Characterization of LY2228820 Dimesylate, a Potent and Selective Inhibitor of p38 MAPK with Antitumor Activity

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Abstract

p38α mitogen-activated protein kinase (MAPK; α, β, δ, and γ isoforms) is a member of the MAPK family, which also includes JNK and ERK (1). The α isoform of p38α, known as p38α MAPK or MAPK14, is activated in response to environmental factors such as lipopolysaccharide (LPS), cytokines, heat/osmotic shock, 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 TNF-α, interleukin-1β (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 trisubstituted imidazole derivative, is a potent and selective, ATP-competitive inhibitor of the α- and β-isoforms of p38 MAPK in vitro (IC50 = 5.3 and 3.2 nmol/L, respectively). In cell-based assays, LY2228820 potently and selectively inhibited phosphorylation of MK2 (Thr334) in anisomycin-stimulated HeLa cells (at 9.8 nmol/L by Western blot analysis) and anisomycin-induced mouse RAW264.7 macrophages (IC50 = 35.3 nmol/L) with no changes in phosphorylation of p38α MAPK, JNK, ERK1/2, c-Jun, ATF2, or c-Myc ≤ 10 nmol/L. LY2228820 also reduced TNF-α secretion by lipopolysaccharide/IFN-γ-stimulated macrophages (IC50 = 6.3 nmol/L). In mice transplanted with B16-F10 melanoma, tumor phospho-MK2 (p-MK2) was inhibited by LY2228820 in a dose-dependent manner [threshold effective dose (TED)70 = 11.2 mg/kg]. Significant target inhibition (>40% reduction in p-MK2) was maintained for 4 to 8 hours following a single 10 mg/kg oral dose. LY2228820 produced significant tumor growth delay in multiple in vivo cancer models (melanoma, non–small cell lung cancer, 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. Mol Cancer Ther; 13(2); 364–74. ©2013 AACR.

Introduction

p38α mitogen-activated protein kinase (MAPK; α, β, δ, and γ isoforms) is a member of the MAPK family, which also includes JNK and ERK (1). The α isoform of p38α, 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 chemotherapy (2). Activation is accomplished by phosphorylation of the Thr180 and Tyr182 residues by chemotherapy (2).

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and proangiogenic 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 that support both direct (tumor-mediated) and indirect (via TME) modes of action for p38 MAPK. It has been implicated in 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 TNF-α 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 VEGF, basic fibroblast growth factor (bFGF), 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 short hairpin RNA (shRNA) reduces cord formation in a tumor/adipocyte-derived stem/endothelial colony-forming cell coculture 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 that likely plays a profound role in tumor-host dynamics. In multiple myeloma, p38 MAPK inhibitors downregulate IL-6 secretion by bone marrow stromal cells (BMSC), 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 by BMSCs in vitro (28). In response to neutrophil elastase, lung cancer cells produce CXCL8, which stimulates the growth of lung cancers 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, 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.

Materials and Methods

Crystallography

For crystallographic studies, human p38α was expressed in Escherichia coli and purified by affinity and gel filtration chromatography. Purified protein was in a solution containing 150 mmol/L NaCl, 20 mmol/L Hepes pH 7.5, 10 mmol/L methionine, 5 mmol/L dithiothreitol, and 10% glycerol. Protein at 16.2 mg/mL was mixed in 1% BSA and 1.25 mmol/L 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 P2₁2₁2₁ with unit cell parameters a = 66.55 Å, b = 74.45 Å, c = 78.69 Å. The diffraction data (resolution of 1.97 Å) were collected on beam line ID-31 (then SGC-CAT) at the advanced photon source (APS; 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 CNS2000 first and then using Refmac5 (Rwork = 0.2303, Rfree = 0.2666).

Kinase assays

For p38-MAPK enzymatic assays (enzyme sourced from either Millipore or Roche), 32P-ATP radiometric filter binding (Millipore MAPH plates) was used with an EGFR peptide substrate, KRELVEPTSPCEANQALLR (Multiple Peptide Systems), and 100 μmol/L (p38α) or 20 μmol/L (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; ref. 37; all human sequence) to assess the relative kinase selectivity in vitro. The initial screen was conducted at 20 μmol/L followed by 10-point concentration–response curves (1:3 serial dilutions from 20 μmol/L to 1 nmol/L). All assays were either 96-well radiometric filter binding (32P-ATP phospho-cellulose or glass fiber) or fluorescence polarization format under linear velocity conditions, at or below the Km[ATP] (typically 30–120 minutes resulting in ≤10% ATP conversion) using 1% to 4% dimethyl sulfoxide (DMSO) final (depending on enzyme tolerance for solvent). IC₅₀ values were calculated using 4-parameter nonlinear regression (ActivityBase software, IDBS).

Cell lines

All human cell lines were obtained from American Type Culture Collection (ATCC; A549, U-87MG, HeLa, and...
MK2 capture ELISA
RAW 264.7 cells were treated with compound (range of 20 μmol/L to 1 nmol/L, 10 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 capture ELISA (cELISA) using a phospho-MK2 (Thr 334; Cell Signaling Technology) antibody. Briefly, 5 μL of a 20 μg/mL concentration of anti-MK2 antibody (Cat. No. KAP-MA015; Stressgen) was placed into a 96-well high-binding MesoScale Discovery (MSD) plate and incubated at 4°C overnight. The plate was blocked for 1 hour at room temperature (RT), washed, and 25 μL of detection antibody, anti-p-MK2 (Cat. No. 3041; Cell Signaling, Inc.) conjugated to ruthenium, was added and incubated for 2 hours at RT. The plate was washed, 150 μL of 1× ReadT buffer was added per well and the plate read on an MSD Sector 6000 instrument.

LPS/IFN-γ-stimulated TNF-α release by mouse peritoneal macrophages in vitro
Mouse peritoneal macrophages were activated in vivo by 3% thioglycollate injection (intraperitoneal, administered 4 days before harvest), then harvested and plated in 96-well microtitrator plates. The cells were treated with LY2228820 (range of 20 μmol/L to 1 nmol/L, 10 1:3 serial dilutions) for 0.5 hours, and then incubated with LPS/IFN-γ for 2 hours (to stimulate p38-MAPK) and the media measured for TNF-α by ELISA (R&D Systems).

Cytokine analysis
A549 cells (3 × 10⁴) were seeded into 24-well tissue culture dishes in Roswell Park Memorial Institute (RPMI, Buffalo, NY) media/10% FBS (Invitrogen). 24 hours later, cells were pretreated for 30 minutes with 0.01% DMSO or LY2228820 dimesylate (range of 20 μmol/L to 1 nmol/L, 10 1:3 serial dilutions) before 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 analysis)
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 μg/mL, trypsine–chymotrypsin inhibitor 10 μg/mL, N-α-Tosyl-l-phenylalalanine chloromethyl ketone 10 μg/mL, aprotinin 10 μg/mL, N-α-Tosyl-l-arginine methyl ester hydrochloride 2 mmol/L, benzamidine hydrochloride hydrate 5 mmol/L) and phosphatase inhibitors (sodium meta-vanadate anhydrous 1 mmol/L, p-nitrophenyl phosphate 15 mmol/L, microcystin 1 μmol/L), okadaic acid 1 μmol/L; 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 (Thr172), phospho-JNK (Thr183/Tyr185), phosphoc-Jun (Ser63), phospho-p44/42 MAPK (Thr202/Tyr204), and phospho-c-Myc (Thr58/ser62).

Phosphorylation of MK2 in mouse B16-F10 melanoma tumors
In vivo target inhibition. Murine B16-F10 melanoma cells were cultured in Dulbecco’s Modified Eagle Medium 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 × 10⁶), and when tumors reached approximately 200 mm³ in size, were dosed orally with LY2228820 in 1% carboxymethylcellulose/0.25% Tween 80. Two hours postdose, tumors were excised, homogenized, and lysed for Western blot analysis. MK2 phosphorylation (p-Thr334), normalized to total glyceraldehyde-3-phosphate dehydrogenase, was quantified by chemiluminescent detection. The 50% or 70% threshold effective dose (TED50 and TED70, respectively) was calculated (JMP software, SAS) to approximate effective dose ranges for testing of LY2228820 in xenograft models, that is, 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 with 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 phospho-MK2 (p-MK2) over various time points.

In vivo phosphorylation of MK2 in mouse and human peripheral blood mononuclear cells
See Supplementary Methods (Supplementary Fig. S1).
shRNA knockdown of p38α MAPK in U-87MG glioma cells

See Supplementary Methods (Supplementary Fig. S2).

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 before xenograft implantation. Cells used in xenograft studies were pathogen tested and authenticated by STR analysis. Banked master stocks were returned to within approximately 6 months, or if inconsistencies in growth behavior were observed. Cells originated from the 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 × 10⁶ cells for U-87MG, SK-OV-3-luc#1 (M. Harrington and S. Brutkiewicz, Indiana University, Indianapolis, IN), 786-O, and 1 × 10⁷ cells for A549 were implanted into athymic nude mice (Harlan); 2 × 10⁶ for A-2780 (NCI DCTD) into CD1 nu/nu mice (Charles River); and 5 × 10⁶ for OPM-2 (DSMZ GmBH) 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. Tumors were allowed to establish for at least 1 week before randomization into treatment groups; treatments began with tumors of 50 to 250 mm³. 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, v/v). 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 to 2 times per week. Tumor size was determined by caliper and tumor volume (mm³) was estimated using the formula: V = l × w² × 0.536, where l is the larger and w is the smaller of the perpendicular diameters. Tumor data were 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 (20–25 g; Harlan) received intraperitoneal injections (2 × 10⁶ cells in 0.2 mL PBS) of SK-OV-3 luciferase-labeled tumor cells or luciferase-
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 LY2228820 (Fig. 1), was found to be a very potent, ATP-competitive inhibitor of both the α and β isofoms of p38 MAPK in vitro (IC50 = 5.3 and 3.2 nmol/L, respectively) with >1,000-fold selectivity for p38α MAPK versus 178 other kinases tested (Table 1 and Supplementary Table S1). Within the MAPK family, LY2228820 was >1,000-fold more selective for p38α versus p38β, 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 p38β.

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

<table>
<thead>
<tr>
<th>Kinase enzyme</th>
<th>IC50 (nmol/L) ± SEM</th>
<th>Cell-based assay</th>
<th>IC50 (nmol/L) ± SEM</th>
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<tbody>
<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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<tr>
<td>p38β MAPK</td>
<td>3.2 ± 0.3</td>
<td>phosphorylation in RAW264.7 cells</td>
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<tr>
<td>p38γ MAPK</td>
<td>&gt;20,000</td>
<td>LPS/IFN-γ-stimulated TNF-α production by mouse peritoneal macrophages</td>
<td>6.3 ± 2.4</td>
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<tr>
<td>ERK1</td>
<td>&gt;20,000</td>
<td>LPS-induced CXCL8 production by A549 cells</td>
<td>144.9 ± 51.8</td>
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<tr>
<td>ERK2</td>
<td>&gt;20,000</td>
<td>by A549 cells</td>
<td></td>
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<tr>
<td>JNK1</td>
<td>894 ± 43</td>
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<tr>
<td>JNK2</td>
<td>80 ± 11</td>
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<tr>
<td>JNK3</td>
<td>158 ± 21</td>
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</table>

NOTE: LY2228820 was >50-fold selective vs. CK1β, MKK6, MKK7β, EGFR(L858R), and >500-fold selective vs. EGFR.

Kinase assays were conducted in either 96-well filter binding (32P-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% to 4% DMSO final. IC50 values were calculated from 10-point concentration–response curves (1:3 serial dilutions from 20 μmol/L to 1 nmol/L). 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 intraperitoneal thioglycollate injection, harvested, and plated. Cells were treated with LY2228820 for 0.5 hours, incubated with LPS/IFN-γ for 2 hours, and the media measured for TNF-α by ELISA (R&D Systems). A549 cells (3 × 104) were seeded into 24-well tissue culture dishes in RPMI media/10% FBS (Invitrogen). Twenty-four hours later, cells were pretreated for 30 minutes with DMSO or 1 μmol/L LY2228820 before 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).

Mouse B16-F10 melanoma lung metastasis model

B16-F10 melanoma cells (50,000) were injected into the tail vein of nude mice 1 day before treatment. Mice were orally dosed by gavage with vehicle or LY2228820 (either 10 or 30 mg/kg) 3 times a day on a 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 LY2228820 (Fig. 1), was found to be a very potent, ATP-competitive inhibitor of both the α and β isofoms of p38 MAPK in vitro (IC50 = 5.3 and 3.2 nmol/L, respectively) with >1,000-fold selectivity for p38α MAPK versus 178 other kinases tested (Table 1 and Supplementary Table S1). Within the MAPK family, LY2228820 was >1,000-fold more selective for p38α versus p38β, 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 p38β.

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 isofoms of p38 MAPK in vitro (IC50 = 5.3 and 3.2 nmol/L, respectively) with >1,000-fold selectivity for p38α MAPK versus 178 other kinases tested (Table 1 and Supplementary Table S1). Within the MAPK family, LY2228820 was >1,000-fold more selective for p38α versus p38β, 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 p38β.
more selective for p38α versus JNK2. In mode of action experiments, LY2228820 was ATP competitive with a $K_a$ (apparent) = 4.5 ± 0.5 nmol/L (radiometric filter binding assay) and with tight-binding kinetics ($k_{on} = 0.0025 ± 0.0005$ s$^{-1}$, $k_{off} = 1.22 × 10^6 ± 0.08$ M$^{-1}$s$^{-1}$; by surface plasmon resonance; data not shown). LY2228820 was also tested in a wide array of assays to assess any off-target effects on nonkinase 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; data not shown). To confirm p38α MAPK–dependent biologic activity, LY2228820 was tested in a series of cell-based assays (Table 1 and Fig. 2). LY2228820 potently inhibited phosphorylation of the p38α MAPK substrate, MK2 (Thr334), in anisomycin-stimulated mouse RAW264.7 macrophages [IC$_{50}$ = 35.3 ± 5.0 nmol/L (n = 4) by ELISA]. In cervical carcinoma (HeLa) cells, phospho-Thr334-MK2 was inhibited by LY2228820 at 9.8 nmol/L and completely ablated by 156 nmol/L; no changes in phosphorylation of p38α MAPK, JNK, ERK1/2, c-Jun, ATF2, or cMyc were observed at concentrations up to 10 μmol/L (Fig. 2). LY2228820 was active in cell-based functional assays, blocking TNF-α secretion by LPS/IFN-γ–stimulated mouse peritoneal macrophages [IC$_{50}$ = 6.3 ± 2.4 nmol/L (n = 4)] and LPS-induced CXCL8 secretion by non–small cell lung cancer (NSCLC; A549) cells in vitro [IC$_{50}$ = 144.9 ± 51.8 nmol/L (n = 3)].

**Figure 2.** Effect of LY2228820 on phosphorylation of various MAP kinase substrates in HeLa cells in vitro. HeLa cells (ATCC) were pretreated with LY2228820 (10 μmol/L = 9.8 nmol/L; 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 2 lanes represent HeLa lysates with and without anisomycin treatment (in absence of p38 inhibitor) serve as controls.

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 antiproliferative effects were not observed across the cell lines and EC$_{50}$ values were greater than 2 μmol/L in all cases (data not shown).

**In vivo tumor target inhibition**

LY2228820 was orally bioavailable in the mouse, with a $T_{1/2} = 2.8$ 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 (TED$_{50}$ = 1.95 mg/kg; TED$_{70}$ = 11.2 mg/kg; Fig. 3A). Significant target inhibition (>40% inhibition of phospho-MK2) was maintained for approximately 4 to 8 hours following a single 10 mg/kg oral dose (Fig. 3B).
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 3 times a day from days 4 to 13. Starting day 14, one LY2228820-treated group continued with the daily dosing regimen (● “Daily”) whereas a second group switched to an intermittent 3 days on/3 days off drug treatment schedule (△ “Intermittent”). Comparable vehicle control groups (○, △) 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 2 schedules. A2780 ovarian xenograft: nude mice bearing xenografts began treatment 15 days after implantation. Vehicle (○) or LY2228820 (●) 10 mg/kg was given orally, 3 times a day, 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 (○) or LY2228820 (●) twice a day at 14.7 mg/kg continuously on days 11 to 28; n = 10 animals/group. (Continued on the following page.)
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% to 51% tumor growth inhibition with both 10 and 30 mg/kg 3 times a day intermittent dosing (measured by area under the curve 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 3 times a day 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 trisubstituted 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 p38\( \alpha \) MAPK. LY2228820 is a potent and selective ATP-competitive inhibitor of the \( \alpha \) and \( \beta \) isoforms of p38 MAPK in vitro. In cell-based assays, LY2228820 potently and selectively inhibited p38\( \alpha \) MAPK substrate phosphorylation (p-Thr334-MK2) with no effects on phosphorylation of p38\( \alpha \) MAPK, JNK, ERK1/2, c-Jun, ATF2, or cMyc. Consistent with established p38\( \alpha \) MAPK signaling activity, LY2228820 also potently reduced LPS-stimulated cytokine secretion by both macrophages and A549 NSCLC cells.

LY2228820 did not demonstrate direct antiproliferative activity in the tumor cell lines tested in vitro despite clear repression of downstream signaling (Fig. 2 and data not shown) but did demonstrate antitumor activity in vivo. In tumor models, a tight pharmacokinetic/
pharmacodynamic relationship between compound exposure, MK2 phosphorylation, and in vivo antitumor 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 TME. Recent data describing a role for p38 MAPK in angiogenesis support this tumor–TME interaction. Using both an in vitro measure of cord formation as well as an in vivo vascular development model, Tate and colleagues 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 this study, LY2228820-induced tumor growth delay in U-87MG tumor xenografts in vivo was phenocopied by p38α MAPK knockdown (Supplementary Fig. S2).
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. Among its many activities, p38α MAPK stabilizes the message of various cytokines including TNF-α 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 survival of patient with shorter lung cancer (40). In addition, p38α MAPK is highly activated in NSCLC relative to normal lung tissue (41). CXCL8 is also higher in the serum and cystic fluid from patients with ovarian cancer as compared with 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, LY2288280 is a potent and selective inhibitor of p38 MAPK with antitumor 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). Although 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 LY2288280 in vitro 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).

Disclosure of Potential Conflicts of Interest
E.M. Chan has ownership interest (including patents) in Eli Lilly and Company. J.R. Graff has ownership interest (including patents) in Eli Lilly and Company. No potential conflicts of interest were disclosed by the other authors.

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