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Identification of ponatinib and other known kinase inhibitors with potent MEKK2 inhibitory activity

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The kinase MEKK2 (MAP3K2) may play an important role in tumor growth and metastasis for several cancer types. Thus, targeting MEKK2 may represent a novel strategy for developing more effective therapies for cancer. In order to identify small molecules with MEKK2 inhibitory activity, we screened a collection of known kinase inhibitors using a high throughput MEKK2 intrinsic ATPase enzyme assay and confirmed activity of the most potent hits with this primary assay. We also confirmed activities of these known kinase inhibitors with an MEKK2 transphosphorylation slot blot assay using MKK6 as a substrate. We observed a good correlation in potencies between the two orthogonal MEKK2 kinase activity assay formats for this set of inhibitors. We report that ponatinib, AT9283, AZD7762, JNJ-7706621, PP121 and hesperadin had potent MEKK2 enzyme inhibitory activities ranging from 4.7 to 60 nM IC_{50}. Ponatinib is an FDA-approved drug that potently inhibited MEKK2 enzyme activity with IC_{50} values of 10–16 nM. AT9283 is currently in clinical trials and produced MEKK2 IC_{50} values of 4.7–18 nM. This set of known kinase inhibitors represents some of the most potent *in vitro* MEKK2 inhibitors reported to date and may be useful as research tools. Although these compounds are not selective for MEKK2, the structures of these compounds give insight into pharmacophores that potently inhibit MEKK2 and could be used as initial leads to design highly selective inhibitors of MEKK2.

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1. Introduction

The ERK5 signaling pathway is one of the mitogen-activated protein kinases (MAPK) signaling pathways that translate extracellular stimuli into gene expression changes in the cell [1,2]. This pathway is activated in response to oxidative stress, hyperosmolarity, and growth factors, including 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). How MEKK2 is activated by external stimuli is still not fully understood, but MEKK2 autophosphorylates in response to stimuli and this autophosphorylation is required for activation of MEKK2 [4]. MEKK2 also activates the JNK MAPK pathway via phosphorylation of the MAP2K MEK7 which activates JNK [5,6]. MEKK2 knock-out mice displayed normal development and fertility [5,7]. In contrast, disruption of the MEKK3 gene in mice results in embryonic lethality due to cardiac development defects [8]. Thus, MEKK2 and MEKK3 integrate different stimuli. MEKK2 has been reported to be activated by cell attachment to fibronectin and to subsequently localize to focal adhesions. MEKK2 knockdown in breast cancer cell lines was shown to stabilize focal adhesions and inhibit cell migration *in vitro* [9,10].

The role of the MEKK2 in cancer has only relatively recently been explored. In one study linking MEKK2 to cancer, MEKK2 was expressed at 4.4-fold higher level in prostate cancer tissue versus benign tissue [11]. Even higher levels of MEKK2 were observed in prostate cancer cell lines. The microRNA miR-520b suppresses tumor formation in breast cancer and hepatocellular carcinoma cells by targeting MEKK2 and Cyclin D1 [12].

Abbreviations: MAPK, mitogen-activated protein kinase; EGF, epidermal growth factor; DMSO, dimethyl sulfoxide; ATP, adenosine triphosphate; ADP, adenosine diphosphate; SAR, structure-activity relationship; SD, standard deviation; FDA, Food and Drug Administration.

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Knock-down of only MEKK2 expression was able to inhibit the growth of hepatocarcinoma cells *in vitro* and *in vivo*. The methylation of MEKK2 by SMYD3 was shown to increase MAP kinase signaling and promote the formation of Ras-dependent carcinomas [13]. Restoration of the expression of the tumor suppressor miR17/20a was shown to enhance tumor cell sensitivity to natural killer cell activity through suppressing MEKK2 [14]. A survey of primary colorectal cancer (CRC) lesions for MEKK2 expression level by western blotting indicated that MEKK2 was highly expressed in CRC compared to normal mucosa [15]. We used a mouse xenograft model of breast cancer to assess the role of MEKK2 in tumor growth and metastasis [16]. Using the breast cancer cell line MDA-MB-231, we observed that shRNA-mediated knockdown of MEKK2 inhibited activation of ERK5 in response to EGF. Knockdown of MEKK2 expression had no observable impact on the growth of the cells in culture, but strongly inhibited both tumor growth and metastasis in the animal model. The tumors formed by the MEKK2 knock-down cells had increased apoptosis compared to size-matched control tumors. MEKK2 shRNA knockdown in the BT474 breast cancer cell line also resulted in reduced tumor growth *in vivo*. Since ERK5 is activated by MEKK2 (via activation of MEK5), we determined whether shRNA knockdown of ERK5 in MDA-MB-231 cells would show similar inhibition of tumor growth and metastasis as the MEKK2 knockdown. The knockdown of ERK5 in these cells inhibited their ability to metastasize without significantly impacting tumor growth. Thus, MEKK2-mediated activation of ERK5 appeared to regulate metastasis, while another MEKK2-dependent pathway, possibly the JNK pathway, was important for tumor growth.

Overexpression or over-activation of ERK5 protein in humans coupled with *in vitro* and animal model data has begun to implicate a role for ERK5 in breast and prostate cancer as well as neuroblastoma, myeloma and oral squamous cell carcinoma [17–21]. The discovery of a potent and selective small molecule ERK5 inhibitor has been reported that inhibited tumor growth in mouse models of cancer [22]. MEK5 may also play a role in tumor development. Elevated tissue expression of MEK5 correlated with bone metastasis and poor prognosis in cases of prostate cancer and benign prostatic hypertrophy [23]. Two relatively selective MEK5 inhibitors have been reported, but no *in vivo* efficacy data were shown [24].

Taken together, literature data supports MEKK2 as a novel drug target for certain cancers. Targeting MEKK2 may be advantageous over inhibiting single MAPK pathways since an MEKK2 inhibitor may blunt activation of both the Erk5 and JNK pathways leading to enhanced anti-tumor efficacy. However, no specific and potent small molecule inhibitors of MEKK2 have been reported to date. Even without regard to selectivity, few potent inhibitors have been reported for MEKK2. Herein, we report the identification of 6 well-characterized kinase inhibitors that have previously un-reported potent MEKK2 inhibitory activity.

2. Materials and methods

2.1. Materials

Common reagents such as HEPES, $MgCl_2$ and dimethyl sulfoxide (DMSO) were reagent grade quality and obtained from Sigma–Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Waltham, MA). The kinase inhibitor library (cat #L1200) and individual kinase inhibitors for confirmation were obtained from Selleck Chemicals (Houston, TX).

2.2. MEKK2 enzyme activity assays

The MEKK2 intrinsic ATPase activity assay and the transphosphorylation assay were performed as previously described [25]. MEKK2 activity assays were performed using ATP concentrations at the apparent Km for ATP of the assay format. The Km for ATP was previously reported for the ATPase assay to be 34 uM and therefore the assay employed 30 uM ATP. The Km for ATP in the transphosphorylation assay was previously reported to be 3.3 uM and therefore we used 3 uM ATP for this assay.

2.3. IC_{50} value determinations

IC_{50} was defined as the concentration of inhibitor that generates a 50% reduction in the specific signal of the assay. Compounds were dissolved at 10 mM in 100% DMSO to make stock solutions. Serial dilutions of compounds were performed in 100% DMSO then subsequently diluted into assay buffer and used in the MEKK2 enzymatic assay. The slot blot transphosphorylation digital bands were quantified using software on the Kodak 4000R Pro imaging station. For both MEKK2 assay formats, compound concentration response curves were generated using data points that represent the average of 2 or 3 determinations per concentration and 10 compound concentrations tested. All IC_{50} values provided are averages of at least three (ATPase assay) or two (transphosphorylation assay) independent determinations. The IC_{50} values were calculated from concentration response data using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA) employing either a four- or three-parameter curve fit.

3. Results and discussion

We have 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 [25]. This assay takes advantage of intrinsic ATPase activity of MEKK2 wherein MEKK2 alone, in the absence of any protein substrates, converts ATP to ADP. After stopping the reaction, relative ADP levels were then measured, with the ADP-Glo kit (Promega, Inc.). We have used this assay to screen a commercial library of 195 known and well-characterized kinase inhibitors. We chose the 7 most potent hits for further

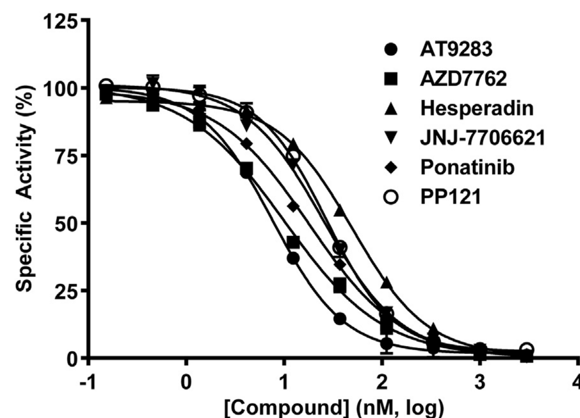

Fig. 1. Inhibitory activity of 6 known kinase inhibitors in the MEKK2 intrinsic ATPase assay. The compounds were tested at the indicated concentrations with the resultant raw signal normalized to maximum (DMSO only) and minimum (no MEKK2) controls, representing 100% and 0% enzyme activity, respectively. Data points represent the average of two determinations per concentration and error bars represent standard deviation (SD). This plot is representative of three independent experiments.

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