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Inhibition of Tpl2 kinase and TNF-α production with 1,7-naphthyridine-3-carbonitriles: Synthesis and structure–activity relationships

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Abstract—The synthesis and structure–activity studies of a series of 6-substituted-4-anilino-[1,7]-naphthyridine-3-carbonitriles as inhibitors of Tpl2 kinase are described. The early exploratory work described here may lead to the discovery of compounds with significant therapeutic potential for treating rheumatoid arthritis and other inflammatory diseases.

Tumor necrosis factor-α (TNF-α), a pro-inflammatory cytokine, is involved in inflammation in a number of disease states, most notably in the autoimmune disease rheumatoid arthritis (RA). RA occurs in nearly 1% of the population with an annual incidence of 0.04%.[1] The debilitating aspects of RA are associated with the erosion of cartilage and bone leading to joint pain, swelling, stiffness, and restricted mobility. The protein therapeutic ENBREL®/etanercept, a soluble form of human recombinant TNF-α receptor (sTNFR-α), is currently available to patients with RA and has been a major advance in treating the disease; however, an orally available small molecule that inhibits TNF-α synthesis and/or signaling would have widespread therapeutic potential.[2]

Tpl2 (Cot/MAP3K8) is a serine/threonine kinase in the MAP3K family that is upstream of MEK in the ERK pathway.[3] Recent studies using Tpl2 knock-out mice indicate an important role for Tpl2 in the LPS-induced production of TNF-α and other pro-inflammatory cytokines.[4] Tpl2 is also required for TNF-α signaling (i.e., the cellular response to ligation of the TNF-α receptor), and thus an inhibitor of Tpl2 would have the double benefit of blocking both TNF-α production *and* signaling.[5] Furthermore, the unique features of Tpl2 presumably increase the potential for discovering a selective Tpl2 inhibitor. Tpl2 is not inhibited by staurosporine, a non-specific kinase inhibitor, and it is the only human kinase that has a proline instead of a conserved glycine in the glycine-rich ATP binding loop.[6]

Screening of our in-house kinase library provided us with several classes of reversible and ATP-competitive hits (Fig. 1), one of which, a 6-substituted-4-anilino-[1,7]-naphthyridine-3-carbonitrile **1**, originated in

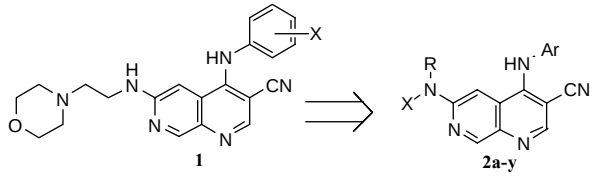

Figure 1. Optimization of 6-substituted-4-anilino-[1,7]-naphthyridine-3-carbonitriles.

Keywords: Tpl2 kinase; 1,7-Naphthyridine-3-carbonitriles.
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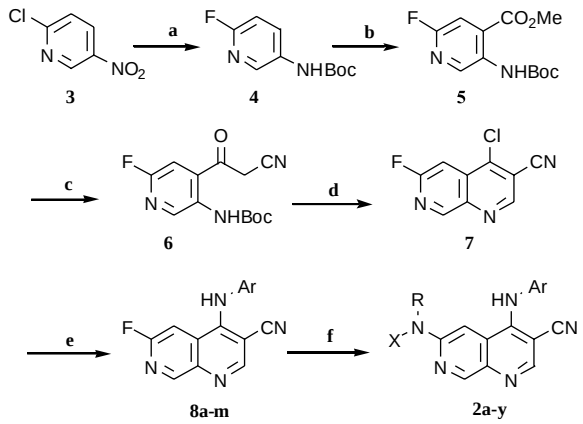


Scheme 1. Reagents and conditions: (a) i—KF, DMSO, 70 °C, 18 h; ii—$SnCl_2$–H_2O, EtOAc; iii—Boc_2O, *t*-BuOH, 40 °C, 3 h, 53%, 3 steps. (b) i—*n*-BuLi, TMEDA, ether, −78 °C then CO_2; ii—$TMSCH_2N_2$, $CHCl_3$/MeOH, 51%, 2 steps. (c) CH_3CN, *n*-BuLi, THF −78 °C, 76%. (d) i—DMF–DMA, rt, 1 h; (ii) oxalyl chloride, DMF, CH_2Cl_2, 59%, 2 steps. (e) $ArNH_2$, EtOH, reflux 6–12 h, 80–94% or $ArNH_2$, DME, 110 °C microwave, 15–45 min, 95%. (f) RXNH, pyridine, 80 °C, 3 days, 30–50% or RXNH, THF or neat, 120–180 °C microwave, 0.5–2 h, 12–60%.

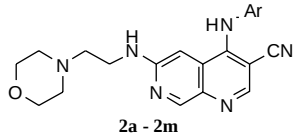
another kinase program targeting a receptor tyrosine kinase. In this manuscript, we wish to describe the early-stage lead exploration of a series of 6-substituted-4-anilino-[1,7]-naphthyridine-3-carbonitriles **2a**–**y**. The synthesis, structure–activity relationships, and in vitro TNF-α inhibition studies of Tpl2 inhibitors are described herein.

Scheme 1 illustrates the synthesis of 6-substituted-4-anilino-[1,7]-naphthyridine-3-carbonitriles. The synthesis of the key intermediate 4-chloro-6-fluoro-[1,7]-naphthyridine-3-carbonitrile **7** has been previously described.[7] Compound **7** was condensed with a variety of anilines ('headpieces') either in refluxing ethanol or via microwave irradiation using a slight excess of the aniline to give the penultimate intermediates **8a**–**m**.

Displacement of the C-6-fluoro atom in **8a**–**m** with various amino-containing groups ('tailpieces') via heating of the mixture in pyridine for 3–5 days gave the target compounds **2a**–**y** in moderate yields.[7] Microwave irradiation dramatically reduced the reaction times for the conversions and in some cases the final two steps were carried out in one pot without purification.[8] The yields for these microwave transformations were variable (on average 35–60%) and dependent on the amine nucleophile with more nucleophilic amines giving higher yields and fewer by-products.

Naphthyridine derivatives **2a**–**y** were first studied for inhibition of isolated Tpl2 enzyme, via quantification of MEK phosphorylation in an ELISA format.[9] For the purpose of independently comparing the effects of the 4-aminoaryl and 6-amino groups on activity, the data are separated into Table 1 [4-(arylamino)-6-(2-morpholinoethylamino)-naphthyridine carbonitriles (**2a**–**m**)] and Table 2 [4-(3-chloro-4-fluorophenylamino)-6-(alkylamino)-naphthyridine-3- carbonitriles (**2a** and **2n**–**y**)]. In the 6-(2-morpholinoethylamino) series (Table 1), large non-rigid substituents in the *para* position of the 4-anilino ring, such as benzyl, thiophenyl, and phenoxy, greatly improved Tpl2 inhibition with IC_{50}s of 2–380 nM (**2b**, **2c**, and **2d**). In contrast, the addition of a more polar sulfonyl or carbonyl group between the two aromatic rings had reduced inhibition (**2f**, 4600 nM) or loss of inhibition (**2e**, >40,000 nM). Despite the tolerance of Tpl2 for large hydrophobic groups on the 4-anilino headpiece, a derivative with a methylene group inserted between the 4-anilino nitrogen atom and the aromatic ring (3-chloro-4-fluorobenzyl derivative **2g**,

Table 1. Inhibition of Tpl2 kinase by 4-(arylamino)-6-(2-morpholinoethylamino)-3-cyano-[1,7]-naphthyridines (**2a**–**m**)

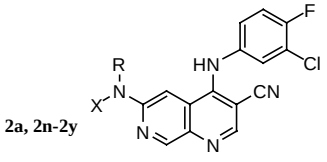


2a - 2m

Compounds	Ar	IC_{50} (nM)[a]
2a	3-Chloro-4-fluorophenyl	1,200
2b	4-Benzylphenyl	2
2c	4-(Phenylthio)phenyl	6
2d	4-Phenoxyphenyl	380
2e	4-(Phenylsulfonyl)phenyl	>40,000
2f	4-(Phenylmethanone)phenyl	4,600
2g	3-Chloro-4-fluorobenzylamine	>40,000
2h	4-Trifluoromethylphenyl	>40,000
2i	4-Chlorophenyl	>40,000
2j	4-Chloro-2-fluorophenyl	10,000
2k	3,4-Dichlorophenyl	10,000
2l	3-Bromo-2-methylphenyl	>40,000
2m	3,5-Dichlorophenyl	4,200

[a] Average of at least two experiments (see Ref. 9).

Table 2. Inhibition of Tpl2 kinase by 4-(3-chloro-4-fluorophenylamino)-6-amino-3-cyano-[1,7]-naphthyridines (**2a** and **2n**–**y**)



2a, 2n-2y

Compounds	R (amine)	X	IC_{50} (nM)[a]
2a	2-(Morpholino)CH_2CH–	H	1,200
2n	3-(Morpholino)$CH_2CH_2CH_2$–	H	3,400
2o	4-Me-(piperazin-l-yl)$CH_2CH_2CH_2$–	H	21,000
2p	(Pyridin-3-yl)CH_2–	H	50
2q	Benzyl	H	73
2r	2-Morpholino-l-phenylethyl–	H	1,800
2s	(1*S*)-1-phenylethylamino	H	27,000
2t	(1*R*)-1-phenylethylamino	H	12
2u	(1*R*)-1-phenylpropylamino	H	414
2v	(1*S*)-1-phenylpropylamino	H	>40,000
2w	Benzyl	Me	>40,000
2x	Cyclopentyl	H	>40,000
2y	3-(Pyrrolidinyl)propyl–	H	3,565

[a] Average of at least two experiments.

>40,000 nM) gave dramatically reduced inhibition. In the absence of a large hydrophobic group at the *para* position of the headpiece, a 3-halo group appears to be necessary for good inhibitory activity, as exemplified by the two inactive analogs **2h** and **2i**. Other analogs containing *meta*-substituted aniline headpieces bearing such groups as alkyl, carbonyl, phenoxy, sulfonyl, and amino were also inactive (not shown).

Structure–activity relationship data previously reported by Wyeth for 4-anilinonaphthyridine-3-carbonitriles,[10] and by other research groups for 4-anilinoquinazolines,[11] indicated that substituents at the C-6 and/or C-7 positions have less influence on isolated kinase activity than the headpiece unit. Thus, the C-6 and/or C-7 positions have been used to introduce groups that improve the solubility and permeability of inhibitors. In the present case, it was found that for the [1,7]-naphthyridine-3-carbonitrile series, a large range of inhibitory activity was dependent on the length, the hybridization, and the stereochemistry of the tailpiece moiety (Table 2). For example, **2a** with a 2-(morpholino)ethylamino group was nearly three times as potent as the 3-methylene containing analogs **2n** and **2y**, and more than 17 times as potent as the 4-piperazinyl analog **2o** (IC_{50} of 21 μM).

A significant improvement in inhibitory activity was achieved with the installation of CH_2-aryl and CH_2-heteroaryl groups at the 6-amino position. The 3-pyridylmethylamino analog **2p** and the benzyl analog **2q** were potent inhibitors of Tpl2 (50 and 73 nM, respectively).

Alkyl and cycloalkyl analogs, represented by derivative **2x**, were not active. Racemic 2-morpholino-1-phenylethylamino derivative **2r**[12] was less potent than **2a** with an IC_{50} of only 1.8 μM. The stereochemical configuration of the tailpiece affects the inhibition of Tpl2 (enantiomeric pairs **2s/2t** and **2u/2v**). In these two cases, the *R*-isomer was 100- to >2000 times more potent than the corresponding *S*-isomer. The importance of a secondary rather than a tertiary amino group at C-6 was seen; when the 6-amino group of **2q** was N-methylated to give **2w**, activity was lost.

Selectivity studies were carried out with Tpl2 inhibitors against a panel of kinases, including kinases known to be involved in TNF-α production (p38 and MK2), and other serine-threonine and tyrosine kinases (Table 3). Analog **2a** showed moderate selectivity over most kinases; however, in a cell-based assay, **2a** showed strong inhibition of EGFR at 5 μM.[10] Analogs with the same tailpiece as **2a**, but with larger hydrophobic headpieces, showed improved selectivity against MEK (**2b**, **2c**, and **2d**) and had reduced inhibition of cellular EGFR, although **2b** and **2c** showed poor selectivity against Src. Of the five compounds tested in this panel, **2l** showed the best combination of potency and selectivity.

With information on the selectivity profiles of these [1,7]-naphthyridine-3-carbonitriles, they were then evaluated in both cellular[13] and blood environments (Table 4).[14] Included in Table 4 are the physicochemical properties that may influence the activity of these derivatives in these assays, specifically solubility, permeability, and protein binding.[15] TNF-α inhibition was diminished in human blood compared to human monocytes for all the compounds tested. The 2-(morpholino)ethylamino tailpiece analog **2a** showed the lowest potency in human monocytes, and yet, apparently due to lower protein binding, high solubility, and higher permeability it was the most active in blood. Analogs **2p** and **2b** were the most potent in monocytes. Low solubility and low permeability, coupled with higher protein binding, were presumably why these two analogs were weaker than **2a** in blood.

Table 3. In vitro selectivity profiles of selected 6-amino-4-anilino-3-cyano-[1,7]-naphthyridines[a]

Compounds	Tpl2	MEK	p38	Src	MK2	PKC	EGFR[b]
2a	1.200	15	58	40	>400	30	100%–5 μM
2p	0.050	>40	180	>400	110	>400	50%–5 μM
2b	0.002	0.63	>400	4	>400	>400	50%–5 μM
2d	0.380	0.80	>400	90	>400	200	50%–5 μM
2c	0.006	0.96	31	3	>400	>400	10%–5 μM

[a] Average of at least two experiments (μM).
[b] A431 cell-based assay from an average of three experiments.

Table 4. In vitro inhibition of TNF-α production with selected inhibitors[a]

Compounds	Human monocytes[a,b]	HWB[b]	Solubility (μg/mL)	Permeability ($\times 10^6$ m/s)	Plasma protein binding[c]
2a	4.5	6.8	64	3.78	85
2p	0.7	8.5	2	0.1	98
2b	0.8	13.6	BLD[d]	BLD	99
2c	2.7	20.1	1	0.01	>99
2d	1.4	14.9	1	BLD	>99

[a] Lipopolysaccharide-induced TNF-α from primary human monocytes and human whole-blood (IC_{50} μM).
[b] Average of at least two experiments.
[c] Percent bound to human serum albumin.
[d] Below limit of detection.

Derivatives **2d** and **2c** were the least potent in monocytes and both demonstrated high protein binding, and poor solubility and permeability. The poor physicochemical properties of **2d** and **2c** most likely precluded TNF-α inhibition in blood.

We have explored Tpl2 inhibition and functional TNF-α inhibition with a class of 6-alkylamino-4-anilino-[1,7]-naphthyridine-3-carbonitriles. In this study, it was found that a large range of inhibitory activity depended on the length, the hybridization, and the stereochemistry of the tailpiece moiety, while headpiece modifications resulted in potent Tpl2 inhibitors with less selectivity over other kinases than the 3-chloro-4-fluorophenyl headpiece analogs. This initial SAR study was used to exploit continued optimization toward the discovery of Tpl2 inhibitors for the treatment of TNF-α-driven diseases such as rheumatoid arthritis. Further studies and modifications in physicochemical properties and improvements in in vitro and in vivo selectivity profiles for this series will be reported in due course.

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8. A typical preparation of target compounds **2b**–**t** is illustrated by the following procedure for **2h**: in a 100 mL round-bottomed flask fitted with a condenser, 4-chloro-6-fluoro-[1,7]-naphthyridine-3-carbonitrile (0.250 g, 1.20 mmol) and 4-chloroaniline (0.168 g, 1.32 mmol) were taken up in 20 mL of 2-ethoxyethanol and heated at reflux for 1 h, until t.l.c. analysis (20% EtOAc in hexanes) showed complete disappearance of the 4-chloronaphthyridine. After cooling to room temperature, the reaction mixture was partitioned between 40 mL of each EtOAc and 5% Na_2CO_3. The aqueous layer was extracted twice more with EtOAc, and the combined organic layers were washed three times with brine, dried over anhydrous $MgSO_4$, filtered, and evaporated to give 4-(4-chlorophenylamino)-6-fluoro-[1,7]-naphthyridine-3-carbonitrile as a crystalline yellow solid, which was of sufficient purity to be used directly in the next step: ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.41 (d, J = 8.6 Hz, 2H) 7.47–7.58 (m, 2H) 8.18 (s, 1H) 8.69 (s, 1H) 9.07 (s, 1H) 10.11 (s, 1H). The product of the first step (0.179 g, 0.600 mmol assuming 100% yield of step 1) was taken up in a microwave vial in 3.4 mL THF, with 4-(2-aminoethyl)morpholine (1.6 mL, 1.6 g, 12 mmol). The sealed vial was heated in a microwave reactor at 150 °C for 1 h, until t.l.c. analysis showed complete disappearance of the starting material. Then THF was removed under reduced pressure, and the crude product was purified by flash chromatography over silica gel (7% MeOH in CH_2Cl_2) and lyophilized to give a fluffy, bright yellow solid (105 mg, 43% yield of 2 steps): ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.43 (br s, 4H) 2.56 (t, J = 6.2 Hz, 2H) 3.37 (q, J = 6.6 Hz, 2 H) 3.49–3.64 (m, 4H) 6.65 (t, J = 5.8 Hz, 1H) 7.01 (s, 1H) 7.31 (d, J = 8.6 Hz, 2 H) 7.46 (d, J = 8.8 Hz, 2H) 8.28 (s, 1H) 8.85 (s, 1H) 9.63 (s, 1H); HRMS (ESI+) calcd for $C_{21}H_{22}ClN_6O$ (MH+) 409.1538, found 409.1542.
9. Tpl2/Cot activity was directly assayed using GST-MEK1 as a substrate. The phosphorylation on serine residues 217 and 221 of GST-MEK1 was detected by an ELISA. Briefly, 0.4 nM Tpl2 was incubated with 35 nM GST-MEK1 in a kinase reaction buffer containing 20 mM Mops, pH 7.2, 50 μM ATP, 20 mM $MgCl_2$, 1 mM DTT, 25 mM β-glycerophosphate, 5 mM EGTA, and 1 mM sodium orthovanadate for 1 h at 30 °C. Compounds solubilized in 100% DMSO were pre-diluted in assay buffer so that the final concentration of DMSO in the reaction was 1%. The kinase reaction was carried out in 100 μl volume on 96-well plates. The kinase reaction was then stopped with the addition of 100 mM EDTA. The entire reaction mix was then transferred to the detection plate, a 96-well Immunosorb plate that had been pre-coated with anti-GST antibody (Amersham). After 1 h incubation at room temperature, the detection plate was washed four times with TBST (TBS + 0.05% Tween 20) and then incubated for another hour at room temperature with anti- phospho-MEK1 antibody (Cell Signaling) 1:1000 in 10 mM Mops 7.5, 150 mM NaCl, 0.05% Tween 20, 0.1% gelatin, 0.02% NaN_3, and 1% BSA. The detection plate was washed again and incubated for 30 min with DELFIA Europium (Eu) labeled goat anti-rabbit IgG (Perkin-Elmer), 1:4000 in the same buffer used for the primary incubation. After a final wash, Eu detection solution was added to each well and the Eu signal was measured in a Wallac Victor Multilabel Counter. Data were imported into Excel and IC50 calculations were performed using the Xlfit (IDBS) software package.
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13. Inhibition of TNF-α in primary human monocytes: human blood buffy coat (containing 1 mM EDTA) is incubated with RosetteSep human monocyte enrichment antibody cocktail (Stem Cell Technologies #15068). This mixture is then diluted with an equal volume of PBS/2% FBS/1 mM EDTA, layered onto an equal volume of Histopaque-1077 (Sigma #H8889), and centrifuged for 20 min at 1500 rpm. The monocytes are then recovered from the interface, diluted, and reisolated on a Histopaque-1077 cushion. After this second round of purification, the monocytes are washed twice in PBS/2% FBS/1 mM EDTA, resuspended in RPMI/0.5% FBS to 2×10^6 cells/mL, plated at 800,000 cells per well in 48-well formats, and incubated at 37 °C/5% CO_2. Thirty minutes prior to LPS stimulation, compounds (in 100% DMSO) are added. LPS is then added to 10 ng/mL and the cultures are allowed to incubate for an additional ~3 h. Media supernatants are then harvested and analyzed for TNF-α by standard ELISA or electrochemiluminescence on a Sector6000 reader (Meso Scale Discovery).
14. Inhibition of TNF-α in human blood: blood is drawn from healthy male volunteers into heparin tubes. At 37 °C, the blood is diluted 1-to-5 with pre-warmed RPMI/3% FCS, and then dispensed into 96-well-formatted 200 μL tube strips and capped. Thirty minutes prior to LPS stimulation compounds (in 100% DMSO) are added. Final DMSO concentrations equal 1.0%. LPS is then added to 10 ng/mL and the cultures are allowed to incubate for an additional ~3 h. Samples are centrifuged in a strip rotor at 6500 rpm, and the plasma supernatants are analyzed by standard ELISA or electrochemiluminescence on a Sector6000 reader (Meso Scale Discovery).
15. For an example see: Cherney, R. J.; Duan, J. J. W.; Voss, M. E.; Chen, L.; Wang, L.; Meyer, D. T.; Wasserman, Z. R.; Hardman, K. D.; Liu, R. Q.; Covington, M. B.; Qian, M.; Mandlekar, S.; Christ, D. D.; Trzaskos, J. M.; Newton, R. C.; Magolda, R. L.; Wexler, R. R.; Decicco, C. *J. Med. Chem.* **2003**, *46*, 1811.