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Discovery of Macrocyclic Pyrimidines as MerTK-specific Inhibitors

Dr. Andrew L. McIver^{#†}, Dr. Weihe Zhang^{#†}, Dr. Qingyang Liu[†], Dr. Xinpeng Jiang[†], Michael A. Stashko[†], James Nichols, Dr. Michael J Miley[§], Dr. Jacqueline Norris-Drouin[†], Dr. Mischa Machius[§], Prof. Deborah DeRyckere^{||}, Dr. Edgar Wood, Prof. Douglas K. Graham^{||}, Prof. H Shelton Earp^{¶,§}, Prof. Dmitri Kireev[†], Prof. Stephen V. Frye^{†,¶}, and Prof. Xiaodong Wang^{†,*}

[†]Center for Integrative Chemical Biology and Drug Discovery, Division of Chemical Biology and Medicinal Chemistry, Eshelman School of Pharmacy

Meryx, Inc., USA

[§] Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

^{||}Aflac Cancer and Blood Disorders Center of Children's Healthcare of Atlanta, Department of Pediatrics, School of Medicine, Emory University, Atlanta, GA 30322, USA

[¶]Lineberger Comprehensive Cancer Center, Department of Medicine, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

[#] These authors contributed equally to this work.

Abstract

Macrocycles have attracted significant attention in drug discovery recently. In fact, a few *de novo* designed macrocyclic kinases inhibitors are currently in clinical trials with good potency and selectivity for their intended target. In this paper, we have successfully engaged a structure-based drug design approach to discover macrocyclic pyrimidines as potent Mer tyrosine kinase (MerTK)-specific inhibitors. An enzyme-linked immunosorbent assay (ELISA) in a 384-well format was employed to evaluate the inhibitory activity of macrocycles in a cell-based assay assessing tyrosine phosphorylation of MerTK. Through SAR studies, analogue **11** (UNC2541) was identified as a potent and MerTK-specific inhibitor and exhibits sub-micro molar inhibitory activity in the cell-based ELISA assay. In addition, an X-ray structure of MerTK protein in complex with **11** was resolved to show that these macro-cycles bind in the MerTK ATP pocket.

*Corresponding Author Tel: 919-843-8456. Fax: 919-843-8465. xiaodonw@unc.edu.

Present Addresses [†] Andrew McIver: Chimerix, Inc., 2505 Meridian Pkwy, Suite 100, Durham, NC 27713; Weihe Zhang: BioCryst Pharmaceuticals, Inc., 2100 Riverchase Center, Suite 200, Birmingham, AL 35244; Qingyang Liu: Department of chemistry, Montgomery College, 51 Mannakee Street, Rockville, MD 20850; Xinpeng Jiang: School of Pharmacy, Zhejiang University of Technology, Jiangshan, Zhejiang, China.

Author Contributions

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Supporting Information

Experimental details and characterization of all compounds and biological methods. The Supporting Information is available, in the online version, at

Graphical abstract

A structure-based drug design approach led to macrocyclic pyrimidines as potent Mer tyrosine kinase (MerTK)-specific inhibitors. Through SAR studies, analogue **11** (UNC2541) was identified as a key compound and exhibits sub-micro molar inhibitory activity in the cell-based enzyme-linked immunosorbent assay (ELISA). In addition, an X-ray structure of MerTK protein in complex with **11** was resolved to show that these macrocycles bind in the MerTK ATP pocket.

Keywords

MerTK; MerTK-specific inhibitors; macrocycles; pyrimidines; intramolecular hydrogen bonds

Macrocycles, especially *de novo* designed macrocycles, have recently gained attention in drug discovery due to the new physicochemical properties and broader intellectual property (IP) that they may provide.¹⁻³ In macrocycles, cyclization leads to a structural preorganization which can increase both binding affinity and selectivity by engaging targets through numerous and spatially distributed binding interactions.^{1,4} A few macrocyclic kinase inhibitors are currently in clinical trials with good potency and selectivity for their intended target.⁵⁻⁷ We have been interested in Mer tyrosine kinase (MerTK) as a therapeutic target^{8,9} and have developed several MerTK inhibitors with varying selectivity profiles.¹⁰⁻¹⁴ The most advanced compound among these inhibitors is UNC2025 which is a potent and highly orally bioavailable MerTK inhibitor.¹⁴ It is also equally potent against FMS-like tyrosine kinase (Flt3). This dual inhibitory activity of UNC2025 is desirable for certain diseases such as AML, however, inhibition of Flt3 has been associated with hematopoietic toxicity^{15,16} and is therefore inadvisable for other applications of MerTK inhibitors. The new pyrrolopyrimidine macrocycles that we have developed recently share the same undesired selectivity profile.¹⁷ In this paper, we will present a new type of MerTK-specific inhibitor—macrocyclic pyrimidines.

We have recently discovered substituted-pyrimidines as novel MerTK specific inhibitors via a structure-based drug design approach.¹² Based on our published X-ray crystal structure of MerTK in complex with **1** (Figures 1a and 1b), the butyl side chain and the cyclohexyl alcohol are close to each other and well-positioned to form a macrocycle. One example of this design is compound **2** (Figure 1c), which has a hydrogen donor, an amino group, at the same position as the hydroxyl group in **1**. The macrocycle is connected by an amide bond and the cyclohexyl ring has been opened to eliminate the chance to introduce new stereogenic centers. Compound **2** fits the MerTK docking model and is predicted to retain three key hydrogen bonds with MerTK protein (Figure 1d, two with the hinge area (F673

and P672) and one with either D741, R727, or N728). Since substituted-pyrimidines show some selectivity for MerTK over Flt3 (e.g. compound **1** is 14-fold more active against MerTK versus Flt3),¹² we were interested to see if macrocycles based on this scaffold could improve MerTK inhibitory activity and/or the selectivity profile over other TAM family members and Flt3.

The syntheses of the designed macrocyclic compounds are straight forward. A general synthetic route is shown in Scheme 1 (see Supporting Information for details). Commercially available 2,4-dichloropyrimidine-5-carbonyl chloride reacted with an amine or alcohol to form the amide/ester **I**. In a one-pot reaction Boc protected amino acids with differing length alkyl chains and various diamines were attached to the pyrimidine core to provide intermediate **II**. The macrocycle was closed using an intramolecular amide coupling reaction, followed by cleavage of the Boc protecting group to yield the desired macrocycles **III**. To explore SAR at the R' position, the starting acid chloride was first converted into an ethyl ester. After the formation of the desired macrocycle, the ethyl ester was hydrolyzed under basic conditions and the resulting free acid was converted to the final amide **IV** via an amide bond coupling reaction and cleavage of the Boc protecting group.

Compound **2** was synthesized using the route presented in Scheme 1 and was tested using in-house microfluidic capillary electrophoresis (MCE) assays at the ATP Kms (details see supporting information Table S1).¹⁸⁻²⁰ As shown in Table 1, compound **2** demonstrated exceptional selectivity over Flt3 (110-fold vs 14-fold for compound **1**) along with weaker activity against MerTK (12-fold lower IC₅₀ than compound **1**). To improve the MerTK activity of compound **2**, we initially explored the ring size of the macrocycle since this would simultaneously vary the position of the hydrogen-bond donor, the amino group, and the flexibility of the ring. As shown in Table 1, when $m = 1$, the inhibitory activity of macrocycles varied depending on the ring size. Compound **2** ($n = 2$) was 3-fold more potent than compound **3** ($n = 1$), however, compound **4** ($n = 3$) was 12-fold less active than compound **2**. When $n \geq 4$, the potency of the macrocycles was improved as the macrocyclic ring size was increased (compounds **4-7**). The trend was also evident when $m = 2$. Compound **8** ($n = 1$) was a weak MerTK inhibitor with micromolar potency while compound **11** (UNC2541) ($n = 4$) was very potent with a low nanomolar IC₅₀ against MerTK. The MerTK inhibitory activities of compound **9** ($n = 2$) and **10** ($n = 3$) were intermediate. When the macrocyclic ring became larger ($n = 5$ or 6), the MerTK activity of the corresponding analogues **12** and **13** was retained, however, the selectivity over Flt3 decreased (73-fold for **11** vs 59-fold for **12** & 19-fold for **13**). In general, most of the analogues had excellent selectivity for MerTK over Flt3 and good selectivity over Axl and Tyro3. To evaluate the inhibitory activity of these compounds in a cell-based assay of tyrosine phosphorylated MerTK (pMerTK), we used a 384-well format enzyme-linked immunosorbent assay (ELISA),¹⁷ in which HEK293 cells are transfected with a cDNA encoding chimeric protein consisting of the extracellular and transmembrane domains from the epidermal growth factor receptor (EGFR) and the MerTK intracellular kinase domain. Activity is then stimulated with EGF. A chimeric protein was utilized because consistent stimulation of native MerTK kinase with the complex, natural ligand (GAS6 plus PtdSer) was not practical. Compound **11** proved to be the most active in this pMerTK ELISA assay

with an EC₅₀ 510 nM. The 100-fold IC₅₀ shift from the MCE assay to the pMerTK ELISA assay isn't unexpected due to the ATP concentration difference (5.0 μM in the MCE assay vs low millimolar range in the pMerTK ELISA assay).

With the optimal ring size and linker of macrocycle **11**, the SAR at the R' position was explored using the synthetic route presented in Scheme 1 (Table 2). Introducing a larger and basic dimethyl amine group in the *para*-position of the benzyl group at the R' position didn't affect the activity or selectivity of the corresponding analogue **14** in either the MCE or pMerTK ELISA assays. However, 3,5-dimethoxyl benzyl group at the R' position led to a 6-fold less active analogue **15**. A *para*-substituted phenyl group at the same position (**16–18**) yielded analogues with dramatically diminished selectivity for MerTK over Flt3 although their MerTK enzymatic activities were similar to **11**. No MerTK cellular activity was observed for these derivatives at a concentration of 1.0 μM in the pMerTK ELISA assay. When placing heterocycles such as 3-pyridyl and 4-pyridyl at the R' position, the resulting analogues **19** and **20** had similar inhibitory enzymatic activity and selectivity for MerTK over Flt3 compared to **11**, and again, no cellular activity was observed at a concentration of 1.0 μM. 2-Pyridyl at the same position led to a 5-fold less active analogue **21**. However, when 3-pyridyl at the R' site was further substituted at the *para*-position with 1-pyrazole, the resulting analogue **22** was 4-fold more active than the parental analogue **19** and exhibited improved Flt3 selectivity (193-fold for **22** vs 152-fold for **19**) and cellular activity (IC₅₀ 420 nM). Saturated heterocycles such as 4-piperidine (**23**) and 4-tetrahydropyran (**24**) were also tolerated at this position. Analogues **23** and **24** were equally potent to **19** in either the MCE or pMerTK ELISA assays. Similarly, further substitution on the nitrogen of 4-piperidine with a benzyl group at the R' position increased the activity of the corresponding analogue **25**, especially in the pMerTK ELISA assay (IC₅₀ 250 nM).

The weak cellular activity of these analogues may be due to the polar α-amino amide linker. Therefore, we varied the linker of the macrocycle to explore if any other linker would improve the cellular activity (Table 3). When the primary amine was protected by a Boc group (**26**) or removed (**27**), the activity against MerTK was dramatically reduced (over 10-fold in the MCE assay compared to compound **11**) due to lack of the key hydrogen bond with either D741 or R727 or N728. On the other hand, a similarly active compound **28** was obtained by substitution of the primary amine to yield a secondary amino group. Unfortunately, the cellular activity of **28** wasn't improved by the reducing the polarity and removing a hydrogen bond donor of the amino group because these changes decreased the MerTK inhibitory activity of **28** (3-fold) as well. In addition, replacement of the amide group in **11** with a less polar ester group such as in **29**, preserved activity in either the MerTK MCE or pMerTK ELISA assays. Another attempt to reduce the polarity while retaining the hydrogen bond donor property of the α-amino amide linker was to replace it with a secondary hydroxyl group. Three analogues were prepared (**30–32**). The ring size and the position of the hydrogen bond donor of analogue **30** was the same as analogue **10**. Both analogues had similar MerTK activity, however, the Flt3 selectivity of **30** was diminished from 89-fold (**10**) to 24-fold (**30**). The activity and selectivity were worse for analogue **31**. Although the ring size and the position of the hydrogen donor of **31** was the same as **12**, analogue **31** was 34-fold less active than **12** in the MerTK MCE assay and only had 5-fold

selectivity over Flt3. Interestingly, analogue **32** had the same ring size and the same position of its hydrogen bond donor as **3**, was 10-fold more active than **3** in the MCE assay. It also had weak activity in the pMerTK ELISA assay (IC_{50} 600 nM). Linkers containing oxygen as the hydrogen bond acceptor such as epoxide (**33**) or ether (**34** & **35**), showed much lower MerTK inhibitory activity. More rigid macrocycles containing a phenyl ring resulted in analogues **36** and **37**. Analogue **36** had the same-sized macrocycle with its hydrogen bond acceptor, an amino group, at the same position as **22**. However, it was 27-fold less active than **22** in the MCE assay. Similarly, analogue **37** was 7-fold weaker than **25** with similar selectivity over Flt3. Overall, the amide linker was optimal in this series.

To confirm our initial design strategy and gain better insight into ligand-protein interactions, we solved the X-ray structure of MerTK in complex with **11** at a resolution of 2.23 Å. Overall, the structure corroborated the preliminary docking hypothesis (Figure 1). However, there was one notable difference. In the crystal structure (Figure 2), both the carbonyl and primary amine groups of the macrocycle pointed outward with respect to the residues R727, N728 and D741, while we had hypothesized that the main interaction in this region would occur between only the primary amine and the above three residues. Our docking hypothesis was based on previous non-macrocyclic amino-pyridines, such as **1**, which all needed either a hydroxyl or a primary amino group to gain potency through interactions with R727, N728 or D741.

In addition, we used a panel of 30 kinases which emphasized tyrosine kinases along with selected serine/threonine kinases to assess the selectivity of promising compounds over other kinase families.⁸⁻⁹ Analogue **11** was evaluated in this panel at a concentration 100 fold above its Mer IC_{50} (Figure 3) (detailed in supplemental materials). Similar to its open-chained analogue (substituted pyrimidines),⁸ five tyrosine kinases were inhibited greater than 50% in the presence of 430 nM **11**. However, three additional serine/threonine kinases were inhibited greater than 50% and were not inhibited by **1** under the same conditions.

In summary, novel and potent macrocyclic pyrimidines have been discovered as MerTK kinase inhibitors via a structure-based drug design approach. The binding mode of these macrocycles was confirmed by an X-ray structure of MerTK protein complexed with analogue **11**. A high-throughput ELISA assay was also employed to evaluate the inhibitory activity of the compounds in a cell-based assay of tyrosine phosphorylated MerTK. These macrocycles proved to be MerTK-selective within the TAM family and over Flt3. However, the selectivity over other kinase families and the cellular activity of these macrocycles need to be further improved to yield a lead compound worthy of further progression in this series.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

TAM	Tyro3-Axl-Mer
AML	acute myeloid leukemia
MCE	microfluidic capillary electrophoresis
IC₅₀	half maximal inhibitory concentration
pMerTK	phosphorylated Mer tyrosine kinase
ELISA	enzyme-linked immunosorbent assay
EC₅₀	half maximal effective concentration

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Figure 1.

a. Structure of **1**; b. X-ray crystal structure of **1** in complex with MerTK (kinase domain) (PDB ID code 4MHA); c. Structure of **2**; d. Docking model of macrocyclic pyrimidine **2**.

Scheme 1.
The synthetic route for macrocyclic compounds

Figure 2.

a. Structure of **11**; b&c. X-ray crystal structure of **11** in complex with Mer (kinase domain) (PDB ID code 5K0X).

Figure 3.
Kinase Tree

Table 1

Preliminary SAR of macrocycle ring size

Compound	n	m	IC ₅₀ (nM) ^a			EC ₅₀ (nM) ^a	
			MerTK	Axl	Tyro3	Flt3	pMerTK ELISA
2	2	1	61 ± 46	1600 ± 370	1800 ± 970	6700 ± 6300	>1000
3	1	1	200 ± 140	21000 ± 7300	8200 ± 7000	inactive	>1000
4	3	1	760 ± 330	28000 ± 2900	inactive	inactive	>1000
5	4	1	230 ± 54	7800 ± 2800	8500 ± 5300	14000 ± 14000	>1000
6	5	1	91 ± 28	2700 ± 1000	3400 ± 1500	10000 ± 1800	>1000
7	6	1	62 ± 25	1100 ± 340	2700 ± 2000	2100 ± 560	>1000
8	1	2	3200 ± 2500	inactive	24000 ± 3300	inactive	>1000
9	2	2	140 ± 96	4000 ± 1400	6900 ± 5800	inactive	>1000
10	3	2	36 ± 14	1700 ± 350	1600 ± 910	3200 ± 610	>1000
11	4	2	4.4 ± 0.5	120 ± 60	220 ± 82	320 ± 11	510 ± 300
12	5	2	4.1 ± 0.2	130 ± 16	150 ± 4.4	240 ^b	ND
13	6	2	6.2 ± 3.3	170 ± 13	160 ± 110	120 ± 36	790 ± 110

^aValues are the mean of two or more independent assays ± SD.^bOnly test once.

Table 2

SAR Study of the R' position

Compound	R'	IC ₅₀ (nM) ^a				EC ₅₀ (nM) ^a
		MerTK	Axl	Tyro3	Flt3	pMerTK ELISA
14		6.4 ± 1.3	180 ± 98	330 ± 110	740 ± 210	470 ± 79
15		25 ± 21	500 ± 30	920 ± 470	5900 ± 470	>1000
16		6.0 ± 2.8	100 ± 34	340 ± 210	49 ± 11	>1000
17		9.5 ± 2.9	300 ± 130	800 ± 340	38 ± 43	>1000
18		4.3 ± 1.1	83 ± 54	150 ± 27	22 ± 3.1	>1000
19		6.3 ± 3.3	330 ± 270	340 ± 230	960 ± 410	>1000
20		4.4 ± 4.1	110 ± 41	360 ± 230	780 ± 110	>1000
21		22 ± 16	540 ± 170	680 ± 470	890 ± 260	>1000

Compound	R'	IC ₅₀ (nM) ^a				EC ₅₀ (nM) ^a
		MerTK	Axl	Tyro3	Flt3	pMerTK ELISA
22		1.5 ± 0.4	72 ± 31	86 ± 2.1	290 ± 25	420 ^b
23		6.5 ± 2.0	150 ± 66	290 ± 220	850 ± 280	>1000
24		14 ± 8.5	300 ± 89	970 ± 680	860 ± 220	>1000
25		2.9 ± 1.9	50 ± 22	150 ± 200	730 ± 260	250 ± 28

^aValues are the mean of two or more independent assays ± SD.

^bOnly test once.

Table 3

Exploration of different macrocycle linkers

Compound	Structure	IC ₅₀ (nM) ^a				EC ₅₀ (nM) ^a
		Mer	Axl	Tyro3	Flt3	pMerTK ELISA
26		54 ± 2.7	1300 ± 190	2200 ± 350	inactive	>1000
27 ^b		59	670	2300	4500	750
28		11 ± 2.7	210 ± 92	850 ± 350	840 ± 350	410 ± 140
29		3.9 ± 0.1	220 ± 11	190 ± 49	65 ± 13	430 ± 42
30 ^b		68	1600	1500	1600	>1000
31 ^b		140	1300	2500	630	>1000
32		21 ^b	920 ^b	1100 ^b	900 ^b	600 ± 76
33		480 ± 150	4400 ± 1100	9200 ± 4100	3100 ± 1700	ND
34		770 ± 600	20170 ± 17020	17350 ± 15500	20080 ± 17190	ND
35		14000 ± 10000	inactive	inactive	inactive	ND

Compound	Structure	IC ₅₀ (nM) ^a			EC ₅₀ (nM) ^a	
		Mer	Axl	Tyro3	Flt3	pMerTK ELISA
36		40 ± 13	300 ± 150	670 ± 470	9700 ± 6500	>1000
37		21 ± 7.0	590 ± 340	1200 ± 1500	15140 ± 21010	>1000

^aValues are the mean of two or more independent assays ± SD.

^bOnly test once.