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Aromatic 2-(Thio)ureidocarboxylic Acids As a New Family of Modulators of Multidrug Resistance-Associated Protein 1: Synthesis, Biological Evaluation, and Structure−Activity Relationships

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Four series of aromatic carboxylic acids were prepared with a urea or thiourea moiety at the neighboring position to the carboxyl group and benzene or thiophene as aromatic scaffold. Using a calcein AM assay, these compounds were evaluated as inhibitors of multidrug resistance-associated protein 1 (MRP1) and selected compounds were examined toward P-glycoprotein (P-gp) as well as breast cancer resistance protein (BCRP) to assess selectivity for MRP1. Two 2-thioureidobenzo[*b*]-thiophene-3-carboxylic acids (**48**, **49**) were identified as particularly potent inhibitors of MRP1, with IC_{50} values of around 1 μM. The structural features of this new family of nontoxic MRP1 inhibitors include a (thio)urea disubstituted with preferentially two alkyl groups at the terminal nitrogen and an additional fused aromatic ring.

Introduction

Failure to respond to chemotherapy is a serious impediment in the treatment of cancer. In this regard, the development of multidrug resistance (MDR[a]) is frequently observed, which leads to a reduced sensibility of cancer cells toward a broad spectrum of cytostatics. The mostly involved mechanism of MDR is associated with the overexpression of transporters of the ATP binding cassette (ABC) family. These efflux pumps are localized in the cell membrane and reduce the intracellular concentration of multiple structurally and functionally unrelated drugs by an active extrusion at the cost of ATP hydrolysis.[1−5]

P-glycoprotein (P-gp), the first discovered member of ABC efflux transporters, possesses their typical architecture with two transmembrane domains usually consisting of six transmembrane helices and two cytoplasmic nucleotide-binding sites.[6] P-gp directed modulators belong to various chemical classes, most of them being well-established drugs in different pharmacological areas. Considerable effort has been devoted to identify new P-gp modulators,[7−14] and pharmacophore patterns of P-gp ligands addressing different binding sites have been derived.[15−19]

Besides the well-known role of P-gp in multidrug resistance, other ABC transporters, e.g., the multidrug resistance-associated proteins (MRPs) and the breast cancer resistance protein (BCRP), can confer multidrug resistance to cancer cells.[1−3,20]

Subsequent to the discovery of P-gp, MRP1 was identified in 1992 as the first member of the ABCC subfamily.[21] This 190 kDa membrane-spanning protein is encoded by the *ABCC1* gene mapped to chromosome 16p13.1. MRP1 shares the common structural motif of ABC proteins. Similar to P-gp, MRP1 is composed of the large core segment containing the nucleotide binding sites and two transmembrane domains but additionally possesses a third transmembrane domain with five helices and an extracellular *N*-terminus.[22−24] Its amino acid sequence resembles that of P-gp only to a modest extent of ∼15%.[25]

The ubiquitous expression of MRP1 in human tissues, e.g., in endothelial cells of brain capillaries, epithelial cells of the digestive, urogenital, and respiratory tracts, endocrine glands, and the hematopoietic system implies an important role for MRP1 in the protection against xenobiotics and endogenous toxins.[24,25] Since its discovery in the H69AR cancer cell line, MRP1 has been found in multidrug-resistant cell lines derived from different tissues and tumor types, for example, in lung, colon, breast, bladder, and prostate cancer as well as leukemia.[21,26−28] MRP1 has unusually broad substrate specificity. Like P-gp, it is capable of transporting a wide variety of anticancer agents, e.g., anthracyclines, *Vinca* alkaloids, and methotrexate. In contrast to P-gp, MRP1 is facilitating the extrusion of negatively charged compounds such as numerous glucuronate, sulfate, and glutathione conjugates, including the eicosanoid leukotriene C_4.[22,24,29−31]

Much effort has been focused on the finding of inhibitors for MRP1, but the number of published compounds is considerably lower than those available for P-gp.[3,32,33] Whereas most P-gp inhibitors failed to affect MRP1, cyclosporin A and verapamil proved active against MRP1. Additionally, several specific inhibitors have been described within the last 15 years.

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[a] Abbreviations: MRP, multidrug resistance-associated protein; calcein AM, acetoxymethyl ester of calcein; P-gp, P-glycoprotein; MDR, multidrug resistance; ABC, ATP binding cassette; BCRP, breast cancer resistance protein; WT, wild-type; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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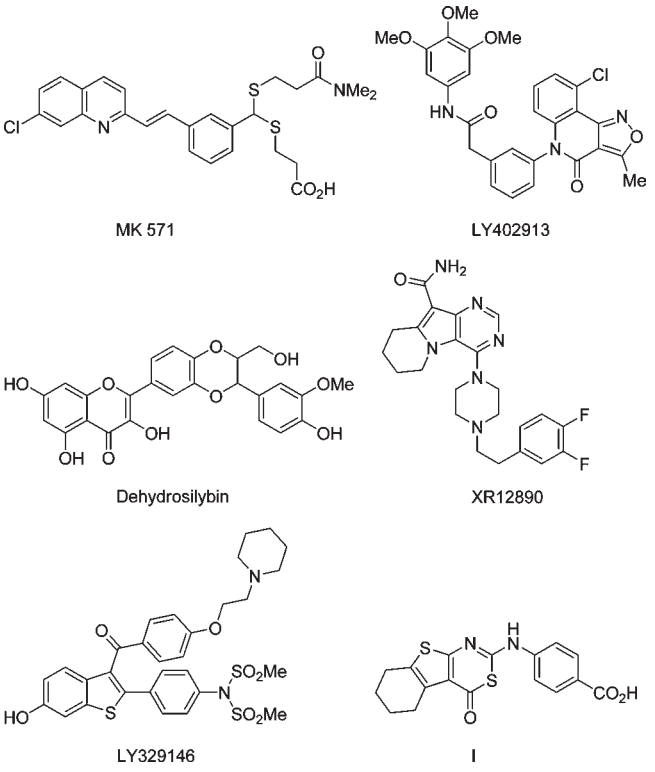


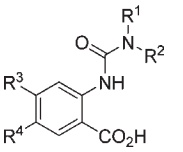
Figure 1. Structures of known MRP1 inhibitors.

The leukotriene D_4 receptor antagonist MK 571 inhibited the leukotriene C_4 transport by membrane vesicles from MRP1-transfected cells,[34,35] and it was frequently used in recent pharmacological studies (Figure 1).[36−43] MK 571 exhibited an K_i value of 0.6 μM in MRP1-mediated leukotriene C_4 transport,[34] IC_{50} values of 3.3 μM in ATP-dependent [^{3}H] *para*-aminohippurate transport,[44] and 7.6 μM in the calcein AM assay.[45]

Among various flavonoids,[46] dehydrosilybin was a particularly active inhibitor of leukotriene C_4 transport.[47] The selective estrogen receptor modulator raloxifene was used as a lead structure to conceive inhibitors of MRP1-mediated MDR. The analogue LY329146 with a bis(methylsulfonyl) amino group in place of one hydroxyl group in raloxifene showed an improved activity.[48] The tricyclic isoxazole LY402913 was identified as a potent and selective modulator by reversing drug resistance to different MRP1 substrates in HeLa-T5 and A2780 cells. Furthermore, LY402913 delayed tumor progression in a xenograft mouse model.[49,50] Another series of selective and highly efficient MRP1 inhibitors derived from a pyrrolo[3,2-*d*]pyrimidine template was reported by Wang et al. Structural modifications led to pyrrolo- and indolopyrimidines, exhibiting IC_{50} values of approximately 100 nM. Some derivatives such as XR12890 displayed good pharmacokinetic profiles, e.g., high oral bioavailability and limited interactions with cytochromes P450.[51,52] Recently, we identified a thieno[2,3-*d*][1,3]thiazin-4-one derivative **I** being active at MRP1 and 2.[45]

The starting point for this study was the evaluation of an in-house library of low molecular weight compounds for modulating effects on MRP1 in a fluorometric calcein AM assay.[45,53] Initially, the selection was focused on carboxylic acids with respect to the anionic nature of the MRP1 substrates. More than 75 structurally diverse carboxylic acids were inspected. Among them, some aromatic 2-(thio)ureido carboxylic acids showed modulating activity on MRP1. On the basis of these initial results, it was intended to provide four series of aromatic carboxylic acids. All compounds bear either a substituted urea or thiourea moiety at the neighboring position to the carboxyl group. As an aromatic scaffold, either a (substituted) benzene or thiophene ring was chosen. The evaluation of these compounds as MRP1 inhibitors is presented herein.

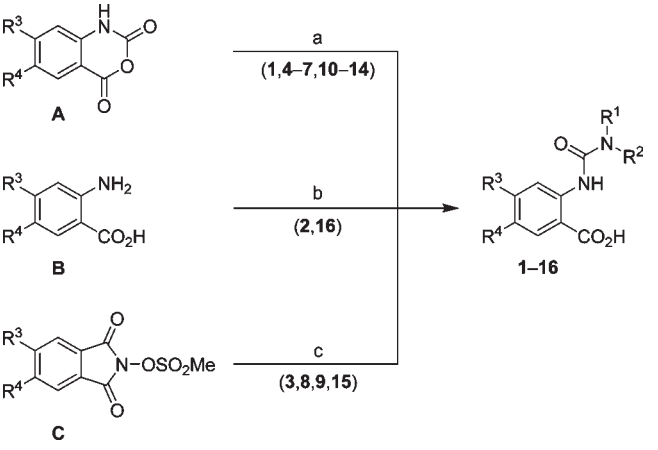
Table 1. 2-Ureidobenzoic Acids **1**−**16**



compd	NR^1R^2	R^3	R^4	prepared from	$IC_{50} \pm SD$ (μM)
1	NHBn	H	H	**A**	ni[d]
2	$N(Et)_2$	H	H	**B**	26.4 ± 10.1
3	$N(Me)CH_2CO_2Me$	H	H	**C**	ni
4[a,b,c]	N(Me)cyclohexyl	H	H	**A**	12.3 ± 2.8
5	N(Me)Ph	H	H	**A**	ni
6	N(Me)Bn	H	H	**A**	42.6 ± 1.6
7	$N(Me)CH_2CH_2Ph$	H	H	**A**	ni
8	1-pyrrolidinyl	H	H	**C**	43.1 ± 11.1
9	4-morpholinyl	H	H	**C**	ni
10	N(Me)cyclohexyl	H	Me	**A**	22.8 ± 6.2
11	N(Me)Ph	H	Me	**A**	ni
12	N(Me)Bn	H	Me	**A**	ni
13	$N(Me)CH_2CH_2Ph$	H	Me	**A**	ni
14	4-morpholinyl	H	Me	**A**	ni
15	4-morpholinyl	Me	Me	**C**	43.4 ± 13.3
16[a,b,c]	$N(Et)_2$	−CH=CH−CH=CH−		**B**	5.38 ± 0.68

[a] Compounds showing no effect in the P-gp assay. [b] Compounds (at 31.6 μM) inhibited BCRP between 25% and 65% relative to the inhibitory effect of XR9577 (at 10 μM). [c] No cytotoxicity (IC_{50} > 100 μM). [d] ni, no inhibition of the MRP1-mediated transport.

Scheme 1[a]

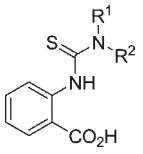


[a] Reagents and conditions: (a) HNR^1R^2, H_2O, room temperature, or HNR^1R^2, EtOH, H_2O, reflux. (b) (1) *N*,*N*′-carbonyldiimidazole, CH_2Cl_2 or THF, room temperature; (2) HNR^1R^2, room temperature. (c) (1) HNR^1R^2, acetone, reflux; (2) 0.25 M HCl, 5 °C.

Chemistry

The final 2-ureidobenzoic acids **1**−**16** are listed in Table 1, their preparation is shown in Scheme 1. The majority of

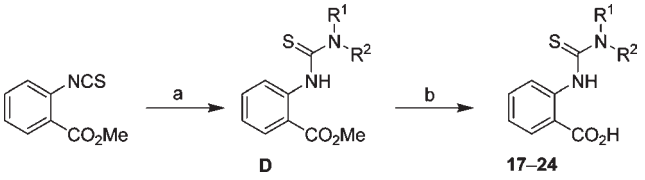
Table 2. 2-Thioureidobenzoic Acids **17**−**24**



compd	NR^1R^2	$IC_{50} \pm SD$ (μM)
17	$N(Et)_2$	21.6 ± 5.3
18[a,b,c]	N(Me)cyclohexyl	16.2 ± 4.2
19	N(Me)Ph	198 ± 29
20	N(Me)Bn	29.1 ± 9.1
21	$N(Me)CH_2CH_2Ph$	24.0 ± 6.9
22	1-pyrrolidinyl	18.3 ± 4.6
23	1-piperidinyl	20.7 ± 2.5
24	4-morpholinyl	ni[d]

[a] Compound showing no effect in the P-gp assay. [b] Compound (at 31.6 μM) inhibited BCRP less than 20% relative to the inhibitory effect of XR9577 (at 10 μM). [c] No cytotoxicity (IC_{50} > 100 μM). [d] ni, no inhibition of the MRP1-mediated transport.

Scheme 2[a]



[a] Reagents and conditions: (a) HNR^1R^2, CH_2Cl_2, room temperature. (b) (1) 1 M NaOH, EtOH (1:1) reflux; (2) H_2O; (3) 2 M HCl.

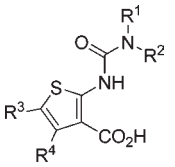
such compounds was obtained from the reaction of isatoic anhydrides **A** with benzylamine or appropriate secondary amines.[54] To prepare 2-(3,3-diethylureido)benzoic acid (**2**), anthranilic acid (**B**, $R^3 = R^4 = H$) was pretreated with *N*,*N*′-carbonyldiimidazole followed by the addition of diethylamine. Similarly, the naphthalene derivative **16** was obtained from 3-amino-2-naphthalenecarboxylic acid (**B**, R^3R^4 = −CH=CH−CH=CH−). The preparation of further 2-ureidobenzoic acids was accomplished when *N*-(mesyloxy)phthalimides **C** were reacted with secondary amines.[55]

The two-step synthetic route to the 2-thioureidobenzoic acids **17**−**24** (Table 2) is depicted in Scheme 2. The methyl benzoate precursors **D** were prepared from secondary amines and methyl 2-isothiocyanatobenzoate[56] in dichloromethane and subsequently saponified with aqueous ethanolic sodium hydroxide.

2-Ureidothiophene-3-carboxylic acids **25**−**28** (Table 3) were synthesized by two alternative routes (Scheme 3). Thieno[2,3-*d*][1,3]oxazine-4-ones **E** underwent ring-opening upon treatment with sodium hydroxide. Because a disubstituted amino group prevented Dimroth rearrangement to pyrimidinediones, the desired noncyclized ureas **25**, **27**, and **28** were obtained. Trifluoroacetic acid promoted the cleavage of the *tert*-butyl ester group in **F** to give the corresponding isopropylurea **26**. Both routes started with the Gewald thiophene synthesis[57,58] to alkyl 2-aminothiophene-3-carboxylates, which were converted either with isopropyl isocyanate to **F** or in five reaction steps to the heterocycles **E**.[59]

The preparation of thiophenecarboxylic acids with a thiourea moiety at the neighboring position is shown in Scheme 4. Compounds **30**−**50** (Table 4) were either obtained

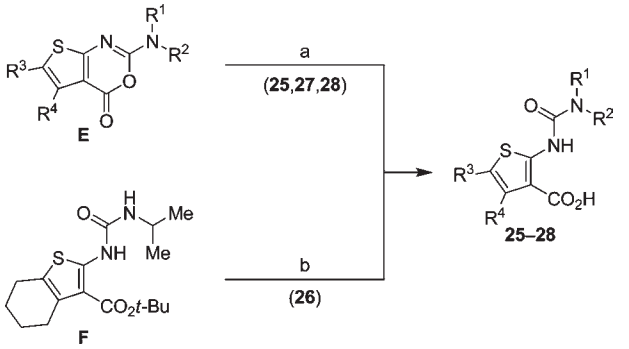
Table 3. 2-Ureidothiophene-3-carboxylic Acids **25**−**28**



compd	NR^1R^2	R^3	R^4	prepared from	$IC_{50} \pm SD$ (μM)
25[a,b,c]	$N(Et)_2$	$-(CH_2)_3-$		**E**	11.6 ± 3.1
26	$NHCH(Me)_2$	$-(CH_2)_4-$		**F**	ni[d]
27[a,b,c]	$N(Et)_2$	$-(CH_2)_4-$		**E**	8.29 ± 4.22
28	4-morpholinyl	$-(CH_2)_4-$		**E**	ni

[a] Compounds showing no effect in the P-gp assay. [b] Compounds (at 31.6 μM) inhibited BCRP between 25% and 65% relative to the inhibitory effect of XR9577 (at 10 μM). [c] No cytotoxicity (IC_{50} > 100 μM). [d] ni, no inhibition of the MRP1-mediated transport.

Scheme 3[a]



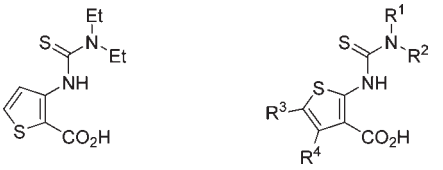
[a] Reagents and conditions: (a) (1) 1.5 M NaOH, acetone (2:1), reflux; (2) 3 M HCl. (b) TFA, 0 °C to room temperature.

by saponification of the ethyl esters **H** or by sodium hydroxide-promoted cleavage of the thiazinone ring of compounds **I**. With respect to R^3 and R^4, either non-, mono-, and disubstituted thiophenes (**30**−**39**) or fused compounds (**40**−**50**) have been synthesized. All compounds were accessible by an initial Gewald reaction. To obtain the aromatic fused ring system in **48**−**50**, the corresponding isothiocyanatobenzo[*b*]-thiophene **G** (R^3R^4 = −CH=CH−CH=CH−) afforded three additional steps. The final compound **29** was achieved following an analogous route, i.e., conversion of methyl 3-aminothiophene-2-carboxylate to the isothiocyanate and the diethylthioureido derivative, which was cyclized with concentrated sulfuric acid to the corresponding 1,3-thiazine-4-one and reopened to **29**.[59]

Biology

An accumulation assay using the MRP1 substrate calcein AM was performed to determine the influence of the test compounds on the transporter protein MRP1.[33,45,53] Calcein AM is a lipophilic, nonfluorescent acetoxymethyl ester of calcein that easily penetrates cellular membranes. Intracellular calcein AM can either be transported out of the cell by MRP1 or cleaved by unspecific cytosolic esterases to produce the fluorescent calcein. The human ovarian cancer cell line 2008 stably expressing MRP1 was used. In 2008 MRP1 cells, the efflux of calcein AM leads to a decreased intracellular fluorescence, compared to the corresponding wild-type cell

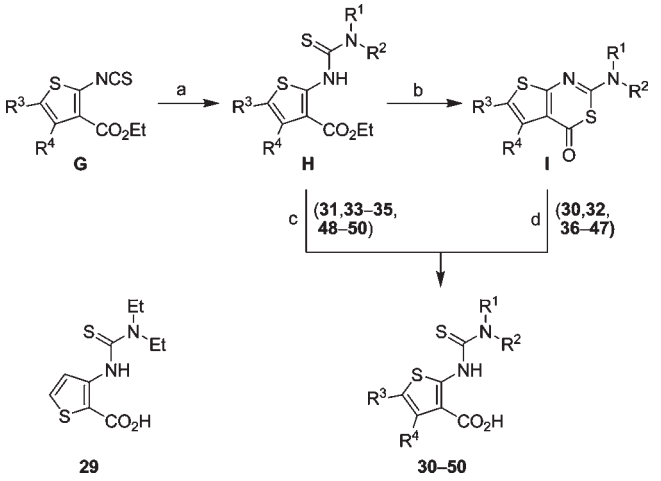
Table 4. 3-(Diethylthioureido)thiophene-2-carboxylic Acid (**29**, Left) and 2-(Thioureido)thiophene-3-carboxylic Acids **30**−**50** (Right)



compd	NR^1R^2	R^3	R^4	prepared from	$IC_{50} \pm SD$ (μM)
29[a,b,c]					4.18 ± 0.70
30[a,b,c]	$N(Et)_2$	H	H	**I**	4.46 ± 0.58
31[a,c,d]	N(Me)cyclohexyl	H	H	**H**	4.51 ± 0.49
32	$N(Et)_2$	H	$CH(Me)_2$	**I**	14.5 ± 5.5
33	4-morpholinyl	H	Ph	**H**	ni[h]
34[c,e]	$N(Et)_2$	Ph	H	**H**	14.3 ± 4.7
35	4-morpholinyl	Ph	H	**H**	27.5 ± 3.3
36	$N(Et)_2$	Me	Me	**I**	20.4 ± 2.1
37[a,b,c]	N(Me)cyclohexyl	Me	Me	**I**	4.19 ± 1.51
38	4-morpholinyl	Me	Me	**I**	18.3 ± 4.5
39	$N(Et)_2$	$CONH_2$	Me	**I**	> 100
40[a,b,c]	$N(Et)_2$	$-(CH_2)_3-$		**I**	2.56 ± 1.11
41	$N(Me)_2$	$-(CH_2)_4-$		**I**	ni
42[a,b,c]	$N(Et)_2$	$-(CH_2)_4-$		**I**	2.71 ± 0.35
43	N(Me)cyclohexyl	$-(CH_2)_4-$		**I**	nd[i]
44	4-morpholinyl	$-(CH_2)_4-$		**I**	ni
45	4-methyl-1-piperazinyl	$-(CH_2)_4-$		**I**	ni
46[a,c,f]	$N(Et)_2$	$-(CH_2)_3CH(CH_3)-$		**I**	4.06 ± 1.05
47[a,b,c]	$N(Et)_2$	$-(CH_2)_5-$		**I**	5.97 ± 3.83
48[a,c,g]	$N(Et)_2$	$-CH=CH-CH=CH-$		**H**	0.932 ± 0.041
49[a,b,c]	N(Me)cyclohexyl	$-CH=CH-CH=CH-$		**H**	1.23 ± 0.29
50	4-morpholinyl	$-CH=CH-CH=CH-$		**H**	ni

[a] Compounds showing no effect in the P-gp assay. [b] Compounds (at 31.6 μM) inhibited BCRP between 25% and 65% relative to the inhibitory effect of XR9577 (at 10 μM). [c] No cytotoxicity (IC_{50} > 100 μM). [d] Partial inhibition of BCRP with $IC_{50app} = 10.2 \pm 3.6$ μM. [e] 20% inhibition at 31.6 μM, 40% inhibition at 100 μM in the P-gp assay. [f] Partial inhibition of BCRP with $IC_{50app} = 9.10 \pm 1.71$ μM. [g] Partial inhibition of BCRP with $IC_{50app} = 6.16 \pm 1.36$ μM. [h] ni, no inhibition of the MRP1-mediated transport. [i] nd, not detectable due to concentration-dependent decrease of fluorescence in both wild-type and MRP1-transfected cells.

Scheme 4[a]



[a] Reagents and conditions: (a) HNR^1R^2, CH_2Cl_2, room temperature. (b) (1) Concd H_2SO_4, room temperature; (2) H_2O; or (1) polyphosphoric acid, 170 °C; (2) ice, EtOH; (c) (1) 1 M NaOH, EtOH (1:1) reflux; (2) H_2O; (3) 2 M HCl. (d) (1) 3 M NaOH, dioxane (1:2), (2) ice, H_2O; (3) 3 M HCl.

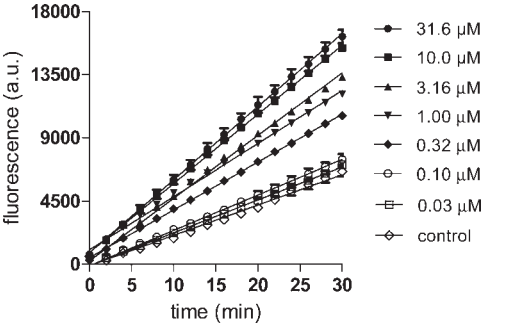


Figure 2. Fluorescence−time curves for different concentrations of compound **48** determined with the calcein AM assay in 2008 MRP1 cells. Presented data are averages from a typical experiment with two replicates belonging to a series of three independent experiments.

line, 2008 WT. An MRP1 inhibitor blocks calcein AM efflux, leading to an enhancement of calcein concentration and intracellular fluorescence (Figure 2). In contrast, as the wild type does not exhibit MRP1, addition of an inhibitor to 2008 WT cells has no effect on fluorescence (Figure 3). When performing the assay, XR9577,[60] a selective blocker of P-gp and BCRP, having no effect on MRP1,[61] was added to inhibit P-gp, which is present to a minor degree in both MRP1-expressing and wild-type cells. The assay medium contained cobalt ions to quench extracellular calcein fluorescence so that only the fluorescence of intracellular calcein is measured.

A subset of active compounds was defined and tested for the ability to affect P-gp in a similar calcein AM assay

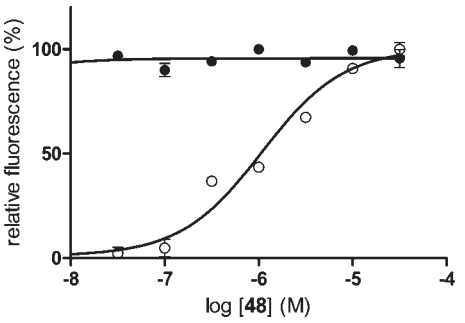


Figure 3. Concentration−effect curve of compound **48** in MRP1-transfected cells (open circles) in comparison to the wild-type cells (closed circles). Curves represent the average of at least five independent experiments. For normalization of data, slopes from fluorescence−time curves were transformed to relative units by subtracting the lowest determined single value from all other data and thus setting it to 0%. The highest measured single value was defined as 100%.

using P-gp overexpressing A2780/ADR cells. Furthermore, these compounds were investigated for modulation of BCRP in MCF-7 MX cells in an accumulation assay with the fluorescent substrate Hoechst 33342.[62] Cytotoxicity of the subset of compounds was determined in 2008 WT cells by measuring viability via MTT assay.[63] The MTT assay was also used to assess potentiating effects of two exemplary 2-ureidothiophene-3-carboxylic acids (**31**, **48**) in combination with the cytotoxic drugs vinblastine and daunorubicin.

Results

In search of MRP1 inhibitors, we identified active representatives of 2-ureidobenzoic acids. All 2-ureidobenzoic acids (**1**−**16**) investigated are outlined in Table 1. The presence of a diethylureido and a cyclohexylmethylureido moiety provided active compounds (**2**, **4**, **10**, **16**). A replacement of the cyclohexyl residue by a phenyl, benzyl, or phenethyl group mostly led to a loss of potency (**4** versus **5**, **7**; **10** versus **11**−**13**). While the introduction of a 5-methyl substituent (R^4 = Me) did not improve biological activity, an additional fused benzene ring was advantageous (**2** versus **16**). The naphthalene derivative **16** (IC_{50} = 5.38 μM) was the only potent substance among the 2-ureidobenzoic acids. Compound **16** did not inhibit P-gp, showed a weak inhibition of BCRP and was not cytotoxic. Whereas seven of the 16 2-ureidobenzoic acids inhibited MRP1, no active compound was identified from a series of 13 related anthranilic acid derivatives (see Supporting Information, Chart S1), i.e., benzoic acids with a 2-alkylamino, 2-arylamino, 2-acylamino, 2-alkoxycarbonylamino, and 2-(3-aroylureido) substituent.

Compounds **17**−**24** are benzoic acids with a thiourea moiety at position 2 (Table 2). As in the ureidobenzoic acid series, a cyclohexylmethylureido moiety in **18** (IC_{50}=16.2 μM) accounted for activity. This substance did not inhibit P-gp but showed also a weak inhibition of BCRP. No cytotoxicity was observed for **18**. Toward MRP1, all compounds, except of **19** and **24**, had IC_{50} values below 30 μM. When comparing analogous ureas with thioureas, a clear effect of the oxygen−sulfur exchange could not be observed (**2**, **4**−**9** versus **17**−**22**, **24**). Five benzoic acids with a 2-(3-aroylthioureido) residue and optional aromatic substituents have also been investigated and proved to be inactive. The replacement of the carboxyl group in **17** by a cyano function abolished activity, indicating the importance of carboxyl group (see Supporting Information, Chart S2).

IC_{50} values of 2-ureidothiophene-3-carboxylic acids **25**−**28** are in given in Table 3. A 2-(3-isopropylureido) or 2-[(4-morpholinylcarbonyl)amino] residue gave inactive compounds. The activities of the two diethylurea derivatives **25** and **27** (IC_{50} = 11.6 and 8.29 μM) were between those of the two benzoic acid-derived diethylureas **2** and **16**. Both compounds, **25** and **27**, did not affect P-gp-mediated transport but were also weak inhibitors of BCRP. They did not exhibit cytotoxicity. Four thiophene-3-carboxylic acids with 2-acylamino or 2-alkoxycarbonylamino groups in place of the 2-ureido function were also evaluated but had no effect on MRP1-mediated transport (see Supporting Information, Chart S3).

Thiophenecarboxylic acids **29**−**50** with an *ortho*-thioureido substituent are outlined in Table 4. Again, the carboxyl group was required for activity as the corresponding methyl ester of **29** and the corresponding carbonitrile of **42** did not affect MRP1 (see Supporting Information, Chart S4). Within the thioureido residue, a diethylamino or cyclohexylmethylamino substitution pattern was advantageous over a more polar one, i.e., morpholino or methylpiperazino. The two isomeric thiophenes **29** and **30** were equipotent.

Starting from 2-(3-diethylthioureido)thiophene-3-carboxylic acid (**30**), the influence of substituents in positions 4 and 5 was examined. The potency was somewhat diminished by introducing two methyl groups (**30** versus **36**), or an isopropyl group (**32**), but was lost by replacing the 5-methyl by a polar carboxamide moiety (**39**). A fused cycloaliphatic ring was tolerated in most cases and provided slightly improved inhibitors, i.e., the tri- and tetramethylene compounds (**30** versus **40** and **42**). Introduction of a methyl group into the tetramethylene chain of **42** resulted in a slight decrease of activity (**46**). As in the 2-ureidobenzoic acid series (compound **16**) a bicyclic aromatic system was advantageous. An additional fused benzene ring in the benzothiophene **48** accounted for the most potent MRP1 inhibitor of our study with an IC_{50} value of 0.932 μM. Increased potency of **48** compared to the analogous cycloaliphatic derivative **42** could be due to planarity of the fused aromatic system. Removing the steric fixation of the annelated benzene ring in favor of a substituted benzene ring considerably dropped activity (**48** versus **34**). Although it was demonstrated by means of X-ray crystallography that the biaryl structure in **34** is nearly in plane (data not shown), the extended aromatic system might lead to steric repulsion. An exchange of the diethylamino group in **48** for a cyclohexylmethylamino substituent in the thioureido residue led to the other potent benzothiophene **49** (IC_{50} = 1.23 μM). Again, the exchange of the dialkyl moiety for a polar morpholino substitutent in **50** resulted in a loss of activity.

When comparing the MRP1-inhibiting properties of analogously substituted thiophenes (**25**, **27** versus **40**, **42**), thioureas were superior to ureas. The bioisosteric benzene−thiophene exchange improved the activity as can be seen from the comparison of the parent diethylthioureidocarboxylic acids (**17** versus **29** and **30**).

Selected MRP1 inhibitors of this series (Table 4) were evaluated as modulators of P-gp, but only **34** showed a weak inhibitory activity. However, inhibition of BCRP was observed in all these cases. Compounds **31**, **46**, and **48** behaved as partial inhibitors of BCRP with a maximum inhibitory effect of 60% relative to XR9577 and apparent IC_{50} values between 6 and 10 μM. The selected MRP1 inhibitors of this series were not cytotoxic. In the modified MTT assay, MRP1-expressing cells were treated with **31** or **48** (in a fixed

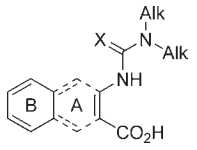


Figure 4. Structural features for bioactive aromatic 2-(thio)ureidocarboxylic acids.

concentration of 31.6 μM) and vinblastine or daunorubicin (in different concentrations). These combinations did not result in an increased cytotoxicity of vinblastine and daunorubicin, respectively (see Supporting Information, Figures S1−S4).

Discussion

On the basis of the structure−activity relationships detailed above, we combined the advantageous structural features as can be deduced from the biological activities of the four series of aromatic carboxylic acids (Figure 4). The best MRP1 inhibitors of the present series are more potent than verapamil, indomethacin, MK 571, and cyclosporin A for which IC_{50} values between 5 and 12 μM had been determined in the calcein AM assay.[45] They have the same order of activity as dehydrosilybin (IC_{50} = 1.1 μM),[32] LY329146 (IC_{50} = 0.8 μM),[32] LY402913 (IC_{50} = 1.8 μM),[64] and compound **I** (IC_{50} = 1.2 μM)[45] but are less potent than the recently reported pyrrolo- and indolopyrimidines such as XR12890.[51,52] The structural features derived for the aromatic carboxylic acids of this study (Figure 4) reflects their differences to other classes of MRP1 inhibitors (Figure 1). The essential components are a carboxyl group at a (hetero)aromatic scaffold with a (thio)urea function in *ortho*-position. The terminal nitrogen of the (thio)urea should be disubstituted, preferentially with two alkyl groups (e.g., diethyl or cyclohexylmethyl). The (hetero)aromatic ring (A) consists of benzene or thiophene, while the orientation of the sulfur seems to have minor influence on activity. An additional fused benzene ring (B) improves the inhibitory activity against MRP1. One the basis of this study, further investigations are ongoing in our laboratories. Structural modifications of the title compounds will include an exchange of rings A and/or B for other heteroaromatics, additional modifications at *N*-3 of the (thio)urea (e.g., cyclohexylethyl or di(iso)propyl), and a bioisosteric replacement of the carboxyl group against a tetrazole moiety.[65]

We evaluated toxicity for 16 selected compounds and did not observe cytotoxic properties. However, compounds **31** and **48** failed to reverse multidrug resistance of MRP1-expressing cells against vinblastine and daunorubicin, respectively. Although different transport mechanisms of substrates are well characterized for MRP1, the mechanism of inhibition is poorly understood.[2,5,32,33,66] Perrotton et al. investigated modulating effects of (*R*)- and (*S*)-verapamil on MRP1.[67] Both enatiomers induced an increase in calcein AM accumulation in MRP1-overexpressing cells, thus implying effective inhibition of the efflux pump. In potentiation assays, only (*R*)-verapamil reverted resistance of MRP1-BHK-21 cells to vincristine. As **31** and **48** in our case, (*S*)-verapamil did not show a cytotoxicity-potentiating effect. The different modulation of MRP1 by both enatiomers was discussed as explanation of controversial results of verapamil in reversion of chemoresistance.[40,67−69]

The most potent representatives of the four series were evaluated for modulating effects on BCRP- and P-gp-mediated transport. All selected substances showed a weak inhibition of Hoechst 33342 efflux mediated by BCRP. Overlapping inhibitors of different ABC transporter are discussed in the literature.[3,23,70−72] Our finding that carboxylic acids are capable of an interaction with BCRP is in agreement with a recently published report revealing MK571 to be a BCRP inhibitor.[71] On the other hand, all MRP1 inhibitors with IC_{50} values less than 10 μM did not affect P-gp. As P-gp generally does not transport negatively charged compounds, it might be concluded that the presence of a carboxylic acid moiety accounts for this selectivity.

Experimental Section

General Methods and Materials. Thin-layer chromatography was carried out on Merck aluminum sheets, silica gel 60 F_{254}. Preparative column chromatography was performed on Merck silica gel 60 (70−230 mesh). Melting points were determined by a Boëtius melting point apparatus (PHMK, VEB Wägetechnik Rapido, Radebeul, Germany) and are uncorrected. ^{1}H NMR (300 MHz) and ^{13}C NMR spectra (75 MHz) were recorded on a Varian Gemini 300. ^{1}H NMR (500 MHz) and ^{13}C NMR spectra (125 MHz) were recorded on a Bruker Avance DRX 500. Chemical shifts δ are given in ppm referring to the signal center using the solvent peaks for reference: $CDCl_3$ 7.26/77.0 ppm and DMSO-d_6 2.49/39.7 ppm. Elemental analyses were performed with a Vario EL apparatus. Solvents and reagents were obtained from Acros (Geel, Belgium), Fluka (Taufkirchen, Germany), or Sigma-Aldrich (Steinheim, Germany). Cobalt sulfate was purchased from Merck (Darmstadt, Germany). XR9577[60] (see Supporting Information, Chart S5) was prepared following the conditions reported by Roe et al.[73] Synthetic procedures and analytical data for compounds **1**, **5**−**16**, **18**−**24**, **26**−**30**, precursor for **34**, precursor for **35**, precursor for **49**, precursor for **50**, as well as compounds **32**−**47**, **49**, and **50** are listed in the Supporting Information. All tested compounds possessed a purity of not less than 95%.

2-(3,3-Diethylureido)benzoic Acid (2). Anthranilic acid (**B**, R^3 = R^4 = H) (688 mg, 5.0 mmol) was added in portions to a stirring solution of *N*,*N*′-carbonyldiimidazole (811 mg, 5.0 mmol) in CH_2Cl_2 (50 mL). After 30 min, diethylamine (731 mg, 10.0 mmol) was added dropwise and the mixture was stirred overnight. The organic layer was washed with 1 M HCl (2 × 5 mL), filtered, and evaporated to dryness to yield **2** (637 mg, 54%): mp 144−148 °C (lit.[55] 151 °C). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.24 (t, J = 7.3 Hz, 6H), 3.43 (q, J = 7.3 Hz, 4H), 6.96 (ddd, J = 8.2, 7.2, 1.3 Hz, 1H), 7.52 (ddd, J = 8.5, 7.2, 1.6 Hz, 1H), 8.05 (dd, J = 8.2, 1.6 Hz, 1H), 8.62 (dd, J = 8.5, 1.0 Hz, 1H), 10.50 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 13.67, 41.73, 112.60, 119.56, 120.52, 131.58, 135.56, 144.40, 154.55, 172.83. Anal. ($C_{12}H_{16}N_2O_3$) C, H, N.

2-[3-(Methoxycarbonylmethyl)-3-methylureido]benzoic Acid (3). A solution of *N*-(mesyloxy)phthalimide (**C**, R^3 = R^4 = H) (1.81 g, 7.5 mmol) in anhydrous acetone (25 mL) was stirred under argon and heated to reflux. A solution of sarcosine methyl ester hydrochloride (3.14 g, 22.5 mmol) and triethylamine (2.28 g, 22.5 mmol) in acetone (7 mL) was added dropwise over 10 min. The mixture was refluxed for additional 15 min and evaporated to dryness. The residue was stirred with 0.25 M HCl (100 mL) and filtrated. The filtrate was kept a 5 °C for 14 days and the precipitate was collected by filtration to obtain **3** (400 mg, 20%): mp 147−149 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 3.07 (s, 3H), 3.66 (s, 3H), 4.16 (s, 2H), 7.02 (ddd, J = 8.2, 7.1, 1.3 Hz, 1H), 7.52 (ddd, J = 8.5, 7.2, 1.6 Hz, 1H), 7.96 (dd, J = 8.0, 1.4 Hz, 1H), 8.42 (dd, J = 8.5, 1.0 Hz, 1H), 10.67 (s, 1H), 13.56 (br s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 35.30, 50.01, 51.89, 114.69, 118.65, 120.92, 131.15, 134.28, 154.89, 170.45, 170.49. Anal. ($C_{12}H_{14}N_2O_5$) C, H, N.

2-(3-Cyclohexyl-3-methylureido)benzoic Acid (4). General Procedure for 2-Ureidobenzoic Acids from Isatoic Anhydride A. A mixture of isatoic anhydride (**A**, R^3 = R^4 = H) (3.26 g, 20 mmol) and 50 mL of an ethanolic solution of *N*-methylcyclohexylamine (4 M) was refluxed for 15 min, diluted with H_2O (200 mL), kept at

8 °C overnight, and filtrated. The filtrate was acidified with 1 M H_2SO_4, and the precipitate was filtered off. It was partitioned between ethyl acetate (50 mL) and 0.5 M NaOH (50 mL). The aqueous layer was washed with ethyl acetate (2 × 50 mL), cooled, and acidified with 1 M H_2SO_4. The precipitate was collected by suction filtration to obtain **4** (3.92 g, 71%): mp 146−147 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.05−1.18 (m, 10H), 2.83 (s, 3H), 3.90−3.98 (m, 1H), 6.97 (ddd, J = 8.2, 7.1, 1.3 Hz, 1H), 7.50 (ddd, J = 8.5, 7.2, 1.6 Hz, 1H), 7.94 (dd, J = 8.2, 1.6 Hz, 1H), 8.47 (dd, J = 8.5, 1.0 Hz, 1H), 10.83 (s, 1H), 13.51 (br s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 25.09, 25.58, 28.12, 30.07, 53.79, 114.44, 188.80, 120.39, 131.11, 134.16, 143.67, 154.27, 170.56. Anal. ($C_{15}H_{20}N_2O_3$) C, H, N.

2-(3,3-Diethylthioureido)benzoic Acid (17). General Procedure for 2-Thioureidobenzoic Acids 17−24. A mixture of methyl 2-(3,3-diethylthioureido)benzoate (**D**, $R^1 = R^2$ = Et)[74] (533 mg, 2.0 mmol), 1 M NaOH (10 mL), and EtOH (10 mL) was refluxed for 1 h. The reaction was allowed to cool to room temperature, and H_2O (30 mL) was added. The solution was filtered, cooled to 0 °C and acidified with 2 M HCl. The precipitate was removed by suction filtration and washed with H_2O (50 mL) to obtain **17** (394 mg, 78%): mp 114−116 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.23 (t, J = 7.1 Hz, 6H), 3.76 (q, J = 7.0 Hz, 4H), 7.11 (ddd, J = 8.2, 7.1, 1.3 Hz, 1H), 7.50 (ddd, J = 8.5, 7.1, 1.6 Hz, 1H), 7.90 (dd, J = 7.9, 1.6 Hz, 1H), 8.49 (dd, J = 8.5, 1.0 Hz, 1H), 10.62 (s, 1H), 13.53 (br s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 12.50, 45.03, 119.10, 122.65, 123.93, 130.49, 132.40, 142.97, 169.83, 178.41. Anal. ($C_{12}H_{16}N_2O_2S$) H, N. C: calcd, 57.12; found, 56.55.

2-(3,3-Diethylureido)-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxylic Acid (25).[75] General Procedure for 2-Ureidothiophene-3-carboxylic Acids from Thieno[2,3-*d*][1,3]oxazin-4-ones E. A mixture prepared from 2-(diethylamino)-6,7-dihydro-4*H*,5*H*-cyclopenta[4,5]thieno[2,3-*d*][1,3]oxazin-4-one (**E**, $R^1 = R^2$ = Et, R^3R^4 = −$(CH_2)_3$−) (132 mg, 0.50 mmol), 1.5 M NaOH (14 mL), and acetone (7 mL) was refluxed for 5 min. After being cooled, the mixture was filtrated into 3 M HCl (14 mL), and the precipitate was collected by filtration to yield **25** (110 mg, 78%): mp 171−173 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.17 (t, J = 6.8 Hz, 6H), 2.23−2.37 (m, 2H), 2.69−2.89 (m, 4H), 3.36 (q, J = 6.8 Hz, 4H), 10.88 (s, 1H), 12.77 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 14.28, 28.03, 29.07, 30.60, 41.86, 106.43, 129.56, 141.52, 152.75, 155.77, 168.34. Anal. ($C_{13}H_{18}N_2O_3S\cdot H_2O$) C, N. H: calcd, 6.71; found, 6.27.

Ethyl 2-(3-Cyclohexyl-3-methylthioureido)thiophene-3-carboxylate (H, R^1 = Me, R^2 = Cyclohexyl, $R^3 = R^4$ = H). General Procedure for 2-Thioureidothiophene-3-carboxylates H from 2-(isothiocyanato)thiophene-3-carboxylates G. *N*-Methylcyclohexylamine (736 mg, 6.5 mmol) was added dropwise to a stirring solution of ethyl 2-(isothiocyanato)thiophene-3-carboxylate (**G**, $R^3 = R^4$ = H)[59] (1.07 g, 5.0 mmol) in CH_2Cl_2 (20 mL). The reaction mixture was stirred at room temperature for 3 h. The organic layer was washed with 0.5 M HCl (2 × 5 mL), dried over Na_2SO_4, filtered, and evaporated to dryness. Recrystallization from EtOH yielded **H** (R^1 = Me, R^2 = cyclohexyl, $R^3 = R^4$ = H) (1.26 g, 77%): mp 143−145 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.11−1.82 (m, 13H), 3.16 (s, 3H), 4.32 (q, J = 7.0 Hz, 2H), 4.71−5.09 (m, 1H), 6.83 (d, J = 5.7 Hz, 1H), 7.18 (d, J = 5.7 Hz, 1H), 11.70 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 14.30, 24.92, 25.38, 29.22, 31.75, 59.32, 60.80, 112.46, 115.90, 123.12, 152.85, 165.91, 175.17. Anal. ($C_{15}H_{22}N_2O_2S_2$) C, H, N.

Ethyl 2-(3,3-Diethylthioureido)benzo[*b*]thiophene-3-carboxylate (H, $R^1 = R^2$ = Et, R^3R^4 = −CH=CH−CH=CH−). General Procedure for Ethyl 2-Thioureidobenzo[*b*]thiophene-3-carboxylates H (R^3R^4 = −CH=CH−CH=CH−). Diethylamine (549 mg, 7.5 mmol) was added dropwise to a solution of ethyl 2-(isothiocyanato)benzo[*b*]thiophene-3-carboxylate (**G**, R^3R^4 = −CH=CH−CH=CH−)[59] (1.3 g, 5.0 mmol) in CH_2Cl_2 (5 mL) at 0 °C. The mixture was stirred at room temperature for 2 h and acidified with 1 M HCl/EtOH. The precipitate was collected by suction filtration and recrystallized from EtOH to yield (**H**, $R^1 = R^2$ = Et, R^3R^4 = −CH=CH−CH=CH−) (1.05 g, 65%): mp 136−138 °C (lit.[59] 137−138 °C). ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.26 (t, J = 6.8 Hz, 6H), 1.42 (t, J = 7.1 Hz, 3H), 3.79−3.86 (m, 4H), 4.46 (q, J = 7.1 Hz, 2H), 7.29 (ddd, J = 8.0, 7.0, 1.1 Hz, 1H), 7.41 (ddd, J = 8.2, 7.3, 1.3 Hz, 1H), 7.88 (d, J = 7.9 Hz, 1H), 8.20 (dt, J = 8.2, 1.0 Hz, 1H), 12.56 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 12.25, 14.28, 46.06, 61.27, 105.21, 122.00, 122.49, 123.80, 125.72, 132.70, 133.18, 156.96, 167.18, 174.77. Anal. ($C_{16}H_{20}N_2O_2S_2$) C, H, N.

2-(3-Cyclohexyl-3-methylthioureido)thiophene-3-carboxylic Acid (31). General Procedure for 2-Thioureidothiophene-3-carboxylic Acids from 2-Thioureidothiophene-3-carboxylates H. A mixture of ethyl 2-(3-cyclohexyl-3-methylthioureido)thiophene-3-carboxylate (**H**, R^1 = Me, R^2 = cyclohexyl, $R^3 = R^4$ = H) (653 mg, 2.0 mmol), 1 M NaOH (10 mL), and EtOH (10 mL) was refluxed for 1 h. The reaction was allowed to cool to room temperature, and H_2O (30 mL) was added. The mixture was filtered, cooled to 0 °C, and acidified with 2 M HCl. The precipitate was removed by suction filtration and washed with H_2O (50 mL) to obtain **31** (480 mg, 78%): mp 174−175 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.06−1.87 (m, 10H), 3.15 (s, 3H), 4.42−5.19 (m, 1H), 6.79 (d, J = 5.7 Hz, 1H), 7.15 (d, J = 6.0 Hz, 1H), 12.08 (s, 1H), 13.20 (br s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 24.91, 25.40, 29.32, 32.29, 59.18, 113.21, 115.40, 123.69, 152.73, 167.88, 175.20. Anal. ($C_{13}H_{18}N_2O_2S_2$) C, H, N.

2-(3,3-Diethylthioureido)benzo[*b*]thiophene-3-carboxylic Acid (48). General Procedure for 2-Thioureidobenzo[*b*]thiophene-3-carboxylic Acids 48−50. A mixture of ethyl 2-(3,3-diethylthioureido)benzo[*b*]thiophene-3-carboxylate (**H**, $R^1 = R^2$ = Et, R^3R^4 = −CH=CH−CH=CH−) (672 mg, 2.0 mmol), 1 M NaOH (10 mL), and EtOH (10 mL) was refluxed for 1 h. The reaction was allowed to cool to room temperature, and H_2O (30 mL) was added. The solution was cooled to 0 °C and acidified with 2 M HCl. The precipitate was removed by suction filtration and washed with H_2O (35 mL). Recrystallization from EtOH yielded **48** (250 mg, 41%): mp 153−155 °C (lit.[59] 142−144 °C). ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.26 (t, J = 6.8 Hz, 6H), 3.70−3.90 (m, 4H), 7.27 (ddd, J = 8.2, 7.1, 1.3 Hz, 1H), 7.38 (ddd, J = 8.4, 7.2, 1.3 Hz, 1H), 7.86 (ddd, J = 7.9, 1.3, 0.6 Hz, 1H), 8.23 (dt, J = 8.2, 1.0 Hz, 1H), 13.02 (s, 1H), 13.78 (br s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 12.28, 46.13, 105.85, 121.88, 122.54, 123.58, 125.53, 132.67, 133.84, 156.85, 169.09, 174.86. Anal. ($C_{14}H_{16}N_2O_2S_2$) C, H, N.

Cell Lines. The human ovarian cancer cell line 2008 WT[76] and the multidrug-resistant cell line 2008 MRP1[76] were kindly provided by Piet Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands). The ovarian carcinoma cell line (A2780) and its P-gp-overexpressing subline (A2780/ADR) were purchased from European Collection of Animal Cell Cultures (ECACC, no. 93112519 [A2780], no. 93112520 [A2780adr], United Kingdom) and were used for P-gp determination. The breast cancer resistant cell line (MCF-7 MX) and the parental cell line (MCF-7) were kindly provided by Dr. E. Schneider, Wadsworth Center, Albany, NY.

The cell lines were cultured in RPMI 1640 medium, supplemented with 10% fetal calf serum (FCS) and 1% antibiotics (100000 units/L penicillin and 100 mg/L streptomycin). The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Expression of MRP1, P-gp, and BCRP was confirmed and determined by Western blot (data not shown).

Calcein AM Accumulation assays.[45,53,77] For estimation of MRP1 activity, the cell line 2008 MRP1 and the corresponding wild-type cell line were used. Cells were harvested by trypsination and centrifuged. After resuspension in medium, the cells were counted with a Casy I cell counter model TT (Schaerfe System, Reutlingen, Germany). The cell density was adjusted to 300000 per mL. The cell suspension (70 μL) containing 21000 cells were transferred into a 96-well microplate. One column of wells was without cells, only containing buffer, the next two following columns contained the cell suspension, either wild-type or transfected cells. Into each well, 10 μL of solutions of the test compounds in Krebs−HEPES buffer at different

concentrations, 10 μL of a 10 μM cobalt sulfate solution in Krebs−HEPES buffer, and 10 μL of a 100 μM solution of XR9577 in Krebs−HEPES buffer were added. After a 30-min preincubation, each well was supplied with 33 μL of a calcein AM solution in Krebs−HEPES buffer to obtain a final concentration of 0.5 μM. Fluorescence was measured over 30 min. Indomethacin (100 μM) was used as positive control. All compounds showing more than 50% inhibition at a concentration of 100 μM were considered to be active, and their inhibitory potency was characterized by determination of complete concentration−response curves. All other compounds were rated inactive (ni, no inhibition). Different concentrations of indomethacin (0.316−178 μM) were generally included in the measurements to obtain IC_{50} values for the test compounds.

For measuring P-gp activity, A2780/ADR cells and the corresponding wild-type cells were used and the calcein AM accumulation assay was performed as described above, except that XR9577 and cobalt ions were omitted. Calcein is not effluxed by P-gp, thus a quenching of extracellular fluorescence by cobalt is not necessary.[78,79] Instead, XR9577 (10 μM) served as a positive control. A concentration−response curve for XR9577 was generated with concentrations 0.010−10 μM. A detailed description is given elswhere.[63] Selected MRP1 inhibitors were evaluated at two different concentrations (100 and 31.6 μM). Compounds showing less than 15% inhibition at a concentration of 100 μM were classified as inactive.

Hoechst 33342 Assay.[62] For the determination of BCRP activity, MCF-7 and MCF-7 MX cells were cultivated in T175 flasks. After a confluence of 80−90% was reached, cells were harvested by gentle trypsination (0.05% trypsin/0.02% EDTA) and then carried over into a 50 mL tube. After this step, cells were centrifugated (1200 rpm, 4 °C, 4 min). Subsequently, the cell pellet was resuspended in fresh culture medium, and the cell density was determined using a Casy I model TT cell counter device (Schaerfe System, Reutlingen, Germany). Followed by another centrifugation, cells were washed three times with Krebs−HEPES buffer and seeded into black 96-well plates (Greiner, Frickenhausen, Germany) at a density of approximately 20000 cells per well in a volume of 90 μL. The plate was divided into two parts: the first, third, seventh, and tenth column of the 12 columns of the 96-well plate (32 wells) only contained 90 μL of Krebs−HEPES buffer, the remaining eight columns (64 wells) were filled with 90 μL cell suspension. The 32 wells containing buffer were defined as "background" wells. Ten μL of various test compounds in different concentrations was added to a total volume of 100 μL so that each concentration contained one "background" well and two wells including cell suspension. The prepared 96-well plate was kept under 5% CO_2 and 37 °C for 30 min. After this preincubation period, 20 μL of a 30 μM Hoechst 33342 solution (protected from light) was added to each well. Fluorescence was measured immediately in constant intervals (120 s) up to 2400 s at an excitation wavelength of 355 nm and an emission wavelength of 460 nm applying a 37 °C tempered BMG POLARstar microplate reader. XR9577 (10 μM) served as positive control in all measurements. A concentration−response curve for XR9577 was generated with concentrations between 0.316 nM and 10 μM (see Supporting Information, Figure S2).

Cytotoxicity (MTT) Assays.[63] Ovarian cancer cells (2008 WT) were seeded into 96-well plates (Greiner, Frickenhausen, Germany) at a density of 20% per well in a fixed volume of 90 μL and kept under 5% CO_2 at 37 °C for 6 h. The seeding density depended on the growth characteristics of the cells and was chosen to avoid a 100% confluency of untreated cells. After 6 h, cells were attached as controlled by microscopy. The assay was performed at a FLUOstar microplate reader (BMG Labtechnologies, Offenburg, Germany). The cells were treated with the test compounds in three different concentrations, resulting in a final volume of 100 μL and final concentrations of the compounds of 3.16, 10, and 31.6 μM per well. After 72 h incubation, 20 μL of MTT solution (5 mg/mL) was added to each well. Incubation with MTT was terminated after 50−70 min (before cell-damaging formazan needles were formed) by injecting 150 μL of a mixture of 2-propanol:concd HCl (50 mL:165 μL) to each well. Then, the 96-well plates were kept at 5 °C for 1−2 h. Finally, the absorption was measured at 595 nm (test wavelength) and 690 nm (reference wavelength). Absorption at the reference wavelength was subtracted from the absorption at the test wavelength.

In a second series of experiments, effects of combinations of selected compounds (**31**, **48**) with the cytostatic drugs vinblastine and daunorubicin were evaluated. A fixed concentration (31.6 μM) of **31** or **48** was combined with distinct concentrations of vinblastine or daunorubicin (0.1 nM−100 μM) using 2008 MRP1 cells. Indomethacin (31.6 μM) served as positive control in this setting. After 72 h, the MTT assay was applied as described above. The two compounds **31** and **48** were omitted in the control experiments, where 2008 MRP1 and 2008 WT cells were treated with different concentrations of the cytostatic drugs.

Assay Data Analyses. The fluorescence measured in the "background" wells was subtracted from the fluorescence measured in the corresponding wells which were supplemented with cells. These data were applied for the following analysis: the slope of each fluorescence−time curve was calculated by linear regression and used as a dependent parameter. From these data, concentration−response curves were generated by nonlinear regression using the four-parameter logistic equation with variable Hill slope (GraphPad Prism 5.0 software, San Diego, CA). For normalization of data, slopes from absorption−time curves or fluorescence−time curves were transformed to relative units by subtracting the lowest determined single value from all other data and thus setting it to 0%. The highest measured single value was defined as 100%.

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Supporting Information Available: Synthetic procedures and analytical data for the remaining compounds, elemental analyses, structures of inactive compounds and of XR9577, cytotoxicity data, as well as exemplary concentration−effect curves from the BCRP assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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