

1,1-Difluoroethyl-substituted triazolothienopyrimidines as inhibitors of a human urea transport protein (UT-B): New analogs and binding model

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ABSTRACT

The kidney urea transport protein UT-B is an attractive target for the development of small-molecule inhibitors with a novel diuretic ('urearetic') action. Previously, two compounds in the triazolothienopyrimidine scaffold (**1a** and **1c**) were reported as UT-B inhibitors. Compound **1c** incorporates a 1,1-difluoroethyl group, which affords improved microsomal stability when compared to the corresponding ethyl-substituted compound **1a**. Here, a small focused library (**4a–4f**) was developed around lead inhibitor **1c** to investigate the requirement of an amidine-linked thiophene in the inhibitor scaffold. Two compounds (**4a** and **4b**) with nanomolar inhibitory potency ($IC_{50} \approx 40$ nM) were synthesized. Computational docking of lead structure **1c** and **4a–4f** into a homology model of the UT-B cytoplasmic surface suggested binding with the core heterocycle buried deep into the hydrophobic pore region of the protein.

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Kidney urea transporters (UTs) are required for the formation of a concentrated urine.^{1,2} Epithelial cells in some kidney tubules express UT-A proteins, which are encoded by the *SLC14A2* gene, and endothelial cells in some microvessels express UT-B, which is encoded by the *SLC14A1* gene.^{3–5} Knockout mice lacking various UTs manifest impaired urinary concentrating ability, suggesting the application of UT inhibitors as 'urearetics' with a novel diuretic mechanism of action.^{6–9}

Early high-throughput screening using a human erythrocyte lysis assay identified phenylsulfoxoxazole inhibitors of human UT-B with $IC_{50} \approx 100$ nM.¹⁰ Limitations of the original compounds included poor metabolic stability and poor activity against rodent UT-B. Further screening using a mouse erythrocyte lysis assay yielded triazolothienopyrimidine UT-B inhibitors (Fig. 1), with the most potent compound, **1a**, having $IC_{50} \approx 11$ nM for inhibition of human UT-B and 25 nM for inhibition of mouse UT-B.¹¹ Administration of **1a** to mice reduced urine osmolality and increased urine output. Compound **1a** and related analogs inhibit UT-B by a competitive mechanism.

Though **1a** was effective in mice when administered in high concentration, it underwent rapid oxidation by hepatic enzymes, mostly at the benzylic position of the ethyl substituent. A collection of 273 commercially available analogs of **1a** (exploring the

R¹ position as well as variations at the amidine linkage) were evaluated, with no improved leads identified.¹² One interesting analog (**1b**) was synthesized containing a trifluoromethyl group at position R¹, which resisted microsomal degradation, but suffered significant loss of potency against UT-B. Reasoning that a two carbon atom substituent was required for potency, but that benzylic hydrogens are prone to radical abstraction, we synthesized the 1,1'-difluoroethyl inhibitor (**1c**), and found that the inhibitor resisted microsomal degradation with little effect on UT-B inhibition activity ($IC_{50} \approx 14$ nM) of the original lead **1a**.¹² We chose to incorporate the amidine-linked thiophene-2-methyl group into **1c**, which has generally been associated with more potent and stable inhibitors in this class. Herein, we prepared a small library of analogs of **1c**, in which alternative amidine linked groups were evaluated for UT-B inhibitory potency and hepatic microsomal stability. Indeed, while the stability of **1c** was enhanced greatly by the incorporation of the 1,1'-difluoroethyl substituent, a slow microsomal oxidation reaction could still be observed by LCMS, which we speculate might be modulated by variation of the amidine substituent.

The synthetic approach to generate this library of inhibitors, with differing amidine substituents tethered from the core heterocycle is summarized in Scheme 1. As was described previously, 4-bromo-(1,1-difluoroethyl)-benzene (**2**) was transformed to the corresponding triazolothienopyrimidine (**3**) by [2+3] cycloaddition with an azidothiophene ester.^{12,11} Subsequently, lactam **3**

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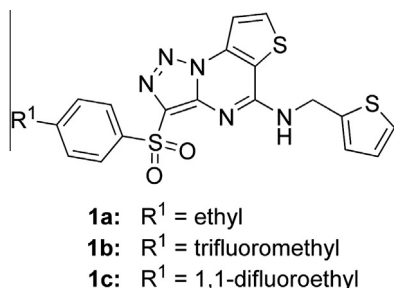
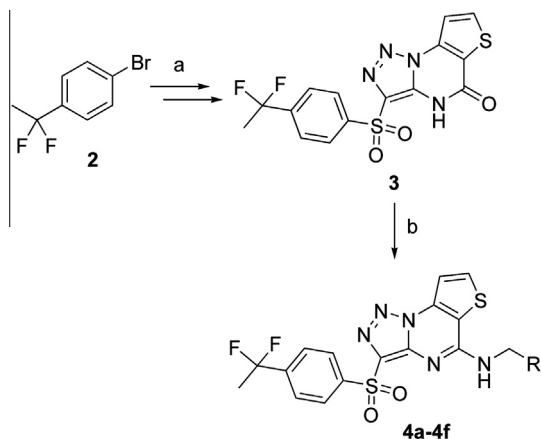


Figure 1. Previously characterized triazolothienopyrimidine inhibitors of UT-B.



Scheme 1. Synthesis of amidine analogs of triazolothienopyrimidine lead structure **1c**. Reagents and conditions: (a) described previously;¹² (b) 1° amines, PyBOP, CH₃CN, DBU, microwave irradiation, 100 °C, 30 min.

was coupled with various primary amines, using PyBOP and DBU under microwave irradiation, to generate a library of amidine derivatives (**4a–4f**). While amidine formation using PyBOP and DBU has been described previously,^{13,14} we observed that microwave irradiation was necessary to effect quantitative conversion in this class of molecules. Typically the compounds could be isolated with simple aqueous workup, and purified by trituration with cold methanol. All of the compounds were characterized by ¹H NMR and HRMS, and had a characteristic triplet (³J = ~18 Hz) due to coupling between the terminal methyl and neighboring difluoromethylene groups. Yields and spectroscopic characterization of these compounds are provided in [Supplementary data](#).

The library was designed to explore the effect of the amidine substituent on UT-B inhibition potency and on microsomal stability ([Table 1](#)). Compounds **1a** and **1c** were the previously characterized inhibitors.^{11,12} The new inhibitors synthesized in this library had amidine substituents designed to explore: methyl substitution on the thiophene (**4a**), an isomeric thiophene connection point (**4b**), replacement of the thiophene heterocycle for furan (**4c**) or benzyl (**4d**), and the use of small hydrophobic alkyl ring systems such as cyclopropylmethyl (**4e**) or cyclobutylmethyl (**4f**).

UT-B inhibition potency was determined by an erythrocyte lysis assay, as previously described¹⁰ in which lysis is measured in acetamide-loaded erythrocytes following dilution into isosmolar PBS ([Supplementary data](#)). Erythrocytes express the UT-B protein, which facilitates the transmembrane transport of urea, acetamide and other small polar solutes. UT-B inhibition increases hypotonic lysis, as measured by near-infrared light absorbance, because water influx is not opposed by acetamide efflux. Microsomal stability was determined by incubation of compounds in the presence of

Table 1
UT-B inhibition activity^a and microsomal stability^b of synthesized compounds

Compd	R ¹	R ²	IC ₅₀ (nM)	% Remaining ^b
1a			11	<5
1c			14	96
4a			38	22
4b			43	47
4c			353	14
4d			>800	79
4e			>800	75
4f			>800	24

^a Determined by mouse erythrocyte lysis assay (*n* = 3). IC₅₀ determined by fit to a single-site saturation model.

^b Percentage of parent compound remaining after 30 min incubation with rat liver microsomes.

rat liver microsomes and NADPH, followed by organic extraction and LCMS analysis, as described¹² ([Supplementary data](#)).

In general, modification of the amidine substituent reduced inhibition potency and microsomal stability. Two of the more potent new inhibitors evaluated were **4a** (containing 4-methylthiophene) and **4b** (thiophene connected at the 3 position). Both of these inhibitors had modest potency, but suffered in terms of microsomal stability when compared to **1c**. An analog containing furan (**4c**) instead of thiophene (**1c**) showed poor potency and stability, which was surprising due to the similarity between the thiophene and furan heterocycles. This is consistent with another observation, in which the furan analog of **1a** was found to have greatly reduced potency.¹² Replacement of the amidine-linked thiophene with non-heterocyclic rings such as benzyl (**4d**), cyclopropylmethyl (**4e**), and cyclobutylmethyl (**4f**) also greatly reduced potency. In the case of **4d** and **4e**, these modifications mildly improved microsomal stability compared to **1a**.

Molecular modeling and docking was done to investigate the potential inhibitor binding site. While a high-resolution structural model of UT-B has not been reported, we constructed a homology model for UT-B using the SWISS MODEL utility, using the full human UT-B1 protein sequence, and have previously used this model to postulate a mode of binding of **1a**.¹¹ Compounds **1c** and **4a–4f**

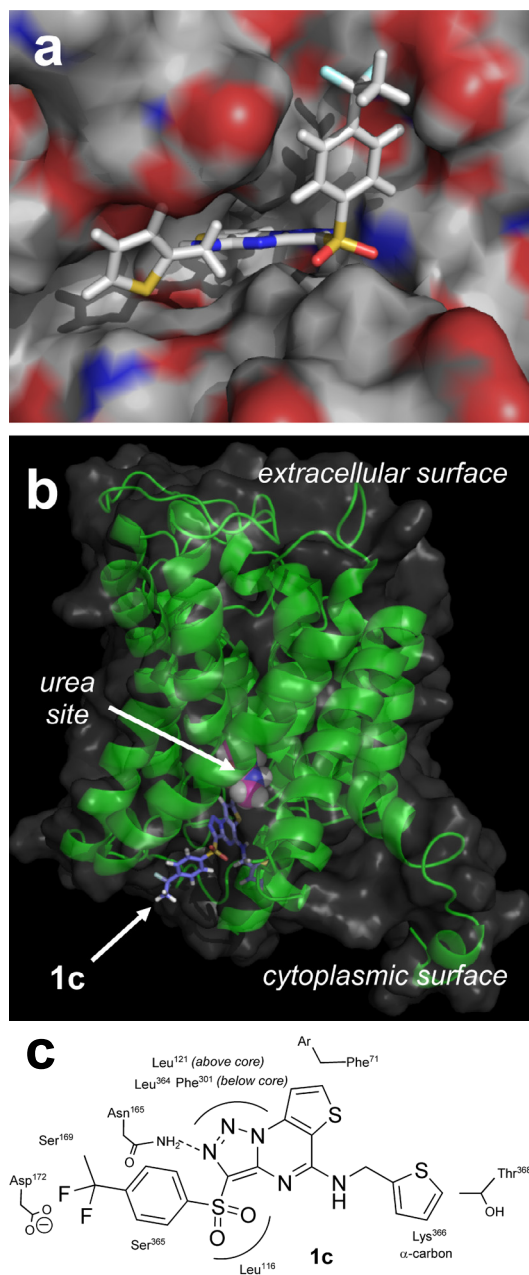


Figure 2. Model of the binding of **1c** with the cytoplasmic channel region of human UT-B based on a homology model: (a) zoomed-in and (b) zoomed-out depictions; (c) potential interactions between **1c** and UT-B cytoplasmic surface residues.

were docked into the same homology model using FRED v2.2.5 (OpenEye Scientific).^{15,16} Figure 2 shows a tentative model for binding of inhibitor **1c**, zoomed in and out, with the key interactions shown. We offer several observations from the modeling:

(a) The computed mode of binding of inhibitor **1c** is consistent with our previously reported model of **1a**. A similar mode of binding was found for **4a** and **4c–4f**, with the orientation of the core heterocycle, aryl sulfone, and amidine substituents being consistent (Supplementary Fig. S1). In the postulated binding mode, the core heterocycle is buried deep into a hydrophobic pocket surrounded by Leu¹²¹, Leu³⁶⁵, Phe³⁰¹, Phe⁷¹, and other amino acids. There is a potential hydrogen bonding interaction between the Asn¹⁶⁵ sidechain and an exposed triazole nitrogen. The aryl sulfone is surrounded by Ser¹⁶⁹, Ser³⁶⁵, and Asp¹⁷², while the thiophene group resides near the Thr³⁶⁸ methyl group and Lys³⁶⁶. Overall,

the inhibitor has a good complementary fit to the surface of UT-B, and aside from the one potential hydrogen bonding interaction, most likely is bound due to hydrophobic interactions with non-polar amino acid residues. Inhibitor **4b** is an exception compared to the other compounds, in that the lowest energy conformation presented the thiophene deep into the pocket.

(b) The docking software used has six independent scoring functions that evaluate protein–ligand interactions using different algorithms. Of the scoring functions available, five reliably distinguished the relatively active inhibitors (**1a**, **1c**, **4a** and **4b**) from the relatively inactive inhibitors (**4c–4f**): Shapegauss, PLP, Chemgauss3, OEChemScore, and Screenshot. The sixth scoring function (ZapBind), which weights heavily toward electrostatic interactions, was not useful at discriminating active from inactive inhibitors in this series, which is expected given the limited electrostatic interactions in our postulated binding mode.

(c) Deciphering the structural determinants of the active and inactive compounds is challenging. The thiophenemethyl amidine compounds (**1a** and **1c**) appear to have the most complementary overlap, giving rise to the most potent compounds. Alkylation of the thiophene (**4a**) or simple use of an isomerically connected thiophene (**4b**) decreases potency, albeit not catastrophically. Most puzzling is the severe drop in potency in the furan-based inhibitor (**4c**), for which docking identified a similar mode of binding as the more potent thiophene-based inhibitor **1c**. Nevertheless, with the FRED scoring functions named above (excluding ZapBind) all correctly ranked **1c** as a better scoring ligand than **4c**. The remaining less active inhibitors (**4d–4f**) were scored poorly by FRED, and apparently the amidine linked substituent had less complementary fit compared to the more active compounds.

In summary, a focused library of six analogs of UT-B inhibitor **1c** was prepared to characterize the structural requirements for potency and microsomal stability. Two additional compounds with modest potency (**4a** and **4b**) were discovered, but none were able to match **1c**. Computational docking into a homology model of UT-B gave a tentative mode of binding for **1c** and **4a–4f**. While caution should be used in over-interpreting docking results from homology models, we were gratified to see that docking with multiple independent scoring functions effectively distinguished the active from inactive analogs, and that with exception of one compound (**4b**), the docked conformations were consistent.

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Supplementary data

Supplementary data (these data include synthetic procedures and spectroscopic characterization of compounds **4a–4f**, and an additional figure related to the computational modeling experiments.) associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmcl.2013.03.089>.

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