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Discovery and characterization of an iminocoumarin scaffold as an inhibitor of MEKK2 (MAP3K2)

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Abstract

The kinase MEKK2 (MAP3K2) activates the MEK5/ERK5 cell signaling pathway and may play an important role in tumor growth and metastasis. Thus, MEKK2 may represent a novel kinase target for cancer. In order to identify inhibitors of MEKK2, we screened a library of compounds using a high throughput MEKK2 intrinsic ATPase enzyme assay. We identified two hits with validated structures and confirmed activity in the primary assay (IC₅₀ values = 322 nM and 7.7 μM) and two orthogonal MEKK2 biochemical assays. Compound 1, the more potent hit, was the subject of further investigation. Limited structure-activity relationship (SAR) studies were performed on this iminocoumarin hit which resulted in 20-fold more potent analogs (e.g. 8 and 16 nM IC₅₀). Two analogs had improved selectivity in a 50-member kinase profiling panel compared to the hit. These studies suggested that substitutions around the phenoxy ring of this scaffold can impart improved potency and selectivity for MEKK2. Analog Compound 1s (16 nM IC₅₀) was further verified by external testing to inhibit MEKK2 and MEKK3 with similar potencies. Compound 1s displayed activity in cell-based assays in which it inhibited ERK5 pathway activation in cells and inhibited cell migration in a scratch assay. Thus, we have identified a scaffold that has promising potential to be developed into a highly selective and potent inhibitor of MEKK2. Information from these SAR studies provides specific guidance for the future design of MEKK2 inhibitor probes.

Keywords

MEKK2; MAP3K2; kinase; inhibitor

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Introduction

The ERK5 signaling pathway is one of the mitogen-activated protein kinase (MAPK) signaling pathways that alter gene expression in response to extracellular stimuli. [1,2]. The ERK5 pathway is activated by oxidative stress, hyperosmolarity and growth factors such as epidermal growth factor (EGF) [3]. ERK5 is activated through phosphorylation by the MAPK kinase MEK5 (MAP2K5). MEK5 in turn is activated through phosphorylation by the MAPK kinase kinases MEKK2 (MAP3K2) and MEKK3 (MAP3K3). The mechanism leading to MEKK2 activation is still not fully understood. However, it is known that MEKK2 autophosphorylates in response to stimuli and this autophosphorylation is required for activation [4]. In addition to the ERK5 pathway, MEKK2 also activates the JNK MAPK pathway by phosphorylation of the MAP2K MEK7 which in turn activates JNK [5,6]. Mouse gene knock-out studies have provided clues to the biological function of the MEKK2/3-MEK5-ERK5 pathway. ERK5 knock-out in mice resulted in embryonic lethality due to defects in cardiac development and angiogenesis [7]. ERK5 appears to protect many cell types from stress-induced apoptosis [3,8,9]. MEK5 gene knock out in mice resulted in lethality apparently due to abnormal cardiac development, similar to the ERK5 knockout mouse [10]. MEKK2 knock-out mice displayed normal development and fertility [5,11]. However, knock out of the MEKK3 gene in mice resulted in embryonic lethality due to cardiac development defects [12]. These animal knock out studies indicate that MEKK2 and MEKK3 signal in response to different stimuli. MEKK2 is activated by cell attachment to fibronectin and then localizes to focal adhesions. MEKK2 knockdown by RNAi in breast cancer cell lines was shown to stabilize focal adhesions and inhibit cell migration in scratch assays [13,14].

A potential role of MEKK2 in cancer has been explored to a limited extent. In a study linking MEKK2 to prostate cancer, MEKK2 was expressed at 4.4-fold higher levels in prostate cancer tissue compared to benign tissue [15]. Prostate cancer cell lines expressed even higher levels of MEKK2. The microRNA miR-520b has been shown to suppress tumor growth in breast cancer and hepatocellular carcinoma cells via suppressing expression of MEKK2 and Cyclin D1 [16]. Importantly, knock-down of just the MEKK2 expression by siRNA was able to reduce the growth of hepatocarcinoma cells *in vitro* and *in vivo*. Furthermore, restoration of the expression of another tumor suppressor, miR17/20a, enhanced tumor cell sensitivity to lysis by natural killer cells via suppressing MEKK2 [17]. MEKK2 has been shown to be highly expressed in primary colorectal cancer compared to normal mucosa [18]. An *in vivo* mouse xenograft model of breast cancer was used to assess the role of MEKK2 in tumor growth and metastasis [19]. shRNA-mediated knockdown of MEKK2 inhibited activation of ERK5 in response to EGF using the breast cancer cell line MDA-MB-231. Surprisingly, MEKK2 knock down had no observable impact on cell growth *in vitro*, but strongly inhibited both tumor growth and metastasis *in vivo*. The smaller tumors resulting from the MEKK2 knock-down cells displayed increased apoptosis over size-matched control tumors. Knockdown of MEKK2 in the BT474 breast cancer cell line also resulted in inhibited tumor growth *in vivo*. In contrast, the knockdown of ERK5 inhibited metastasis without significantly impacting tumor growth. In this animal model, activation of ERK5 by MEKK2 appeared to regulate metastasis, while another MEKK2-dependent

pathway, potentially the JNK pathway, was important for tumor growth. In addition, the downstream components activated by MEKK2, MEK5 and ERK5, have also been implicated in cancer progression by numerous studies [20]. An inhibitor of MEKK2 may reduce activation of both the ERK5 and JNK pathways leading to improved anti-tumor efficacy.

Thus, the current literature data supports the concept of MEKK2 as a novel drug target. However, no specific *and* potent small molecule inhibitors of MEKK2 have been reported to date. We have previously reported the discovery of approved drugs (e.g. ponatinib), clinical trial drugs and other research tool compounds that potently inhibit MEKK2 activity in biochemical assays [21,22]. However, all of these lack selectivity for MEKK2 and it is unknown if any of them inhibit MEKK2 activity in cells.

Materials and Methods

Materials

Common reagents such as HEPES, dithiothreitol (DTT), MgCl_2 and dimethyl sulfoxide (DMSO) were reagent grade quality purchased from Thermo Fisher Scientific (Waltham, MA) or Sigma-Aldrich (St. Louis, MO). Organic synthesis and structure confirmation of Compound 1 and its analogs 1b – 1s are described in Supplementary Material. Compounds 1, 1a and 2 were purchased from Life Chemicals (Niagara-on-the-Lake ON, Canada) and Enamine (Monmouth Jct., NJ). All synthesized and purchased compounds were verified for purity (95%) and mass by LC/MS. The structures of all synthesized compounds were confirmed by ^1H and ^{13}C NMR. Compounds were dissolved in DMSO and DMSO was used as solvent control and concentration of DMSO was kept constant under all experimental conditions.

Cell culture

MDA-MB-231 cell line (ATCC, Manassas, VA) was cultured at 37°C in an atmosphere of 5% CO_2 and 95% humidity in DMEM-high glucose media supplemented with 10% FBS, 200 mM L-glutamine, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and 15 mM HEPES. All reagents and plasticware for cell culture were obtained from ThermoFisher Scientific.

MEKK2 Biochemical Assays

The MEKK2 intrinsic ATPase activity assay, MEKK2 TR-FRET binding assay, MEKK2 transphosphorylation activity assay and IC_{50} determinations were performed as previously described [21]. MEKK2 activity assays were performed using ATP concentrations at the apparent K_m of the assay format. For all MEKK2 assay formats, compound concentration response curves were generated using data points that represent the average of 2 or 3 determinations per concentration and 8–10 compound concentrations tested.

Kinase Profiling

Kinase profiling of a serine/threonine and tyrosine kinase panel was performed by Carna Biosciences, Inc. (Kobe, Japan) using a mobility shift assay format as previously reported [23]. In this 50-member kinase assay panel, compounds were tested at 1 μM concentration in duplicate and average percent inhibition reported. The IC_{50} data generated by Carna

Biosciences was performed using target enzyme and another kinase as a substrate in an enzyme-linked immunosorbent assay (ELISA)-based format to detect product. In these assays, each concentration was tested in duplicate and data averaged before calculating the IC₅₀ values. Carina Biosciences kinase assays were performed using ATP concentrations at the apparent K_m for ATP in each kinase assay.

Cell-Based Assays

For ERK5 pathway inhibition analysis, MDA-MB-231 cells were incubated in serum-free media overnight and then treated with compound (or DMSO as solvent control) for 1.5 h prior to stimulating cells for 30 min with 0.2 M sorbitol to activate the ERK5 pathway. Cell lysates were made and standard western blot analysis was performed as previously reported [24], using anti-phosphoERK5 antibody (Cell Signaling, Danvers, MA, cat# 3371) and then the membrane was stripped and re-probed with anti-ERK5 recognizing total ERK5 (Cell Signaling). The scratch cell migration assay was performed according to published protocol [25]. After a confluent cell monolayer was reached and the scratch in the cell monolayer created, compound or 1% DMSO was added to the normal growth media and the same area of the scratch photographed at 0 and 18–24 hr later.

Results and Discussion

We previously reported the development and validation of a novel intrinsic ATPase activity assay for MEKK2 and demonstrated its utility as a high throughput assay for the discovery of small molecule inhibitors of MEKK2 [21]. We used this assay to screen a collection of approximately 15,000 commercially available compounds ([21] and data not shown). Herein, we report the structures and activities of two confirmed hits from this screen. Hit compound 1 generated IC₅₀ values in the MEKK2 ATPase activity assay of 322 ± 161 nM (Table 1). Compound 1 was further confirmed as a hit by re-synthesis. Two completely different MEKK2 assay formats were used to confirm the activity observed in the ATPase assay. The MEKK2 TR-FRET binding assay measured binding to MEKK2 via competitive displacement of a labeled ATP-site binding small molecule. An additional MEKK2 activity assay was employed that detects the native transphosphorylation activity of MEKK2 using kinase-inactive MKK6 as a substrate. Phosphorylation of MKK6 was detected by western-style quantitative slot blot using a phospho-MKK6 specific antibody [21]. Compound 1 produced average potencies of 355 ± 134 nM in the binding assay and 261 ± 14 nM in the transphosphorylation assay format (Table 1). Thus, compound 1 confirmed activity in all three MEKK2 assay formats. Compound 2 (hit #2) generated IC₅₀ values in the MEKK2 ATPase activity assay of 7.7 ± 1.6 μ M and similar potency in the MEKK2 binding assay with an ave. IC₅₀ of 7.9 ± 3.6 μ M (Table 1). Thus, both hits were confirmed as MEKK2 inhibitors with multiple assay formats. Since compound 1 was significantly more potent, we focused further work on this scaffold.

We sought to perform limited SAR on compound 1 to explore the potential of this iminocoumarin scaffold for improved potency and selectivity. We focused our efforts on substitution around the phenol ring and modifying the methoxy group on the chromene ring and assessing activity using the ATPase assay (Table 2). Replacing the hydroxy group of the

phenol ring with a methoxy group (Table 2, compound 1a) resulted in complete loss in inhibitory activity. Many other substitutions were tested (data not shown), but all resulted in a complete loss in activity, indicating that the hydroxy group is absolutely required for activity. This finding may be analogous to a reported LRRK2 inhibitor in which modeling and SAR suggested that a critical 4-hydroxy on a phenol group may act as donor and acceptor with conserved residues [26]. At the R'₂ position of the phenol ring, we substituted with a chlorine, methyl, hydroxy and a fluorine group (Table 2, compounds 1b–e). The presence of a methyl or hydroxy group had little impact on the potency. In contrast, the chlorine or fluorine group resulted in enhanced potencies with IC₅₀s of 60 and 115 nM. At the R'₁ position on the phenol group, we substituted with CF₃, methyl and fluorine groups (compounds 1f–h) which resulted in dramatic enhancement of MEKK2 potency with IC₅₀ values of 38, 28 and 39 nM, respectively.

Since fluorine at the R'₁ and R'₂ positions was shown to enhance the potency, we sought to determine if difluorinated analogs of compound 1 would further improve the potency. Difluorinated compounds 1i–j (Table 2,) with fluorines at the R'₁/R'₂ and R'₂/R'₄ positions generated IC₅₀ values of 36 and 27 nM. These compounds were similar in potency as monofluorinated compound 1h. However, substitution of fluorines at the R'₁ and R'₅ positions of the phenolic ring generated compound 1k with an IC₅₀ of 8 nM. The enhancement in potency of the mono and difluorinated compounds may be due to the electron withdrawing ability of this group to modulate the hydrogen bonding donor/acceptor of the phenol hydroxyl group. Limited SAR was also explored at the methoxy position of the chromene ring (Table 2). Replacement of the methoxy group with an ethoxy, propoxy, isopropoxy, fluorine, bromine and hydroxy groups resulted in compounds 1l–q which gave IC₅₀s of 130, 371, 502, 2102, 151 and 766 nM respectfully. Thus, the ethoxy group at this position resulted in >2-fold improvement in potency which was the best among the tested substitutions. Since a methyl or CF₃ group at the R'₁ position on the phenol group dramatically enhanced potency of the hit compound 1, we synthesized analogs of these compounds containing an ethoxy group on the chromene ring. The methyl and CF₃ substituted compounds 1r–s generated IC₅₀s that were reduced to 16 and 18 nM, respectively (Table 2).

In order to assess the selectivity of this scaffold, we profiled the inhibitory activity of selected analogs against a panel of diverse kinase activity assays performed by Carna Biosciences. Selected compounds were tested at 1 μM compound against a panel of 50 serine/threonine and tyrosine kinase activity assays. Compound 1 inhibited 50% of the activity in 40% of the kinase panel, thus indicating low selectivity for this hit (Fig. 1). The R'₂ fluoro analog of compound 1 (1e), inhibited 50% of the activity in 42% of the kinase panel (Fig. 1). Of note, >95% inhibition was obtained in 7 kinase assays whereas the original hit compound produced >95% inhibition in only 3 kinases. These data suggested that the 2' F substitution resulted in reduced selectivity. In contrast, profiling of compound 1f with the R'₁ CF₃ substitution on the phenol group resulted in 50% inhibition of only 8% of the kinase panel (Fig. 1). Thus, the CF₃ group appeared to impart enhanced selectivity while enhancing activity against MEKK2 by over 8-fold. Profiling of the dual modified analog compound 1s resulted in hitting 16% of the kinases in the panel (Fig. 1), but it's also

twice as potent against MEKK2 compared to compound 1f. Overall, the activity and selectivity profiles appeared similar between compounds 1f and 1s.

We sought to further validate the MEKK2 inhibitory activity of compound 1s using a different source of MEKK2 and a different assay format while also further assessing selectivity of other kinases in the MAPK family (Fig. 2). An MEKK2 transphosphorylation assay was performed by Carna Biosciences using MAP2K7 as a substrate and an ELISA for detection of phosphorylated product. In this assay, compound 1s generated an IC_{50} of 60 nM against MEKK2 which is similar (<4-fold) to the IC_{50} value determined by our ATPase assay format. The discrepancy in potencies could be due to the use of only the catalytic domain of MEKK2 in the CarnaBio assay, as opposed to the full-length MEKK2 in our assays. This compound also produced an IC_{50} of 53 nM against the closely related MEKK3, making it a dual inhibitor of MEKK2/MEKK3. This was not unexpected since the catalytic domains of MEKK2 and MEKK3 are 94% homologous [27]. Activity against MAP2K5 (MEK5) was also tested and 50% inhibition was not achieved, possibly due to solubility problems in this assay. Based on the dose response curve, we estimated an IC_{50} of >1 μ M against MAP2K5. No activity (>10 μ M IC_{50}) was observed against MAP3K5 (ASK1) and TAK1-TAB1 (MAP3K7).

We further investigated the activity of Compound 1s in cell-based assays. Initially, we tested compound 1s for cell cytotoxicity. This compound had no effect on the viability of MDA-MB-231 cells in a 24 hr exposure resazurin cell viability assay (data not shown). We sought to determine whether Compound 1s could inhibit the ERK5 pathway using the appearance of phosphorylated ERK5 (pERK5) as an indicator of pathway activation. We treated cells with 10 μ M compound for 1.5 hr and then stimulated the cells with 0.2 M sorbitol for 30 min (Fig. 3A). Western blot analysis of the cell lysates indicated that Compound 1s inhibited the phosphorylation of ERK5, an indicator of inhibition of the MEKK2/MEK5/ERK5 pathway. Furthermore, we tested Compound 1s in the scratch cell migration assay to determine if it could inhibit cell migration which has been reported to involve MEKK2 for these cells (Fig. 3B). The data indicated that an inactive analog of compound 1 had no effect compared to DMSO treated control, while the MEK5 inhibitor BIX02188 (1 μ M) and Compound 1s (10 and 1 μ M) showed inhibition of scratch healing. Thus, compound 1s was able to inhibit the ERK5 pathway activation in cells and cell migration in the scratch assay.

We report the identification and confirmation of 2 HTS hits with *in vitro* MEKK2 inhibitory activity using our previously reported MEKK2 ATPase HTS assay. Initial SAR studies for one of the hits resulted in 20-fold more potent analogs with the most potent one having an IC_{50} of 8 nM for MEKK2. This small set of analogs also led to the identification of analogs with improved selectivity in a 50-member kinase profiling panel. These SAR studies suggested that substitutions around the phenoxy ring can impart improved potency and selectivity for MEKK2. We also demonstrated that one of these analogs displayed cell-based assay activity where it inhibited EKR5 pathway activation and cell migration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

DMSO	dimethyl sulfoxide
MAPK	mitogen-activated protein kinase
MS	mass spectrometry
ATP	adenosine triphosphate
ADP	adenosine diphosphate
SAR	structure-activity relationship
SD	standard deviation
TR-FRET	time-resolved fluorescence resonance energy transfer
HTS	high throughput screening
ELISA	enzyme-linked immunosorbent assay

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Highlights

- Two novel small molecule inhibitors of MEKK2 discovered from screening
- Structures validated and activities confirmed in multiple MEKK2 biochemical assays
- Synthesis of analogs for iminocoumarin scaffold led to 20-fold more potent analogs
- Kinase profiling indicated that scaffold modifications could increase selectivity
- One analog inhibited ERK5 pathway activation in cells and cell migration

Figure 1. Selectivity of Compound 1 and derivatives in a panel of 50 kinase activity assays
Kinase profiling was performed by Carna Biosciences. Compounds 1, 1e, 1f and 1s were tested at 1 μ M in duplicate in each assay and the average percent inhibition was plotted for the indicated kinase. Results were sorted by activity with the highest percent inhibition on the left side of the graphs. Dashed line indicates 50% inhibition. The number and percentage of kinase assays resulting in 50% inhibition is provided for each compound.

Figure 2. IC₅₀ determinations for Compound 1s in selected kinase activity assays

IC₅₀ determinations for Compound 1s were performed by Carna Biosciences using the indicated kinase activity assays. Biochemical kinase activity assays were performed using the indicated target kinase and another kinase as substrate in ELISA-based activity assays where the [ATP] was at or near the respective K_m values for each kinase assay. Compound 1s was tested in duplicate at each of the 10 indicated concentrations for each assay and the mean $\pm$ SD percent activity was plotted. Calculated IC₅₀ values were 60 nM for MEKK2 (●), 53 nM for MEKK3 (■), >1 μ M for MAP2K5 (▼) and >10 μ M for TAK1-TAB1 (◆) and MAP3K5 (▲).

Figure 3. Activity of Compound 1s in cell-based assays

(A) MDA-MB-231 cells were treated with DMSO or Compound 1s (10 μ M) and then stimulated with sorbitol, as indicated. Cell lysates were analyzed by western blot for phosphoERK5 (pERK5) and total ERK5. Data are representative of two independent experiments. (B) A cell migration “scratch” assay was employed using MDA-MB-231 cells. After scratching the cell monolayers, cells were treated at 0 h with 1% DMSO (solvent), an inactive analog of compound 1, the MEK5 inhibitor BIX02188 or Compound 1s (10 or 1 μ M). Photographs show the scratch at 0 and 18 hr as indicated. White areas are cell monolayers, while black areas are zones without cells. Each condition was performed in duplicate or singlet wells and data are representative of four independent experiments.

Table 1

Structures and activities of screening hits in multiple MEKK2 assay formats

Compound	Structure	IC ₅₀ ± SD (nM) for MEKK2 ^a		
		Intrinsic ATPase Assay	Binding Assay	Transphos. Assay
1		322 ± 161	355 ± 134 ^b	261 ± 14 ^b
2		7,710 ± 1,610	7,930 ± 3,600	ND ^c

^aIC₅₀ values are the mean ± SD of at least three independent determinations using the indicated assay.^bValues as reported in [21].^cNot determined

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Table 2

MEKK2 inhibitory activity for Compound 1 derivatives

Compound	R'1	R'2	R'3	R'4	R'5	R	IC50 (nM) ^a
1a	H	H		H	H	OMe	>10,000
1b	H		OH	H	H	OMe	60 ^b
1c	H		OH	H	H	OMe	362
1d	H		OH	H	H	OMe	361
1e	H		OH	H	H	OMe	115
1f		H	OH	H	H	OMe	38
1g		H	OH	H	H	OMe	28
1h		H	OH	H	H	OMe	39
1i			OH	H	H	OMe	36
1j	H		OH		H	OMe	27
1k		H	OH	H		OMe	8

Compound	R'1	R'2	R'3	R'4	R'5	R	IC50 (nM) ^a
1l	H	H	OH	H	H		130 ^b
1m	H	H	OH	H	H		371 ^c
1n	H	H	OH	H	H		502 ^b
1o	H	H	OH	H	H		2102
1p	H	H	OH	H	H		152 ^b
1q	H	H	OH	H	H		766
1r		H	OH	H	H		18 ^c
1s		H	OH	H	H		16

^aIC₅₀ values are the mean of two to five independent determinations using the intrinsic ATPase MEKK2 activity assay.

^b Apparent solubility problems observed at compound concentrations >1 μM.

^c Apparent solubility problems observed at compound concentrations >0.3 μM.