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RESEARCH ARTICLE

Synthesis and evaluation of 5,6-disubstituted thiopyrimidine aryl aminothiazoles as inhibitors of the calcium-activated chloride channel TMEM16A/Ano1

Katarzyna A. Piechowicz[1], Eric C. Truong[1], Kashif M. Javed[1], Rachelle R. Chaney[1], Johnny Y. Wu[1], Puay W. Phuan[2], Alan S. Verkman[2], and Marc O. Anderson[1]

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Abstract

Transmembrane protein 16A (TMEM16A), also called Ano1, is a Ca^{2+} activated Cl^- channel expressed widely in mammalian epithelia, as well as in vascular smooth muscle and some tumors and electrically excitable cells. TMEM16A inhibitors have potential utility for treatment of disorders of epithelial fluid and mucus secretion, hypertension, some cancers and other diseases. 4-Aryl-2-amino thiazole **T16A$_{inh}$-01** was previously identified by high-throughput screening. Here, a library of 47 compounds were prepared that explored the 5,6-disubstituted pyrimidine scaffold found in **T16A$_{inh}$-01**. TMEM16A inhibition activity was measured using fluorescence plate reader and short-circuit current assays. We found that very little structural variation of **T16A$_{inh}$-01** was tolerated, with most compounds showing no activity at 10 μM. The most potent compound in the series, **9bo**, which substitutes 4-methoxyphenyl in **T16A$_{inh}$-01** with 2-thiophene, had IC_{50} ~1 μM for inhibition of TMEM16A chloride conductance.

Keywords

Aminothiazole, anoctamin, calcium-activate chloride channel, thiopyrimidine, thiouracil, transmembrane protein 16A

History

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Introduction

Transmembrane protein 16A (TMEM16A) (also known as anoctamin1, ANO1, DOG1, ORAOV2, TAOS-2) is a Ca^{2+}-activated Cl^- channel (CaCC) that is expressed widely in mammalian tissues, including secretory epithelial cells, smooth muscle cells in the airways and reproductive tract, interstitial cells of Cajal and nociceptive neurons[1,2]. TMEM16A is overexpressed in some human cancers and its expression has been correlated with tumor grade[3,4]. Studies in TMEM16A knockout mice have implicated its involvement in tracheal development[5,6] and mucociliary clearance[7], with knockout mice showing mucus accumulation in the airways[8]. TMEM16A knockout or knockdown is associated with diminished rhythmic contraction of gastric smooth muscle cells[5], defective protein reabsorption in kidney proximal tubule[9] and attenuated pain response[10]. TMEM16A knockout mice also manifest reduced blood pressure and decreased hypertensive response following vasoconstrictor treatment[11].

TMEM16A contains eight putative transmembrane domains with intracellular NH_2 and COOH termini and two calmodulin binding domains[1,2]. Putative Ca^{2+} binding sites are located at E702 and E705[12]. The TMEM16A protein appears to be structured as a homodimer[13,14]. TMEM16A is expressed in multiple splice variants that have variable sensitivity to cytosolic Ca^{2+}[15,16]. An X-ray crystal structure (3.4 Å resolution) was recently solved of a fungal TMEM16 isoform with Ca^{2+}-activated lipid scramblase activity (nhTMEM16), which has 39–42% homology to mammalian TMEM16A[17]. nhTMEM16 contains 10 transmembrane segments per subunit and a region of six residues (including glutamate and aspartate), surrounding bound Ca^{2+} ions, providing a potential structural explanation for Ca^{2+}-activation.

Pharmacological inhibition of TMEM16A has been proposed to be of utility for inflammatory and reactive airways diseases and hypertension, and perhaps for pain and cancer[1]. TMEM16A activation has been considered as a therapeutic strategy to treat cystic fibrosis, gastrointestinal hypomotility and salivary gland hypofunction[18–20]. TMEM16A has recently been proposed as a target in chronic inflammatory disease[21]. Non-selective CaCC inhibitors, which inhibit TMEM16A as well as non-TMEM16A (as yet unidentified) CaCCs have been identified by high-throughput screening[22]. TMEM16A-selective inhibitors have been identified from functional screens using TMEM16A-transfected cells, which include aminothiazole linked to a disubstituted pyrimidine (**T16A$_{inh}$-A01**; Figure 1)[23]. **T16A$_{inh}$-A01** has been used in studies of TMEM16A function in vascular smooth muscle cells and mammalian blood vessels[24], models of chronic hypoxic pulmonary hypertension[25], epithelial fluid transport[26] and cancer cell proliferation[27].

Herein, we present a systematic structural elaboration of the lead inhibitor **T16A$_{inh}$-A01**, including a variation of its pyrimidine (alkyl, small cycloalkyl and fluoroalkyl) and aminothiazole

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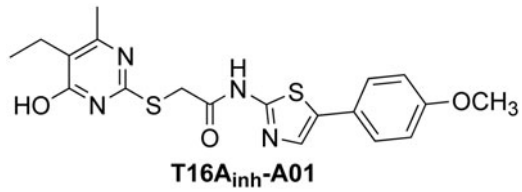


Figure 1. The structure of lead inhibitor **T16A$_{inh}$-A01**.

substituent (aromatic and heteroaromatic). On the pyrimidine, alkyl and cycloalkyl substituents were chosen to probe a possible hydrophobic pocket in the binding site, noting the presence of ethyl and methyl in the lead inhibitor. Fluoroalkyl substituents were considered given their resistance to oxidative metabolism, and ability to form electrostatic interactions. We hypothesized a more substantial interaction with the disubstituted pyrimidine and the binding site, while assuming the role of the aminothiazole substituent less important, and as a possible position for solubilizing groups. To probe this hypothesis, we designed inhibitors replacing 4-methoxyphenyl in **T16A$_{inh}$-A01** with other aromatic rings, as well as a small selection of mono- and bicyclic heterocycles.

Materials and methods

Cell lines and culture

Fischer rat thyroid (FRT) cells were stably transfected with human TMEM16A (the abc isoform) and halide sensor YFP-H148Q/I152L/F46L. Cells were plated in 96-well black-walled microplates (Corning Inc., Corning, NY) at a density of 20 000 cell/well in Coon's modified F-12 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Assays were done 24 h after plating[20,23].

TMEM16A functional assay

Each well of a 96-well plate containing the cultured cells was washed twice with phosphate-buffered saline (PBS) leaving 50 μl. Test compounds (0.5 μl in dimethyl sulfoxide (DMSO)) were added to each well at specified concentration. After 10 min, each well was assayed individually for TMEM16A-mediated I^- influx by recording fluorescence continuously (400 ms/point) for 2 s (baseline), then 50 μl of 140 mM I^- solution was added at 2 s, and then 50 μl of 70 mM I^- solution containing 300 μM adenosine triphosphate (ATP) was added at 6.4 s. The 70 mM I^- solution consisted of a 1:1 mixture of PBS and the 140 mM I^- solution. The initial rate of I^- influx following each of the solution additions was computed from fluorescence data by non-linear regression[20,23,28].

Short-circuit current assay

FRT-TMEM16A cells were grown on Snapwell inserts as described[20] and mounted in Ussing chambers (Physiologic Instruments, San Diego, CA). The basolateral membrane was permeabilized with amphotericin B (250 μg/ml) for 30 min, and a chloride gradient was applied in which the basolateral membrane was bathed with the HCO_3-buffered solution, and in the apical solution 120 mM NaCl was replaced by sodium gluconate. Compounds were added to the apical solution. Cells were bathed for a 10-min stabilization period and aerated with 95% O_2/5% CO_2 at 37 °C before addition of 100 μM ATP. Short-circuit current was measured using an EVC4000 Multi-Channel V/I Clamp (World Precision Instruments, Sarasota, FL).

Chemistry: general

Unless otherwise indicated, all reaction solvents were anhydrous and obtained as such from commercial sources. All other reagents were used as supplied. Reverse-phase high-pressure liquid chromatography (RP-HPLC) analysis was performed using a Dionex Ultimate 3000 system, using a C_{18} column [3 × 150 mm]. Low-resolution electrospray ionization (ESI)-liquid chromatography mass spectrometry (LCMS) was carried out with an Agilent 1100 HPLC coupled to an Agilent 1956B mass spectrometry detector (MSD). RP-HPLC runs typically employed gradients of two solvents: [A] = H_2O (0.05% trifluoroacetic acid (TFA)) and [B] CH_3CN (0.05% TFA); RP-LCMS used the same solvent system with TFA replaced with formic acid (88% aq). The standard HPLC and LCMS gradients proceeded with [A:B] = 95:5 to [A:B] = 5:95 over 10 min. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on either a Bruker 300 or 500 MHz instrument. ^1H NMR chemical shifts are relative to tetramethylsilane (TMS) (δ = 0.00 ppm), $CDCl_3$ (δ 7.26), CD_3OD (δ = 4.87 and 3.31), acetone-d_6 (δ 2.05), or DMSO-d_6 (δ 2.5). ^{13}C NMR chemical shifts are relative to CD_3OD (δ 49.2) or $CDCl_3$ (δ 77.2). Microwave-assisted organic synthesis was performed using a Biotage Initiator instrument. Several compounds were prepared but also had a commercial supplier or were known: **2a–c** (via general procedure 1); **7a**, **b**, **e** and **f** (via general procedure 4); **8a–c**, **e**, **h–m**, **o** and **p** (via general procedure 6); **9ag**, **ai**, **aj** and **ax** (via general procedure 7).

*General procedure 1: 4-aryl-2-aminothiazole bromoacetamides (**2a–c**) prepared from 4-aryl-2-aminothiazoles (**1a–c**)*

Substituted 4-aryl-2-aminothiazole (1.0 eq, 2.5 mmol) (**1a–c**) was dissolved in anhydrous methylene chloride (0.3 M), followed by treatment with triethylamine (1.2 eq) and placed into an ice bath. The reaction mixture was stirred under argon until internal temp was about 0 °C and bromoacetyl bromide (1.05 eq) dissolved in dichloromethane (DCM) was added dropwise. Next, the reaction mixture was stirred under argon for 1 h at room temperature (RT). LCMS indicated consumption of starting material and formation of a product. The crude product was treated with HCl (0.1 M aq; 50 ml), transferred to a separatory funnel and extracted with 1:1 mixture of ethyl acetate and diethyl ether (50 ml). Then, the organic phase was washed with additional HCl (0.1 M aq), brine and was then dried over Na_2SO_4 and concentrated *in vacuo*.

*General procedure 2: 2-amino heteroaryl thiazoles (**4b–d**) prepared from heteroaryl methyl ketones (**3b–d**)*

Heteroaryl methyl ketone (1.0 eq; 8 mmol) (**3b–d**) was dissolved in EtOAc (0.1 M), followed by the addition of $CuBr_2$ (2.0 eq). This reaction mixture was refluxed at 100 °C for 1 h. LCMS indicated consumption of starting material and the formation of the desired bromoketone intermediate. The reaction mixture was then left to cool to RT. Upon reaching RT, the reaction mixture was filtered by using a Buchner funnel, to remove excess precipitated $CuBr_2$, and the filtrate was then added to a fresh round bottom flask (RBF). Thiourea (2.0 eq) was then added into the reaction mixture, which was then heated again for 1 h at 100 °C. Reaction mixtures typically changed from green to orange during the course of the reaction, with the formation of a precipitate. The mixture was then allowed to cool to RT. After reaching RT, the mixture was filtered with a Buchner funnel. The precipitate was then rinsed with ethyl acetate, in order to remove excess thiourea, which generated a crude product. The identity and purity of the product was confirmed by LCMS.

*General procedure 3: 4-heteroaryl-2-aminothiazole chloroacetamide (**5a–d**) prepared from 4-heteroaryl-2-aminothiazoles (**5a–d**)*

4-Heteroaryl 2-aminothiazole (**4a**–**d**) (1.0 eq; 0.4 mmol) was dissolved in 1,2-dichloroethane (DCE):dimethylformamide (DMF) (4:1 mixture, 0.1 M). Bromoacetic acid (7.0 eq), 4-dimethylaminopyridine (4-DMAP) (0.10 eq) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI HCl) (7.0 eq) were added sequentially. The reaction mixture was then refluxed at 100 °C for 1 h, and LCMS confirmed consumption of starting material (SM) and formation of the product. The reaction mixture was cooled to RT, and then taken up into Et_2O:EtOAc (1:1; 50 ml), and washed with HCl (0.1 M aq.; 3×50 ml), then sat. aq. NaCl (50 ml), dried over Na_2SO_4 and concentrated *in vacuo*. Bromoacetamide intermediates were converted to chloroacetamide **(3a–d)** through the course of the reaction, presumably from chloride present in EDCI HCl. Identity as chloroacetamide and purity was confirmed by LCMS. The products were generally pushed to the next step without additional purification or characterization.

*General procedure 4: α-substituted β keto esters (**7**) using potassium carbonate*

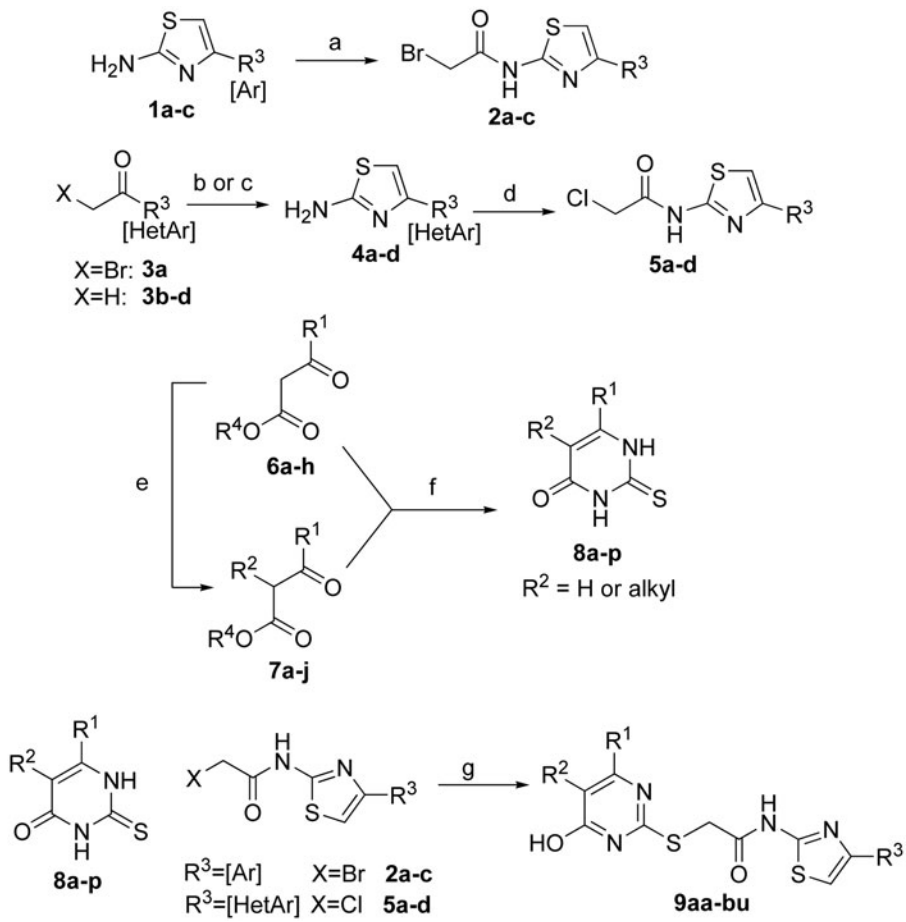
To a mixture of an unsubstituted β-keto ester (**6**) (1 eq) and iodo- or bromoalkane (1.05 eq) in DMF (0.1 M) was added K_2CO_3 (1.5 eq) and the mixture was allowed to briefly stir at RT under argon. Then, the reaction mixture was heated to 60 °C for 30 min. Some products (**7f** and **g**) were formed more effectively with the use of microwave irradiation (110 °C, 10 min). HPLC showed consumption of a starting material and formation of the product. The reaction mixture was taken up in H_2O, extracted with DCM, washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. Crude products were subjected to the subsequent cyclization reactions without additional purification.

*General procedure 5: α-substituted β keto isobutyl esters (**7**) using sodium tert-butoxide*

To a mixture of an unsubstituted β-keto ester (**6**) (1 eq) and bromoalkane (1.05 eq) in *tert*-butanol (0.5 M) was added sodium *tert*-butoxide (1.2 eq) and the mixture was allowed to briefly stir at RT under argon, and was then heated to 90 °C for 24 h. HPLC showed consumption of a starting material and formation of the product. The reaction mixture was taken up in water, extracted with DCM, washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. Crude products were subjected to the subsequent cyclization reactions without additional purification.

*General procedure 6: thiouracils (**8a–p**) generated by cyclization of unsubstituted (**6**) or substituted (**7**) β keto esters*

A freshly prepared solution of sodium ethoxide was obtained by dissolving Na (10 eq) in EtOH (0.1 M), which was treated with a substituted or unsubstituted β-keto ester (**6** or **7**) (1 eq) followed by thiourea (2 eq). The reaction mixture was stirred under argon, heated to 100 °C in an oil bath, and allowed to reflux overnight. LCMS indicated consumption of starting material and formation of a product. The solvent was removed and the crude reaction mixture was acidified with 1 M HCl to pH = 3, extracted with DCM, dried over Na_2SO_4 and concentrated *in vacuo* to give crude thiouracil (**8a**–**p**) products, which were subjected to the coupling reaction without additional purification. Alternatively, the reactions could be affected by microwave irradiation (15 min at 150 °C).



Scheme 1. Synthesis of 4-aryl/heteroaryl-2-aminothiazole inhibitor candidates. *Reagents and conditions*: (a) bromoacetyl bromide, Et_3N, DCM, 0 °C; (b) for bromoketone **6a**: thiourea, THF, 50 °C; (c) for methyl ketones **6b–d**: $CuBr_2$, EtOAc, 100 °C; then thiourea 100 °C; (d) bromoacetic acid, EDCI HCl, cat. 4-DMAP, DCE:DMF (1:1), 100 °C; (e) R-X, base, DMF, 60 °C or MW 110 °C (see experimental); (f) Na/EtOH, thiourea, 100 °C; (g) K_3PO_4-H_2O, DMF. For chloroacetamides (X = Cl), NaI was added to facilitate substitution.

*General procedure 7: substituted thiopyrimidine aryl aminothiazoles (**9aa–bu**) from conjugation of thiouracils (**8a–p**) with 2-aminothiazole haloacetamides (**2a–c** or **5a–d**)*

To a 20 ml scintillation vial was added 4-aryl or 4-heteroaryl 2-aminothiazole haloacetamide (1.0 eq, typically 10–50 mg) (**2** or **5**), in DMF (0.1 M) followed by the addition of a substituted thiouracil (**8**) (1.0–1.2 eq). The reaction mixture was placed in an oil bath pre-heated to 60 °C. In the case of less reactive chloroacetamide (**5a–d**), NaI was added to facilitate the reaction (1 eq). Then, K_3PO_4 monohydrate (3 eq) was added and the vial was heated for 1 h. LCMS indicated consumption of starting materials and formation of product. The crude reaction mixture was diluted with EtOAc (20 ml) and washed five times with brine (20 ml), dried over Na_2SO_4 and concentrated *in vacuo*. Next, the crude reaction mixtures were purified by trituration with Et_2O to give final products (**9aa–bu**). As specified individually, some compounds needed additional purification by preparative HPLC.

*2-Chloro-N-(4-thiophen-2-yl-thiazol-2-yl)-acetamide (**5b**)*

Utilizing general procedure 2, 1-thiophen-2-yl-ethanone (**3b**) (1000 mg, 7.92 mmol) was converted to 4-thiophen-2-yl-thiazol-2-ylamine (**4b**) which was isolated as a white solid (1980 mg, 95%). This was utilized in the next step, utilizing general procedure 3, to generate the title compound (**5b**) as a pink solid (28.4 mg, 25%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 4.39 (s, 2H), 7.10 (t, $J = 2$ Hz, 1H), 7.49 (d, $J = 5$, 1H), 7.51 (s, 1H), 7.52 (d, $J = 5$ Hz, 1H).^{13}C NMR (125 MHz, DMSO-d_6) δ 42.7, 107.4, 124.3, 126.1, 128.5, 138.7, 144.4, 157.9, 165.6. ESI-LCMS (low resolution) m/z calculated for $C_9H_7ClN_2OS_2$ [M + H] 259.7, found [M + H] 259.3.

*2-(4-Hydroxy-5-methyl-6-trifluoromethyl-pyrimidin-2-ylsulfanyl)-N-(4-phenyl-thiazol-2-yl)-acetamide (**9ao**)*

Utilizing general procedure 7 with thiouracil **8p** (20 mg, 0.095 mmol) and aminothiazole bromoacetamide **2a** (28 mg, 0.095 mmol), yellow solid was obtained (5 mg, 12%) after required preparative HPLC purification. ^{1}H NMR (500 MHz, *acetone-d_6*) δ 2.12 (s, 3H), 4.34 (s, 2H), 7.30 (t, $J = 7$ Hz, 1H), 7.40 (t, $J = 7$ Hz, 2H), 7.48 (s, 1H), 7.93 (d, $J = 7$ Hz, 2H). ESI-LCMS (low resolution) m/z calculated for $C_{17}H_{13}F_3N_4O_2S_2$ [M + H] 427.0, found [M + H] 427.2.

*2-(4-Hydroxy-5,6-dimethyl-pyrimidin-2-ylsulfanyl)-N-(4-thiophen-2-yl-thiazol-2-yl)-acetamide (**9bs**)*

Utilizing general procedure 7 with thiouracil **8l** (15.0 mg, 0.097 mmol) and aminothiazole chloroacetamide **5b** (25.00 mg, 0.097 mmol), brown solid was obtained (5.4 mg, 14.7%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.84 (s, 3H), 2.12 (s, 3H), 4.06 (s, 2H), 7.10 (t, $J = 3$ Hz, 1H), 7.44 (s, 1H), 7.85 (d, $J = 5$ Hz, 1H), 7.95 (d, $J = 4$ Hz, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 10.4, 20.8, 33.6, 106.3, 114.0, 123.6, 125.4, 127.9, 136.9, 138.3, 143.6, 157.9, 167.2, 177.9, 221.9. ESI-LCMS (low resolution) m/z calculated for $C_{15}H_{14}N_4O_2S_3$ [M + H] 379.5, found [M + H] 379.3.

*2-(5-Ethyl-4-hydroxy-6-methyl-pyrimidin-2-ylsulfanyl)-N-(4-thiophen-2-yl-thiazol-2-yl)-acetamide (**9bo**)*

Utilizing general procedure 7 with thiouracil **8h** (16 mg, 0.097 mmol) and aminothiazole chloroacetamide **5b** (25 mg, 0.097 mmol), light brown solid was obtained (4.3 mg, 11%). ^{1}H NMR (500 MHz, acetone-d_6) δ 1.06 (t, $J = 7$ Hz, 3H), 2.41 (s, 3H), 2.50 (q, $J = 8$ Hz, 2H), 4.17 (s, 2H), 7.07 (t, $J = 7$ Hz, 1H), 7.33 (s, 1H), 7.40 (d, $J = 4$, 1H), 7.49 (d, $J = 3$ Hz, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 13.0, 18.5, 32.9, 34.1, 107.0, 115.8, 124.3, 126.0, 128.5, 138.8, 144.28, 158.4, 162.0, 164.0 167.4, 174.4. ESI-LCMS (low resolution) m/z calculated for $C_{16}H_{16}N_4O_2S_3$ [M + H] 393.5, found [M + H] 393.3.

Table 1. Yields from preparation of 4-aryl 2-aminothiazole bromoacetamides (**2a–c**) and 4-heteroaryl 2-aminothiazole chloroacetamides (**5a–d**). The heteroaryl aminothiazole intermediates were prepared from either a heteroaryl bromoketone (**3a**) or methylketones (**3b–d**).

Product	SM	R^3	Amino-thiazole formation (% yield)	Halo-acetamide formation (% yield)
4-aryl 2-aminothiazole bromoacetamides				
2a	**1a**	Ph	n/a	31
2b	**1b**	4-Cl-Ph	n/a	86
2c	**1c**	4-MeO-Ph	n/a	95
4-heteroaryl 2-aminothiazoles chloroacetamides				
5a	**3a**	2-benzofuran	65*	91
5b	**3b**	2-thiophene	95†	25
5c	**3c**	3-indole	98†	81
5d	**3d**	4-isoquinoline	25†	39

*Yield after cyclization of the commercially available bromoketone (**3a**) with thiourea.

†Two step yield after bromination of methyl ketones (**3b–d**) followed by cyclization with thiourea.

Table 2. Yields from α-alkylation of β-keto esters (**6** → **7**).

Product	SM	R^1	R^2	R^4	Alkylation (% yield)
7a	**6a**	Me	Pr	Me	48
7b	**6a**	Me	Bu	Me	32
7c	**6b**	Me	CH_2CyPr	*i*Bu	30
7d	**6b**	Me	CH_2CyBu	*i*Bu	19
7e	**6c**	CyPr	Me	Me	quant
7f	**6c**	CyPr	Et	Me	89
7g	**6c**	CyPr	Pr	Me	78

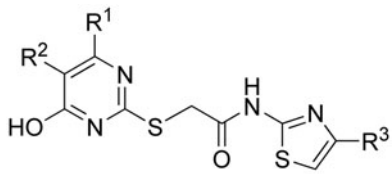
*i*Bu, isobutyl; CyPr, cyclopropyl; CyBu, cyclobutyl.

Table 3. Yields from cyclization of β-keto esters (**6** or **7**) to mono- and di-substituted thiouracils (**8a–p**) using thiourea.

Product	SM	R^1	R^2	R^4	Thiouracil formation (% yield)
Cyclization of prepared α-substituted β-keto esters					
8a	**7a**	Me	Pr	Me	50
8b	**7b**	Me	Bu	Me	31
8c	**7c**	Me	CH_2CyPr	*i*Bu	33
8d	**7d**	Me	CH_2CyBu	*i*Bu	50
8e	**7e**	CyPr	Me	Me	86
8f	**7f**	CyPr	Et	Me	40
8g	**7g**	CyPr	Pr	Me	30
Cyclization of commercially obtained β-keto esters					
8h	**7h**	Me	Et	Me	37
8i	**6c**	CyPr	H	Me	67
8j	**6d**	Et	H	Me	35
8k	**6e**	Pr	H	Me	33
8l	**7i**	Me	Me	Et	74
8m	**6f**	CHF_2	H	Et	50
8n	**6g**	C_2F_5	H	Et	65
8o	**6h**	CF_3	H	Me	85
8p	**7j**	CF_3	Me	Me	48

CyPr, cyclopropyl; CyBu, cyclobutyl.

Table 4. Coupling yields and TMEM16A inhibition of a library of thiopyrimidine aryl aminothiazoles (**9aa–bu**). Yields (%) are of the isolated or purified products. IC_{50} (μM) for inhibition of TMEM16A anion conductance using a fluorescence plate reader assay. The purity of active compounds was >95% based on HPLC-LCMS analysis at 254 nm, combined with the absence of impurities observed by inspection of ^{1}H NMR spectra.



9aa-9bu

Product	SM thiouracil	SM aminothiazole	R^1	R^2	R^3	Isolated yield (%)	IC_{50} (μM)
T16A$_{inh}$-A01	**n/a**	**n/a**	Me	Et	4-MeO-Ph	n/a	1.8
9aa	**8a**	**2a**	Me	Pr	Ph	29	>10
9ab	**8b**	**2a**	Me	Bu	Ph	19	>10
9ac	**8c**	**2a**	Me	CH_2CyPr	Ph	71	>10
9ad	**8e**	**2a**	CyPr	Me	Ph	73	>10
9ae	**8f**	**2a**	CyPr	Et	Ph	87	>10
9af	**8g**	**2a**	CyPr	Pr	Ph	75	>10
9ag	**8h**	**2a**	Me	Et	Ph	35	>10
9ah	**8i**	**2a**	CyPr	H	Ph	68	>10
9ai	**8j**	**2a**	Et	H	Ph	70	>10
9aj	**8k**	**2a**	Pr	H	Ph	70	>10
9ak	**8l**	**2a**	Me	Me	Ph	65	>10
9al	**8m**	**2a**	CHF_2	H	Ph	62	>10
9am	**8n**	**2a**	C_2F_5	H	Ph	10	>10
9an	**8o**	**2a**	CF_3	H	Ph	76	>10
9ao	**8p**	**2a**	CF_3	Me	Ph	12	6.2
9ap	**8a**	**2b**	Me	Pr	4-Cl-Ph	98	>10
9aq	**8b**	**2b**	Me	Bu	4-Cl-Ph	57	>10
9ar	**8c**	**2b**	Me	CH_2CyPr	4-Cl-Ph	94	>10
9as	**8d**	**2b**	Me	CH_2CyBu	4-Cl-Ph	57	>10
9at	**8e**	**2b**	CyPr	Me	4-Cl-Ph	90	>10
9au	**8f**	**2b**	CyPr	Et	4-Cl-Ph	85	>10
9av	**8g**	**2b**	CyPr	Pr	4-Cl-Ph	59	>10
9aw	**8i**	**2b**	CyPr	H	4-Cl-Ph	75	>10
9ax	**8j**	**2b**	Et	H	4-Cl-Ph	14	>10
9ay	**8k**	**2b**	Pr	H	4-Cl-Ph	87	>10
9az	**8l**	**2b**	Me	Me	4-Cl-Ph	72	>10
9ba	**8m**	**2b**	CHF_2	H	4-Cl-Ph	58	>10
9bb	**8n**	**2b**	C_2F_5	H	4-Cl-Ph	3	>10
9bc	**8o**	**2b**	CF_3	H	4-Cl-Ph	33	>10
9bd	**8p**	**2b**	CF_3	Me	4-Cl-Ph	38	>10
9be	**8e**	**2c**	CyPr	Me	4-MeO-Ph	97	>10
9bf	**8f**	**2c**	CyPr	Et	4-MeO-Ph	81	>10
9bg	**8g**	**2c**	CyPr	Pr	4-MeO-Ph	67	>10
9bh	**8i**	**2c**	CyPr	H	4-MeO-Ph	69	>10
9bi	**8l**	**2c**	Me	Me	4-MeO-Ph	78	>10
9bj	**8m**	**2c**	CHF_2	H	4-MeO-Ph	75	>10
9bk	**8n**	**2c**	C_2F_5	H	4-MeO-Ph	12	>10
9bl	**8o**	**2c**	CF_3	H	4-MeO-Ph	91	>10
9bm	**8p**	**2c**	CF_3	Me	4-MeO-Ph	97	>10
9bn	**8h**	**5a**	Me	Et	2-benzofuran	10	>10
9bo	**8h**	**5b**	Me	Et	2-thiophene	11	3.5
9bp	**8h**	**5c**	Me	Et	3-indole	10	>10
9bq	**8h**	**5d**	Me	Et	4-isoquinoline	7	>10
9br	**8l**	**5a**	Me	Me	2-benzofuran	37	>10
9bs	**8l**	**5b**	Me	Me	2-thiophene	15	2.9
9bt	**8l**	**5c**	Me	Me	3-indole	6	>10
9bu	**8l**	**5d**	Me	Me	4-isoquinoline	8	>10

Results and discussion

Chemistry

The targeted 5,6-disubstituted pyrimidine-linked aminothiazole scaffold was approached through the synthetic strategy outlined in Scheme 1. The synthesis commenced with the preparation of aminothiazole haloacetamide. Bromoacetylation of simple substituted 4-aryl-2-aminothiazoles (**1a–c**) was accomplished with bromoacetic bromide to generate the corresponding bromoacetamide (**2a–c**). Bromoketone **3a** was commercially available and directly subjected to cyclization to aminothiazole **4a**. Other 4-heteroaryl-2-aminothiazoles were not available, and were prepared in a one-pot two-step bromination/cyclization process from heteroaryl methyl ketones (**3b–d**) using $CuBr_2$ followed by reaction with thiourea, generating aminothiazole products (**4b–d**) in good yields. Surprisingly, our attempts to form bromoacetamides of heteroaryl aminothiazoles **4a–d** using highly reactive bromoacetyl bromide were not successful. Therefore, we

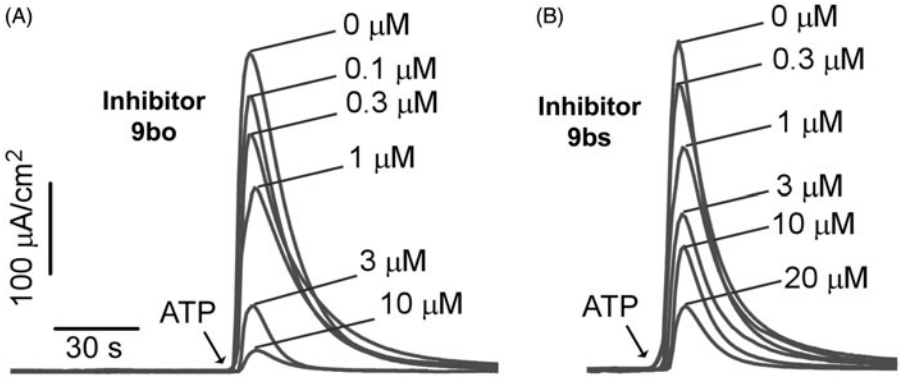


Figure 2. Short-circuit current measured in TMEM16A-expressing FRT cells. Inhibitors were added 5 min prior to TMEM16A activation by 100 μM ATP. Concentration-dependent inhibition by (A) **9bo** ($IC_{50} \sim 1\,\mu M$); (B) **9bs** ($IC_{50} \sim 3\,\mu M$).

coupled **4a–d** with bromoacetic acid in the presence of EDCI HCl. Interestingly, transient bromoacetamides were converted to chloroacetamides (**5a–d**) through the course of the reaction presumably due to chloride present in EDCI HCl, as confirmed by LCMS. Fortunately, chloride was a sufficient leaving group in the subsequent alkylation reactions, albeit with the assistance of sodium iodide. The products of both routes are listed in Table 1, with the reactions generally occurring in good yield.

α-substituted β-keto esters were prepared for cyclization with thiourea to generate thiouracils, with the results of alkylation summarized in Table 2. Methyl acetoacetate (**6a**) and isobutyl acetoacetate (**6b**) were alkylated to β-keto esters (**7a–d**) by simple substitution. Isobutyl esters were used to decrease the transesterification during the reaction, and also to decrease the volatility of β-keto ester products, aiding in isolation. Methyl 3-cyclopropyl-3-oxopropionate (**6c**) was used to prepare a small homologous series (**7e–g**) of β-keto esters. Isolated yields were fair to quantitative.

Upon generation of a small library of α-substituted β-keto esters (**7a–g**), the compounds were cyclized to the corresponding thiouracils (**8a–g**) by treatment of with thiourea under basic conditions (Table 3). Additionally, a selection of commercially available α-substituted β-keto esters (**7h–j**) and α-non-substituted species (**6c–h**) were also cyclized to the corresponding thiouracils (**8h–p**).

The final synthetic task was coupling of the mono- and disubstituted thiouracils (**8a–p**) with 4-aryl and 4-heteroaryl 2-aminothiazole haloacetamides (**2a–c** and **5a–d**) to generate the inhibitor candidates (**9aa–bu**). Each of the thiouracils was coupled with one or more 2-aminothiazole haloacetamides in the presence of K_3PO_4 monohydrate in DMF at 60 °C, with the results summarized in Table 4. Poorly electrophilic 2-aminothiazole chloroacetamide (**5a–d**) required the addition of sodium iodide to facilitate the alkylation, by the Finkelstein mechanism. The reactions generally worked well, giving acceptable isolated yields of product, allowing construction of the 47-member library of inhibitor candidates. While the coupling reactions proceeded to completion, the slight impurity of a small number of products necessitated purification by preparative HPLC (see Supplementary material).

Biological characterization

Compounds **9aa–bu** were evaluated for inhibition of TMEM16A anion channel function using a cell-based functional assay as described previously[20,23]. The compounds were added to FRT cells stably expressing human TMEM16A and the iodide-sensitive fluorescent protein YFP-H148Q/I152L/F46L and assayed from the kinetics of iodide uptake using a fluorescence plate reader. Initial testing was done at 10 μM. IC_{50} values for active compounds were determined from concentration-inhibition measurements, as summarized in Table 4. The fluorescence plate reader results were used to select candidates for the more definitive, albeit lower throughput, short-circuit (apical membrane) current assay.

Surprisingly, most of the synthesized compounds were inactive at 10 μM showing little tolerance for variation of the thiouracil or aromatic ring in **T16A$_{inh}$-A01**. Cycloalkyl or fluoroalkyl substituents at R^1, or alkyl or cycloalkyl substituents at R^2, generally produced inactive compounds. An exception was **9ao**, which incorporated a trifluoromethyl group at R^1 and methyl at R^2, but with reduced potency ($IC_{50} = 6.2\,\mu M$) compared to **T16A$_{inh}$-A01**. Previously, it was shown that the R^3 substituent could be varied as different substituted aromatic rings, with preservation of potency[23]. For the majority of compounds reported herein, R^3 was Ph, 4-Cl-Ph, or 4-MeO-Ph, with nearly all compounds inactive at 10 μM. A small series of compounds explored replacement of R^3 with heterocycles (**9bn–bu**), while keeping the thiouracil substitution found in **T16A$_{inh}$-A01** (R^1 = methyl, R^2 = ethyl) or a homolog ($R^1 = R^2$ = methyl). Of these compounds, all with bicyclic heterocycles were inactive. Gratifyingly, two inhibitors with R^3 = 2-thiophene (**9bo**, $IC_{50} = 3.5\,\mu M$; **9bs,** $IC_{50} = 2.9\,\mu M$) were active.

Compounds **9bs** and **9bo** were evaluated by a short-circuit current electrophysiological assay of TMEM16A function[23], with concentration-dependence shown in Figure 2. IC_{50} values were ~1 μM for **9bo** and ~3 μM for **9bs**. The IC_{50} for **T16A$_{inh}$-A01** is ~1 μM as reported previously[23].

Conclusion

In conclusion, a library of 47 5,6-disubstituted pyrimidine analogs (**9aa–bu**) of the lead 4-aryl-2-aminothiazole inhibitor (**T16A$_{inh}$-A01**) was synthesized in a modular strategy utilizing haloacetamide (**2a–c** or **5a–d**) and thiouracil (**8a–p**) building blocks. This study currently represents the first systematic exploration of 4-aryl-2-aminothiazoles as inhibitors of TMEM16A. **T16A$_{inh}$-A01** is a good starting point for optimization due to its applications in studying TMEM16A function in smooth muscle cells, hypertension and cancer, and because of its low micromolar potency[23]. Most of the compounds synthesized here were inactive at 10 μM, while three compounds showed measurable activity (**9ao**, **9bo** and **9bs**). The most potent compound, **9bo**, with IC_{50} ~1 μM, may serve as an alternative to lead compound **T16A$_{inh}$-A01**.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Supplementary material available online