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From High-Throughput Screening to Target Validation: Benzo[*d*]isothiazoles as Potent and Selective Agonists of Human Transient Receptor Potential Cation Channel Subfamily M Member 5 Possessing In Vivo Gastrointestinal Prokinetic Activity in Rodents

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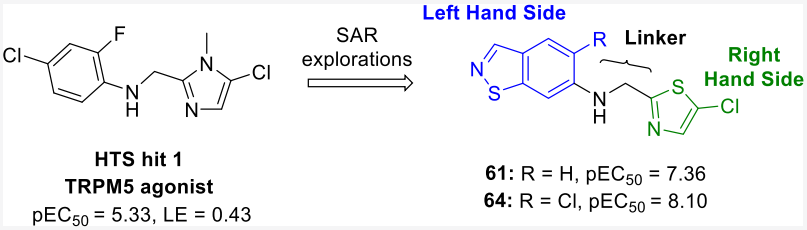

ABSTRACT: Transient receptor potential cation channel subfamily M member 5 (TRPM5) is a nonselective monovalent cation channel activated by intracellular Ca^{2+} increase. Within the gastrointestinal system, TRPM5 is expressed in the stoma, small intestine, and colon. In the search for a selective agonist of TRPM5 possessing in vivo gastrointestinal prokinetic activity, a high-throughput screening was performed and compound **1** was identified as a promising hit. Hit validation and hit to lead activities led to the discovery of a series of benzo[*d*]isothiazole derivatives. Among these, compounds **61** and **64** showed nanomolar activity and excellent selectivity (>100-fold) versus related cation channels. The in vivo drug metabolism and pharmacokinetic profile of compound **64** was found to be ideal for a compound acting locally at the intestinal level, with minimal absorption into systemic circulation. Compound **64** was tested in vivo in a mouse motility assay at 100 mg/kg, and demonstrated increased prokinetic activity.

INTRODUCTION

Transient receptor potential (TRP) cation channels have been described as polymodal cell sensors,[1−4] activated by a vast array of stimuli in the environment, ranging from temperature, natural chemicals and toxins, to mechanical stimuli. TRP cation channels also respond to endogenous agents and messengers produced during tissue injury and inflammation. They are grouped into six subfamilies: (i) TRPC for "canonical" (TRPC1−7), (ii) TRPV for "vanilloid" (TRPV1−6), (iii) TRPM for "melastatin" (TRPM1−8), (iv) TRPP for "polycystin" (TRPP2, TRPP3, and TRPP5), (v) TRPML for "mucolipin" (TRPML1−3), and (vi) TRPA for "ankyrin" (TRPA1).

The TRP family gene products are intrinsic membrane proteins with six transmembrane-spanning domains (S1−S6) and a cation-permeable pore region between S5 and S6. The length of the intracellular amino (N) and carboxy (C) termini and structural domains varies significantly between members of the TRP channel subfamilies.[5] The cytoplasmic domains are involved in the regulation and modulation of channel function and trafficking. Functional TRP channels consist of four identical or similar TRP subunits.

The TRPM subfamily members are broadly expressed in a variety of cells and tissues, such as sensory ganglia, pancreatic β cells, immune cells, tongue, heart, and kidneys, and are critical for sensory physiology, insulin release, magnesium homeostasis, ischemic injury, and inflammatory responses.[6]

TRP cation channel subfamily M member 5 (TRPM5) has been reported to be expressed in mammalian taste buds, in a subset of cells that coexpress receptors for bitter, sweet, and umami tastes.[7,8] Using a mouse in which the TRPM5 promoter drives the expression of GFP[9] or direct antibody labeling, TRPM5 expression has been detected in distinct subsets of olfactory neurons and in the vomeronasal organ, in the upper and lower gastrointestinal tract, and in the respiratory system.[10,11] TRPM5 is also expressed in pancreatic islets,

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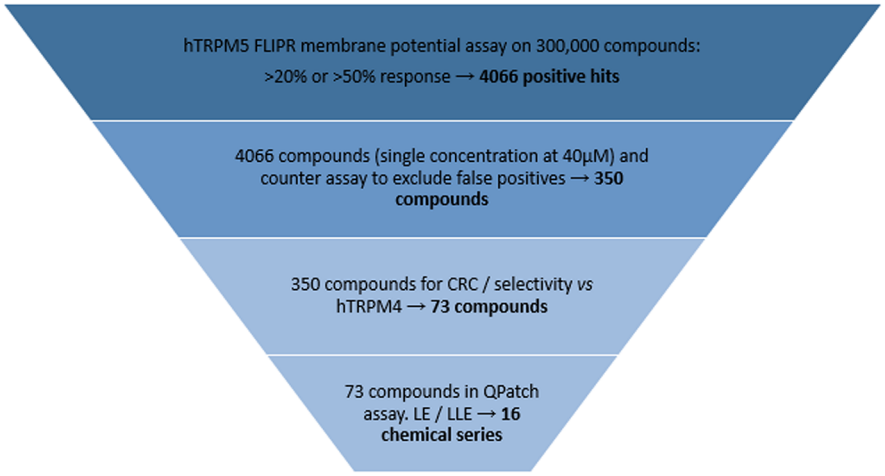

Figure 1. HTS workflow.

where it is localized to insulin-secreting β-cells and mediates depolarizing currents after glucose stimulation.[12,13] Recently, it has been reported that potentiation of TRPM5 with steviol glycosides stimulates glucose-induced insulin release,[14] therefore TRPM5 modulators may potentially be useful as insulin secretagogue modulators. TRPM5 is also expressed in tuft cells, which are taste-chemosensory epithelial cells of the small intestine and are suggested to be critical for protection during enteric infections and inflammatory responses.[15] Chemosensory tuft cells are involved in type-2 immune response initiation through secretion of the interleukin (IL)-25 by a TRPM5-dependent mechanism and mice lacking TRPM5 showed a significant reduction in IL-25 production compared to wild-type (WT), resulting in the inability to mount an immune response against helminths.[16]

TRPM5 is voltage-sensitive, monovalent cation-selective, and activated by the intracellular Ca^{2+} increase, producing a transient depolarizing current.[17] Currently, the TRPM5 pharmacology is poorly understood and a very limited number of compounds that selectively interact with this ion channel are known. TRPM5-modulating molecules range from steviol glycosides such as stevioside, rebaudioside A, and their aglycon steviol, which potentiates the activity of TRPM5.[14] Flufenamic acid is described to reduce TRPM5 currents but in the micromolar range of concentrations[18] and is active on other ion channels such as calcium-activated chloride channels and potassium, calcium, and sodium channels,[19] and displays a 10-fold higher potency for TRPM4.[20] Nicotine inhibits TRPM5 currents with an effective inhibitory concentration in the millimolar range.[21] Several azole-based antifungal compounds (clotrimazole, ketoconazole, econazole, and miconazole) inhibit TRPM5 activity in the micromolar range[18] and miconazole and ketoconazole strongly inhibit the cardiac potassium and calcium channels in the micromolar range corresponding to clinically relevant concentrations.[22]

With the aim of identifying new selective TRPM5 activators that would help in understanding the physiological and pathological role of this channel, a high-throughput screening (HTS) was run. TRP channels in general have been screened using different methods such as a Ca^{2+}-sensitive fluorescent dye for TRPA1[23] and a membrane potential dye for TRPC.[24] TRPV1 has been screened applying different technologies such as the Ca^{2+}-sensitive fluorescent dye, membrane potential dye, and atomic absorption spectroscopy-based rubidium flux assay.[25] Fluorescent Ca^{2+} detection with precise temperature manipulation such as heat for TRPV1 and cold for TRPM8[26] and fluorescent dye-based assay measuring the fluorescence quench of fura-2 by TRPM7-mediated Mn^{2+} influx were utilized.[27]

TRPM5 channel activity cannot be assessed with a Ca^{2+}-based screening method because it is selective for monovalent cations. Membrane potential dye[28] and thallium flux-based assay using a fluorescent thallium dye were reported.[18] Herein, we decided to use a membrane potential assay technology, followed by automated patch clamp methods to identify new selective TRPM5 activators.

RESULTS AND DISCUSSION

In the search for novel chemical matter to be utilized as a starting point for the development of potent and selective agonists of hTRPM5, a HTS approach was selected. More specifically, we decided to screen a diversity-based compound library with a fluorometric imaging plate reader (FLIPR) membrane potential (FMP) assay protocol. According to the protocol developed, the test compound is first added to CHO-K1 cell lines stably expressing hTRPM5 at a concentration of 40 μM to see if it is able to open the TRPM5 channel resulting in depolarization of the cell membrane. In a second addition, 200 nM adenosine 5′-triphosphate (ATP) (EC_{20}) is added to increase the intracellular Ca^{2+} concentration, activating the channel by 20% of the maximal achievable activation. The EC_{20} concentration of ATP was selected according to preliminary experiments, and in each plate 200 nM ATP was added as second addition also in the wells where, as first addition, was used the vehicle corresponding to the 0% control. With this double addition protocol, both direct agonists and potentiators (i.e., compounds increasing depolarization only if the channel is partially activated in the presence of ATP) may be identified.

The HTS campaign was conducted on a library of 300,000 compounds (Figure 1) resulting from the combination of part of the Aptuit screening collection (250,000 compounds) and a subset of compounds selected from Takeda internal library (50,000 compounds). This HTS campaign afforded 4066 positive hits (compounds with >20 or >50% response at the first or second addition, respectively), including both agonists and potentiators. Hits were progressed to hit confirmation (at single concentration, 40 μM in duplicate) and to two counter assays: an assay protocol measuring Ca^{2+}-flux in CHO-

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hTRPM5, to determine if potential agonists act on TRPM5 directly, or whether the observed activity was indirect, due to an intracellular Ca^{2+} release, and an FMP assay using CHO-WT cells to identify compounds, which elicit a membrane potential change independent of TRPM5. The aim of these counter assays is to identify and exclude false positive compounds.

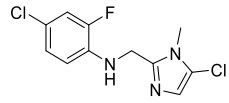
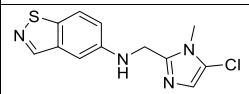
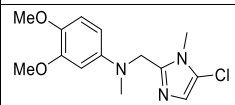
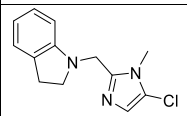
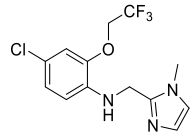
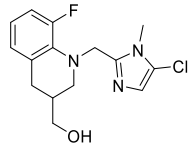
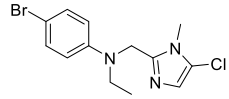
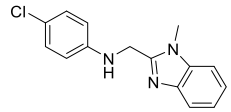
While a good overlap was generally observed between hit identification and hit confirmation, a large proportion of compounds active in agonist mode (1st addition) were found to be also active in CHO-WT cells (false positives) and were, therefore, discharged. A selection of compounds for follow up with concentration−response curve (CRC) measurements was made based on the following criteria: (i) TRPM5 activity in the FMP assay [>30% for compounds active in the first addition or >50% for compounds active in the potentiator mode (second ATP addition) and a difference in activity in the FMP assay between TRPM5 and WT >30% for both classes of hits], (ii) low activity in the Ca^{2+}-flux assay (<20% for agonist mode hits or <50% for potentiator mode hits), and (iii) low activity on the CHO-WT in the FMP assay (<30%). A total of 350 compounds were selected for follow-up in CRCs and hTRPM4 selectivity (FMP assay). For all test samples subjected to CRC, compound identity and purity were assessed by liquid chromatography (LC)−mass spectrometry (MS) analysis.

The 73 most promising compounds in terms of hTRPM5 activity and selectivity were selected to be further profiled in electrophysiology using a QPatch assay, the vast majority of them (63 out of 73) confirming activity. No difference in the electrophysiology behavior could be highlighted between agonists and potentiators and, because it was decided to utilize electrophysiology as the primary screening assay in the following hit to lead phase, no further differentiation was made between these two classes. However, compounds tested in nominally free intracellular calcium did not show activity (data not shown), and therefore could be classified as potentiators. The hits were then clustered and further prioritized based on the calculated properties such as ligand efficiency (LE)[29,30] and lipophilic LE (LLE)[31] to give 16 chemical series, which were further explored by confirming the activity from solid samples and performing structure−activity relationship (SAR) expansion mainly through compound acquisition.

In this article, we will describe in detail the optimization of one of these chemical series, which is represented by compound **1**. This analogue emerged as a moderate activator of hTRPM5 showing a pEC_{50} = 5.22 when tested in QPatch and pEC_{50} = 5.33 when tested in SyncroPatch (LE = 0.43 and LLE = 2.91). During the following optimization process and after a validation phase, compounds were tested only with a SyncroPatch instrument, which allowed a higher throughput. The validation phase showed a good correlation between the QPatch and SyncroPatch data, although a shift of potency to higher values for the SyncroPatch assay was observed, likely due to the higher intracellular calcium concentration used (data not shown).

To perform the initial SAR expansion, the examples shown in Table 1 were selected by means of substructure and similarity searches on commercially available databases. As could be expected, the initial potency of the analogues was very low, with activity mostly between 1 and 10 μM, but these encouraging first results, notably the activity of compound **2** (pEC_{50} 6.48, LE = 0.49 and LLE = 4.56), prompted us to further explore this chemical class through synthesis. At first, three regions were identified in this scaffold for exploration: a right hand side, consisting of the imidazole moiety, a left hand side, an aryl moiety, and a third region was identified in the linker between the right and the left hand sides. The screening of the first analogues of hit compound **1** suggested some preliminary considerations, which we wanted to further explore. In the left hand side region, electron-withdrawing groups (EWG) seemed to be more tolerated than electron-donating groups as can be observed comparing compounds **3** and **4** (pEC_{50} <4.5 and 4.64, respectively) with compounds **1**, **5**, and **7** (pEC_{50}, respectively, 5.33, 5.60, and 5.29). The presence of a hydrogen atom on the nitrogen in the linker did not seem crucial for activity as can be observed in compound **6** (pEC_{50} 5.30), where the substitution on the linker was incorporated in a cycle, and compound **7** (pEC_{50} 5.29). So far, little information could be acquired on the right hand side region of the molecule: the presence of the chlorine atom on the imidazole did not appear crucial for activity (compound **5**, pEC_{50} 5.60) and a major steric hindrance in that part of the molecule was detrimental for activity (compound **8**,

Table 1. Hit Expansion/Validation by Commercially Available Compounds

Cmpd		TRPM5 pEC_{50}[a,b]
1		5.22±0.18 (QPatch HTX system) 5.33±0.15
2		6.48±0.16
3		<4.5
4		4.64±0.25
5		5.60±0.21
6		5.30±0.45
7		5.29±0.09
8		<4.5

[a]Reported values are the means of at least two independent experiments. [b]All data were obtained using the SyncroPatch 384 Patch Engine except where stated otherwise.

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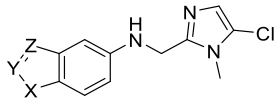
Table 2. Exploration of Benzofused Moieties on the Left Hand Side



compd	X	Y	Z	TRPM5 pEC_{50} [a,b]
2	S	N	CH	6.48 ± 0.16
9	O	N	CH	5.52 ± 0.17
10	NH	N	CH	5.22 ± 0.18
11	NMe	N	CH	5.28 ± 0.16
12	S	CH	N	4.87 ± 0.05
13	O	CH	N	4.79 ± 0.19
14	NH	CH	N	<4.5
15	NMe	N	N	<4.5

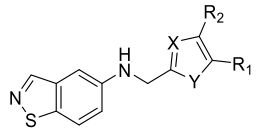
[a]Reported values are the means of at least two independent experiments. [b]All data were obtained using the SyncroPatch 384 Patch Engine.

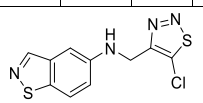
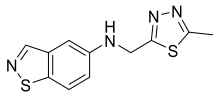
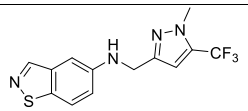
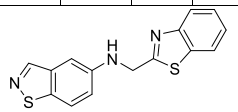
pEC_{50} < 4.5). The most interesting analogue discovered up to this point was the benzo[*d*]isothiazole derivative **2** (pEC_{50} 6.48), which was further explored.

A brief exploration of potential alternatives for the benzo[*d*]-isothiazole ring is described in Table 2. The replacement of the sulfur atom of compound **2** with an oxygen or a nitrogen atom led to less-active compounds, with benzo[*d*]isoxazole (compound **9**, pEC_{50} 5.52) being slightly more active than the corresponding indazoles (compound **10**, pEC_{50} 5.22 and compound **11**, pEC_{50} 5.28). The isomers of the corresponding bicyclic systems (compounds **12** and **13**) showed lower potency, while compounds **14** and **15** were completely inactive.

The exploration of the imidazole moiety (right hand side) is detailed in Table 3. In this case, the removal of the chlorine atom (compound **18**, pEC_{50} 5.29) resulted in a 10-fold loss of activity. The removal of the methyl group (compound **16**, pEC_{50} < 4.5) and the removal of one nitrogen atom to give a pyrrolo derivative (compound **19**, pEC_{50} < 4.5) resulted in complete loss of activity. The insertion of an additional chlorine atom on the heteroaromatic ring was not tolerated (compound **17**, pEC_{50} < 4.5). The only substitution that retained most of the activity was in compound **20** (pEC_{50} 6.34), where the chlorine atom was replaced by a bromine. The most interesting result was that the thiazole derivative **25** (pEC_{50} 7.05, LE = 0.57 and LLE = 4.31) was found to be more active than compound **2**. Following this result, some more exploration of this new heterocyclic ring was performed. Removing the nitrogen atom on the five membered ring to give thiophene **26** (pEC_{50} 5.56) resulted in loss of activity, as was the case of the corresponding imidazole−pyrrole pair shown in Table 3 (compounds **2** and **19**), indicating the importance of a nitrogen atom in that position. The substitution of the chlorine atom with another EWG such as $-CF_3$ was tolerated (compound **27**, pEC_{50} 6.31), as was the substitution of the chlorine atom with a methyl group (compound **28**, pEC_{50} 6.24). Interestingly, shifting the position of the methyl group led to an inactive compound (compound **30**, pEC_{50} < 4.5). While the electronic contribution of the substituent seemed to be of no particular importance, compound **29** (pEC_{50} < 4.5) showed that a more hindered substituent such as the isopropyl led to an inactive compound, suggesting that the allowed space around this region of the molecule was somewhat limited. This was confirmed when the benzo-fused derivative **33** was tested.

Table 3. Exploration of the Right Hand Side Moiety



Cmpd	X	Y	R_1	R_2	TRPM5 pEC_{50} [a,b]
2	N	NMe	Cl	H	6.48±0.16
16	N	NH	Cl	H	<4.5
17	N	NMe	Cl	Cl	<4.5
18	N	NMe	H	H	5.29±0.19
19	CH	NMe	Cl	H	<4.5
20	N	NMe	Br	H	6.34±0.06
21					5.28±0.21
22					4.72±0.14
23					<4.5
24	CH	S	CN	H	<4.5
25	N	S	Cl	H	7.05±0.10
26	CH	S	Cl	H	<4.5
27	N	S	CF_3	H	6.31±0.09
28	N	S	Me	H	6.24±0.21
29	N	S	iPr	H	<4.5
30	N	S	H	Me	<4.5
31	N	S	Me	Me	<4.5
32	N	S	H	H	5.33±0.20
33					<4.5

[a]Reported values are the means of at least two independent experiments. [b]All data were obtained using the SyncroPatch 384 Patch Engine.

At this stage, we wanted to acquire more information on the most promising analogues discovered so far, and the results for compounds **2** and **25** are summarized in Table 4. Both compounds showed activity on the orthologue mouse channel similar to that shown for the human channel and very good selectivity versus TRPM8, TRPV1, TRPV4, TRPA1, and TRPM4. Kinetic solubility measured from 10 mM stock solution in dimethyl sulfoxide (DMSO) showed a difference between compounds **2** and **25**. The imidazole moiety of compound **2** seemed beneficial for solubility (215 μM), while the thiazole **25** analogue showed very low solubility (50 μM). Another difference between the imidazole and the thiazole series was the plasma protein binding. For compound **2**, the fraction

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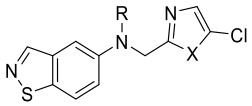
Table 4. In Vitro Characterization of 2 and 25

	compound **2**	compound **25**
hTRPM5 pEC_{50}[a,b]	6.48 ± 0.16	7.05 ± 0.10
mTRPM5 pEC_{50}[a,b]	6.50 ± 0.05	7.07 ± 0.08
MiC (human) μL/min/mg protein[c]	109	193
MiC (mouse) μL/min/mg protein[c]	446	462
Caco-2 permeability efflux ratio (nm/s)[c]	219, 1.03	126, 0.97
solubility pH 7.4 (μM)[c]	215	50
hTRPM8/hTRPV1/hTRPV4/hTRPA1/hTRPM4 (pEC_{50})[a,d]	<4.8/<4.8/<4.8/<4.8/<4.2	<4.8/<4.8/<4.8/5.5/<4.2
plasma protein binding (as fraction unbound) human/rat[c]	10.3/16.5	<1/<1

[a]Reported values are the means of at least two independent experiments. [b]All data were obtained using the SyncroPatch 384 Patch Engine. [c]See the Experimental Part section for details. [d]All data were obtained by FLIPR.

Table 5. Exploration of Linkers



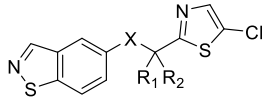
compd	X	R	TRPM5 pEC_{50}[a,b]
2	NMe	H	6.48 ± 0.16
25	S	H	7.05 ± 0.10
34	NMe	Me	6.80 ± 0.36
35	S	Me	6.35 ± 0.06
36	NMe	cyclopropylmethyl	6.80 ± 0.20
37	NMe	*i*-Pr	5.29 ± 0.09
38	NMe	cyclohexylmethyl	<4.5
39	NMe	benzyl	<4.5
40	NMe	Et	6.33 ± 0.09
41	NMe	acetyl	<4.5

[a]Reported values are the means of at least two independent experiments. [b]All data were obtained using the SyncroPatch 384 Patch Engine.

unbound was 10.3% in human and 16.5% in rat, while for compound **25**, both values were lower than 1%. For both compounds, permeability measured with Caco-2 cells was found to be high (219 nm/s for compound **2** and 126 nm/s for compound **25**). Initially, our goal was to find a compound that could act as a systemic drug, thus some of our preliminary efforts were directed toward increasing the metabolic stability, as both compounds showed very high clearance both in human and mouse microsomes [for compound **2**, MiC (h) = 109 μL/min/mg protein and MiC (m) = 446 μL/min/mg protein, while for compound **25**, MiC (h) = 193 μL/min/mg protein and MiC (m) = 462 μL/min/mg protein]. In order to better understand the possible metabolic pathways of compound **2** and help us address the issue of microsomal clearance, a Met ID experiment was performed. Two major metabolites were observed, M1 (30%, M/Z = 295) and M2 (10%, M/Z = 151). These two metabolites suggested an oxidation process on the left hand side of the molecule (+"O") and a dealkylation process on the right hand side region. From this first exploration, some conclusions could be made: two series were identified, the thiazole series, which showed greater potency, and the imidazole series, which showed better overall drug metabolism and pharmacokinetics (DMPK) parameters for compounds acting as systemic drugs.

The first exploration of the linker entailed the substitution on the nitrogen atom, as shown in Table 5. The presence of the hydrogen atom did not seem crucial for the activity as can be observed by comparing the NH derivative (compound **2**, pEC_{50} 6.48) with the corresponding *N*-methyl derivative (compound **34**, pEC_{50} 6.80) on the imidazole series. Because the thiazole moiety proved to be more active, we thought that the introduction of a methyl group on the nitrogen of compound **25** could lead to a more potent derivative. However, the thiazole *N*-methyl derivative (compound **35**, pEC_{50} 6.35) exhibited less activity than the corresponding thiazole NH derivative (compound **25**, pEC_{50} 7.05). For this reason, the exploration of potential substituents on the nitrogen atom was performed on the imidazole series, and the most interesting results are presented in Table 5. The *N*-cyclopropylmethyl derivative (compound **36**, pEC_{50} 6.80) retained the same activity as the *N*-methyl derivative, but upon increasing the size of the carbon ring, the activity was completely lost (compounds **38** and **39**, pEC_{50} < 4.5 for both). An interesting finding was that while the *N*-ethyl derivative still showed some activity (compound **40**, pEC_{50} 6.33), the *N*-acetyl derivative (compound **41**, pEC_{50} < 4.5) was completely inactive showing that the acetyl group was not tolerated.

Table 6. Exploration of Linkers



compd	X	R_1	R_2	TRPM5 pEC_{50}[a,b]	human MiC (μL/min/mg protein)[c]	mouse MiC (μL/min/mg protein)[c]
25	NH	H	H	7.05 ± 0.10	193	462
42	NH	*C═O		<4.5		
43	NH	H	Me	5.12 ± 0.09		
44	NH	Me	Me	<4.5	173	250
45	O	H	H	6.08 ± 0.17	157	763

[a]Reported values are the means of at least two independent experiments. [b]All data were obtained using the SyncroPatch 384 Patch Engine. [c]See the Experimental Part section for details.

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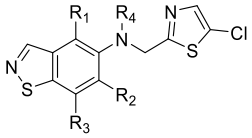
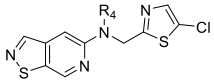
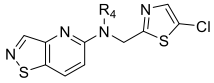
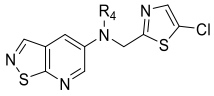
Table 7. Exploration of the Benzene Ring



Cmpd	R_1	R_2	R_3	R_4	TRPM5 pEC_{50} [a,b]	Human MiC (μL/min/mg protein) [c]	Mouse MiC (μL/min/mg protein) [c]
25	H	H	H	H	7.05±0.10	193	462
46	H	Me	H	H	7.41±0.14	157	148
47	H	Cl	H	H	7.61±0.07	149	527
48	H	F	H	H	7.01±0.27	142	312
49					6.33±0.09	155	93
50	Me	H	H	H	5.76±0.11	178	508
51	Cl	H	H	H	5.80±0.07	174	258
52	F	H	H	H	6.03±0.12	247	448
53					5.93±0.10	172	421
54	H	H	Me	H	<4.5	169	434
55	H	H	Cl	H	<4.5	150	221
56	H	H	F	H	5.86±0.18	202	612
57					5.18±0.16	84	163
58	H	Cl	H	Me	5.79±0.11	/	/
59	H	F	H	Me	5.55±0.2	/	/
60	H	Me	H	Me	5.28±0.16	562	748

[a]Reported values are the means of at least two independent experiments. [b]All data were obtained using the SyncroPatch 384 Patch Engine. [c]See the Experimental Part section for details.

Because the Met-ID experiment of compound **2** showed that one possible path of metabolism entails the dealkylation of the right hand side moiety, further exploration was performed on the carbon atom of the linker, mainly to assess if improvement could be achieved in microsomal stability, as briefly described in Table 6. The introduction of one or two methyl groups on the linker proved to be detrimental for activity (compound **43**, pEC_{50} 5.12, compound **44**, pEC_{50} < 4.5), and did not give any improvement in terms of microsomal stability. The replacement of the amine linker with an oxygen (compound **45**, pEC_{50} 6.08) lowered the potency by 10-fold while increasing clearance. Finally, the replacement of the methylene group with a carbonyl was detrimental for the activity confirming that a basic nitrogen is required (compound **42**, pEC_{50} < 4.5).

The next exploration was on the benzene ring of the benzo[*d*]isothiazole scaffold, and different substitutions were evaluated on all the available positions. This exploration served two potential goals: on the one hand, we wanted to further increase the potency, and on the other hand we wanted to understand if some of these substitutions could improve the ADME parameters. As can be observed in Table 7, the most interesting substitution is at position 6, with the chloro substituent (compound **47**, pEC_{50} 7.61) leading to an increase in activity compared to the methyl substituent (compound **46**, pEC_{50} 7.41). Both compounds were more active than the corresponding unsubstituted analogue (compound **25**, pEC_{50} 7.05). As can be observed, fluoro substitution did not lead to any improvement in terms of potency (compound **48**, pEC_{50} 7.01) nor the introduction of the nitrogen atom in the same position (compound **49**, pEC_{50} 6.33). Shifting the substituents on the other two positions of the benzene ring led to a decrease in potency, with the analogues substituted in position 4 (compounds **50**, **51**, **52**, and **53**) being slightly more potent than the same analogues substituted in position 7 (compounds **54**, **55**, **56**, and **57**). As can be observed in Table 7, microsomal stability did not improve with any of these substitutions, indicating that the very high intrinsic clearance measured in vitro is potentially related to the benzo[*d*]isothiazole core itself.

As far as potency is concerned, an unexpected result was observed when the substitution at position 6 was combined with the methylation of the nitrogen on the linker. We already described how the *N*-methyl derivatives are more active than the NH derivatives on the imidazole series, so it was expected that the introduction of a substituent on the benzene ring could be beneficial with these derivatives. However, the -chloro, -fluoro, and -methyl derivatives (compounds **58**, pEC_{50} 5.79; **59**, pEC_{50} 5.55; and **60**, pEC_{50} 5.28) all exhibited less activity than the unsubstituted derivative (compound **34**, pEC_{50} 6.80), thus suggesting a possible steric clash between the methyl group on the nitrogen atom with the ortho substituent on the benzene ring.

The observation that higher activity could be achieved by shifting the position of a substituent on the benzene ring prompted us to explore the best position for the linker on the benzo[*d*]isothiazole moiety. This brief exploration (described in Table 8) showed that activity was completely lost when the linker was moved to position 4 (see compound **62**, pEC_{50} < 4.5), was reduced when it was moved to position 7 (see compound **63**, pEC_{50} 5.84), and was increased when the linker was moved to position 6 (see compound **61**, pEC_{50} 7.36). In the light of these results, we wanted to combine the information obtained on the most active position for the linker with the most interesting substituents on the benzene ring.

This exploration is depicted in Table 9, where the most interesting ortho substitutions (fluoro, chloro, and methyl) were applied to both the thiazole and the imidazole series. As can be observed, all the substitutions led to more active derivatives, with compound **64** (pEC_{50} 8.10, LE = 0.62 and LLE = 4.8) bearing a chlorine at position 5, the linker at position 6, and the thiazole on the right hand side being the most active compound obtained from this chemical series. Figure 2 shows the details of the electrophysiology experiment performed on compound **64**.

All the previous findings obtained with the linker at position 5 were confirmed with the linker at position 6: the thiazole series proved to provide more active compounds than the imidazole

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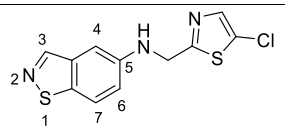
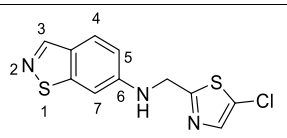
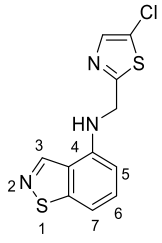
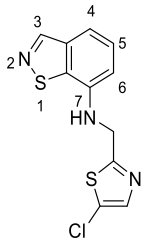
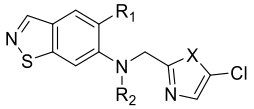
Table 8. Exploration of the Linker Position

Cmpd		TRPM5 pEC_{50} [a,b]	Cmpd		TRPM5 pEC_{50} [a,b]
25		7.05±0.10	**61**		7.36±0.14
62		<4.5	**63**		5.84±0.27

[a]Reported values are the means of at least two independent experiments. [b]All data were obtained using the SyncroPatch 384 Patch Engine.

Table 9. Exploration of the Benzene Ring



compd	R_1	R_2	X	TRPM5 pEC_{50} [a,b]
61	H	H	S	7.36 ± 0.14
64	Cl	H	S	8.10 ± 0.21
65	Cl	h	NMe	7.95 ± 0.20
66	Me	H	S	7.75 ± 0.15
67	F	H	S	7.54 ± 0.11
68	Me	h	NMe	7.42 ± 0.20
69	H	Me	NMe	7.07 ± 0.16
70	F	h	NMe	6.99 ± 0.16
71	H	h	NMe	6.53 ± 0.05
72	Cl	Me	NMe	6.51 ± 0.13

[a]Reported values are the means of at least two independent experiments. [b]All data were obtained using the SyncroPatch 384 Patch Engine.

series; the methylation of the nitrogen atom on the linker on the imidazole series led to a more potent compound (compare compound **69**, pEC_{50} 7.07 with compound **71**, pEC_{50} 6.53); finally, as described for the derivatives in Table 9, the combination of the N-methylation on the linker with the ortho substitution on the benzene ring proved to be detrimental for the activity also with these isomers (compare compound **72** pEC_{50} 6.51 with compound **65** pEC_{50} 7.95).

A full DMPK and selectivity profile for compound **64** is shown in Table 10, where it is compared with compound **61**.

While the whole exploration performed allowed us to obtain a significant increase in potency, it was clear that the metabolic stability remained an unresolved issue. In order to confirm if the in vitro data were predictive of the actual behavior in the animal model, compound **61** was profiled in an in vivo pharmacokinetic (PK) experiment following PO and IV administration in mouse, and the data are summarized in Table 11. The results showed that compound **61** displays a moderate volume of distribution, moderate clearance (as predicted from the in vitro experiments), a short half-life, and a very low oral bioavailability of 1.5% in mouse.

The results of the in vivo experiment showed that compound **61** was not suitable for a systemic approach due to the very low bioavailability. Because the whole chemical series suffered from a low microsomal stability, we considered to shift from a systemic to a local approach.

Compound **64** emerged as the most suitable for this new approach due to its greater potency (hTRPM5 pEC_{50} 8.10) and lower microsomal stability [MiC (mouse) = 520 μL/min/mg protein] compared to compound **61**.

Although a full PK profile is not available, when compound **64** was dosed orally in mouse at 1, 3, and 100 mg/kg, the exposure in plasma was very limited and could be quantified only at 100 mg/kg at 6 h after administration. However, the exposure measured in the target organs of the gastrointestinal tract (colon and ileum) showed a very high exposure at 6 h (colon: 2620 ng/g; ileum: 7379 ng/g), and some detectable levels at 24 and 48 h, at the highest dose. These data suggested that compound **64** could be considered as a luminally restricted compound acting at the intestinal level. See Table 12 for all the relevant data.

In light of this PK behavior, compound **64** was selected as a compound to evaluate the in vivo effect of a locally acting TRPM5 agonist at the intestinal level to enhance gastrointestinal motility in mice. This stimulatory effect is considered clinically relevant to the management of disorders characterized by constipation or impaired motility in general. Following the method described by Zhou et al.,[32] we investigated the stimulatory effect of **64** on gastrointestinal transit in normal mice, measuring fecal parameters during the time after administration (Tables 13 and 14, Figures 3 and 4). The advantage of this model is the possibility to measure the fecal parameters during the time of the experiment in the same mice; the use of single cages provided with grid and removable tray on the bottom permitted to remove feces at the different time points without disturbing the mice.

The test compound was administered orally, in CD-1 mice, at the doses of 1, 10, and 100 mg/kg and the number and weight of feces were evaluated 1, 2, 3, and 6 h after treatment with the tested compound or with the vehicle of formulation. In addition, the wet feces for each mouse were dried at 105 °C for 24 h to calculate the percentage of water of feces. At the same time points, the general health status of mice was monitored for evaluating the potential side effect on locomotion and general

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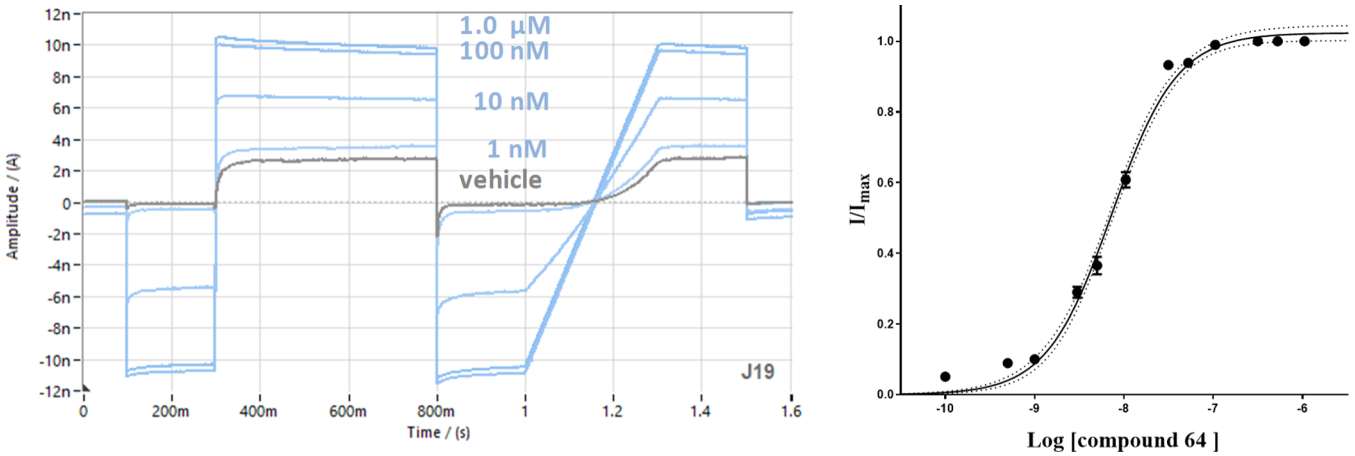

Figure 2. hTRPM5 current recorded in SyncroPatch potentiated by compound **64**. (Left) Current traces recorded in the absence (black trace) and in the presence of increasing concentrations of compound **64** (blue traces, concentrations indicated) obtained on a representative cell. (Right) Current amplitudes measured in the presence of increasing concentrations of compound **64** and subtracted from the vehicle current amplitude were normalized to the max value obtained for each cell. Normalized values obtained for the same concentration were averaged and plotted vs the log of concentration. Vertical bars correspond to the standard error of the mean. The number of averaged values for each concentration ranges from 3 to 12. The estimated pEC_{50} value was 8.10. The 95% confidence interval for the fitting is included in the dotted lines.

Table 10. DMPK Characterization of 61 and 64

	compound **61**	compound **64**
hTRPM5 pEC_{50} [a,b]	7.36 ± 0.14	8.10 ± 0.21
mTRPM5 pEC_{50} [a,b]	7.36 ± 0.07	7.80 ± 0.15
MiC (human) µL/min/mg protein[c]	146	172
MiC (mouse) µL/min/mg protein[c]	164	520
Caco-2 permeability efflux ratio (nm/s)[c]	106.8, 1.08	41, 1.6
solubility pH 7.4 (µM)[c]	511	30
hTRPM8/hTRPV1/hTRPV4/hTRPA1/hTRPM4 (pEC_{50})[a,d]	<4.8/<4.8/<4.8/5.2/<4.2	<4.8/<4.8/<4.8/5.5/5.5
plasma protein binding (as fraction unbound %)[c] human/mouse	<1/<1	<1/<1

[a]Reported values are the means of at least two independent experiments. [b]All data were obtained using the SyncroPatch 384 Patch Engine. [c]See the Experimental Part section for details. [d]All data were obtained by FLIPR.

Table 11. Compound 61 In Vivo PK Studies[a]

compd	adm	dose (mg/kg)	Cl_{obs} (mL/min/kg)	C_{last} (ng/mL)	V_{ss} (L/kg)	$t_{1/2}$ (h)	AUC_{last} (µg h/mL)	F %
61	IV	1	31.2 ±4.98	2.99 ± 1.18	1.12 ± 0.52	0.81	541 ± 92.6	
61	PO	10		2.76 ± 1.24			94.3 ± 19.4	1.54

[a]See Experimental Part section for details.

Table 12. Compound 64 In Vivo PK Studies

mean exposure after oral administration in mouse				
dose (mg/kg)	collection time (h)	plasma (ng/mL)	colon (ng/g)	ileum (ng/g)
1	6	BQL[a]	63.8	BQL
	24	BQL	BQL	BQL
	48	BQL	BQL	BQL
3	6	BQL	143	BQL
	24	BQL	BQL	BQL
	48	BQL	BQL	BQL
100	6	22.6	2620	7379
	24	BQL	164	202
	48	BQL	58	BQL
	72	BQL	BQL	BQL

[a]BQL = 20 ng/mL for plasma and all tissues.

behaviors. Compound **64** demonstrated to increase fecal parameters in mice, reaching a statistically significant effect at 100 mg/kg between 1 and 2 h after treatment, both for the fecal count ($p < 0.01$) and for the fecal weight ($p < 0.01$). An increase of the percentage of water in feces statistically significant at 100 mg/kg was observed, considering the total fecal wet weight during the 6 h of observation (56.9% with respect to 44.7% of the vehicle group). The general status of the mice indicated a normal locomotor and general behavioral condition.

The positive results obtained for compound **64** indicate a potential role for TRPM5 agonists as gastroprokinetic agents.

CHEMISTRY

Compound **2** and the analogues **9**−**15** were synthesized, as described in Scheme 1, through reductive amination of the commercially available heteroaryl amines **73a**−**h** with 5-chloro-1-methyl-1*H*-imidazole-2-carbaldehyde.

The derivatives **16**−**33** bearing various right hand side moieties were all prepared from benzo[*d*]isothiazol-5-amine **73a** (Scheme 2). Compounds **18** and **25**−**33** were synthesized using $NaBH_4$, while for derivatives **16**, **17**, **19**, **20**, **22**, and **24**, the reductive amination was performed with $NaBH(OAc)_3$. For the

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Table 13. Results of In Vivo Experiment of Compound 64[a]

		total number of feces/mouse after treatment (mean ± SEM)			
treatment	dose (mg/kg)	1 h	2 h	3 h	6 h
1-vehicle		3 ± 0.8	5.3 ± 0.7	9.1 ± 1.1	20.1 ± 1.4
2-compound **64**	1	4.3 ± 0.8	7.5 ± 1.1	11.8 ± 1.1	24.3 ± 1.8
3-compound **64**	10	3.4 ± 0.8	7.3 ± 1.2	10.3 ± 0.9	18.5 ± 1.1
4-compound **64**	100	4.0 ± 0.9	10.2 ± 1.5**	13.0 ± 1.6*	16.3 ± 1.7

[a]Statistical analysis was performed by Dunnett's test. N = 12/group. *p < 0.05; **p < 0.01 treated group vs vehicle.

Table 14. Results of In Vivo Experiment of Compound 64[a]

		total weight (g) feces/mouse after treatment (mean ± SEM)			
treatment	dose (mg/kg)	1 h	2 h	3 h	6 h
1-vehicle		0.08 ± 0.02	0.13 ± 0.02	0.21 ± 0.03	0.43 ± 0.05
2-compound **64**	1	0.09 ± 0.02	0.15 ± 0.02	0.23 ± 0.02	0.45 ± 0.04
3-compound **64**	10	0.08 ± 0.02	0.18 ± 0.04	0.23 ± 0.03	0.39 ± 0.03
4-compound **64**	100	0.12 ± 0.02	0.31 ± 0.05**	0.41 ± 0.05**	0.49 ± 0.05

[a]Statistical analysis was performed by Dunnett's test. N = 12/group. **p < 0.01 treated group vs vehicle.

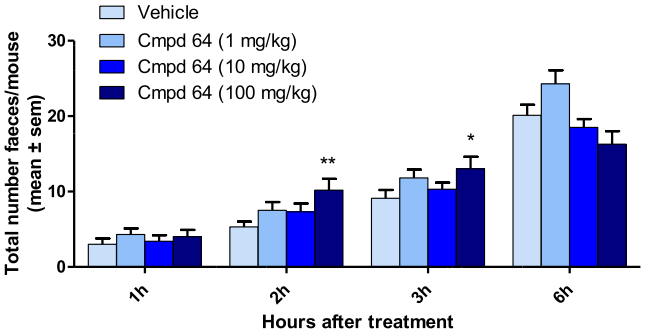

Figure 3. Total number of feces per mouse after treatment with compound **64**. The test compound was administered orally, in CD-1 mice, at the doses of 1, 10, and 100 mg/kg and the total number of feces was evaluated 1, 2, 3, and 6 h after treatment. Statistical analysis was performed by Dunnett's test. N = 12/group. *p < 0.05; **p < 0.01 treated group vs vehicle.

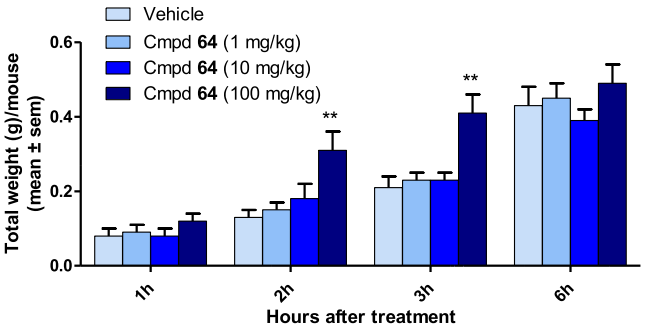

Figure 4. Total weight of feces (g) per mouse after treatment with compound **64**. The test compound was administered orally, in CD-1 mice, at the doses of 1, 10, and 100 mg/kg and the total weight of feces (g) was evaluated 1, 2, 3, and 6 h after treatment. Statistical analysis was performed by Dunnett's test. N = 12/group. **p < 0.01 treated group vs vehicle.

synthesis of compound **16**, the *N*-trityl-protected aldehyde **75** was prepared according to the known procedures[33] and the final deprotection of the trityl group was accomplished with TFA.[34] Derivatives **21** and **23** were obtained reacting **73a** with the appropriate chloromethyl-heterocycles.

As shown in Scheme 3, the synthesis of the N-substituted analogues **34**–**41** started with the corresponding NH derivatives **2** and **25**. While compounds **34** and **35** were prepared using MeI, the majority of derivatives (**36**–**40**) were synthesized through reductive amination with the corresponding aldehydes or with acetone (compound **37**). Compound **41** was obtained by reacting compound **2** with acetyl chloride.

The synthetic procedure for the derivatives **42**–**44** is outlined in Scheme 4. The reaction of **73a** with 5-chlorothiazole-2-carbonyl chloride generated in situ provided amide **42**.[35] Derivatives **43** and **44** were obtained by condensation of **73a** with aldehyde **76** or ketone **77**, respectively, followed by addition of MeMgBr.[36] 5-Chlorothiazole-2-ethanone **77** was prepared according to the literature.[37]

The synthesis of the O-linked compound **45** (Scheme 5) started with the alkylation of benzo[*d*]isothiazol-5-ol **78**, readily available from the corresponding amine.[38]

As described in Scheme 6, the substituted derivatives **46**–**57** and **82**–**84** were obtained from the commercial *ortho*-fluoro/chloro arylaldehydes bearing a nitro group or a bromine atom in position 5. The isothiazole ring closure was afforded using sulfur and aq NH_4OH[39] or with a three-step procedure involving BnSH, SO_2Cl_2, and NH_3.[40,41] Amino intermediates **81a**–**l** were obtained by nitro reduction or copper-mediated amination[42,43] or with halogenation of benzo[*d*]isothiazol-5-amine **73a**. The subsequent reaction of **81a**–**l** with the appropriate aldehydes yielded the final products **46**–**57** and **82**–**84**. Finally, compounds **58**–**60** were obtained by reductive N-methylation of **82**–**84** using formaldehyde.[44]

As depicted in Schemes 7 and 8, the variously substituted derivatives **61**–**72** were prepared in a similar manner to the compounds described in Scheme 6.

CONCLUSIONS

In conclusion, the objective of this study was to find a suitable compound that could selectively interact with the hTRPM5 ion channel. After a HTS, 73 potential hits were discovered and in particular in this article the exploration of one of them is described. After optimization of three distinct regions of hit compound **1**, guided by SAR analysis, compounds **61** and **64** were selected as potentially useful tool compounds for an in vivo model due to their nanomolar TRPM5 agonist activity and

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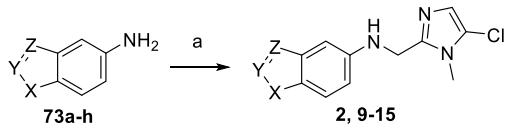
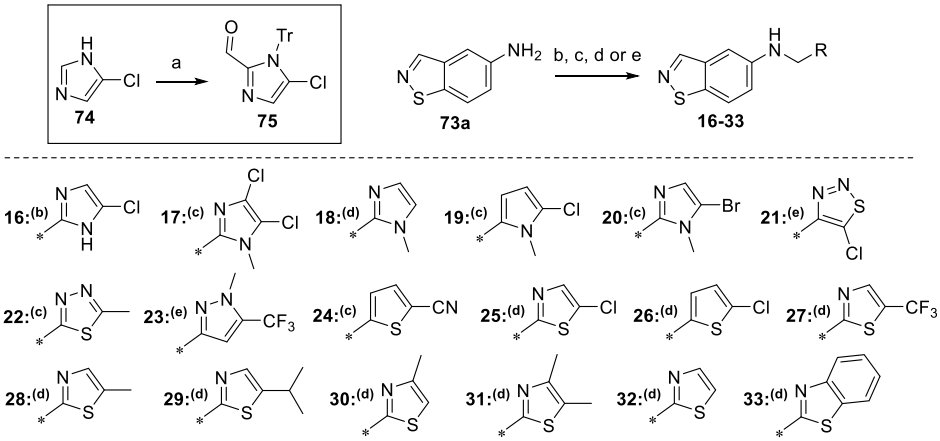
Scheme 1. Synthesis of Compounds 2, 9−15[a]



	X	Y	Z
73a, 2:	S	N	CH
73b, 9:	O	N	CH
73c, 10:	NH	N	CH
73d, 11:	NMe	N	CH
73e, 12:	S	CH	N
73f, 13:	O	CH	N
73g, 14:	NH	CH	N
73h, 15:	NMe	N	N

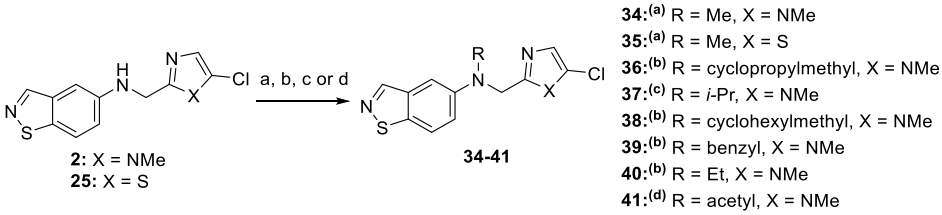
[a]Reagents and conditions: (a) (i) 5-chloro-1-methyl-1*H*-imidazole-2-carbaldehyde, MeOH, 60 °C, 18 h, (ii) $NaBH_4$, 0 °C−room temperature (rt), 2 h, 7−96%, two steps.

Scheme 2. Synthesis of Analogues 16−33[a]



[a]Reagents and conditions: (a) (i) triphenylmethyl chloride, Et_3N, CH_2Cl_2, rt, 20 h, (ii) *n*-BuLi, dimethylformamide (DMF), tetrahydrofuran (THF), −78 °C, 4 h, 57%, two steps; (b) (i) **75**, $NaBH(OAc)_3$, CH_2Cl_2, AcOH cat., rt, 16 h, (ii) TFA, CH_2Cl_2, rt, 2 h, 42%, two steps; (c) RCHO, $NaBH(OAc)_3$, CH_2Cl_2, AcOH cat., rt, 16 h, 28−74%; (d) (i) RCHO, MeOH, 60 °C, 16 h, (ii) $NaBH_4$, 0 °C−rt, 2 h, 16−91%, two steps (e) RCH_2Cl, Et_3N, EtOH, 80 °C, 6 h, 15−17%.

Scheme 3. Synthesis of N-Substituted Derivatives 34−41[a]



[a]Reagents and conditions: (a) NaH, MeI, DMF, rt, 1−16 h, 19−55%; (b) corresponding aldehydes, $NaBH(OAc)_3$, CH_2Cl_2, AcOH cat., rt, 16 h, 42−59%; (c) acetone, $Na(OAc)_3BH$, CH_2Cl_2, AcOH cat., rt−reflux, 22 h, 24%; (d) CH_3COCl, Et_3N, CH_2Cl_2, rt, 4 h, 74%.

excellent selectivity. In particular, compound **64** was investigated for its locally acting stimulatory effect on gastrointestinal transit in mice due to its very low absorption and high gastrointestinal tract exposure as shown by in vivo PK studies in mice. Compound **64** showed a statistically significant increase of the fecal parameters after oral administration at 100 mg/kg. The positive results obtained for compound **64** indicate a potential role for TRPM5 agonists as gastroprokinetic agents.

EXPERIMENTAL PART

Materials and Methods. Chemicals were obtained from Sigma-Aldrich, and cell culture reagents were purchased from Thermo Fisher Scientific Spa (Italy), GE Healthcare Bio-Sciences (Austria). Cell lines were purchased from Scottish Biomedical Drug Discovery (Glasgow, Scotland). Four compounds described as "taste enhancers" in the Patent WO2008/024364 as examples 2, 3, 1, and 10, respectively, were synthesized in Aptuit. Compound 10 was used as a positive modulator. A compound described as a blocker in Patent WO2010/132615 as example 43 was obtained from Takeda laboratories.

Cell Culture. CHO-K1 cell lines stably expressing human TRPM5 (CHO-hTRPM5) or mouse TRPM5 (CHO-mTRPM5) or human TRPM4 (CHO-hTRPM4) and the CHO-K1 cell line (CHO) used as the cell background for the generation of stable cell lines were cultured in minimum essential medium supplemented with 2 mM L-glutamine and 8 μg/mL blasticidin for CHO-hTRPM5 or F12-HAM (Ham's F-12 nutrient mix) supplemented with 250 μg/mL hygromycin B for CHO-mTRPM5 and CHO-hTRPM4 or F-12-HAM for the CHO cell line. All media were supplemented with 10% heat inactivated fetal bovine serum and 1% penicillin−streptomycin. Cells were passaged twice a week and kept in a humidified incubator at 37 °C and 5% cO_2.

QPatch HTX Assay. The QPatch HTX (Sophion Bioscience A/S, Ballerup, Denmark), an automated electrophysiology platform described in detail by Mathes et al.,[45] was used for confirmation of selective and specific hits. The extracellular solution was (in mM) NaCl (137); KCl (4); $CaCl_2$ (1.8); $MgCl_2$ (1); *N*-(2-hydroxyethyl)-

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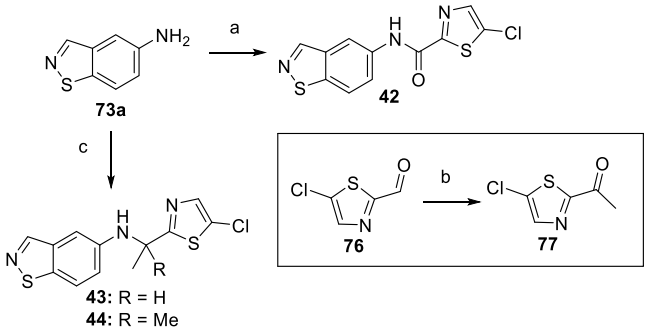
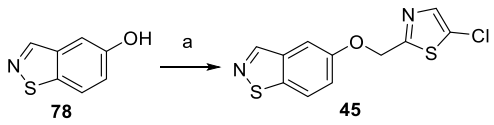
Scheme 4. Synthesis of Compounds 42−44[a]



[a]Reagents and conditions: (a) (i) 5-chlorothiazole-2-carboxylic acid, $(COCl)_2$, CH_2Cl_2, DMF cat., 0 °C−rt, 2 h, (ii) Et_3N, CH_2Cl_2, 0 °C−rt, 2 h, 53%, two steps; (b) (i) MeMgBr, THF, 0 °C−rt, 1 h, (ii) Dess−Martin periodinane, CH_2Cl_2, rt, 1 h, 48%, two steps; (c) (i) **76** (for **43**) or 77 (for **44**), toluene, reflux, 20 h, (ii) MeMgBr, rt, 3 h, 8−35%, two steps.

Scheme 5. Synthesis of the O-Linked Compound 45[a]



[a]Reagents and conditions: (a) 5-chloro-2-(chloromethyl)-1,3-thiazole, K_2CO_3, DMF, rt, 16 h, 62%.

piperazine-*N*′-ethanesulfonic acid (HEPES) (10); and D-glucose (10); and the pH was adjusted to 7.4 with NaOH. The intracellular solution was (in mM) KCl (120); NaCl (10); $CaCl_2$ (*x*); $MgCl_2$ (4.28), hydroxyethylethylenediaminetriacetic acid (HEDTA) (10); and HEPES (10); and the pH was adjusted to 7.2 with KOH. Value *x* corresponds to 0.9 mM and it is the concentration calculated with MaxChelator software to control the intracellular free calcium concentration and obtain 1 μM free concentration. Test compounds were prepared in DMSO 100% at a concentration 1000-fold greater than test concentrations. Stock solutions were diluted in glass vials with extracellular solution to final perfusion concentrations of 0.1, 1, 10, and 30 suitable for application to cells in the QPatch HTX system. Cells were detatched with Triple and mixed with the SFM II serum-free medium supplemented with 25 mM HEPES and then placed in a cell storage tank in the QPatch machine 5 min before starting the experiment. Electrophysiological recordings were carried out at rt with currents recorded in a whole cell configuration patch clamp. Sophion QPatch assay software was used for cell capture, seal formation, and obtaining whole cell and data acquisition. Once whole cell formation was obtained, cells were maintained at a holding potential of 0 mV and the following voltage protocol was applied at a frequency of 0.1 Hz: a step to −80 mV for 200 ms, followed by a depolarizing step to 80 mV for 500 ms and an hyperpolarizing step to −80 mV for 200 ms, followed by a voltage ramp to 80 mV for 300 ms, and after additional 300 ms at 80 mV, a final step to the holding potential. No leak subtraction was applied. Current amplitude was measured at the end of the first depolarizing voltage step.

SyncroPatch Assay. The SyncroPatch 384 Patch Engine (Nanion Technologies, Munich, Germany), an automated multiwell planar patch-clamp system described in detail by Obergrussberger,[46] was used for focussed library screening and confirmation of selective and specific hits. Chips with single-hole medium resistance (5−8 MΩ) were used. The extracellular solution contained (in mM) NaCl (140); KCl (4); $BaCl_2$ (5); $MgCl_2$ (1); HEPES (10); and D-glucose (5); and the pH was adjusted to 7.4 with NaOH. The intracellular solution contained (in mM) K_2SO_4 (75); KCl (30); NaCl (10); $CaCl_2$ (*x*); $MgCl_2$ (4.28), HEDTA (10); HEPES (10); Na-ATP (2); and the pH was adjusted to 7.2 with KOH. Value *x* corresponds to 2.5 mM and it is the concentration calculated with MaxChelator software to control the intracellular free calcium to obtain 5 μM free concentration.[47] Test compounds were prepared from 6 mM 100% DMSO stocks and dose−response compound plates were prepared by serially diluting the stock compound solutions into DMSO, then 200× well plates were obtained on an Echo 550 (Labcyte) instrument. Assay plates were diluted in extracellular solution, using a Multidrop dispenser (Thermo Scientific), and tested in cumulative protocol at final concentrations of 0.03, 0.3, 3.1, and 31.6 μM in 0.5% DMSO, followed by an addition of a positive modulator at 3 μM and a final addition of a blocker at 10 μM. The SyncroPatch liquid protocol consists of 180 s for vehicle incubation and 150 s for compound incubation at each concentration tested, followed by additional 150 s for positive modulator incubation and additional 150 s for blocker incubation. Cells were harvested and resuspended to 0.5×10^6 cell/mL in 30 mL of extracellular solution without $BaCl_2$ and maintained in a cell hotel reservoir at 10 °C with a shaking speed of 200 rpm for 10 min before starting the experiment. After initiating the experiment, cell catching, sealing, whole-cell formation, liquid application, recording, and data acquisition were performed sequentially. Electrophysiological recordings were carried out at rt with currents recorded on a whole cell configuration patch clamp. Once whole cell formation was obtained, cells were maintained at a holding potential of 0 mV and the following voltage protocol was applied at a frequency of 0.1 Hz: a step to −80 mV for 200 ms, followed by a depolarizing step to 80 mV for 500 ms and an hyperpolarizing step to −80 mV for 200 ms, followed by a voltage ramp to 80 mV for 300 ms, and after additional 300 ms at 80 mV a final step to the holding potential. No leak subtraction was applied. Current amplitude was measured at the end of the first depolarizing voltage step.

Data Analysis. The QPatch and SyncroPatch experiments were performed by measuring the current amplitude in vehicle and in the presence of compound at the end of each incubation period. The percentage of current increase was calculated considering the value recorded in vehicle as 100%. In QPatch data acquisition and analysis was performed using Sophion QPatch assay software 5.6. In SyncroPatch data acquisition and analysis was performed using PatchControl and DataController384 software (Nanion, Inc).

FMP Assay for the hTRPM4 Receptor. The described assay provides the in vitro agonist potency (pEC_{50}) of compounds at the hTRPM4 receptor stably expressed in CHO cells by measuring the membrane potential changes using FLIPR (Molecular Devices) and the red component of the FMP kit (R8126, Molecular Devices). CHO-hTRPM4 cells were plated at 10,000 cells/well in black, clear-bottom 384-well plates (781092, Greiner Bio-One GmbH) 24 h before the assay and incubated at 37 °C, 5% CO_2. In the assay, cells are washed twice in Hank's balanced salt solution (HBSS), 20 mM HEPES, pH 7.4, leaving 10 μL/well after the final aspiration. Cells are incubated with the 10 μL/well loading solution 2× for 30 min at 37 °C and in 0% CO_2. Compounds were characterized in a concentration-dependent manner (11 concentrations, 1:3 serial dilutions) for their ability to increase fluorescence counts and a pEC_{50} was calculated. 10 μL/well of test compound solutions, buffer (0% control), and 30 μM A23187 (10 μM as the final concentration, 100% control) are added to the loaded cells and membrane potential responses are measured. The final DMSO concentration in the assay was 0.5%.

hTRPM8 FLIPR Calcium Assay. The described assay provides the in vitro agonist potency (pEC_{50}) and antagonist potency (pIC_{50}) of compounds at the hTRPM8 channel stably expressed in HEK cells by measuring changes in the intracellular Ca^{2+} concentration using FLIPR technology (Molecular Devices) and the calcium 6 dye (Molecular Devices, Sunnyvale, CA). HEK-hTRPM8 cells were plated at 20,000 cells/well in black, clear-bottom, poly-D-lysine-coated 384-well plates (781946, Greiner Bio-One GmbH) 24 h before the assay and incubated at 37 °C, 5% CO_2. On the day of the experiment, the medium was removed from cells and 30 μL/well loading solution (HBSS buffer with calcium-6 dye and 2.5 mM probenecid) was added for 1 h at 37 °C and 1 h at rt. A dual addition and read-out FLIPR protocol was applied allowing for agonist and antagonist characterization. In the first addition, compounds were characterized in a concentration-dependent manner (11 point CRC, 1:3 serial dilutions) for their ability to increase

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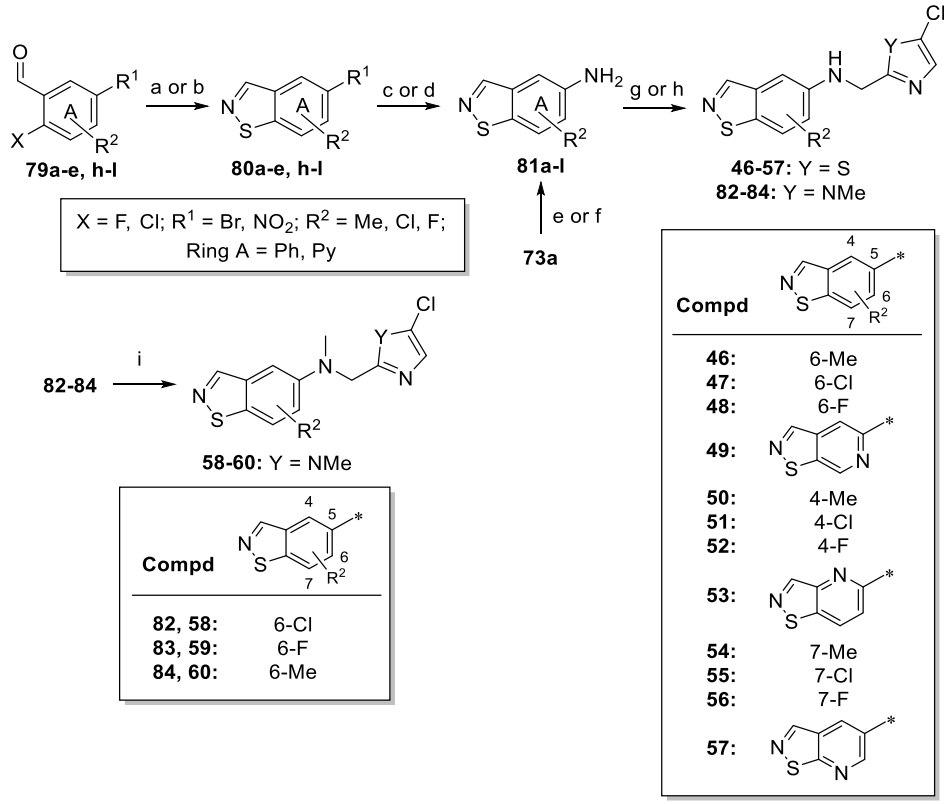
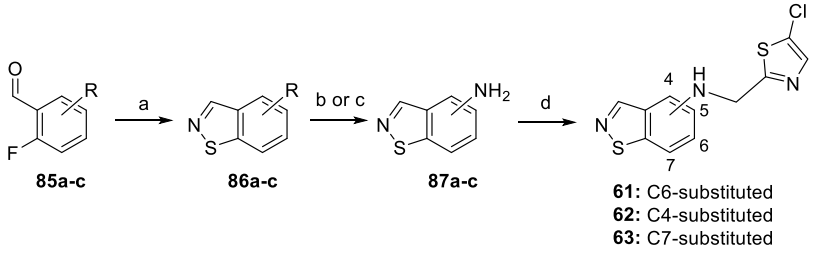
Scheme 6. Synthesis of Derivatives 46−57, 82−84, and 58−60[a]



[a]Reagents and conditions: (a) sulfur, NH_4OH, DMF, 90 °C, 16 h, 10−69%; (b) (i) BnSH, KO^{t}Bu, THF, rt, 2 h, (ii) SO_2Cl_2, CH_2Cl_2, rt, 1 h, (iii) 7 M NH_3 in MeOH, THF, rt, 1 h, 15−58%, three steps; (c) NaN_3, Cu_2O, L-proline, DMSO, 100 °C, 1−16 h, 9−78%; (d) Fe, NH_4Cl, EtOH, 80 °C, 3 h, 60−77%; (e) NCS, THF, rt, 16 h, 57%; (f) Selectfluor, DMF, rt, 3 h, 11%; (g) 5-chloro-1-methyl-1*H*-imidazole-2-carbaldehyde or 5-chlorothiazole-2-carbaldehyde, $NaBH(OAc)_3$, CH_2Cl_2, AcOH cat., rt, 16 h, 4−67%; (h) (i) 5-chloro-1-methyl-1*H*-imidazole-2-carbaldehyde or 5-chlorothiazole-2-carbaldehyde, MeOH, 60 °C, 16 h, (ii) $NaBH_4$, 0 °C−rt, 2−3 h, 18−55%, two steps; (i) $HO(CH_2O)_nH$, $NaBH(OAc)_3$, CH_2Cl_2, AcOH cat., rt, 24−72 h, 43−56%.

Scheme 7. Synthesis of Compounds 61−63[a,b]



[a]R = Br, NO_2. [b]Reagents and conditions: (a) sulfur, NH_4OH, DMF, 90 °C, 16 h, 43−67%; (b) NaN_3, Cu_2O, L-proline, DMSO, 100 °C, 1−16 h, 50−80%; (c) Fe, NH_4Cl, EtOH, 80 °C, 3 h, 40%; (d) (i) 5-chlorothiazole-2-carbaldehyde, MeOH, 60 °C, 16 h, (ii) $NaBH_4$, 0 °C−rt, 2 h, 13−20%, two steps.

intracellular calcium levels with respect to the standard agonist (−) menthol (100 μM as the final concentration, 100% control), and if a concentration-dependent effect was observed, the pEC_{50} value was calculated. Immediately after the 5 min recording of this first addition, a second addition containing 20 μM (−) menthol was performed. Inhibition of the agonist evoked signal, recorded for 3 min, indicated antagonist activity of the compound and allowed the calculation of the compound pIC_{50} value. The final DMSO concentration in the assay was 0.5%. The assay was performed at rt.

hTRPA1 FLIPR Calcium Assay. The described assay provides the in vitro agonist potency (pEC_{50}) and antagonist potency (pIC_{50}) of compounds at the hTRPA1 channel stably expressed in HEK cells by measuring changes in the intracellular Ca^{2+} concentration using FLIPR technology (Molecular Devices) and the calcium 6 dye (Molecular Devices, Sunnyvale, CA). HEK-hTRPA1 cells were plated at 20,000 cells/well in black, clear-bottom, poly-D-lysine-coated 384-well plates (781946, Greiner Bio-One GmbH) 24 h before the assay and incubated at 37 °C, 5% CO_2. On the day of the experiment, the medium was removed from cells and 30 μL/well loading solution (HBSS buffer with calcium-6 dye and 2.5 mM probenecid) was added for 2 h at 37 °C. A dual addition and read-out FLIPR protocol was applied allowing for agonist and antagonist characterization. In the first addition, compounds were characterized in a concentration-dependent manner (11 point CRC, 1:3 serial dilutions) for their ability to increase intracellular calcium levels with respect to the standard agonist (−) menthol (1 mM as final concentration, 100% control), and if a concentration-dependent effect was observed, the pEC_{50} value was calculated. Immediately after the 5 min recording of this first addition, a

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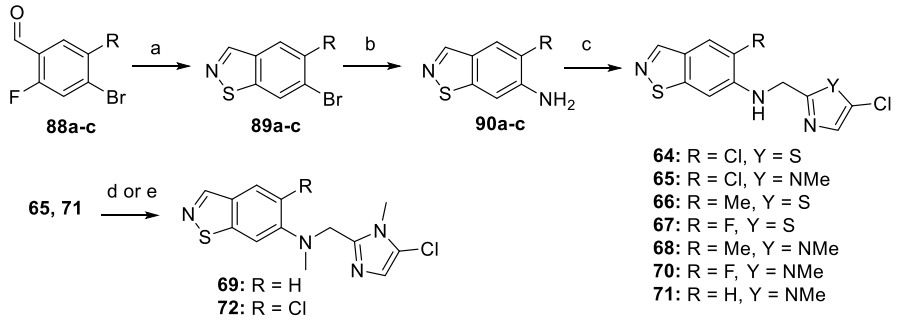
Scheme 8. Synthesis of Analogues 64−72[a]



[a]Reagents and conditions: (a) (i) BnSH, KO^{t}Bu, THF, rt, 2 h, (ii) SO_2Cl_2, CH_2Cl_2, rt, 1 h, (iii) 7 M NH_3 in MeOH, THF, rt, 1 h, 20−53%, three steps; (b) NaN_3, Cu_2O, L-proline, DMSO, 100 °C, 16 h, 35−77%; (c) 5-chloro-1-methyl-1*H*-imidazole-2-carbaldehyde or 5-chlorothiazole-2-carbaldehyde, $NaBH(OAc)_3$, CH_2Cdichloroethane (DCE), AcOH cat., rt−70 °C, 8−36 h, 39−54%; (d) NaH, MeI, DMF, 0 °C−rt, 3 h, 11%; (e) $HO(CH_2O)_nH$, $NaBH(OAc)_3$, DCE, AcOH cat., 40 °C, 48 h, 16%.

second addition containing 100 μM (−) menthol was performed. Inhibition of the agonist-evoked signal, recorded for 3 min, indicated the antagonist activity of the compound and allowed the calculation of the compound pIC_{50} value. The final DMSO concentration in the assay was 0.5%. The assay was performed at 37 °C.

hTRPV1 FLIPR Calcium Assay. The described assay provides the in vitro agonist potency (pEC_{50}) and antagonist potency (pIC_{50}) of compounds at the hTRPV1 channel stably expressed in CHO cells by measuring changes in the intracellular Ca^{2+} concentration using FLIPR technology (Molecular Devices) and the calcium 6 dye (Molecular Devices, Sunnyvale, CA).

CHO-hTRPV1 cells were plated at 10,000 cells/well in black, clear-bottom 384-well plates (781092, Greiner Bio-One GmbH) 24 h before the assay and incubated at 37 °C, 5% CO_2. On the day of the experiment, the medium was removed from cells and 30 μL/well loading solution (HBSS buffer with calcium-6 dye and 2.5 mM probenecid) was added 1 h at 37 °C and 1 h at rt. A dual addition and read-out FLIPR protocol was applied allowing for agonist and antagonist characterization. In the first addition, compounds were characterized in a concentration-dependent manner (11 point CRC, 1:3 serial dilutions) for their ability to increase intracellular calcium levels with respect to the standard agonist capsaicin (30 μM as final concentration, 100% control), and if a concentration-dependent effect was observed, the pEC_{50} value was calculated. Immediately after the 5 min recording of this first addition, a second addition containing 100 nM capsaicin was performed. Inhibition of the agonist evoked signal, recorded for 3 min, indicated antagonist activity of the compound and allowed the calculation of the compound pIC_{50} value. The final DMSO concentration in the assay was 0.5%. The assay was performed at rt.

hTRPV4 FLIPR Calcium Assay. The described assay provides the in vitro agonist potency (pEC_{50}) and antagonist potency (pIC_{50}) of compounds at the hTRPV4 channel stably expressed in HEK cells by measuring changes in the intracellular Ca^{2+} concentration using FLIPR technology (Molecular Devices) and the calcium 6 dye (Molecular Devices, Sunnyvale, CA). HEK-hTRPV4 cells were plated at 20,000 cells/well in black, clear-bottom, poly-D-lysine-coated 384-well plates (781946, Greiner Bio-One GmbH) 24 h before the assay, in the presence of 1 μg/mL tetracycline, and incubated at 37 °C, 5% CO_2. On the day of the experiment, the medium was removed from cells and 30 μL/well loading solution (HBSS buffer with calcium-6 dye and 2.5 mM probenecid) was added for 1 h at 37 °C and 1 h at rt. A dual addition and read-out FLIPR protocol was applied allowing for agonist and antagonist characterization. In the first addition, compounds were characterized in a concentration-dependent manner (11 point CRC, 1:3 serial dilutions) for their ability to increase intracellular calcium levels with respect to the standard agonist GSK1016790A (100 nM as the final concentration, 100% control), and if a concentration-dependent effect was observed, the pEC_{50} value was calculated. Immediately after the 5 min recording of this first addition, a second addition containing 20 nM GSK1016790A was performed. Inhibition of the agonist-evoked signal, recorded for 3 min, indicated the antagonist activity of the compound and allowed the calculation of the compound pIC_{50} value. The final DMSO concentration in the assay was 0.5%. The assay was performed at rt.

Fluorescent Membrane Potential Assay for hTRPM5. Cells were resuspended in the culture medium and seeded onto 384-well assay plates at 5000 cells/well, and after an incubation of >18 h, they were washed twice with 50 μL of assay buffer (20 mM HEPES, 1× HBSS, pH 7.4), leaving 10 μL. The FMP dye (FMP kit cat # R8123, Molecular Devices, Wokingham, UK) dissolved in the assay buffer was then added (10 μL) and incubated at 37 °C for 30 min. The plates of a batch were placed in stacker at rt. Compound intermediate plates contained compound at 3× concentration in the assay buffer with 1.5% DMSO. In FLIPR, 10 μL of 3× compound or controls were transferred to the cell plate (first addition) and the relative fluorescence unit (RFU) was measured for 2 min (40 μM was the final compound concentration; 0.5% DMSO was the 0% control; and 10 μM A23187 was the 100% control). After such time, 10 μL from 4× ligand plate (200 nM ATP final concentration, and appropriate controls such as buffer for 0% effect and 10 μM A23187 as 100% control) were transferred to the cell plate and RFU was measured for 4 min. The averaged RFU of the baseline and the max RFU values after first and second addition were exported. For agonist and positive modulator assessment, the ratio between the first addition and the baseline and the ratio between second addition and the baseline were calculated, respectively. Upon treatment of cells with an agonist of TRPM5, or alternatively with a compound that will lead to an elevation of intracellular Ca^{2+} levels, the opening of the TRPM5 channel will result in a depolarization of the cell membrane and consequently in a change in the RFU to higher values.

Counter Assays. Two counter assays were set up: an assay measuring Ca^{2+}-flux in CHO-hTRPM5, to determine if potential agonists act on TRPM5 directly, or on intracellular Ca^{2+} release, and a membrane potential blue dye assay using CHO-K1 cells, to identify compounds that elicit a membrane potential change independent of TRPM5.

Chemistry. Reagents and solvents were obtained from commercial sources and used as supplied. The ^{1}H NMR spectra were recorded on either a Bruker Avance 400, a Bruker Avance III 400, or an Agilent Direct Drive spectrometer. The spectra were acquired in the stated solvent, and chemical shifts (δ) are reported in ppm relative to the residual solvent peak. Chemical shifts are given in parts per million using conventional abbreviations for designation of major peaks, for example, s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublets; dt, doublet of triplets; and br, broad. MS was performed using an ultraperformance LC/mass spectrometry (UPLC/MS) Acquity system equipped with a photodiode array detector and coupled to a Waters single quadrupole mass spectrometer. Compound UPLC/MS retention times (R_t) were obtained using the following methods. Method 1: Acquity UPLC CSH C18 column (50 mm × 2.1 mm, 1.7 μm particle size) at 40 °C, a linear gradient from 3 to 100% B over 2 min [A = 0.1% v/v solution of HCOOH in water, B = 0.1% v/v solution of

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HCOOH in acetonitrile (ACN)] and a flow rate of 1 mL/min. Method 2: Kinetex column EVO C18 100 Å (50 mm × 2.1 mm, 1.7 μm particle size) at 40 °C, a linear gradient from 3 to 100% B over 2 min (A = 10 mM ammonium bicarbonate aqueous solution adjusted to pH 10 with ammonia, B = ACN) and a flow rate of 1 mL/min. Thin-layer chromatography analyses were carried out on alumina sheets precoated with silica gel 60 F_{254} and visualized with UV light. Flash chromatography purifications were performed using KP-Sil 32−63 μm 60 Å cartridges. When high-performance liquid chromatography (HPLC) purification was performed, compounds were purified using a semipreparative AutoPurification HPLC System from Waters. AutoPurification System was equipped with a Waters 2525 binary pump, a Waters 2996 diode array detector (DAD), and a Micromass ZQ MS detector. A Waters 2767 sample manager fraction collector has been used for sample injection and fraction recovery. Two different types of reversed phase methods were used. Method A: XSelect semipreparative column C-18 CSH (30 × 100 mm, 5 μm, from Waters), linear gradient from 3 to 100% B over 15 min (A = 0.1% v/v solution of HCOOH in water, B = 0.1% v/v solution of HCOOH in ACN), and a flow rate of 40 mL/min; method B: Gemini C-18 semipreparative column (30 × 100 mm, 5 μm, from Phenomenex), a linear gradient from 3 to 100% B over 15 min (A = 10 mM ammonium bicarbonate aqueous solution adjusted to pH 10 with ammonia, B = ACN), a flow rate of 40 mL/min. Purities of all target compounds were greater than 95% as determined by HPLC analysis. Compounds were analyzed for and found not to contain pan assay interference substructural features.

*General Procedure for the Synthesis of Compounds **2** and **9**−**15**.* A solution of appropriate heteroaryl amine **73a**−**h** (1 equiv) and 5-chloro-1-methyl-1*H*-imidazole-2-carbaldehyde (2 equiv) in MeOH (0.1 M) was heated at 60 °C for 16 h. After cooling to 0 °C, $NaBH_4$ (3 equiv) was added and the reaction mixture was allowed to warm to rt and stirred for 2 h. The mixture was treated with a saturated aqueous $NaHCO_3$ solution and extracted with dichloromethane (DCM). The organic layer was washed with brine, dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by reverse-phase HPLC or as stated to provide the target compounds.

*N-((5-Chloro-1-methyl-1H-imidazol-2-yl)methyl)benzo[d]-isothiazol-5-amine (**2**).* Compound **2** was prepared from benzo[*d*]-isothiazol-5-amine **73a** (2 mg, 0.013 mmol) according to the general procedure. The residue was purified by preparative HPLC (method B). White solid (3.5 mg, 96% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.77 (s, 1H), 7.75 (d, J = 8.80 Hz, 1H), 7.35−7.19 (m, 1H), 7.02 (dd, J = 8.80, 1.96 Hz, 1H), 6.95 (s, 1H), 4.58 (br s, 1H), 4.41 (d, J = 4.89 Hz, 2H), 3.63 (s, 3H). LC−MS method 2: R_t = 0.79 min. MS-ESI (m/z): calcd for $C_{12}H_{11}ClN_4S$ [M + H]$^+$, 279.0; found, 279.1.

*N-((5-Chloro-1-methyl-1H-imidazol-2-yl)methyl)benzo[d]-isoxazol-5-amine (**9**).* Compound **9** was prepared from benzo[*d*]-isoxazol-5-amine **73b** (23 mg, 0.17 mmol) according to the general procedure. The residue was purified by preparative HPLC (method B). White solid (8.4 mg, 19% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.59 (s, 1H), 7.46 (d, J = 9.05 Hz, 1H), 7.02 (dd, J = 8.93, 2.32 Hz, 1H), 6.97−6.90 (m, 2H), 4.42 (br s, 1H), 4.37 (d, J = 4.89 Hz, 2H), 3.63 (s, 3H). LC−MS method 2: R_t = 0.73 min. MS-ESI (m/z): calcd for $C_{12}H_{11}ClN_4O$ [M + H]$^+$, 263.1; found, 263.2.

*N-((5-Chloro-1-methyl-1H-imidazol-2-yl)methyl)-1H-indazol-5-amine (**10**).* Compound **10** was prepared from 1*H*-indazol-5-amine **73c** (23 mg, 0.17 mmol) according to the general procedure. The residue was purified by preparative HPLC (method B). White solid (17.90 mg, 40% yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.61 (br s, 1H), 7.77 (s, 1H), 7.28 (d, J = 8.77 Hz, 1H), 6.92 (s, 1H), 6.89 (dd, J = 8.88, 2.08 Hz, 1H), 6.86 (s, 1H), 5.87 (t, J = 5.48 Hz, 1H), 4.29 (d, J = 5.48 Hz, 2H), 3.58 (s, 3H). LC−MS method 2: R_t = 0.60 min. MS-ESI (m/z): calcd for $C_{12}H_{12}ClN_5$ [M + H]$^+$, 262.1; found, 262.2.

*N-((5-Chloro-1-methyl-1H-imidazol-2-yl)methyl)-1-methyl-1H-indazol-5-amine (**11**).* Compound **11** was prepared from 1-methyl-1*H*-indazol-5-amine **73d** (34 mg, 0.23 mmol) according to the general procedure. The residue was purified by preparative HPLC (method B). White solid (57.40 mg, 90% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.82 (s, 1H), 7.26 (d, J = 9.78 Hz, 1H), 6.98−6.88 (m, 3H), 4.36 (s, 2H), 4.32−4.07 (m, 1H), 4.03 (s, 3H), 3.62 (s, 3H). LC−MS method 2: R_t = 0.69 min. MS-ESI (m/z): calcd for $C_{13}H_{14}ClN_5$ [M + H]$^+$, 276.1; found, 276.1.

*N-((5-Chloro-1-methyl-1H-imidazol-2-yl)methyl)benzo[d]-thiazol-5-amine (**12**).* Compound **12** was prepared from benzo[*d*]-thiazol-5-amine **73e** (51 mg, 0.34 mmol) according to the general procedure. The residue was purified by column chromatography on silica gel (0−50% EtOAc/cyclohexane). White solid (48 mg, 51% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.93 (s, 1H), 7.73 (d, J = 8.56 Hz, 1H), 7.40 (d, J = 2.20 Hz, 1H), 6.98−6.89 (m, 2H), 4.56 (br s, 1H), 4.41 (d, J = 3.67 Hz, 2H), 3.62 (s, 3H). LC−MS method 1: R_t = 0.48 min. MS-ESI (m/z): calcd for $C_{12}H_{11}ClN_4S$ [M + H]$^+$, 279.0; found, 279.0.

*N-((5-Chloro-1-methyl-1H-imidazol-2-yl)methyl)benzo[d]-oxazol-5-amine (**13**).* Compound **13** was prepared from benzo[*d*]-oxazol-5-amine **73f** (23 mg, 0.17 mmol) according to the general procedure. The residue was purified by preparative HPLC (method B). White solid (3 mg, 7% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.01 (s, 1H), 7.40 (d, J = 8.80 Hz, 1H), 7.06 (d, J = 2.20 Hz, 1H), 6.93 (s, 1H), 6.81 (dd, J = 8.68, 2.32 Hz, 1H), 4.41 (t, J = 3.90 Hz, 1H), 4.36 (d, J = 4.60 Hz, 2H), 3.62 (s, 3H). LC−MS method 2: R_t = 0.68 min. MS-ESI (m/z): calcd for $C_{12}H_{11}ClN_4O$ [M + H]$^+$, 263.1; found, 263.2.

*N-((5-Chloro-1-methyl-1H-imidazol-2-yl)methyl)-1H-benzo[d]-imidazol-5-amine (**14**).* Compound **14** was prepared from 1*H*-benzo[*d*]imidazol-5-amine **73g** (23 mg, 0.17 mmol) according to the general procedure. The residue was purified by preparative HPLC (method B). White solid (38 mg, 85% yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.05−11.87 (m, 1H), 7.99−7.79 (m, 1H), 7.35−7.18 (m, 1H), 6.91 (s, 1H), 6.95−6.74 (m, 1H), 6.72−6.59 (m, 1H), 6.05−5.72 (m, 1H), 4.30 (d, J = 5.26 Hz, 2H), 3.58 (s, 3H). LC−MS method 2: R_t = 0.54 min. MS-ESI (m/z): calcd for $C_{12}H_{12}ClN_5$ [M + H]$^+$, 262.1; found, 262.2.

*N-((5-Chloro-1-methyl-1H-imidazol-2-yl)methyl)-1-methyl-1H-benzo[d][1,2,3]triazol-5-amine (**15**).* Compound **15** was prepared from 1-methyl-1*H*-benzo[*d*][1,2,3]triazol-5-amine **73h** (47 mg, 0.32 mmol) according to the general procedure. The residue was purified by preparative HPLC (method B). White solid (54.6 mg, 62% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.34 (d, J = 8.88 Hz, 1H), 7.13 (d, J = 1.43 Hz, 1H), 7.01 (dd, J = 8.82, 1.92 Hz, 1H), 6.95 (s, 1H), 4.57 (br t, J = 4.70, 4.70 Hz, 1H), 4.38 (d, J = 4.49 Hz, 2H), 4.25 (s, 3H), 3.63 (s, 3H). LC−MS method 2: R_t = 0.62 min. MS-ESI (m/z): calcd for $C_{12}H_{13}ClN_6$ [M + H]$^+$, 277.1; found, 277.2.

*Synthesis of 5-Chloro-1-trityl-1H-imidazole-2-carbaldehyde (**75**).* A solution of 5-chloro-1*H*-imidazole **74** (2.0 g, 19.5 mmol), triphenylmethyl chloride (5.44 g, 19.5 mmol), and Et_3N (2.7 mL, 19.5 mmol) in DCM (150 mL) was stirred at rt for 20 h. The mixture was washed with brine, dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was dissolved in THF (100 mL), cooled to −78 °C, and *n*-BuLi (2.5 M in hexanes, 2.55 mL, 6.38 mmol) was added dropwise. After 1 h at −78 °C, DMF (1.35 mL, 17.4 mmol) was added dropwise and the reaction mixture was stirred at the same temperature for 4 h. A saturated aqueous NH_4Cl solution was added and the mixture was allowed to reach rt and extracted with DCM. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and evaporated to dryness to afford 5-chloro-1-trityl-1*H*-imidazole-2-carbaldehyde **75** (2.0 g, 57% yield over two steps). White solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.22 (s, 1H), 7.32 (m, 9H), 7.12 (m, 6H), 7.10 (s, 1H).

*N-((5-Chloro-1H-imidazol-2-yl)methyl)benzo[d]isothiazol-5-amine (**16**).* A mixture of benzo[*d*]isothiazol-5-amine **73a** (80 mg, 0.52 mmol) and **75** (200 mg, 0.52 mmol) in DCM (15 mL) and catalytic AcOH was stirred at rt for 30 min. $NaBH(OAc)_3$ (330 mg, 1.56 mmol) was added and the reaction mixture was stirred at rt for additional 16 h, then treated with a saturated aqueous $NaHCO_3$ solution and extracted with DCM. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by column chromatography on silica gel (0−50% EtOAc/cyclohexane) to provide *N*-((5-chloro-1-trityl-1*H*-imidazol-2-yl)methyl)benzo[*d*]-isothiazol-5-amine (190 mg, 72% yield). LC−MS method 1: R_t = 1.48 min. MS-ESI (m/z): calcd for $C_{30}H_{23}ClN_4S$ [M + H]$^+$, 507.1; found, 507.1. A solution of *N*-((5-chloro-1-trityl-1*H*-imidazol-2-yl)methyl)-

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benzo[*d*]isothiazol-5-amine (80 mg, 0.16 mmol) in DCM (3 mL) and TFA (1.5 mL) was stirred at rt for 2 h and then concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (20−100% EtOAc/cyclohexane). White solid (17.7 mg, 42% yield over two steps). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.73 (s, 1H), 7.77 (d, J = 8.55 Hz, 1H), 7.12 (d, J = 1.97 Hz, 1H), 7.02−6.95 (m, 1H), 6.88 (s, 1H), 4.56 (s, 2H). LC−MS method 1: R_t = 0.66 min. MS-ESI (m/z): calcd for $C_{11}H_9ClN_4S$ $[M + H]^+$, 265.0; found, 265.0.

*General Procedure for the Synthesis of Compounds **17**, **19**, **20**, **22**, and **24**.* A mixture of benzo[*d*]isothiazol-5-amine **73a** (1 equiv) and the appropriate aldehyde (1−1.5 equiv) in DCM (0.1 M) and catalytic AcOH was stirred at rt for 30 min. $NaBH(OAc)_3$ (1.5−3 equiv) was added and the reaction mixture was stirred at rt for additional 16 h, then treated with a saturated aqueous $NaHCO_3$ solution and extracted with DCM. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was purified as described for each compound.

*N-((4,5-Dichloro-1-methyl-1H-imidazol-2-yl)methyl)benzo[d]-isothiazol-5-amine (**17**).* Compound **17** was prepared using **73a** (100 mg, 0.68 mmol) and 4,5-dichloro-1-methyl-1*H*-imidazole-2-carbaldehyde (170 mg, 0.95 mmol) according to the general procedure. The crude material was purified by reverse-phase column chromatography (5−70% MeCN/0.1% v/v HCOOH in water). Pale yellow solid (60 mg, 28% yield). ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.86 (s, 1H), 7.88 (d, J = 8.78 Hz, 1H), 7.27 (s, 1H), 7.10 (d, J = 8.78 Hz, 1H), 6.51 (t, J = 5.35 Hz, 1H), 4.38 (d, J = 5.76 Hz, 2H), 3.62 (s, 3H). LC−MS method 1: R_t = 0.99 min. MS-ESI (m/z): calcd for $C_{12}H_{10}Cl_2N_4S$ $[M + H]^+$, 313.0; found, 313.0.

*N-((5-Chloro-1-methyl-1H-pyrrol-2-yl)methyl)benzo[d]-isothiazol-5-amine (**19**).* Compound **19** was prepared using **73a** (100 mg, 0.68 mmol) and 5-chloro-1-methyl-1*H*-pyrrole-2-carbaldehyde (100 mg, 0.68 mmol) according to the general procedure. The crude material was purified by column chromatography on silica gel (50% EtOAc/cyclohexane). Pale yellow solid (92 mg, 47% yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.85 (s, 1H), 7.85 (d, J = 8.77 Hz, 1H), 7.24 (d, J = 1.97 Hz, 1H), 7.10 (dd, J = 8.77, 2.19 Hz, 1H), 6.28 (t, J = 5.26 Hz, 1H), 6.11 (d, J = 3.51 Hz, 1H), 6.00 (d, J = 3.73 Hz, 1H), 4.24 (d, J = 5.26 Hz, 2H), 3.53 (s, 3H). LC−MS method 2: R_t = 1.00 min. MS-ESI (m/z): calcd for $C_{13}H_{12}ClN_3S$ $[M + H]^+$, 278.0; found, 278.1.

*N-((5-Bromo-1-methyl-1H-imidazol-2-yl)methyl)benzo[d]-isothiazol-5-amine (**20**).* Compound **20** was prepared using **73a** (150 mg, 0.99 mmol) and 5-bromo-1-methyl-1*H*-imidazole-2-carbaldehyde (283 mg, 1.5 mmol) according to the general procedure. The crude material was purified by column chromatography on silica gel (5−100% EtOAc/cyclohexane). White solid (238 mg, 74% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.77 (s, 1H), 7.75 (d, J = 8.77 Hz, 1H), 7.27−7.22 (m, 1H), 7.13−6.95 (m, 2H), 4.60 (br s, 1H), 4.43 (s, 2H), 3.66 (s, 3H). LC−MS method 1: R_t = 0.54 min. MS-ESI (m/z): calcd for $C_{12}H_{11}BrN_4S$ $[M + H]^+$, 323.0; found, 323.0.

*N-((5-Methyl-1,3,4-thiadiazol-2-yl)methyl)benzo[d]isothiazol-5-amine (**22**).* Compound **22** was prepared using **73a** (50 mg, 0.33 mmol) and 5-methyl-1,3,4-thiadiazole-2-carbaldehyde (63 mg, 0.5 mmol) according to the general procedure. The crude material was purified by column chromatography on silica gel (5−100% EtOAc/cyclohexane). Pale yellow solid (38 mg, 44% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.72 (s, 1H), 7.75 (d, J = 8.78 Hz, 1H), 7.19 (s, 1H), 6.99 (dd, J = 8.71, 1.58 Hz, 1H), 4.83 (s, 2H), 4.92−4.66 (m, 1H), 2.74 (s, 3H). LC−MS method 1: R_t = 0.72 min. MS-ESI (m/z): calcd for $C_{11}H_{10}N_4S_2$ $[M + H]^+$, 263.0; found, 263.0.

*5-((Benzo[d]isothiazol-5-ylamino)methyl)thiophene-2-carbonitrile (**24**).* Compound **24** was prepared using **73a** (50 mg, 0.33 mmol) and 5-formylthiophene-2-carbonitrile (56 mg, 0.41 mmol) according to the general procedure. The crude material was purified by reverse-phase column chromatography (5−70% MeCN/0.1% v/v HCOOH in water). Pale yellow solid (38 mg, 41% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.72 (s, 1H), 7.76 (d, J = 8.55 Hz, 1H), 7.54 (d, J = 3.73 Hz, 1H), 7.13 (d, J = 1.97 Hz, 1H), 7.07 (d, J = 3.73 Hz, 1H), 6.97 (dd, J = 8.66, 2.30 Hz, 1H), 4.67 (d, J = 3.51 Hz, 2H), 4.47 (br s, 1H). LC−MS method 1: R_t = 1.01 min. MS-ESI (m/z): calcd for $C_{13}H_9N_3S_2$ $[M + H]^+$, 272.0; found, 272.0.

*General Procedure for the Synthesis of Compounds **18** and **25**−**33**.* A solution of benzo[*d*]isothiazol-5-amine **73a** (1 equiv) and the appropriate aldehyde (1.2−1.5 equiv) in MeOH (0.1 M) was heated at 60 °C for 16 h. After cooling to 0 °C, $NaBH_4$ (3 equiv) was added and the reaction mixture was allowed to warm to rt and stirred for 2 h. The mixture was treated with a saturated aqueous $NaHCO_3$ solution and extracted with DCM. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by reverse-phase HPLC or as stated to provide the target compounds.

*N-((1-Methyl-1H-imidazol-2-yl)methyl)benzo[d]isothiazol-5-amine (**18**).* Compound **18** was prepared from **73a** (50 mg, 0.33 mmol) and 1-methyl-1*H*-imidazole-2-carbaldehyde (55 mg, 0.5 mmol) according to the general procedure. The residue was purified by preparative HPLC (method B). White solid (50 mg, 62% yield). ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.84 (s, 1H), 7.85 (d, J = 8.80 Hz, 1H), 7.33 (d, J = 1.71 Hz, 1H), 7.14 (dd, J = 8.80, 1.96 Hz, 1H), 7.09 (s, 1H), 6.81 (d, J = 0.98 Hz, 1H), 6.41 (t, J = 5.01 Hz, 1H), 4.34 (d, J = 5.38 Hz, 2H), 3.66 (s, 3H). LC−MS method 2: R_t = 0.63 min. MS-ESI (m/z): calcd for $C_{12}H_{12}N_4S$ $[M + H]^+$, 245.1; found, 245.1.

*N-((5-Chlorothiazol-2-yl)methyl)benzo[d]isothiazol-5-amine (**25**).* Compound **25** was prepared from **73a** (50 mg, 0.33 mmol) and 5-chlorothiazole-2-carbaldehyde (73 mg, 0.5 mmol) according to the general procedure. The residue was purified by preparative HPLC (method B). White solid (15 mg, 16% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.73 (s, 1H), 7.76 (d, J = 8.51 Hz, 1H), 7.56 (s, 1H), 7.17 (d, J = 1.92 Hz, 1H), 7.00 (dd, J = 8.78, 2.20 Hz, 1H), 4.72−4.65 (m, 3H). LC−MS method 1: R_t = 1.05 min. MS-ESI (m/z): calcd for $C_{11}H_8ClN_3S_2$ $[M + H]^+$, 282.0; found, 282.1.

*N-((5-Chlorothiophen-2-yl)methyl)benzo[d]isothiazol-5-amine (**26**).* Compound **26** was prepared from **73a** (40 mg, 0.27 mmol) and 5-chlorothiophene-2-carbaldehyde (59 mg, 0.4 mmol) according to the general procedure. The residue was purified by preparative HPLC (method B). White solid (62 mg, 81% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.73 (s, 1H), 7.74 (d, J = 8.78 Hz, 1H), 7.18 (d, J = 1.92 Hz, 1H), 6.96 (dd, J = 8.78, 2.20 Hz, 1H), 6.87−6.81 (m, 1H), 6.79 (d, J = 3.84 Hz, 1H), 4.51 (d, J = 5.49 Hz, 2H), 4.30 (br t, J = 4.70, 4.70 Hz, 1H). LC−MS method 2: R_t = 1.06 min. MS-ESI (m/z): calcd for $C_{12}H_9ClN_2S_2$ $[M + H]^+$, 281.0; found, 281.2.

*N-((5-(Trifluoromethyl)thiazol-2-yl)methyl)benzo[d]isothiazol-5-amine (**27**).* Compound **27** was prepared from **73a** (40 mg, 0.27 mmol) and 5-(trifluoromethyl)thiazole-2-carbaldehyde (69 mg, 0.32 mmol) according to the general procedure. The residue was purified by preparative HPLC (method A). White solid (17 mg, 20% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.72 (s, 1H), 8.16−7.97 (m, 1H), 7.77 (d, J = 8.78 Hz, 1H), 7.14 (d, J = 1.92 Hz, 1H), 7.00 (dd, J = 8.78, 2.20 Hz, 1H), 4.77 (s, 3H). LC−MS method 1: R_t = 1.09 min. MS-ESI (m/z): calcd for $C_{12}H_8F_3N_3S_2$ $[M + H]^+$, 316.0; found, 316.0.

*N-((5-Methylthiazol-2-yl)methyl)benzo[d]isothiazol-5-amine (**28**).* Compound **28** was prepared from **73a** (40 mg, 0.27 mmol) and 5-methylthiazole-2-carbaldehyde (51 mg, 0.4 mmol) according to the general procedure. The residue was purified by preparative HPLC (method B). White solid (14 mg, 20% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.72 (s, 1H), 7.74 (d, J = 9.00 Hz, 1H), 7.40 (d, J = 1.17 Hz, 1H), 7.18 (d, J = 1.96 Hz, 1H), 7.00 (dd, J = 8.80, 2.15 Hz, 1H), 4.80−4.61 (m, 3H), 2.43 (d, J = 0.78 Hz, 3H). LC−MS method 1: R_t = 0.91 min. MS-ESI (m/z): calcd for $C_{12}H_{11}N_3S_2$ $[M + H]^+$, 262.0; found, 262.1.

*N-((5-Isopropylthiazol-2-yl)methyl)benzo[d]isothiazol-5-amine (**29**).* Compound **29** was prepared from **73a** (50 mg, 0.33 mmol) and 5-isopropylthiazole-2-carbaldehyde (51 mg, 0.33 mmol) according to the general procedure. The residue was purified by preparative HPLC (method B). White solid (50 mg, 52% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.73 (s, 1H), 7.74 (d, J = 8.55 Hz, 1H), 7.43 (s, 1H), 7.20 (d, J = 1.53 Hz, 1H), 7.01 (dd, J = 8.77, 2.19 Hz, 1H), 4.82−4.58 (m, 3H), 3.17 (spt, J = 6.80 Hz, 1H), 1.32 (d, J = 6.80 Hz, 6H). LC−MS method 2: R_t = 0.97 min. MS-ESI (m/z): calcd for $C_{14}H_{15}N_3S_2$ $[M + H]^+$, 290.1; found, 290.1.

*N-((4-Methylthiazol-2-yl)methyl)benzo[d]isothiazol-5-amine (**30**).* Compound **30** was prepared from **73a** (40 mg, 0.27 mmol) and 4-methylthiazole-2-carbaldehyde (51 mg, 0.4 mmol) according to the

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general procedure. The residue was purified by preparative HPLC (method B). White solid (18 mg, 25% yield). ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.83 (d, J = 0.82 Hz, 1H), 7.88 (d, J = 8.78 Hz, 1H), 7.13 (d, J = 1.92 Hz, 1H), 7.12−7.07 (m, 2H), 6.91 (t, J = 6.04 Hz, 1H), 4.61 (d, J = 6.04 Hz, 2H), 2.35 (d, J = 1.10 Hz, 3H). LC−MS method 2: R_t = 0.81 min. MS-ESI (m/z): calcd for $C_{12}H_{11}N_3S_2$ [M + H]$^+$, 262.0; found, 262.1.

*N-((4,5-Dimethylthiazol-2-yl)methyl)benzo[d]isothiazol-5-amine (**31**).* Compound **31** was prepared from **73a** (50 mg, 0.33 mmol) and 4,5-dimethylthiazole-2-carbaldehyde (61 mg, 0.43 mmol) according to the general procedure. The crude material was purified by column chromatography on silica gel (5−100% EtOAc/cyclohexane). White solid (30 mg, 33% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.72 (s, 1H), 7.73 (d, J = 8.61 Hz, 1H), 7.18 (d, J = 1.96 Hz, 1H), 6.99 (dd, J = 8.71, 2.25 Hz, 1H), 4.95−4.33 (m, 3H), 2.34 (s, 3H), 2.31 (s, 3H). LC−MS method 1: R_t = 0.92 min. MS-ESI (m/z): calcd for $C_{13}H_{13}N_3S_2$ [M + H]$^+$, 276.1; found, 276.0.

*N-(Thiazol-2-ylmethyl)benzo[d]isothiazol-5-amine (**32**).* Compound **32** was prepared from **73a** (40 mg, 0.27 mmol) and thiazole-2-carbaldehyde (45 mg, 0.4 mmol) according to the general procedure. The residue was purified by preparative HPLC (method B). White solid (61 mg, 91% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.72 (d, J = 0.82 Hz, 1H), 7.79 (d, J = 3.29 Hz, 1H), 7.75 (d, J = 8.78 Hz, 1H), 7.30 (d, J = 3.29 Hz, 1H), 7.18 (d, J = 1.92 Hz, 1H), 7.02 (dd, J = 8.65, 2.33 Hz, 1H), 4.85−4.66 (m, 3H). LC−MS method 2: R_t = 0.74 min. MS-ESI (m/z): calcd for $C_{11}H_9N_3S_2$ [M + H]$^+$, 248.0; found, 248.1.

*N-(Benzo[d]thiazol-2-ylmethyl)benzo[d]isothiazol-5-amine (**33**).* Compound **33** was prepared from **73a** (40 mg, 0.27 mmol) and benzo[d]thiazole-2-carbaldehyde (65 mg, 0.4 mmol) according to the general procedure. The residue was purified by preparative HPLC (method B). White solid (16.6 mg, 20% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.70 (s, 1H), 8.04 (d, J = 8.22 Hz, 1H), 7.89−7.81 (m, 1H), 7.76 (d, J = 8.61 Hz, 1H), 7.51 (td, J = 7.83, 1.17 Hz, 1H), 7.43−7.34 (m, 1H), 7.21 (d, J = 1.96 Hz, 1H), 7.05 (dd, J = 8.80, 2.15 Hz, 1H), 4.99−4.77 (m, 3H). LC−MS method 1: R_t = 1.06 min. MS-ESI (m/z): calcd for $C_{15}H_{11}N_3S_2$ [M + H]$^+$, 298.0; found, 298.1.

*General Procedure for the Synthesis of Compounds **21** and **23**.* To a solution of benzo[d]isothiazol-5-amine **73a** (1 equiv) and Et_3N (2 equiv) in EtOH (0.15 M), the appropriate chloromethyl-heterocycle (0.8 equiv) was added and the reaction mixture was heated at reflux for 6 h. The mixture was diluted with water and extracted with EtOAc (2×). The combined extracts were washed with brine, dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by reverse-phase column chromatography (5−80% MeCN/0.1% v/v HCOOH in water) and then by reverse-phase HPLC (method B) to provide the target compounds.

*N-((5-Chloro-1,2,3-thiadiazol-4-yl)methyl)benzo[d]isothiazol-5-amine (**21**).* Compound **21** was prepared from **73a** (100 mg, 0.66 mmol) and 5-chloro-4-(chloromethyl)-1,2,3-thiadiazole (94 mg, 0.55 mmol) according to the general procedure (23 mg, 15% yield). Pale yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.76 (s, 1H), 7.75 (d, J = 8.55 Hz, 1H), 7.32 (d, J = 2.19 Hz, 1H), 7.04 (dd, J = 8.77, 2.19 Hz, 1H), 4.85−4.67 (m, 3H). LC−MS method 1: R_t = 0.96 min. MS-ESI (m/z): calcd for $C_{10}H_7ClN_4S_2$ [M + H]$^+$, 283.0; found, 283.0.

*N-((1-Methyl-5-(trifluoromethyl)-1H-pyrazol-3-yl)methyl)benzo[d]isothiazol-5-amine (**23**).* Compound **23** was prepared from **73a** (90 mg, 0.6 mmol) and 3-(chloromethyl)-1-methyl-5-(trifluoromethyl)-1H-pyrazole (100 mg, 0.5 mmol) according to the general procedure (26 mg, 17% yield). Pale yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.84 (s, 1H), 7.85 (d, J = 8.77 Hz, 1H), 7.19 (d, J = 1.97 Hz, 1H), 7.09 (dd, J = 8.77, 2.19 Hz, 1H), 6.81 (s, 1H), 6.43 (t, J = 5.92 Hz, 1H), 4.28 (d, J = 5.92 Hz, 2H), 3.93 (s, 3H). LC−MS method 2: R_t = 1.03 min. MS-ESI (m/z): calcd for $C_{13}H_{11}F_3N_4S$ [M + H]$^+$, 313.1; found, 313.2.

*General Procedure for the Synthesis of Compounds **34** and **35**.* NaH (60% w/w in mineral oil, 2 equiv) was added to a solution of **2** or **25** (1 equiv) in dry DMF (0.1 M) and cooled to 0 °C. The reaction mixture was stirred at rt for 15 min, then MeI (2 equiv) was added and stirring was continued for 1 or 16 h, respectively. The mixture was treated with a saturated aqueous NH_4Cl solution and extracted with EtOAc (3×). The combined extracts were washed with brine, dried over Na_2SO_4, filtered, and evaporated to dryness. The crude material was purified as described for each compound.

*N-((5-Chloro-1-methyl-1H-imidazol-2-yl)methyl)-N-methylbenzo[d]isothiazol-5-amine (**34**).* Compound **34** was prepared using compound **2** (24 mg, 0.09 mmol) according to the general procedure. The crude material was purified by column chromatography on silica gel (10−100% EtOAc/cyclohexane). White solid (15 mg, 55% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.79 (s, 1H), 7.82 (d, J = 8.78 Hz, 1H), 7.45 (d, J = 2.26 Hz, 1H), 7.30 (dd, J = 8.91, 2.38 Hz, 1H), 6.93 (s, 1H), 4.55 (s, 2H), 3.54 (s, 3H), 2.99 (s, 3H). LC−MS method 1: R_t = 0.58 min. MS-ESI (m/z): calcd for $C_{13}H_{13}ClN_4S$ [M + H]$^+$, 293.2; found, 293.2.

*N-((5-Chlorothiazol-2-yl)methyl)-N-methylbenzo[d]isothiazol-5-amine (**35**).* Compound **35** was prepared using compound **25** (29 mg, 0.1 mmol) according to the general procedure. The crude material was purified by reverse-phase HPLC (method B). White solid (5.8 mg, 19% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.78 (s, 1H), 7.81 (d, J = 8.78 Hz, 1H), 7.55 (s, 1H), 7.33 (d, J = 2.47 Hz, 1H), 7.17 (dd, J = 9.06, 2.47 Hz, 1H), 4.78 (s, 2H), 3.18 (s, 3H). LC−MS method 2: R_t = 1.03 min. MS-ESI (m/z): calcd for $C_{12}H_{10}ClN_3S_2$ [M + H]$^+$, 296.0; found, 296.1.

*General Procedure for the Synthesis of Compounds **36** and **38**−**40**.* A mixture of compound **2** (50 mg, 0.18 mmol) and the appropriate aldehyde (0.36 mmol) in DCM (4 mL) and catalytic AcOH was stirred at rt for 30 min. $NaBH(OAc)_3$ (114 mg, 0.54 mmol) was added and the reaction mixture was stirred at rt for 16 h, then treated with a saturated aqueous $NaHCO_3$ solution and extracted with DCM (2×). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by column chromatography on silica gel (0−50% EtOAc/cyclohexane and then 0−30% MeCN/DCM) affording the target compounds.

*N-((5-Chloro-1-methyl-1H-imidazol-2-yl)methyl)-N-(cyclopropylmethyl)benzo[d]isothiazol-5-amine (**36**).* Compound **36** was prepared using cyclopropanecarbaldehyde (25 mg, 0.36 mmol) according to the general procedure (25 mg, 42% yield). White solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.79 (s, 1H), 7.81 (d, J = 8.99 Hz, 1H), 7.57 (d, J = 1.97 Hz, 1H), 7.33 (dd, J = 8.88, 2.30 Hz, 1H), 6.90 (s, 1H), 4.62 (s, 2H), 3.55 (s, 3H), 3.23 (d, J = 6.58 Hz, 2H), 1.15−0.94 (m, 1H), 0.58−0.00 (m, 4H). LC−MS method 1: R_t = 0.73 min. MS-ESI (m/z): calcd for $C_{16}H_{17}ClN_4S$ [M + H]$^+$, 333.1; found, 333.0.

*N-((5-Chloro-1-methyl-1H-imidazol-2-yl)methyl)-N-(cyclohexylmethyl)benzo[d]isothiazol-5-amine (**38**).* Compound **38** was prepared using cyclohexanecarbaldehyde (40 mg, 0.36 mmol) according to the general procedure (39 mg, 58% yield). White solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.77 (s, 1H), 7.78 (d, J = 8.77 Hz, 1H), 7.43 (d, J = 2.19 Hz, 1H), 7.23 (dd, J = 8.88, 2.30 Hz, 1H), 6.90 (s, 1H), 4.59 (s, 2H), 3.49 (s, 3H), 3.23 (d, J = 6.80 Hz, 2H), 1.81−1.62 (m, 6H), 1.33−1.02 (m, 3H), 0.99−0.82 (m, 2H). LC−MS method 1: R_t = 1.07 min. MS-ESI (m/z): calcd for $C_{19}H_{23}ClN_4S$ [M + H]$^+$, 375.1; found, 375.1.

*N-Benzyl-N-((5-chloro-1-methyl-1H-imidazol-2-yl)methyl)benzo[d]isothiazol-5-amine (**39**).* Compound **39** was prepared using benzaldehyde (38 mg, 0.36 mmol) according to the general procedure (29 mg, 44% yield). White solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.75 (s, 1H), 7.80 (d, J = 8.99 Hz, 1H), 7.49 (d, J = 2.19 Hz, 1H), 7.39−7.13 (m, 6H), 6.90 (s, 1H), 4.61 (s, 2H), 4.56 (s, 2H), 3.43 (s, 3H). LC−MS method 1: R_t = 0.84 min. MS-ESI (m/z): calcd for $C_{19}H_{17}ClN_4S$ [M + H]$^+$, 369.1; found, 369.0.

*N-((5-Chloro-1-methyl-1H-imidazol-2-yl)methyl)-N-ethylbenzo[d]isothiazol-5-amine (**40**).* Compound **40** was prepared using acetaldehyde (16 mg, 0.36 mmol) according to the general procedure (32 mg, 59% yield). White solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.78 (s, 1H), 7.81 (d, J = 8.99 Hz, 1H), 7.49 (d, J = 2.19 Hz, 1H), 7.32−7.24 (m, 1H), 6.92 (s, 1H), 4.51 (s, 2H), 3.53 (s, 3H), 3.44 (q, J = 7.02 Hz, 2H), 1.11 (t, J = 7.13 Hz, 3H). LC−MS method 1: R_t = 0.66 min. MS-ESI (m/z): calcd for $C_{14}H_{15}ClN_4S$ [M + H]$^+$, 307.1; found, 307.0.

*Synthesis of N-((5-Chloro-1-methyl-1H-imidazol-2-yl)methyl)-N-isopropylbenzo[d]isothiazol-5-amine (**37**).* A mixture of compound **2** (50 mg, 0.18 mmol) and acetone (26 μL, 0.36 mmol) in DCM (4 mL) and catalytic AcOH was stirred at rt for 30 min. $NaBH(OAc)_3$ (114 mg, 0.54 mmol) was added and the reaction mixture was stirred at rt for 16

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h. More acetone (52 μL, 0.72 mmol) was added and the reaction mixture was heated to reflux for additional 6 h. After cooling to rt, the mixture was treated with a saturated aqueous $NaHCO_3$ solution and extracted with DCM (2×). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by reverse-phase HPLC (method B). White solid (14 mg, 24% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.78 (s, 1H), 7.78 (d, J = 8.80 Hz, 1H), 7.64 (d, J = 2.20 Hz, 1H), 7.30 (dd, J = 8.80, 2.20 Hz, 1H), 6.83 (s, 1H), 4.43 (s, 2H), 3.91 (quin, J = 6.66 Hz, 1H), 3.58 (s, 3H), 1.22 (d, J = 6.60 Hz, 6H). LC−MS method 2: R_t = 0.95 min. MS-ESI (m/z): calcd for $C_{15}H_{17}ClN_4S$ $[M + H]^+$, 321.1; found, 321.1 $[M + H]^+$.

*Synthesis of N-(Benzo[d]isothiazol-5-yl)-N-((5-chloro-1-methyl-1H-imidazol-2-yl)methyl)acetamide (**41**).* Acetyl chloride (13 μL, 0.18 mmol) was added to a solution of compound **2** (50 mg, 0.18 mmol) and Et_3N (26 μL, 0.19 mmol) in DCM (5 mL) and cooled to 0 °C. The reaction mixture was allowed to reach rt and stirred for 4 h, then treated with a saturated aqueous NH_4Cl solution and extracted with DCM (2×). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by column chromatography on silica gel (50−100% EtOAc/cyclohexane). White solid (42.6 mg, 74% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.91 (d, J = 1.00 Hz, 1H), 7.99 (d, J = 8.53 Hz, 1H), 7.87 (d, J = 1.51 Hz, 1H), 7.30 (dd, J = 8.53, 2.01 Hz, 1H), 6.81 (s, 1H), 5.01 (s, 2H), 3.72 (s, 3H), 1.91 (s, 3H). LC−MS method 1: R_t = 0.55 min. MS-ESI (m/z): calcd for $C_{14}H_{13}ClN_4OS$ $[M + H]^+$, 321.1; found, 321.0.

*Synthesis of N-(Benzo[d]isothiazol-5-yl)-5-chlorothiazole-2-carboxamide (**42**).* Oxalyl chloride (10 μL, 0.22 mmol) was added to a solution of 5-chlorothiazole-2-carboxylic acid (25 mg, 0.15 mmol) in dry DCM (0.7 mL) and catalytic DMF cooled to 0 °C. The reaction mixture was allowed to reach rt and stirred at rt for 2 h, then the solvent was evaporated under vacuum. The residue was suspended in dry DCM (0.5 mL) and added dropwise to a solution of benzo[d]isothiazol-5-amine **73a** (27 mg, 0.18 mmol) and Et_3N (42 μL, 0.3 mmol) in DCM (0.5 mL) and cooled to 0 °C. The reaction mixture was allowed to reach rt and stirred at rt for 2 h. It was diluted with DCM and washed with brine. The organic layer was dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by reverse-phase column chromatography (5−80% MeCN/0.1% v/v HCOOH in water). Yellow solid (25 mg, 53% yield over two steps). ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.16 (s, 1H), 9.14 (s, 1H), 8.77 (d, J = 1.97 Hz, 1H), 8.25−8.17 (m, 2H), 7.99 (dd, J = 8.77, 1.75 Hz, 1H). LC−MS method 1: R_t = 1.09 min. MS-ESI (m/z): calcd for $C_{11}H_6ClN_3OS_2$ $[M + H]^+$, 296.0; found, 296.0.

*Synthesis of 1-(5-Chlorothiazol-2-yl)ethan-1-one (**77**).* MeMgBr (3 M solution in Et_2O, 0.66 mL, 2.0 mmol) was added to a solution of 5-chlorothiazole-2-carbaldehyde **76** (200 mg, 1.35 mmol) in dry THF (6 mL) and cooled to 0 °C. The reaction mixture was allowed to reach rt and stirred for 1 h, then treated with a saturated aqueous NH_4Cl solution and extracted with EtOAc. The organic layer was dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by column chromatography on silica gel (0−50% EtOAc/cyclohexane) affording 1-(5-chlorothiazol-2-yl)ethan-1-ol (220 mg, quantitative yield). A mixture of 1-(5-chlorothiazol-2-yl)ethan-1-ol (220 mg, 1.35 mmol) and Dess−Martin periodinane (859 mg, 2.03 mmol) in DCM (5 mL) was stirred at rt for 1 h. The solvent was evaporated and the residue was purified by column chromatography on silica gel (0−50% EtOAc/cyclohexane). White solid (120 mg, 48% yield over two steps). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.29 (s, 1H), 2.68 (s, 3H).

*General Procedure for the Synthesis of Compounds **43** and **44**.* A mixture of benzo[d]isothiazol-5-amine **73a** (1 equiv) and intermediate **76** or **77** (1 equiv) in toluene (0.15 M) was heated at reflux for 20 h. After cooling to rt, MeMgBr (3 M solution in Et_2O, 1.2 equiv) was added and the reaction mixture was stirred at rt for 3 h. The mixture was quenched with a saturated aqueous NH_4Cl solution and extracted with EtOAc. The organic layer was dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was purified as described for each compound.

*N-(1-(5-Chlorothiazol-2-yl)ethyl)benzo[d]isothiazol-5-amine (**43**).* Compound **43** was prepared using **73a** (100 mg, 0.67 mmol) and **76** (99 mg, 0.67 mmol) according to the general procedure. The crude material was purified by reverse-phase HPLC (method B). Yellow solid, racemic mixture (70 mg, 35% yield). ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.83 (s, 1H), 7.92−7.89 (m, 1H), 7.76 (s, 1H), 7.15−7.08 (m, 2H), 6.82 (d, J = 5.87 Hz, 1H), 4.81 (quin, J = 6.48 Hz, 1H), 1.58 (d, J = 6.85 Hz, 3H). LC−MS method 2: R_t = 0.98 min. MS-ESI (m/z): calcd for $C_{12}H_{10}ClN_3S_2$ $[M + H]^+$, 296.0; found, 296.1.

*N-(2-(5-Chlorothiazol-2-yl)propan-2-yl)benzo[d]isothiazol-5-amine (**44**).* Compound **44** was prepared using **73a** (56 mg, 0.37 mmol) and **77** (60 mg, 0.67 mmol) according to the general procedure. The crude material was purified by column chromatography on silica gel (0−20% EtOAc/cyclohexane). White solid (9.6 mg, 8% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.65 (s, 1H), 7.70 (d, J = 8.77 Hz, 1H), 7.53 (s, 1H), 6.92 (d, J = 1.97 Hz, 1H), 6.85 (dd, J = 8.77, 2.19 Hz, 1H), 4.38 (s, 1H), 1.78 (s, 6H). LC−MS method 1: R_t = 1.17 min. MS-ESI (m/z): calcd for $C_{13}H_{12}ClN_3S_2$ $[M + H]^+$, 310.0; found, 310.0.

*Synthesis of 5-((5-Chlorothiazol-2-yl)methoxy)benzo[d]isothiazole (**45**).* A mixture of benzo[d]isothiazol-5-ol **78** (50 mg, 0.33 mmol), 5-chloro-2-(chloromethyl)-1,3-thiazole (37 μL, 0.33 mmol), and K_2CO_3 (91 mg, 0.66 mmol) in dry DMF (2 mL) was stirred at rt for 16 h. It was treated with a saturated aqueous NH_4Cl solution and extracted with EtOAc (2×). The combined organic layers were dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by column chromatography on silica gel (50% EtOAc/cyclohexane). Yellow solid (58 mg, 62% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.83 (s, 1H), 7.88 (d, J = 8.78 Hz, 1H), 7.61 (s, 1H), 7.55 (d, J = 2.47 Hz, 1H), 7.29 (dd, J = 8.78, 2.47 Hz, 1H), 5.38 (s, 2H). LC−MS method 1: R_t = 1.16 min. MS-ESI (m/z): calcd for $C_{11}H_7ClN_2OS_2$ $[M + H]^+$, 283.0; found, 283.1.

*General Procedure for the Synthesis of Intermediates **80a,b,d,h,j−l** and **86a−c**.* A mixture of compound **79a,b,d,h,j−l** or **85a−c** (1 equiv), sulfur (1 equiv), and NH_4OH aqueous solution (0.7 M) in DMF (0.7 M) was heated at 90 °C for 16 h. After cooling to rt, the mixture was treated with a saturated aqueous NH_4Cl solution and extracted with EtOAc (2×). The combined organic layers were dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by column chromatography as described for each intermediate.

*6-Methyl-5-nitrobenzo[d]isothiazole (**80a**).* Compound **80a** was prepared using 2-fluoro-4-methyl-5-nitrobenzaldehyde **79a** (1 g, 5.46 mmol) according to the general procedure. The residue was purified by column chromatography on silica gel (0−100% EtOAc/cyclohexane) (478 mg, 45% yield). LC−MS method 1: R_t = 0.97 min. MS-ESI (m/z): calcd for $C_8H_6N_2O_2S$ $[M + H]^+$, 195.0; found, 195.1.

*6-Chloro-5-nitrobenzo[d]isothiazole (**80b**).* Compound **80b** was prepared using 2-chloro-4-fluoro-5-nitrobenzaldehyde **79b** (1 g, 4.9 mmol) according to the general procedure. The residue was diluted in MeCN (40 mL) and added to a solution of isopentyl nitrite (1.38 mL, 10.25 mmol) and $CuCl_2$ (1 g, 7.68 mmol) in MeCN (46 mL). The resulting mixture was stirred at 65 °C for 16 h. After cooling to rt, the mixture was treated with 6 M HCl (aq) and extracted with EtOAc. The organic layers were dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by column chromatography on silica gel (10% EtOAc/cyclohexane) (529 mg, 48% yield). LC−MS method 1: R_t = 0.99 min. MS-ESI (m/z): calcd for $C_7H_3ClN_2O_2S$ $[M + H]^+$, 215.0; found, 215.0.

*5-Bromoisothiazolo[5,4-c]pyridine (**80d**).* Compound **80d** was prepared using 2-bromo-5-fluoropyridine-4-carbaldehyde **79d** (1.3 g, 6.4 mmol) according to the general procedure. The residue was purified by column chromatography on silica gel (0−50% EtOAc/cyclohexane) (136 mg, 10% yield). LC−MS method 1: R_t = 0.81 min. MS-ESI (m/z): calcd for $C_6H_3BrN_2S$ $[M + H]^+$, 214.9; found, 215.0.

*5-Bromoisothiazolo[4,5-b]pyridine (**80h**).* Compound **80h** was prepared using 2-bromo-5-fluoropyridine-4-carbaldehyde **79h** (640 mg, 3.2 mmol) according to the general procedure. The residue was purified by column chromatography on silica gel (0−50% EtOAc/cyclohexane) (345 mg, 51% yield). LC−MS method 1: R_t = 0.82 min. MS-ESI (m/z): calcd for $C_6H_3BrN_2S$ $[M + H]^+$, 214.9; found, 214.9.

*5-Bromo-7-chlorobenzo[d]isothiazole (**80j**).* Compound **80j** was prepared using 5-bromo-3-chloro-2-fluorobenzaldehyde **79j** (400 mg, 1.68 mmol) according to the general procedure. The residue was

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purified by column chromatography on silica gel (10−80% EtOAc/cyclohexane) (136 mg, 32% yield). LC−MS method 1: R_t = 1.27 min. MS-ESI (m/z): calcd for $C_7H_3BrClNS$ $[M + H]^+$, 247.9; found, 247.9.

*5-Bromo-7-fluorobenzo[d]isothiazole (**80k**).* Compound **80k** was prepared using 5-bromo-2,3-difluorobenzaldehyde **79k** (200 mg, 0.9 mmol) according to the general procedure. The residue was purified by column chromatography on silica gel (10% EtOAc/cyclohexane) (145 mg, 69% yield). LC−MS method 1: R_t = 1.16 min. MS-ESI (m/z): calcd for C_7H_3BrFNS $[M + H]^+$, 231.9; found, 231.9.

*5-Bromoisothiazolo[5,4-b]pyridine (**80l**).* Compound **80l** was prepared using 5-bromo-2-fluoro-3-pyridinecarboxaldehyde **79l** (1.3 g, 6.4 mmol) according to the general procedure. The residue was purified by column chromatography on silica gel (0−50% EtOAc/cyclohexane) (365 mg, 27% yield). LC−MS method 1: R_t = 0.89 min. MS-ESI (m/z): calcd for $C_6H_3BrN_2S$ $[M + H]^+$, 214.9; found, 214.9.

*6-Bromobenzo[d]isothiazole (**86a**).* Compound **86a** was prepared using 4-bromo-2-fluorobenzaldehyde **85a** (10 g, 49.3 mmol) according to the general procedure. The residue was purified by column chromatography on silica gel (0−50% EtOAc/cyclohexane) (5.8 g, 55% yield). LC−MS method 1: R_t = 1.08 min. MS-ESI (m/z): calcd for C_7H_4BrNS $[M + H]^+$, 213.9; found, 213.9.

*4-Bromobenzo[d]isothiazole (**86b**).* Compound **86b** was prepared using 2-bromo-6-fluorobenzaldehyde **85b** (500 mg, 2.46 mmol) according to the general procedure. The residue was purified by column chromatography on silica gel (0−50% EtOAc/cyclohexane) (226 mg, 43% yield). LC−MS method 1: R_t = 1.09 min. MS-ESI (m/z): calcd for C_7H_4BrNS $[M + H]^+$, 213.9; found, 213.9.

*7-Nitrobenzo[d]isothiazole (**86c**).* Compound **86c** was prepared using 2-chloro-3-nitrobenzaldehyde (500 mg, 2.69 mmol) according to the general procedure. The residue was purified by column chromatography on silica gel (0−30% EtOAc/cyclohexane) (325 mg, 67% yield). LC−MS method 1: R_t = 0.89 min. MS-ESI (m/z): calcd for $C_7H_4N_2O_2S$ $[M + H]^+$, 181.0; found, 181.0.

*General Procedure for the Synthesis of Intermediates **80c,e,i** and **89a−c**.* Benzyl mercaptan (1 equiv) was added dropwise to a solution of potassium *tert*-butoxide (1 equiv) in THF (0.15 M). After 5 min at rt, a solution of substituted benzaldehydes **79c,e,i** or **88a−c** (1 equiv) in THF (0.5 M) was added. The reaction was stirred at rt for 2 h and consumption of starting material was followed by LC−MS. Upon completion, the reaction mixture was quenched with water, phases were separated, and the aqueous layer was extracted with EtOAc. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was dissolved in DCM (0.18 M) and SO_2Cl_2 (2 equiv) was added dropwise. After stirring 1 h at rt, the reaction was concentrated under reduced pressure. The residue was dissolved in THF (0.1 M), cooled to 0 °C, and treated with ammonia (7 M in MeOH, 1.5 equiv). After stirring 1 h at rt, water was added and the reaction mixture was extracted with EtOAc (2×). The combined organic layers were washed with water, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified as described for each compound.

*5-Bromo-6-fluorobenzo[d]isothiazole (**80c**).* Compound **80c** was prepared using 5-bromo-2,4-difluorobenzaldehyde **79c** (4 g, 18.4 mmol) according to the general procedure. The residue was purified by column chromatography on silica gel (20% EtOAc/cyclohexane) (620 mg, 15% yield over three steps). LC−MS method 1: R_t = 1.06 min. MS-ESI (m/z): calcd for C_7H_3BrFNS $[M + H]^+$, 231.9; found, 231.9.

*5-Bromo-4-methylbenzo[d]isothiazole (**80e**).* Compound **80e** was prepared using 3-bromo-6-chloro-2-methylbenzaldehyde **79e** (840 mg, 3.6 mmol) according to the general procedure. The residue was purified by column chromatography on silica gel (0−10% EtOAc/cyclohexane) (247 mg, 30% yield over three steps). LC−MS method 1: R_t = 1.16 min. MS-ESI (m/z): calcd for C_8H_6BrNS $[M + H]^+$, 227.9; found, 227.9.

*5-Bromo-7-methylbenzo[d]isothiazole (**80i**).* Compound **80i** was prepared using 5-bromo-2-fluoro-3-methylbenzaldehyde **79i** (672 mg, 3.11 mmol) according to the general procedure. The residue was purified by column chromatography on silica gel (0−10% EtOAc/cyclohexane) (413 mg, 58% yield over three steps). LC−MS method 1: R_t = 1.19 min. MS-ESI (m/z): calcd for C_8H_6BrNS $[M + H]^+$, 227.9; found, 227.9.

*6-Bromo-5-chlorobenzo[d]isothiazole (**89a**).* Compound **89a** was prepared using 4-bromo-5-chloro-2-fluorobenzaldehyde **88a** (655 mg, 2.76 mmol) according to the general procedure. The residue was purified by column chromatography on silica gel (0−20% EtOAc/cyclohexane) (360 mg, 53% yield over three steps). LC−MS method 1: R_t = 1.17 min. MS-ESI (m/z): calcd for $C_7H_3BrClNS$ $[M + H]^+$, 247.9; found, 247.9.

*6-Bromo-5-methylbenzo[d]isothiazole (**89b**).* Compound **89b** was prepared using 4-bromo-2-fluoro-5-methylbenzaldehyde **88b** (600 mg, 2.76 mmol) according to the general procedure. The residue was purified by reverse-phase column chromatography (5−50% MeCN/0.1% v/v HCOOH in water) (120 mg, 20% yield over three steps). LC−MS method 1: R_t = 1.18 min. MS-ESI (m/z): calcd for C_8H_6BrNS $[M + H]^+$, 227.9; found, 227.9.

*6-Bromo-5-fluorobenzo[d]isothiazole (**89c**).* Compound **89c** was prepared using 4-bromo-2,5-difluorobenzaldehyde **88c** (476 mg, 2.15 mmol) according to the general procedure. The residue was purified by column chromatography on silica gel (0−20% EtOAc/cyclohexane) (235 mg, 47% yield over three steps). LC−MS method 1: R_t = 1.07 min. MS-ESI (m/z): calcd for C_7H_3BrFNS $[M + H]^+$, 231.9; found, 231.9.

*General Procedure for the Synthesis of Intermediates **81c−e,h−l**, **87a,b**, and **90a−c**.* A mixture of compound **80c−e,h−l** or **86a,b** or **89a−c** (1 equiv), NaN_3 (2 equiv), Cu_2O (1.1 equiv), and L-proline (1.3 equiv) in DMSO (0.1 M) was heated at 100 °C for 1−16 h. Consumption of starting material was followed by LC−MS. After cooling to rt, the mixture was quenched with a saturated aqueous NH_4Cl solution, stirred for 1 h, and then filtered through a pad of Celite, washing with EtOAc and water. Phases were separated and the organic one was washed with a saturated aqueous $NaHCO_3$ solution and brine, dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by column chromatography or used in the next step without any further purification as described for each intermediate.

*6-Fluorobenzo[d]isothiazol-5-amine (**81c**).* Compound **81c** was prepared using **80c** (230 mg, 0.99 mmol) according to the general procedure. The residue was used in the next step without any further purification (130 mg, 78% yield). LC−MS method 1: R_t = 0.67 min. MS-ESI (m/z): calcd for $C_7H_5FN_2S$ $[M + H]^+$, 169.0; found, 168.9.

*Isothiazolo[5,4-c]pyridin-5-amine (**81d**).* Compound **81d** was prepared using **80d** (136 mg, 0.64 mmol) according to the general procedure. The residue was used in the next step without any further purification (37 mg, 38% yield). LC−MS method 1: R_t = min. MS-ESI (m/z): calcd for $C_6H_5N_3S$ $[M + H]^+$, 152.0; found, 152.1.

*4-Methylbenzo[d]isothiazol-5-amine (**81e**).* Compound **81e** was prepared using **80e** (247 mg, 1.08 mmol) according to the general procedure. The residue was purified by column chromatography on silica gel (10−50% EtOAc/cyclohexane) (30 mg, 17% yield). LC−MS method 1: R_t = 0.27 min. MS-ESI (m/z): calcd for $C_8H_8N_2S$ $[M + H]^+$, 165.0; found, 165.0.

*Isothiazolo[4,5-b]pyridin-5-amine (**81h**).* Compound **81h** was prepared using **80h** (345 mg, 1.61 mmol) according to the general procedure. The residue was used in the next step without any further purification (22 mg, 9% yield). LC−MS method 1: R_t = 0.53 min. MS-ESI (m/z): calcd for $C_6H_5N_3S$ $[M + H]^+$, 152.0; found, 151.9.

*7-Methylbenzo[d]isothiazol-5-amine (**81i**).* Compound **81i** was prepared using **80i** (413 mg, 1.81 mmol) according to the general procedure. The residue was purified by column chromatography on silica gel (10−50% EtOAc/cyclohexane) (131 mg, 44% yield). LC−MS method 1: R_t = 0.47 min. MS-ESI (m/z): calcd for $C_8H_8N_2S$ $[M + H]^+$, 165.0; found, 165.0.

*7-Chlorobenzo[d]isothiazol-5-amine (**81j**).* Compound **81j** was prepared using **80j** (413 mg, 1.81 mmol) according to the general procedure. The residue was purified by column chromatography on silica gel (10−50% EtOAc/cyclohexane) (19 mg, 19% yield). LC−MS method 1: R_t = 0.82 min. MS-ESI (m/z): calcd for $C_7H_5ClN_2S$ $[M + H]^+$, 185.0; found, 185.0.

*7-Fluorobenzo[d]isothiazol-5-amine (**81k**).* Compound **81k** was prepared using **80k** (145 mg, 0.63 mmol) according to the general procedure. The residue was purified by column chromatography on silica gel (10−50% EtOAc/cyclohexane) (19 mg, 19% yield). LC−MS

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method 1: R_t = 0.71 min. MS-ESI (m/z): calcd for $C_7H_5FN_2S$ [M + H]$^+$, 169.0; found, 168.9.

*Isothiazolo[5,4-b]pyridin-5-amine (**81l**).* Compound **81l** was prepared using **80l** (365 mg, 1.7 mmol) according to the general procedure. The residue was used in the next step without any further purification (171 mg, 67% yield). LC−MS method 1: R_t = 0.43 min. MS-ESI (m/z): calcd for $C_6H_5N_3S$ [M + H]$^+$, 152.0; found, 152.0.

*Benzo[d]isothiazol-6-amine (**87a**).* Compound **87a** was prepared using **86a** (400 mg, 1.87 mmol) according to the general procedure. The residue was purified by reverse-phase column chromatography (0−50% MeCN/water) (226 mg, 80% yield). LC−MS method 1: R_t = 0.54 min. MS-ESI (m/z): calcd for $C_7H_6N_2S$ [M + H]$^+$, 151.0; found, 151.0.

*Benzo[d]isothiazol-4-amine (**87b**).* Compound **87b** was prepared using **86b** (226 mg, 1.06 mmol) according to the general procedure. The residue was purified by reverse-phase column chromatography (5−80% MeCN/water) (80 mg, 50% yield). LC−MS method 1: R_t = 0.55 min. MS-ESI (m/z): calcd for $C_7H_6N_2S$ [M + H]$^+$, 151.0; found, 151.0.

*5-Chlorobenzo[d]isothiazol-6-amine (**90a**).* Compound **90a** was prepared using **89a** (350 mg, 1.4 mmol) according to the general procedure. The residue was used in the next step without any further purification (200 mg, 77% yield). LC−MS method 1: R_t = 0.81 min. MS-ESI (m/z): calcd for $C_7H_5ClN_2S$ [M + H]$^+$, 185.0; found, 185.0.

*5-Methylbenzo[d]isothiazol-6-amine (**90b**).* Compound **90b** was prepared using **89b** (120 mg, 0.52 mmol) according to the general procedure. The residue was purified by column chromatography on silica gel (0−30% EtOAc/cyclohexane) (30 mg, 35% yield). LC−MS method 1: R_t = 0.73 min. MS-ESI (m/z): calcd for $C_8H_8N_2S$ [M + H]$^+$, 165.0; found, 165.1.

*5-Fluorobenzo[d]isothiazol-6-amine (**90c**).* Compound **90c** was prepared using **89c** (223 mg, 0.96 mmol) according to the general procedure. The residue was purified by column chromatography on silica gel (0−30% EtOAc/cyclohexane) (80 mg, 50% yield). LC−MS method 1: R_t = 0.65 min. MS-ESI (m/z): calcd for $C_7H_5FN_2S$ [M + H]$^+$, 169.0; found, 169.2.

*General Procedure for the Synthesis of Intermediates **81a,b** and **87c**.* A mixture of compound **80a,b** or **86c** (1 equiv), iron powder (3 equiv), and NH_4Cl (1 equiv) in EtOH (0.2 M) was heated at reflux for 3 h. After cooling to rt, the mixture was filtered through a pad of Celite, washing with EtOH, and the filtrate was concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (0−10% MeOH/DCM).

*6-Methylbenzo[d]isothiazol-5-amine (**81a**).* Compound **81a** was prepared using **80a** (360 mg, 1.86 mmol) according to the general procedure (186 mg, 60% yield). LC−MS method 1: R_t = 0.53 min. MS-ESI (m/z): calcd for $C_8H_8N_2S$ [M + H]$^+$, 165.0; found, 165.0.

*6-Chlorobenzo[d]isothiazol-5-amine (**81b**).* Compound **81b** was prepared using **80b** (300 mg, 1.40 mmol) according to the general procedure (158 mg, 77% yield). LC−MS method 1: R_t = 0.82 min. MS-ESI (m/z): calcd for $C_7H_5ClN_2S$ [M + H]$^+$, 185.0; found, 185.0.

*Benzo[d]isothiazol-7-amine (**87c**).* Compound **87c** was prepared using **86c** (325 mg, 1.80 mmol) according to the general procedure (107 mg, 40% yield). LC−MS method 1: R_t = 0.62 min. MS-ESI (m/z): calcd for $C_7H_6N_2S$ [M + H]$^+$, 151.0; found, 150.9.

*Synthesis of 4-Chlorobenzo[d]isothiazol-5-amine (**81f**).* *N*-Chlorosuccinimide (178 mg, 1.33 mmol) was added to a solution of benzo[*d*]isothiazol-5-amine **73a** (200 mg, 1.33 mmol) in THF (10 mL) and the mixture was stirred at rt for 16 h. The mixture was treated with a saturated aqueous $NaHCO_3$ solution and extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by column chromatography on silica gel (10−50% EtOAc/cyclohexane) (141 mg, 57% yield). LC−MS method 1: R_t = 0.83 min. MS-ESI (m/z): calcd for $C_7H_5ClN_2S$ [M + H]$^+$, 185.0; found, 185.0.

*Synthesis of 4-Fluorobenzo[d]isothiazol-5-amine (**81g**).* Selectfluor (700 mg, 1.99 mmol) was added to a solution of benzo[*d*]-isothiazol-5-amine **73a** (200 mg, 1.33 mmol) in DMF (4 mL) and cooled to 0 °C. The reaction mixture was stirred at rt for 3 h, treated with a saturated aqueous $NaHCO_3$ solution, and extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by column chromatography on silica gel (10−30% EtOAc/cyclohexane) (24 mg, 11% yield). LC−MS method 1: R_t = 0.68 min. MS-ESI (m/z): calcd for $C_7H_5FN_2S$ [M + H]$^+$, 169.0; found, 169.0.

*General Procedure for the Synthesis of Compounds **46**, **49**−**57**, and **84**.* A mixture of the appropriate intermediate **81** (1 equiv) and 5-chloro-1-methyl-1*H*-imidazole-2-carbaldehyde or 5-chlorothiazole-2-carbaldehyde (1−1.5 equiv) in DCM (0.1 M) and catalytic AcOH was stirred at rt for 30 min. $NaBH(OAc)_3$ (2−3 equiv) was added and the reaction mixture was stirred at rt for 16 h, then treated with a saturated aqueous $NaHCO_3$ solution and extracted with DCM. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was purified as described for each compound.

*N-((5-Chlorothiazol-2-yl)methyl)-6-methylbenzo[d]isothiazol-5-amine (**46**).* Compound **46** was prepared using **81a** (90 mg, 0.55 mmol) and 5-chlorothiazole-2-carbaldehyde (81 mg, 0.55 mmol) according to the general procedure. The crude material was purified by reverse-phase column chromatography (5−60% MeCN/0.1% HCOOH in water). White solid (39 mg, 24% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.70 (s, 1H), 7.68 (s, 1H), 7.57 (s, 1H), 7.13 (s, 1H), 4.73 (d, J = 5.70 Hz, 2H), 4.54 (d, J = 5.04 Hz, 1H), 2.40 (s, 3H). LC−MS method 1: R_t = 1.11 min. MS-ESI (m/z): calcd for $C_{12}H_{10}ClN_3S_2$ [M + H]$^+$, 296.0; found, 296.0.

*N-((5-Chlorothiazol-2-yl)methyl)isothiazolo[5,4-c]pyridin-5-amine (**49**).* Compound **49** was prepared using **81d** (37 mg, 0.25 mmol) and 5-chlorothiazole-2-carbaldehyde (37 mg, 0.25 mmol) according to the general procedure. The crude material was purified by reverse-phase column chromatography (5−50% MeCN/0.1% HCOOH in water). White solid (10 mg, 14% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.90 (s, 1H), 8.76 (s, 1H), 7.53 (s, 1H), 6.98 (d, J = 1.10 Hz, 1H), 5.36 (t, J = 5.15 Hz, 1H), 4.84 (d, J = 6.14 Hz, 2H). LC−MS method 1: R_t = 0.94 min. MS-ESI (m/z): calcd for $C_{10}H_7ClN_4S_2$ [M + H]$^+$, 283.0; found, 283.0.

*N-((5-Chlorothiazol-2-yl)methyl)-4-methylbenzo[d]isothiazol-5-amine (**50**).* Compound **50** was prepared using **81e** (30 mg, 0.18 mmol) and 5-chlorothiazole-2-carbaldehyde (27 mg, 0.18 mmol) according to the general procedure. The crude material was purified by column chromatography on silica gel (10−50% EtOAc/cyclohexane). Yellow solid (17.8 mg, 33% yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.09 (d, J = 0.88 Hz, 1H), 7.77 (d, J = 8.77 Hz, 1H), 7.75 (s, 1H), 6.96 (d, J = 8.77 Hz, 1H), 6.26 (t, J = 6.03 Hz, 1H), 4.69 (d, J = 6.14 Hz, 2H), 2.53 (s, 3H). LC−MS method 1: R_t = 1.10 min. MS-ESI (m/z): calcd for $C_{12}H_{10}ClN_3S_2$ [M + H]$^+$, 296.0; found, 295.9.

*4-Chloro-N-((5-chlorothiazol-2-yl)methyl)benzo[d]isothiazol-5-amine (**51**).* Compound **51** was prepared using **81f** (141 mg, 0.76 mmol) and 5-chlorothiazole-2-carbaldehyde (113 mg, 0.76 mmol) according to the general procedure. The crude material was purified by column chromatography on silica gel (10−50% EtOAc/cyclohexane) and by reverse-phase column chromatography (5−60% MeCN/0.1% HCOOH in water). White solid (49 mg, 20% yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.95 (d, J = 0.78 Hz, 1H), 7.95 (d, J = 9.00 Hz, 1H), 7.76 (s, 1H), 7.13 (d, J = 8.61 Hz, 1H), 6.74 (t, J = 6.26 Hz, 1H), 4.77 (d, J = 6.26 Hz, 2H). LC−MS method 1: R_t = 1.20 min. MS-ESI (m/z): calcd for $C_{11}H_7Cl_2N_3S_2$ [M + H]$^+$, 316.0; found, 316.0.

*N-((5-Chlorothiazol-2-yl)methyl)-4-fluorobenzo[d]isothiazol-5-amine (**52**).* Compound **52** was prepared using **81g** (24 mg, 0.14 mmol) and 5-chlorothiazole-2-carbaldehyde (31.6 mg, 0.21 mmol) according to the general procedure. The crude material was purified by column chromatography on silica gel (5% EtOAc/DCM). Yellow solid (14.4 mg, 34% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.91 (d, J = 0.66 Hz, 1H), 7.55 (s, 1H), 7.56 (d, J = 8.55 Hz, 1H), 7.07 (dd, J = 8.33, 7.67 Hz, 1H), 4.75−4.84 (m, 1H), 4.73 (d, J = 5.90 Hz, 2H). LC−MS method 1: R_t = 1.11 min. MS-ESI (m/z): calcd for $C_{11}H_7ClFN_3S_2$ [M + H]$^+$, 300.0; found, 300.0.

*N-((5-Chlorothiazol-2-yl)methyl)isothiazolo[4,5-b]pyridin-5-amine (**53**).* Compound **53** was prepared using **81h** (22 mg, 0.15 mmol) and 5-chlorothiazole-2-carbaldehyde (21.5 mg, 0.15 mmol) according to the general procedure. The crude material was purified by reverse-phase column chromatography (5−90% MeCN/0.1% HCOOH in water). White solid (1.5 mg, 4% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.87 (s, 1H), 8.00 (d, J = 8.77 Hz, 1H), 7.51 (s, 1H),

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6.71 (d, J = 8.99 Hz, 1H), 5.38 (t, J = 5.81 Hz, 1H), 4.94 (d, J = 5.92 Hz, 2H). LC−MS method 1: R_t = 0.92 min. MS-ESI (m/z): calcd for $C_{10}H_7ClN_4S_2$ $[M + H]^+$, 283.0; found, 283.0.

*N-((5-Chlorothiazol-2-yl)methyl)-7-methylbenzo[d]isothiazol-5-amine (**54**).* Compound **54** was prepared using **81i** (131.3 mg, 0.80 mmol) and 5-chlorothiazole-2-carbaldehyde (118 mg, 0.80 mmol) according to the general procedure. The crude material was purified by column chromatography on silica gel (10−50% EtOAc/cyclohexane). Yellow solid (159.2 mg, 67% yield). ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.87 (s, 1H), 7.77 (s, 1H), 7.01 (s, 1H), 6.86−6.96 (m, 2H), 4.61 (d, J = 6.04 Hz, 2H), 2.45 (s, 3H). LC−MS method 1: R_t = 1.11 min. MS-ESI (m/z): calcd for $C_{12}H_{10}ClN_3S_2$ $[M + H]^+$, 296.0; found, 296.0.

*7-Chloro-N-((5-chlorothiazol-2-yl)methyl)benzo[d]isothiazol-5-amine (**55**).* Compound **55** was prepared using **81j** (19 mg, 0.10 mmol) and 5-chlorothiazole-2-carbaldehyde (15 mg, 0.10 mmol) according to the general procedure. The crude material was purified by column chromatography on silica gel (10−50% EtOAc/cyclohexane) and by reverse-phase column chromatography (5−60% MeCN/0.1% HCOOH in water). White solid (9.7 mg, 30% yield). ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.96 (s, 1H), 7.78 (s, 1H), 7.23 (d, J = 1.96 Hz, 1H), 7.19−7.22 (m, 2H), 4.66 (d, J = 6.36 Hz, 2H). LC−MS method 1: R_t = 1.21 min. MS-ESI (m/z): calcd for $C_{11}H_7Cl_2N_3S_2$ $[M + H]^+$, 316.0; found, 316.0.

*N-((5-Chlorothiazol-2-yl)methyl)-7-fluorobenzo[d]isothiazol-5-amine (**56**).* Compound **56** was prepared using **81k** (48 mg, 0.29 mmol) and 5-chlorothiazole-2-carbaldehyde (63 mg, 0.43 mmol) according to the general procedure. The crude material was purified by column chromatography on silica gel (7% EtOAc/DCM). White solid (42 mg, 49% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.70 (d, J = 4.17 Hz, 1H), 7.56 (s, 1H), 6.98 (d, J = 1.75 Hz, 1H), 6.69 (dd, J = 10.74, 1.75 Hz, 1H), 4.75 (t, J = 5.30 Hz, 1H), 4.63−4.70 (m, 2H). LC−MS method 1: R_t = 1.14 min. MS-ESI (m/z): calcd for $C_{11}H_7ClFN_3S_2$ $[M + H]^+$, 300.0; found, 300.0.

*N-((5-Chlorothiazol-2-yl)methyl)isothiazolo[5,4-b]pyridin-5-amine (**57**).* Compound **57** was prepared using **81l** (96.65 mg, 0.64 mmol) and 5-chlorothiazole-2-carbaldehyde (93.86 mg, 0.64 mmol) according to the general procedure. The crude material was purified by reverse-phase column chromatography (5−50% MeCN/0.1% HCOOH in water). White solid (51.80 mg, 16% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.77 (s, 1H), 8.39 (d, J = 2.74 Hz, 1H), 7.57 (s, 1H), 7.39 (d, J = 2.74 Hz, 1H), 4.87 (br s, 1H), 4.69 (d, J = 6.04 Hz, 2H). LC−MS method 1: R_t = 0.88 min. MS-ESI (m/z): calcd for $C_{10}H_7ClN_4S_2$ $[M + H]^+$, 283.0; found, 283.0.

*N-((5-Chloro-1-methyl-1H-imidazol-2-yl)methyl)-6-methylbenzo[d]isothiazol-5-amine (**84**).* Compound **84** was prepared using **81a** (100 mg, 0.60 mmol) and 5-chloro-1-methyl-1*H*-imidazole-2-carbaldehyde (97 mg, 0.67 mmol) according to the general procedure. The crude material was purified by column chromatography on silica gel (0−30% EtOAc/cyclohexane). White solid (50 mg, 29% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.76 (s, 1H), 7.67 (s, 1H), 7.26 (s, 1H), 6.97 (s, 1H), 4.43 (s, 3H), 3.65 (s, 3H), 2.38 (s, 3H). LC−MS method 1: R_t = 0.56 min. MS-ESI (m/z): calcd for $C_{13}H_{13}ClN_4S$ $[M + H]^+$, 293.1; found, 293.0.

*General Procedure for the Synthesis of Compounds **47**, **48**, **82**, and **83**.* A solution of the appropriate intermediate **81** (1 equiv) and 5-chlorothiazole-2-carbaldehyde or 5-chloro-1-methyl-1*H*-imidazole-2-carbaldehyde (1.5−2 equiv) in MeOH (0.1 M) was heated at 60 °C for 16 h. After cooling to 0 °C, $NaBH_4$ (2 equiv) was added and the reaction mixture was allowed to warm to rt and stirred for 2−3 h. The mixture was quenched with a saturated aqueous $NaHCO_3$ solution and extracted with DCM. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and evaporated to dryness. The crude material was purified by column chromatography on silica gel (5% EtOAc/DCM).

*6-Chloro-N-((5-chlorothiazol-2-yl)methyl)benzo[d]isothiazol-5-amine (**47**).* Compound **47** was prepared using **81b** (18 mg, 0.10 mmol) and 5-chlorothiazole-2-carbaldehyde (21 mg, 0.15 mmol) according to the general procedure. Pale yellow solid (16.9 mg, 55% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.70 (s, 1H), 7.93 (s, 1H), 7.57 (s, 1H), 7.19 (s, 1H), 5.32 (t, J = 5.37 Hz, 1H), 4.75 (d, J = 5.70 Hz, 2H). LC−MS method 1: R_t = 1.18 min. MS-ESI (m/z): calcd for $C_{11}H_7Cl_2N_3S_2$ $[M + H]^+$, 315.9; found, 315.9.

*N-((5-Chlorothiazol-2-yl)methyl)-6-fluorobenzo[d]isothiazol-5-amine (**48**).* Compound **48** was prepared using **81c** (50 mg, 0.30 mmol) and 5-chlorothiazole-2-carbaldehyde (88 mg, 0.59 mmol) according to the general procedure. White solid (31.2 mg, 35% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.70 (s, 1H), 7.59 (d, J = 10.43 Hz, 1H), 7.57 (s, 1H), 7.20 (d, J = 7.68 Hz, 1H), 4.94 (br s, 1H), 4.72 (d, J = 5.76 Hz, 2H). LC−MS method 1: R_t = 1.08 min. MS-ESI (m/z): calcd for $C_{11}H_7ClFN_3S_2$ $[M + H]^+$, 300.0; found, 300.0.

*6-Chloro-N-((5-chloro-1-methyl-1H-imidazol-2-yl)methyl)benzo[d]isothiazol-5-amine (**82**).* Compound **82** was prepared using **81b** (70 mg, 0.38 mmol) and 5-chloro-1-methyl-1*H*-imidazole-2-carbaldehyde (110 mg, 0.76 mmol) according to the general procedure. White solid (26.2 mg, 22% yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.89 (d, J = 0.66 Hz, 1H), 8.25 (s, 1H), 7.57 (s, 1H), 6.95 (s, 1H), 6.26 (t, J = 5.15 Hz, 1H), 4.49 (d, J = 5.26 Hz, 2H), 3.62 (s, 3H). LC−MS method 1: R_t = 0.66 min. MS-ESI (m/z): calcd for $C_{12}H_{10}Cl_2N_4S$ $[M + H]^+$, 313.0; found, 313.0.

*N-((5-Chloro-1-methyl-1H-imidazol-2-yl)methyl)-6-fluorobenzo[d]isothiazol-5-amine (**83**).* Compound **83** was prepared using **81c** (80 mg, 0.48 mmol) and 5-chloro-1-methyl-1*H*-imidazole-2-carbaldehyde (144.5 mg, 0.95 mmol) according to the general procedure. White solid (25.9 mg, 18% yield). ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.82−8.90 (m, 1H), 7.95 (d, J = 11.25 Hz, 1H), 7.55 (d, J = 7.96 Hz, 1H), 6.93 (s, 1H), 6.35−6.45 (m, 1H), 4.44 (d, J = 5.49 Hz, 2H), 3.61 (s, 3H). LC−MS method 1: R_t = 0.56 min. MS-ESI (m/z): calcd for $C_{12}H_{10}ClFN_4S$ $[M + H]^+$, 297.0; found, 297.0.

*General Procedure for the Synthesis of Compounds **58**−**60**.* A mixture of compounds **82**, **83**, or **84** (1 equiv) and paraformaldehyde (3 equiv) in DCM (0.05 M) and catalytic AcOH was stirred at rt for 30 min. $NaBH(OAc)_3$ (3 equiv) was added and the reaction mixture was stirred at rt for 24−72 h. Consumption of starting material was followed by LC−MS. Upon completion, the mixture was treated with water and extracted with DCM. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and evaporated to dryness. The crude material was purified as described for each compound.

*6-Chloro-N-((5-chloro-1-methyl-1H-imidazol-2-yl)methyl)-N-methylbenzo[d]isothiazol-5-amine (**58**).* Compound **58** was prepared using **82** (50 mg, 0.16 mmol) and paraformaldehyde (14.3 mg, 0.48 mmol) according to the general procedure. The crude material was purified by column chromatography on silica gel (5% EtOAc/DCM). White solid (29.3 mg, 56% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.82 (s, 1H), 8.01 (s, 1H), 7.83 (s, 1H), 6.87 (s, 1H), 4.37 (s, 2H), 3.61 (s, 3H), 2.77 (s, 3H). LC−MS method 1: R_t = 0.74 min. MS-ESI (m/z): calcd for $C_{13}H_{12}Cl_2N_4S$ $[M + H]^+$, 327.0; found, 327.0.

*N-((5-Chloro-1-methyl-1H-imidazol-2-yl)methyl)-6-fluoro-N-methylbenzo[d]isothiazol-5-amine (**59**).* Compound **59** was prepared using **83** (56 mg, 0.19 mmol) and paraformaldehyde (17 mg, 0.57 mmol) according to the general procedure. The crude material was purified by column chromatography on silica gel (40% EtOAc/DCM) and then by reverse-phase column chromatography (5−60% MeCN/0.1% HCOOH in water). Colorless oil (32 mg, 54% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.81 (d, J = 0.98 Hz, 1H), 7.69 (d, J = 7.83 Hz, 1H), 7.64 (d, J = 11.25 Hz, 1H), 6.91 (s, 1H), 4.35 (s, 2H), 3.65 (s, 3H), 2.79 (s, 3H). LC−MS method 1: R_t = 0.65 min. MS-ESI (m/z): calcd for $C_{13}H_{12}ClFN_4S$ $[M + H]^+$, 311.1; found, 311.1.

*N-((5-Chloro-1-methyl-1H-imidazol-2-yl)methyl)-N,6-dimethylbenzo[d]isothiazol-5-amine (**60**).* Compound **60** was prepared using **84** (27 mg, 0.09 mmol) and paraformaldehyde (8 mg, 0.27 mmol) according to the general procedure. The crude material was purified by column chromatography on silica gel (0−50% EtOAc/cyclohexane). White solid (12.2 mg, 43% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.81 (s, 1H), 7.90−7.65 (m, 2H), 6.90 (s, 1H), 4.28 (s, 2H), 3.53 (s, 3H), 2.64 (s, 3H), 2.51 (s, 3H). LC−MS method 1: R_t = 0.67 min. MS-ESI (m/z): calcd for $C_{14}H_{15}ClN_4S$ $[M + H]^+$, 307.1; found, 307.0.

*General Procedure for the Synthesis of Compounds **61**−**63**.* A solution of the appropriate intermediate **87** (1 equiv) and 5-chlorothiazole-2-carbaldehyde (1 equiv) in MeOH (0.1 M) was heated

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at 60 °C for 16 h. After cooling to 0 °C, $NaBH_4$ (3 equiv) was added and the reaction mixture was allowed to warm to rt and stirred for 2 h. The mixture was quenched with a saturated aqueous $NaHCO_3$ solution and extracted with DCM. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and evaporated to dryness. The crude material was purified by reverse-phase HPLC to give the target compounds.

*N-((5-Chlorothiazol-2-yl)methyl)benzo[d]isothiazol-6-amine (**61**).* Compound **61** was prepared using **87a** (100 mg, 0.66 mmol) and 5-chlorothiazole-2-carbaldehyde (97 mg, 0.66 mmol) according to the general procedure. White solid (25 mg, 13% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.68 (s, 1H), 7.84 (d, J = 8.77 Hz, 1H), 7.56 (s, 1H), 7.03 (d, J = 1.32 Hz, 1H), 6.82 (dd, J = 8.66, 2.08 Hz, 1H), 4.90 (t, J = 5.59 Hz, 1H), 4.69 (d, J = 5.92 Hz, 2H). LC−MS method 1: R_t = 1.03 min. MS-ESI (m/z): calcd for $C_{11}H_8ClN_3S_2$ $[M + H]^+$, 282.0; found, 282.1.

*N-((5-Chlorothiazol-2-yl)methyl)benzo[d]isothiazol-4-amine (**62**).* Compound **62** was prepared using **87b** (80 mg, 0.53 mmol) and 5-chlorothiazole-2-carbaldehyde (78 mg, 0.53 mmol) according to the general procedure. White solid (30 mg, 20% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.94 (s, 1H), 7.56 (s, 1H), 7.43−7.30 (m, 2H), 6.61−6.48 (m, 1H), 5.32 (br s, 1H), 4.79 (d, J = 5.70 Hz, 2H). LC−MS method 1: R_t = 1.08 min. MS-ESI (m/z): calcd for $C_{11}H_8ClN_3S_2$ $[M + H]^+$, 282.0; found, 282.1.

*N-((5-Chlorothiazol-2-yl)methyl)benzo[d]isothiazol-7-amine (**63**).* Compound **63** was prepared using **87c** (107 mg, 0.71 mmol) and 5-chlorothiazole-2-carbaldehyde (105 mg, 0.71 mmol) according to the general procedure. Yellow solid (28.6 mg, 14% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.90 (s, 1H), 7.59−7.53 (m, 2H), 7.33 (t, J = 7.78 Hz, 1H), 6.72 (d, J = 7.45 Hz, 1H), 4.80 (d, J = 5.70 Hz, 2H), 4.55 (br s, 1H). LC−MS method 1: R_t = 1.08 min. MS-ESI (m/z): calcd for $C_{11}H_8ClN_3S_2$ $[M + H]^+$, 282.0; found, 282.1.

*General Procedure for the Synthesis of Compounds **64**−**68**, **70**, and **71**.* A mixture of the appropriate intermediate **90** (1 equiv) and 5-chloro-1-methyl-1*H*-imidazole-2-carbaldehyde or 5-chlorothiazole-2-carbaldehyde (1−3 equiv) in DCM or DCE (0.1 M) and catalytic AcOH was stirred at rt for 30 min. $NaBH(OAc)_3$ (2−3 equiv) was added and the reaction mixture was stirred at rt or heated at 70 °C for 8−48 h. Consumption of starting material was followed by LC−MS. Upon completion, the mixture was treated with a saturated aqueous $NaHCO_3$ solution and extracted with DCM. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and evaporated to dryness. The crude material was purified as described for each compound.

*5-Chloro-N-((5-chlorothiazol-2-yl)methyl)benzo[d]isothiazol-6-amine (**64**).* Compound **64** was prepared using **90a** (70 mg, 0.38 mmol) and 5-chlorothiazole-2-carbaldehyde (112 mg, 0.76 mmol) according to the general procedure using DCE as a solvent and heating the reaction mixture at 70 °C for 8 h. The crude material was purified by column chromatography on silica gel (0−30% EtOAc/cyclohexane). Yellow solid (58 mg, 48% yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.77 (d, J = 0.66 Hz, 1H), 8.20 (s, 1H), 7.78 (s, 1H), 7.35 (s, 1H), 7.06 (t, J = 6.14 Hz, 1H), 4.75 (d, J = 6.14 Hz, 2H). LC−MS method 1: R_t = 1.14 min. MS-ESI (m/z): calcd for $C_{11}H_7Cl_2N_3S_2$ $[M + H]^+$, 316.0; found, 316.0.

*5-Chloro-N-((5-chloro-1-methyl-1H-imidazol-2-yl)methyl)benzo-[d]isothiazol-6-amine (**65**).* Compound **65** was prepared using **90a** (120 mg, 0.65 mmol) and 5-chloro-1-methyl-1*H*-imidazole-2-carbaldehyde (188 mg, 1.3 mmol) according to the general procedure using DCE as a solvent and heating the reaction mixture at 70 °C for 20 h. The crude material was purified by column chromatography on silica gel (20−50% EtOAc/cyclohexane). Yellow solid (90 mg, 45% yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.76 (s, 1H), 8.17 (s, 1H), 7.49 (s, 1H), 6.94 (s, 1H), 6.63 (t, J = 5.26 Hz, 1H), 4.51 (d, J = 5.26 Hz, 2H), 3.61 (s, 3H). LC−MS method 1: R_t = 0.66 min. MS-ESI (m/z): calcd for $C_{12}H_{10}Cl_2N_4S$ $[M + H]^+$, 313.0; found, 313.0.

*N-((5-Chlorothiazol-2-yl)methyl)-5-methylbenzo[d]isothiazol-6-amine (**66**).* Compound **66** was prepared using **90b** (15 mg, 0.09 mmol) and 5-chlorothiazole-2-carbaldehyde (26.5 mg, 0.18 mmol) according to the general procedure using DCM as a solvent and stirring the reaction mixture at rt for 36 h. The crude material was purified by column chromatography on silica gel (0−30% EtOAc/cyclohexane). White solid (12.7 mg, 47% yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.72 (s, 1H), 7.81 (s, 1H), 7.77 (s, 1H), 7.11 (s, 1H), 6.67 (t, J = 6.03 Hz, 1H), 4.70 (d, J = 5.92 Hz, 2H), 2.30 (s, 3H). LC−MS method 1: R_t = 1.07 min. MS-ESI (m/z): calcd for $C_{12}H_{10}ClN_3S_2$ $[M + H]^+$, 296.0; found, 296.0.

*N-((5-Chlorothiazol-2-yl)methyl)-5-fluorobenzo[d]isothiazol-6-amine (**67**).* Compound **67** was prepared using **90c** (36 mg, 0.21 mmol) and 5-chlorothiazole-2-carbaldehyde (93 mg, 0.63 mmol) according to the general procedure using DCE as a solvent and heating the reaction mixture at 70 °C for 20 h. The crude material was purified by column chromatography on silica gel (0−30% EtOAc/cyclohexane). Yellow solid (33 mg, 54% yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.77 (s, 1H), 7.88 (d, J = 11.62 Hz, 1H), 7.78 (s, 1H), 7.33 (d, J = 7.45 Hz, 1H), 7.28−7.18 (m, 1H), 4.70 (d, J = 6.14 Hz, 2H). LC−MS method 1: R_t = 1.05 min. MS-ESI (m/z): calcd for $C_{11}H_7ClFN_3S_2$ $[M + H]^+$, 300.0; found, 300.0.

*N-((5-Chloro-1-methyl-1H-imidazol-2-yl)methyl)-5-methylbenzo[d]isothiazol-6-amine (**68**).* Compound **68** was prepared using **90b** (15 mg, 0.09 mmol) and 5-chloro-1-methyl-1*H*-imidazole-2-carbaldehyde (14.5 mg, 0.10 mmol) according to the general procedure using DCM as a solvent and stirring the reaction mixture at rt for 36 h. The crude material was purified by column chromatography on silica gel (0−50% EtOAc/cyclohexane). White solid (10.3 mg, 39% yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.73 (d, J = 0.66 Hz, 1H), 7.80 (s, 1H), 7.32 (br s, 1H), 7.23 (s, 1H), 6.31 (br s, 1H), 4.60 (s, 2H), 3.69 (s, 3H), 2.29 (s, 3H). LC−MS method 1: R_t = 0.57 min. MS-ESI (m/z): calcd for $C_{13}H_{13}ClN_4S$ $[M + H]^+$, 293.1; found, 293.1.

*N-((5-Chloro-1-methyl-1H-imidazol-2-yl)methyl)-5-fluorobenzo-[d]isothiazol-6-amine (**70**).* Compound **70** was prepared using **90c** (36 mg, 0.21 mmol) and 5-chloro-1-methyl-1*H*-imidazole-2-carbaldehyde (91 mg, 0.63 mmol) according to the general procedure using DCE as a solvent and heating the reaction mixture at 70 °C for 20 h. The crude material was purified by column chromatography on silica gel (20−60% EtOAc/cyclohexane). Yellow solid (32 mg, 52% yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.75 (s, 1H), 7.83 (d, J = 11.84 Hz, 1H), 7.47 (d, J = 7.67 Hz, 1H), 6.93 (s, 1H), 6.82 (td, J = 5.26, 2.19 Hz, 1H), 4.46 (d, J = 5.48 Hz, 2H), 3.60 (s, 3H). LC−MS method 1: R_t = 0.55 min. MS-ESI (m/z): calcd for $C_{12}H_{10}ClFN_4S$ $[M + H]^+$, 297.0; found, 297.1.

*N-((5-Chloro-1-methyl-1H-imidazol-2-yl)methyl)benzo[d]-isothiazol-6-amine (**71**).* Compound **71** was prepared using **87a** (64 mg, 0.43 mmol) and 5-chloro-1-methyl-1*H*-imidazole-2-carbaldehyde (62 mg, 0.43 mmol) according to the general procedure using DCM as a solvent and stirring the reaction mixture at rt for 24 h. The crude material was purified by column chromatography on silica gel (15% MeOH/DCM). White solid (35 mg, 30% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.68 (s, 1H), 7.83 (d, J = 8.55 Hz, 1H), 7.11 (d, J = 1.10 Hz, 1H), 6.96 (s, 1H), 6.84 (dd, J = 8.77, 1.97 Hz, 1H), 4.88 (br s, 1H), 4.42 (d, J = 4.60 Hz, 2H), 3.62 (s, 3H). LC−MS method 1: R_t = 0.48 min. MS-ESI (m/z): calcd for $C_{12}H_{11}ClN_4S$ $[M + H]^+$, 279.0; found, 279.0.

*Synthesis of N-((5-Chloro-1-methyl-1H-imidazol-2-yl)methyl)-N-methylbenzo[d]isothiazol-6-amine (**69**).* NaH (60% w/w in mineral oil, 4 mg, 0.2 mmol) was added to a solution of **71** (25 mg, 0.09 mmol) in dry DMF (1 mL) and cooled to 0 °C. The reaction mixture was stirred at rt for 15 min, MeI (6 μL, 0.1 mmol) was added, and stirring was continued for 3 h. The mixture was treated with a saturated aqueous NH_4Cl solution and extracted with EtOAc. The combined extracts were washed with brine, dried over Na_2SO_4, filtered, and evaporated to dryness. The crude material was purified by column chromatography on silica gel (5% MeOH/DCM). White solid (3 mg, 11% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.70 (s, 1H), 7.89 (d, J = 8.77 Hz, 1H), 7.25 (d, J = 1.32 Hz, 1H), 7.10 (dd, J = 8.99, 1.97 Hz, 1H), 6.93 (s, 1H), 4.63 (s, 2H), 3.51 (s, 3H), 3.05 (s, 3H). LC−MS method 1: R_t = 0.56 min. MS-ESI (m/z): calcd for $C_{13}H_{13}ClN_4S$ $[M + H]^+$, 293.1; found, 293.0.

*Synthesis of 5-Chloro-N-((5-chloro-1-methyl-1H-imidazol-2-yl)-methyl)-N-methylbenzo[d]isothiazol-6-amine (**72**).* A mixture of the compound **65** (60 mg, 0.19 mmol) and paraformaldehyde (29 mg, 0.95 mmol) in DCE (3 mL) and catalytic AcOH was stirred at rt for 30 min.

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$NaBH(OAc)_3$ (121 mg, 0.57 mmol) was added and the reaction mixture was heated at 40 °C for 48 h. The mixture was treated with water and extracted with DCM. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and evaporated to dryness. The crude material was purified by column chromatography on silica gel (20−50% EtOAc/cyclohexane). White solid (10 mg, 16% yield). ^1H NMR (400 MHz, $CDCl_3$): δ 8.75 (s, 1H), 8.08 (s, 1H), 7.68 (s, 1H), 6.89 (s, 1H), 4.41 (s, 2H), 3.57 (s, 3H), 2.81 (s, 3H). LC−MS method 1: R_t = 0.75 min. MS-ESI (m/z): calcd for $C_{13}H_{12}Cl_2N_4S$ $[M + H]^+$, 327.0; found, 327.1.

Solubility Assay. Equipment and Chemical Reagents. Control compounds: ibuprofen for high solubility and progesterone for low solubility.

DMSO evaporation device: Genevac HT-4X.

Shaking device: DVX 2500 Multi Tube Vortex VWR.

Filtration device: MultiScreen Resist vacuum manifold Millipore Phenomenex 96 well glass fiber filter 0.45 μm plate Agilent HPLC system with DAD, UV detection provided at 230 and 254 nm.

Sample plate: NUNC 400.

Experimental Design. 10 μL of a 10 mM compound stock solution in DMSO was dispensed in a well in duplicate and dried under vacuum. 200 μL of phosphate-buffered saline buffer with pH = 7.4 was added in each well, sonicated for 5 min, and then mixed with heavy shaking at rt for 2 h. The final concentration was 500 μM. Then, the solutions were filtered, 160 μL of filtrate was transferred in a well, and 40 μL of MeOH was added. The sample was analyzed by HPLC/UV.

In Vitro Clearance in Liver Microsomes. Equipment and Chemical Reagents. All chemicals and solvents were of reagent or LC grade. Water was purified in house (Milli-Q, Millipore) and used in the preparation of solutions.

50 mM phosphate buffer at pH 7.4: 11.2 mL of potassium phosphate monobasic and 38.8 mL of potassium phosphate dibasic diluted to 1 L with Milli-Q water.

Regenerating system: reduced nicotinamide adenine dinucleotide phosphate (NADPH) regenerating system solution prepared by dissolving 1.7 mg of nicotinamide adenine dinucleotide phosphate, 7.8 mg of glucose 6-phosphate (G6P), and 6 units of G6P dehydrogenase in 1 mL of 2% sodium bicarbonate solution (prepared by dissolving 20 g of $NaHCO_3$ in 1 L of Milli-Q water).

Liver microsomes were supplied by an accredited supplier.

Experimental Design. Liver microsomes were rapidly thawed in a water bath at 37 °C and kept on ice until use. Liver microsomes were diluted with 50 mM potassium phosphate buffer pH 7.4 to a protein concentration of 0.56 mg/mL.

5 μL of the test compound and positive control working solutions (WS) (to give a final concentration of 0.5 μM) were added to 445 μL of microsomes and 50 μL of the regenerating system.

Automated assay steps were performed using RSP Freedom EVO (Tecan) and Hamilton Systems:

150 μL of quenching solution was taken in each well of 96 deep well 1 mL plates.

800 μL aliquots of NADPH regenerating system were prewarmed at 37 °C for 5 min.

5 μL of 50 μM test items and control were added to 445 μL of the 0.56 mg/mL microsome solution and the incubation mixture was prewarmed in a 96-deep-well 2 mL plate (incubation plate) at 37 °C for 5 min.

Incubation reactions were initiated by adding 50 μL of the prewarmed NADPH regenerating system to the incubation mixtures.

50 μL aliquots were taken from incubation mixtures at: 0, 3, 6, 9, 15, and 30 min.

Samples were centrifuged at 3000 rpm for 10 min and further diluted in order to optimize the analytical conditions prior to the LC−MS/MS analysis.

Peak areas for test and control items were integrated using Integrator software from Agilent or MultiQuant Software from AB Sciex and exported to XLFit or Morphit (The Edge) designed to calculate in vitro metabolic stability parameters. The observed rate constant (k_{obs}) for parent degradation was calculated by determining the slope of the line of the graph of the natural log of percentage parent remaining versus incubation time. Clearance was estimated from the rate of depletion k (min^{-1}), the volume of the incubation V (mL), and the amount of microsomal proteins in the incubation M (mg) scaled for the protein in the incubation relative to that in the liver.

$$\text{Clint} = k \times V/M$$

CLi values were expressed as μL/min/mg liver microsomes.

Plasma Protein Binding. Equipment and Chemical Reagents. Unless otherwise stated, all analytical reagents were of at least standard analytical laboratory reagent grade.

Plasma from different species, according to sponsor requirements, was obtained in-house or purchased from external suppliers (Sera Laboratories International Ltd. or B&K Universal Ltd.).

Rapid equilibrium dialysis device (Thermo Scientific RED Device) or 96 well dialyzer apparatus (HTDialysis LLC).

RSP Microlab STARlet (Hamilton).

Refrigerated centrifuge, GS 6KR (Beckman).

Microtiter stirrer with thermostatic Cupola mod. 715 CT+ (Asal).

Experimental Design. The dialysis buffer was prepared as follows: Na_2HPO_4 8.69 g, KH_2PO_4 1.90 g, and NaCl 4.11 g were dissolved in 1 L of Milli-Q water and the pH was adjusted to pH 7.4 with H_3PO_4.

An appropriate amount of test item was dissolved in DMSO (or an appropriate solvent) to give a 10 mM solution. Further dilutions, to obtain 50 μM WS, were prepared using 50% ACN in Milli-Q water. 50 μM WS used to spike the plasma to obtain a final concentration of 0.5 μM.

350 μL of test item-free isotonic phosphate buffer was dispensed in the buffer compartment and 200 μL of the spiked matrix was loaded in the matrix compartment of the RED Device.

Alternatively, 150 μL of test item-free buffer was dispensed in the buffer compartment and 150 μL of the spiked matrix was loaded in the matrix compartment of a 96-well dialyzer apparatus (HTDialysis LLC).

At the end of the equilibration period (device is sealed and shaken at 500 rpm for 5 h at 37 °C), 50 μL of the dialyzed matrix was added to 50 μL of the corresponding test item-free dialyzed buffer, and vice versa for buffer, such that the volume of buffer to matrix to be the same. Samples were extracted by protein precipitation with 300 μL of ACN containing rolipram (for positive ionization mode) or diclofenac (for negative ionization mode) as the internal standard and centrifuged for 10 min at 2800 rpm. Supernatants collected (100 μL), diluted with 18% ACN in Milli-Q water (200 μL), and injected into an HPLC MS/MS or UPLC MS/MS system.

Plasma protein binding was determined with the following formulas:

$$\text{Afu} = \text{buffer}/\text{plasma}$$

where afu = the apparent fraction unbound; buffer = the analyte/internal standard ratio determined in the buffer compartment; and plasma = the analyte/internal standard ratio determined in the plasma compartment.

$$\text{Fucr} = (1/D)/([(1/\text{Afu} - 1) + 1/D])$$

where fucr = the fraction unbound corrected; D = the matrix dilution factor (D = 1 for plasma); and % binding = (1 − fucr) × 100; % unbound = 100 − % bound.

Caco-2 Cell Permeability. Equipment and Chemical Reagents. Digoxin at a final target concentration of 10 μM.

Atenolol (low permeable reference) at a final concentration of 3 μM.

Metoprolol (high permeable reference) or propranolol at a final concentration of 3 μM.

Palmitoyl carnitine at a final concentration of 0.3 mM.

Elacridar HCl (GF120918) at a final concentration of 10 μM.

DMSO.

Lucifer yellow at a final concentration 100 μM.

HBSS with 12.5 mM HEPES.

Caco-2 cells used originated from American Type Culture Collection and cultured in house.

Experimental Design. Test item stock solutions and reference compound solutions were diluted with DMSO to generate a 200× solution.

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200× solutions were then diluted 1:200 with transport buffer to have a 3 or 10 μM final concentration (0.5% DMSO).

On the day of the experiment, the culture medium present in the feeder tray was replaced with prewarmed transport buffer and the apical wells were refilled with transport buffer. Incubation of the cells was carried out at 37 °C for 30 min.

During preincubation, Trans Epithelial Electrical Resistance (TEER) measurements were taken from selected wells (at least 20%) using a Millicell ERS meter (Electrical Resistance System, from Millipore).

After preincubation, the transport of the test items was measured in two directions, [$A > B$] and [$B > A$], in the absence or presence of palmitoyl carnitine at 0.3 mM or 10 μM GF120918, where applicable.

At the end of the incubation, aliquots from receiver, donor, and dosing solutions were transferred into the MS plate. Samples were extracted by protein precipitation with ACN, centrifuged for 10 min at 3000 rpm, and then injected into an LC−MS/MS system.

The apparent permeability (P_{app}) was determined in [$A > B$] and [$B > A$] directions in the absence or in the presence of palmitoyl carnitine or GF120918.

The following parameters were calculated:

1. P_{app} (apparent permeability) values calculated for directions [$A > B$] and [$B > A$], according to the following equation

$$P_{app} = \left(\frac{dQ}{dt}\right) \times \left(\frac{1}{C_0}\right) \times \left(\frac{1}{A}\right)$$

where dQ/dt = the permeability rate (dQ/dt is the amount of test/control items within the incubation period); C_0 = the initial concentration of test/control items in the donor compartment; and A = the surface area of the filter, corresponding to the surface area of the cell monolayer. The P_{app} value (nm/s) reported as average P_{app} (nm/s) ± standard deviation from three monolayers for [$A > B$] and for [$B > A$] directions, where applicable.

2. Monolayer efflux ratios were derived using the mean P_{app} [$A > B$] and [$B > A$] direction according to the following equation

$$\text{Efflux ratio} = \left(\frac{B - AP_{app}\ (\text{nm/s})}{A - BP_{app}\ (\text{nm/s})}\right)$$

PK Studies. Animals. All experiments involving animals were carried out in accordance with the European Directive 2010/63/UE governing animal welfare and protection, which is acknowledged by the Italian Legislative Decree no. 26/2014 and according to the company policy on the care and use of laboratories animals. All the studies were revised by the Animal Welfare Body and approved by Italian Ministry of Health (authorization no. 181/2018-PR, authorization no. 67/2014-PR).

Animals were housed in groups on a 12 h light/dark schedule with food and water ad libitum and fed with Mouse House and iso-BLOX until the day of experiment.

*Compound **61** PK Profile after Intravenous (IV) and Oral (PO) Administration in Male CD-1 Mouse: Experimental Design.* The study was conducted on two groups of animals:

Group 1 (M1−M3): 3 male CD-1 mice each received a single IV administration of test item at a nominal dose level of 1 mg/kg. Serial blood samples were collected from each animal at up to 8 time points of 50 μL each over 24 h postdose. After the completion of the last time point, mice were killed with CO_2.

Group 2 (M4−M6): 3 male CD-1 mice each received a single oral administration of test item at a nominal dose level of 10 mg/kg. Serial blood samples were collected from each animal at up to 8 time points of 50 μL each over 24 h postdose. After the completion of the last time point, mice were killed with CO_2.

Group 1 (IV): 0.083, 0.25, 0.5, 1, 2, 4, 8, and 24 h postdose.

Group 2 (PO): 0.25, 0.5, 1, 2, 4, 6, 8, and 24 h postdose.

*Compound **64** PK Profile after Oral (PO) Administration in Male C57BL6 Mouse: Experimental Design.* The study was conducted on three groups of animals:

Group 1 (M1−M18): 18 male C57BL6 mice each received a single oral administration of test item at a nominal dose level of 1 mg/kg. Blood, colon, and ileum were collected at 6, 24, and 48 h postdose (n = six animals per time point).

Group 2 (M19−M36): 18 male C57BL6 mice each received a single oral administration of test item at a nominal dose level of 3 mg/kg. Blood, colon, and ileum were collected at 6, 24, and 48 h postdose (n = six animals per time point).

Group 3 (M37−M54): 18 male C57BL6 mice each received a single oral administration of test item at a nominal dose level of 100 mg/kg. Blood, colon, and ileum were collected at 6, 24, and 48 h postdose (n = six animals per time point).

Experimental Procedure. 50 μL blood samples were collected from tail vein into tubes containing anticoagulant (e.g., K3EDTA, lithium heparin), stored on ice, and centrifuged to prepare plasma.

Plasma, colon, ileum, and feces samples were stored at approximately −20 °C, pending analysis.

Plasma and tissue samples were assayed using a method based on protein precipitation with ACN, followed by LC−MS/MS analysis.

Results for test substances in plasma were subjected to non-compartmental PK analysis using Phoenix WinNonlin 6.3.

This experiment was carried out in accordance with the PHS Policy, the National Research Council Guide for the Care and Use of Laboratory Animals (8th edition), and National Laboratory Animal Management Regulations (2017), and according to the company policies, SOPs, and guidelines on the care and use of laboratory animals. All the studies were approved by the Institutional Animal Care and Use.

*Assessment of Compound **64** on the Gastromotility Model.* Male CD-1 mice (6 weeks old; Charles River, Calco, Como, Italy) were housed in groups on a 12 h light−dark cycle (light on at 0600 h) and handled 3 days before the test, to minimize the stress due to manipulation on the day of the experiment; access to food and water was allowed ad libitum. On the night before the experiment, the mice were housed in single cages provided with grid and removable tray on the bottom, for the habituation to the experimental environment. On the day of the test, mice were treated with test compound (1−10−100 mg/kg) or vehicle by oral administration and returned to their cage and allowed access to their standard food and top water ad libitum. Twelve mice for each group of treatment were included, and treatments were randomly distributed on the rack of cages. The group size of 12 was selected as the minimal number necessary to measure a statistical difference (at least $p < 0.05$) fixing the statistical power of 0.8.

1, 2, 3, and 6 h after treatment, feces for each mouse were collected, counted, and weighted.

The number and weight of feces were expressed in terms of the total number and dry weight per mouse (drying at 105 °C, 24 h). After recording the weight, the wet feces for each mouse were dried at 105 °C for 24 h to calculate the percentage of water of feces as follows

$$\text{fecal water content (\%)} = (\text{feces weight before dried} - \text{feces weight after dried}) / \text{feces weight before dried} \times 100$$

Statistical analysis was performed by one-way ANOVA followed by Dunnett's test.

Mice were also monitored for the general health status at the same time points of feces collection, in particular coat conditions, posture, locomotion, and general behavior. After the completion of the experiment, mice were killed with CO_2.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.1c00065.

Molecular formula string (CSV)

LC-MS traces and ^{1}H NMR spectra of compounds **25**, **61**, and **64**; compound **64** safety scan panel; and compound **64** cytotoxicity data (cell viability ATPlite assay (PDF)

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This manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

CHO, Chinese hamster ovary cells; CRC, concentration−response curve; EDG, electron-donating groups; EWG, electron-withdrawing group; FLIPR, fluorometric imaging plate reader; FMP, FLIPR membrane potential; LE, ligand efficiency; LLE, lipophilic ligand efficiency; Met ID, metabolite identification; TRPA, transient receptor potential cation channel subfamily A; TRPC, transient receptor potential cation channel subfamily C; TRPM, transient receptor potential cation channel subfamily M; TRPP, transient receptor potential cation channel subfamily P; TRPV, transient receptor potential cation channel subfamily V

REFERENCES

(1) Gees, M.; Colsoul, B.; Nilius, B. The role of transient receptor potential cation channels in Ca^{2+} signaling. *Cold Spring Harbor Perspect. Biol.* **2010**, *2*, a003962.

(2) Nilius, B.; Owsianik, G. The transient receptor potential family of ion channels. *Genome Biol.* **2011**, *12*, 218−229.

(3) Wu, L.-J.; Sweet, T.-B.; Clapham, D. E. International union of basic and clinical pharmacology. LXXVI. Current progress in the mammalian TRP ion channel family. *Pharmacol. Rev.* **2010**, *62*, 381−404.

(4) Gees, M.; Owsianik, G.; Nilius, B.; Voets, T. TRP channels. *Compr. Physiol.* **2012**, *2*, 563−608.

(5) Owsianik, G.; D'hoedt, D.; Voets, T.; Nilius, B. Structure-function relationship of the TRP channel superfamily. *Rev. Physiol., Biochem. Pharmacol.* **2006**, *156*, 61−90.

(6) Hasan, R.; Zhang, X. Ca^{2+} regulation of TRP ion channels. *Int. J. Mol. Sci.* **2018**, *19*, 1256.

(7) Pérez, C. A.; Huang, L.; Rong, M.; Kozak, J. A.; Preuss, A. K.; Zhang, H.; Max, M.; Margolskee, R. F. A transient receptor potential channel expressed in taste receptor cells. *Nat. Neurosci.* **2002**, *5*, 1169−1176.

(8) Zhang, Y.; Hoon, M. A.; Chandrashekar, J.; Mueller, K. L.; Cook, B.; Wu, D.; Zuker, C. S.; Ryba, N. J. P. Coding of sweet, bitter, and umami tastes: different receptor cells sharing similar signalling pathways. *Cell* **2003**, *112*, 293−301.

(9) Clapp, T. R.; Medler, K. F.; Damak, S.; Margolskee, R. F.; Kinnamon, S. C. Mouse taste cells with G protein-coupled taste receptors lack voltage-gated calcium channels and SNAP-25. *BMC Biol.* **2006**, *4*, 7.

(10) Kaske, S.; Krasteva, G.; König, P.; Kummer, W.; Hofmann, T.; Gudermann, T.; Chubanov, V. TRPM5, a taste-signaling transient receptor potential ion-channel, is a ubiquitous signalling component in chemosensory cells. *BMC Neurosci.* **2007**, *8*, 49.

(11) Wyatt, A.; Wartenberg, P.; Candlish, M.; Krasteva-Christ, G.; Flockerzi, V.; Boehm, U. Genetic strategies to analyze primary TRP channel-expressing cells in mice. *Cell Calcium* **2017**, *67*, 91−104.

(12) Brixel, L. R.; Monteilh-Zoller, M. K.; Ingenbrandt, C. S.; Fleig, A.; Penner, R.; Enklaar, T.; Zabel, B. U.; Prawitt, D. TRPM5 regulates glucose-stimulated insulin secretion. *Pflügers Arch.* **2010**, *460*, 69−76.

(13) Colsoul, B.; Schraenen, A.; Lemaire, K.; Quintens, R.; Van Lommel, L.; Segal, A.; Owsianik, G.; Talavera, K.; Voets, T.; Margolskee, R. F.; Kokrashvili, Z.; Gilon, P.; Nilius, B.; Schuit, F. C.; Vennekens, R. Loss of high-frequency glucose-induced Ca^{2+} oscillations in pancreatic islets correlates with impaired glucose tolerance in Trpm5-/- mice. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 5208−5213.

(14) Philippaert, K.; Pironet, A.; Mesuere, M.; Sones, W.; Vermeiren, L.; Kerselaers, S.; Pinto, S.; Segal, A.; Antoine, N.; Gysemans, C.; Laureys, J.; Lemaire, K.; Gilon, P.; Cuypers, E.; Tytgat, J.; Mathieu, C.; Schuit, F.; Rorsman, P.; Talavera, K.; Voets, T.; Vennekens, R. Steviol glycosides enhance pancreatic beta-cell function and taste sensation by potentiation of TRPM5 channel activity. *Nat. Commun.* **2017**, *8*, 14733.

(15) Steele, S. P.; Melchor, S. J.; Petri, W. A., Jr. Tuft cells: new players in colitis. *Trends Mol. Med.* **2016**, *22*, 921−924.

(16) Zierler, S.; Hampe, S.; Nadolni, W. TRPM channels as potential therapeutic targets against pro-inflammatory diseases. *Cell Calcium* **2017**, *67*, 105−115.

(17) Prawitt, D.; Monteilh-Zoller, M. K.; Brixel, L.; Spangenberg, C.; Zabel, B.; Fleig, A.; Penner, R. TRPM5 is a transient Ca^{2+}-activated

cation channel responding to rapid changes in [Ca^{2+}]i. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 15166−15171.
(18) Philippaert, K.; Kerselaers, S.; Voets, T.; Vennekens, R. A thallium-based screening procedure to identify molecules that modulate the activity of Ca^{2+}-activated monovalent cation-selective channels. *SLAS Discovery* **2018**, *23*, 341−352.
(19) Guinamard, R.; Simard, C.; Del Negro, C. Flufenamic acid as an ion channel modulator. *Pharmacol. Ther.* **2013**, *138*, 272−284.
(20) Ullrich, N. D.; Voets, T.; Prenen, J.; Vennekens, R.; Talavera, K.; Droogmans, G.; Nilius, B. Comparison of functional properties of the Ca^{2+}-activated cation channels TRPM4 and TRPM5 from mice. *Cell Calcium* **2005**, *37*, 267−278.
(21) Gees, M.; Alpizar, Y. A.; Luyten, T.; Parys, J. B.; Nilius, B.; Bultynck, G.; Voets, T.; Talavera, K. Differential effects of bitter compounds on the taste transduction channels TRPM5 and IP_3 receptor type 3. *Chem. Senses* **2014**, *39*, 295−311.
(22) Sung, D.-J.; Kim, J.-G.; Won, K. J.; Kim, B.; Shin, H. C.; Park, J.-Y.; Bae, Y. M. Blockade of K^+ and Ca^{2+} channels by azole antifungal agents in neonatal rat ventricular myocytes. *Biol. Pharm. Bull.* **2012**, *35*, 1469−1475.
(23) Luo, J.; Zhu, Y.; Zhu, M. X.; Hu, H. Cell-based calcium assay for medium to high throughput screening of TRP channel functions using FlexStation 3. *J. Visualized Exp.* **2011**, *54*, No. e3149.
(24) Miller, M.; Wu, M.; Xu, J.; Weaver, D.; Li, M.; Zhu, M. X. High-throughput screening of TRPC channel ligands using cell-based assays. *TRP Channels*; CRC Press/Taylor & Francis: Boca Raton, FL, 2011; Chapter 1.
(25) Zicha, S.; Radresa, O.; Laplante, P.; Morton, M.; Jones, K.; Main, M.; Trivedi, S.; Julien, R. P.; Griffin, A.; Labrecque, J.; Ahmad, S.; Brown, W. Novel methodology to identify TRPV1 antagonists independent of Capsaicin activation. *J. Biomol. Screening* **2012**, *18*, 544−555.
(26) Reubish, D. S.; Emerling, D. E.; Defalco, J.; Steiger, D.; Victoria, C. L.; Vincent, F. Functional assessment of temperature-gated ion-channel activity using a real-time PCR machine. *Biotechniques* **2009**, *47*, iii−ix.
(27) Castillo, B.; Pörzgen, P.; Penner, R.; Horgen, F. D.; Fleig, A. Development and optimization of a high-throughput bioassay for TRPM7 ion channel inhibitors. *J. Biomol. Screening* **2010**, *15*, 498−507.
(28) Bryant, R. W.; Lee, S. P.; Palmer, R. K.; Yang, Q.; Buber, M. N. T. High throughput screening assay for the TRPM5 ion channel. WO 2007056160 A2, 2007.
(29) Hopkins, A. L.; Groom, C. R.; Alex, A. Ligand efficiency: a useful metric for lead selection. *Drug Discovery Today* **2004**, *9*, 430−431.
(30) Abad-Zapatero, C. Ligand efficiency indices for effective drug discovery. *Expert Opin. Drug Discovery* **2007**, *2*, 469−488.
(31) Tarcsay, Á.; Nyíri, K.; Keserű, G. M. Impact of lipophilic efficiency on compound quality. *J. Med. Chem.* **2012**, *55*, 1252−1260.
(32) Zhou, M.; Ping, J.; Chen, J.; Xiu, A.; Zhao, Y.; Zhan, Y.; Chen, P.; Zhang, J. Laxative effects of Salecan on normal and two models of experimental constipated mice. *BMC Gastroenterol.* **2013**, *13*, 52.
(33) Kirk, K. L. Facile synthesis of 2-substituted imidazoles. *J. Org. Chem.* **1978**, *43*, 4381−4383.
(34) Ramirez, S. M.; Layman, J. M.; Bissel, P.; Long, T. E. Ring-opening polymerization of imidazole epoxides for the synthesis of imidazole-substituted poly(ethylene oxides). *Macromolecules* **2009**, *42*, 8010−8012.
(35) Kwon, S.; Han, Y. T.; Jung, J.-W. Studies on the synthesis of indothiazinone and its derivatives via direct 3-acylation of indole. *Synth. Commun.* **2015**, *45*, 1662−1668.
(36) Kouznetsov, V. V.; Vargas Méndez, L. Y.; Sortino, M.; Vásquez, Y.; Gupta, M. P.; Freile, M.; Enriz, R. D.; Zacchino, S. A. Antifungal and cytotoxic activities of some N-substituted aniline derivatives bearing a hetaryl fragment. *Bioorg. Med. Chem.* **2008**, *16*, 794−809.
(37) Fache, F.; Cros, F.; Piva, O.; Lefebvre, F. Easy access to 2-alkyl and 2-arylthiazolines using a modified heteropolyacid catalysis: application to the synthesis of bacillamide A. *Synth. Commun.* **2012**, *42*, 2098−2109.
(38) Bingham, M. J.; Huggett, M.; Kiyoi, Y.; Napier, S. E.; Nimz, O. Preparation of 9-azabicyclo[3.3.1]nonane derivatives for therapeutic use as dopamine and serotonin reuptake inhibitors. WO 2007039563 A1, 2007.
(39) Jin, B.; Dong, Q.; Hung, G. Preparation of substituted pyrazoles and imidazoles as hemoglobin modifier compounds and uses thereof. WO 2017218960 A1, 2017.
(40) Fink, D. M.; Strupczewski, J. T. Preparation of 6-fluorobenzisothazoles via a regioselective nucleophilic aromatic substitution reaction. *Tetrahedron Lett.* **1993**, *34*, 6525−6528.
(41) Bollinger, S. R.; Engers, D. W.; Panarese, J. D.; West, M.; Engers, J. L.; Loch, M. T.; Rodriguez, A. L.; Blobaum, A. L.; Jones, C. K.; Gray, A. T.; Conn, P. J.; Lindsley, C. W.; Niswender, C. M.; Hopkins, C. R. Discovery, structure−activity relationship, and biological characterization of a novel series of 6-((1H-Pyrazolo[4,3-b]pyridin3-yl)amino)-benzo[d]isothiazole-3-carboxamides as positive allosteric Modulators of the metabotropic glutamate receptor 4 (mGlu4). *J. Med. Chem.* **2019**, *62*, 342−358.
(42) Messaoudi, S.; Brion, J.-D.; Alami, M. Copper-catalyzed direct amination of halo(hetero)arenes with sodium azide as the amino source. *Mini-Rev. Org. Chem.* **2011**, *8*, 448−454.
(43) Markiewicz, J. T.; Wiest, O.; Helquist, P. Synthesis of primary aryl amines through a copper-assisted aromatic substitution reaction with sodium azide. *J. Org. Chem.* **2010**, *75*, 4887−4890.
(44) Holzer, P.; Masuya, K.; Furet, P.; Kallen, J.; Valat-Stachyra, T.; Ferretti, S.; Berghausen, J.; Bouisset-Leonard, M.; Buschmann, N.; Pissot-Soldermann, C.; Rynn, C.; Ruetz, S.; Stutz, S.; Chène, P.; Jeay, S.; Gessier, F. Discovery of a dihydroisoquinolinone derivative (NVP-CGM097): a highly potent and selective MDM2 inhibitor undergoing phase 1 clinical trials in p53wt tumors. *J. Med. Chem.* **2015**, *58*, 6348−6358.
(45) Mathes, C.; Friis, S.; Finley, M.; Liu, Y. QPatch: the missing link between HTS and ion channel drug discovery. *Comb. Chem. High Throughput Screening* **2009**, *12*, 78−95.
(46) Obergrussberger, A.; Stölzle-Feixet, S.; Becker, N.; Brüggemann, A.; Möller, C. Novel screening techniques for ion channel targeting drugs. *Channels* **2015**, *9*, 367−375.
(47) Bers, D. M.; Patton, C. W.; Nuccitelli, R. A practical guide to the preparation of Ca^{2+} buffers. *Methods Cell Biol.* **2010**, *99*, 1−26.

https://doi.org/10.1021/acs.jmedchem.1c00065
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