Complete Inhibition of Anisomycin and UV Radiation but Not Cytokine Induced JNK and p38 Activation by an Aryl-substituted Dihydropyrrolopyrazole Quinoline and Mixed Lineage Kinase 7 Small Interfering RNA*\[S\]

Mixed lineage kinase 7 (MLK7) is a mitogen-activated protein kinase kinase kinase (MAP3K) that activates the pro-apoptotic signaling pathways p38 and JNK. A library of potential kinase inhibitors was screened, and a series of dihydropyrrolopyrazole quinolines was identified as highly potent inhibitors of MLK7 in vitro catalytic activity. Of this series, an aryl-substituted dihydropyrrolopyrazole quinoline (DHP-2) demonstrated an IC_{50} of 70 nM for inhibition of pJNK formation in COS-7 cell MLK7/JNK co-transfection assays. In stimulated cells, DHP-2 at 200 nM or MLK7 small interfering RNA completely blocked anisomycin and UV induced but had no effect on interleukin-1β or tumor necrosis factor-α-induced p38 and JNK activation. Additionally, the compound blocked anisomycin and UV-induced apoptosis in COS-7 cells. Heart tissue homogenates from MLK7 transgenic mice treated with DHP-2 at 30 mg/kg had reduced JNK and p38 activation with no apparent effect on ERK activation, demonstrating that this compound can be used to block MLK7-driven MAPK pathway activation in vivo. Taken together, these data demonstrate that MLK7 is the MAPKKK required for modulation of the stress-activated MAPKs downstream of anisomycin and UV stimulation and that DHP-2 can be used to block MLK7 pathway activation in cells as well as in vivo.

The mitogen-activated protein kinases (MAPKs)\[1\] are a highly conserved family of signal transduction molecules that transmits extracellular signals from the membrane to the nucleus. There are three major branches of MAPK signaling that include ERK, c-Jun N-terminal kinase (JNK) and p38. The JNK and p38 branches of the MAPK family are activated by stress stimuli including cytokines, osmotic stress, mitogens, UV irradiation, chemotherapeutic agents, and anisomycin (1, 2). There are three kinases that form a MAPK signaling module where a MAPK is activated by a MAPK kinase (MAPKK), which in turn is regulated by a MAPKK kinase (MAPKKK) (Fig. 1). The upstream activation of JNK and p38 is complex, allowing for activation of this pathway in multiple cells and by multiple stimuli. Cellular and receptor specificity of the pathway is conferred by protein-protein interactions where the MAPK and a MAPKKK assemble with a specific MAPKKK on scaffold proteins such as JNK-interacting protein (3, 4) or β-arrestin (5). The resulting signaling module acts as a bridge joining the appropriate receptor to the downstream effectors, enabling activation of the stress-activated MAPK.

The most distal point at which signal and cell specificity for JNK and p38 activation is conferred is at the MAPKKK level. Mixed lineage kinases (MLKs) are a family of MAPKKKs activating JNK and p38. There are currently seven mammalian kinases belonging to the MLK family that have recently been reviewed (6, 7). These kinases can be divided into three subclasses based on sequence similarity and domain structure, and they include MLK1--4, dual leucine zipper kinases, and the zipper sterile α-motif kinases (ZAKs). Although much is known about the mechanisms regulating MLK interaction with downstream MAPK and MAPK substrates, specific stimuli that activate MLKs and the physiologic roles of distinct MLKs are not as well characterized. Within the MLK family of enzymes, a physiological role for MLK3 has been the most extensively studied. Overexpression studies with a dominant/negative MLK3 inhibitor and gene-silencing studies with MLK3 small interfering RNA (siRNA) suggest a role for this kinase, respectively, in transforming growth factor-β-induced apoptosis (8) and cytokine- and mitogen-induced JNK and p38 activation (9). A role for the MLKs from subclass 1 (MLK1, 2, and 3) in neuronal cell death has been suggested through the use of overexpression and dominant/negative inhibitor mutants in neuronal cell culture (6, 7, 10). This role for MLKs has been further substantiated through the use of CEP-1347, a potent small molecule inhibitor of MLK1--3 (11, 12). This inhibitor has been shown to protect against JNK-induced apoptosis in cellular models of neuronal apoptosis and animal models of Parkinson disease (13, 14).

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\[1\] The abbreviations used are: MAPK, mitogen-activated protein kinase; MAPKKK, mitogen-activated protein kinase kinase kinase; MAPKK, mitogen-activated protein kinase kinase; IL, interleukin; ERK, extracellular signal-regulated kinase; TNF, tumor necrosis factor; siRNA, small interfering RNA; JNK, c-Jun N-terminal kinase; MLK, mixed lineage kinase; MBP, myelin basic protein; PBS, phosphate-buffered saline; PBST, PBS + Tween 20; ZAK, zipper sterile α-motif kinase; PBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; ELISA, enzyme-linked immunosorbent assay; Tg, transgenic; DHP, dihydropyrrolopyrazole quinone; GST, glutathione S-transferase; MRK, mixed lineage kinase-related kinase; MTLK, MLK-like MAPKKK; MAPKAP K2, MAPK-activated protein kinase 2.
MLK7 (also called MRK-β and MLTK-β) (15–17) is a recently described member of the subclass 3 of MLKs (ZAKs) that has enriched expression in skeletal muscle and heart tissue. MLK7 is a splice variant of the first identified member of this class, a leucine zipper, and ZAK (also called MRK-α or MLTK-α) (16–18). Studies to define the activating stimuli and physiological functions for MLK7 utilized overexpression of wild type and dominant/negative mutants as well as siRNA to demonstrate MLK7 activation by hyperosmotic stress (16) and by ionizing radiation where it may play a role in G2 phase checkpoint control-induced cell cycle arrest stimulated by ionizing radiation (17, 19–21). Another study using ectopic expression in JB6C11 skin epidermal cells suggests a role for the enzyme in neoplastic transformation (8). We have previously shown that transgenic mice with cardiac-restricted overexpression of MLK7 (MLK7 Tg) have increased p38 pathway activation and characteristics of a cardiac compensatory phenotype. When exposed to chronic β-adrenergic stimulation, the Tg mice rapidly deteriorated and demonstrated increased mortality compared with wild type controls. Simultaneous activation of JNK and p38 in heart tissue from the MLK7 Tg mice suggested that stress associated with adrenergic stimulation increased the activity of MLK7 and may contribute to cardiac decompensation during periods of acute cardiac stress (22).

To further define the requirement of MLK7 for MAPK activation in the presence of cellular stresses and identify potential physiological roles for the enzyme, we identified small molecule inhibitors of MLK7 catalytic function. The study described here reports on the discovery of an aryl-substituted dihydropyrrrol-opyrazole quinoline (DHP-2) as a potent inhibitor of MLK7 catalytic function. This compound, along with gene silencing utilizing MLK7 siRNA analysis, demonstrates that MLK7 is the MAPKKK responsible for JNK and p38 pathway activation downstream of anisomycin and UV stimulation. However, the inhibitory effects were not obtained when the pathway was activated by either TNF-α or IL-1β. Additionally, MLK7 inhibition with DHP-2 protected cells from apoptotic cell death induced by these stimuli. Furthermore, treatment of MLK7 transgenic mice resulted in significant reduction of p38 and JNK pathway activation in heart tissue, suggesting that this compound will be a useful tool to further define the physiological role of MLK7.

EXPERIMENTAL PROCEDURES

Compounds and Reagents—The DHP compounds were prepared as reported by Sawyer et al. (23) and Toth and co-workers (24) with the key synthetic intermediate being DHP bromide (7-bromo-2-(dihydro-4H-pyrrolo[1,2-b]pyrazol-3-yl)-quinoline). The DHP bromide was reacted with various aryl boronic acids in dioxane and 2 M aqueous K2CO3 in the presence of catalytic Pd(dba)3 (tris(dibenzylideneacetone)dipalladium(0)) and triphenylphosphine to provide the DHP products. Alternatively, the DHP bromide was converted to its corresponding boronic acid and reacted with commercially available aryl boronides under the same conditions of palladium catalysis. Complete synthetic details and reaction scheme are found under Supplemental Data. Products were purified by high pressure liquid chromatography.

Protein Expression and Purification—An N-terminal glutathione S-transferase (GST)-tagged MLK7 was expressed and purified from Sf9 insect cells as described previously (25). For MLK2 expression and purification, the cDNA corresponding to the catalytic domain (amino acids 1–444) was amplified from Human Brain Marathon Ready cDNA Library (Clontech) using the primers P1 (5′-CTGTTGTAAGACGGCTCC- CGCGAGCC-3′) and P2 (5′-CCTCTCCTGCTCTGCTGACATG-3′) and the nested primer P3 (5′-GAGGGCGCGCGACATGAGGAG- GAGGGG-3′). The PCR fragment was cloned into pCRSCRIPT SK−, and the resulting plasmid was digested with NcoI/NotI, the 1357-bp fragment purified and cloned into pACCHLT-B baculoviral transfer vector (BD Biosciences) that was modified using two synthetic linkers (5′-CATGGGACCTAAAGCGCGCAGCCGGCTCATTATAA-3′ and 5′-GATCTATTAGTGAGCGGCGCGCGCGCGTTAATGTC-3′). The resulting plasmid coded for an N-terminal GST-tagged MLK2 from residue 1–444 with nine additional C-terminal residues (GLERPHGSH) followed by two stop codons. The transfer vector encoding GST-MLK2(1–444) was co-transfected with Bacculovirus DNA (BD Biosciences) into SF9 cells using Lipofectin reagent (Invitrogen) to form recombinant baculovirus. Purification of the overexpressed GST-MLK2 was similar to that described for MLK7.

In Vitro Kinase Activity Measurement—MLK7 and MLK2 kinase activities were measured using a filter binding assay as described previously (25). Kinase inhibitory activity of compounds was evaluated from 10-point dose response curves (0.001–20 μM), single well for each dose. Data were analyzed using a 4-parameter sigmoidal dose response equation and IC50 values calculated from the resulting curve. For inhibitor modality analysis, a pentahistidine-tagged version of MLK7 (50 nM) (15) was incubated in a reaction mixture containing DHP-8 (0.001–20 μM), 5 μM myelin basic protein (MBP), 4 mM dithiothreitol, 5 mM MgCl2, 0.5 μg/ml of γ-32P[ATP] and cold ATP (3.75–120 μM ATP). Incubation was carried out for 2 h at room temperature, stopped with 10% acetic acid, transferred to a Millipore MAPHN0B50 filter, and aspirated. The filter plates were washed with 0.5% H2PO4 three times and counted on a Packard Top Count. Data were analyzed using double-reciprocal plots of velocity expressed as picomole phosphate/minute versus ATP concentration. K50 was determined from a plot of the slopes obtained from the double reciprocal plots at each concentration of DHP-8.

MLK7 Inhibitor Activity Assay—MLK7 Cell-based Assay—The MLK7 inhibitory activity of compounds in cells was determined using a co-transfection system of MLK7 with JNK. Transfection of cells was described previously with the exception that transfections were carried out in 96-well plates and medium was removed after 6 h of transfection and replaced with DMEM, 0.5% FBS (25). On the second day after transfection, medium was again aspirated and 100 μl of fresh medium (DMEM, 0.5% FBS) containing serial dilution of compound (0.0005–10 μM) were added, each dose in duplicate. Incubation was continued for 2 h at 37 °C, at which time medium was removed and pJNK levels in the cells were determined using phosphospecific JNK antibodies formatted either as the BioPlex pJNK assay kit (Bio-Rad) according to the manufacturer's protocol or as a cellular ELISA. For cellular ELISA, medium was removed and cells were fixed in PBS supplemented with 8% formaldehyde for 20 min followed by four washes with PBS supplemented with 0.1% Triton X-100, 5 min each wash with shaking. Plates were incubated with 0.6% H2O2 in PBS supplemented with 0.1% Tween 20 (PBST) for 20 min at room temperature with shaking followed by four washes in PBST. Blocking was performed at room temperature for 1.5 h with 10% FBS in PBST, and the plates were rinsed once with PBST and then incubated with 1:500 dilution of pJNK antibody (Cell Signaling) at 4 °C overnight. The following day, plates were washed four times with PBS, incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-rabbit IgG, and subsequently washed three times in PBST and twice with PBS, 5-min wash each with shaking. Antibody binding was detected using FEMTO Super Signal (Pierce), and luminescence was measured on a Dynex MCX microtiter plate luminometer. Data were acquired and IgG was determined using a four-parameter sigmoidal dose response equation. The variability of pJNK quantification assays was <10%, and a bridging study of 30 compounds taken through both methods of detection gave highly correlated IC50 values (r2 = 0.903 within the expected variability of the assays (minimum significant ratio = 3).

Cell Stimulation and siRNA—COS-7 cells were maintained in DMEM, 5% FBS. For siRNA transfections, DMEM (1.5 ml) was incubated with 60 μl of TransIT-TKO (Mirus, MIR2150) at room temperature for 8 min and then mixed with 37.5 μl of 40 μM MLK7 siRNA (5′-CCUCAGAAUCAACGUGUACdTdT-3′ and 5′-AACCUUUCUGGACUUGGTTd-3′) or nonspecific siRNA (Control X, Dharmacon) and incubation continued at room temperature for an additional 8–16 min. Cells were harvested and adjusted to 3.2×105 cells/well of a 24-well plate in DMEM, 5% FBS. For siRNA TransIT-TKO mixture was added to 9.0 ml of cells, mixed, and dispensed in a 24-well poly-l-lysine dish (500 μl/well). The following day, the siRNA mixture was removed and replaced with fresh medium, and incubation continued for 2 more days and then cells were stimulated as described in the following paragraph. After stimulation, cells were lysed and subjected to Western blot analysis for phosphorylated JNK as described previously (15).

For cellular stimulation in the presence of DHP-2, COS-7 cells were plated at a density of 2.0×105 cells/well of a 24-well plate in DMEM supplemented with 5% FBS and incubated for 6 h at 37 °C followed by incubation overnight in DMEM supplemented with 0.5% FBS and treated for 90 min with 200 nM DHP-2 prior to stimulation. Cells were stimulated with anisomycin (10 μg/ml, 30 min), TNF-α (10 ng/ml, 15 min), IL-1β (10 ng/ml, 15 min), 2.0 mM H2O2 (15 min), 80 or 800 μM FeSO4.
UV irradiation (wave length = 254 nm), or 0.5 M NaCl (5 min).

DNA Fragmentation Assay—COS-7 cells were plated in a 24-well plate using DMEM supplemented with 5% FBS. After overnight incubation at 37 °C, cells were pretreated with DHP-2 at 200 nM for 1 h and stimulated with either 800 J/M² UV light or anisomycin at 10 μg/ml followed by overnight incubation. The following day, DNA fragmentation analysis was performed using the Cell Death Detection ELISA Kit (Roche Applied Science) according to the manufacturer’s suggested protocol. Means ± S.E. were determined, and the data were analyzed by a two-tailed Student’s t test.

MLK7 Transgenic in Vivo Analysis—MLK7 Tg were generated as described previously (22). Mice were dosed by oral gavage with vehicle (0.5% carboxymethyl cellulose, 0.1% polysorbate 80) or the indicated amount of compound and were sacrificed 1 h post-dose by CO₂ asphyxiation after which heart ventricles were harvested and frozen in liquid nitrogen. For isoproterenol stimulation, animals were dosed with intraperitoneal injection of 15 mg/kg isoproterenol in sterile saline 45 min after oral gavage of either vehicle or compound and heart tissues were harvested 15 min after isoproterenol stimulation. Tissue homogenates were prepared and analyzed by Western blot as described previously. The use of mice was approved by the Institutional Animal Care and Use Committees of the American Association for Accreditation of Laboratory animal Care-accredited institutions (Eli Lilly and Company).

RESULTS

Identifying MLK7 Inhibitors—We have previously demonstrated that GST-fused MLK7 has kinase activity when over-expressed and purified from Sf9 cells (25). For compound screening, GST-MLK7 was purified to homogeneity and a filter binding assay for MLK7 catalytic function with MBP as substrate was developed. Using this assay, DHP-1 (Table I) was identified as a potent inhibitor of MLK7 catalytic function with an IC₅₀ of 48 nM. This compound was unsubstituted on the pendant aryl ring. A series of 7-aryl-substituted dihydropyrrolopyrazole quinolines was synthesized (Table I) with variation at the ortho, meta, and para positions for comparison with DHP-1. Ortho substitution by hydroxyl, amino, or carboxylic acid groups (DHP-5, DHP-6, and DHP-7, respectively) resulted in a 6–20-fold decreased activity in the MLK7 filter binding assay. However, the carboxylic acid group was well tolerated in the para position (DHP-3, MLK7 IC₅₀ 15 nM), as was a carboxamide (DHP-4, MLK7 IC₅₀ 23 nM). DHP-2, substituted at the meta position by fluorine and para by amino, was found to be comparably active in MLK7 to the monosubstituted carboxylic acid and carboxamide derivatives (IC₅₀ 17 nM). DHP-8, in which a thiophene rather than a phenyl ring substituted the 7-position of the quinoline, was also found to be active (MLK7 IC₅₀ 11 nM). The thiophene can be regarded as an isostere of the phenyl ring in that the enzymatic activity of DHP-8 is comparable to that of DHP-1. The compounds all demonstrated IC₅₀...
MLK7 Inhibitor

Fig. 1. MLK7 signaling cascade. Schematic diagram of the known MLK7 and inflammatory cytokine signaling cascade from the cellular membrane to transcription factor activation in the nucleus. EGF, epidermal growth factor.

> 20 μM in a MLK2 filter binding assay using MBP as substrate, suggesting that the dihydropyrrolopyrazole quinolines are specific for MLK7 over other members of the mixed lineage family of kinases. Inhibitor potency analysis of this class of compounds was determined using the MBP filter binding assay with varying concentrations of DHP-8 (IC_{50} 11 nM) and ATP. The Lineweaver-Burke plot of the resulting data revealed the dihydropyrrolopyrazole quinolines to be ATP-competitive inhibitors of MLK7 catalytic function (K_{i} = 1.5 nM) (Fig. 2A).

Compounds with IC_{50} < 500 nM in the filter binding assay were tested for the ability to inhibit MLK7 catalytic activity in the cell. COS-7 cells were co-transfected with MLK7 and JNK, and the amount of pJNK formed was measured using a pJNK cellular ELISA. We have demonstrated previously that there is no formation of pJNK in this co-transfection assay without the presence of active MLK7. The IC_{50} data shown in Table I reveal DHP-2 and DHP-8 to be the most potent inhibitors of MLK7 cellular activity with a mean IC_{50} of 0.075 and 0.052 μM, respectively. In contrast, the unsubstituted DHP, DHP-1, and the 4-carboxyl-substituted aryls, DHP-3 and DHP-4, demonstrated IC_{50} values of 1.0, 2.1, and 0.45 μM, respectively. As expected, based on the relative in vitro potencies, 2-OH-substituted aryl had the least activity with a IC_{50} of 6.2 μM.

MLK7 resides at the MAPKK level of JNK and p38 signal transduction pathways, and as shown in Fig. 1, a MAPKK resides between JNK and MLK7. To confirm that the compounds were acting at the MAPKK level and not at the MAPKK level, MLK7 was co-transfected with MKK4 and the resulting pMKK4 formed was measured by cellular ELISA. The IC_{50} values generated for compounds in this assay were in good agreement with those generated from the JNK co-transfection, suggesting that the compounds are active upstream of MAPKK (r^2 = 0.907). Since DHP-2 demonstrated the most potent MLK7 cellular inhibition as well as the most ideal solubility and metabolic stability in human microsomal studies (data not shown), this compound was chosen for further analysis. Representative individual experiments determining dose response curves of DHP-2 in MLK7 in vitro kinase assay, as well as MLK7/JNK and MLK7/MKK4 co-transfection assays, are shown in Fig. 2, B–D, respectively.

MLK7 is the MAPKKK Responsible for Anisomycin and UV-induced p38 and JNK Activation—Although it is clear that MLK7 activates p38 and JNK, cellular stresses that stimulate MLK7 activation have not been well defined. In the following set of experiments, DHP-2 was used as a tool to identify cellular stressors that may be activating JNK and p38 through the MAPKKK MLK7. Thus, cultured COS-7 cells were pretreated with 200 nM DHP-2 and then stimulated with anisomycin, IL1-β, TNF-α, H_{2}O_{2}, NaCl, or ultraviolet radiation. Cellular lysates were collected, and Western blots were analyzed with antibodies to phosphorylated or total JNK and p38. The results shown in Fig. 3 demonstrate that the MLK7 inhibitor DHP-2 at 200 μM completely blocked both JNK and p38 activation downstream of anisomycin and UV radiation. In contrast, the compound had no effect on activation of the stress-activated MAPKs downstream of cytokine stimulation. The compound partially blocked p38 activation in response to hyperosmotic stress but had no effect on H_{2}O_{2}-induced p38 activation. The effect of DHP-2 on JNK activation by these two stresses could not be determined due to low level of activation in COS-7 cells. These data suggest that MLK7 is the MAPKKK involved in anisomycin and UV-induced signal transduction but not cytokine activation of JNK and p38. Additionally, the data further demonstrate the specificity of DHP-2 at the MAPKKK level, because all four of these stresses are believed to activate JNK and p38 activation through the same MAPKKs but different MAPKKks (Fig. 1). To further demonstrate the specificity of DHP-2 for MLK7, 10-point dose response curves (0.15 nM–3.0 μM) for the inhibition of anisomycin, UV, IL-1β, or TNF-α-phosphorylated JNK and p38 were determined using the appropriate cellular ELISAs. The resulting IC_{50} values for anisomycin and UV pJNK formation of 0.018 and 0.026 μM, respectively (Table II), are nearly identical and have similar potency to the IC_{50} reported for pJNK inhibition in the MLK7/JNK co-transfection assay (IC_{50} = 0.075 μM). The IC_{50} values for phosphorylated p38 formation downstream of anisomycin (0.014 μM) and UV radiation (0.020 μM) are nearly identical to those of pJNK inhibition for the same stimuli. The similar IC_{50} values for all of these assays provide further evidence that DHP-2 is blocking MAPK activation by inhibiting MLK7, the kinase common to both p38 and JNK activation by these stimuli in COS-7 cells. Additionally, the dose response curves suggest IC_{50} of >3.0 μM for IL1-β and TNF-α, further demonstrating the specificity of the inhibitor for the UV and anisomycin signal transduction pathway.

To confirm the role of MLK7 in anisomycin and UV signaling, gene-silencing studies with control or MLK7 siRNA were performed in COS-7 cells. Cells were transfected with either nonspecific or MLK7 siRNA and subsequently stimulated, and cellular lysates were collected for Western blot analysis. The results in Fig. 4 demonstrate that MLK7 siRNA efficiently reduced protein expression levels as compared with control siRNA. Similar to DHP-2 treatment, the reduction of MLK7 expression completely blocked JNK and p38 activation induced by anisomycin and UV radiation but had no effect on the signal transduction pathways activated by IL-1β or TNF-α. MLK7 blocked p38 activation in response to hyperosmotic stress but had no effect on JNK activation and had no effect on either pathway when stimulated by H_{2}O_{2}. For all of the stimuli, the pattern generated with MLK7 siRNA is identical to that generated with DHP-2 at 200 nM and further confirms the specificity of the compound for MLK7 and that MLK7 catalytic activity is required for anisomycin and UV radiation signal transduction pathway to activate p38 and JNK. Results from studies using DHP-2 or MLK7 siRNA in stimulated human umbilical vein endothelial cells, HEK293 cells, and HeLa cells were similar to those in COS-7 cells and suggest that MLK7 is the MAPKKK involved in anisomycin- and UV-induced p38 and JNK activation in multiple cell types (data not shown).

DHP-2 Inhibits Anisomycin- and UV-induced Cell Death—Previous studies have demonstrated that UV radiation and anisomycin stimulate apoptosis in cell culture and that this programmed cell death requires JNK expression and activation
We utilized DHP-2 to determine whether inhibition of MLK7 activity was sufficient to block anisomycin or UV-induced apoptosis. Cells pretreated with either vehicle or 200 nM DHP-2 were stimulated with anisomycin or UV radiation. The level of apoptosis was determined using a DNA fragmentation assay. Treatment of COS-7 cells with either anisomycin or UV radiation significantly increased DNA fragmentation (83%) relative to unstimulated cells ($p < 0.01$) (Fig. 5). DHP-2 (200 nM) completely inhibited the anisomycin-induced DNA fragmentation ($100\%$; $p < 0.01$) and inhibited 75% UV-induced DNA fragmentation ($75\%$; $p < 0.01$; Fig. 5). Additionally, MLK7 siRNA protected from UV and anisomycin induced apoptosis (data not shown), further dem-
A similar experiment was performed to determine the effect of the control level for both the 30 and 100 mg/kg dose (binding to the two bands representing pMAPKAP K2 to 20% of shown in Fig. 6 demonstrate that DHP-2 significantly reduced antibody gave superior signals in heart tissue homogenates, we heart tissue was harvested 1 h after dosing. Heart tissue homogenates were submitted to SDS-PAGE followed by Western blot analysis utilizing antibodies for phosphorylated or total JNK. The results in Fig. 7 demonstrate that the 10 mg/kg dose did not reduce pJNK levels. However, the 30 and 100 mg/kg doses significantly reduced pJNK to 20% that of the vehicle control. DHP-2 also reduced pMAPKAP K2 to a similar level in the isoproterenol-stimulated mice. The compound had no effect on ERK pathway activation (data not shown). These data demonstrate that MLK7 activated by physiological effects of isoproterenol in heart tissue from these transgenic mice can be inhibited by DHP-2.

DISCUSSION

This screen for inhibitors of MLK7 identified 7-aryl-substituted dihydropyrrolopyrazole quinolines as potent competitive inhibitors of MLK7 catalytic activity in vitro and in cell-based assays. Using this compound along with MLK7 siRNA gene silencing, we demonstrated that the MLK7 catalytic domain functions as the MAPKKK required for JNK and p38 signal transduction in response to anisomycin and UV radiation and has some effect on pathway activation in response to hyperosmotic stress. In these studies, we clearly demonstrate that MLK7 is involved in JNK and p38 activation in response to hyperosmotic stress. This is in conflict with studies by Gotoh et al. (16) where overexpression of MLK7 (MLTK-β) suggested that the kinase was activated by hyperosmotic stress but not anisomycin or UV radiation. The discrepancy may be due to the use of overexpression in the Gotoh study where protein partners linking the kinase to extracellular stimuli may be limiting and the majority of the overexpressed protein would not be partnered with the appropriate docking proteins. MLK7 is constitutively active when overexpressed (15, 25). Consequently, immunoprecipitation followed by in vitro kinase analysis in transfected cells will predominantly reflect the activity of the overexpressed kinase that is not properly coupled to membrane receptors, whereas the minor, properly partnered, and activated enzyme will not be detectable. In our experiments, we evaluated effects on the endogenous and appropriately coupled protein where artifacts due to overexpression are minimized. Additionally, we have used two independent methods to demonstrate that MLK7 is the MAPKKK responsible for anisomycin and UV MAPK signaling.

DHP-2 had no inhibitory activity against another MLK subclass 1 enzyme, MLK2. Further demonstration of DHP-2 specificity for the subclass 3 over 1 is evidenced by the lack of inhibition against TNF-α-induced JNK and p38 activation, a pathway where a role for MLK3 has recently been defined using siRNA techniques (9). Experiments utilizing K252A and other indolecarbazoles derivatives, compounds similar in structure to the potent MLK1, 2, and 3 inhibitor, CEP-1347 (12), have little or no effect on MLK7 catalytic activity (K252A IC₅₀ = 6.62 μM, >20 μM IC₅₀ for related molecules). MLK7 is a member of the most divergent subclass of MLKs with 40% homology overall and 62% homology in the catalytic domain compared with the other family members, whereas MLK1–4 share 78% homology in the catalytic domain. Both the dihydropyrrolopyrazole quinolines and the indolecarbazoles inhibit kinase activity by competing with ATP substrate binding. Taken together, the data suggest the ATP binding domains of these two MLK subclasses are quite divergent, allowing for subclass-specific molecules to be generated.

![Fig. 4. Effect of MLK7 siRNA on agonist-induced p38 and JNK activation in COS-7 cells.](image_url)

![Fig. 5. DHP-2 blocks anisomycin- and UV-induced cell death.](image_url)
Although there is evidence that DHP-2 does not inhibit MLK subclass 1 family members, the specificity against another MLK, ZAK, is less likely. MLK7 is a splice variant of ZAK, having an identical N terminus through the kinase domain and the leucine zipper but with a different C-terminal domain such that MLK7 codes for a 55-kDa protein and ZAK codes for a 91-kDa protein (15, 18). Because the catalytic domains between the two spliced forms are identical, DHP-2 will probably be a nanomolar inhibitor of ZAK as well as MLK7. Additionally, the siRNA primers used in this study are designated to a region that is shared between the two variants. Therefore, these experiments do not distinguish between contributions of ZAK or MLK7 to anisomycin- or UV radiation-induced p38 and JNK activation. MLK7 Western blots suggested some cross-reactivity with a 91-kDa protein and that this protein was also reduced by MLK7 siRNA, suggesting that both forms were silenced by the N-terminal specific siRNA as expected. Additional studies utilizing siRNA to the variant-specific C-terminal domain will be required to define the contribution of each individual splice variant.

There is growing evidence that p38 and/or JNK activation may be implicated in cardiac hypertrophy, congestive heart failure, and ischemia/reperfusion injury (27–33). Previous studies of transgenic animals with cardiac-restricted overexpression of MLK7 demonstrated increased β-adrenergic stimulated p38 and JNK activation relative to littermate controls that is associated with negative effects on contractility (22), suggesting MLK7 to be a MAPKKK involved in negative inotropic effects of stress pathway activation in this tissue. Other studies utilizing an activated mutant form of MKK3 have also suggested that p38 activation results in decreased contractility (34). Additionally, we have found that MLK7 inhibition with DHP-2 can partially prevent the profound reduction in left ventricular contractility in a rat model of congestive heart failure (3). The MLK7 activation and subsequent effect on contractility in the stimulated transgenic mice are believed to be a secondary response to physiological changes associated with adrenergic stim-

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**FIG. 6.** Effect of DHP-2 on p38 activation in heart tissue from MLK7 Tg mice. A, heart tissue was harvested from MLK7 Tg mice 1 h after dosing with vehicle, 30 or 100 mg/kg DHP-2. Homogenates of tissue were prepared and submitted to SDS-PAGE and Western blot analysis using antibodies against phospho-MAPKAP K2 (pMK2) and visualized using ECL. B, densitometric scan of the Western blot was performed, and upper and lower pMAPKAP K2 band values were normalized to p36 and expressed as percent signal in vehicle-treated mice. In all of the cases, means ± S.E. are reported, and each lane represents an individual animal. *, p < 0.001 relative to vehicle control from the same gel.

**FIG. 7.** Effect of DHP-2 on JNK activation in heart tissue from isoproterenol-challenged MLK7 Tg mice. A, MLK7 Tg mice were dosed orally with vehicle, 10, 30, or 100 mg/kg DHP-2. Mice were given intraperitoneal injection of isoproterenol (15 mg/kg) 45 min after oral dosing, and heart tissue was harvested 15 min after isoproterenol stimulation. Tissue homogenates were prepared and submitted to SDS-PAGE and Western blot analysis using antibodies to phosphorylated JNK (pJNK) or JNK as indicated to the left of the panel. B, densitometric scan of the Western blot was performed and pJNK values were normalized to total JNK and expressed as percent of signal achieved with isoproterenol stimulation with vehicle treatment. Means ± S.E. are reported, and each lane represents an individual animal. *, p < 0.001 relative to vehicle control from the same gel.

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2 X. Wang and L. J. Bloem, unpublished observations.

3 M. E. Christe and L. J. Bloem, unpublished observation.
ulation, because studies in our laboratory using isolated cardiac myocytes demonstrated activation of the enzyme by hypertosmotic stress but not by adrenergic stimulation (data not shown). Further studies will be necessary to define how anisomycin activation of the enzyme in tissue culture relates to activation induced by the physiological changes associated with β-adrenergic stimulation in vivo.

Multiple cellular responses and physiological conditions have been linked to JNK or p38 pathway activation. As such, there has been great interest in identifying small molecules that specifically block these kinases for the treatment of disease. To this end, several molecules that block either JNK or p38 have been identified and have entered clinical trials (3, 35). A diverse array of environmental stressors and physiological stimuli activate JNK and p38 signal transduction pathways. Because of this complexity, there is potential that targeting at the MAPK level will result in blocking p38 and JNK response to all of these conditions. Targeting at a more upstream point such as at the MAPKKK level as we have done here affords the strategy that this strategy is possible by showing that MLK7 gene transduction, leaving the pathway open to activation by other stimuli such as at the MAPKK level as we have done here affords the possible that targeting at the MAPKK level will result in blocking p38 and JNK response to all of these conditions. Targeting at a more upstream point such as at the MAPKKK level as we have done here affords the possibility that targeting at the MAPKKKK level will result in blocking p38 and JNK response to all of these conditions.

In summary, a potent and selective inhibitor of MLK7 in vitro, cellular, and in vivo activity has been identified. This compound will be a useful tool for further investigation on the role of this kinase in other cellular and physiological functions.

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