Synthesis and Investigation of S-Substituted
2-Mercaptobenzoimidazoles as Inhibitors of Hedgehog SignalingSimone Gräßle,[†] Steven Susanto,[†] Sonja Sievers,[§] Emel Tavsan,[†] Martin Nieger,^{||} Nicole Jung,^{*,†,‡,§} and Stefan Bräse^{*,†,‡,§}[†]Institute of Toxicology and Genetics, Karlsruhe Institute of Technology, Campus North, Hermann-von-Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen, Germany[‡]Institute of Organic Chemistry, Karlsruhe Institute of Technology, Fritz-Haber-Weg 6, 76131 Karlsruhe, Germany[§]Max Planck Institute of Molecular Physiology, Otto-Hahn-Str. 11, 44227 Dortmund, Germany^{||}Department of Chemistry, University of Helsinki, P.O. Box 55, FI-00014 Helsinki, Finland

S Supporting Information

ABSTRACT: Due to the arising resistance of common drugs targeting the Hedgehog signaling pathway, the identification of new compound classes with inhibitory effect is urgently needed. We were able to identify S-alkylated 2-mercaptobenzoimidazoles as a new compound class that exhibits Hedgehog signaling activity in a low micromolar range. The scope of the 2-mercaptobenzoimidazole motif has been investigated by the syntheses of diverse derivatives, revealing that the elongation of the linker unit and the exchange of particular substitution patterns are tolerable with respect to the activity of the compound class.

KEYWORDS: Hedgehog signaling, cancer, small molecules, 2-mercaptobenzoimidazoles

The Hedgehog (Hh) signaling pathway acts as a key mediator of many fundamental processes, such as cell fate specification, proliferation, and patterning, as well as tissue morphogenesis and homeostasis.¹ Pathway activation is initiated by the binding of one of the three secreted and lipid-modified ligands to Patched (PTCH1),² a 12-pass transmembrane-spanning receptor. In the absence of a ligand, PTCH1 constitutively represses the activity of Smoothened (SMO), a 7-pass transmembrane-spanning protein with homology to G-protein-coupled receptors.³ Following Hh ligand binding to PTCH1, the repression on SMO is released, and the expression and post-translational processing of the three GLI zinc-finger transcription factors is modulated. Misregulation of Hh signaling has been described in many malignancies, including cancers of the skin (basal cell carcinoma) and the brain (medulloblastoma) where activating mutations of Hh signaling components have been found.⁴ Furthermore, there are emerging roles for Hh signaling also in other cancer types such as breast, ovarian, and prostate cancer, while the particular contribution and effect of Hh signaling on different cancer types is still unclear.^{5–7} Synthetic small molecule inhibitors of Hh signaling like Vismodegib (1),^{8–11} Sonidegib (2),^{12,13} and BMS-833923 (3)¹⁴ (Figure 1) with a high chemical diversity are in clinical use or in clinical trials. In addition, natural small molecules like Berberine,¹⁵ Calcitriol,¹⁶ and others,^{17–19} which have been recently found to influence Hh signaling, are of interest for the search of new inhibitors that may overcome the developing resistance against currently available drugs.^{20,21} Searching for new structures with potential

Figure 1. Known inhibitors of the Hedgehog pathway.^{8–9,10,11,12,13,14}

to inhibit Hh signaling, an in-house library of 940 compounds²² has been screened resulting in the identification of 2-mercaptobenzoimidazoles as new lead structures. The class of 2-mercaptobenzoimidazoles has been shown to include potent heterocycles in medicinal chemistry due to their presence in drugs like omeprazole (4)^{23–25} or fabomotizole (5).²⁶ In particular, 2-(phenoxyalkylthio)-1H-benzimidazoles were reported as a promising compound class toward the targeting of Binding Function 3 (BF3)-site of the androgen receptor (Figure 2, 6).^{27,28} To the best of our knowledge, no activity of 2-mercaptobenzoimidazoles with respect to the Hh signaling pathway has been reported before.

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Figure 2. Structures of selected drugs including a 2-mercaptobenzimidazole core (4, 5),^{23–26} the potent BF3-inhibitor 6,²⁸ and a selected library member 7 [$R^1 = (\text{CH}_2)_3\text{CO}_2\text{Et}$].²⁹

Recently, we were able to present a solid supported strategy for the synthesis of diverse *S,N*-dialkylated 2-mercaptobenzimidazoles by immobilization and derivatization of mercaptobenzimidazole on solid supports.²⁹ The cleavage of the immobilized precursors yielded *N*-alkylated target compounds with different lengths of the alkyl side chain (7) (Figure 2), which were investigated toward their activity in Hh signaling along with a diverse set of compounds from academic research projects. Most of the tested *N,S*-dialkylated 2-mercaptobenzimidazoles showed no significant reduction of the Hh signaling; only the results of the derivative 7 suggested a slight inhibitory effect (12% inhibition). In order to find 2-mercaptobenzimidazoles with higher activity than given for the *N*-alkylated compound 7, a library of *S*-alkylated mercaptobenzimidazoles was synthesized.³⁰ Therefore, a literature-known procedure without functionalization of the NH-group was successfully used.²⁸ The library consisted of five main types of compounds (10 to 14) (Tables 1–3), which differ in the type or position of the heteroatoms N, O, and S and the length of the bridging linker unit (Scheme 1). All compounds were tested toward their activity in the Hedgehog signaling pathway and have been evaluated in comparison to the Hh inhibitor Vismodegib (1),

Scheme 1. General Scheme for the Synthesis of Compounds 10–14 and Crystal Structures for Selected Examples (10c, 11b)^a

^a(A) Two different conformers of 10c. (B) Asymmetric unit of 11b, showing the two crystallographic independent molecules (displacement parameters are drawn at 50% probability level).

being a benchmark drug for the inhibition of Hh signaling with an IC_{50} of approximately 0.1 μM in the herein described osteogenesis assay (Table 1).

For investigating signal transduction through the Hh pathway, mouse embryonic mesodermal fibroblast C3H/10T1/2 cells were used in the osteogenesis assay. These

Table 1. *O*-Aryl-Type C2-Based Mercaptobenzimidazoles 10a–10q

	R^1	R^2	R^3	R^4	R^5	Hh IC_{50} [μM] ^a	VIA IC_{50} [μM] ^{b,c}
10a	Me	H	H	H	Me	1.76 ± 0.07	inact
10b	H	Me	H	H	H	2.08 ± 0.15	inact
10c	H	H	H	H	H	2.22 ± 0.09	inact
10d	H	H	OH	H	H	2.23 ± 0.41	7.12
10e	F	H	H	H	H	2.32 ± 0.13	inact
10f	H	Me	Cl	H	H	2.57 ± 0.02	inact
10g	OMe	H	H	H	H	2.61 ± 0.08	inact
10h	Me	Me	H	H	Me	2.90 ± 0.50	inact
10i	Me	H	H	H	H	4.26 ± 0.86	inact
10j	H	NO_2	H	H	H	4.47 ± 0.18	inact
10k	H	$-(\text{CH}_2)_4-$		H	H	4.49 ± 0.44	inact
10l	Me	H	H	Me	H	6.41 ± 1.41	inact
10m	H	H	H	$i\text{Pr}$	H	9.17 ± 1.23	inact
10n	H	H	Ph	H	H	inact	inact
10o	$i\text{Pr}$	H	H	H	H	>10	inact
10p	NO_2	H	Me	H	H	inact	inact
10q	H	H	H	CO_2Me	H	inact	inact
1						0.14 ± 0.1	inact

^aHh inhibiting activity was determined using mesodermal stem cells C3H/10T1/2 in the osteogenesis assay. ^bVIA = viability, inact = inactive; cell viability was determined using Cell Titer Glo assay. ^cCompounds were scored inactive if the viability of the cells was higher than 80% (compared to the DMSO control).

multipotent mesenchymal progenitor cells can differentiate into osteoblasts upon treatment with the SMO agonist Purmorphamine.^{31,32} During differentiation, osteoblast specific genes such as alkaline phosphatase (ALK), which plays an essential role in bone formation, are highly expressed. The activity of ALK can be monitored by following substrate hydrolysis yielding a highly luminescent product. An inhibition of the pathway results in reduction of luminescence. The screening of the library of 2-(aryloxyalkylthio)-1*H*-benzimidazoles **10**–**14** revealed a significantly higher inhibition of the Hh pathway for almost all compounds in comparison to the initially identified *S,N*-dialkylated 2-mercaptobenzimidazole **7b**. Especially, several derivatives of the series of 2-((2-aryloxyethyl)thio)-1*H*-benzimidazoles **10a**–**q** were found to inhibit Hh signaling in the low micromolar range (Table 1). The activity of the individual compounds within the targeted substance class strongly depends on the substitution pattern of the phenolic ring system. While the 2,6-dimethylphenoxy derivative **10a** and the 3-methylphenoxy derivative **10b** gave the best results in the screening of the 2-mercaptobenzimidazole library, a 3-fold decrease of the inhibition was detected for very similar compounds like the 1,4-dimethylphenoxy-derivative **10l** (Table 1). Altogether, eight compounds of the series of type **10** yielding IC₅₀ values ≤ 3 μM (**10a**–**10h**) were identified as potent new candidates for the inhibition of Hedgehog signaling. Thereby, compound **10a** showed the strongest inhibition of the Hh signal (Figure 3 shows the dose-dependent inhibition of the

Figure 3. Dose–response curve of the most promising compound **10a**. Half-logarithmic plot of Hh activity against compound concentration.

Hh signal). While all compounds **10** rely on the same core structure, compounds of type **11**–**14** were examined to elucidate the influence of structural changes on the Hh signaling activity. The effect of structural changes on the main compound core was exemplarily investigated in order to define the scope of further modifications of the given 2-

mercaptobenzimidazole lead structure. The results for compounds **11a**–**11c** (Table 2) show, at least for this small set of synthesized compounds, that the exchange of the phenolic part with a thiophenolic part does not induce a loss of activity if a suitable substitution pattern is chosen. Contrary, the exchange of the benzimidazole core (e.g., **10c**, Table 1) with a methyl-benzothiazol-2-amine based core as given in compounds **12a,b** (Table 2) caused loss of activity.

In addition to the synthesis of ethylthio-substituted benzimidazoles **10**, a small set of propylthio-substituted benzimidazoles (**13**, **14a**–**14c**) (Table 3) has been inves-

Table 3. *S*- and *O*-Aryl-Type C3-Based Mercaptobenzimidazoles **13** and **14**

	Z	R ¹	R ²	R ³	R ⁴	R ⁵	Hh IC ₅₀ [μM] ^a	VIA IC ₅₀ [μM] ^{b,c}
13	S	H	H	Cl	H	H	2.85 ± 0.1	inact
14a	O	Me	H	H	H	Me	5.60 ± 0.31	inact
14b	O	H	H	Me	H	H	6.29 ± 1.26	inact
14c	O	H	Me	H	H	H	inact	inact

^aHh inhibiting activity was determined using mesodermal stem cells C3H/10T1/2 in the osteogenesis assay. ^bVIA = viability, inact = inactive; cell viability was determined using Cell Titer Glo assay. ^cCompounds were scored inactive if the viability of the cells was higher than 80% (compared to the DMSO control).

tigated to determine the effect of the aliphatic linker part on the inhibition of Hh signaling. It was found that the inhibition of the Hh signaling can also be achieved with compounds bearing a propyl linker part. Activity in a low micromolar range could be detected for compound **13** (Table 3), which has been synthesized with a thiophenolic moiety, while other derivatives bearing a phenolic building block (like compounds **14a**–**14c**) (Table 3) showed weaker inhibition values in comparison to the originally investigated ethylthio-substituted benzimidazoles (e.g., compound **10a** in direct comparison to compound **14a**). Except from one example (**10d**), none of the compounds affected the viability of the cells (see Figure 4). In addition, an inhibition of the alkaline phosphatase could also be excluded

Table 2. *S*-Aryl-Type C2-Based Mercaptobenzimidazoles **11a**–**c** and **12a,b**

	X	Y	Z	R ¹	R ²	R ³	R ⁴	R ⁵	Hh IC ₅₀ [μM] ^a	VIA IC ₅₀ [μM] ^{b,c}
11a	NH	S	S	H	Me	H	H	H	3.06 ± 0.18	inact
11b	NH	S	S	H	OMe	H	H	H	5.63 ± 0.73	inact
11c	NH	S	S	Cl	Cl	Cl	H	H	5.71 ± 0.91	inact
12a	S	NH	O	Me	H	H	Me	Me	>10	inact
12b	S	NH	O	H	H	H	H	Me	inact	inact

^aHh inhibiting activity was determined using mesodermal stem cells C3H/10T1/2 in the osteogenesis assay. ^bVIA = viability, inact = inactive; cell viability was determined using Cell Titer Glo assay. ^cCompounds were scored inactive if the viability of the cells was higher than 80% (compared to the DMSO control).

Figure 4. Structure–activity relationship for 2-(aryloxyalkylthio)-1H-benzimidazoles as Hh inhibitors.

(Supporting Information). A summary of the structure–activity relationship for 2-(aryloxyalkylthio)-1H-benzimidazoles as Hh inhibitors is presented in Figure 4.

To validate the biological activity of the mercaptobenzimidazoles, a selection of compounds was tested for their Hh inhibition in the Shh-LIGHT 2 assay. The Shh-LIGHT 2 cells carry a firefly luciferase reporter under the control of a GLI-dependent promoter. Luciferase gene activity can be specifically induced by treatment of the cells with the Hh agonist Purmorphamine; while treatment with inhibitors leads to a reduction in the luminescent signal. The activity against Hh signaling of the most important compounds was confirmed using this orthogonal assay system. Just as already seen in the osteogenesis results, compound **10a** was found to be the most active compound, while, e.g., compound **10q** was inactive (Table 4). Again, compound **10d** was the only compound that affected the viability of the Shh-LIGHT 2 cells.

Table 4. Results of the Shh-LIGHT 2 Assay

	Shh light IC ₅₀ [μM] ^a	VIA IC ₅₀ [μM] ^{b,c}		Shh light IC ₅₀ [μM] ^{a,c}	VIA IC ₅₀ [μM] ^{b,c}
10a	3.16 ± 0.12	inact	10k	7.29 ± 2.15	inact
10b	5.11 ± 1.05	inact	10q	inact	inact
10c	8.92 ± 1.51	inact	11a	3.48 ± 0.12	inact
10d	8.12 ± 2.31	7.70	11b	3.55 ± 0.23	inact
10f	7.89 ± 2.19	inact	13	>10	inact
10i	6.93 ± 2.22	inact	14a	4.12 ± 0.54	inact
10j	6.49 ± 2.50	inact	1	0.98 ± 0.04	inact

^aHh inhibiting activity was validated in a subset of compounds using Shh-LIGHT 2 assay. ^bVIA = viability, inact = inactive; cell viability was determined using Cell Titer Glo assay. ^cCompounds were scored inactive if the remaining activity or viability after compound treatment was higher than 80% (compared to the DMSO control).

While several small molecules targeting the Hh signaling pathway are well-known drugs in clinical use, the arising resistance toward those established compounds enforces the identification of new compound classes with inhibitory effect for the development of new lead structures. We discovered S-alkylated 2-mercaptobenzimidazoles as a new compound class that exhibits activity in the low micromolar range. Although all of the tested compounds were found to have lower activities in the osteogenesis assay in comparison to the established Hh inhibitor Vismodegib currently in clinical use, they serve as beneficial starting points for novel modes of Hh inhibition to overcome resistance. The scope of the 2-mercaptobenzimidazole motif been investigated by the synthesis of further derivatives, showing that the elongation of the linker unit and the exchange of particular substitution patterns are tolerable without a noteworthy loss of activity. These promising results will be used for further modifications of the 2-mercaptobenzimidazole core structure aiming for the enhancement of the compound activities into a nanomolar range in the future.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmmedchemlett.7b00100.

Syntheses of the 2-mercaptobenzimidazoles **10–14** and their precursor **9** as well as the analytical data, copies of the spectra of all synthesized compounds, and crystallographic data of **10c** and **11b**, and biological methods (PDF)

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Notes

The authors declare no competing financial interest. CCDC 1561471 (**10c**) and CCDC 1561472 (**11b**) contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

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■ ABBREVIATIONS

Hh, Hedgehog; ALK, alkaline phosphatase; SMO, smoothened; Ptch1, patched; BF3, binding function 3; VIA, viability; IC, inhibitory concentration; inact, inactive

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