

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Abstract

p38α mitogen-activated protein kinase (MAPK) is activated in cancer cells in response to environmental factors, oncogenic stress, radiation, and chemotherapy. p38α MAPK phosphorylates a number of substrates, including MAPKAP-K2 (MK2), and regulates the production of cytokines in the tumor microenvironment, such as TNFα, IL-1β, IL-6 and CXCL8 (IL-8). p38α MAPK is highly expressed in human cancers and may play a role in tumor growth, invasion, metastasis and drug resistance. LY2228820 dimesylate (hereafter LY2228820), a tri-substituted imidazole derivative, is a potent and selective, ATP-competitive inhibitor of the α- and β- isoforms of p38 MAPK in vitro (IC$_{50}$ = 5.3 nM and 3.2 nM, respectively). In cell-based assays, LY2228820 potently and selectively inhibited phosphorylation of MK2 (Thr334) in anisomycin-stimulated HeLa cells (at 9.8 nM by Western blot) and anisomycin-induced mouse RAW264.7 macrophages (IC$_{50}$ = 35.3 nM) with no changes in phosphorylation of p38α MAPK, Jnk, Erk1/2, c-Jun, ATF2 or c-Myc < 10 μM. LY2228820 also reduced TNFα secretion by LPS/IFNγ-stimulated macrophages (IC$_{50}$ = 6.3 nM). In mice transplanted with B16-F10 melanoma, tumor phospho-MK2 (p-MK2) was inhibited by LY2228820 in a dose-dependent manner (TED$_{70}$ = 11.2 mg/kg). Significant target inhibition (>40% reduction in p-MK2) was maintained for 4-8 hours following a single 10 mg/kg oral dose. LY2228820 produced significant tumor growth delay in multiple in vivo cancer models (melanoma, NSCLC, ovarian, glioma, myeloma, breast). In summary, LY2228820 is a p38 MAPK inhibitor which has been optimized for potency, selectivity, drug-like properties (such as oral bioavailability), and efficacy in animal models of human cancer.

Introduction

p38 MAPK (α, β, δ, γ isoforms) is a member of the mitogen-activated protein kinase family which also includes Jnk and Erk (1). The α isoform of p38, known as p38α MAPK or MAPK14, is activated in response to environmental factors such as lipopolysaccharide (LPS), cytokines, heat/osmotic shock, radiation, and chemotherapy (2). Activation is accomplished
by phosphorylation of the Thr180 and Tyr182 residues by upstream MAP kinase kinases, including MKK3 and MKK6 (3). p38α MAPK phosphorylates a number of substrates, including MAPKAP-K2 (MK2), which then phosphorylates tristetraprolin, regulating the post-translational processing of various proteins (4).

p38 MAPK plays a key regulatory role in the production of cytokines such as tumor necrosis factor alpha (TNFα), interleukin-1β (IL-1β), interleukin-6 (IL-6) and interleukin-8 (IL-8, CXCL8) (4-6). Given the critical role of p38α MAPK in regulating these cytokines, there is keen interest in identifying p38 MAPK inhibitors for use in inflammatory diseases such as rheumatoid arthritis, Crohn’s disease, and the pain associated with inflammation (7, 8). The role of inflammation in cancer initiation and progression has been widely studied. Soluble factors produced by the tumor and by the tumor microenvironment (TME), can activate p38 MAPK and promote tumorigenesis, angiogenesis, invasion, metastasis and resistance to anti-cancer agents (2, 6, 9, 10). For example, tumor-infiltrating macrophages, which possess abundant amounts of activated p38α MAPK, are associated with adverse prognosis in cancer (11, 12). We have previously shown that pharmacologic inhibition of p38 MAPK activity in tumor infiltrating macrophages in vivo is associated with an anti-tumor effect (13). Similarly, IL-6 and CXCL8 have proliferative and pro-angiogenic properties; importantly, elevated levels of these cytokines are correlated with poor prognosis in breast and ovarian cancers (14, 15).

The actions of p38 MAPK in cancer involve aberrant interactions between the tumor and its microenvironment, and these interactions may differ among tumor types. There are data which support both direct (tumor-mediated) and indirect (via TME) modes of action for p38 MAPK in oncogenesis with a complex interplay between the two likely important for disease progression. For example, both melanoma cells and the associated tumor-infiltrating leukocytes secrete abundant amounts of cytokines (such as IL-1, IL-6, CXCL8 and Gro-α) which are known to promote angiogenesis, growth, invasion and metastasis (16-20). Ovarian cancer cells produce TNFα and CXCL8 upon p38α MAPK activation, and these cytokines may act in an autocrine manner to promote peritoneal colonization and tumor vascularization (21). The U-87MG glioma, A-2780 and SK-OV-3 ovarian cancers, and PC-3 prostate cancer all secrete
vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and IL-6; and importantly, secretion of these cytokines can be significantly reduced by p38 MAPK inhibition (6, 22). Furthermore, pretreatment of tumor cells with LY2228820 or p38α MAPK shRNA reduces cord formation in a tumor/adipocyte-derived stem/endothelial colony forming cell co-culture system, supporting a p38 MAPK mediated effect of the tumor on the TME (6).

p38 MAPK activity also influences TME biology, an often overlooked aspect of cancer therapy, but one which likely plays a profound role in tumor-host dynamics. In multiple myeloma, p38 MAPK inhibitors down-regulate IL-6 secretion by bone marrow stromal cells (BMSCs), inhibit myeloma cell proliferation (23-26), and reduce osteoclastic bone destruction (24, 27). Inhibition of p38 MAPK also reduces production of VEGF and platelet-derived growth factor (PDGF) by BMSCs in vitro (28). In response to neutrophil elastase, lung cancer cells produce CXCL8 which stimulates the growth of lung cancer cells in vitro (29-31) in a p38α MAPK dependent manner, indicating that neutrophils in the TME may activate the p38 MAPK signaling pathway in tumor cells to promote lung cancer growth (32). Furthermore, secretion of CXCL8 by both the tumor and TME increases proliferation of ovarian cancer cells in vitro and in vivo (30, 32). IL-6, secreted from adipose stromal cells to promote migration and invasion of breast cancer cells (33), likely requires p38 MAPK because its direct substrate, MK2, contributes to IL-6 mRNA stabilization (34). Finally, from the perspective of immune surveillance, activation of p38 MAPK is associated with enhanced dendritic cell tolerance during melanoma progression (35). Conversely, p38 MAPK inhibition restores the function of monocyte-derived dendritic cells in myeloma, limiting evasion of the tumor from immune surveillance (36).

Given the substantial body of evidence supporting a role for p38 MAPK in cancer progression, we developed a potent and selective p38 MAPK inhibitor, LY22288220 dimesylate (hereafter LY2228820), and characterized its activity using in vitro biochemical and cellular systems and in vivo xenograft models in which tumor and/or TME p38 MAPK activity has been implicated. These results demonstrate that LY2228820, a novel p38 MAPK inhibitor,
provides an opportunity for the treatment of cancer through modulation of aberrant interactions between the tumor and its supportive microenvironment, a therapeutic strategy that merits further clinical evaluation.

**Material and Methods**

**Crystallography.**
For crystallographic studies human p38 was expressed in *E. coli* and purified by affinity and gel filtration chromatography. Purified protein was in a solution containing 150 mM NaCl, 20 mM Hepes pH 7.5, 10 mM methionine, 5 mM DTT and 10% glycerol. Protein at 16.2 mg/ml was mixed in 1% βOG and 1.25 mM LY2228820 before crystallization drops were set up. Diffraction-quality crystals of p38/LY2228820 were grown by the vapor diffusion technique at 21°C under the reservoir condition 0.1 M Na cacodylate, pH=6.2, 10% PEG 3350. Crystals belong to space group P2₁₂₁ with unit cell parameters *a*=66.558 Å, *b*=74.456 Å, *c*=78.691 Å. The diffraction data (resolution of 1.97 Å) were collected on beam line ID-31 (then SGX-CAT) at the APS (Advanced Photon Source, Argonne National Laboratories). The crystal structure was determined by the method of molecular replacement and was refined using a maximum likelihood target as incorporated in the program CNX2000 first and then using Refmac5 (R<sub>work</sub>=0.2303, R<sub>free</sub>=0.2666).

**Kinase assays.** For p38-MAPK enzymatic assays (enzyme sourced from either Millipore or Roche), ³³P-ATP radiometric filter binding (Millipore MAPH plates) was used with an EGFR peptide substrate, KRELVEPTPSGEAPNQALLR (Multiple Peptide Systems) and 100 µM (p38α) or 20 µM (p38β, p38γ) ATP run under linear velocity conditions. LY2228820 was also tested against a panel of enzymatic kinase assays (internal assays and Merck Millipore KinaseProfiler™(37) ; all human sequence) to assess the relative kinase selectivity *in vitro*. The initial screen was conducted at 20 µM followed by 10-point concentration-response curves (1:3 serial dilutions from 20 µM to 1 nM). All assays were either 96-well radiometric filter binding (³³P-ATP phospho-cellulose or glass fiber) or fluorescence polarization formats.
under linear velocity conditions, at or below the Km[ATP] (typically 30-120 minutes resulting in ≤ 10% ATP conversion) using 1-4% DMSO final (depending on enzyme tolerance for solvent). IC₅₀ values were calculated using 4-parameter non-linear regression (ActivityBase software, IDBS).

**Cell Lines:** All human cell lines were obtained from ATCC (A549, U-87MG, HeLa, MDA-MB-468, 786-O), DSMZ (OPM-2) or the NCI DCTD Tumor Repository (A2780) between 2004-2012 and pathogen tested (PCR): Human immunodeficiency virus (HIV1, HIV2), Hepatitis viruses (A, B, C), Human T-lymphotropic virus (HTLV 1, HTLV 2), Epstein Barr virus, Hantaviruses (Hantaan, Seoul, Sin Nombre), Herpes simplex 1, Herpes simplex 2, Human cytomegalovirus, Human herpes virus 6, Human herpes virus 8, Human adenovirus, Varicella virus, Lymphocytic choriomeningitis virus, and Mycoplasma spp. These lines were authenticated by STR-based DNA profiling and multiplex PCR (IDEXX RADIL CellCheck™, IDEXX Laboratories). Rodent cell lines (B16-F10, RAW264.7) were purchased from ATCC (between 2000-2002) and pathogen tested (PCR) against Mycoplasma spp. and Mycoplasma pulmonis.

**MK2 capture ELISA (cELISA):** RAW 264.7 cells were treated with compound (range of 20µM to 1nM, ten 1:3 serial dilutions) for 2 hours at 37°C/5% CO₂. Anisomycin (10 µg/mL) was added into the media to activate the p38 pathway. After 30 minutes incubation, cells were fixed and p38 activity was assessed in an electrochemiluminescent cELISA using a phospho-MK2 (Thr 334, Cell Signaling Technologies) antibody. Briefly, 5 µl of a 20 µg/ml concentration of anti-MAPKAP-K2 antibody (Cat.# KAP-MA015, Stressgen, Ann Arbor, MI) was placed into a 96 well high binding MesoScale Discovery (MSD, Gaithersburg, MD) plate and incubated at 4°C overnight. The plate was blocked for 1 hr at RT, washed, and 25 µl of detection antibody, anti-p-MAPKAP-K2 (Cat.# 3041, Cell Signaling, Inc., Beverly, MA) conjugated to ruthenium, was added and incubated for 2 hours at room temperature. The plate was washed, 150 µl of 1X ReadT buffer was added per well and the plate read on an MSD Sector 6000 instrument.

**LPS/IFNγ-stimulated TNFα Release by Mouse Peritoneal Macrophages in vitro:** Mouse peritoneal macrophages were activated in vivo by 3% thioglycollate injection (intraperitoneal, administered four days prior to harvest), then harvested and plated in 96-well microtiter
plates. The cells were treated with LY2228820 (range of 20uM to 1nM, ten 1:3 serial dilutions) for 0.5 hours, and then incubated with LPS/IFNγ for 2 hours (to stimulate p38-MAPK) and the media measured for TNFα by ELISA (R&D Systems).

**Cytokine Analysis.** A549 cells (3x 10^4) were seeded into 24-well tissue culture dishes in RPMI (Roswell Park Memorial Institute) media/10% FBS (Invitrogen). 24 hours later, cells were pre-treated for 30 minutes with 0.01% DMSO or LY2228820 dimesylate (range of 20uM to 1nM, ten 1:3 serial dilutions) prior to the addition of 100 ng/mL LPS (Millipore). Conditioned media was collected 72 hours post-LPS treatment and total viable cells were counted by Coulter Counter, (Beckman Coulter). Samples were analyzed for CXCL8 secretion with Quantikine® Colorimetric Sandwich ELISAs (R&D Systems) according to the manufacturer’s recommendations.

**Anisomycin-stimulated HeLa cell kinase selectivity assay (Western Blot):** HeLa cells were pretreated with LY2228820 for 1 hour before stimulation with anisomycin (10 µg/mL) for 45 minutes. Cells were then lysed in a protein lysis buffer (containing protease [Leupeptin 10 ug/ml, Trypsin-Chymotrypsin inhibitor 10 ug/ml, TPCK (N-p-Tosyl-L-phenylalanine chloromethyl ketone) 10 ug/ml, Aprotinin 10 ug/ml, TAME (Na-p-Tosyl-L-arginine methyl ester hydrochloride) 2mM, Benzamidine hydrochloride hydrate 5 mM] and phosphatase inhibitors [Sodium Metavanadate anhydrous 1 mM, PNPP (p-Nitrophenyl phosphate) 15 mM, Microcystin 1 uM], Okadaic acid 1uM, Sigma-Aldrich) and proteins were analyzed by Western blotting for p38, JNK, and p44/42 (ERK1/2) pathway activities. All antibodies were obtained from Cell Signaling Technologies: phospho-MK-2 (Thr334); phospho-p38 MAP kinase (Thr180/Tyr182); total p38a MAPK antibody; phospho-ATF2 (Thr71); phospho-JNK (Thr183/Tyr185); phospho-cJun (Ser63); phospho-p44/42 MAP kinase (Thr202/Tyr204); phospho-cMyc (Thr58/ser62).

**Phosphorylation of MK2 in mouse B16-F10 melanoma tumors.**

**In Vivo Target Inhibition:** Murine B16-F10 melanoma cells were cultured in DMEM supplemented with L-Glutamine, high glucose and 10% FBS [GIBCO 11965-092]. C57/bl6 mice (Charles River) were implanted in the rear flank with B16-F10 cells (2 X 10^6), and when tumors
reached approx. 200 mm³ in size, were dosed orally with LY2228820 in 1% carboxymethylcellulose/ 0.25% Tween® 80. Two hours post-dose, tumors were excised, homogenized and lysed for Western Blot. MK2 phosphorylation (p-Thr334), normalized to total GAPDH, was quantified by chemiluminescent detection. The 50% or 70% threshold effective dose (TED₅₀ and TED₇₀, respectively) was calculated (JMP software, SAS) in order to approximate effective dose ranges for testing of LY2228820 in xenograft models, i.e., where significant target inhibition was observed. The TED₅₀ or TED₇₀ is defined as the dose where a statistically significant effect was achieved, and there was at least 50% or 70% inhibition, respectively, compared to vehicle control.

**In Vivo Pharmacodynamics:** Mice bearing B16-F10 tumors were given a single dose of LY2228820 approximating the TED₇₀ (10 mg/kg p.o.) and sampled for compound exposure and tumor p-MK2 over various time points.

**In vivo Phosphorylation of MK2 in mouse and human peripheral blood mononuclear cells (PBMC).** See Supplemental Methods (Supplemental Figure 1).

**shRNA knockdown of p38α MAPK in U-87MG glioma cells.** See Supplemental Methods (Supplemental Figure 2).

**In vivo tumor models.** All studies were done in accordance with AALAC-accredited institutional guidelines. Female immunocompromised mice received food and water ad libitum and were acclimated for at least 1 week prior to xenograft implantation. Cells used in xenograft studies were pathogen tested and authenticated by short tandem-repeat analysis. Banked master stocks were returned to within approximately 6 months, or if inconsistencies in growth behavior were observed. Cells originated from the American Type Culture Collection (ATCC) unless otherwise indicated.

Subcutaneous cell-based inoculations were performed in a 1:1 volume (200 µL total) with Matrigel® (BD Biosciences) and were injected in the right rear flank. Inoculums and host animals were as follows: 5 X 10⁶ cells for U-87MG, SK-OV-3x-luc#1 (M. Harrington and S.
Brutkiewicz, Indiana University), 786-O, and $1 \times 10^7$ cells for A549 were implanted into athymic nude mice (Harlan); $2 \times 10^6$ for A-2780 (NCI DCTD) into CD1 nu/nu mice (Charles River); and $5 \times 10^6$ for OPM-2 (DSMZ GmbH, Germany) into CB-17 SCID mice (Taconic) irradiated with 2.5 Gy within 24 hours of implant. MDA-MB-468 breast cancer xenografts were initiated as implants of established cell-derived tumors in athymic nude mice at Oncotest GmbH (Germany). Tumors were allowed to establish for at least one week prior to randomization into treatment groups; treatments began with tumors of 50-250 mm$^3$. LY2228820 was prepared in 1% CMC/0.25% Tween® 80 or HEC 1% / Tween® 80 0.25% / AF 0.05% and delivered by oral gavage (10 mL/kg volume). Comparable vehicle control groups were run in parallel. Dosages, dosing schedules and cohort sizes are described in the legend for each study. Tumor size and body weight were recorded 1-2 times per week. Tumor size was determined by caliper and tumor volume (mm$^3$) was estimated using the formula: $l \times w^2 \times 0.536$, where $l$ is the larger and $w$ is the smaller of the perpendicular diameters. Tumor data was analyzed by Repeated Measures using ANOVA with a Tukey post-hoc test.

**SK-OV-3 and 786-O orthotopic xenograft models:**

Female athymic nude mice (20 to 25 g, Harlan) received intraperitoneal injections ($2 \times 10^6$ cells in 0.2 mL PBS) of SK-OV-3 luciferase-labeled tumor cells or luciferase-labeled 786-O cells ($2 \times 10^6$ in 0.025 mL PBS) implanted into the renal capsule of the surgically accessed left kidney. On days 10 and 21 respectively, animals with a signal demonstrating tumor presence were randomized into study groups and started on drug treatments. Anesthetized animals were imaged weekly with an IVIS Spectrum (Caliper Life Sciences) following intraperitoneal injection 150 μL of 15 mg/mL of D-luciferin (Sigma Aldrich). Data review and analysis was performed with the Living Image 3.0 program. Tumor growth was measured for each animal as luminescent light intensity or Total Flux (P/S) with fixed ROI data from the IVIS Spectrum. Statistical comparisons employed Dunnett’s ANOVA and t-test measures between the treatment groups.
**Mouse B16-F10 melanoma lung metastasis model:** B16-F10 melanoma cells (50,000) were injected into the tail vein of nude mice one day prior to treatment. Mice were orally dosed by gavage with vehicle or LY2228820 (either 10 mg/kg or 30 mg/kg) TID, on a schedule of 4 days on/3 days off for 14 consecutive days (n = 10 animals/group). Lung tissues were collected and placed in 10% neutral buffered formalin followed by 70% ethanol storage. Individual lung metastases were counted visually. Data were analyzed by ANOVA.

**Results.**

Utilizing molecular modeling to guide medicinal chemistry efforts, a series of molecules were designed from early screening actives to optimize binding to the ATP pocket of p38α MAPK, reduce potential off-target kinase activity, achieve drug-like properties, and minimize the risk of drug-drug interactions. The candidate compound 2 was identified from iterative SAR on hits found in the Lilly compound collection, as represented by the generic structure 1 (Fig 1A). Awareness of potential CYP activity associated with the imidazole central core led to the difluoroaryl substitution at C-2 with other sterically bulky groups such as tert-butyl as found in the candidate (38). The contribution of the 4-fluorophenyl group to enzymatic affinity can be understood by recognizing that it occupies a hydrophobic pocket of the ATP binding site near the gatekeeper Thr106, as seen in the x-ray co-crystal structure (Fig 1B). In addition, modifications of the benzimidazole substituent at C-5 and the aromatic group, R2, at C-4 of the imidazole were made in consideration of improving cell-based potency as well as pharmacokinetic properties.

**In vitro characterization data**

One compound from the benzimidazole SAR, LY2228820 (Fig 1), was found to be a very potent, ATP-competitive inhibitor of both the α and β isoforms of p38 MAPK in vitro (IC$_{50}$ = 5.3 nM and 3.2 nM, respectively) with > 1000-fold selectivity for p38α MAPK versus 178 other kinases tested (Table 1, Suppl. Table 1). Within the MAPK family, LY2228820 was >1000-fold
more selective for p38α versus p38δ, p38γ, ERK1 and ERK2; >50-fold more selective for p38α versus JNK1; 30-fold more selective for p38α versus JNK3; and 15-fold more selective for p38α versus JNK2. In mode of action experiments, LY2228820 was ATP-competitive with a Ki [apparent] = 4.5 ± 0.5 nM (radiometric filter binding assay) and with tight-binding kinetics (kd = 0.0025 ± 0.0005 s⁻¹, ka = 1.22 x 10⁶ ± 0.08 M⁻¹s⁻¹; by Surface Plasmon Resonance, SPR; data not shown). LY2228820 was also tested in a wide array of assays to assess any off-target effects on non-kinase enzymes, ion channels, transporters, G-protein coupled receptors, nuclear hormone receptors, and others; and importantly, no significant off-target effects were observed (Cerep profiling service, France; data not shown).

To confirm p38α MAPK-dependent biological activity, LY2228820 was tested in a series of cell-based assays (Table 1, Fig 2). LY2228820 potently inhibited phosphorylation of the p38α MAPK substrate, MK2 (Thr334), in anisomycin-stimulated mouse RAW264.7 macrophages (IC₅₀ = 35.3 ± 5.0 nM [n=4] by ELISA). In cervical carcinoma (HeLa) cells, phospho-Thr334-MK2 was inhibited by LY2228820 at 9.8 nM and completely ablated by 156 nM; no changes in phosphorylation of p38α MAPK, JNK, ERK1/2, c-Jun, ATF2 or cMyc were observed at concentrations up to 10 μM (Fig 2). LY2228820 was active in cell-based functional assays, blocking TNFα secretion by LPS/IFNγ-stimulated mouse peritoneal macrophages (IC₅₀ = 6.3 ± 2.4 nM [n=4]) and LPS-induced CXCL8 secretion by non-small cell lung cancer (A549) cells in vitro (IC₅₀ = 144.9 ± 51.8 nM [n=3]).

LY2228820 was screened for cytotoxicity across > 50 cancer cell lines using both standard monolayer and soft agar culture conditions. Despite evidence of p38 MAPK inhibition and consequent changes in cellular cytokines cited above, single agent anti-proliferative effects were not observed across the cell lines and EC₅₀ values were greater than 2 µM in all cases (data not shown).

**In Vivo Tumor Target Inhibition:** LY2228820 was orally bioavailable in the mouse, with a T1/2 = 2.8 hours (single oral dose of 20 mg/kg). In mice implanted with B16-F10 melanoma, tumor phospho-MK2 was effectively inhibited by LY2228820 in a dose-dependent manner (TED₅₀ = 1.95 mg/kg, TED₇₀ = 11.17 mg/kg, Fig 3a). Significant target inhibition (>40% inhibition of
phospho-MK2) was maintained for approx. 4-8 hours following a single 10 mg/kg oral dose (Fig 3b).

**In Vivo PD Biomarker:** To understand whether peripheral blood monocytes (PBMC) could be used as a potential surrogate biomarker for p38 MAPK inhibition, PBMC’s were collected from mice dosed orally with LY2228820 or from patients with multiple myeloma (treated with LY2228820 *ex vivo*). With either mouse or human PBMC, LY2228820 inhibited MK2 phosphorylation: mouse *in vivo* TED<sub>50</sub> = 1.01 mg/kg [compound exposure approx. 100 nM] and human *ex vivo* IC<sub>50</sub> = 0.12 µM (Suppl Fig 1a, b).

**In Vivo Efficacy:** Based on pharmacokinetic and pharmacodynamic data, subsequent *in vivo* subcutaneous xenograft studies were conducted at doses which were predicted to yield >50% target inhibition (ranging from 10-30 mg/kg p.o., either BID or TID). LY2228820 up to 30 mg/kg TID was well tolerated in all studies and no significant weight loss (<10%) or animal death attributable to drug treatment was observed.

LY2228820-treated A549 NSCLC xenografts demonstrated significant tumor growth inhibition under two different dosing schedules. LY2228820 (20 mg/kg TID) was administered for 34 days on a continuous daily schedule (days 4-38) or an intermittent schedule (3 day on, then 3 day off) starting on day 16. Both the continuous and intermittent treatment schedules resulted in significant tumor growth delay after three weeks of treatment compared to the vehicle control (p<0.05, days 27-38). Tumor growth was not significantly different on the two LY2228820 treatment schedules. A number of other solid tumor xenograft models demonstrated highly significant tumor growth inhibition when LY2228820 was administered orally BID or TID in a range from 10-30 mg/kg. A complete attenuation of tumor growth occurred in the U-87MG glioma model during the treatment period (days 11-28, p<0.001); the A-2780 ovarian, MDA-MB-468 breast and OPM-2 myeloma xenografts were inhibited 72% (p<0.01), 60% (p<0.001) and 42% (p<0.05), respectively, on the last day of study treatment (Fig 4). Importantly, shRNA knockdown of p38α MAPK led to significant U-87MG tumor growth delay, further substantiating a role for p38 MAPK in tumorigenesis (Suppl. Fig. 2). Additionally, LY2228820 dosed intermittently at 10 and 30 mg/kg reduced the number of lung
metastases in the B16-F10 syngeneic mouse melanoma model; metastases were significantly reduced by 57% at 30 mg/kg (p=0.012, Fig 4).

Several other cell-based and patient-derived subcutaneous xenograft models did not demonstrate tumor growth inhibition with LY2228820 treatment. The effect of LY2228820 was compared in two of these xenograft models with both flank and orthotopic implantation. Luciferase-expressing SK-OV-3 ovarian cell xenografts implanted into the peritoneal cavity were highly responsive to LY2228820, demonstrating not only 50-51% tumor growth inhibition with both 10 and 30 mg/kg TID intermittent dosing (measured by AUC for bioluminescence; p<0.05; Fig 5) but also lower total excised tumor weight and reduced ascites in a separate study with 10 mg/kg on a daily schedule. Subcutaneously implanted SK-OV-3 xenografts were unresponsive to LY2228820 on the same schedules (Fig. 5). Similarly, LY2228820-induced tumor growth inhibition was not observed in the subcutaneously implanted 786-O renal carcinoma xenograft employing a daily 30 mg/kg TID schedule (Fig 5).

However, luciferase-labeled 786-O cell xenografts orthotopically implanted into the renal capsule of the left kidney demonstrated a 44% decrease in luminescence after 3 weeks (p<0.05), and 35% reduced average excised tumor weight (p<0.05) when treated on the same schedule with the same dose (Fig. 5).

Discussion

Following an extensive medicinal chemistry effort to identify potent, selective and orally active inhibitors of p38 MAPK (38, 39), a series of tri-substituted imidazole derivatives were synthesized and tested. This effort resulted in the synthesis of LY2228820 and, for the first time, characterization of its crystal structure in complex with p38α MAPK. LY2228820 is a potent and selective ATP-competitive inhibitor of the α and β isoforms of p38 MAPK in vitro. In cell-based assays, LY2228820 potently and selectively inhibited p38α MAPK substrate phosphorylation (p-Thr334-MK2) with no effects on phosphorylation of p38α MAPK, JNK, ERK1/2, c-jun, ATF2 or cMyc. Consistent with established p38α MAPK signaling activity,
LY2228820 also potently reduced LPS-stimulated cytokine secretion by both macrophages and A549 non-small cell lung cancer cells.

LY2228820 did not demonstrate direct anti-proliferative activity in the tumor cell lines tested in vitro despite clear repression of downstream signaling (Fig 2 and data not shown) but did demonstrate anti-tumor activity in vivo. In tumor models, a tight pharmacokinetic (PK)/pharmacodynamic (PD) relationship between compound exposure, MK2 phosphorylation and in vivo anti-tumor activity was observed. The fact that tumor growth inhibition by LY2228820 was observed in some, but not all, xenograft models tested supports the concept that p38α MAPK activity is cell and/or context dependent. Responsive xenografts are derived from several different histologies, have a variety of commonly considered mutations such as p53, KRAS, PTEN, PIK3CA, CDKN2A and APC, and have cytokine profiles that range from little to no cytokine secretion (e.g. A2780) to high expressors of cytokines such as IL-6, CXCL8 and VEGF (e.g. U-87MG and 786-O). Yet both responsive and unresponsive subcutaneous xenograft tumors demonstrate p38 MAPK inhibition with LY2228820 treatment, as evidenced by reduced MK2 and HSP27 phosphorylation (data not shown).

Further support for the cell/context dependent influence of p38 MAPK on cancer development and tumor growth is the observation that unresponsive tumors in the subcutaneous environment are responsive in the orthotopic environment. These findings are most readily understood if the p38 MAPK mechanism of action is primarily derived through tumor interaction with the tumor micro-environment (TME). Recent data describing a role for p38 MAPK in angiogenesis supports this tumor-TME interaction. Using both an in vitro measure of cord formation as well as an in vivo vascular development model, Tate et al demonstrate that p38 MAPK inhibition by LY2228820 reduces angiogenesis both at the level of the stroma and at the level of the tumor cell itself (6). The effects of LY2228820 on VEGF, cytokine levels, and angiogenesis in vitro were phenocopied by p38α MAPK shRNA, but not p38β MAPK shRNA, confirming the p38α MAPK dependency (6). Similarly in the present studies, LY2228820-induced tumor growth delay in U-87MG tumor xenografts in vivo was phenocopied by p38α MAPK knock-down (Suppl. Fig. 2).
In addition to the preclinical data, there is compelling clinical evidence suggesting p38 MAPK activity contributes to, or drives, several cancers by modulating the release of soluble, tumor supportive factors. Amongst its many activities, p38α MAPK stabilizes the message of various cytokines including TNFα and CXCL8 and is required for IL-1 induction of IL-6 (2). Higher serum and alveolar lavage IL-6 and CXCL8 levels are associated with shorter lung cancer patient survival (40). In addition, p38α MAPK is highly activated in non-small cell lung cancer relative to normal lung tissue (41). CXCL8 is also higher in the serum and cystic fluid from ovarian cancer patients as compared to healthy patients or those with benign cysts (14) and increased CXCL8 expression correlated with more advanced stages of disease. In breast cancer, high levels of activated p38 MAPK have been correlated with invasive disease, tamoxifen resistance, and poor survival (42-45). Given these clinical observations, and the cumulative in vitro and in vivo preclinical data, it is possible that therapeutic inhibition of p38 MAPK could be effective clinically in reducing tumor growth, invasion, and metastasis.

In summary, LY2228820 is a potent and selective inhibitor of p38 MAPK with anti-tumor activity. Further studies are ongoing to determine the potential of this compound in other tumor types and in combination with standard-of-care agents to determine, for example, whether p38 MAPK may be involved in drug resistance to traditional cytotoxic therapy (44, 45, 46). While there are a multitude of data linking p38 MAPK to tumor growth and metastasis via microenvironment interactions, it is still unclear how these effects manifest themselves across tumor types. The differences observed in response to LY228820 in vivo suggest there is likely more than one mechanism by which p38 MAPK drives tumor growth. The preclinical activity profile, drug-like properties, and pharmacodynamic effect on p-MK2, provide a compelling rationale to explore the clinical utility of this molecule in human cancer (47).
Acknowledgements:

The authors wish to thank the following individuals for their excellent technical and/or managerial support: Li Fan, Lisa Green, Steve Hatch, Lysiane Huber, Blake Neubauer, Neal Roehm, Scott Watkins, and Jeff Wolos.

References:

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**Table 1. In vitro kinase and cell-based activity of LY2228820**

<table>
<thead>
<tr>
<th>Kinase Enzyme</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (nM) +/- SEM</th>
<th>Cell-based Assay</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (nM) +/- SEM</th>
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<tr>
<td>p38α MAPK</td>
<td>5.3 ± 1.6</td>
<td>Anisomycin-stimulated MK2</td>
<td>35.3 ± 5.0</td>
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<td>p38β MAPK</td>
<td>3.2 ± 0.3</td>
<td>phosphorylation in RAW264.7 cells</td>
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<td>p38δ MAPK</td>
<td>&gt;20,000</td>
<td>LPS/INFγ-stimulated TNFα production</td>
<td>6.3 ± 2.4</td>
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<td>p38γ MAPK</td>
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<td>ERK1</td>
<td>&gt;20,000</td>
<td>LPS-induced CXCL8 production</td>
<td>144.9 ± 51.8</td>
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<tr>
<td>ERK2</td>
<td>&gt;20,000</td>
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<td>JNK1</td>
<td>894 ± 43</td>
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<td>JNK2</td>
<td>80 ± 11</td>
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<td>JNK3</td>
<td>158 ± 21</td>
<td>LPS-induced CXCL8 production</td>
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</table>

Note: LY2228820 was >50-fold selective vs. CK1δ, MKK6, MKK7β, EGFR[L858R], and >500-fold selective vs. EGFR.

Kinase assays were conducted in either 96-well filter binding (³³P-ATP phospho-cellulose or glass fiber) or fluorescence polarization formats under linear velocity conditions, at or below the Km[ATP] (30-120 minutes with < 10% ATP conversion) using 1-4% DMSO final. IC50 values were calculated from 10-point concentration-response curves (1:3 serial dilutions from 20 µM to 1 nM). RAW 264.7 cells (ATCC) were treated with compound for 2 hours at 37°C/5% CO2. Anisomycin (10 µg/mL) was added to the media to activate p38. After 30 minutes incubation, cells were fixed and phospho-MK2 was quantified (Thr 334, Cell Signaling Technologies). Mouse peritoneal macrophages were activated *in vivo* by i.p. thioglycollate injection, harvested and plated. Cells were treated with LY2228820 for 0.5 hours, incubated with LPS/INFγ for 2 hours and the media measured for TNFα by ELISA (R&D Systems). A549 cells (3x 10⁴) were seeded into 24-well tissue culture dishes in RPMI media/10% FBS (Invitrogen). 24 hours later, cells were pre-treated for 30 minutes with DMSO or 1 µM LY2228820 prior to the addition of 100 ng/mL LPS (Millipore). Conditioned media was collected 72 hours post-LPS treatment and total viable cells were counted. Samples were analyzed for CXCL8 secretion with Quantikine® Colorimetric Sandwich ELISAs (R&D Systems).
Figure legends

Figure 1.

A. Depiction of SAR progression from initial actives (1) to candidate molecule, LY2228820 (2). The candidate compound 2 was identified from iterative SAR on hits found in the Lilly compound collection, as represented by the generic structure 1. Molecules were designed to optimize binding to the ATP pocket of p38α MAPK, reduce the potential of off-target cross-reactivity and minimize CYP P450 activity. B. Crystal structure of LY2228820 in the ATP-binding site of p38α MAPK. The amino aza-benzimidazole moiety in LY2228820 forms two hydrogen bonds with the hinge backbone. The fluoro-phenyl group projected from the center imidazole is buried in a hydrophobic pocket near the gate-keeper residue Thr106, which has close van der waals contact between its side chain and the phenyl ring. The fluoro group has Bürgi-Dunitz interaction with two backbone carbonyls and also has close interaction with the side chain of Leu104. Compared to the binding conformation of inhibitors like SB203580, LY2228820 has a more extended warhead group and hence shifted hinge position. Though the fluoro-phenyl group is similarly bound, the lower position of the P-loop in the LY2228820 structure creates a smaller cavity (closer distance) between the N-terminal lobe and the C-terminal lobe.

Figure 2. Effect of LY2228820 on phosphorylation of various MAP kinase substrates in HeLa cells in vitro. HeLa cells (ATCC) were pretreated with LY2228820 (10uM -> 9.8nM, 1:2 serial dilutions) for 1 hour before stimulation with anisomycin (10 μg/mL) for 45 minutes. Cells were then lysed in a protein lysis buffer (containing protease and phosphatase inhibitors) and proteins were analyzed by Western blotting for p38, JNK, and p44/42 (ERK1/2) pathway activities. The first two lanes represent HeLa lysates with and without Anisomycin treatment (in absence of p38 inhibitor) serve as controls.
Figure 3. **A) Dose-response effect of LY2228820 on phosphorylation of MAPKAP-K2 (p-MK2) in mouse B16-F10 melanoma tumors.** Murine B16-F10 melanoma cells were implanted in the rear flank of C57BL/6 mice; when tumors reached approx. 200 mm, animals were dosed orally with vehicle or LY2228820 (0.1, 0.3, 1, 3, 10 and 30 mg/kg). Two hours post-dose, tumors were excised, homogenized and lysed for Western Blot. MK2 phosphorylation (p-Thr334) was quantified by chemiluminescent detection. Symbols represent % inhibition of p-MK2 in tumors of individual animals (n = 3 independent experiments). The 50% or 70% threshold effective dose (TED<sub>50</sub> and TED<sub>70</sub>, respectively) ± Standard Error of the Mean (SEM) were calculated to approximate effective dose ranges for testing of LY2228820 in xenograft models. **B) Time course effect of LY2228820 on phosphorylation of MK2 (phospho-MK2) in mouse B16-F10 melanoma tumors.** Time-course response to a single 10 mg/kg p.o. dose of LY2228820 in the mouse B16-F10 tumor model (n=6 per group); tumor p-MK2 (open circles) and plasma compound concentration (closed circles) were assessed at various time points over a 24 hour period.

Figure 4. **Effect of LY2228820 in solid tumor xenograft models in vivo.**

**A549 NSCLC xenograft:** Nude mice were treated orally with vehicle (open symbols) or LY2228820 (closed symbols) at 20 mg/kg TID from days 4-13. Starting day 14, one LY2228820-treated group continued with the daily dosing regimen (● “Daily”) while a second group switched to an intermittent 3 days on/3 days off drug treatment schedule (▲ “Intermittent”). Comparable vehicle control groups (o, △) were run in parallel. N = 10 animals/group. A significant decrease in tumor volume occurred with both continuous and intermittent dosing strategies; efficacy of LY2228820 was not significantly different on the two schedules.

**A2780 ovarian xenograft:** Nude mice bearing xenografts began treatment 15 days after implantation. Vehicle (o) or LY2228820 (●) 10 mg/kg was given orally, TID, on a schedule of 4 days on/ 3 days off for 3 weeks. N=10 animals per group. Significant tumor growth inhibition occurred throughout the treatment phase.
U-87MG Glioma xenograft model: Nude mice were treated orally with vehicle (o) or LY2228820 (●) BID at 14.7 mg/kg continuously on days 11-28, N = 10 animals/group. Significant tumor growth inhibition was observed on days 21-35.

OPM-2 Myeloma xenograft: Nude mice were treated orally with vehicle (o) or LY2228820 (●) BID at 30 mg/kg continuously, N = 10 and 9 animals/group for vehicle and LY2228820 respectively. Significant tumor growth inhibition was observed with LY2228820 after 10 days of treatment and throughout the remainder of the study.

MDA-MB-468 xenograft: Subcutaneous MDA-MB-468 breast cancer xenografts were implanted into nude mice. Vehicle (o) or LY2228820 (●) was administered orally, 30 mg/kg BID (n=7 per group). LY2228820 treatment resulted in significant tumor growth inhibition.

B16-F10 melanoma syngeneic mouse model: Cells were injected into the tail vein of nude mice one day prior to compound treatment. Vehicle or LY2228820 was administered orally at 10 or 30 mg/kg on a 4 days on/3 days off schedule for 14 days (n=10 animals per group). All animals were examined for lung metastases at the end of study. A significant reduction in lung mets was observed following treatment with 30 mg/kg LY2228820.

Statistics: *p< 0.05, **p<0.01, ***p<0.001 by ANOVA.

Figure 5. Effect of LY2228820 in flank vs. orthotopic tumor xenograft models.

SK-OV-3 ovarian xenograft models: Luciferase-expressing SK-OV-3 cells were implanted either subcutaneously in the flank or intraperitoneally as an orthotopic model. Both were treated orally with vehicle (o) or LY2228820 at 30 mg/kg (●) or 10 mg/kg (▲) TID on a 3 day on/ 3 day off schedule for 3 weeks. Subcutaneous xenografts did not demonstrate tumor growth inhibition with LY2228820; orthotopic xenografts demonstrated significant tumor growth inhibition at both doses of LY2228820.

786-O renal carcinoma xenograft models: 786-O cells were implanted subcutaneously in the flank; luciferase-expressing 786-O were implanted into the renal capsule of the left kidney as an orthotopic model. Both were treated orally with vehicle (o) or LY2228820 (●) at 30mg/kg
TID on a continual schedule for 3 weeks. Subcutaneous 786-O xenografts did not demonstrate tumor growth inhibition with LY2228820; orthotopic 786-O xenografts treated with LY2228820 demonstrated significant tumor growth inhibition on the final day of measurement.
Figure 1

A.

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**Figure 2**

Phosphorylation of various protein kinases in response to Anisomycin treatment with different concentrations of LY2288820 (nM).
Figure 3

A

% inhibition of p-MK2/GAPDH

Log dose of LY2228820 (mg/kg)

TED_{50} = 1.95

TED_{70} = 11.2

B

LY2228820, ng/ml (mouse)

% Inhibition (tumor p-MK2)

Time (Hours)
Figure 4

**A549**

- Vehicle (daily)
- Vehicle (intermittent)
- LY2228820, 20mg/kg (intermittent)
- LY2228820, 20mg/kg (daily)

**B16-F10**

- Vehicle
- 10mg/kg
- 30mg/kg

(p=0.012)

**A2780**

- Vehicle
- LY2228820

**U87MG**

- Vehicle
- LY2228820

**MDA-MB-468**

- Vehicle
- LY2228820

**OPM-2**

- Vehicle
- LY2228820
Characterization of LY2228820 dimesylate, a potent and selective inhibitor of p38 MAPK with anti-tumor activity


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