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A 4-aminobenzoic acid derivative as novel lead for selective inhibitors of multidrug resistance-associated proteins

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ABSTRACT

We present a novel lead for inhibitors of multidrug resistance-associated proteins (MRPs). Compound **1** (4-[(5,6,7,8-tetrahydro-4-oxo-4*H*-[1]benzothieno[2,3-*d*][1,3]thiazin-2-yl)amino]benzoic acid) was about six times more potent than the known inhibitor MK571 at MRP1, while at MRP2 its effect was similar to that of MK571. Structural analogs were also evaluated. Among them, compound **2**, sharing the 4-aminobenzoic acid substructure with **1**, also inhibited MRP1. Both derivatives were inactive against P-gp. It can be concluded that their carboxyl group is needed for inhibition of MRPs and accounts for the selectivity of these compounds.

Multidrug resistance (MDR) represents a major problem in the chemotherapeutic treatment of cancer.[1] A multidrug-resistant tumor is mainly characterized by an increased cellular efflux of a large number of structurally different endo- and xenobiotics and a reduced intracellular drug accumulation. This can be related to the overexpression of ATP-binding cassette (ABC) transporters, conferring resistance to a broad spectrum of cytostatic drugs by active transport mechanisms.[2] The best-studied ABC transporter so far is the ABCB1 gene-encoded efflux pump referred to as multidrug resistance protein 1 (MDR1) or P-glycoprotein (P-gp).[3] Its preferred substrates are hydrophobic and cationic compounds.[4]

Multidrug resistance-associated protein 1 (MRP1), the first discovered member of the ABCC subfamily, was identified in 1992.[5] This 190-kDa membrane-spanning protein shares only 15% amino acid homology with P-gp.[6] Both MRP1 and P-gp are capable of transporting a wide range of anticancer agents, including anthracyclines, *Vinca* alkaloids, and the folic acid analog methotrexate.[7] In contrast to P-gp, MRP1 preferentially transports negatively charged compounds, including glutathione conjugates, sulfates, or glucuronides.[8]

Multidrug resistance-associated protein 2 (MRP2) represents the transporter most closely related to MRP1. It was discovered 4 years after MRP1 and has a somewhat different substrate spectrum. However, like MRP1, MRP2 is able to extrude endogenous compounds such as LTC_4, billirubin, and glutathione conjugates indicating a similar role in detoxification.[9,10] In contrast to MRP1, MRP2 is capable of transporting cisplatin,[11,12] and thus represents a candidate for causing cisplatin resistance in cancer chemotherapy.[13]

There are only few specific inhibitors of ABC transporters known so far, and thus the challenge for medicinal chemists has been to develop potent and specific inhibitors, particularly for MRPs. In this study, we present a new inhibitor of MRPs with a remarkable selectivity for MRP1.

A focussed screening approach was applied to identify candidates from our in-house library. With respect to the substrate specificity of MRP1, carboxylic acid derivatives were selected. More than 75 structurally diverse carboxylic acids were screened for MRP1 inhibitory activity. Compound **1** (4-[(5,6,7,8-tetrahydro-4-oxo-4*H*-[1]benzothieno[2,3-*d*][1,3]thiazin-2-yl)amino]benzoic acid) was identified as a potent inhibitor of MRP1. To specify structural requirements for inhibitory activity against MRP1, three related compounds (**2**–**4**)[14] as well as standard inhibitors[15] were included in the investigations at the ABC transporters MRP1, MRP2, and P-gp. The chemical structures of compounds **1**–**4** are given in Figure 1.

A calcein AM fluorescence assay was performed to determine the influence of the test compounds on the transporter proteins.[16] Calcein AM is a known substrate of both MRPs and P-gp.[17] The human ovarian cancer cell line A2780 stably expressing P-gp as well as the human ovarian cancer cell line 2008 and the Madin–Darby canine kidney cell line MDCKII stably expressing MRP1 and MRP2, respectively, were used.[18] When performing the MRP assays, XR9957,[19] a selective blocker for P-gp and BCRP that has no effect on MRP1 and MRP2,[20] was added to inhibit P-gp which is present to a minor degree in both MRP-expressing cell lines. The

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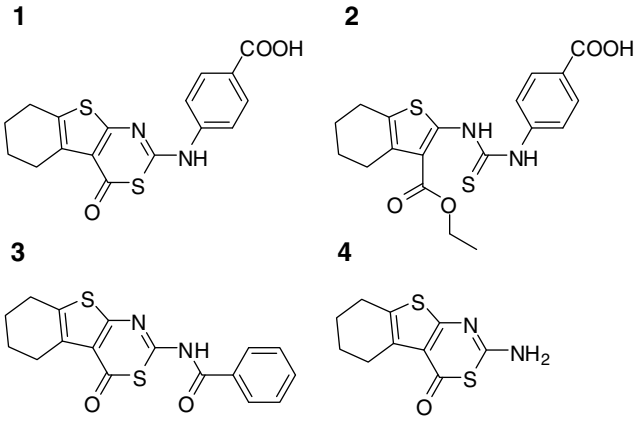

Figure 1. Chemical structures of the investigated compounds.

assay medium contained cobalt ions to quench the extracellular calcein fluorescence so that only the fluorescence of the intracellular calcein is measured.

In Figure 2, exemplary fluorescence–time curves in the presence of varying concentrations of compound **1** are shown. The calcein AM fluorescence within the cells increased in response to the inhibitor concentration in MRP1-, as well as MRP2-transfected cells. By plotting the slopes of the fluorescence–time curves *versus* the logarithmic concentrations of the inhibitors, dose–response curves were obtained and are shown for test compound **1** in Figure 3 to illustrate the effects on MRP1- and MRP2-transfected cells, respectively.

In Table 1, IC_{50} values for compounds **1**–**4** are given together with those of some standard inhibitors of MRP1, MRP2, and P-gp. Compound **1** was the most potent inhibitor of MRP1 with an IC_{50} value of 1.2 μM. It was four times more effective than cyclosporin A, the best inhibitor of MRP1 among the reference compounds. In case of MRP2, cyclosporin A exhibited the strongest inhibitory activity, followed by the equipotent inhibitors **1** and MK571. Indomethacin, verapamil, and inhibitor **2** had weak, compounds **3** and **4** no detectable effects on MRP2 function. In this series, none of the tested substances was more active at MRP2 than at MRP1, indicating a reduced susceptibility of MRP2 to inhibition.

The two known P-gp inhibitors cyclosporin A and verapamil showed similar activity against P-gp. Their IC_{50} values were in the same range as determined at MRP1. Cyclosporin A was the only tested compound of this series which inhibited the three ABC

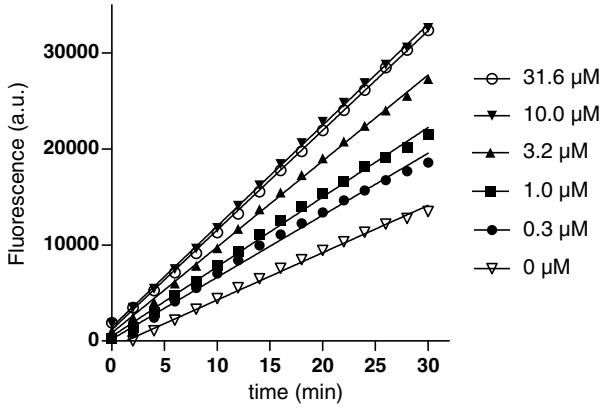

Figure 2. Fluorescence–time curves for different concentrations of compound **1** determined with the calcein accumulation assay in 2008 MRP1 cells. Data are mean values from a typical experiment with two replicates belonging to a series of at least three independent experiments.

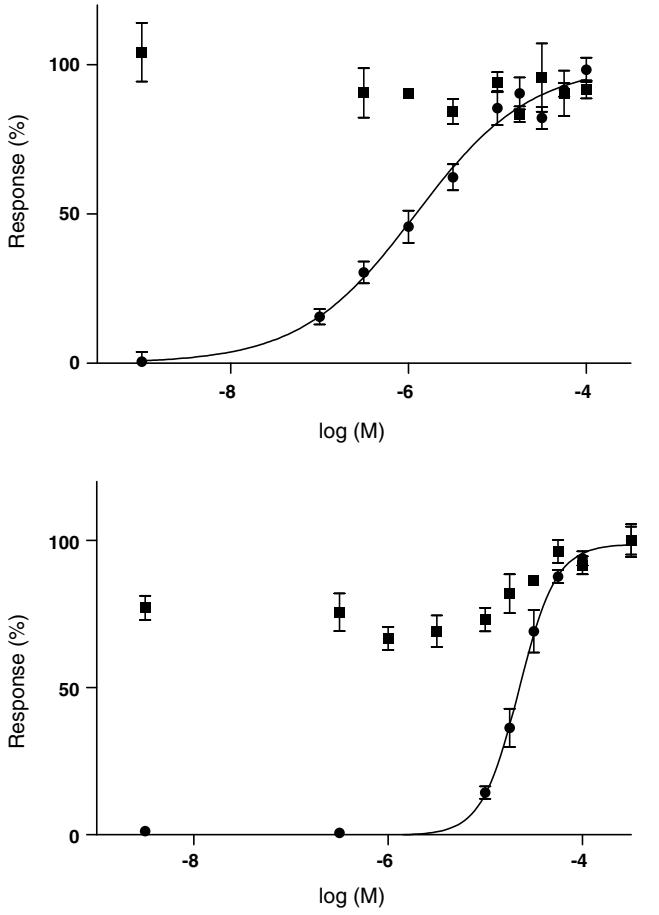

Figure 3. Concentration–effect curves of compound **1** in MRP1- (top) and MRP2 (bottom)-transfected cell lines (circles) in comparison to the wild-type cell lines (squares). Curves represent an average of at least three independent experiments and are normalized in the range from 0% to 100%.

Table 1
Tested compounds with their IC_{50} values

Compounds	IC_{50}[a] (μM)		
	MRP1	MRP2	P-gp
1	1.21 ± 0.29	21.5 ± 5.9	na
2	20.4 ± 3.6	>200	na
3	na	na	na
4	na	na	>200
Verapamil	9.66 ± 2.79	>200	5.42 ± 1.33
Indomethacin	12.0 ± 3.5	235 ± 73	na
MK571	7.57 ± 1.10	20.7 ± 7.3	na
Cyclosporin A	4.78 ± 0.61	13.6 ± 1.9	4.92 ± 0.20

[a] Values are means ± SD of at least three independent experiments carried out on different occasions. na, not active.

transporters, all of them with low micromolar IC_{50} values. On the other hand, compounds with a carboxylic acid moiety (i.e., indomethacin, MK571, **1** and **2**) were inactive in the P-gp assay.

The LTD_4 receptor antagonist MK571, a chloroquinolinyl styrene derivative with dithioacetal structure bearing a carboxamidoalkyl and a carboxyalkyl substituent, is an effective and specific inhibitor of MRPs.[9,21] The newly identified inhibitor **1** showed a similar activity pattern, having no effect on P-gp but potency against MRPs in the low micromolar range. When comparing the data for **1** and MK571, compound **1** (IC_{50}: 1.2 μM) was six times more potent, while the effects on MRP2 were almost identical.

Noteworthy, compound **2** with an opened thiazinone ring was a much less potent inhibitor in both MRP assays than compound **1**. Compared to the more flexible structure of the bicyclic thiophene derivative **2**, the rigid tricyclic thienothiazinone structure of **1** might account for the better activity. Further data are needed to confirm this hypothesis. The related compounds **3** and **4** both share the same ring system as **1**, but were nonetheless inactive as inhibitors of MRP1 and MRP2. In compound **3**, the carboxyl group was deleted and a carbonyl inserted between the substituent's nitrogen atom and phenyl ring. In compound **4**, the carboxyphenyl moiety was removed. This led us to conclude that both the tetrahydrobenzothieno[2,3-*d*][1,3]thiazin-4-one system and the presence of the carboxylic acid moiety were necessary for activity. The latter conclusion agrees with the known preference of MRPs to transport negatively charged compounds.[22]

In conclusion, we present a novel and potent inhibitor of MRP1. The heterocyclic skeleton of compound **1** may now serve as a lead structure for further selective inhibitors of the ABCC subfamily of ABC transporters.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.07.127.

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16. For estimation of MRP1 and MRP2 activity, the cell lines 2008 MRP1 and MDCKII MRP2, respectively, and the corresponding wild-type cell lines were used. Cells were harvested by trypsination and centrifuged. After resuspension in medium the cells were counted with a Casy©1 cell counter Model TT (Schaerfe System, Reutlingen, Germany). The cell density was adjusted to 300,000 per ml. Seventy microliters of the cell suspension containing 21,000 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 KHP at different concentrations, 10 μL of a 10 μM cobalt sulfate solution in KHP, and 10 μL of a 100-μM solution of XR9957 in KHP were added. After a 30-min preincubation, each well was supplied with 33 μL of a calcein AM solution in KHP to obtain a final concentration of 0.5 μM. Indomethacin was used as positive control, for both MRP1 and MRP2 assays. For measuring P-gp activity, the calcein accumulation assay was performed as described above, except that XR9957 and cobalt ions were omitted. Instead, XR9957 served as a positive control. A detailed description of the P-gp assay is given in: Mueller, H.; Kassack, M. U.; Wiese, M. *J. Biomol. Screen.* **2004**, *9*, 506.
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