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COMMUNICATION

Synthesis and Biological Evaluation of 3-Arylindazoles as Selective MEK4 Inhibitors

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Abstract: Herein we report the discovery of a novel series of highly potent and selective mitogen-activated protein kinase kinase 4 (MEK4) inhibitors. MEK4 is an upstream kinase in MAPK signalling pathways that phosphorylates p38 MAPK and JNK in response to mitogenic and cellular stress queues. MEK4 is overexpressed and induces metastasis in advanced prostate cancer lesions. However, the value of MEK4 as an oncology target has not been pharmacologically validated because selective chemical probes targeting MEK4 have not been developed. Optimization of this series via structure activity relationships and molecular modelling led to the identification of compound **6ff** (4-(6-fluoro-2H-indazol-3-yl)benzoic acid), a highly potent and selective MEK4 inhibitor. This series of inhibitors is the first of its kind in both activity and selectivity and will be useful in further defining the role of MEK4 in prostate and other cancers.

MAPK signaling cascades are dysregulated in human cancer and inflammatory diseases, and small molecule inhibitors targeting MAPK signaling components are under intense investigation in the clinic.[1] A majority of MEK inhibitors target MEK1/2 including the FDA approved drug trametinib/Mekinist.[2] Most clinically relevant MEK inhibitors today target the MEK1/2 allosteric site and therefore show no activity against MEK3/4/5/6 or 7. As a result, there is a dearth of chemical matter directed at these other MAPK kinase family members, which is surprising given their roles in a host of biological processes. Consequently, their value as therapeutic targets has not been thoroughly investigated and new compounds that are selective for MEK family kinases beyond MEK1/2 could have tremendous potential. Most clinical studies with MEK inhibitors have yielded disappointing results, due at least in part to the paucity of biomarkers of MEK inhibitor sensitivity and toxicity. Bernards and co-workers recently showed data suggesting that cancers having mutations in MEK4 or its upstream kinase MEKK1, which are frequent in tumors of breast, prostate and colon, may respond to MEK inhibitors.[3] Their findings also suggest that MEKK1 and MEK4 are potential drug targets *in combination* with current MEK inhibitors, in spite of the fact that they are encoded by putative tumor suppressor genes.

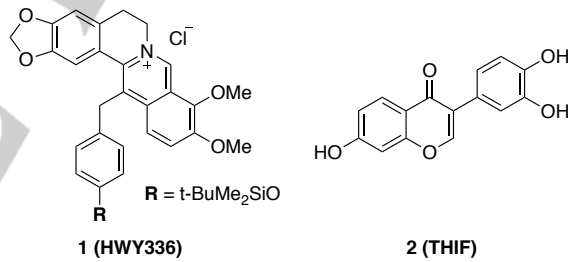


Figure 1. Structures of previously reported MEK4 inhibitors.

MEK4 (also known as MAP2K4, MKK4, SEK1) is a dual-specificity kinase, i.e., it phosphorylates serine/threonine as well as tyrosine residues, and it constitutes a second tier signaling protein of the canonical three-tier MAPK cascade.[4] MEK4 has become a target of interest for the therapeutic inhibition of prostate cancer (PCa) metastasis.[5] Although often described only as an activator of JNK, MEK4 also activates p38α and p38β, which complicates any investigation in this area.[1d] MEK4 is overexpressed in advanced PCa lesions and induces invasion and metastasis in PCa.[5-6] MEK4 also appears to have a similar pro-invasion/pro-metastatic role in several other cancer types, including breast and pancreatic cancers.[7] Through genetic and chemical approaches, MEK4 was shown to increase the invasive potential of PCa cells *in vitro* by upregulating the production of several matrix metalloproteinases (MMP's) in response to TGF-β treatment.[5, 6d] Overexpressing MEK4 increased the number of metastatic deposits observed in a PCa mouse model.[6d] These

findings present MEK4 as a clinically important therapeutic target and underscore the need to develop selective MEK4 probes for *in vivo* target validation in advanced cancer model systems.

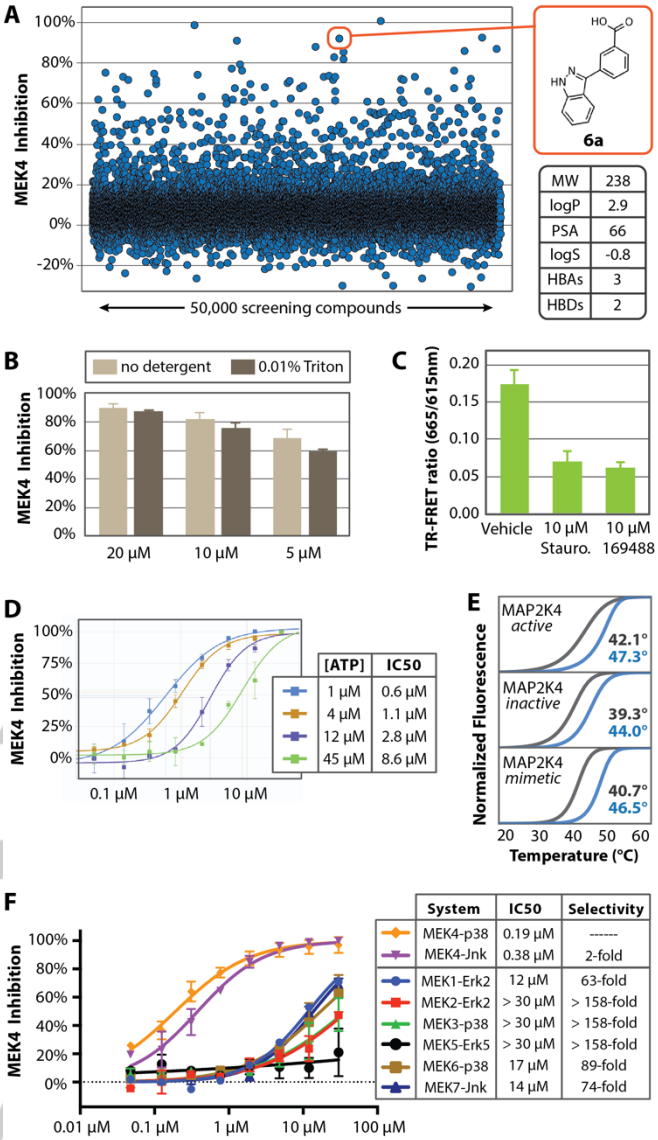
To date, in the literature there has been minimal advancement in MEK4 inhibitor development. HWY336 (**1**), a protoberberine derivative, inhibits both MEK4 and MEK7 (**Figure 1**).[8] HWY336 not only has poor selectivity and only moderate potency, but the pharmacological parameters are not ideal as it is a tetracyclic alkaloid, a compounds class known for promiscuity in biological effects.[9] Trihydroxyisoflavones have also been shown to have effects against MEK4 but not in a selective manner.[10] These isoflavones again are rather non-selective, for example 7, 3', 4'-trihydroxyisoflavone (THIF, **2**) also inhibits Cot activity.[10a] The current landscape of chemical tools to probe this important kinase further stresses the need to develop selective and pharmacologically robust MEK4 inhibitors.

Recognizing that MEK4 represents a novel and validated therapeutic target we sought to identify and characterize selective MEK4 inhibitors. Previously, we developed a platform for mapping the pharmacological relatedness of all seven MEK kinase family members to understand compound selectively.[11] Herein we discuss leveraging that foundational platform to screen compounds and identify a potent and selective hit molecule. Optimization and biological evaluation gave further insight into potential utilization of this series of compounds as selective MEK4 inhibitors.

To discover new inhibitors of MEK4, a library of 50,000 diverse compounds (commercially available ChemBridge DIVERSet-CL) was screened using an enzymatic ADP-Glo assay with active recombinant human MEK4 and full-length p38α substrate. The library was calculated to have a diversity index of 0.73 and determined that >90% of the compounds adhered to drug-like filters including Lipinski, Veber, and Pipeline Pilot SMARTS filters. Several compounds exhibited potent activity, and for this study subsequent work focused on a relatively small hit compound with an indazole core that inhibited MEK4 by 92% at 10 μM in the initial screen (**Figure 2A, 6a**). LC/MS analysis of the compound confirmed its mass and that its purity was > 95% (data not shown).

Compound **6a** was next tested to determine if its activity could be attributed to compound aggregation. A large fraction of hit compounds in wide-ranging HTS campaigns have been found to inhibit the target protein by forming hydrophobic aggregates that nonspecifically interact with the protein.[12] Since detergents have been shown to disrupt compound aggregation, a common way to identify these artifacts is by adding detergent to the assay and looking for a substantial or complete loss of potency. Inhibitor **6a** was tested at several concentrations in the ADP-Glo assay using a buffer with and without 0.01% Triton X-100, and similar MEK4 inhibition profiles were observed in both cases (**Figure 2B**). Additionally, **6a** was examined by dynamic light scattering (DLS), another common way to identify compound aggregates. The compound was tested at 20, 5, and 1 μM in buffer with and without Triton X-100, and under no conditions were aggregates observed (data not shown).

To ensure **6a** does not nonspecifically interfere with the ADP-Glo assay technology (for example, as a luciferase inhibitor) 1μM ADP was spiked in to simulate enzymatic ATP turnover and no reduction in the luminescent signal at 20, 10, and 5 μM of compound was observed (data not shown). To further validate **6a** as a bona fide MEK4 inhibitor, an orthogonal TR-FRET assay was used to determine if the compound could displace a fluorescently-



tagged tracer bound to the MEK4 active site. Inhibitor **6a** at 10 μM reduced the TR-FRET ratio to background levels, similar to 10 μM staurosporine, a low nanomolar MEK4 and pan-kinase tool inhibitor (**Figure 2C**).

Figure 2. Identification and validation of 6a. (A) High throughput chemical screen using a functional ADP-Glo assay revealed **6a** as one of the most active hit compounds against MEK4. **(B) 6a** was tested with and without detergent in the ADP-Glo assay at three doses (n=2-4). **(C)** TR-FRET was used to evaluate competitive binding with an active site tracer to MEK4 (n=6). **(D) 6a** was tested at four different ATP concentrations and potency was determined using a logistic regression curve fit (n=2). **(E)** Thermal stability of various MEK4 constructs was analyzed with vehicle (grey curves) or **6a** (blue curves) using a Boltzmann curve fit. Data is representative of two independent runs. **(F) 6a** was titrated against the seven MEK family proteins to calculate the potency in each system by logistic regression (n=2).

The ability of **6a** to displace the active site TR-FRET probe, along with the potential for kinase hinge binding by the indazole moiety, suggested that the compound likely binds to the ATP pocket of the MEK4 active site. To further characterize the binding mode, an ATP titration experiment was performed. Direct ATP-competitive inhibitors generally experience a loss of potency when tested in the presence of high ATP concentrations, particularly at concentrations above the enzyme's K_M for ATP.[13]

We determined the K_M of MEK4 for ATP to be 3.0 ± 0.4 μM, so **6a** was tested at eight doses each with 1, 4, 12, and 45 μM of ATP. The potency of the inhibitor decreased with each stepwise increase in ATP concentration, suggesting an ATP-competitive binding mode (**Figure 2D**). As expected, a smaller shift was observed from 1 to 4 μM since 4 μM approximates the K_M value. Next, the fluorescence thermal shift (FTS) assay was used to determine the change in thermal stability of MEK4 in the presence of **6a**. The stability of a protein is commonly altered when it binds a ligand, and we previously demonstrated that a hallmark of MEK4 inhibitors is increased thermal stability.[14] Compound **6a** increased the melting temperature of three MEK4 constructs: (1) phosphorylated (active) full-length MEK4 (the same construct used in the ADP-Glo and TR-FRET assays described above), (2) the nonphosphorylated (inactive) kinase domain of MEK4, and (3) a mutant (S257E/T261E) phosphomimetic (partially active) version of the kinase domain, each by 5-6 degrees (**Figure 2E**). This suggests **6a** has little preference for binding the active versus inactive forms of MEK4.

Finally, to investigate if **6a** exhibits specificity for MEK4 relative to the other six MEK isoforms (which have 38-61% homology to MEK4), an ADP-Glo MEK assay panel[11] was used to determine the potency of this inhibitor against all seven MEK enzymes. The assays were run at a saturating concentration of full-length protein substrates (Erk2, Erk5, p38α, or Jnk1ß), and an ATP concentration below the K_M of ATP for each enzyme. The potency of **6a** against MEK4 was 0.19 μM and 0.38 μM using p38α and Jnk1ß substrates, respectively. In comparison, the next most potent target was MEK1, with an IC_{50} of 12 μM, indicating that **6a** has 30- to 60-fold selectivity for MEK4 relative to any other MEK protein (**Figure 2F**).

The identification and validation of **6a** as a novel ATP-competitive MEK4 inhibitor prompted us to model its binding to MEK4 and subsequently carry out medicinal chemistry in an effort to further improve its potency and selectivity. Molecular modeling of compound **6a** bound to MEK4 was carried out using the Glide docking engine implemented in the Schrödinger suite.[15] Several key interactions were identified from the molecular modeling. When analyzing the predicted binding poses of **6a**, critical hydrogen bonding interactions in the hinge region between the small molecule and the backbone of MEK4 residues Leu180 and Met181 were observed (**Figure 3**). The predicted binding poses are consistent with it being an ATP-competitive inhibitor (**Figure 2D**). Modeling also indicated a favorable electrostatic interaction between the **6a** carboxylate and Lys187. In addition, there appeared to be space extending from the 5- and 6- position of the indazole ring that was unoccupied. Replacement of the carboxylate with other hydrogen bond acceptors could maintain or strengthen this interaction while modulating the physiochemical properties of the inhibitors.

Synthesis of the new indazole analogs proceeded as shown in **Table 1** starting with commercially available indazoles **1**. Selective iodination at the 3-position was achieved using NaOH and iodine to provide the intermediate in yields ranging from 90-95% yield. The iodinated heterocycle was then treated with Boc_2O to protect the free N-H group, since it was found that unprotected indazoles either failed in the subsequent Suzuki reaction or had dramatically slower reaction rates. Thus, treatment of iodides **2** with a range of boronic acids (**3**) provided the final 3-aryl indazoles **4** in moderate to good yields. In several cases, the Boc-protecting group was lost during the course of the Suzuki reaction. In others,

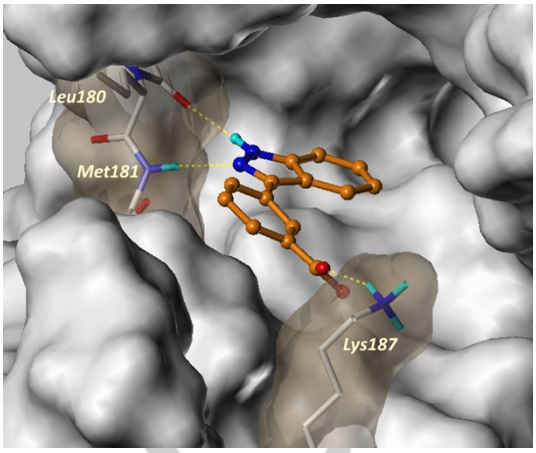


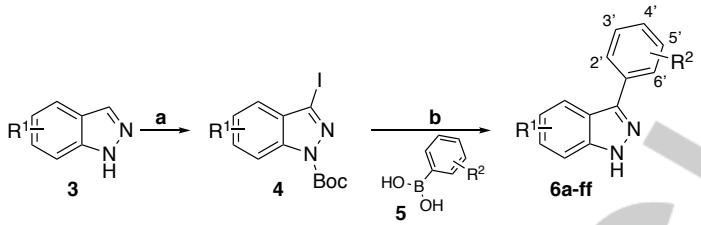
Figure 3. Modelling of 6a. Docked pose of **6a** with MAP2K4. Key interactions are highlighted, including those with hinge residues Leu180 and Met181, as well as an electrostatic interaction between the carboxylate and Lys187.

deprotection was carried out separately using TFA. For final compounds containing carboxylic acids, their methyl ester boronic acid was used in the Suzuki reaction and subsequent hydrolysis was then performed to generate the carboxylic acid.

Based on molecular modeling of unsubstituted indazole **6a**, it was expected that introduction of small hydrophobic groups on the indazole ring could occupy a back hydrophobic pocket. Addition of methyl- or chloro- groups at the indazole 4-position resulted in compounds with significantly decreased potency (**Table 1**). In contrast to unfavorable substitutions on the 4-position, addition of several different small hydrophobic groups on the 5-position resulted in compounds with greater MEK4 potency. Addition of a 5-CF_3 group onto the indazole in combination with 4'-CO_2H (**6n**) or 4'-OH (**6j**) resulted in inhibitors with comparable potency to the original unsubstituted hit compound (**6a**). The inhibitor with a CH_3 on the 5-position and a 4'-OH on the 3-aryl ring (**6q**) was almost 7-times more potent than the analogous 4-CH_3-substituted indazole compound (**6b**). Compounds with a 5-CH_3 on the indazole tended to be more potent when the 3-aryl ring was substituted at the 4'-position. Compounds with a Boc-group on the indazole N-1 were completely inactive, which is consistent with the modeling which shows the indazole N-H forming a key interaction with the kinase hinge region. While exploring the activity of 6-CF_3 analogs, the importance of the 3-aryl carboxylic acid moiety was also examined. Inhibitors with a methyl ester instead of an acid (e.g. **6y**) were completely inactive, while their carboxylic acid analog (**6z**) showed potent inhibition (IC_{50} of 0.72 μM). It was found that chloro-substituted indazoles possessed improved potency compared with their -CH_3 or -CF_3 analogs. Finally, several indazoles with a fluorine on the indazole 6-position were prepared and showed excellent potency for MEK4 (**6ee** and **6ff**) and were 5- and 10-fold more potent (respectively) than their 6-Cl analogs. Based on further molecular docking studies (Figure S1) it is suspected that the fluorine stabilizes most favourable planar confirmation of the compound while being the optimal size for the small polar/solvent expose region.

Having improved the MEK4 potency of our lead series, several of the most potent inhibitors were profiled against the entire MEK family to evaluate selectivity (**Table 2**). It was found that all compounds tested inhibited MEK4 more potently than any other

Table 1. Synthesis and SAR of substituted 3-aryl indazoles.



Compound	R^1	R^2	MEK4-p38, IC_{50}, μM [95% CI]	Compound	R^1	R^2	MEK4-p38, IC_{50}, μM [95% CI]
6a	H	3'-CO_2H	0.64 [0.53, 0.77]	6q	5-CH_3	4'-OH	0.59 [0.46, 0.75]
6b[b]	4-CH_3	4'-OH	4.0 [3.3, 4.8]	6r	5-CH_3	4'-$CONH_2$	0.56 [0.40, 0.77]
6c	4-Cl	3',4'-(OCH_2O)	4.5 [3.9, 5.2]	6s	5-CH_3	4'-CO_2H	0.15 [0.10, 0.23]
6d	4-Cl	4'-$CONH_2$	4.4 [3.9, 5.0]	6t	5-CH_3	3'-pyridyl	1.6 [1.1, 2.3]
6e	4-Cl	3'-pyridyl	> 22	6u	5-CH_3	3'-CH_2OH	1.4 [1.2, 1.8]
6f	4-Cl	3'-CH_2OH	7.2 [6.0, 8.6]	6v	5-CH_3	3'-$CONH_2$	0.97 [0.74, 1.3]
6g	4-Cl	3'-$C(CH_3)OH$	> 22	6w	5-CH_3	4'-CH_2OH	1.3 [1.1, 1.5]
6h	4-Cl	3'-$CONH_2$	1.3 [1.0, 1.6]	6x	6-CF_3	3',4'-(OCH_2O)	6.1 [4.9, 7.7]
6i	4-Cl	4'-CH_2OH	8.1 [7.2, 9.1]	6y	6-CF_3	4'-CO_2CH_3	> 20
6j	5-CF_3	4'-OH	0.76 [0.67, 0.86]	6z	6-CF_3	4'-CO_2H	0.72 [0.56, 0.92]
6k[c]	5-CF_3	4'-OH	> 20	6aa	6-Cl	3',4'-(OCH_2O)	0.46 [0.30, 0.71]
6l[c]	5-CF_3	3'-$CONH_2$	9.9 [7.3, 14]	6bb	6-Cl	4'-CO_2CH_3	2.2 [1.7, 2.9]
6m	5-CF_3	4'-$CONH_2$	1.1 [0.99, 1.3]	6cc	6-Cl	4'-CO_2H	0.12 [0.10, 0.14]
6n	5-CF_3	4'-CO_2H	0.47 [0.39, 0.55]	6dd	6-F	4'-CO_2CH_3	0.42 [0.35, 0.51]
6o[b]	5-CH_3	3'-CH_2OH, 4'-F	1.0 [0.69, 1.5]	**6ee**	**6-F**	**3',4'-(OCH_2O)**	**0.063 [0.029, 0.14]**
6p	5-CH_3	3',4'-(OCH_2O)	0.41 [0.36, 0.48]	**6ff**	**6-F**	**4'-CO_2H**	**0.041 [0.034, 0.049]**

[a] Reagents and Conditions: (a) i. NaOH, I_2, MeOH, rt, 24 h, 90-95%; ii. Boc_2O, rt, 16 h.; (b) Ar-$B(OH)_2$, $PdCl_2(dppf)\cdot DCM$, K_3PO_4, H_2O, 1,4-Dioxane, 90 °C, 1.5 h., 50-70%. [b] Compounds were purchased from ChemBridge. [c] Compound has a Boc-group on the indazole N-1 position.

MEK kinase. In general, compounds had the greatest selectivity against MEK5, 2, and 3. In particular, compound **6ff** displays excellent selectivity across the entire MEK family. This compound is at least 150-fold more potent against MEK4 than any other MEKkinase, and is at least 385-fold selective against three other MEK kinases.

Because compound **6ff** was a potent and selective (among the MEK family) MEK4 inhibitor in the functional assay, it was evaluated against 57 diverse kinases (see supporting information Table S1). These kinases were chosen to be a highly representative set of the broader kinome in accordance with recent work showing this approach provides an efficient way in which to assess overall kinase selectivity while minimizing experimentation.[16] In addition to the 50 kinases from the diversity panel,[16] the 7 MEK family members were also included. The original hit (**6a**) was also evaluated to allow comparison. Inhibitor **6a** was found to have an S(35) value of 0.088, meaning that it inhibited 8.8% of tested kinases > 35%, which in our case was 5 out of 57 kinases tested. Inhibitor **6ff** had an S(35) of 0.21, or 12 out of the 57 kinases. Both compounds were tested at 10 μM and both fully inhibited MEK4. Because both compounds fully inhibited MEK4 at the tested concentration of 10 μM and **6ff** is 3-4 fold more potent than **6a**, it is possible that **6ff** may be more selective than compound **6a** when both compounds are tested at concentrations that give equivalent MEK4 inhibition. Additional work to further define and improve the kinase selectivity of our lead series is underway.

A preliminary evaluation of the cellular efficacy of **6ff** was performed to investigate the potential involvement of MEK4 in cancer cell motility and migration. The results from a wound scratch assay showed that compound **6ff** had no efficacy at the concentrations tested (10 and 25 μM).[10b] Interestingly, compound **6ee** has modest efficacy at 10 μM and performs better at 25 μM, though never achieving the effect of control compound genistein (see Table S2), suggesting differences in cell permeability between the negatively-charged **6ff** carboxylate and the **6ee** ether may be relevant. A trypan blue exclusion for cells treated for 3 days to look at effects on proliferation and viability was also performed (see Figure S2). Interestingly, it was observed that both compounds (**6ff** and **6ee**) had less toxicity

compared to the promiscuous kinase inhibitor genistein (with p values < 0.05 when comparison of **6ff** and **6ee** against genistein

Table 2. MEK family profiling. All data are IC_{50} values in μM.

	MEK Isoform							
ID	**MEK 4 (p38)**	**MEK 4 (Jnk)**	**1**	**2**	**3**	**5**	**6**	**7**
6a	0.18	0.46	7.2	> 27	16	> 27	10	8.3
6b	0.92	0.88	11	15	>27	> 27	21	12
6j	0.25	0.24	4.6	5.3	2.3	> 27	1.6	2.2
6n	0.13	0.06	10	> 27	2.9	> 27	6.0	2.0
6o	0.39	0.75	4.0	5.4	6.0	> 27	3.4	4.9
6p	0.11	0.56	6.6	9.1	8.5	16	3.2	6.1
6q	0.21	0.41	7.7	7.2	5.5	25	2.2	5.4
6r	0.20	0.51	3.9	6.1	4.1	19	2.1	3.7
6s	0.18	0.22	17	> 27	12	> 27	8.7	11
6z	0.36	0.10	17	> 27	>27	0.35	8.8	6.9
6ee	0.26	1.5	6.6	9.3	21	> 27	5.8	14
6ff	**0.066**	**0.10**	**15**	**> 27**	**>27**	**> 27**	**11**	**12**

[a] Sample size n=2 and 95% confidence intervals were calculated for each compound tested.

was performed at 10 μM). The results could be indicative of the necessity to improve compound solubility and cell permeability.

In summary, we have evaluated and optimized the potency of a series of indazoles selective for MEK4 *in vitro*. To the best of our knowledge this is the first potent and selective (among the MEK family) inhibitor of MEK4. Analysis of the subtle differences of ligand binding in conjunction with the previous known trends of MEK family selectively that we have described allowed for the discovery of a potent and selective MEK4 inhibitor. The empirical binding affinities and functional potencies, along with sequential *in silico* docking studies, were used to predict the molecular features of the ligands responsible for activity and selectivity across this kinase family. The structure−activity relationship described herein indicates that the hit compounds are amenable to further optimization as they progress into more advanced *in vivo* and preclinical studies. The optimized compound **6ff** has strong potency and moderate selectivity which will be improved in the future. Further evaluation of the lead inhibitors is underway to validate and pursue MEK4 as a relevant cancer target.

Experimental Section

General methods for the synthesis and characterization of all compounds, NMR spectra, and methods for the in vitro protocols can be found in the supplemental information (PDF).

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Keywords: mitogen-activated protein kinase kinase 4 • MEK4 • MAP2K4 • indazoles

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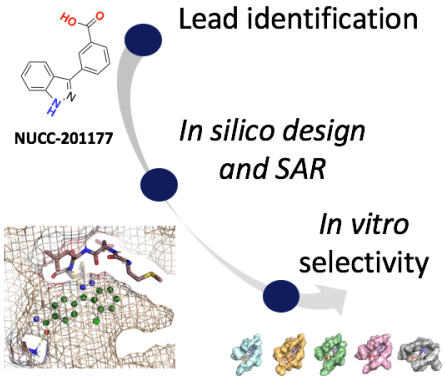

MEK4 is overexpressed and induces metastasis in advanced prostate cancer lesions. Herein we leveraged a MEK kinase platform we previously developed, for mapping the pharmacological relatedness of MEK kinase family members, to identify new potent and selective MEK4 inhibitors. We utilized computational docking, in vitro testing and preliminary cellular data to guide the optimization of this novel series of compounds as the first of its kind in both activity and selectivity.