Bioorganic & Medicinal Chemistry Letters 19 (2009) 1722–1725

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Identification of a selective thieno[2,3-*c*]pyridine inhibitor of COT kinase and TNF-α production

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ARTICLE INFO

Article history:
Received 2 December 2008
Revised 23 January 2009
Accepted 27 January 2009
Available online 30 January 2009

Keywords:
Thienopyridine
COT
Tpl2
Kinase
TNF
RA
Rheumatoid arthritis

ABSTRACT

COT (Tpl2 in mice) is a serine/threonine MAP3 kinase that regulates production of TNF-α and other pro-inflammatory cytokines such as IL-1β via the ERK/MAP kinase pathway. As TNF-α and IL-1β are clinically validated targets for therapeutic intervention in rheumatoid arthritis (RA), blocking COT provides a potential avenue for amelioration of disease. Herein we describe identification of a cellular active selective small molecule inhibitor of COT kinase.

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Elevated levels of TNF-α and IL-1β have been implicated as mediators of a number of autoimmune diseases, in particular, the pain and joint destruction characteristic of rheumatoid arthritis (RA).[1] COT kinase (Tpl2 in mice) is a serine/threonine MAP3 kinase that regulates production of TNF-α and other pro-inflammatory cytokines such as IL-1β via the ERK-MAP kinase pathway.[2] In particular, COT/Tpl2 is essential for the activation of the MAPK pathway in macrophages stimulated by TLR agonists such as lipopolysaccharide (LPS)[3] and has also been suggested to be required for TNF-R signaling.[4] COT KO mice are viable and display a significantly delayed onset of Crohn's disease when crossed with ΔARE mutant mice.[5] In addition, downstream cytokines such as TNF-α and IL-1β are clinically validated targets for therapeutic intervention in RA.[6] Therefore blocking COT could provide a potential avenue for amelioration of disease.

Therapies that have successfully targeted TNF-α or IL-1β in man include biologics directed towards these cytokines such as adalimumab (Humira), etanercept (Enbrel) and infliximab (Remicade) which have demonstrated significant efficacy in the treatment of RA.[6] However these agents require administration via injection or infusion and identification of an orally available small molecule therapy would provide an additional benefit to patients. Identification of an inhibitor of COT kinase would alter production of pro-inflammatory cytokines such as TNF-α and IL-1β and as such have the potential to be a novel and effective small molecule therapy for the treatment of RA. To date, 1,7-napthyridine-3-carbonitriles, quinoline-3-carbonitriles, and thienopyridines have been reported as COT kinase inhibitors.[7,8] Herein we describe additional small molecule inhibitors of COT kinase from within the thieno[2,3-*c*]pyridine series and the first confirmed cell active, selective small molecule inhibitor of COT kinase which may serve as an important tool for further investigation of this target.

COT has a low homology to other kinases which provides a unique opportunity to achieve selectivity across the kinome. However low homology coupled with an inability to obtain crystal structures can also present a challenge in proceeding with a structure-based or in silico based drug design strategy as surrogate structures may not be predictive enough to translate to the target protein. Efforts to obtain a crystal structure of this protein have been unsuccessful to date. Contributing to this difficulty may be the stability of free COT. COT is now known to be part of a ternary complex with p105 and ABIN2 in the resting state[9] while free COT is believed to be the active form and is rapidly degraded in vitro.

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0960-894X/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2009.01.088

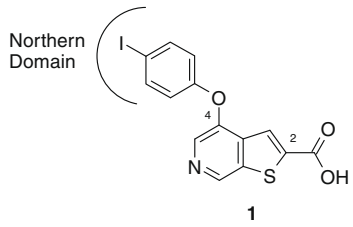
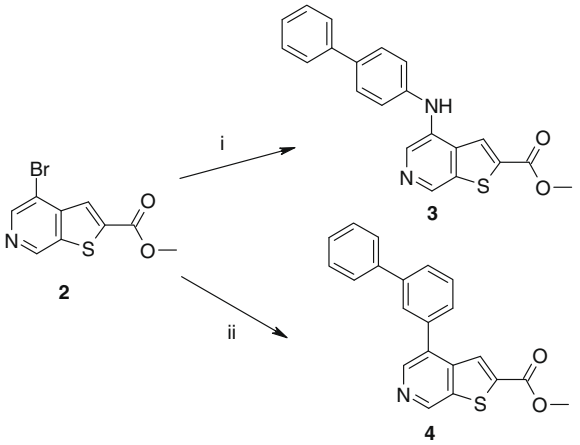


Figure 1. High throughput screening (HTS) hit.



Scheme 1. Installation of direct attached and N-linked northern domains. Reagents and conditions: (i) 2.4 equiv Cs_2CO_3, *p*-PhPhNH$_2$, Pd_2dba_3/Xanthphos (1:2, 6 mol%), dioxane, 100 °C, 18 h; (ii) 2 equiv Cs_2CO_3, *m*-PhPhB(OH)$_2$, 4 mol% Pd FibreCat® (Strem), EtOH/H_2O, 110 °C, 15 min, microwave.

The program has therefore had to rely on molecular modeling and classical SAR in order to rationalize interactions with the protein.

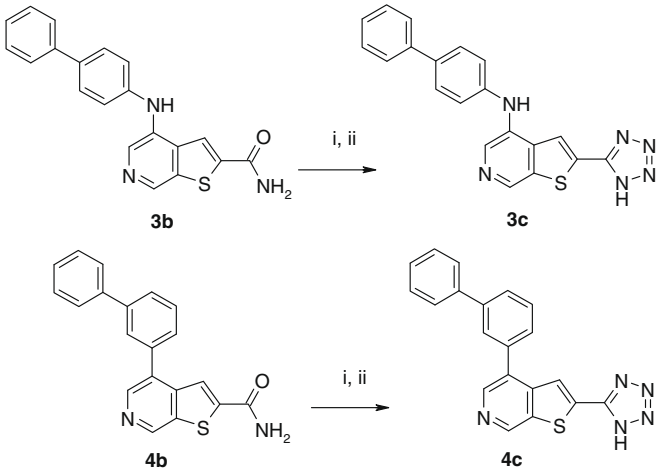
Thieno[2,3-*c*]pyridine, **1** (Fig. 1), was identified as our lead structure following screening of the Abbott compound collection (IC_{50} = 1 μM).[8]

Previous exploration of SAR around **1** revealed a preference for a C-4 biphenyl ether moiety and a slight preference for an acidic C-2 group.[8] Therefore the biphenyl system was held constant while exploration around the linker ether oxygen and C-2 carboxylate was initiated. Synthesis was initiated from previously described 4-bromo-thienopyridine **2**.[10] Identification of optimal conditions for palladium mediated Buchwald and Suzuki type coupling reactions (Scheme 1) allowed utilization of intermediate **2** for installation of both N-linked or direct attached biphenyl northern domains, respectively.

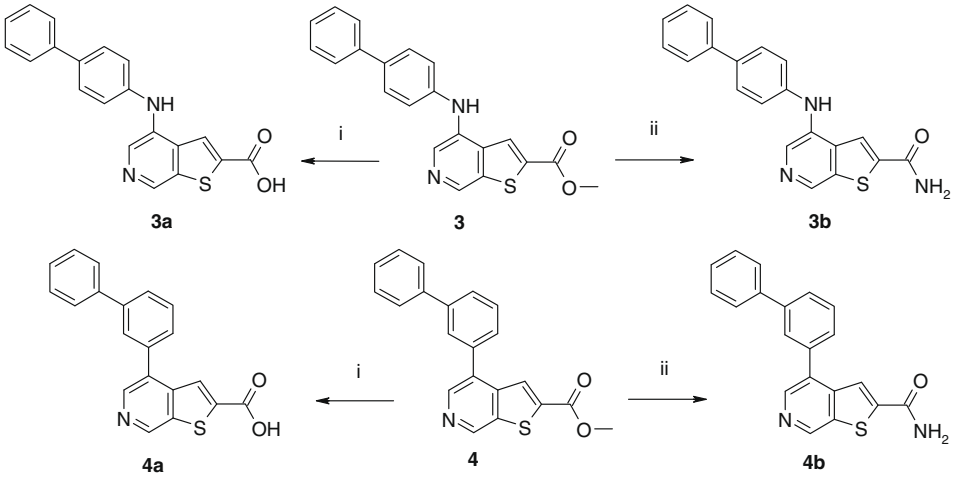
Of the ligands evaluated, Xanthphos proved to be superior for Buchwald coupling to bromoester **2**. Use of Cs_2CO_3 as base allowed the reaction to proceed without significant hydrolysis of the methyl ester. Suzuki coupling was facilitated by Pd on solid support allowing simple removal of Pd via filtration following reaction in the microwave. The methyl ester was either hydrolyzed to the corresponding carboxylate or converted to the corresponding amide with ammonia (Scheme 2).

The C-2 amides could be further dehydrated then reacted with sodium azide to form the corresponding C-2 tetrazoles (Scheme 3). Isolation of the tetrazole is based on a procedure from Wittenberger et al., whereby the reaction is quenched with a large excess of acetonitrile.[11] CH_3CN sequesters excess azide as methyl tetrazole (soluble in the aqueous layer during workup) providing a safer, more efficient workup procedure.

Compounds were evaluated for inhibition of COT kinase activity and selectivity versus a limited kinase panel using an HTRF-based assay.[12] SAR indicated acidic C-2 groups were well tolerated in agreement with O-linked analogs previously described (included

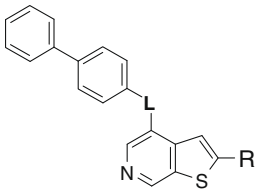


Scheme 3. Conversion of the amide head group to the corresponding tetrazole. Reagents and conditions: (i) TFAA, pyridine, 25 °C. 6 h; (ii) 1.3 equiv NaN_3, 1.3 equiv NH_4Cl, DMF, 80 °C, 18 h.



Scheme 2. Conversion of the ester head group. Reagents and conditions: (i) NaOH (1 N), 70 °C, 12 h; (ii) NH_3, MeOH, 110 °C.

Table 1
SAR of C-2 and linker for biaryl analogs **3–7**



Compound	Linker	R	COT HTRF IC_{50}[a] (μM)
3a	N	CO_2H	0.14
3b	N	$C(O)NH_2$	0.28
3c	N	Tetrazole	0.21
4a	Bond	CO_2H	0.05
4b	Bond	$C(O)NH_2$	2.9
4c	Bond	Tetrazole	0.08
5	O	CO_2H	0.31
6	O	$C(O)NH_2$	>50
7	O	Tetrazole	0.13

[a] Details are provided in Supplemental material.

in Table 1 as comparators, entries 5 and 7) while the C-2 amide group appeared to be less consistently tolerated (Table 1, entries 3b, 4b and 6). Direct attachment of the northern domain biphenyl provided slightly more potent C-2 acidic analogs (Table 1, entries 4a and 4b) and led to further exploration of this subseries. Thienopyridines in general showed good selectivity over MEK and ERK, MAP kinases downstream of COT.[8]

In order to probe additional interactions with the protein, an amino group ortho to the pyridine nitrogen of the thienopyridine core was targeted. The direct attached series was chosen in order to avoid a 1,4-heteroatom relationship across the aromatic ring.

A model of compound **10a** bound to COT kinase is shown in Figure 2. The biphenyl moiety projects into the solvent exposed region adjacent to the hinge, making VdW contact with the Gly-rich loop in accord with previously reported interactions between the ligand and the ATP-binding.[8] A C5-amino analog (inactive, data not shown) does not fit into the active site well because of a) a mis-

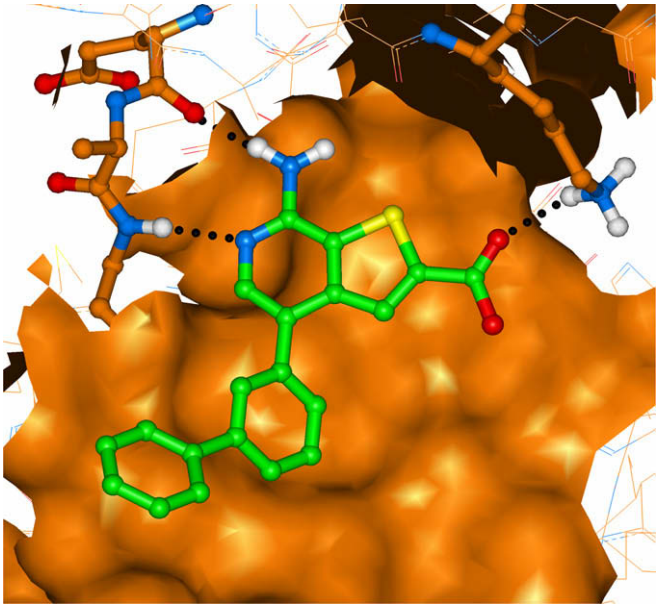
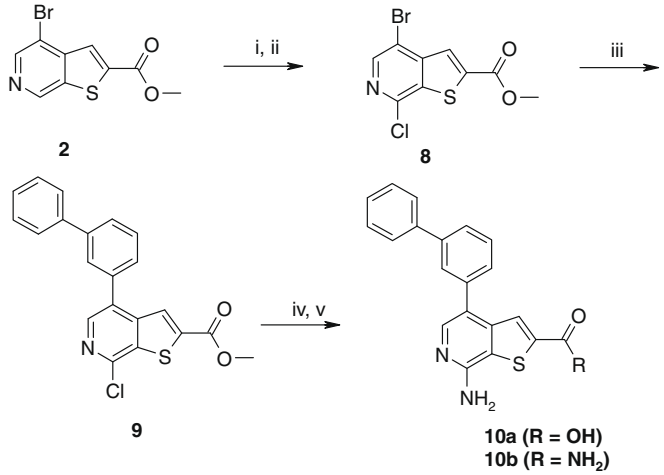


Figure 2. Model of compound **10a** bound to homology model of COT/Tpl-2 kinase. Hydrogen bonds between the ligand and Gly 210 N–H (3.2 Å), Glu 208 C=O (3.0 Å), and Lys 167 NH^{3+} (3.0 Å) are shown as black dotted lines.



Scheme 4. Introduction of a C-7 amino group. Reagents and conditions: (i) mCPBA, CH_2Cl_2, (ii) $POCl_3$, (iii) 3-biphenylboronic acid, $PdCl_2$ dppf, Cs_2CO_3, 10:1 DME:H_2O, (iv) Benzophenone imine, Pd_2dba_3, Xanthphos, Cs_2CO_3, dioxane, 100 °C, 5 h, then 2 N HCl, rt, (v) NaOH (1 N) 70 °C, 12 h (R = OH), or 7 M NH_3, 70 °C (R = NH_2).

aligned H-bond with either of Gly 210 or Lys 167, and/or b) an increased biaryl torsion of the pendent phenyl ring due to the ortho amino group which results in a poor steric fit into the active site.

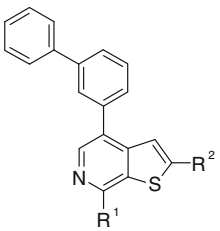
Starting from intermediate **2**, the aza-nitrogen was oxidized to the corresponding *N*-oxide with mCPBA (Scheme 4). Reaction with phosphorous oxychloride resulted in regioselective C-7 ortho chlorination to provide intermediate **8**. Suzuki coupling to install the biphenyl moiety followed by Pd-mediated coupling with benzophenone imine and acidic hydrolysis provided the aminopyridine ester. The ester was either hydrolyzed to the corresponding acid **10a** or converted to the corresponding amide **10b** via aminolysis.

Analog **10a** makes a productive hydrogen bond between the amino group and the hinge (Fig. 2) resulting in a 2–3× gain in potency versus **4a** (Table 2). In addition a corresponding 18-fold gain in potency is observed for amino analog **10b** versus **4b**.

The most potent analogs were further profiled in a cellular assay measuring the inhibition of TNF production in peripheral human blood monocytic cells (PBMCs) stimulated with LPS (Table 2).[12] Analogs bearing a C-2 acidic group, while potent against the enzyme, did not translate to equipotent cell active analogs. However, analog **10b** (Table 2) with a neutral C-2 amide demonstrated a good correlation between enzyme and cellular potency.

In order to understand the cellular selectivity profile analog **10b** was further assessed in macrophages. Two different forms of COT

Table 2
SAR of C-7 amino biaryl direct attached analogs



Compound	R^1	R^2	COT IC_{50}[a] (μM)	PBMC TNF inhibition[a] (μM)
4a	H	CO_2H	0.05	3.7
10a	NH2	CO_2H	0.02	2.3
4b	H	$C(O)NH_2$	2.9	ND[b]
10b	NH2	$C(O)NH_2$	0.16	0.37

[a] Details are provided in Supplemental material.
[b] Low solubility and non-reproducible results.

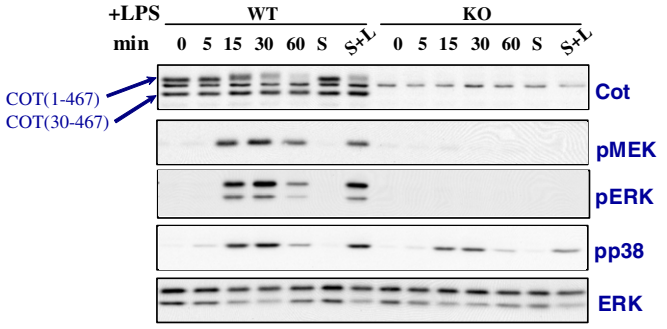


Figure 3. Bone marrow derived macrophages (BMDM) from wild type and COT deficient mice. Cells are plated and serum starved (0.5% FBS) for 16 h prior to stimulation. Cells are stimulated with LPS (100 ng/ml) (L) from 5 to 60 min or 20% FBS (S) for 15 min. Western blots are carried out with indicated antibodies. Long form of COT (1–467) is phosphorylated and degraded with kinetics similar to phosphorylation/activation of ERK and MEK in the wild type animal BMDMs while the alternatively translated form of COT (30–467) is not degraded upon LPS stimulation. COT KO cells show complete failure to induce MEK/ERK phosphorylation upon LPS stimulation. p38 kinase is phosphorylated in both wild-type and COT deficient cells with little quantitative defect.

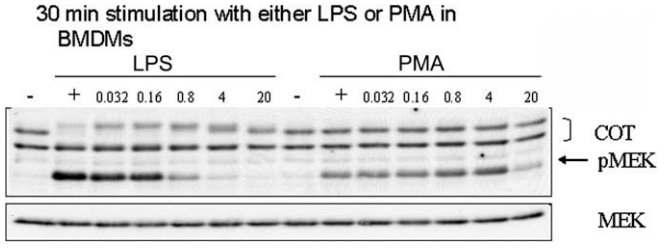


Figure 4. BMDM cells were stimulated with LPS or PMA in the presence of increasing concentration of compound **10b** (μM). PMA is able to stimulate MEK activation and TNF production in a PKC dependent manner but is not able to induce phosphorylation/degradation of COT (1–467). LPS induces phosphorylation/degradation of COT (1–467) as well as COT dependent MEK phosphorylation and both processes are inhibited by compound **10b** in a dose dependent manner.

Table 3
Profile of compound **10b**

Compound	COT IC_{50}[a] (μM)	MEK IC_{50}[a] (μM)	ERK IC_{50}[a] (μM)	MK2 IC_{50}[a] (μM)	p38 IC_{50}[a] (μM)	PBMC TNF IC_{50}[a] (μM)	Hu WB TNF IC_{50}[a] (μM)
10b	0.17	13	4.3	>50	>50	0.37	34

[a] Details are provided in the Supplemental material.

are present due to alternative translation initiation (M-1 or M-30). The long form (COT (1–467)) is activated and degraded following LPS stimulation (Fig. 3). This effect is not observed when 20% FBS is added as stimulus following starvation. The kinetics of activation and degradation of COT (1–467) are consistent with the activation of MEK and ERK in COT WT cells. MEK and ERK activation is totally absent in COT KO cells upon LPS stimulation with little effect on p38 activation. Alternatively, stimulation with PMA activates TNF-α production via a COT independent pathway and as such serves as a cellular readout of selectivity (Fig. 4). Compound **10b** shows COT pathway specificity via lack of inhibition of PMA induced MEK phosphorylation.[13]

Finally, COT **10b** was assessed for selectivity and its ability to inhibit TNF-α production in human whole blood (Table 3). Though selective and potent in isolated cells, **10b** was not potent in human whole blood due in part to high plasma protein binding.

In summary, a novel series of 7-amino substituted thieno [2,3-*c*]pyridines have been identified as COT enzyme inhibitors. Analog **10b** is demonstrated to selectively inhibit the COT pathway following LPS stimulation in macrophages and provides an important tool molecule for further studies.

Acknowledgement

The authors thank Kent Stewart for helpful discussions about the COT model.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.01.088.

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