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Aminocyanopyridine inhibitors of mitogen activated protein kinase-activated protein kinase 2 (MK-2)

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Abstract—A class of inhibitors of mitogen-activated protein kinase-activated protein kinase 2 (MK-2) was discovered. These compounds have demonstrated activity against the enzyme with IC_{50} values as low as 130 nM and suppress the expression of TNFα in U937 cells. These represent the first small molecule inhibitors of MK-2 to be reported.

TNFα has been implicated in several inflammatory disease states in humans.[1] Biologic anti-TNFα therapy has been shown to be effective in the treatment of diseases such as rheumatoid and psoriatic arthritis.[2] Numerous potential biological targets have been identified for inhibition to attenuate the biosynthesis of TNFα and hence be efficacious in the treatment of inflammatory disease. Among the best documented of these targets is p38 MAPK.[3,4] It was reported that the p38 kinase inhibitor VX-745 has demonstrated efficacy in rheumatoid arthritis patients in a clinical study.[5] Mitogen-activated protein kinase-activated protein kinase 2 (MK-2) is a direct substrate of p38 kinase and is linked to TNFα production.[6,7] An MK-2 knockout mouse has been reported to be resistant to disease in arthritis models.[6] Administration of lipopolysaccharide (LPS) to MK-2 knockout mice results in the rise of serum TNFα, but only to a maximum of 10–20% of the wild type control animals. Moreover, the MK-2 knockout mice were reported to be fertile and healthy. These reports suggest that MK-2 is an attractive target for the safe and effective treatment of TNFα mediated disease.

Through high throughput screening and early exploratory synthesis, we identified a series of benzopyranopyridines represented by structure **1** as modest inhibitors of MK-2 (Fig. 1). For example, compound **1a** exhibited an IC_{50} value of 1.94 μM[8] and was determined to bind in a competitive manner with ATP. Likewise, closely related analogs **1b–e** (Table 1) also showed MK-2 inhibition with low micromolar IC_{50} values.

Compounds **1a–e** were readily accessible via stepwise condensation of salicylaldehydes with 3 equiv of malononitrile as shown in Scheme 1. Although these were interesting lead molecules, the malononitrile group in the 5-position was deemed undesirable as a potential

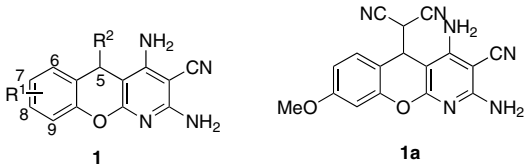

Figure 1. Benzopyranopyridine MK-2 inhibitors.

Keywords: MK-2; Kinase inhibitor; TNFα.
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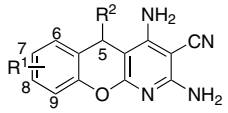
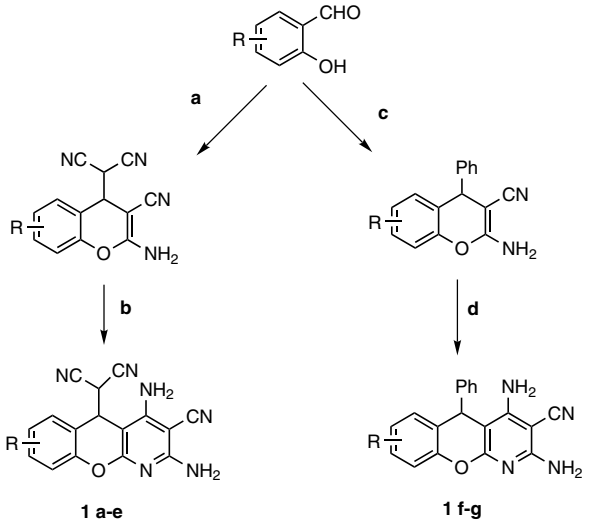
Table 1. MK-2 inhibition for compounds **1a–g**



1 a-g

Compd	R^1	R^2	MK-2 Inhibition IC_{50} (μM)[a]
1a	8-OMe	$CH(CN)_2$	1.94
1b	8-OH	$CH(CN)_2$	12.8
1c	7-OMe	$CH(CN)_2$	6.18
1d	7-Br	$CH(CN)_2$	4.22
1e	7-OH	$CH(CN)_2$	3.8
1f	8-OH	Ph	>200
1g	8-OMe	Ph	>200

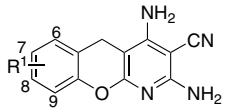
[a] IC_{50} values determined by method in Ref. 8.



1 a-e **1 f-g**

Scheme 1. Reagents and conditions: (a) $CH_2(CN)_2$ (2 equiv), cat. piperidine/EtOH, rt; (b) $CH_2(CN)_2$, cat. piperidine/EtOH, reflux; (c) Ph–C═C$(CN)_2$, cat. piperidine, EtOH, reflux; (d) $CH_2(CN)_2$, pyridine, reflux.

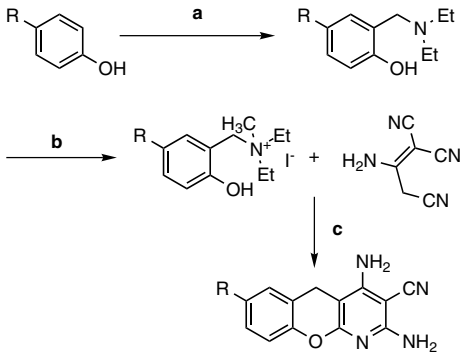
Table 2. IC_{50} values for aminocyanopyridine inhibitors against MK-2 and cellular TNFα expression



2-a-m

Compd	R^1	MK-2 Inhibition IC_{50} (μM)[a]	U937 TNFα IC_{50} (μM)[b]
2a	7,8-Di-OH	0.13	0.92
2b	8-OH	0.18	1.08
2c	7-OH	0.47	25.4
2d	7,8-Di-OMe	0.33	64.8
2e	9-OH	0.46	7.12
2f	7,8-O-CH_2CH_2O-	0.49	55.7
2g	7-NH_2	0.53	>100
2h	H	0.56	>100
2i	7-OH,8-Et-O-$(CH_2)_2$O	0.62	11.9
2j	6,8-Di-OH	0.69	>100
2k	7-OH,8-EtO	0.77	9.4
2l	8,9-Di-OH	0.85	6.92
2m	8-O-$(CH_2)_2$-pyrrolodine	1.68	2.91

[a] IC_{50} values determined by method in Ref. 8.
[b] IC_{50} values determined by method in Ref. 9.

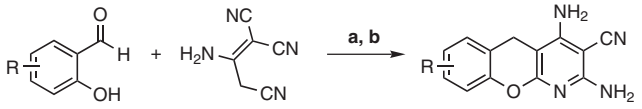


Scheme 2. Reagents and conditions: (a) Et_2NH, CH_2O; (b) CH_3I/acetone; (c) EtOH, cat. piperidine, heat.

toxicophore as well as a chemically reactive functional group. Hence our initial efforts of optimizing structure **1** were focused on either replacing the malononitrile group with alkyl or aryl groups or excising it completely. Analogs **1f** and **1g** bearing a phenyl group in the 5-position were prepared via sequential condensation of salicylaldehydes with benzylidenemalononitrile and malononitrile as shown in Scheme 1. Attempts to prepare 5-alkyl substituted analogs using this methodology were unsuccessful. Both **1f** and **1g** were completely devoid of MK-2 inhibition (Table 1).

Significant improvement in MK-2 inhibition was achieved with 5-unsubstituted analogs. For example, compound **2d** exhibited an MK-2 IC_{50} value of 0.33 μM (Table 2). These compounds can be prepared by the method described in Scheme 2. The Mannich reaction of a *para* substituted phenol with diethylamine and formaldehyde is used to introduce a diethylaminomethyl group in the *ortho* position. Conversion of the tertiary amine to a quaternary salt was followed by reaction with malononitrile dimer to produce the desired target compounds.

This method was limited to the *para* substituted phenols as starting materials, however, and alternative methods were sought to prepare a wider range of analogs. A modified synthetic scheme outlined in Scheme 3 allows for the incorporations of a wider variety of substitution patterns. Thus, malononitrile dimer was condensed with substituted salicyaldehydes in acetic acid and ethanol (1:1). The solvents were evaporated and the residue taken up in trifluoroacetic acid and reduced with triethylsilane. The products usually precipitated out of the reaction mixture as pure TFA salts. In cases where the products were not pure, the final products were purified by reverse phase chromatography. This method allowed for the rapid synthesis of analogs using parallel synthesis.

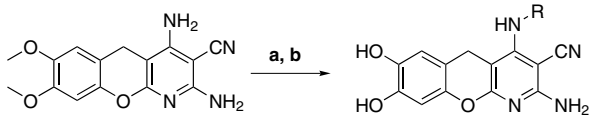


Scheme 3. Reagents and conditions: (a) EtOH/AcOH (1:1); (b) TFA/Et_3SiH.

Using these routes, potent inhibitors of MK-2 were prepared. Table 2 provides some illustrative examples. The most potent compounds discovered in this series have a hydroxyl group at either the 8- (compound **2b**) or at both the 7- and 8-positions (compound **2a**) of the phenyl ring. The corresponding 7-hydroxy analog (compound **2c**) was less potent, as was the 9-hydroxy analog (compound **2e**). It appears that hydroxyl groups in the 7- and 8-positions are involved in hydrogen bonding to the target. Compound **2e** apparently cannot engage in any such interaction because it has the same potency as the unsubstituted analog **2h**. Methoxy substitution is also tolerated as demonstrated by example **2d**, but an amine (compound **2g**) is essentially equipotent with the unsubstituted phenyl. Similarly, tying the oxygens of compound **2a** into a ring resulted in a compound (**2f**) no more potent than the unsubstituted phenyl (**2h**). Further elaboration of the phenols resulted in losses in potency of MK-2 inhibition. For example, appending a 1-pyrrolidinylethyl group to the 8-hydroxy compound (compound **2m**) resulted in a large drop in potency (~10×). The most potent analogs found were also active in a cellular assay (Table 2). U937 cells were treated with compound **2a** for 1 h and then challenged with LPS.[9] Several compounds in this series were found to be active in this assay. These results indicate that small molecule MK-2 inhibitors are effective in reducing cellular TNFα expression.

Substitution at the 4-amino position with small alkyl groups can be accomplished by treating **2d** with sodium hydroxide in DMSO with an alkyl halide (Scheme 4). The methoxy groups were demethylated with BBr_3 to produce the final compounds. The activities of these compounds are reported in Table 3.

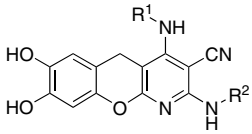
In general these compounds are only slightly less active than the unsubstituted analogs, suggesting that both hydrogens of the 4-amino group are not involved in hydrogen bonding and that there may be some space for further elaboration. Substitution at both the 4- and 2-amino groups (compound **3d**) resulted in a compound with no measurable activity. This suggests that either both hydrogens of the 2-amino group are involved in



2d **3a-d**

Scheme 4. Reagents and conditions: (a) NaOH, DMSO, R-Br; (b) BBr_3/DMSO.

Table 3. IC_{50} values for aminocyanopyridine inhibitors against MK-2



3a-d

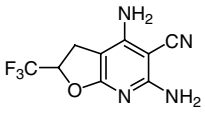
Compd	R^1	R^2	MK-2 Inhibition IC_{50} (μM)[a]
3a	CH_2-CH_2-OH	H	0.237
3b	Pr	H	0.403
3c	Et	H	0.419
3d	Et	Et	>200

[a] Values determined by method in Ref. 8.

critical hydrogen bonding to the target or there is no room to accommodate an alkyl group at this position.

The heteroatom of the central ring was also replaced. The nitrogen and sulfur analogs were prepared starting from the corresponding *ortho*-amino or mercapto benzaldehydes in accordance with Scheme 3. The nitrogen analog required further substitution to avoid aromatization. It was found that neither of these substitutions was well tolerated (Table 4). The sulfur analog was inactive against the target and the *N*-methyl analog (compound **4c**) was only weakly active compared to compound **2h**.

The core of these compounds can be truncated as well. Commercially available **5** was also found to be an inhibitor of MK-2 (IC_{50} = 2 μM). This suggests that the amino cyano pyridine substructure is an important part of the MK-2 binding pharmacophore. While the benzopyran portion of the active structures described herein also appears to contribute significantly to binding, the above result further suggests that it may be optimized in a variety of ways.



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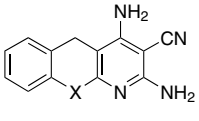
Compounds that were active in the cellular assay were also tested in vivo. Compound **2a** was found to be active in a rat LPS assay (20 mpk, IP, 68% inhibition) and **2m**

Table 4. IC_{50} values for aminocyanopyridine inhibitors against MK-2



4a-c

Compd	X	MK-2 Inhibition IC_{50} (μM)[a]
4a	S	>200
4b	SO_2	>200
4c	N-Me	70

[a] Values determined by method in Ref. 8.

was found to be orally active (20 mpk, PO, 60% inhibition).[10]

In conclusion, a class of small molecule MK-2 inhibitors has been discovered and the basic SAR of the series has been described. These inhibitors are effective in attenuating TNFα production in cellular assays and are active in vivo as well.

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8. MK-2 IC_{50} value determination: Recombinant MAPKAPK2 was phosphorylated at a concentration of 42–78 mM by incubation with 0.23 mM of active p38a in 50 mM HEPES, 0.1 mM EDTA, 10 mM magnesium acetate, and 0.25 mM ATP, pH 7.5 for 1 h at 30 °C. The phosphorylation of HSP-peptide (KKKALSRQLSVAA) by MAPKAPK2 was measured using an anion exchange resin capture assay method. The reaction was carried out in 50 mM β-glycerolphosphate, 0.04% BSA, 10 mM magnesium acetate, 2% DMSO, and 0.8 mM dithiotheritol, pH 7.5 in the presence of the HSP-peptide with 0.2 μCi [g33P]ATP and 0.03 mM ATP. The reaction was initiated by the addition of 15 nM MAPKAPK2 and was allowed to incubate at 30 °C for 30 min. The reaction was terminated and [g33P]ATP was removed from solution by the addition of 150 mL of AG 1X8 ion exchange resin in 900 mM sodium formate pH 3.0. A 50 mL aliquot of head volume was removed from the quenched reaction mixture and added to a 96-well plate, 150 mL of Microscint-40 (Packard) was added and the amount of phosphorylated-peptide was determined. The assay is performed at a final concentration of 2% DMSO. The error for this assay is taken to be 0.2 log units.
9. The human monocyte-like cell line, U937 (ATCC #CRL-1593.2), is cultured in RPMI1640 media with 10% heat-inactivated fetal calf serum (GIBCO), glutamine and pen/strep at 37 °C and 5% CO_2. Differentiation of U937 to monocytic/macrophage-like cells is induced by the addition of phorbol12-myristate 13-acetate (Sigma) at final concentration of 20 ng/mL to a culture of U937 cells at ~0.5 million cells/mL and incubated for 24 h. The cells are centrifuged, washed with PBS and resuspended in fresh media without PMA and incubated for 24 h. Cells adherent to the culture flask are harvested by scraping, centrifugation, and resuspended in fresh media to 2 million cells/mL, and 0.2 mL is aliquoted to each of 96 wells in flat-bottom plate. Cells are then incubated for an additional 24 h to allow for recovery. The media is removed from the cells, and 0.1 mL of fresh media is added per well. 0.05 mL of serially diluted compound or control vehicle (Media with DMSO) is added to the cells. The final DMSO concentration does not exceed 1%. After 1 h incubation, 0.05 mL of 400 ng/mL LPS (*E. coli* serotype 0111:B4, Sigma) in media is added for final concentration of 100 ng/mL. Cells are incubated at 37 °C for 4 h. After 4 h incubation, supernatants are harvest and assayed by ELISA for the presence of TNFα. The error for this assay is taken to be 0.5 log units.
10. Adult male 225–250 g Lewis rats (Harlan Sprague–Dawley) were used. Rats were fasted 18 h prior to oral dosing, and allowed free access to water throughout the experiment. Each treatment group consisted of five animals. Compounds were prepared as a suspension in a vehicle consisting of 0.5% methylcellulose, 0.025% Tween-20 in PBS. Compounds or vehicle were orally administered in a volume of 1 mL using an 18-gauge gavage needle. LPS (*E. coli* serotype 0111:B4, Lot #39H4103, Cat. # L-2630, Sigma) was administered 1–4 h later by injection into the penile vein at a dose of 1 mg/kg in 0.5 mL sterile saline. Blood was collected in serum separator tubes via cardiac puncture 1.5 h after LPS injection, a time point corresponding to maximal TNFα production. After clotting, serum was withdrawn and stored at −20 °C until assay by ELISA.