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

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Abstract

Transmembrane protein 16A (TMEM16A), also called Ano1, is a Ca²⁺ 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 IC₅₀ ~1 μM for inhibition of TMEM16A chloride conductance.

Keywords

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

Introduction

Transmembrane protein 16A (TMEM16A) (also known as anoctamin1, ANO1, DOG1, ORAOV2, TAOS-2) is a Ca²⁺-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

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Declaration of interest

The authors report no conflicts of interest.

Supplementary material available online

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 clearance⁷, with knockout mice showing mucus accumulation in the airways⁸. TMEM16A knockout or knockdown is associated with diminished rhythmic contraction of gastric smooth muscle cells⁵, defective protein reabsorption in kidney proximal tubule⁹ and attenuated pain response¹⁰. TMEM16A knockout mice also manifest reduced blood pressure and decreased hypertensive response following vasoconstrictor treatment¹¹.

TMEM16A contains eight putative transmembrane domains with intracellular NH₂ and COOH termini and two calmodulin binding domains^{1,2}. Putative Ca²⁺ binding sites are located at E702 and E705¹². 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 Ca²⁺^{15,16}. An X-ray crystal structure (3.4 Å resolution) was recently solved of a fungal TMEM16 isoform with Ca²⁺-activated lipid scramblase activity (nhTMEM16), which has 39–42% homology to mammalian TMEM16A¹⁷. nhTMEM16 contains 10 transmembrane segments per subunit and a region of six residues (including glutamate and aspartate), surrounding bound Ca²⁺ ions, providing a potential structural explanation for Ca²⁺-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 cancer¹. 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 disease²¹. Non-selective CaCC inhibitors, which inhibit TMEM16A as well as non-TMEM16A (as yet unidentified) CaCCs have been identified by high-throughput screening²². 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 vessels²⁴, models of chronic hypoxic pulmonary hypertension²⁵, epithelial fluid transport²⁶ and cancer cell proliferation²⁷.

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 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, 2mM 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 described²⁰ 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 HCO₃⁻-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% O₂/5% CO₂ 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 C₁₈ 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] = H₂O (0.05% trifluoroacetic acid (TFA)) and [B] CH₃CN (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 ¹³C 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), CDCl₃ (δ 7.26), CD₃OD (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; 50ml), transferred to a separatory funnel and extracted with 1:1 mixture of ethyl acetate and diethyl ether (50ml). 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 $\text{Et}_2\text{O}:\text{EtOAc}$ (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 iodoor 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).

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 Et₂O 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%). ¹H 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). ¹³C 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 C₉H₇ClN₂OS₂ [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. ¹H 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 C₁₇H₁₃F₃N₄O₂S₂ [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%). ¹H 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). ¹³C 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 C₁₅H₁₄N₄O₂S₃ [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%). ¹H 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). ¹³C 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 C₁₆H₁₆N₄O₂S₃ [M + H] 393.5, found [M + H] 393.3.

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 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 thioracils, 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 thioracils (**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 thioracils (**8h–p**).

The final synthetic task was coupling of the mono- and disubstituted thioracils (**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 thioracils 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 ($\text{IC}_{50} = 6.2 \mu\text{M}$) 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 potency²³. 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** ($\text{R}^1 = \text{methyl}$, $\text{R}^2 = \text{ethyl}$) or a homolog ($\text{R}^1 = \text{R}^2 = \text{methyl}$). Of these compounds, all with bicyclic heterocycles were inactive. Gratifyingly, two inhibitors with $\text{R}^3 = 2\text{-thiophene}$ (**9bo**, $\text{IC}_{50} = 3.5 \mu\text{M}$; **9bs**, $\text{IC}_{50} = 2.9 \mu\text{M}$) were active.

Compounds **9bs** and **9bo** were evaluated by a short-circuit current electrophysiological assay of TMEM16A function²³, with concentration-dependence shown in Figure 2. IC_{50} values were $\sim 1 \mu\text{M}$ for **9bo** and $\sim 3 \mu\text{M}$ for **9bs**. The IC_{50} for **T16A_{inh}-A01** is $\sim 1 \mu\text{M}$ as reported previously²³.

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 potency²³. 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 $\text{IC}_{50} \sim 1 \mu\text{M}$, may serve as an alternative to lead compound **T16A_{inh}-A01**.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors alone are responsible for the content and writing of this article.

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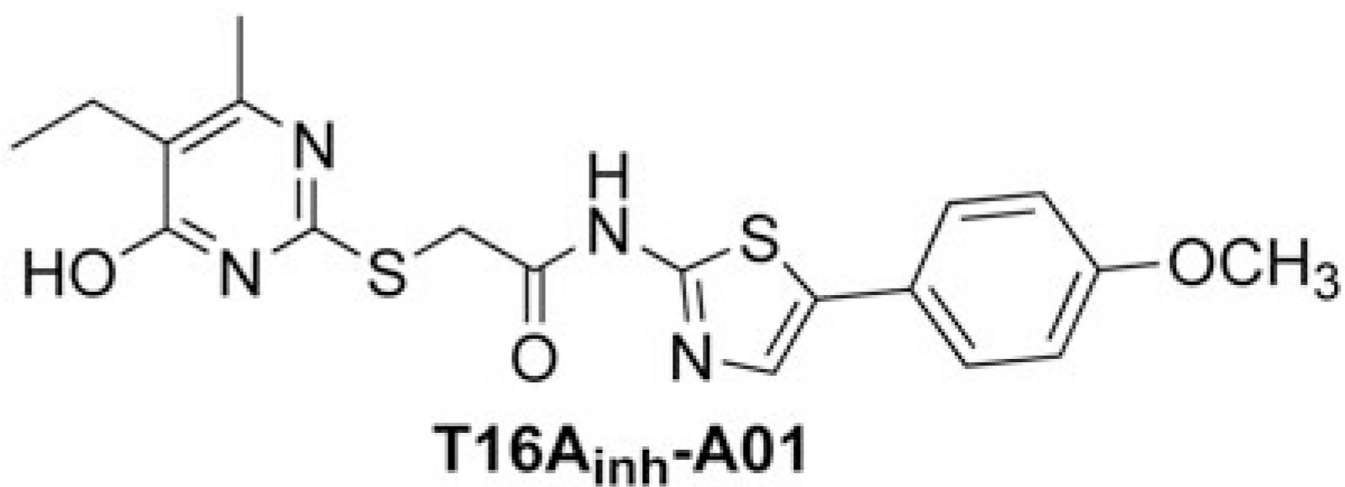


Figure 1.
The structure of lead inhibitor T16A_{inh}-A01.

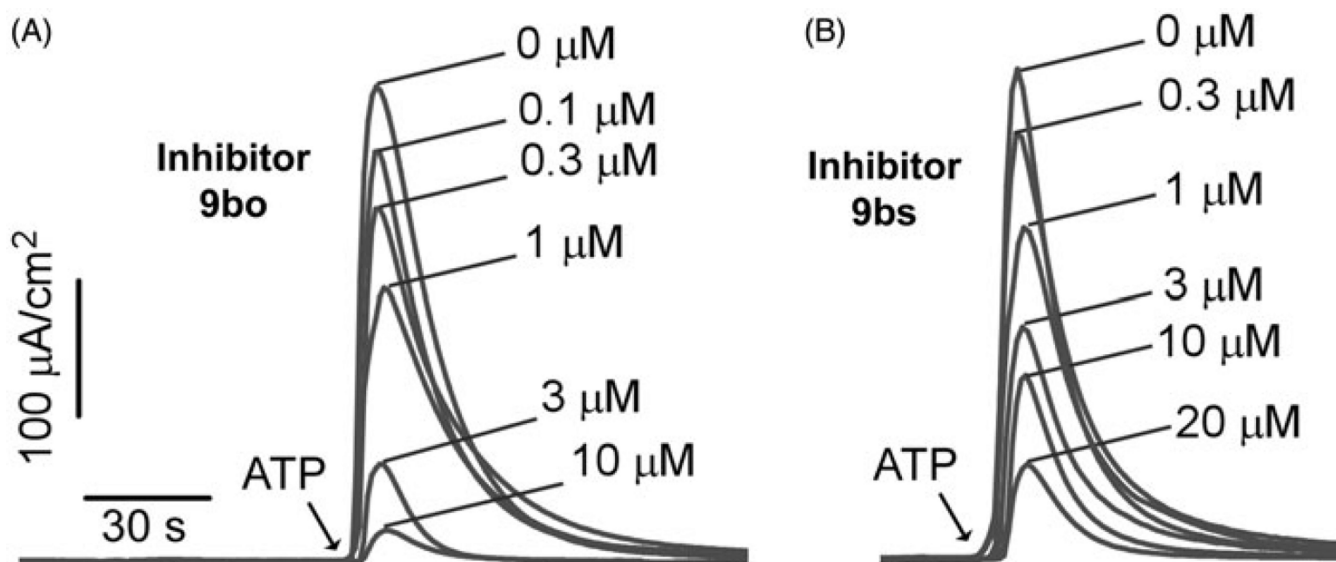
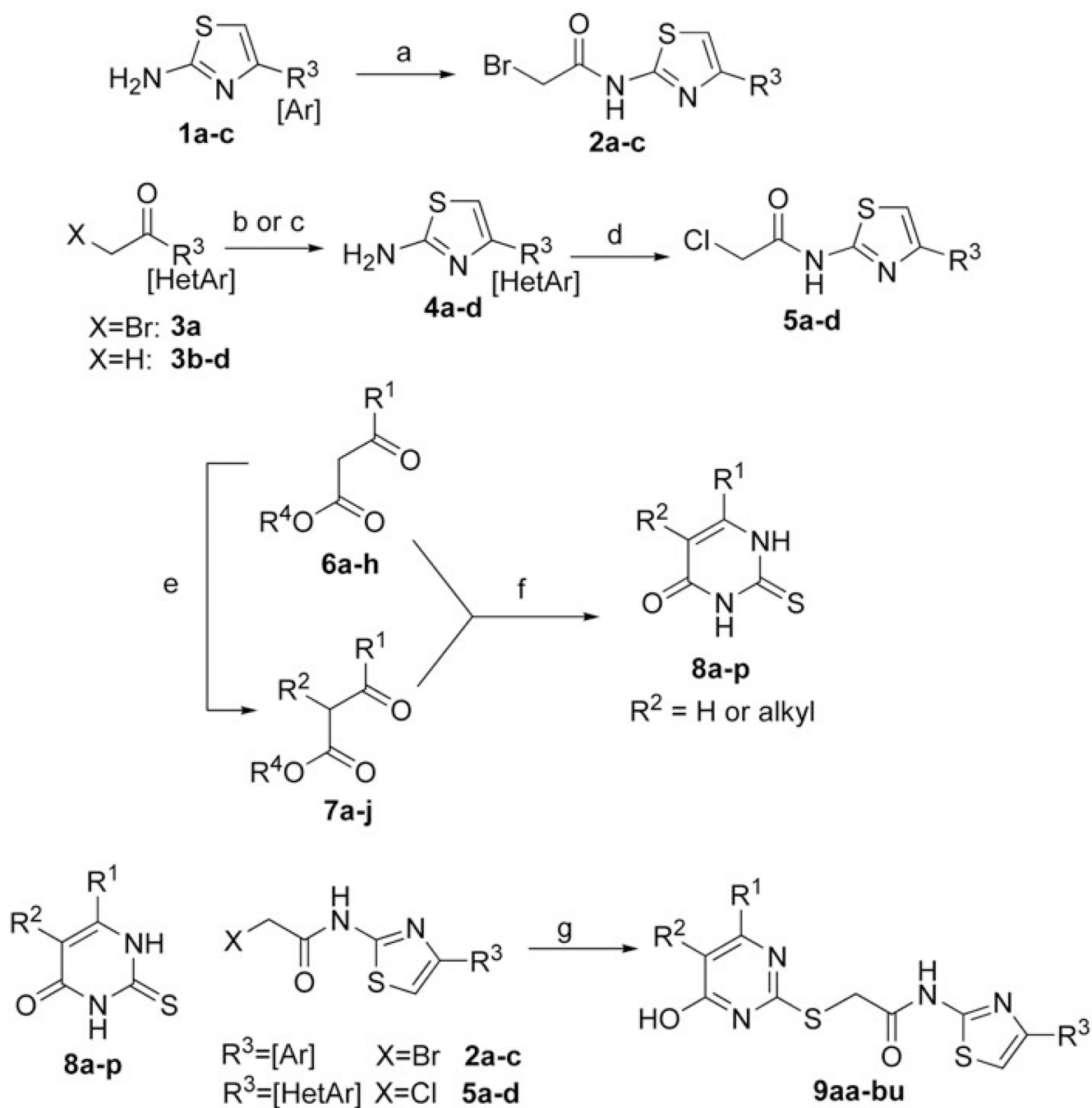


Figure 2. Short-circuit current measured in TMEM16A-expressing FRT cells. Inhibitors were added 5 min prior to TMEM16A activation by 100 μM ATP. Concentration-dependent inhibition by (A) **9bo** ($IC_{50} \sim 1 \mu\text{M}$); (B) **9bs** ($IC_{50} \sim 3 \mu\text{M}$).

**Scheme 1.**

Synthesis of 4-aryl/heteroaryl-2-aminothiazole inhibitor candidates. *Reagents and conditions:* (a) bromoacetyl bromide, Et₃N, DCM, 0 °C; (b) for bromoacetone **6a**: thiourea, THF, 50 °C; (c) for methyl ketones **6b-d**: CuBr₂, 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) K₃PO₄-H₂O, DMF. For chloroacetamides (X = Cl), NaI was added to facilitate substitution.

Table 1

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

Product	SM	R ³	Amino-thiazole formation (% yield)	Halo-acetamide formation (% yield)
4-aryl2-aminothiazolebromoacetamides				
2a	1a	Ph	n/a	31
2b	1b	4-Cl-Ph	n/a	86
2c	1c	4-MeO-Ph	n/a	95
4-heteroaryl2-aminothiazoleschloroacetamides				
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** \rightarrow **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 ₂ CyPr	<i>t</i> Bu	30
7d	6b	Me	CH ₂ Cy <i>t</i> Bu	<i>t</i> Bu	19
7e	6c	CyPr	Me	Me	quant
7f	6c	CyPr	Et	Me	89
7g	6c	CyPr	Pr	Me	78

*t*Bu, isobutyl; CyPr, cyclopropyl; Cy*t*Bu, cyclobutyl.

Table 3

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

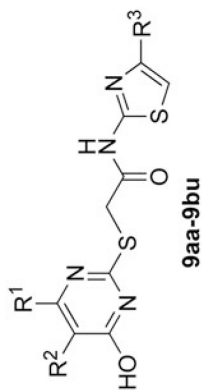
Product	SM	R ¹	R ²	R ⁴	Thiouracil formation (% yield)
Cyclization of prepared α -substituted β -keto esters					
8a	7a	Me	Pr	Me	50
8b	7b	Me	Bu	Me	31
8c	7c	Me	CH ₂ CyPr	<i>i</i> Bu	33
8d	7d	Me	CH ₂ CyBu	<i>i</i> Bu	50
8e	7e	CyPr	Me	Me	86
8f	7f	CyPr	Et	Me	40
8g	7g	CyPr	Pr	Me	30
Cyclization of commercially 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 ₂ F ₅	H	Et	65
8o	6h	CF ₃	H	Me	85
8p	7j	CF ₃	Me	Me	48

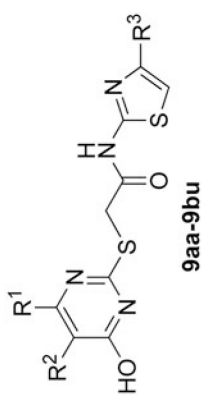
CyPr, cyclopropyl; CyBu, cyclobutyl.

Table 4

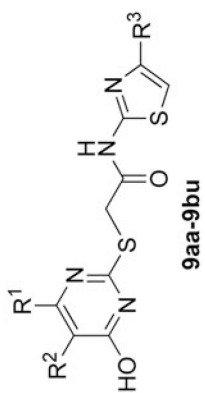
Coupling yields and TMEM16A inhibition of a library of thiopyrimidine aryl aminothiazoles (**9aa–9bu**). Yields (%) are of the isolated or purified products. IC₅₀ (μM) for inhibition of TMEM16A anion conductance using a fluorescence plate reader assay. The purity of active compounds was >95% based on HPLC-LCMS analysis at 254 nm, combined with the absence of impurities observed by inspection of ¹H NMR spectra.

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 ₂ CyPr	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 ₂ F 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 ₂ CyPr	4-Cl-Ph	94	>10





Product	SM thiouracil	SM aminothiazole	R ¹	R ²	R ³	Isolated yield (%)	IC ₅₀ (μM)
9as	8d	2b	Me	CH ₂ CyBu	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 ₂ F 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 ₂ F 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



Product	SM thiouracil	SM aminothiazole	R ¹	R ²	R ³	Isolated yield (%)	IC ₅₀ (μM)
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