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The discovery of potent blockers of the canonical transient receptor channels, TRPC3 and TRPC6, based on an anilino-thiazole pharmacophore

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ABSTRACT

Lead optimization of piperidine amide HTS hits, based on an anilino-thiazole core, led to the identification of analogs which displayed low nanomolar blocking activity at the canonical transient receptor channels 3 and 6 (TRPC3 & 6) based on FLIPR (carbachol stimulated) and electrophysiology (OAG stimulated) assays. In addition, the anilino-thiazole amides displayed good selectivity over other TRP channels (TRPA1, TRPV1, and TRPV4), as well as against cardiac ion channels (CaV1.2, hERG, and NaV1.5). The high oxidation potential of the aliphatic piperidine and aniline groups, as well as the lability of the thiazole amide group contributed to the high clearance observed for this class of compounds. Conversion of an isoquinoline amide to a naphthyridine amide markedly reduced clearance for the bicyclic piperidines, and improved oral bioavailability for this compound series, however TRPC3 and TRPC6 blocking activity was reduced substantially. Although the most potent anilino-thiazole amides ultimately lacked oral exposure in rodents and were not suitable for chronic dosing, analogs such as **14**–**19**, **22**, and **23** are potentially valuable in vitro tool compounds for investigating the role of TRPC3 and TRPC6 in cardiovascular disease.

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Calcium and sodium ion flux across the plasma membrane is a powerful signaling mechanism that mediates a wide range of physiologic functions.[1] In general, changes in intracellular calcium are controlled by two processes, (1) release from stores such as the endoplasmic and sarcoplasmic reticulum, and (2) through influx across the plasma membrane. The canonical transient receptor potential channels (TRPC) are believed to play a role in both types of calcium signaling processes.[2] In recent years one prominent mechanism of activation identified for TRPC channels has been through $G\alpha_q$-protein receptor signaling. Upstream of TRPC channels, the key step in the $G\alpha_q$-signaling cascade is the generation of 1,4,5-inositol triphosphate (IP3) and diacylglycerol (DAG) through phospholipase C (PLC) activation. Subsequent to PLC activation, TRPC channels are activated by a direct interaction with DAG.[2] The TRPC3, TRPC6, and TRPC7 subfamily of TRPC channels are calcium/sodium permeable, show very high protein sequence homology, and are able to function as store operated channels (SOC) as well as receptor operated channels (ROC). An added element to their complexity is that they can form heteromultimeric complexes with each other, as well as with TRPC1, TRPC4, and TRPC5.[3] The functional relevance of the multimeric complexes is largely unknown, and may likely contribute to multiple signaling pathways. For example, TRPC3 and TRPC6 have been shown to serve as receptor operated channels in vascular smooth muscle cells, and likely contribute to regulation of vascular tone.[4] In addition TRPC3 and TRPC6 channels have also been shown to regulate

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calcium (Ca^{2+}) flux in cardiac myocytes, and promote cardiac hypertrophy through activation of calcineurin and NFAT.[5]

As a result TRPC3 and TRPC6 are associated with cardiovascular pathophysiology including cardiac hypertrophy/myopathy, and hypertension.[4,6] Due to the association of both TRPC3 and TRPC6 with cardiac hypertrophy, and the unmet medical need associated with cardiovascular disease, a small molecule drug discovery program was undertaken which focused on identifying blockers of both TRPC3 and TRPC6.

There have been few reports of small molecule blockers of TRPC3 or TRPC6 in the literature.[7] In general, the tool compounds identified to date suffer from poor selectivity, weak potency, and/or questionable tractability as potential starting points for drug discovery. Therefore a TRPC3 and TRPC6 high-throughput screening (HTS) effort was carried out to identify small molecule blockers of these channels as starting points for lead optimization and target validation. This article will highlight the SAR around blocker activity and pharmacokinetics for this new class of potent, dual TRPC3 and TRPC6 blockers. A preliminary selectivity profile will also be detailed.

Among the TRPC3 or TRPC6 blockers identified to date, one of the most extensively profiled has been a compound referred to in the literature as Pyr3 or ethyl-1-(4-(2,3,3-trichloroacrylamide)phenyl)-5-(trifluoro-methyl)-1H-pyrazole-4-carboxylate (**1**; Fig. 1).[7b] Within the TRPC family, Pyr3 has been shown to be a selective inhibitor of TRPC3-mediated Ca^{2+} influx as evidenced by studies in human embryonic kidney (HEK) cells over-expressing the TRPC family members (TRPC1, 3, 4, 5, 6, and 7). In addition, Pyr3 inhibits hypertrophic signaling in cardiac myocytes and hypertrophic growth in a pressure overload model in mice.[7] However one noteworthy characteristic of the class of 5-(trifluoro-methyl)-1H-pyrazoles to which Pyr3 belongs is that they are known to be blockers of Ca^{2+} release-activated calcium (CRAC) channels such as Orai.[8] This cross-activity and the potential for other cross-activities raises questions about the utility of this class of compounds for TRPC3 and TRPC6 target validation studies. Our efforts in this area began with the discovery of two anilino-thiazole hits (**2** and **3**; Fig. 1) identified from high throughput screening, with an overall objective of identifying a tool molecule with good potency, selectivity, and oral PK for study in chronic models of heart failure.

Target molecules **2**–**25** were synthesized either through Method 1 or Method 2 described in Scheme 1. The 4-carboxy-anilino-thiazole core was readily accessible from two starting points, bromopyruvate **i** or ethyl 2-bromo-thiazole-4-carboxylate **ii**. Condensation of a phenyl-thiourea **iv** with the appropriately substituted bromopyruvate gave rise to the anilino-thiazole **iii** shown in Scheme 1 (Method 1). Amide formation with the requisite amine delivered the target molecules. Substitution of bromo-thiazole **ii** with the appropriate aniline, followed by hydrolysis, gave rise to intermediate **iii** (Method 2).

Intermediate **iii** was once again subjected to amine coupling to provide the target molecules. The synthesis of compound **17** was accomplished by treating the requisite anilino-thiazole carboxamide substrate with N-chloro-succinimide.[9]

HTS and primary screening was conducted using a FLIPR assay. TRPC3 or TRPC6 was overexpressed in HEK cells using BacMam gene transfer. TRPC3 and TRPC6 channel opening causes an influx of calcium and sodium cations that results in a change in electrical potential across the cell membrane. This change in membrane potential was monitored using membrane potential dyes with measurements recorded using a FLIPR Tetra instrument. The $G\alpha_q$-coupled receptor agonist carbachol was used as an agonist challenge, creating the baseline change in membrane potential.[2b,c] Inhibitors were evaluated based on their ability to block $G\alpha_q$-receptor driven calcium/sodium ion influx which occurs through TRPC3 or TRPC6 activation by virtue of the carbachol challenge. Anilino-thiazoles **2** and **3** were among the most potent hits identified from a TRPC6 HTS (Fig. 1). HTS hit **2** inhibited carbachol-induced TRPC3 and TRPC6 mediated Ca^{2+}/Na^{+} flux with an IC_{50} of about half-micromolar in the FLIPR assay (Table 1).

The dual activity observed at both channels was not surprising given the high sequence homology between TRPC3 and TRPC6. Initial lead optimization efforts around **2** focused on the aniline ring since substitution was more readily probed due to the wide variety of commercially available anilines. A combination of electron donating and withdrawing groups were introduced on the aniline to determine their effect on activity. Replacement of the 4-methyl-phenyl group in example **2** with 4-chloro-phenyl (**4**) or 4-methoxy-phenyl (**5**) did not result in a significant activity change. Installation of a 2-chloro-substituent (**6**) led to a substantial drop in activity (8- to 10-fold) at both TRPC3 and TRPC6 relative to HTS hit **2**. The combination of two deactivating groups, 2-fluoro-4-chloro (**7**), also resulted in almost no change in TRPC3 or TRPC6 activity. In addition, meta-substitution of the aniline ring did not change activity as compared to *para*-substitution (3-Cl-phenyl and 3-methoxy-phenyl were synthesized—data not shown). The combination of donorgroups at the 3- and 4-positions (3,4-methylenedioxy; **8**) did not have a significant effect on activity either. Contrary to the flat SAR observed around the aniline, SAR around the piperidine group proved to be fairly sensitive, with mainly small alkyl groups tolerated at the 2- and 4-positions (**2** and **9**); introduction of larger groups such as phenyl (**10**–**12**) and cyclohexyl (**13**) resulted in little or no activity irrespective of the point of attachement to the piperidine. The addition of two methyl groups, in the form of a 2,3-dimethyl-piperidine moiety (**14**), proved to be the most potent substitution pattern, resulting in a slight increase in TRPC3 activity, and a five fold improvement in TRPC6 activity relative to screening hit **2**. The combination of 2,3-dimethyl-piperidine and 3,4-methylenedioxy-aniline (**15**) offered an even greater improvement in potency, as TRPC3 and

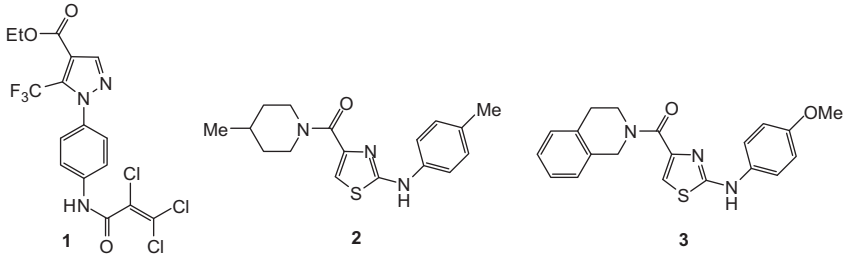
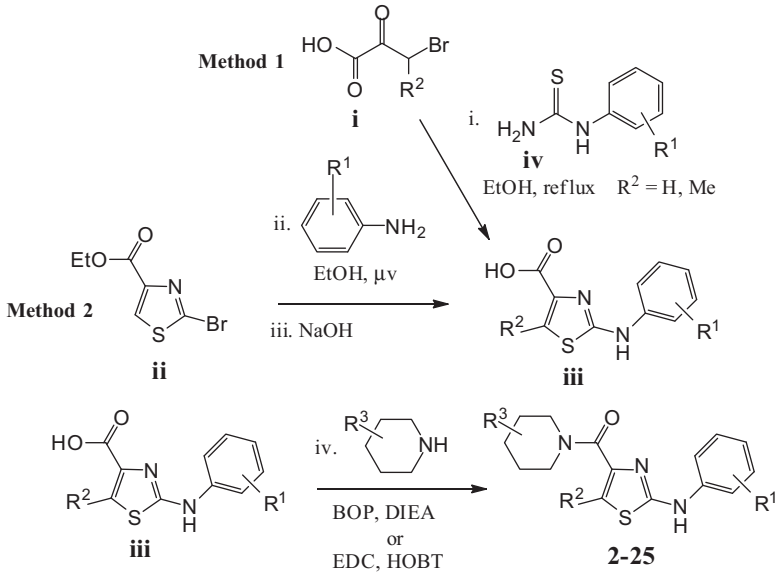
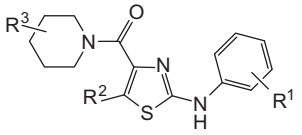


Figure 1. Pyr3 and anilino-thiazole HTS hits.



Scheme 1. Synthesis of 2-anilino-thiazole amides. Reagents and conditions: (i) **iv**, EtOH, reflux (60–100%). (ii) **aniline**, EtOH, μv (20–50%). (iii) NaOH, EtOH, reflux (90–100%). (iv) **amine**, BOP, DIEA, DMF, rt or **amine**, EDC, HOBT, CH_2Cl_2, rt (60–80%).

Table 1
FLIPR SAR of anilino-thiazole piperidine amides



Ex	R^1	R^2	R^3	hTRPC3 IC_{50}[a] (nM)	hTRPC6 IC_{50}[a] (nM)
2	4-Me	H	4-Me	630	400
4	4-Cl	H	4-Me	800	800
5	4-OMe	H	4-Me	800	800
6	2-Cl	H	4-Me	6300	3160
7	2-F,4-Cl	H	4-Me	1600	630
8	3,4-(O-CH_2-O)	H	4-Me	400	160
9	4-Me	H	2-Me	800	630
10	4-Me	H	2-Ph	12600	15800
11	4-Me	H	3-Ph	>25,000	>25,000
12	4-OMe	H	4-Ph	2500	1600
13	4-Me	H	4-Cyclohexyl	6300	4000
14	4-Me	H	2,3-Me	250	80
15	3,4-(O-CH_2-O)	H	2,3-Me	80	16
16	2-F-4,5-(O-CH_2-O)	Me	2,3-Me	160	25
17	2-F-4,5-(O-CH_2-O)	Cl	2,3-Me	32	4
18	2-F,4-Cl	Me	2,3-Me	250	50
19	2-F,4-Cl	Me	(*S,S*)-2,3-Me	100	16
20	2-F,4-Cl	Me	(*R,R*)-2,3-Me	1000	1600

[a] Values are means (±2- to 3-fold) from at least three independent experiments; h = human.

TRPC6 potency increased 8- and 20-fold respectively compared to hit **2**. In an effort to deactivate the aromatic system towards oxidation and block potential sites of metabolism on the thiazole, a 2-fluoro-group was inserted at the aniline *ortho*-position and a methyl-group was incorporated at the 5-position of the thiazole **15**. Analog **16** did not have a significant effect on potency and unfortunately little effect on pharmacokinetics (vide infra). Replacement of the methyl-group with a chlorine atom (**17**) at the thiazole 5-position led to a dramatic improvement in potency relative to hit **2**, especially in terms of TRPC6, as potency improved 100-fold. Replacement of the 2-fluoro-4,5-methylenedioxy-aniline in **16** with 2-fluoro-4-chloro-aniline (**18**), an aniline group that was believed to offer reduced iv clearance relative to the methylenedioxy-aniline, did not have a significant effect on potency. The (*S,S*)-2,3-dimethyl-piperidine enantiomer (**19**) offered comparable TRPC3/6 potency to the racemate (**18**), while the (*R,R*)-2,3-dimethyl-piperidine enantiomer (**20**) resulted in a four fold loss of TRPC3 activity and almost a 30-fold loss of TRPC6 activity.

Although the robust blocking activity observed in the FLIPR assay provided evidence that anilino-thiazole leads were blocking TRPC3- or TRPC6-mediated Ca^{2+}/Na^+ flux in cells, more direct evidence was sought to further validate the mechanism of action. Since carbachol-mediated $G\alpha_q$ activation is upstream of TRPC3/6, a more downstream activator was targeted. It has been well established that the endogenous activator of TRPC3 and TRPC6, diacylglycerol (DAG), does not have suitable stability for electrophysiology studies. Therefore a surrogate glycerol, 1-oleoyl-2-acetyl-glycerol (OAG), was used to activate TRPC3 or TRPC6 in cultured cells.[2] As shown

in Figure 2, OAG (10 μM) produced a significant increase in both inward and outward TRPC3 and TRPC6 currents in HEK cells heterologously expressing TRPC3 or TRPC6.[2]

The resulting TRPC3 or TRPC6 current was inhibited by the potent blocker example **14** providing direct evidence that the anilino-thiazoles were blocking OAG-stimulated TRPC3 or TRPC6 current.

In general, most piperidine amides displayed modest to high clearance, a short mean residence time (MRT), and poor oral bioavailability as described in Table 2. The most promising analog in terms of bioavailability (64%) was **19**, however clearance and MRT were inadequate to produce drug levels to support chronic dosing. The high in vivo clearance observed for this series was bourne out in microsomal intrinsic clearance studies, as nearly all piperidine amides screened displayed high clearance (>100 ml/min/kg). It is likely that both gut instability of the amide functionality and cytochrome P450 oxidation of aliphatic/aromatic groups contributed to the moderate to high clearance observed for the piperidine amide subclass of anilino-thiazoles.

Anilino-thiazole selectivity against other notable TRP families was also explored (Table 3).[10] Analog **14** was found to be 100-fold selective over TRPA1, 15-fold selective over TRPV1, and about 80-fold selective against TRPV4. The 2-fluoro-4-chloro-aniline **19** proved to be more selective than the 4-methyl-aniline **14**. Analog **19** was over 1500-fold selective over TRPA1, 390-fold selective over TPRV1, and 780-fold selective over TRPV4. Selectivity against non-TRP ion channels which impact cardiovascular safety was also examined.[11] Amide examples **14** and **19** were screened against the cardiac calcium (CaV1.2), sodium (NaV1.5), and potassium (hERG) channels. While both compounds displayed excellent cardiac ion channel selectivity in general, the 2-fluoro-4-chloro-aniline **19** displayed only modest (100-fold) selectivity over the sodium channel.

In addition to our efforts to improve potency and oral PK in the piperidine amide series, we also attempted to address those same parameters in the tetrahydroisoquinoline amide series (**3**). The 4-methoxy-aniline (**3**) in the isoquinoline amide series displayed

Table 3
Selectivity against TRP families and cardiac ion channels

Ex	TRPA1	TRPV1	TRPV4	CaV1.2	hERG	NaV1.5
14	8000	1260	6300	10,000	>50,000	50,000
19	25,000	6300	12,500	10,000	>50,000	3300

Values are a means(±2- to 3-fold) of at least two experiments. IC_{50}s (nM) shown.

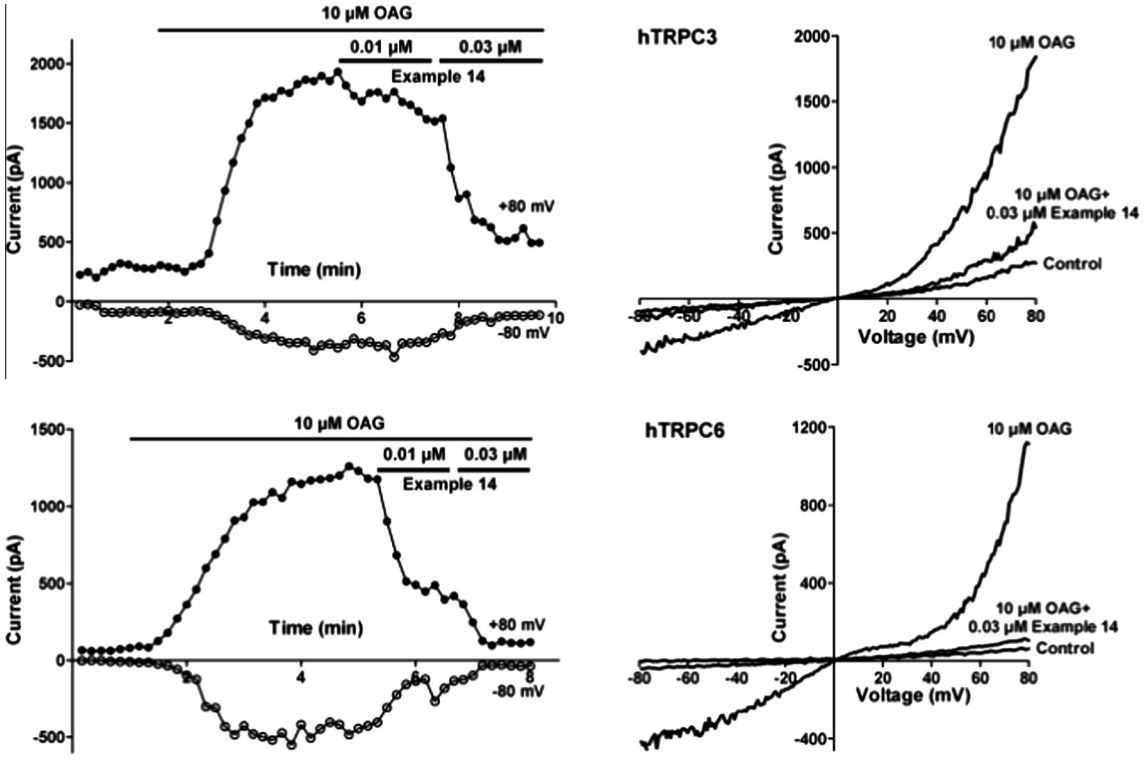


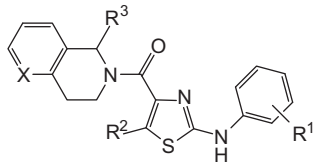
Figure 2. Activation and blockade of TRPC3 and TRPC6 channels heterologously expressed in HEK cells. Top left: time course of inhibition of OAG (10 μM) activated hTRPC3 current by example **14** (0.01 and 0.03 μM); current amplitudes were measured at −80 mV and +80 mV. Top right: current/voltage relationship of OAG-activated hTRPC3 current in the presence and absence of example **14**. Bottom left: time course of inhibition of OAG (10 μM) activated hTRPC6 current by example **14** (0.01 and 0.03 μM); current amplitudes were measured at −80 mV and +80 mV. Bottom right: current/voltage relationship of OAG-activated hTRPC6 current in the presence and absence of example **14**.

Table 2
Piperidine amide pharmacokinetic parameters in the rat

Ex	Dose (iv) (mg/kg)	Cmax (iv) (ng/ml)	CL (iv) (ml/min/kg)	MRT (iv) (h)	DNAUC (iv) (h·kg·L)	%*F*
14	1.04	310	75	2.8	0.2	<1
15	1.05	340	86	2.1	0.2	<1
17	0.86	490	46	0.4	0.3	7
19	0.82	120	150	0.7	0.1	64

Values are means ±10%, from 2 to 3 animals; Cmax—maximum concentration; CL—clearance; DNAUC—dose normalized area under the curve;%*F*—percent oral bioavailability.

Table 4
FLIPR SAR of anilino-thiazole isoquinoline analogs



Compd	R^1	R^2	R^3	X	hTRPC3 IC_{50}[a] (nM)	hTRPC6 IC_{50}[a] (nM)
3	4-OMe	H	H	CH	1260	500
21	3,4-(O-CH_2-O)	H	H	CH	500	160
22	3,4-(O-CH_2-O)	H	Me	CH	316	63
23	3,4-(O-CH_2-O)	Me	Me	CH	316	32
24	3,4-(O-CH_2-O)	Me	Me	N	10,000	1260
25	2-F,4-Cl	Me	H	N	20,000	6300

[a] Values are means (±2- to 3-fold) from at least three independent experiments.

Table 5
Isoquinoline amide pharmacokinetic parameters in the rat

Compd	Dose (iv) (mg/kg)	Cmax (iv) (ng/ml)	CL (iv) (ml/min/kg)	MRT (iv) (h)	DNAUC (iv) (h·kg/L)	%*F*
3	0.93	250	120	0.3	0.14	~1
24	1.06	1800	9.3	1.3	1.86	65
25	0.92	510	22	1.6	0.85	100

Vdss ranged from 0.7 to 2 L/kg.
Values are means ±10%, from 2 to 3 animals.

comparable TRPC3/6 activity to the corresponding 4-methoxy-aniline (**5**) in the piperidine amide series. In general, substitution of the 'benzo' portion of the tetrahydroisoquinoline was unremarkable. Groups such as cyano, chloro, fluoro, or methyl (irrespective of their position on the 'benzo' ring) did not change activity relative to **3**. The 3,4-methylenedioxy-aniline (**21**) group did not significantly alter TRPC3/6 potency compared to 4-methoxy-aniline (**3**) (Table 4). Introduction of a methyl group at the 2-position (R3) of the isoquinoline ring (**22**) led to an eight fold improvement in TRPC6 potency relative to hit **3**. This could be due in part to the R3 methyl's ability to bias a particular amide conformer. Interestingly, TRPC6 potency was further enhanced with analog **23** when methyl substitution at both the isoquinoline 2-position and the thiazole 5-position was introduced. These changes delivered a 15-fold improvement over (**3**).

In an attempt to decrease the lipophilicity of the isoquinoline system, carbon-5 of the isoquinoline was replaced with a nitrogen creating a 5,6,7,8-tetrahydro-1,6-naphthyridine ring system. The 5,6,7,8-tetrahydro-1,6-naphthyridine was selected to test this hypothesis due to ease of synthesis. A significant decrease in activity was observed for 5,6,7,8-tetrahydro-1,6-naphthyridine **24** as compared to isoquinoline **23**, suggesting that the benzo-portion of the molecule resides in a highly lipophilic region of the TRPC3 and TRPC6 ion channels. Similarly, the 2-fluoro-4-chloro-aniline analog **25** also displayed markedly reduced TRPC3 and TRPC6 blocking activity. Although the naphthyridine analogs displayed reduced TRPC3/6 potency, oral pharmacokinetic (PK) properties were markedly improved over initial lead **3**. Especially in the case of analog **24**, where all PK parameters were enhanced by about one order of magnitude (Table 5). Despite numerous attempts to introduce substitution in combination with the 5,6,7,8-tetrahydro-1,6-naphthyridine, no additional analogs were identified which possessed submicromolar potency and acceptable oral pharmacokinetics.

In conclusion, lead optimization of piperidine and isoquinoline amide HTS hits, based on an anilino-thiazole core, led to the identification of analogs which displayed low nanomolar potency in FLIPR (carbachol stimulated) and electrophysiology (OAG stimulated) assays. In addition, anilino-thiazole amides **14** and **19** displayed good selectivity over other TRP channels (TRPA1, TRPV1, and TRPV4), as well as over cardiac ion channels (CaV1.2, hERG, and NaV1.5). The significant oxidation potential of the aliphatic piperidine and aniline groups, as well as the lability of the thiazole amide group likely contributed to the high clearance observed for the anilino-thiazole class of compounds. While the conversion of the isoquinoline amide to a naphthyridine amide reduced clearance and improved oral bioavailability, TRPC3 and TRPC6 blocking activity was reduced substantially. Although the more potent anilino-thiazole amides lacked oral exposure in rodents suitable for chronic dosing, analogs such as **14**–**19**, **22**, and **23** are potentially valuable in vitro tool compounds useful for investigating the role of TRPC3 and TRPC6 in disease models. Evaluation of this class of TRPC3/6 tool compounds in cardiac myocytes will be the subject of a forthcoming article.[12]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.06.047.

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