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Design and Synthesis of Novel Macrocyclic Mer Tyrosine Kinase Inhibitors

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KEYWORDS. Mer inhibitors, MerTK, macrocycle, pyrrolopyrimidine, TAM kinase, structure-based drug design.

ABSTRACT: Mer tyrosine kinase (MerTK) is aberrantly elevated in various tumor cells and has a normal anti-inflammatory role in the innate immune system. Inhibition of MerTK may provide dual effects against these MerTK-expressing tumors through reducing cancer cell survival and redirecting the innate immune response. Recently, we have designed novel and potent macrocyclic pyrrolopyrimidines as MerTK inhibitors using a structure-based approach. The most active macrocycles had an EC_{50} below 40 nM in a cell-based MerTK phosphor-protein ELISA assay. The X-ray structure of macrocyclic analogue **3** complexed with MerTK was also resolved and demonstrated macrocycles binding in the ATP binding pocket of the MerTK protein as anticipated. In addition, the lead compound **16** (UNC3133) had a 1.6 hr half-life and 16% oral bioavailability in a mouse PK study.

Mer tyrosine kinase (MerTK) belongs to the TAM (Tyro3, Axl, MerTK) family and regulates adult tissues and organ systems that are subject to continuous challenge and renewal throughout life.[1,2] Aberrant expression of MerTK in various hematological and solid tumors promotes cancer cell survival, chemoresistance and invasive motility.[3,4] In addition, MerTK triggers macrophage engulfment of apoptotic material (efferocytosis) and promotes an anti-inflammatory response, preventing excessive inflammation and autoimmune disease. Unfortunately, within the tumor microenvironment this suppresses anti-tumor innate immune responses.[5-9] Therefore, inhibition of MerTK may provide dual therapeutic effects against MerTK-expressing tumors by reducing cancer cell survival, invasion, and metastasis as well as stimulating anti-tumor immune responses.

In addition, TAM family members are used by many enveloped viruses, such as West Nile (WNV), Dengue (DENV), HIV-1, Ebola, Marburg, Zika, and Chikungunya, to attach and gain access to cells through apoptotic mimicry (a phosphatidylserine (PtdSer)-dependent process).[10-12] Inhibition of MerTK may also lower the infectivity of an important class of enveloped viruses.[13]

Recently, we reported UNC2025 (**1**), a potent MerTK/Flt3 dual inhibitor.[14] Although, our attempt to obtain an X-ray structure of a complex of MerTK protein with UNC2025 failed, the X-ray structure of compound UNC569 (**2**) complexed with MerTK protein kinase domain was successfully resolved at a resolution of 2.69 Å (Figure 1a & 1b).[15] UNC569 formed three hydrogen bonds with MerTK protein, two with the hinge region of the protein and the third one with R727. UNC2025 is expected to bind MerTK in a similar fashion.[14] One interesting observation from this X-ray structure was that thermal motions of the butyl amine side chain and the 4-aminocylcohexylmethyl group could bring them close enough to be connected by a short linker, thus forming a macrocycle. However, connecting these two groups directly was synthetically challenging. Retaining the cyclohexyl ring and the hydroxyl group, the key hydrogen bond donor, would introduce new stereogenic centers in the newly formed macrocycles. To provide a proof of concept for the macrocycle hypothesis, a simplified macrocycle **3** with an amide linker was proposed (Figure 1c). Although UNC569 belongs to the pyrazolopyrimidine series, we have previously shown that pyrrolopyrimidines have similar structure-activity relationships (SAR) and better solubility (solubility of the HCl salt of **1** is 47 mg/mL at pH 7.4).[13] Therefore, we used a pyrrolopyrimidine core to form macrocycle **3**.

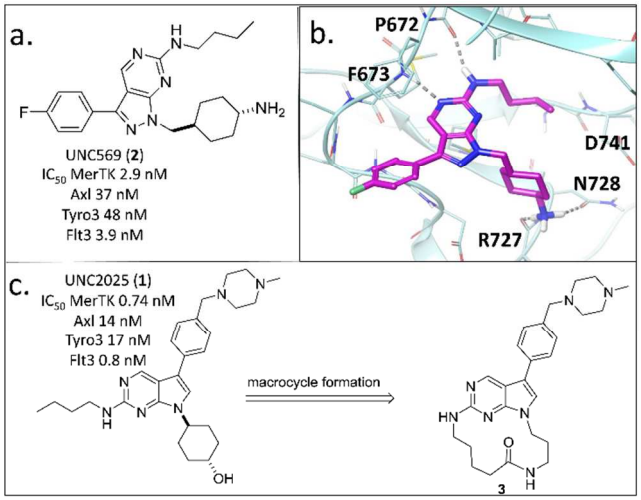


Figure 1. a. The structure of UNC569; b. X-ray crystal structure of UNC569 in complex with MerTK (kinase domain) (PDB code 3TCP); c. The structure of proposed macrocycle **3**.

Macrocycles have been prominent in drug structures for decades although most macrocyclic drugs on the market are macrolides or cyclic peptides derived from natural products. The structural preorganization and incomplete rigidity provided by cyclization with flexible linkers may facilitate interactions with target protein through favorable entropies and numerous, spatially distributed binding interactions, thus, increasing both binding affinity and selectivity.[16] Furthermore, some reports suggest that cyclization has a favorable impact on other essential properties required for drugs, such as membrane permeability,[17] metabolic stability, and overall pharmacokinetics.[18,19] Currently, a handful *de novo* designed macrocyclic kinase inhibitors are in clinical development.[16] However, limited opportunities in lead optimization, potential increased costs for scale up, and poor understanding of macrocycle absorption, distribution, metabolism, execration, and toxicity (ADMET) are still concerns for drug discovery efforts in this field. With this background, we explored introduction of macrocycles into MerTK inhibitors.[20]

Indeed, compound **3** proved active against MerTK (IC_{50} 65 nM) with 20- and 30-fold selectivity over Axl (IC_{50} 1300 nM) and Tyro3 (IC_{50} 2000 nM), respectively, and similar activity against Flt3 (IC_{50} 160 nM) in our in-house microfluidic capillary electrophoresis (MCE) assay.[21-23] The X-ray structure of **3** complexed with the MerTK kinase domain was also resolved at a 2.55 Å resolution (Figure 2). Overall, the structure was in agreement with the preliminary docking hypothesis (Figure 1). The major difference between MerTK binding by **3** and **2**, was that, the amide group of **3**, was only able to form a hydrogen bond with N728 because of its limited freedom of motion, while the 4-aminocylcohexyl substituent in **2** could simultaneously bind to three residues, R727, N728 and D741 (Figure 2).

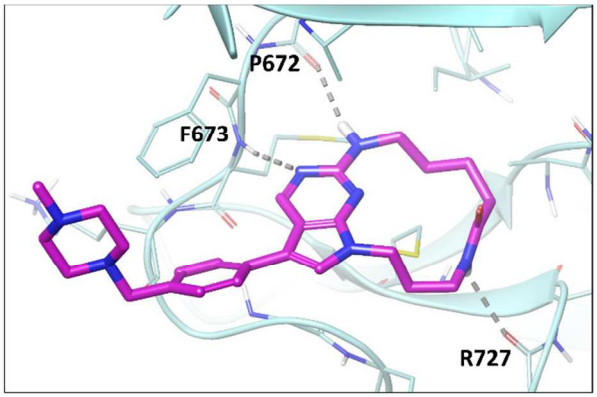
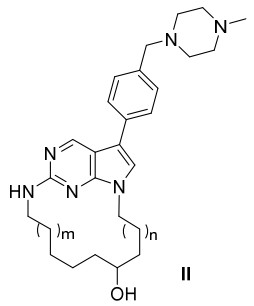


Figure 2. X-ray structure of macrocyclic compound **3** complexed with Mer protein (kinase domain) (PDB code 5K0K).

To improve the hydrogen bonding capability of **3**, we installed an amino or a hydroxyl group in the macrocycle as shown in structures **I** & **II** (Table 1). The closest analogues to **3** from structures **I** & **II** were compounds **4** & **10** respectively which had a similar ring size while **4** had an amino group and **10** had a hydroxyl group attached to the macrocycles (inseparable racemic mixture). As proof of concept, both compounds were prepared, and indeed were 15-fold more active against MerTK than **3**. Changing the ring size of the macrocycles would affect not only the conformation of the molecule but also the location of the amino/hydroxyl group, which is crucial for forming the desired hydrogen bond. Thus, we next explored the SAR of the ring size of molecules **I** & **II**.

Table 1. SAR of the ring size

Structure	Compound	m	n	IC_{50} (nM)[a]				EC_{50} (nM)[a]
				MerTK	Axl	Tyro3	Flt3	pMerTK ELISA
	4	2	1	3.9 ± 2.7	33 ± 17	39 ± 16	20 ± 9.6	>1000
	5	4	1	17 ± 1.9	300 ± 170	110 ± 26	100 ± 11	>1000
	6	6	1	30 ± 3.8	170 ± 31	170 ± 28	70 ± 2.6	>1000
	7	2	2	8.9 ± 4.4	150 ± 90	130 ± 74	130 ± 45	>1000
	8	4	2	2.2 ± 0.8	11 ± 8.2	13 ± 6.1	12 ± 3.2	34 ± 26
	9	6	2	0.8 ± 0.1	4.2 ± 1.9	7.5 ± 9.2	2.5 ± 2.6	160 ± 43



II

	m	n	MerTK	Axl	Tyro3	Flt3	pMerTK ELISA
10	1	1	4.1 ± 1.4	40 ± 10	53 ± 32	12 ± 1.3	39 ± 5.9
11	2	1	5.6 ± 3.4	50 ± 24	60 ± 40	13 ± 9.4	240 ± 190
12	3	1	1.8 ± 0.5	14 ± 11	25 ± 19	7.5 ± 2.0	43 ± 9.2
13	4	1	9.6 ± 9.4	26 ± 15	54 ± 5.7	8.9 ± 0.4	190 ± 30
14	1	2	8.0 ± 3.2	120 ± 36	170 ± 130	29 ± 1.1	150 ± 0.8
15[b]	2	2	4.0	36	94	12	ND
16	3	2	3.0 ± 1.1	17 ± 6.2	31 ± 9.2	6.8 ± 2.7	110 ± 69
17	4	2	2.6 ± 1.9	9.3 ± 7.1	22 ± 22	8.1 ± 5.8	72 ± 37
18	1	3	4.1 ± 0.1	28 ± 13	57 ± 41	6.3 ± 2.1	69 ± 17
19	2	3	5.0 ± 0.4	25 ± 8.2	44 ± 29	5.4 ± 1.0	120 ± 1.1
20	3	3	3.1 ± 1.3	18 ± 8.9	38 ± 3.3	9.6 ± 0.9	150 ± 39
21	4	3	4.9 ± 2.5	17 ± 6.9	49 ± 36	13 ± 7.3	400 ± 30

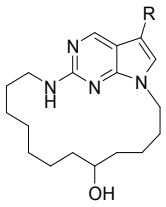
[a] Values are the mean of two or more independent assays ± SD. [b] Only test once.

For structure **I**, when n = 1, ring expansion of the macrocycle led to weaker MerTK inhibitors. As shown in Table 1, analogues **5** (m = 4) and **6** (m = 6) were 4 and 8-fold less active than **4** (m = 2), respectively. However, a larger ring was better when n = 2. Analogue **9** (m = 6) was 10-fold more active against MerTK than analogue **7** (m = 4) and 3-fold more active than **8** (m = 4). Interestingly, analogue **9** was 7-fold more active against Tyro3 compared with UNC2025 although their activities against MerTK were similar. These results also demonstrated the importance of the location of the hydrogen bond donor. For structure **II**, when n = 1, the MerTK inhibitory activity of the analogues varied slightly based on the ring size (compounds **10**–**13**). Analogue **12** with a 16-membered ring (m = 3) was the most active MerTK inhibitor. However, when n = 2, a larger ring led to a more MerTK active analogue (compounds **14**–**17**) with less selectivity among TAM family members (compounds **14** (15-fold over Axl & 21-fold over Tyro3) vs **17** (4-fold over Axl & 8-fold over Tyro3)). For n = 3, analogues **18**–**21** (m = 1–4) were equally potent against Mer independent on the ring size, possibly due to the flexibility of the large ring when the hydroxyl group, the hydrogen bond donor, was at the optimal position. Overall, the ring size had less effect on the activity of macrocycles with structure **II** possibly due to the less restricted nature of the alkyl macrocycles in structure **II** versus the amide linked macrocycles in structure **I**.

To evaluate the inhibitory activity of our compounds in a cell-based assay based on the inhibition of MerTK phosphorylation (pMerTK), a 384-well plate ELISA assay was developed (pMerTK ELISA) (Table 1 and 2). In this assay, HEK293 cells expressing chimeric proteins[24,25] consisting of the extracellular and transmembrane domains from the epidermal growth factor receptor (EGFR) and the intracellular kinase domain from Mer were plated and stimulated with EGF with and without inhibitors. A chimeric protein was utilized because consistent stimulation of native MerTK with the complex, natural ligand (GAS6 plus PtdSer) was not practical. In general, compounds in structure **I** were more polar due to the primary amine group (shorter retention time in LC/MS spectra), likely less cell permeable, and had weaker cellular activity than compounds in structure **II** with the exception of analogue **8**, which was very active in this assay (EC_{50} for UNC2025 was 12 ± 3.1 nM). The reasons for the exception are unclear and still under investigation.

Table 2. SAR Study of R

Structure	Compound	R	IC_{50} (nM)[a]				EC_{50} (nM)[a]
			MerTK	Axl	Tyro3	Flt3	pMerTK ELISA
	22		140 ± 93	810 ± 170	1800 ± 1500	280 ± 130	>1000
	23		150 ± 140	620 ± 320	1300 ± 1100	370 ± 240	>1000



24	86 ± 40	340 ± 13	940 ± 600	250 ± 130	>1000
25	160 ± 34	920 ± 420	1200 ± 810	470 ± 230	>1000
26	170 ± 20	910 ± 250	1100 ± 320	550 ± 78	>1000
27	16 ± 11	93 ± 22	300 ± 250	75 ± 70	160 ± 2.3
28	8.1 ± 7.2	65 ± 37	78 ± 71	26 ± 19	210 ± 23
29	11 ± 8.0	110 ± 62	190 ± 120	32 ± 18	220 ± 39
30	30 ± 30	180 ± 94	300 ± 300	84 ± 87	490 ± 13
31	32 ± 1.7	180 ± 43	350 ± 130	68 ± 7.9	690 ± 48
32	54 ± 9.3	390 ± 24	690 ± 360	63 ± 3.4	>1000
33	13 ± 6.9	180 ± 37	210 ± 53	56 ± 39	380 ± 160
34	80 ± 94	120 ± 3.7	170 ± 98	37 ± 1.1	260 ± 89
35	94 ± 46	640 ± 54	1200 ± 650	1000 ± 15	>1000

[a] Values are the mean of two or more independent assays ± SD.

Next, as shown in Table 2, we explored the SAR at the R position while fixing the macrocycle as a 17-membered ring as in analogue **16** (UNC3133). The R group projects out of the MerTK active site (Figure 2). Although it interacts mostly with the solvent, it can still affect the activity of the inhibitors and be used for tuning solubility and other pharmacokinetic properties. Various groups were introduced to this site. When a phenyl group was at the R position, analogue **22** was active against MerTK but was much weaker than **16** (47-fold) (Table 1) and had no activity in the pMerTK ELISA assay at a concentration of 1.0 μM. A small substituent, such as fluoro or methoxyl group, on the phenyl ring didn't affect the MerTK activity in either the MCE or pMerTK ELISA assays (within 2-fold, see analogues **23**-**26**). However, a larger substituent on the phenyl ring significantly increased the MerTK activity. Analogues **27**-**29** had similar substituents at the R position, one phenyl and one heterocycle connected by a one atom linker (CH_2, CO or SO_2), thus they shared similar activity against MerTK as analogue **16**. A longer linker (an amide bond) and no linker between the phenyl and the heterocycle led to weaker MerTK inhibitors **30** and **31** respectively (10-fold weaker than **16** in the MCE assay and 5-folder weaker in the pELISA assay). Introducing a pyridine ring at the R position led to analogues that could be very active against MerTK depending on the position of the nitrogen in the pyridine ring. Analogue **33** with a 3-pyridinyl group at the R position was more active in the MCE assay than analogues **32** (4-fold) and **34** (6-fold) with a 2-pyridinyl and a 4-pyridinyl group, respectively. However, **34** was equipotent to **33** in the pMerTK ELISA assay while **32** was inactive at a concentration of 1.0 μM. A 4-tetrahydropyran group was also tolerated at the R position and analogue **35** was equally potent as analogue **22**. The change of the MerTK activity based on the size, shape and electron density of the R group could not be explained by the

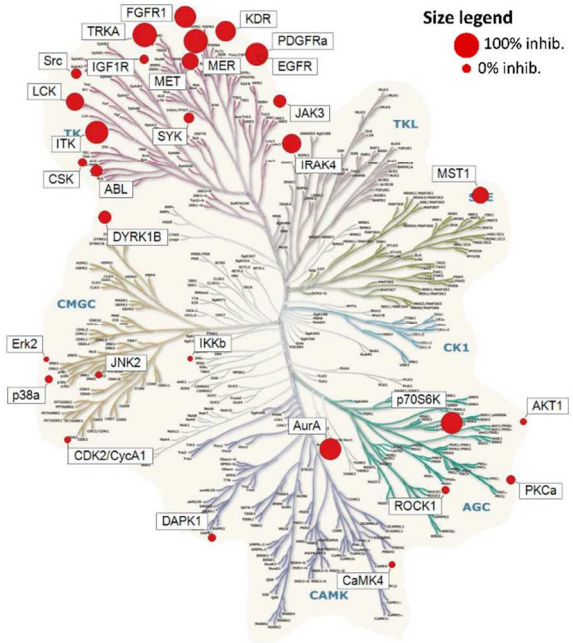


Figure 3. Kinase Tree.

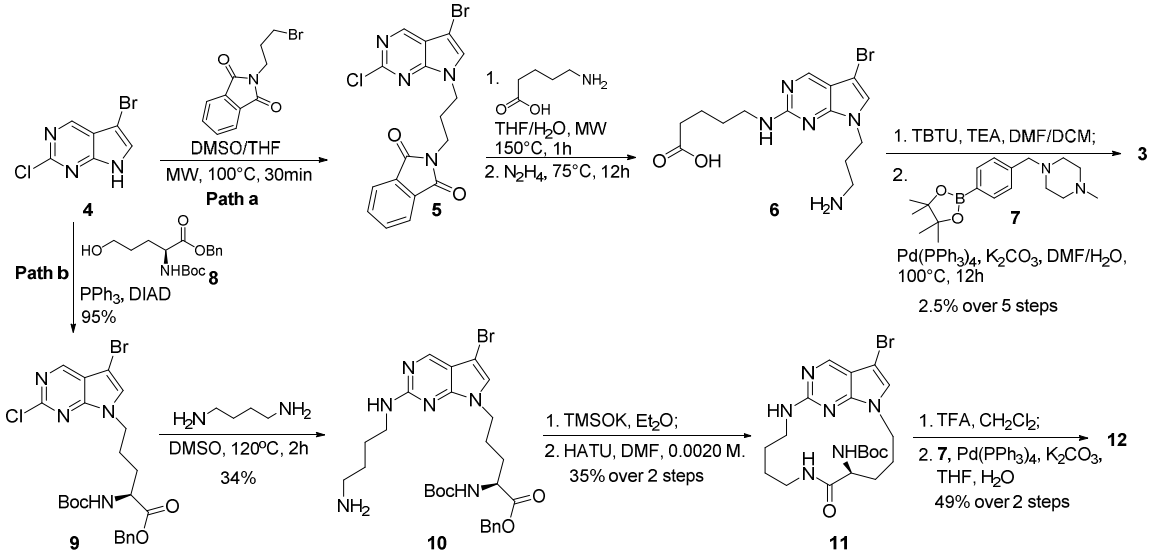
X-ray structure of **3** since the R group was in the solvent front and didn't have close interactions with the MerTK protein. Generally, these analogues have some selectivity over Axl (> 5-fold) and Tyro3 (> 10-fold) and similar activity against Flt3 and Mer. Analogue **16** was still one of the most active MerTK

inhibitors and had a medium-sized ring, thus was chosen for the further study.

Analogue **16** was tested in the *in vivo* pharmacokinetic (PK) study in mice via both intravenous (IV) and oral (PO) administration (details in supplemental materials). It had a 1.6 hr half-life, 16% oral bioavailability, 106 mL/min/kg systemic clearance (1.2 fold of the normal liver blood flow in mice) and 6.8 L/kg volume of distribution (V_{ss}) (9.7 fold higher than the normal volume of total body water) at a dose of 3 mg/kg. These PK properties are not optimal for an *in vivo* tool compound and need to be further improved.

The inhibitory activity mediated by **16** against a panel of 30 kinases was also determined at a concentration 100-fold above its MerTK IC_{50} (Figure 3) (details in supplemental materials). This experiment provides a broad survey of kinase families emphasizing tyrosine kinases along with a selection of serine/threonine kinases. Surprisingly, the selectivity of **16** across the kinome was not very high (worse than UNC2025[14]): eight tyrosine kinases and four serine/threonine kinases were inhibited by greater than 50% in the presence of 300 nM **16**. This may be due to the flexibility of this type of macrocycles, which would allow it to conformationally adapt to the active site of disparate kinases.

Scheme 1. The synthetic routes for macrocyclic compounds



In summary, the first potent macrocyclic MerTK inhibitors were developed using structure-based drug design. An amide bond formation or a Ru-catalyzed ring metathesis reaction was the key step to build up the macrocycles. An X-ray structure of the MerTK protein complexed with analogue **3** showed that these macrocyclic compounds resided in the ATP binding pocket. The most active analogues had an EC_{50} below 40 nM in our newly developed high-throughput phospho-Mer cellular ELISA assay. These macrocycles were not very selective across the kinome. The selectivity and PK properties will need to be improved to develop useful *in vivo* tool compounds.

ASSOCIATED CONTENT

Supporting Information

Representative synthetic routes to macrocyclic compounds are shown in Scheme 1. The synthesis of **4** started with commercially available 5-bromo-2-chloro-7*H*-pyrrolo[2,3-*d*]pyrimidine (**36**) as shown in path a. Mitsunobu reaction between **36** and **37** led to intermediate **38**. An S_NAr replacement of the chloride on the pyrimidine ring with butane-1,4-diamine yielded **39**. The macrocycle was formed by an intra-molecular amide bond coupling reaction of the unprotected acid and the free amine to provide intermediate **40**. The final compound **4** was obtained by deprotection of the Boc protecting group of **40** followed by a Suzuki coupling reaction with boronic ester **41**. In path b, *N*-alkylation of **36** with alkyl bromide **42** under basic conditions followed by an S_NAr replacement of the chloride on the pyrimidine ring with but-3-en-1-amine yielded intermediate **43,** a macrocycle precursor. A ring closing metathesis reaction of **43** was then catalyzed by the second generation of Grubbs' catalyst followed by a Suzuki coupling reaction with boronic ester **41** to yield the intermediate **44** which was a mixture of *cis*- and *trans*-isomers. Reduction of the newly formed double bonds and removal of the TBS protecting group led to the desired analogue **16**.

Experimental details and characterization of all compounds and biological methods.

The Supporting Information is available free of charge on the ACS Publications website.

Accession Codes: The atomic coordinates for the X-ray crystal structure of **3** have been deposited with the RCSB Protein Data Bank under the accession code 5K0K.

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Author Contributions

The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

MerTK, Mer tyrosine kinase; PtdSer, phosphatidylserine; SAR, structure-activity relationships; ADMET, absorption, distribution, metabolism, execration, and toxicity; MCE, microfluidic capillary electrophoresis; IC_{50}, half maximal inhibitory concentration; pMerTK, phosphorylated Mer tyrosine kinase; ELISA, enzyme-linked immunosorbent assay; IC_{50}, half maximal effective concentration.

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Design and Synthesis of Novel Macrocyclic Mer Tyrosine Kinase Inhibitors

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