Identification of Pyrrole[3,4-c]pyrazoles as Potent Tropomyosin Receptor Kinase A (TrkA) Inhibitors

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Tropomyosin receptor kinase A (TrkA) is a receptor tyrosine kinase, encoded with the neurotrophic tyrosine receptor kinase 1 (NTRK1) gene.[1] Its homologs, TrkB and TrkC, are associated with the NTRK2 and NTRK3 genes, respectively. TrkA is the high-affinity catalytic receptor for the neurotrophic, nerve growth factor (NGF) and mediates the NGF function: neuronal differentiation and anti-apoptotic event, and it is mainly expressed in the nervous systems and non-neuronal cells, including monocytes, lung, bone, and pancreatic beta cells.[2] TrkA has recently been highlighted as a novel drug target as its mutations have been reported in many cancers: activating TrkA deletion mutation to acute myeloid leukemia (AML), TrkA alternative splicing variant (TrkAIII) to neuroblastoma (NB), missense somatic mutation NTKS M379I and R577G to melanoma, and oncogenic fusion MPRIP-NTRK1 and CD74-NTRK1 to lung adenocarcinoma.[3–6] Although several TrkA inhibitors are currently under development for various anticancer treatments, many of them are multi-kinase inhibitors and have limited chemical motif diversity (Figure 1).[7] Herein, we wish to report the new finding of pyrrole[3,4-c]pyrazole, RMK-036 as a TrkA inhibitor and disclose its limited *structure-activity relationship*.

The pyrrolo[3,4-c]pyrazoles have previously been investigated for their CDK2, PAK, and Aurora-A kinase activities.[8–10] To make a concise and conclusive result with the previously reported information, the TrkA inhibition study was planned as follows. First, as the pyrrolo[3,4-c] pyrazole C3 amido derivatives are widely explored, the C3 carbamates are designed for novelty consideration; benzyloxycarbonyl (Cbz) was introduced for C3-NH derivatization in this study. Second, *N*-butyl urea was adopted for the pyrrolo[3,4-c]pyrazole N5 substitution based on reported potencies to kinases and synthetic easiness.[8] Finally, as the importance of the substitution at the pyrrolo[3,4-c]pyrazole C6 position, which occupies the hydrophobic buried region of the CDK2, has previously been reported, the C6 unsubstituted cyclopropyl, dimethyl, and monomethyl are introduced into the pyrrolo[3,4-c]pyrazole to explore TrkA potency and selectivity in this report. Right-sized substitution in the C6 hydrophobic area will enjoy maximum hydrophobic interaction; however, the undersized substitution cannot make a full hydrophobic interaction, and the excessively larger displacement may suffer the steric clash in that pocket.

Synthetic chemistry toward RMK-036 is outlined in Scheme 1. Starting from commercially available 1-aminocyclopropane-1-carboxylic acid, a key intermediate pyrazolamine **1** was readily prepared using the reported procedure.[8,10(b)] The pyrazolamine (**1**) was coupled with benzyl chloroformate to provide a benzylcarbamate (**2**). After Boc de-protection of **2** using trifluoroacetic acid, the crude was treated with *n*-butyl isocyanate to form an *n*-butyl urea **3**. The final deprotection of the ethyl carbamate of **3** with methanol under basic atmosphere resulted in RMK-036 (69% three-step yield). The products were purified using silica gel column chromatography and reverse-phase HPLC if necessary. Analogs **4–6** are prepared in a similar manner. Boc deprotection of **1** with trifluoroacetic acid, followed by coupling with stoichiometric *n*-butyl isocyanate and ethyl carbamate deprotection provided **7**.

As the C6 position of the pyrrolo[3,4-c]pyrazole motif displays a unique hydrophobic *structure-activity relationship* to CDK2 and Aurora-A, the cyclopropyl-substituted (RMK-036), unsubstituted (**4**), dimethyl-substituted (**5**), and *racemic* monomethyl-substituted (**6**) derivatives at the C6 position were prepared to examine their activity to TrkA and are summarized in Table 1. The C6 cyclopropyl-substituted RMK-036 is the most potent to TrkA in the biochemical assay (IC_{50} = 0.019 μM).[11] The C6 dimethyl-substituted **5** and *racemic* monomethyl-substituted **6** are

‖ These authors contributed equally to this work.

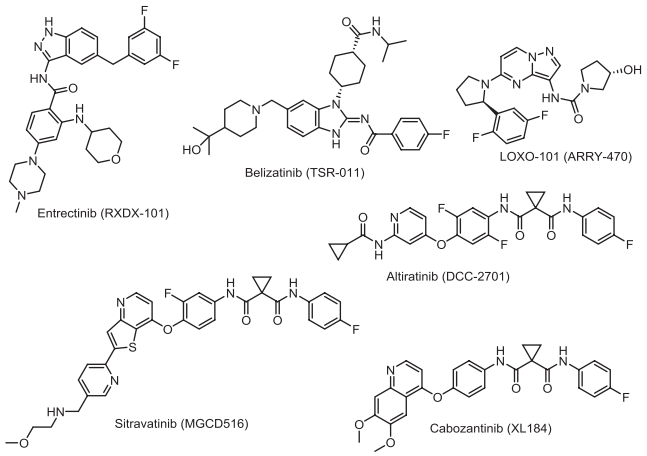
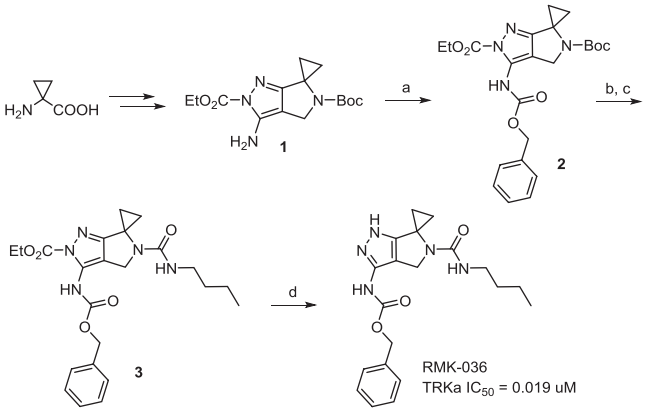
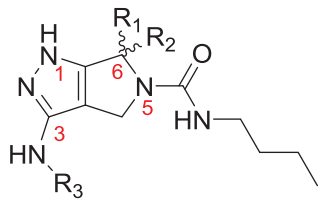


Figure 1. TrkA inhibitors.



Scheme 1. Preparation of RMK-036. Regents and conditions: (a) benzyl chloroformate, DIPEA, THF, 0 °C–rt, 12 h, 20%; (b) TFA/CH_2Cl_2 (1:1), rt, 0.5 h; (c) *n*-butyl isocyanate, Et_3N, CH_2Cl_2, 0 °C–rt, 2 days; (d) Et_3N/MeOH (1:1), rt, overnight, 69% in three steps.

Table 1. Structure and SAR of RMK-036 analogs.



Compounds	R_1	R_2	R_3	TrkA IC_{50} (μM)
RMK-036	Cyclopropyl		Cbz	0.019
4	H	H	Cbz	>10
5	CH_3	CH_3	Cbz	0.21
6	H	CH_3	Cbz	0.39
7	Cyclopropyl		H	>10

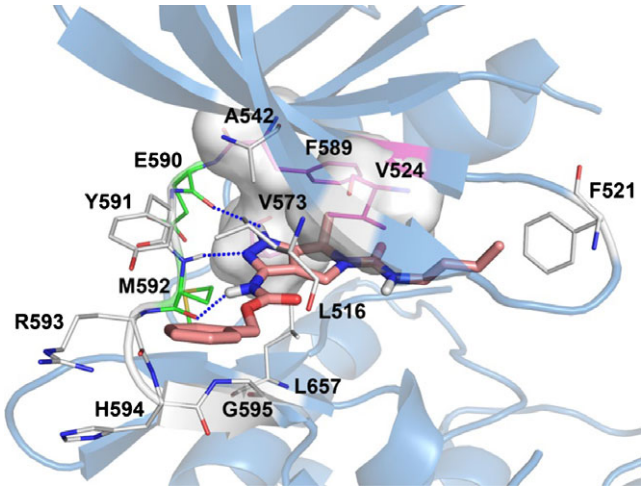


Figure 2. Proposed binding mode of RMK-036 within TrkA. (TrkA is depicted by ribbons, with interacting residues represented as sticks. Residues in green are in the hinge region and magenta in the buried region. Hydrogen-bonding interactions are drawn as blue dashed lines, and the buried region is shown as the surface.)

less potent than RMK-036 to TrkA (IC_{50} = 0.21 and 0.39 μM, respectively). However, the C6 unsubstituted analog **4** is inactive in our assay (IC_{50} > 10 μM). This finding confirms the importance of the hydrophobic interaction at this region, and the pyrrolo[3,4-c]pyrazole C6 cyclopropyl is the optimal size for TrkA inhibition. In addition to this, the pyrrolo[3,4-c]pyrazole C3 Cbz of RMK-036 is confirmed to be essential for the TrkA inhibition. When the C3 Cbz of RMK-036 was removed, compound **7** loses activity to TrkA completely.

To gain insight into a potential binding mode of RMK-036 to TrkA at an atomic level, molecular docking has been studied (Figure 2).[12–16] The pyrrolo[3,4-c]pyrazole of RMK-036 participates in three hydrogen bonds, with E590 and M592 in the hinge region. In addition, this moiety creates a CH-π interaction with A542 as well as hydrophobic interactions with V524 and L657. The C6 cyclopropyl group forms hydrophobic interactions with F589, V524, and V573 in the buried region, which is a small cavity at the back of TrkA's pocket. The previous study shows that the occupation of this buried region by small hydrophobic groups, such as cyclopropyl, dimethyl, and methyl, is beneficial to the ligand–protein binding.[8] In the case of TrkA, it appears that the C6 cyclopropyl group is the optimal size, with a hydrophobic character for RMK compounds to interact with residues in the buried region. The C3 benzyl carbamate group is exposed to the solvent through a hydrophobic interaction with L516. Additional hydrophobic interaction occurs between the N5 urea *N*-butyl group and F521 on the P-loop.

In conclusion, during our ongoing research program, we identified a pyrrole[3,4-c]pyrazole, RMK-036, as a novel class of TrkA inhibitor. Substituted with cyclopropyl at the pyrrole[3,4-c]pyrazole C6 position, RMK-036 inhibits TrkA with 19 nM of IC_{50} value. Various C6 substitution

analogs of RMK-036 reveal dramatic *structure-activity relationship* in TrkA inhibition. The structural basis for the inhibition of TrkA activity by RML-036 at the ATP binding site has also been examined by molecular docking studies. The information on molecular interactions between RMK-036 and TrkA would be valuable during the subsequent lead optimization process.

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Supporting Information. Additional supporting information is available in the online version of this article.

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11. TrkA assay description: Purified TrkA protein was purchased from Carna Biosciences (Kobe, Japan). Kinase, biotinylated substrate peptide, and inhibitors were added for pre-incubation in 384-well plates, and then the reaction was started by the addition of ATP. After 30 min, an anti-phosphotyrosine antibody labeled with europium (Eu) cryptate and streptavidin labeled with the fluorophore XL665 were added to the reaction mixtures. After 1 h incubation, fluorescence of samples was measured using an Envision multi-label reader (Perkin Elmer, Waltham, MA, USA) with 320 nm excitation and 620 nm (Eu-labeled antibody) and 665 nm (XL665-labeled streptavidin) dual emissions. The homogeneous time-resolved fluorescence (FRET) between Eu cryptate and XL665 was measured to quantify the phosphorylation of the substrate peptide. The ratio of both intensities was calculated with 12 different serially diluted concentrations (0.03–10 000 nM) of compounds tested. The data were plotted against inhibitor concentrations to determine IC_{50} values, and the IC_{50} values were calculated by using the Prizm program (La Jolla, CA, USA).
12. Molecular docking was carried out using the Maestro v10.2 (Schrödinger, Inc.)[13] with the crystal structure of TrkA (PDB code: 4PMT).[14] Structural defects of TrkA were fixed by the Protein Preparation Wizard module and low-energy 3D structures of TrkA inhibitor RMK-036 was generated by the LigPrep. Based on a grid box of $30 \times 30 \times 30$ Å^3 centered on ligand in complex with TrkA, RMK-036 was then docked into the TrkA kinase catalytic domain using the Glide module in Extra Precision (XP) mode. The protein-ligand interactions were analyzed using the Discovery Studio Modeling Environment v4.0[15] and the binding models of compounds were displayed using the PyMOL software.[16]
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