Journal of Medicinal Chemistry

Article

9-Deazapurines as Broad-spectrum Inhibitors of the ABC Transport Proteins P-glycoprotein (P-gp, ABCB1), Multidrug Resistance-associated Protein 1 (MRP1, ABCC1), and Breast Cancer Resistance Protein (BCRP, ABCG2)

Katja Stefan, Sven Marcel Schmitt, and Michael Wiese

J. Med. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jmedchem.7b00788 • Publication Date (Web): 10 Oct 2017

ACS Publications

Journal of Medicinal Chemistry is published by the American Chemical Society.
1155 Sixteenth Street N.W., Washington, DC 20036

9-Deazapurines as Broad-spectrum Inhibitors of the ABC Transport Proteins P-glycoprotein (P-gp, ABCB1), Multidrug Resistance-associated Protein 1 (MRP1, ABCC1), and Breast Cancer Resistance Protein (BCRP, ABCG2)

Katja Stefan†, Sven Marcel Schmitt†, Michael Wiese*

University of Bonn, Pharmaceutical Institute, An der Immenburg 4, 53121 Bonn, Germany

*E-mail: mwiese@uni-bonn.de, phone: +49/228/73-5212

† these authors contributed equally

Key words: ABC transporter; P-gp (ABCB1); MRP1 (ABCC1); BCRP (ABCG2); dual / double inhibitor; triple inhibitor; multiple inhibition.

Abstract. P-glycoprotein (P-gp, ABCB1), Multidrug Resistance-associated Protein 1 (MRP1, ABCC1) and Breast Cancer Resistance Protein (BCRP, ABCG2) are the three major ABC transport proteins conferring resistance to many structurally diverse anticancer agents leading to the phenomenon called multidrug resistance (MDR). Much effort was put on the development of clinically useful compounds to reverse MDR. Broad-spectrum inhibitors of ABC transport proteins can be of great use in cancers that simultaneously co-express two or three transporters. In this work we continued our effort to generate new, potent, nontoxic and multiple effective inhibitors of the three major ABC transporters. The best compound was active in very low micromolar concentration range against all three transporters and restored sensitivity

toward daunorubicin (P-gp and MRP1) and SN-38 (BCRP) in A2780/ADR (P-gp), H69AR (MRP1) and MDCK II BCRP (BCRP) cells. Additionally, the compound is a non-competitive inhibitor of daunorubicin (MRP1), calcein AM (P-gp) and pheophorbide A (BCRP) transport.

Introduction. Since the discovery of P-gp in 1976 by *Juliano* and *Ling*,[1] the clinical importance of ABC transport proteins in cancer therapy has been increasingly recognized.[2] These transporters are frequently expressed in several types of human cancers,[2,3,4] often simultaneously.[2] Due to active efflux of antineoplastic agents driven by ATP hydrolysis, their intracellular concentration is decreased, causing a lack of response during chemotherapy called multidrug resistance (MDR). Furthermore, two major impacts in cancer research were the discovery of MRP1 (1992 by *Cole* et al.),[5] and BCRP (1998 by *Doyle* et al.),[6] as these transport proteins have high affinity to structurally diverse anticancer drugs,[7,8] similar to P-gp.[9]

The development of potent and selective inhibitors of one of the three transport proteins was in focus of medicinal chemistry in the last four decades. Many structurally unrelated compounds were found to have high inhibitory power against P-gp,[10] MRP1,[11,12,13] and BCRP.[14,15] Structure-activity relationship (SAR) studies revealed crucial partial structures and substituents being important to inhibit the target transport protein.[16,17,18] Analysis of the inhibition type and the elucidation of the mode of action was also in focus of several studies.[19,20,21]

As MRP1 was the second most prevalent transporter in MDR,[5] most P-gp inhibitors have been tested against MRP1, too.[11,12] This led to some dual inhibitors, e.g. the calcium channel blocker verapamil (**1**; Fig. 1),[22,23] the dihydropyridine derivative bis(pyridin-4-ylmethyl) 2,6-dimethyl-4-(3-methyl-5,6-dihydro-1,4-dithiin-2-yl)-1,4-dihydropyridine-3,5-dicarboxylate (NIK-250; **2**; Fig. 1),[24,25] the immunosuppressant

cyclosporine A[26,27] or the quinoline derivative 4-(diphenylacetyl)-a-[(5-quinolinyloxy)methyl]-1-piperazineethanol-2-butenedioate fumarate (dofequidar, MS-209, **3**; Fig. 1).[28,29,30] The Ely Lilly company spent huge effort in the development of MRP1 inhibitors, some with dual inhibitory feature,[31,32,33] like the tricyclic isoxazole amide isoster No. 13 (**4**; Fig. 1; $IC_{50\ (P\text{-}gp)}$ = 1.33 μM; $IC_{50\ (MRP1)}$ = 0.32 μM).[31] Xenova Ltd. specifically developed dual inhibitors of P-gp and MRP1, like the quinazolinone No. 40 (**5**; Fig. 1; $IC_{50\ (P\text{-}gp)}$ = 0.60 μM; $IC_{50\ (MRP1)}$ = 0.40 μM),[34] the pyrrolopyrimidine No. 47 (**6**; Fig. 1; $IC_{50\ (P\text{-}gp)}$ = 0.105 μM; $IC_{50\ (MRP1)}$ = 1.31 μM),[35] and the indolopyrimidine No. 49 (**7**; Fig. 1; $IC_{50\ (P\text{-}gp)}$ = 3.31 μM and $IC_{50\ (MRP1)}$ = 0.235 μM).[36] We recently reported on the pyrrolopyrimidine No. 31 (**8**; Fig. 1; $IC_{50\ (P\text{-}gp)}$ = 6.82 μM and $IC_{50\ (MRP1)}$ = 0.340 μM).[37]

Since P-gp was the most studied transport protein, many known P-gp inhibitors were investigated for their inhibitory power against BCRP. Several structures have been reported, such as *N*-[4-[2-(3,4-dihydro-6,7-dimethoxy-2(1*H*)-isoquinolinyl)ethyl]phenyl]-9,10-dihydro-5-methoxy-9-oxo-4-acridinecarboxamide (elacridar, GF120918)[38] or *N*-[2-[[[4-[2-(3,4-dihydro-6,7-dimethoxy-2(1*H*)-isoquinolinyl)ethyl]phenyl]amino]carbonyl]-4,5-dimethoxyphenyl]-3-quinolinecarboxamide (tariquidar, XR9576).[39] *Pick* et al. developed tariquidar analogs and found dual inhibitors (No. 16, **9**; Fig. 2; $IC_{50\ (P\text{-}gp)}$ = 0.421 μM and $IC_{50\ (BCRP)}$ = 5.06 μM).[40] *Köhler* et al. showed in 2015 that the tetrazolic HM30181 derivative No. 23 (**10**; Fig. 2; $IC_{50\ (P\text{-}gp)}$ = 7.73 μM and $IC_{50\ (BCRP)}$ = 0.180 μM) has affinity toward both transporters.[41] *Spindler* et al. reported in 2016 on the tetrahydro-*β*-carboline derivative No. 44 (**11**; Fig. 2; $IC_{50\ (P\text{-}gp)}$ = 2.86 μM and $IC_{50\ (BCRP)}$ = 1.10 μM) as dual inhibitor of P-gp and BCRP.[42] Flavonoids and their precursors are also good dual inhibitors as reported by *Pick* et al. (flavonoid No. 21; **12**; Fig. 2; $IC_{50\ (P\text{-}gp)}$ = 9.0 μM and $IC_{50\ (BCRP)}$ = 1.4 μM),[43] *Gu* et al. (bifendate-chalcone 8f; **13**; Fig. 2),[44,45,46] *Juvale* et al. (chalcone

No. 10; **14**; Fig. 2),[47]and *Kraege* and *Stefan* et al. (quinazoline-chalcone No. 24; **15**; Fig. 2; $IC_{50\ (P\text{-}gp)}$ = 0.48 μM and $IC_{50\ (BCRP)}$ = 0.60 μM).[20] But also quinazolines have been reported as dual inhibtors by several authors, e.g. *Juvale* et al. (quinazoline No. 2; **16**; Fig. 2; $IC_{50\ (P\text{-}gp)}$ = 5.85 μM and $IC_{50\ (BCRP)}$ = 0.190 μM),[48,49] and *Krapf* et al. (4-anilino-2-pyridylquinazoline No. 39; **17**; Fig. 2; $IC_{50\ (P\text{-}gp)}$ = 0.334 μM and $IC_{50\ (BCRP)}$ = 0.299 μM).[50] *Kraege* and coworkers found in 2016 the acryloylphenylcarboxamide No. 33 (**18**; Fig. 2; $IC_{50\ (P\text{-}gp)}$ = 0.494 μM and $IC_{50\ (BCRP)}$ = 0.573 μM)[51] and the acryloylphenylcarboxylate No. 29 (**19**; Fig. 2; $IC_{50\ (P\text{-}gp)}$ = 2.16 μM and $IC_{50\ (BCRP)}$ = 1.44 μM) as two good dual P-gp and BCRP inhibitors.[52]

Reports on dual MRP1 and BCRP inhibitors are rare. *Häcker* et al. reported on rather selective MRP1 inhibitors, the aromatic 2-(thio)ureidocarboxylic acids, which showed only partial effects against BCRP at concentrations above 30 μM.[53] *Chow* et al. reported on specific flavonoids with dual inhibitory power, e.g. (4-(2-(2-(4-(4-(4-(3-(4-fluoro-2-hydroxyphenyl)-3-oxoprop-1-en-1-yl)phenoxy)butyl)-1*H*-1,2,3-triazol-1-yl)ethoxy)ethoxy)phenyl)-4*H*-chromen-4-one (AclOAzl; **20**; Fig. 3).[14,54] We recently showed pyrrolopyrimidine derivative No. 16 (**21**; Fig. 3; $IC_{50\ (MRP1)}$ = 2.87 μM and $IC_{50\ (BCRP)}$ = 4.25 μM) as moderately good dual MRP1 and BCRP inhibitors.[37]

Triple inhibitors of P-gp, MRP1 and BCRP with high potency are barely known. *Li* et al. showed piperine (**22**; Fig. 4), a *Piper nigrum*-derived compound to affect the accumulation of rhodamine 123, doxorubicin and mitoxanthrone in MCF7/DOX (P-gp and BCRP) and A-549/DDP (MRP1) cells.[55] The pipecolinate derivative biricodar (VX-710, **23**; Fig. 4), specifically designed for dual P-gp/MRP1 inhibition evaluated in clinical trials,[56,57] is nowadays considered to be a triple inhibitor of P-gp, MRP1 and BCRP, as stated by *Mindermann* et al.[58] (3S,6S,12aS)-1,2,3,4,6,7,12,12a-octahydro-9-methoxy-6-(2-methylpropyl)-1,4-dioxopyrazino[1′,2′:1,6]pyrido[3,4- b]indole-3-

propanoic acid 1,1-dimethylethyl ester hydrate (**24**; Ko143; Fig. 4), one of the most frequently used standard inhibitors of BCRP, was found by *Weidner* et al. not to be specific as it also inhibited murine P-gp and MRP1 at high concentrations above 10 μM.[59] Most recent reports by *Pick* et al. (tariquidar analogue No. 14; **25**; Fig. 4; $IC_{50\ (P\text{-}gp)}$ = 3.63 μM, $IC_{50\ (MRP1)}$ = 20.77 μM, $IC_{50\ (BCRP)}$ = 1.25 μM),[60] *Juvale* et al. (quinazoline No. 29; **26**; Fig. 4; $IC_{50\ (P\text{-}gp)}$ = 3.49 μM, $IC_{50\ (MRP1)}$ = 7.82 μM, $IC_{50\ (BCRP)}$ = 0.42 μM),[61] *Krapf* et al. (4-anilino-quinoline No. 29; **27**; Fig. 4; $IC_{50\ (P\text{-}gp)}$ = 1.42 μM, $IC_{50\ (MRP1)}$ = 2.98 μM, $IC_{50\ (BCRP)}$ = 1.90 μM),[62] and us (pyrrolopyrimidine No. 23 (**28**; $IC_{50\ (P\text{-}gp)}$ = 21.7 μM, $IC_{50\ (MRP1)}$ = 0.981 μM, $IC_{50\ (BCRP)}$ = 4.68 μM)[37] and derivative No. 9 (**29**; $IC_{50\ (P\text{-}gp)}$ = 14.0 μM, $IC_{50\ (MRP1)}$ = 0.790 μM, $IC_{50\ (BCRP)}$ = 20.4 μM); both Fig. 4)[63] are the most noteworthy triple inhibitors.

As one can see, multiple inhibitors can be found in many different structural classes. But broad-spectrum compounds active in low micromolar range or better are rarely known. Pyrrolo- and indolopyrimidines are promising leads with regard to inhibition of ABC transport proteins, and cancer research in general, as different compounds of these substance classes were found to have antiproliferative characteristics with antitumor activity.[64] Since pyrrolopyrimidines[35,37] and indolopyrimidines[36] contain the same 9-deazapurine partial structure, we termed these compounds as stated in the title.

In this work new 9-deazapurines were synthesized and investigated for their inhibitory power against P-gp, MRP1 and BCRP. The most promising triple inhibitor was evaluated with regard to its intrinsic toxicity, sensitization of A2780/ADR (daunorubicin; P-gp), H69AR (daunorubicin; MRP1) and MDCK II BCRP (SN-38; BCRP) cells and inhibition type.

Results and Discussion

Chemistry

The compounds **41**-**61** were prepared as stated in the literature, and the intermediates have already been described.[35,37,63,65]

Preparation of the 3-(2-(piperazine-1-yl)ethyl)-1*H*-indole precursor (**30**, Fig. 5 A) for compounds **41**-**43**, **48**-**55**, and **66** was performed as stated in the literature[66] with minor modifications (Scheme 1 A). 3-(2-bromoethyl)-1*H*-indole was added to a saturated solution of piperazine in acetonitrile and the mixture exposed to diisopropylethylamine for 24 h, yielding the alkylated intermediate 3-(2-(piperazine-1-yl)ethyl)-1*H*-indole **30**.

The intermediates **31a**-**b** to **35a**-**b** for compounds **62**-**65** were prepared as reported before.[37,63] 2-Methoxyaniline or 4-ethoxyaniline was added to a saturated solution of (ethoxylmethylene)malononitrile in ethanol, yielding the malononitrile derivatives **31a**-**b**. The pyrrole derivatives **32a**-**b** were obtained by exposure to ethyl bromoacetate. Addition of dimethyl formamide dimethyl acetal gave the intermediates **33a**-**b**. Ring closure was performed by exposure to gaseous ammonia, yielding the lactam derivatives **34a**-**b**. Chlorination using phosphoryl chloride gave the precursors **35a**-**b**. Compounds **62**-**65** were prepared as described before.[37,63] Scheme 1 B gives an overview of the synthetic steps.

Preparation of the intermediates **36**-**40** of compounds **66**-**74** was performed as stated in the literature[35,36,37,63] with minor modifications. 2-Aminobenzonitrile was exposed to excess acetic anhydride yielding the acylated derivative **36**. Cyclisation to the pyrrole analog **37** was conducted with ethyl bromoacetate. Addition of dimethyl formamide dimethyl acetate yielded derivative **38**. Deacylation and simultaneous cyclisation was achieved by exposure to gaseous ammonia, providing compound **39**. Excess

phosphoryl chloride and reflux gave the chlorinated precursor **40**. Compounds **66**-**74** were prepared as described before.[35,36,37,63,65]

All other compounds were also prepared as stated in the literature.[35,37,63] The identity of the intermediates **30**, **31a**-**b** to **35a**-**b** and **36**-**40** was confirmed by ^{1}H NMR spectroscopy. The identity of compounds **41**-**74** was determined by ^{1}H and ^{13}C NMR spectroscopy and their purity by LC-MS analysis.

Biological Investigation

Calcein, Daunorubicin and Pheophorbide A Assays to Investigate the Compounds for P-gp, MRP1 and BCRP Inhibition. We classified the 9-deazapurines according to their structure into four classes: (i) 7,8-cyclisized 9-deazapurines with nitrogen containing heterocyclic residues at position 6 (table 1); (ii) 9-deazapurines with (2-(1*H*-indole-3-yl)ethyl)piperazine-1-yl residue at position 6 and aliphatic or aliphatic-aromatic variations at position 7 (table 2); (iii) 7,8-cyclisized 9-deazapurines with oxygen containing heterocyclic residues at position 6 and 7 (table 3); (iv) 8,9-annulated 9-deazapurines with variations at position 6 (table 4). Figure 6 shows the obtained results of subclass (i). The starting compound was compound **41** (Fig. 5 A), which had already been described by *Wang* et al.[35] This applies also for standard compound 12 (SC12, **75**; Fig. 5 B), which was established as standard inhibitor of MRP1 by us before.[37] According to the publication of *Wang* et al. in 2004 compound **41** possessed an IC_{50} value of 5.82 µM against P-gp and 0.6 µM for MRP1. In comparison to **75** (Fig. 5 B), which possessed in their work an IC_{50} of 9.63 µM toward P-gp and 0.69 µM against MRP1, the activities of these two compounds can be considered to be equal. *Wang* et al. decided to go on with the phenethylpiperazine structure. The reasons are simple,

since building blocks of this kind are much cheaper and easier to purchase. Additionally, it seemed that the phenethylpiperazine derivative was a bit more selective than the 1*H*-indole derivative. With multiple inhibition toward the three major ABC transport proteins in mind, we took compound **41** as starting structure for our continuing work. In our test system it possessed an IC_{50} value of 5.52 µM toward P-gp and 0.344 µM against MRP1, very similar to the results found by *Wang* et al. Additionally, the compound had also inhibitory activity against BCRP (IC_{50} = 3.41 µM). Compared to the compounds mentioned in the introduction, this was already a good triple inhibitor. It was shown before that increase of the molecular weight can be beneficial with respect to P-gp and MRP1 inhibition.[37] The introducing of a cycloheptyl ring system (compound **42**) at positions 7 and 8 instead of the cyclohexyl ring increased activity toward the two former transport proteins, but erased the affinity to BCRP. This made it a rather potent dual P-gp/MRP1 inhibitor ($IC_{50\ (P\text{-}gp)}$ = 1.71 µM and $IC_{50\ (MRP1)}$ = 0.289 µM, respectively). Interestingly, the elimination of the piperazine partial structure made the resulting compound **43** a selective inhibitor of MRP1 in submicromolar range ($IC_{50\ (MRP1)}$ = 0.957 µM).

A pyridine partial structure with alkyl linker was shown before to be beneficial for MRP1 and P-gp inhibition, as **2**[24,25] or **23**[56,57,58] show. Therefore, we substituted the 4-(2-(1*H*-indole-3-yl)ethyl)piperazine-1-yl residue of compounds **41**-**42** by a 4-(2-(pyridin-4-yl)ethyl)piperazine-1-yl side chain. The resulting compounds **44** and **45** had dual inhibitory power toward MRP1 and BCRP, although the BCRP affinity was in double-digit micromolar range. Higher lipophilicity seemed to be preferred as the latter compound had a cycloheptyl ring system compared to the former with its cyclohexyl ring. Removal of the ethylene linker between the nitrogen heterocycle and the piperazine partial structure gave compound **46**, which was also a good dual inhibitor of MRP1 (IC_{50} = 1.02 µM) and BCRP (IC_{50} = 4.86 µM) with no affinity to P-gp. The

more hydrophobic counterpart compound **47** was unfortunately not analyzable due to solubility issues. This compound promised to have an even better affinity toward MRP1 and BCRP since this correlation of activity and lipophilicity was found by us before.[37]

The compound class (ii) of this work consisted of 4-(2-(1*H*-indole-3-yl)ethyl)piperazine-1-yl derivatives of 9-deazapurines with aliphatic and aliphatic-aromatic variations at position 7. Figure 7 gives the corresponding screening data. Neither the screening nor the detailed biological investigation of MRP1 gave a tendency for a preferred side chain at position 7, as these compounds showed all good inhibitory activity toward this transporter between 0.5 µM and 0.3 µM. This could be expected, as all compounds of this work were derived from known MRP1 inhibitors. But interestingly, we observed a correlation between side chain length and inhibitory power with respect to P-gp and BCRP. One can see the tendency between compounds **48** and **55** (Fig. 5 C). This could be confirmed by the activity data, which is increasing with higher lipophilicity. Compound **52** had no affinity toward BCRP, but good inhibitory power against P-gp and MRP1 ($IC_{50\ (P\text{-}gp)}$ = 1.28 µM and $IC_{50\ (MRP1)}$ = 0.328 µM, respectively). But compounds **53**-**55** showed good inhibitory activity toward all the ABC transporters. While compound **53** had noticeable differences in the activity against P-gp (IC_{50} = 4.48 µM), MRP1 (IC_{50} = 0.405 µM) and BCRP (IC_{50} = 4.07 µM), compounds **54** and **55** were both active in the low micromolar range or even below. It is noteworthy that compound **54** did not reach 100% accumulation in the calcein AM assay, for which we have no explanation until now. Since this test compound did not reach the necessary value of 75% of the maximal inhibition caused by cyclosporine A toward P-gp or **24** toward BCRP, it was not investigated in the MDR reversal-efficacy testing. Compound **55** was the best inhibitor of the three ABC transporters, with IC_{50} values of 1.46 µM (P-gp), 0.501 µM (MRP1) and 1.69 µM (BCRP). This makes it an exceptional triple inhibitor,

and as far as we know there has not been any report of that kind. Therefore, this compound was chosen for ongoing experiments.

As one can see in Figures 1 to 4, many known P-gp inhibitors have oxygen containing substituents, especially methoxy groups,[31,41,42,43,52] like **1** (Fig. 1). Additionally, a piperonyl partial structure was reported to be beneficial with regard to P-gp inhibition.[44,45,46,55] Therefore we synthesized new 9-deazapurine derivatives containing oxygen heterocycles or oxygen containing substituents (compound class (iii)). The corresponding screening results are presented in Figure 8. The piperonyl-containing compounds (**56**-**59**) were good MRP1 inhibitors, but poor ones of P-gp or BCRP. A furanoyl moiety with annulated cyclohexyl (**60**) or cycloheptyl (**61**) at position 7 and 8 gave dual inhibitors of MRP1 and BCRP with rather poor inhibitory activity against the latter transport protein. Derivatization at position 7 with a 2-methoxyphenyl (**62**-**63**) or 4-ethoxyphenyl (**64**-**65**) scaffold gave compound **63** as only representative of a triple inhibitor, but with very poor inhibitory power toward BCRP and P-gp. Altogether, the herein presented oxygen derivatives gave not the expected results with regard to inhibition of P-gp, although compound **65** showed to affect both, P-gp (IC_{50} = 7.29 µM) and MRP1 (IC_{50} = 0.859 µM).

Finally, we synthesized 8,9-annulated 9-deazapurines with variations at position 6, as these were presented to be very potent inhibitors of MRP1 before.[36] This moiety has also similarity to known BCRP inhibitors.[42] Herein we combined these structures with substituents that have previously[37,63] and within this work been shown to affect P-gp. Figure 9 gives a summary of the screening data. Subclass (iv) can be divided into three categories: (a) 9-deazapurines with nitrogen heterocycle side chain (No. **66**-**68**); (b) 9-deazapurines with aliphatic-aromatic variations at position 6 (No. **69**-**72**); (c) 9-deazapurines with oxygen heterocycle containing side chains (No. **73**-**74**). As one can

see already from the screening results (Fig. 9), this compound class had high affinity toward BCRP, while these compounds were only capable to achieve I_{max} values between 30% and 60% with respect to inhibition of P-gp and MRP1, respectively. Within the first category (a), the 2-(pyridin-2-yl)ethyl residue of compound **67** is preferred compared to the 2-(1*H*-indole-3-yl)ethyl residue of compound **66** with respect to MRP1 inhibition. Vice-versa, the inhibitory activity toward the other two transport proteins is diminished. But removal of the alkyl side chain in compound **68** gave a good triple inhibitor with IC_{50} values of 5.00 µM (P-gp), 0.495 µM (MRP1) and 1.81 µM (BCRP). This is again a rare example of a triple inhibitor in the single-digit micromolar range. This tendency can also be observed with respect to the second category (b). While the inhibitory activity toward MRP1 decreases from compound **69** to **72** (similar to previous observations with respect to these residues[37]), it increases with respect to BCRP. Compound **69** is the best compound in this set against P-gp (IC_{50} = 1.11 µM), MRP1 (IC_{50} = 0.405 µM), BCRP (IC_{50} = 0.839 µM), although one might keep in mind that this compound like the other 8,9-annulated 9-deazapurines had a reduced I_{max} value ($I_{max\ (P\text{-}gp)}$ = 26%; $I_{max\ (MRP1)}$ = 61%; $I_{max\ (BCRP)}$ = 28%). Figures 10 A-C show the concentration-effect curves of compound **69** as a representative of the 8,9-annulated 9-deazapurines in comparison to the corresponding standard inhibitors cyclosporine A (P-gp), compound **75** (MRP1) and compound **24** (BCRP). The basic mechanism behind this phenomenon has not been clarified until now and is subject of further investigations.

Although there is a tenfold difference between the activities toward MRP1 (IC_{50} = 0.146 µM) and BCRP (IC_{50} = 1.10 µM), compound **72** is one rare example of a good dual inhibitor of these transport proteins. Finally, among the oxygen heterocycle-bearing derivatives in sub-class (iii) compound **73** was found to be a moderate triple inhibitor in single-digit and submicromolar concentration range. The furanoyl containing

compound **74** is again a rare example of a dual MRP1/BCRP inhibitor with IC_{50} values of 0.169 µM (MRP1) and 6.06 µM (BCRP).

The effect of partial inhibition as described for compounds **53**-**54** (ii) or the 8,9-annulated 9-deazapurines (e.g. compound **69** (Fig. 10)) had already been observed and reported in the literature. The quinazoline-chalcone derivative No. 17 (**76**; Fig. 11) showed a partial effect on A2780/ADR cells overexpressing P-gp.[20] This was also shown for several other compound classes.[67,68] But also for MRP1 reports are available. The natural compound cepharanthine (**77**; Fig. 11) for example was not able to sensitize MRP1 overexpressing IN500 and T98G glioma cells completely toward vincristine, etoposide and doxorubicin.[25] The same observations have been made for the MRP1 inhibitor 2-(4-benzhydrylpiperazin-1-yl)ethyl 5-(4,6-dimethyl-2-oxido-1,3,2-dioxaphosphinan-2-yl)-2,6-dimethyl-4-(3-nitrophenyl)nicotinate (PAK 104P, **78**; Fig. 11) in HL60/ADR cells[69] or the widely used standard MRP1 inhibitor 3-(((3-(2-(7-chloroquinolin-2-yl)vinyl)phenyl)((3-(dimethylamino)-3-oxopropyl)thio)methyl)thio)propanoic acid (MK571, **79**; Fig. 11) in GLC_4/ADR cancer cells.[70] Last but not least, several BCRP inhibitors were shown not to reach the inhibition value of the standard inhibitor **24**, like the tetrazolic HM30181 derivative No. 10 (**80**; Fig. 11) reported by *Köhler* et al.[41] or the 4-anilino-quinazoline No. 4 (**81**; Fig. 11) reported by *Krapf* and coworkers.[62] Even compound **24** itself is a partial inhibitor compared to fumitremorgin C (**82**; Fig. 11), an *Aspergillus fumigatus*-isolated compound from which standard inhibitor **24** was derived. This and several other tariquidar analogs with partial inhibiting feature have been reported by *Kühnle* et al.[71] The basic mechanisms behind these effects are still unknown and the objective of further investigations.

*MTT-viability Assay to Determine Intrinsic Cytotoxicity of Broad-spectrum Inhibitor **55**.* Since compound **55** showed very good inhibitory activity against P-gp, MRP1 and BCRP, and the maximal effect values were comparably high as the corresponding effect values of the standard inhibitors cyclosporine A, **75** and **24**, respectively, we further investigated this compound with respect to its intrinsic toxicity. As can be seen in Figure 12 A, compound **55** had no toxic effect on H69AR, MDCK II BCRP and the corresponding sensitive cell lines, and only a marginal effect on A2780/ADR and the wild type cells. Table 5 gives the corresponding GI_{50} values, which ranged mostly in the low double-digit concentration range. Table 5 gives also the calculated therapeutic ratios ($GI_{50\ (\text{compound } \mathbf{55} \text{ in resistant cell line})}$ divided by the $IC_{50\ (\text{compound } \mathbf{55} \text{ in resistant cell line})}$) of all tested cell lines with respect to compound **55**. It is always at least 9 times more potent than toxic with respect to the three tested cell lines. The corresponding graphics can be taken from Figures 12 B-D.

*MDR Reversal Assay to Determine Capability of Compound **55** to Restore Sensitivity of ABC Transporters.* Figure 13 A to C show the obtained concentration-effect curves of daunorubicin (P-gp and MRP1) and SN-38 (BCRP) in P-gp (A2780/ADR), MRP1 (H69AR) and BCRP (MDCK II BCRP) overexpressing cells in comparison to their parental counterparts (A2780, H69 and MDCK II, respectively). An increase of the concentration of compound **55** from 0.1 µM to 10 µM leads to sensitization of the cell lines as indicated by the shift of the corresponding concentration-effect curve to the left toward the sensitive cell line. Calculation of the resistance factor ($rf_{native} = EC_{50\ (\text{cytotoxic agent; resistant cell line})}$ divided by the $EC_{50\ (\text{cytotoxic agent; sensitive cell line})} = rf_{native}$) without and with supplementation ($rf_{compound} = EC_{50\ (\text{cytotoxic agent; resistant cell line + compound } \mathbf{55})}$ divided by the $EC_{50\ (\text{cytotoxic agent; sensitive cell line})} = rf_{compound}$) of compound **55** of all cell lines is shown in table 6. While the A2780/ADR cell line is 54 times more resistant against daunorubicin than

the sensitive cell line, the resistance factor became only 4.1 in presence of 10 µM of compound **55**. The same holds for MRP1. While the doxorubicin-selected variant was 11 times more resistant than the sensitive counterpart, 10 µM of compound **55** decreased the rf to 1.2, which equals full sensitization. With respect to MDCK II BRCP cells, the transfected variant was 20 times more resistant than the wild type. Here, 10 µM compound **55** fully restored sensitivity, making the transfected cells as sensible toward SN-38 as the wild type. Vice versa, the potentiation factor (pf = $EC_{50\ (\text{cytotoxic agent; resistant cell line})}$ divided by $EC_{50\ (\text{cytotoxic agent; resistant cell line + compound 55})}$) arose with increasing compound concentration (table 6). We also calculated the maximal sensitization (pf divided by the rf_{native}), which represents the percentage sensitization normalized on the resistance factor. While compound **55** was able to fully restore sensitivity of MRP1 and BCRP overexpressing cells, this effect was only partially observed with regard to A2780/ADR cells. This is caused by the resistance factor of 54, which is high compared to the other two cell lines.

To further evaluate the compound with respect to its sensitizing property, we plotted the resultant GI_{50} values of each calculated concentration-effect curve from Figure 13 A-C against the concentration of compound **55**. Minus infinity (bottom value) corresponds to the GI_{50} value of the resistant cell line without compound. Infinity (top value) corresponds to the GI_{50} value of the sensitive cell line. As one can see from Figure 14, the resultant concentration-effect curves for all three tested ABC transporters had EC_{50} values below 1 µM, that are presented in Table 7. This means that only submicromolar concentrations are needed to reduce the resistance of the tested cell lines by half.

Determination of Mode of Inhibition. We analyzed compound **55** with respect to the type of inhibition. As can be seen in Figure 15, in all three cases the type of inhibition is non-competitive as indicated by the reduced transport velocity (v_{max}), while the affinity (K_M) stayed the same. Taken together, compound **55** is a non-competitive inhibitor of P-gp-mediated transport of calcein AM, MRP1-mediated transport of daunorubicin, and BCRP-mediated transport of pheophorbide A.

Conclusion.

In this work we continued our effort to generate new potent inhibitors of the ABC transport proteins P-gp, MRP1 and BCRP. While the literature gives much information about selective and dual inhibitors with respect to these three transporters, the field of triple inhibition is rather poorly investigated.

In this manuscript we report not only on compounds with good dual inhibitory power toward P-gp/MRP1 (**42**, **52**, **65**) and BCRP/MRP1 (**46**, **72**, **74**), but also on compound **55** as a highly potent and efficient modulator of all three investigated transport proteins. With respect to accumulation of the fluorescent substrates calcein (P-gp), daunorubicin (MRP1) and pheophorbide A (BCRP), compound **55** showed to be superior in comparison to the other tested compounds as the resulting I_{max} values were higher than 75% compared to the effect-values of the corresponding standard inhibitors. The IC_{50} values with respect to these three transport proteins were in the low single-digit micromolar range (P-gp and BCRP) or even in the higher nanomolar range (MRP1). Sensitization of the cell lines toward the cytotoxic agents daunorubicin (P-gp and MRP1) as well as SN-38 (BCRP) determined via the MDR reversal assay showed compound **55** to fully sensitize the latter two transporters, while P-gp overexpressing

cells still kept partial resistance toward daunorubicin. Furthermore, we showed that only submicromolar concentrations are needed to reduce the resistance of the corresponding cell lines by half. Up to now there has never been any report on a compound with this ability. The type of inhibition of the P-gp-mediated transport of calcein AM, the MRP1-mediated transport of daunorubicin and the BCRP-mediated transport of pheophorbide A was non-competitive in all three cases as determined by Cornish-Bowden analysis. Due to the fact that compound **55** is at least 9 times more potent than toxic toward A2780/ADR, H69AR and MDCK II BCRP cells, and it sensitizes cancer cells significantly in submicromolar concentrations, it represents a great lead structure for ongoing research in cancer chemotherapy and a good candidate for *in vivo* studies.

Experimental Section.

Chemistry. *Materials*. Chemicals used for synthesis were purchased from Acros Organics (Geel, Belgium), Alfa Aesar (Karlsruhe, Germany), Applichem GmbH (Darmstadt, Germany), Fisher Scientific GmbH (Waltham, MA, USA), Merck Millipore (Billerica, MA, USA), Sigma-Aldrich (St. Louis, MO, USA) and VWR International GmbH (Darmstadt, Germany) and used without further purification. For monitoring reaction progress analytical thin layer chromatography was used with silica gel F_{254} coated aluminum plates (Merck Millipore). Methylene chloride/acetone (18:1 and 9:1, respectively) or methylene chloride/acetone/methanol (9:1:1) was used as eluent for all intermediates **31a**-**b** to **35a**-**b** and **36**-**40** and target compounds **41**-**74**, except for compound **30**, which was analyzed with methylene chloride/acetone/ethanol/ammonia solution (25%; 1:1:1:0.5). UV detection was conducted at 254 nm. The target compounds **41**-**74** were purified by column chromatography with silica gel 60 (43-60

μm, Merck Millipore) and gradient elution (petroleum ether/methylene chloride 1:1, methylene chloride, methylene chloride/acetone 18:1, methylene chloride/acetone 9:1, methylene chloride/acetone/methanol 18:1:1, methylene chloride/acetone/methanol 9:1:1, in each case 200 mL). Intermediate **30** was additionally eluted with 200 mL of methylene chloride/acetone/ethanol/ammonia solution (25%; 1:1:1:0.5 or 1:1:1:1). While the identity of intermediates **30**, **31a**-**b** to **35a**-**b** and **36**-**40** was determined by ^{1}H NMR spectroscopy, the identity and purity of the target compounds **41-74** was confirmed by ^{1}H, as well as ^{13}C NMR spectroscopy and LC-MS analysis. The NMR spectra were recorded in DMSO-d_6 or $CDCl_3$ as stated in the spectroscopic data. Using a Bruker Avance 500 MHz (500/126 MHz) the chemical shifts (δ) are expressed as ppm calibrated to the solvent signal of DMSO (^{1}H NMR δ 2.50; ^{13}C NMR δ 39.5) or $CDCl_3$ (^{1}H NMR δ 7.26; ^{13}C NMR δ 77.1). Distortionless enhancement by polarization transfer (DEPT) and attached proton test (APT) techniques were employed to assign ^{13}C signals. Spin multiplicities of the compounds **30-74** are depicted as singulet (s), doublet (d), doublet of doublets (dd), triplet of doublets (td), triplet (t), doublet of triplets (dt), quartet (q), quintet (quint) and multiplet (m). LC-MS analysis of the target compounds **41**-**74** was conducted using an Agilent 1100 series with photo diode array (DAD) detector (Agilent Technologies, Santa Clara, CA, USA) and a Nucleodur column 100-5 C18 (Macherey-Nagel, Düren, Germany) followed by ESI mass spectrometry using an API 2000 Triple Quadrupole mass spectrometer (Applied Biosystems, Waltham, MA, USA) and Sciex Analyst Software version 1.5.1. The purity of all investigated compounds in biological testing was determined as ≥ 95% unless otherwise stated.

General Procedure for the Preparation of Compound **30**. This synthesis was performed as described[66] with minor modifications. 3-(2-bromoethyl)-1*H*-indole was dissolved in acetonitrile and given to a saturated solution of piperazine (4 eq.) in acetonitrile.

Addition of Hünig base (diisopropylethylamin, 6 eq.) started the reaction at room temperature. After 24 hours, the Hünig base hydrochloride was filtered off and the clear filtrate was evaporated, resulting in an orange oil containing the title compound. Column chromatography performed as stated above resulted in the purified alkylated product.

*3-(2-(piperazine-1-yl)ethyl)-1H-indole (***30***)*. Orange solid; yield: 52%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.74 (s, 1H), 7.49 (d, J = 7.9 Hz, 1H), 7.31 (d, J = 8.1 Hz, 1H), 7.13 (d, 2.3 Hz, 1H), 7.04 (dd, J = 8.1, 7.0 Hz), 6.95 (dd, J = 7.9, 7.0, Hz), 2.85-2.81 (m, 2H), 2.81-2.76 (m, 4H), 2.59-2.53 (m, 2H), 2.48-2.40 (m, 4H), 1.87 (s, 1H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 136.3, 127.4, 122.6, 120.9, 118.4, 118.2, 112.7, 111.4, 59.5, 53.2 (2C), 45.2 (2C), 22.3.

General Procedure for the Preparation of Compounds **36a-b**. The intermediates **31a-b** to **35a-b** for compounds **62-65** were synthesized as described before.[37,63] 2-methoxyaniline or 4-ethoxyaniline was given to a saturated solution of (ethoxymethylene)malononitrile in ethanol. The reaction progress could be followed visibly as the resultant aniline derivative (**31a** or **31b**) precipitated as a yellow solid. After 1 hour, the solid was filtered off, rinsed with ethanol and dried. 2-(((2-methoxyphenyl)amino)methylene)malononitrile (**31a**) or 2-(((4-ethoxyphenyl)amino)methylene)malononitrile (**31b**) was dissolved in dimethyl formamide and heated to 100 °C in the presence of excess potassium carbonate. Ethyl bromoacetate was added dropwise in an equimolar amount and heating was continued for 5 hours. After pouring into ice-cold water, the resulting precipitate containing the pyrrole derivatives **32a** or **32b** was filtered off and dried. Ethyl 3-amino-4-cyano-1-(2-methoxyphenyl)-1*H*-pyrrole-2-carboxylate (**32a**) or ethyl 3-amino-4-cyano-1-(4-ethoxyphenyl)-1*H*-pyrrole-2-carboxylate (**32b**) was dissolved in dimethyl formamide

and 5 eq. of dimethyl formamide dimethyl acetale were added. After 5 hours, the solvent was evaporated and the viscous liquid containing the pyrrole derivatives **33a** or **33b** was used without further purification or characterization. The brown oil containing ethyl 4-cyano-3-(((dimethylamino)methylene)amino)-1-(2-methoxyphenyl)-1*H*-pyrrole-2-carboxylate (**33a**) or ethyl 4-cyano-3-(((dimethylamino)methylene)amino)-1-(4-ethoxyphenyl)-1*H*-pyrrole-2-carboxylate (**33b**) was dissolved in ethanol and gaseous ammonia was passed in for 5 hours. The solvent was evaporated and a sodium hydroxide solution (10% m/m) was added, stirring the reaction mixture for 30 minutes. Neutralization with acetic acid gave the cyclisation product **34a** or **34b**. 5-(2-methoxyphenyl)-4-oxo-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (**34a**) or 5-(4-ethoxyphenyl)-4-oxo-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (**34b**) was dissolved in excess phosphoryl chloride chloride and refluxed overnight. The reaction mixture was poured into ice-cold water leading to a precipitate, which was filtered off, rinsed with fresh water and dried, yielding the title compounds 4-chloro-5-(2-methoxyphenyl)-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (**35a**) or 4-chloro-5-(4-ethoxyphenyl)-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (**35b**) as precursors to generate the target compounds **62-65**.

*2-(((2-Methoxyphenyl)amino)methylene)malononitrile (***31a***)* Yellow needles; yield: 46%. This intermediate was used without further purification or characterization.

*2-(((4-Ethoxyphenyl)amino)methylene)malononitrile (***31b***)* Yellowish powder; yield: 73%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.98 (s, 1H), 8.34 (s, 1H), 7.33 (d, J = 9.1 Hz, 2H), 6.91 (d, J = 9.1 Hz, 2H), 4.00 (q, J = 7.0 Hz, 1H), 1.30 (t, J = 7.0 Hz, 3H).

*Ethyl 3-amino-4-cyano-1-(2-methoxyphenyl)-1H-pyrrole-2-carboxylate (***32a***)* Brown powder; quantitative yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.63 (s, 1H), 7.21 (d, J =

8.9 Hz, 2H), 6.93 (d, *J* = 9.0, 2H), 5.90 (s, 2H), 4.05 (q, *J* = 7.0), 3.99 (q, *J* = 7.1 Hz, 2H), 1.33 (t, *J* = 7.0 Hz, 3H), 0.97 (t, *J* = 7.1 Hz, 3H).

*Ethyl 3-amino-4-cyano-1-(4-ethoxyphenyl)-1H-pyrrole-2-carboxylate (***32b***)* Brown powder; yield: 92%. This intermediate was used without further purification or characterization.

*Ethyl 4-cyano-3-(((dimethylamino)methylene)amino)-1-(2-methoxyphenyl)-1H-pyrrole-2-carboxylate (***33a***)* Brown oil; quantitative yield. This intermediate was used without further purification or characterization.

*Ethyl 4-cyano-3-(((dimethylamino)methylene)amino)-1-(4-ethoxyphenyl)-1H-pyrrole-2-carboxylate (***33b***)* Brown oil, yield: 94%. This intermediate was used without further purification or characterization.

*5-(2-Methoxyphenyl)-4-oxo-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***34a***)* greywhite powder; yield: quantitative. This intermediate was used without further purification or characterization.

*5-(4-Ethoxyphenyl)-4-oxo-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***34b***)* Greywhite powder; yield: quantitative. This intermediate was used without further purification or characterization.

*4-Chloro-5-(2-methoxyphenyl)-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***35a***)* Greywhite powder; yield: not determined. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.94 (s, 1H), 8.89 (s, 1H), 7.63-7.58 (m, 2H), 7.28 (dd, *J* = 8.8, 1.1 Hz, 1H), 7.15 (td, *J* = 7.6, 1.2 Hz, 1H), 3.74 (s, 3H).

4-Chloro-5-(4-ethoxyphenyl)-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (**35b**) Greywhite powder: yield: quantitative. This intermediate was used without further purification or characterization.

General Procedure for the Preparation of Compound **40**. Due to low yields when attempting to react 2-aminobenzonitrile with ethyl bromoacetate, the primary amine was acylated first. Therefore, 2-aminobenzonitrile was dissolved in excess acetic anhydride and heated to 100 °C using microwave irradiation at 200 W for 2 hours. The reaction mixture was poured into water. The resulting precipitate was rinsed with fresh water and filtered off, resulting in the acylated compound **36**. The following reactions were performed as already published[35,36,37,63] but with minor modifications. N-(2-cyanophenyl)acetamide (**36**) was dissolved in dimethyl formamide and heated to 100 °C in the presence of excess potassium carbonate. An equimolar amount of ethyl bromoacetate was added dropwise and heating was continued for 5 hours. Afterwards, the reaction mixture was poured into ice-cold water and stirred for one hour. The resultant precipitate was filtered off and dried, yielding the pyrrole derivative **37**. A solution of compound **37** in dimethyl formamide and 3.5 equivalents of dimethyl formamide dimethyl acetale was heated to 100°C for 5 h. Afterwards the solvent was evaporated resulting in a brown oil, which was used without further purification and characterization. This oil containing ethyl 1-acetyl-3-(((dimethylamino)methylene)amino)-1*H*-indole-2-carboxylate (**38**) was dissolved in ethanol and gaseous ammonia was passed through for 5 hours. Addition of a 10% (m/m) sodium hydroxide solution followed by stirring for 30 minutes. After neutralization with acetic acid, the precipitating solid was rinsed with fresh water and dried, resulting in the cyclisized compound **39**. The dried 3,5-dihydro-4*H*-pyrimido[5,4-*b*]indole-4-one (**39**) was dissolved in excess phosphoryl chloride and refluxed overnight. The reaction

mixture was poured into ice-cold water, yielding the chlorinated precursor 4-chloro-5*H*-pyrimido[5,4-*b*]indole (**40**).

*N-(2-Cyanophenyl)acetamide (***36***)* White needles; yield: 70%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.11 (s, 1H), 7.78 (dd, J = 7.6, 1.4 Hz, 1H), (dd, J = 8.3, 7.5 Hz, 1H), 7.57 (dd, J = 8.4, 0.9 Hz, 1H), 7.32 (td, J = 7.6, 1.2 Hz, 1H), 2.09 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 168.8, 140.5, 133.9, 133.3, 125.7, 125.6, 117.0, 107.4, 23.3.

*Ethyl 3-amino-1H-indole-2-carboxylate (***37***)* Brown powder; yield: 62%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.05 (dt, J = 8.4, 0.8 Hz, 1H), 7.96 (dd, J 7.9, 1.2 Hz, 1H), 7.48 (dd, J = 8.5, 7.2 Hz, 1H), 7.27 (dd, J =8.0, 7.2 Hz, 1H), 6.70 (s, 2H), 4.29 (q, J = 7.0 Hz, 2H), 2.37 (s, 3H), 1.30 (t, J = 7.1 Hz, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 171.3, 162.1, 144.5, 138.5, 130.0, 122.0, 121.8, 121.2, 115.2, 104.3, 59.9, 26.5, 14.6.

*Ethyl 1-acetyl-3-(((dimethylamino)methylene)amino)-1H-indole-2-carboxylate (***38***)* Brown oil; yield: 62%. This intermediate was used without further purification or characterization.

*3,5-Dihydro-4H-pyrimido[5,4-b]indole-4-one (***39***)* greywhite powder; yield: 40%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.32 (s, 1H), 12.03 (s, 1H), 7.98 (s, 1H), 7.98 (dt, J 8.0, 0.9 Hz, 1H), 7.52 (dt, J = 8.3, 0.8, 1H), 7.45 (dd, J = 8.2, 6.9 Hz, 2H), 7.22 (dd, J = 7.9, 6.9 Hz, 1H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 154.7, 141.5, 138.7, 138.3, 127.1, 122.7, 121.3, 120.4, 120.3, 112.9.

*4-Chloro-5H-pyrimido[5,4-b]indole (***40***)* brown powder; yield: 52%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.33 (s, 1H), 8.84 (s, 1H), 8.25 (dt, J 8.0, 1.0 Hz 1H), 7.70 (dd, J = 7.8, 6.6 Hz, 1H), 7.68 (dt, J = 8.3, 1.3 2H), 7.37 (dd, J = 8.0, 6.6 Hz, 1H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 148.7, 147.0, 141.9, 141.4, 130.9, 128.2, 121.7, 121.2, 120.0, 113.1.

The target compounds **41**-**61**, **62**-**65** and **66**-**74** were prepared as already published by us and others.[35,36,37,63,65] A solution of chlorinated precursors,[37] **35a-b** or **40** in 0.5 mL dimethyl formamide and 0.5 mL of piperazine precursor **30**, other piperazine derivatives or tryptamine in 0.5 mL dimethyl formamide was heated to 110 °C with 200 W microwave irradiation for 60 minutes. The resulting product was first purified by column chromatography as stated above and recrystalyzed from ethanol and petroleum ether (1:1).

*4-(4-(2-(1H-Indole-3-yl)ethyl)piperazine-1-yl)-6,7,8,9-tetrahydropyrimido[4,5-b]indolizine-10-carbonitrile (***41***)* Yellow powder; yield: 58%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.76 (s, 1H), 8.47 (s, 1H), 7.52 (d, J = 7.9 Hz, 1H), 7.32 (dt, J = 8.2, 0.8 Hz, 1H), 7.15 (d, J = 2.3 Hz, 1H), 7.05 (dd, J = 8.1, 7.1 Hz, 1H), 6.96 (dd, J = 7.9, 7.0 Hz, 1H), 4.36 (t, J = 5.6 Hz, 2H), 3.37-3.32 (m, 4H), 3.11 (t, J = 6.5 Hz, 2H), 2.89 (t, J = 7.3 Hz, 2H), 2.71-2.65 (m, 6H), 1.98-1.88 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 154.4, 151.3, 151.0, 150.2, 136.3, 127.4, 123.6, 120.9 (2C), 118.4, 118.3, 114.5, 112.7, 111.5, 83.6, 58.9, 52.4 (2C), 50.4 (2C), 46.1, 22.9, 22.6, 22.6, 18.4. LC-MS: (*m*/*z*) calc.: 425.2; found: 426.4 $[M+H]^+$. Purity: 91%.

*4-(4-(2-(1H-Indole-3-yl)ethyl)piperazine-1-yl)-7,8,9,10-tetrahydro-6H-pyrimido[4',5':4,5]pyrrolo[1,2-a]azepine-11-carbonitrile (***42***)* Pale yellow crystals; yield: 99%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.74 (s, 1H), 8.48 (s, 1H), 7.52 (d, J = 7.9 Hz, 1H), 7.32 (d, J = 8.1, 1H), 7.16 (d, J = 2.2 Hz, 1H), 7.05 (dd, J = 8.0, 7.0 Hz, 1H), 6.96 (dd, J = 7.9, 7.1 Hz, 1H), 4.55-4.42 m, 2H), 3.41-3.30 (m, 4H), 3.09 (t, J = 5.5 Hz, 2H), 2.89 (t, J = 7.3 Hz, 2H), 2.75-2.62 (m, 6H), 1.91-1.82 (m, 2H), 1.80-1.71 (m, 4H) ^{13}C NMR (126 MHz, DMSO-d_6) δ 156.6, 153.7, 151.1, 149.6, 136.2, 127.4, 122.6, 120.9, 118.4, 118.3, 117.9, 114.6, 112.6, 111.4, 85.3, 58.9, 52.2 (2C), 49.8 (2C), 47.4, 29.9, 27.9, 27.1, 25.6, 22.5. LC-MS: (*m*/*z*) calc.: 439.3; found: 440.3 $[M+H]^+$. Purity: 99%.

*4-((2-(1H-Indole-3-yl)ethyl)amino)-6,7,8,9-tetrahydropyrimido[4,5-b]indolizine-10-carbonitrile (**43**)* Pale yellow crystals; yield: 13%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.80 (s, 1H), 8.27 (s, 1H), 7.60 (d, J = 7.8 Hz, 1H), 7.33 (d, J = 8.1 Hz, 1H), 7.20 (d, J = 2.1 Hz, 1H), 7.06 (t, J = 7.5 Hz, 1H), 6.97 (t, J = 7.4 Hz, 1H), 6.92 (t, J = 5.6 Hz, 1H), 4.32 (t, J = 6.2 Hz, 2H), 3.75 (dd, J = 14.0, 6.0 Hz, 2H), 3.04-2.98 (m, 4H), 2.03-1.95 (m, 2H), 1.86-1.79 (m, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 152.2, 149.8, 147.6, 147.5, 136.4, 127.6, 122.8, 121.1, 118.5, 118.3, 115.0, 113.9, 112.1, 111.5, 82.1, 45.6, 41.4, 25.0, 23.0, 22.1, 18.1. LC-MS: (m/z) calc.: 356.2; found: 357.2 $[M+H]^+$. Purity: 96%.

*4-(4-(2-(Pyridin-4-yl)ethyl)piperazine-1-yl)-6,7,8,9-tetrahydropyrimido[4,5-b]indolizine-10-carbonitrile (**44**)* Yellow powder; yield: 42%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.46 (s, 1H), 8.44 (dd, J = 4.4, 1.6 Hz, 2H), 7.28 (dd, J = 4.4, 1.6 Hz, 2H), 4.34 (t, J = 5.6 Hz, 2H), 3.38-3.31 (m, 4H), 3.11 (t, J = 6.5 Hz, 2H), 2.80 (t, J = 7.3 Hz, 2H), 2.71-2.60 (m, 6H), 1.98-1.87 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 154.3, 151.2, 151.0, 150.2, 149.5 (2C), 136.3, 124.4 (3C), 118.2, 114.4, 83.6, 58.1, 52.6 (2C), 50.2, 46.0 (2C), 31.8, 22.9, 22.6, 18.4. LC-MS: (m/z) calc.: 387.2; found: 388.3 $[M+H]^+$. Purity: 96%.

*4-(4-(2-(Pyridin-4-yl)ethyl)piperazine-1-yl)-7,8,9,10-tetrahydro-6H-pyrimido[4',5':4,5]pyrrolo[1,2-a]azepine-11-carbonitrile (**45**)* Yellow powder; yield: 42%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.49 (s, 1H), 8.46 (d, J = 4.6 Hz, 2H), 7.29 (d, J = 4.7 Hz, 2H), 4.52-4.45 (m, 2H), 3.20-3.17 (m, 4H), 3.12 (t, J = 6.4 Hz, 2H), 2.82 (t, J = 7.3 Hz, 2H), 2.70-2.59 (m, 6H), 1.91-1.83 (m, 2H), 1.83-1.71 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 156.6, 153.7, 151.1, 149.6, 149.5, 149.5 (2C), 124.4 (3C), 117.9, 114.6, 85.3, 58.2, 52.0 (2C), 49.7 (2C), 47.4, 31.8, 29.9, 27.9, 27.1, 25.6. LC-MS: (m/z) calc.: 401.2; found: 402.2 $[M+H]^+$. Purity: 100%.

*4-(4-(Pyridin-2-yl)piperazine-1-yl)-6,7,8,9-tetrahydropyrimido[4,5-b]indolizine-10-carbonitrile (**46**)* Pale yellow crystals; yield: 55%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.49 (s, 1H), 8.15-8.12 (m, 1H), 7.56 (dd, J = 8.9, 6.9 Hz, 1H), 6.88 (d, J = 8.6 Hz), 6.68 (dd, J = 7.1, 4.9 Hz, 1H), 4.42 (t, J = 5.5 Hz, 2H), 3.67 (t, J = 5.3 Hz, 4H), 3.42 (t, J = 5.2 Hz, 4H), 3.13 (t, J = 6.4 Hz, 2H), 1.99-1.88 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 159.2, 154.4, 151.3, 151.1, 150.3, 147.7, 137.8, 118.4, 114.4, 113.6, 107.5, 83.6, 50.1 (2C), 46.1, 44.5 (2C), 23.0, 22.6, 18.4. LC-MS: (m/z) calc.: 359.2; found: 360.1 $[M+H]^+$. Purity: 93%.

*4-(4-(Pyridin-2-yl)piperazine-1-yl)-7,8,9,10-tetrahydro-6H-pyrimido[4',5':4,5]pyrrolo[1,2-a]azepine-11-carbonitrile (**47**)* Yellow crystals; yield: 58%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.51 (s, 1H), 8.14 (dd, J = 4.8, 1.9 Hz, 1H), 7.57 (dd, J = 8.8, 6.8 Hz, 1H), 6.90 (d, J = 8.6 Hz), 6.68 (dd, J = 7.1, 4.9 Hz, 1H), 4.57-4.50 (m, 2H), 3.76-3.63 (m, 4H), 3.44-3.35 (m, 4H), 3.11 (t, J = 5.0 Hz, 2H), 1.89-1.73 (m, 6H). LC-MS: (m/z) calc.: 373.2; found: 374.2 $[M+H]^+$. Purity: 100%.

*4-(4-(2-(1H-Indole-3-yl)ethyl)piperazine-1-yl)-5-methyl-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (**48**)* yellow crystals; yield: 29%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.74 (s, 1H), 8.51 (s, 1H), 8.44 (s, 1H), 7.52 (d, J = 7.9 Hz, 1H), 7.32 (dt, J = 8.1, 0.9 Hz, 1H), 7.16 (d, J = 2.3 Hz, 1H), 7.05 (dd, J = 8.1, 7.0 Hz, 1H), 6.96 (dd, J = 7.9, 7.0 Hz, 1H), 4.01 (s, 3H), 3.42-3.37 (m, 4H), 2.89 (t, J = 7.3 Hz, 2H), 2.73-2.65 (m, 6H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 154.9, 151.4, 150.1, 141.5, 136.3, 127.4, 122.6, 121.0, 118.6, 118.4, 118.3, 114.4, 112.6, 111.5, 85.4, 58.9, 52.3 (2C), 50.2 (2C), 36.8, 22.5. LC-MS: (m/z) calc.: 385.2; found: 386.3 $[M+H]^+$. Purity: 100%.

*4-(4-(2-(1H-Indole-3-yl)ethyl)piperazine-1-yl)-5-ethyl-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (**49**)* Yellow crystals; yield: 25%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.76 (s, 1H), 8.61 (s, 1H), 8.55 (s, 1H), 7.52 (d, J = 7.9 Hz, 1H), 7.32 (d, J = 8.1 Hz, 1H), 7.16

(d, *J* = 2.2 Hz, 1H), 7.06 (dd, *J* = 8.0, 7.0 Hz, 1H), 6.96 (dd, *J* = 7.9, 7.0 Hz, 1H), 4.35 (q, *J* = 7.2 Hz, 2H) 3.38-3.33 (m, 4H), 2.90 (t, *J* = 7.4 Hz, 2H), 2.76-2.66 (m, 6H), 1.38 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 155.0, 151.4, 150.6, 140.6, 136.3, 127.4, 122.7, 121.0, 118.4, 118.3, 117.9, 114.4, 112.6, 111.5, 86.2, 58.9, 52.4 (2C), 50.2 (2C), 44.1, 22.6, 16.1. LC-MS: (*m*/*z*) calc.: 399.2; found: 400.2 $[M+H]^+$. Purity: 97%.

*4-(4-(2-(1H-Indole-3-yl)ethyl)piperazine-1-yl)-5-propyl-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***50***)* White crystals; yield: 21%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.74 (s, 1H), 8.60 (s, 1H), 8.56 (s, 1H), 7.52 (d, *J* = 7.9 Hz, 1H), 7.32 (dt, *J* = 8.1, 0.8 Hz, 1H), 7.16 (d, *J* = 2.3 Hz, 1H), 7.05 (dd, *J* = 8.1, 7.0 Hz, 1H), 6.96 (dd, *J* = 7.9, 7.0 Hz, 1H), 4.27 (t, *J* = 7.4 Hz, 2H) 3.38-3.32 (m, 4H), 2.90 (t, *J* = 7.3 Hz, 2H), 2.72-2.64 (m, 6H), 1.76 (p, *J* = 7.3 Hz, 2H), 0.72 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR 126 MHz, DMSO-d_6) δ 155.1, 151.4, 150.6, 141.2, 136.3, 127.4, 122.6, 120.9, 118.4, 118.3, 118.0, 114.3, 112.6, 111.5, 85.9, 58.9, 52.5 (2C), 50.8, 50.2 (2C), 24.2, 22.5, 10.7. LC-MS: (*m*/*z*) calc.: 413.2; found: 414.3 $[M+H]^+$. Purity: 91%.

*4-(4-(2-(1H-Indole-3-yl)ethyl)piperazine-1-yl)-5-cyclopropyl-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***51***)* White crystals; yield: 39%. ^{1}H NMR 500 MHz, DMSO-d_6) δ 10.74 (s, 1H), 8.47 (s, 1H), 8.46 (s, 1H), 7.51 (d, *J* = 7.9 Hz, 1H), 7.32 (d, *J* = 8.1, 1H), 7.15 (d, *J* = 2.3 Hz, 1H), 7.04 (dd, *J* = 8.1, 7.1 Hz, 1H), 6.96 (dd, *J* = 7.9, 7.1 Hz, 1H), 3.97 (p, *J* = 3.6 Hz, 1H) 3.60-3.50 (m, 4H), 2.88 (t, *J* = 7.8 Hz, 2H), 2.70-2.62 (m, 6H), 1.15-1.02 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 154.4, 151.3, 150.1, 140.2, 136.3, 127.4, 122.6, 120.9, 118.5, 118.4, 118.2, 114.4, 112.6, 111.4, 85.6, 58.9, 52.5 (2C), 49.8 (2C), 32.1, 22.5, 8.5 (2C). LC-MS: (*m*/*z*) calc.: 411.2; found: 412.2 $[M+H]^+$. Purity: 96%.

*4-(4-(2-(1H-Indole-3-yl)ethyl)piperazine-1-yl)-5-phenyl-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***52***)* Yellow crystals; yield: 11%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.70 (s, 1H), 8.74 (s, 1H), 8.58 (s, 1H), 7.62-7.58 (m, 2H), 7.54-7.50 (m, 3H), 7.43 (d, *J* = 7.8 Hz, 1H), 7.30 (dt, *J* = 8.1, 0.8 Hz, 1H), 7.07 (d, *J* = 2.3 Hz, 1H), 7.03 (dd, *J* = 8.1, 7.1 Hz, 1H), 6.94 (dd, *J* = 7.9, 7.0 Hz, 1H), 3.12-3.06 (m, 4H) 2.71 (t, *J* = 8.3 Hz, 2H), 2.44 (t, *J* = 8.2 Hz, 2H), 2.16-2.08 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 153.2, 151.9, 150.2, 141.4, 137.9, 136.3, 129.6 (2C), 128.5, 127.3, 125.3 (2C), 122.5, 120.9, 118.3, 118.2, 115.3, 114.1, 112.5, 111.4, 88.1, 58.7, 51.5 (2C), 48.8 (2C), 22.2. LC-MS: (*m*/*z*) calc.: 447.2; found: 448.1 $[M+H]^+$. Purity: 93%.

*4-(4-(2-(1H-Indole-3-yl)ethyl)piperazine-1-yl)-5-benzyl-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***53***)* white crystals; yield: 18%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.74 (s, 1H), 8.65 (s, 1H), 8.56 (s, 1H), 7.51 (d, *J* = 7.9 Hz, 1H), 7.33-7.28 (m, 3H), 7.27-7.23 (m, 1H), 7.16-7.14 (m, 1H), 7.14-7.13 (m, 1H), 7.43 (d, *J* = 7.8 Hz, 1H), 7.04 (dd, *J* = 8.1, 7.0 Hz, 1H), 6.96 (dd, *J* = 7.9, 7.0 Hz, 1H), 5.54 (s, 2H), 3.39-3.33 (m, 4H) 2.90-2.85 (m, 2H), 2.71-2.61 (m, 6H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 155.2, 151.7, 150.9, 141.7, 137.0, 136.3, 128.8 (2C), 128.1, 127.4, 127.1 (2C), 122.6, 120.9, 118.4, 118.3, 117.9, 114.00, 112.6, 111.4, 87.0, 58.3, 52.3 (2C), 52.1, 50.0 (2C), 22.5. LC-MS: (*m*/*z*) calc.: 461.2; found: 462.0 $[M+H]^+$. Purity: 97%.

*4-(4-(2-(1H-Indole-3-yl)ethyl)piperazine-1-yl)-5-phenethyl-5H-pyrrolo[3,2-d]pyrimidine-7- (***54***)* white crystals; yield: 9%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.73 (s, 1H), 8.55 (s, 1H), 8.50 (s, 1H), 7.51 (d, *J* = 7.9 Hz, 1H), 7.33 (dt, *J* = 8.1, 0.8 Hz, 1H), 7.18-7.09 (m, 4H), 7.05 (dd, *J* = 8.2, 7.0 Hz, 1H), 7.01-6.95 (m, 3H), 4.56 (t, *J* = 7.2 Hz, 2H), 3.29-3.26 (m, 4H) 3.01 (t, *J* = 7.1 Hz, 2H), 2.88 (t, *J* = 7.2 Hz, 2H), 2.71-2.61 (m, 6H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 155.0, 151.4, 150.4, 141.1, 137.3, 136.3, 128.7 (2C), 128.2 (2C), 127.4, 126.7, 122.6, 121.0, 118.4, 118.3, 118.1, 114.3,

112.6, 111.5, 86.2, 59.0, 52.3 (2C), 50.3, 50.0 (2C), 37.2, 22.4. LC-MS: (*m*/*z*) calc.: 475.3; found: 476.2 $[M+H]^+$. Purity: 97%.

*4-(4-(2-(1H-Indole-3-yl)ethyl)piperazine-1-yl)-5-(3-phenylpropyl)-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***55***)* Pale yellow crystals; yield: 16%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.75 (s, 1H), 8.62 (s, 1H), 8.54 (s, 1H), 7.52 (d, *J* = 7.9 Hz, 1H), 7.33 (d, *J* = 8.1 Hz, 1H), 7.23 (t, *J* = 7.5 Hz, 2H), 7.16 (d, *J* = 2.1 Hz, 1H), 7.14 (t, *J* = 7.4 Hz, 1H), 7.09 (d, *J* = 7.0 Hz, 2H), 7.05 (m, 1H), 6.97 (m, 1H), 4.33 (t, *J* = 7.3 Hz, 2H) 3.27-3.23 (m, 6H), 2.87 (t, *J* = 7.2 Hz, 2H), 2.62 (t, *J* = 7.2 Hz, 2H), 2.59-2.51 (m, 4H), 2.09 (p, *J* = 7.5 Hz, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 155.0, 151.4, 150.4, 140.9, 140.5, 136.3, 128.5 (2C), 128.3 (2C), 127.3, 126.2, 122.6, 120.9, 118.4, 118.3, 118.0, 114.3, 112.6, 111.5, 86.2, 58.8, 52.2 (2C), 50.1 (2C), 48.6, 32.0, 32.0, 22.5. LC-MS: (*m*/*z*) calc.: 489.3; found: 490.3 $[M+H]^+$. Purity: 95%.

*4-(4-(Benzo[d][1,3]dioxol-5-ylmethyl)piperazine-1-yl)-6,7,8,9-tetrahydropyrimido[4,5-b]indolizine-10-carbonitrile (***56***)* White crystals; yield: 46%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.45 (s, 1H), 6.88 (d, *J* = 1.5 Hz, 1H), 6.84 (d, *J* = 7.9 Hz, 1H), 6.77 (dd, *J* = 7.9, 1.5 Hz, 1H), 5.98 (s, 2H), 4.33 (t, *J* = 5.6 Hz, 2H), 3.45 (s, 2H), 3.36-3.29 (m, 4H), 3.10 (t, *J* = 6.4 Hz, 2H), 2.61-2.50 (m, 4H), 1.98-1.84 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 154.3, 151.2, 150.9, 150.2, 147.4, 146.4, 131.9, 122.2, 118.2, 114.4, 109.3, 108.0, 100.9, 83.6, 61.9, 52.1 (2C), 50.4 (2C), 46.1, 22.9, 22.6, 18.4. LC-MS: (*m*/*z*) calc.: 416.20; found: 490.3 $[M+H]^+$. Purity: 98%.

*4-(4-(Benzo[d][1,3]dioxol-5-ylmethyl)piperazine-1-yl)-7,8,9,10-tetrahydro-6H-pyrimido[4',5':4,5]pyrrolo[1,2-a]azepine-11-carbonitrile (***57***)* Pale yellow crystals; yield: 35%.^{1}H NMR (500 MHz, DMSO-d_6) δ 8.46 (s, 1H), 6.88 (d, *J* = 1.5 Hz, 1H), 6.84 (d, *J* = 7.8 Hz, 1H), 6.77 (dd, *J* = 7.9, 1.6 Hz, 1H), 5.98 (s, 2H), 4.54-4.40 (m, 2H), 3.45 (s, 2H), 3.35-3.29 (m, 4H), 3.12-3.04 (m, 2H), 2.61-2.49 (m, 4H), 1.89-1.80 (m, 2H), 180-

168 m, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 156.6, 153.7, 151.1, 149.6, 147.4, 146.3, 131.9, 122.2, 117.9, 114.6, 109.3, 108.0, 100.9, 85.3, 61.8, 51.9 (2C), 49.8 (2C), 47.4, 29.9, 27.9, 27.2, 25.6. LC-MS: (*m*/*z*) calc.: 430.21; found: 431.2 $[M+H]^+$. Purity: 100%.

*4-(4-(Benzo[d][1,3]dioxol-5-ylmethyl)piperazine-1-yl)-5-phenyl-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***58***)* Yellow crystals; yield: 37%. ^{1}H NMR (500 MHz, DMSO-d_6, δ 8.80 (s, 1H), 8.64 (s, 1H), 7.58-7.46 (m, 5H), 707 (s, 1H), 6.97 (s, 1H), 6.90 (s, 1H), 6.06 (s, 1H), 4.17-3.97 (m, 2H), 3.73-3.40 (m, 4H), 315-2.96 (m, 4H)). ^{13}C NMR (126 MHz, DMSO-d_6, δ 151.7, 151.4, 147.5, 141.7, 137.5, 129.5 (3C), 128.6, 125.4 (3C), 115.5, 113.9, 101.6, 88.2, 49.1 (2C), 45.2 (2C)). LC-MS: (*m*/*z*) calc.: 438.2; found: 439.1 $[M+H]^+$. Purity: 98%.

*4-(4-(Benzo[d][1,3]dioxol-5-ylmethyl)piperazine-1-yl)-5-phenethyl-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***59***)* White crystals; yield: 20%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.55 (s, 1H), 8.48 (s, 1H), 7.17-7.11 (m, 3H), 6.98-6.95 (m, 2H), 6.87 (d, *J* = 1.4 Hz, 1H), 6.85 (d, *J* = 7.9 Hz, 1H), 6.76 (dd, *J* = 7.9, 1.5 Hz, 1H), 5.98 (s, 2H) ,4.52 (t, *J* = 7.2 Hz, 2H), 3.44 (s, 2H), 3.26-3.20 (m, 4H), 3.00 (t, *J* = 7.1 Hz, 2H), 2.57-2.51 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 154.9, 151.4, 150.5, 147.4, 146.4, 141.1, 137.3, 131.8, 128.7 (2C), 128.3 (2C), 126.8, 122.3, 118.1, 114.3, 109.3, 108.3, 101.0, 86.2, 61.8, 52.0 (2C), 50.3, 50.0 (2C), 37.2. LC-MS: (*m*/*z*) calc.: 466.2; found: 467.3 $[M+H]^+$. Purity: 99%.

*4-(4-(Furan-2-carbonyl)piperazine-1-yl)-6,7,8,9-tetrahydropyrimido[4,5-b]indolizine-10-carbonitrile (***60***)* Yellow crystals; yield: 37%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.49 (s, 1H), 7.85 (dd, *J* = 1.7, 0.7 Hz, 1H), 7.03 (dd, *J* = 3.5, 0.7 Hz, 1H), 6.64 (dd, *J* = 3.4, 1.8 Hz, 1H), 4.40 (t, *J* = 5.6 Hz, 2H), 3.94-3.82 (m, 4H), 3.39 (t, *J* = 5.0 Hz, 4H), 3.13 (t, *J* = 6.4 Hz, 2H), 2.02-1.86 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 158.7, 153.9, 151.2, 151.1, 150.3, 147.0, 144.9, 118.2, 115.9, 114.4, 111.5, 83.6, 60.4, 46.0 (2C),

40.4 (2C), 23.0, 22.6, 18.3. LC-MS: (*m*/*z*) calc.: 376.2; found: 377.2 $[M+H]^+$. Purity: 99%.

*4-(4-(Furan-2-carbonyl)piperazine-1-yl)-7,8,9,10-tetrahydro-6H-pyrimido[4',5':4,5]pyrrolo[1,2-a]azepine-11-carbonitrile (***61***)* White crystals; yield: 37%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.50 (s, 1H), 7.85 (dd, *J* = 1.8, 0.8 Hz, 1H), 7.04 (dd, *J* = 3.5, 0.8 Hz, 1H), 6.64 (dd, *J* = 3.5, 1.8 Hz, 1H), 4.54-4.46 (m, 2H), 3.95-3.81 (m, 4H), 3.40-3.35 (m, 4H), 3.11 (t, *J* = 5.4 Hz, 2H), 1.89-1.72 (m, 6H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 158.7, 156.9, 153.4, 151.0, 149.8, 147.0, 145.0, 117.9, 116.0, 114.5, 111.5, 85.3, 56.1 (2C), 49.8, 47.5 (2C), 29.9, 27.8, 27.2, 25.6. LC-MS: (*m*/*z*) calc.: 390.2; found: 391.2 $[M+H]^+$. Purity: 96%.

*5-(2-Methoxyphenyl)-4-(4-phenethylpiperazine-1-yl)-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***62***)* Pale yellow crystals; yield: 22%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.57 (s, 1H), 8.56 (s, 1H), 7.54 (dd, *J* = 8.4, 7.5 Hz, 1H), 7.42 (dd, *J* = 7.8, 1.6 Hz, 1H), 7.30 (dd, *J* = 8.4, 1.1 Hz, 1H), 7.26-7.20 (m, 2H), 7.19-7.08 (m, 4H), 3.74 (s, 3H), 3.10-2.59 (m, 4H), 2.63-2.57 (m, 2H), 2.38-2.31 (m, 2H), 2.05-1.93 (m, 4H) ^{13}C NMR (126 MHz, DMSO-d_6) δ 158.5, 153.1, 151.7, 150.9, 141.4, 137.8, 130.9, 128.9 (2C), 128.3 (2C), 127.1, 126.7 (2C), 115.6, 115.2 (2C), 114.2, 87.6, 63.8, 62.1, 51.5 (2C), 48.8 (2C), 14.7. LC-MS: (*m*/*z*) calc.: LC-MS: (*m*/*z*) calc.: 438.2; found: 439.3 $[M+H]^+$. Purity: 96%.

*4-(4-Benzylpiperazine-1-yl)-5-(2-methoxyphenyl)-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***63***)* Yellwow crystals; yield: 20%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.56 (s, 1H), 8.55 (s, 1H), 7.53 (dd, *J* = 8.4, 7.5 Hz, 1H), 7.41 (dd, *J* = 7.8, 1.6 Hz, 1H), 7.32-7.21 (m, 4H), 7.20-7.16 (m, 2H), 7.10 (td, *J* 7.6, 1.2 Hz, 1H), 3.71 (s, 3H), 3.31 (s, 2H), 3.09-2.96 (m, 4H), 1.97-1.87 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 153.9, 153.8, 151.7, 150.2, 141.5, 137.7, 130.7, 128.9 (2C), 128.3 (2C), 128.0, 127.1, 126.6, 120.8,

117.0, 114.0, 112.9, 87.7, 61.9, 56.0, 51.4 (2C), 49.1 (2C). LC-MS: (*m*/*z*) calc.: 424.2; found: 425.2 $[M+H]^+$. Purity: 100%.

*5-(4-Ethoxyphenyl)-4-(4-phenethylpiperazine-1-yl)-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***64***)* Pale yellow crystals; yield: 99%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.66 (s, 1H), 8.55 (s, 1H), 7.41 (d, *J* = 8.8 Hz, 2H), 7.42 (t, *J* = 7.5 Hz, 2H), 7.15 (t, *J* = 7.9 Hz, 3H), 7.11 (d, *J* = 8.8 Hz, 2H), 4.11 (q, *J* = 6.9 Hz, 2H), 3.11-3.04 (m, 4H), 2.60 (t, *J* = 8.3 Hz, 2H), 2.38 (t, *J* = 8.4 Hz, 2H), 2.13-2.07 (m, 4H), 1.33 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 158.5, 153.2, 151.7, 150.9, 141.4, 140.4, 130.9, 128.7 (2C), 128.4 (2C), 126.8 (2C), 126.0, 115.7, 115.2 (2C), 114.2, 87.6, 63.8, 59.7, 51.4 (2C), 48.9 (2C), 32.4, 14.7 LC-MS: (*m*/*z*) calc.: 452.2; found: 453.2 $[M+H]^+$. Purity: 100%.

*4-(4-Benzylpiperazine-1-yl)-5-(4-ethoxyphenyl)-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***65***)* Pale yellow crystals; yield 20%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.64 (s, 1H), 8.54 (s, 1H), 7.40-7.36 (m, 2H), 7.31-7.26 (m, 2H), 7.24-7.20 (m, 1H), 7.20-7.17 (m, 2H), 7.11-7.07 (m, 2H), 4.14 (q, *J* = 7.0 Hz, 2H), 3.31 (s, 2H), 3.14-3.02 (m, 4H), 2.08-1.96 (m, 4H), 1.39 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 158.5, 153.1, 151.7, 150.9, 141.4, 137.8, 130.9, 128.9 (2C), 128.3 (2C), 127.1, 126.7 (2C), 115.6, 115.2 (2C), 114.2, 87.6, 63.8, 62.1, 51.5 (2C), 48.8 (2C), 14.7. LC-MS: (*m*/*z*) calc.: 438.2; found: 439.1 $[M+H]^+$. Purity: 97%.

*4-(4-(2-(1H-Indole-3-yl)ethyl)piperazine-1-yl)-5H-pyrimido[5,4-b]indole (***66***)* White Crystals; yield: 28%.^{1}H NMR (500 MHz, DMSO-d_6) δ 11.70 (s, 1H), 11.34 (s, 1H), 10.97 (s, 1H), 8.57 (s, sH), 8.33 (d, *J* = 7.9 Hz, 1H), 7.65 (t, *J* = 7.8 Hz, 1H), 7.57 (dd, *J* = 8.2, 7.0 Hz, 1H), 7.37 (d, *J* = 8.1 Hz, 1H) ,7.29-7.24 (m, 2H), 7.11-7.07 (m, 1H), 7.03-6.98 (m, 1H), 4.70-4.48 (m, 2H), 3.85-3.60 (m, 4H), 3.49-3.38 (m, 2H), 3.27-3.03 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 150.4, 148.8, 145.1, 140.4, 136.4, 128.8, 126.9,

123.3, 121.4, 120.7, 120.5, 120.2, 118.8, 118.6, 118.5, 112.8, 111.7, 119.4, 55.9, 50.8 (2C), 43.4 (2C), 19.8. LC-MS: (*m*/*z*) calc.: 396.2; found: 397.2 $[M+H]^+$. Purity: 97%.

*4-(4-(2-(Pyridin-2-yl)ethyl)piperazine-1-yl)-5H-pyrimido[5,4-b]indole (***67***)* Pale yellow crystals; yield: 33%. ^{1}H NMR (500 MHz, $CDCl_3$) *δ* 9.15 (s, 1H), 8.65 (s, 1H), 8.51 (d, *J* = 4.5 Hz, 1H), 8.23 (d, *J* = 7.9 Hz, 1H), 7.60 (td, *J* = 7.7, 1.6 Hz, 1H), 7.53-7.47 (m, 2H), 7.27-7.24 (m, 1H), 7.18 (d, *J* = 7.8 Hz, 1H), 7.13 (dd, *J* = 7.1, 5.2 Hz, 1H), 3.87 (t, *J* = 4.5 Hz, 4H), 3.02-2.97 (m, 2H), 2.84-2.80 (m, 2H), 2.64 (t, *J* = 4.6 Hz, 4H). ^{13}C NMR (126 MHz, $CDCl_3$) *δ* 159.7, 151.3, 150.0, 149.2, 146.0, 140.1, 136.6, 128.9, 123.3, 121.6, 121.5, 121.3, 120.8, 119.2, 112.1, 58.1, 52.7 (2C), 46.2 (2C), 35.3. LC-MS: (*m*/*z*) calc.: 358.19; found: 359.0 $[M+H]^+$. Purity: 89%.

*4-(4-(Pyridin-2-yl)piperazine-1-yl)-5H-pyrimido[5,4-b]indole (***68***)* Pale yellow crystals; yield: 46%. ^{1}H NMR (500 MHz, $CDCl_3$) *δ* 8.92 (s, 1H), 8.69 (s, 1H), 8.24 (d, *J* = 7.9 Hz, 1H), 8.19 (dd, *J* = 5.3, 1.6 Hz, 1H), 7.53-7.45 (m, 3H), 7.30-7.25 (m, 1H), 6.66 (t, *J* = 3.4 Hz, 1H), 6.64 (s, 1H), 4.00 (t, *J* = 5.2 Hz, 4H), 3.73 (t, *J* = 5.2 Hz, 4H). ^{13}C NMR (126 MHz, $CDCl_3$) *δ* 159.1, 151.5, 150.1, 148.0, 146.1, 140.0, 137.7, 129.0, 121.6, 121.3, 120.9, 119.3, 113.8, 112.0, 107.1, 46.1 (2C), 44.9 (2C). LC-MS: (*m*/*z*) calc.: 330.2; found: 331.1 $[M+H]^+$. Purity: 97%.

*4-(4-Phenethylpiperazine-1-yl)-5H-pyrimido[5,4-b]indole (***69***)* Pale yellow crystals; yield: 18%. ^{1}H NMR (500 MHz, DMSO-d_6) *δ* 11.27 (s, 1H), 8.46 (s, 1H), 8.09 (d, *J* = 7.8 Hz, 1H), 7.61 (dt, *J* = 8.3, 0.9 Hz, 1H), 7.53 (dd, *J* = 8.2, 7.0 Hz, 1H), 7.30-7.21 (m, 5H), 7.20-7.16 (m, 1H), 3.80 (t, *J* = 4.9 Hz, 4H), 2.80 (dd, *J* = 9.1, 6.6 Hz, 2H), 2.65 (t, *J* = 5.0 Hz, 4H), 2.61 (dd, *J* = 9.0, 6.7 Hz, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) *δ* 151.1, 149.1, 144.8, 140.5, 140.0, 128.8 (2C), 128.4 (3C), 126.0, 120.7, 120.5, 119.9, 118.5, 112.7, 59.8, 52.8 (2C), 46.1 (2C), 32.8. LC-MS: (*m*/*z*) calc.: 357.2; found: 358.1 $[M+H]^+$. Purity: 94%.

*4-(4-Benzylpiperazine-1-yl)-5H-pyrimido[5,4-b]indole (**70**)* Yellow crystals; yield: 36%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.09 (s, 1H), 8.61 (s, 1H), 8.20 (d, J = 7.9 Hz, 1H), 7.50-7.42 (m, 2H), 7.33-7.30 (m, 4H), 7.27-7.25 (m, 1H), 7.24-7.20 (m, 1H), 3.90 (t, J = 4.9 Hz, 4H), 3.55 (s, 2H), 2.61 (t, J = 4.9 Hz, 4H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 151.3, 149.6, 145.1, 139.9, 137.1, 129.3 (2C), 128.9, 128.4 (2C), 127.4, 121.2, 121.2, 120.8, 118.9, 112.1, 62.9, 52.8 (2C), 46.3 (2C). LC-MS: (m/z) calc.: 343.2; found: 344.1 $[M+H]^+$. Purity: 97%.

*4-(4-Benzhydrylpiperazine-1-yl)-5H-pyrimido[5,4-b]indole (**71**)* Pale yellow crystals; yield: 54%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.02 (s, 1H), 8.47 (s, 1H), 8.18 (d, J = 7.9 Hz, 1H), 7.49-7.37 (m, 6H), 7.30-7.25 (m, 4H), 7.21-7.17 (m, 3H), 4.28 (s, 1H), 3.95-3.89 (m, 4H), 2.61-2.54 (m, 4H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 151.2, 142.0, 139.8 (2C), 129.1, 128.7 (4C), 128.7 (4C), 127.2 (3C), 121.3, 121.0, 118.3, 112.2, 76.0, 51.8, 52.8 (2C), 46.7 (2C). LC-MS: (m/z) calc.: 419.2; found: 420.4 $[M+H]^+$. Purity: 97%.

*(4-Phenylpiperazine-1-yl)-5H-pyrimido[5,4-b]indole (**72**)* Pale yellow crystals; yield: 41%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.70 (s, 1H), 8.39 (s, 1H), 8.27 (d, J = 7.9 Hz, 1H), 7.56-7.48 (m, 2H), 7.32-7.26 (m, 3H), 6.97-6.93 (m, 2H), 6.90 (t, J = 7.3 Hz, 1H), 4.01 (t, J = 5.2 Hz, 4H), 3.37 (t, J = 5.1 Hz, 4H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 151.4, 151.0, 150.4, 146.4, 139.9, 129.3 (2C), 129.0, 121.8, 121.4, 121.0, 120.4, 119.3, 116.4 (2C), 111.9, 49.3 (2C), 46.5 (2C). LC-MS: (m/z) calc.: 329.2; found: 330.1 $[M+H]^+$. Purity: 97%.

*4-(4-(Benzo[d][1,3]dioxol-5-ylmethyl)piperazine-1-yl)-5H-pyrimido[5,4-b]indole (**73**)* Pale yellow crystals; yield: 43%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.26 (s, 1H), 8.45 (s, 1H), 8.08 (d, J = 7.9 Hz, 1H), 7.60 (d, J = 8.2 Hz, 1H), 7.55-7.49 (m, 1H), 7.25-7.20 (m, 1H), 6.93-6.89 (m, 1H), 6.86 (d, J = 7.8 Hz, 1H), 6.80 (d, J = 7.9 Hz, 1H), 5.99 (s, 2H), 3.84-3.71 (m, 4H), 3.54-3.43 (m, 2H), 2.64-2.51 (m, 4H). ^{13}C NMR (126 MHz,

DMSO-d_6) δ 151.1, 149.1, 147.4, 146.4, 144.8, 140.0, 131.8, 128.4, 122.3, 120.7, 120.5, 119.9, 118.5, 112.7, 109.4, 198.0, 100.9, 61.8, 52.5 (2C), 46.1 (2C). LC-MS: (*m*/*z*) calc.: 387.2; found: 388.2 $[M+H]^+$. Purity: 98%.

*(4-(5H-Pyrimido[5,4-b]indole-4-yl)piperazine-1-yl)(furan-2-yl)methanone (***74***)* Pale yellow crystals; yield: 75%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.39 (s, 1H), 8.50 (s, 1H), 8.11 (d, *J* = 7.9 Hz, 1H), 7.87 (dd, *J* = 1.7, 0.7 Hz, 1H), 7.63 (d, *J* = 8.2 Hz, 1H), 7.55 (dd, *J* = 8.2, 7.1 Hz, 1H), 7.25 (dd, *J* = 7.8, 7.0 Hz, 1H), 7.06 (dd, *J* = 3.4, 0.7 Hz, 1H), 6.65 (dd, *J* = 3.4, 1.8 Hz, 1H), 3.95-3.90 (m, 4H), 3.90-3.84 (m, 4H)^{13}C NMR (126 MHz, DMSO-d_6) δ 158.6, 150.9, 149.1, 147.1, 145.0, 144.9, 140.1, 128.5, 120.7, 120.6, 120.0, 118.6, 115.9, 112.7, 111.5, 55.7 (2C), 46.0 (2C). LC-MS: (*m*/*z*) calc.: 347.14; found: 348.0 $[M+H]^+$. Purity: 98%.

Biological Investigation. *Chemicals.* The reference compounds cyclosporine A and **24** were purchased from Tocris bioscience (Bristol, IO, USA). **75** was established as MRP1 inhibitor by us before.[37,63] The acetoxymethyl (AM) ester of the fluorescence dye calcein (calcein AM) and the chlorophyll breakdown product pheophorbide A were delivered by Calbiochem (EMD Chemicals (San Diego, CA, USA)) and Frontier Scientific Inc. (Logan, UT, USA), respectively. The antineoplastic drug daunorubicin was provided by Sigma (Oakville, ON, Canada). All other chemicals were purchased from Carl Roth GmbH (Karlsruhe, Germany), Merck KgaA (Darmstadt, Germany), Th. Geyer GmbH Co KG (Renningen, Germany) and Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The reference inhibitors and target compounds were stored as 10 mM stock solutions in DMSO at -20 °C. Dilutions and dilution series were prepared in Krebs-HEPES buffer (KHB, consisting of 1.3 mM $CaCl_2$, 11.7 mM $_D$-Glucose monohydrate, 10.0 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethansulfonic acid), 4.7 mM KCl, 1.2 mM KH_2PO_3, 1.2 mM $MgSO_4$, 118.6 mM NaCl, and 4.2 mM $NaHCO_3$,

adjusted to pH 7.4 with sodium hydroxide solution and sterilized by filtration with membrane filters (Whatman FP 30/0.2 µM CA-S filter units, GE Healthcare UK limited, Buckinghamshire, UK) with Braun Injekt 29 mL syringe (ALMO-Erzeugnisse, Erwin Busch GmbH, Bad Arolsen, Germany), stored in cellstar 50 mL tubes (Greiner bio one, Frickenhausen, Germany).

Cell Culture. The doxorubicin-selected human ovarian carcinoma cell line A2780/ADR overexpressing P-gp as well as its sensitive counterpart A2780 was supplied by the European Collection of Animal Cell Culture (ECACC, No. 931125120 and No. 93112519, respectively). The doxorubicin-selected small cell lung cancer cell line H69AR overexpressing MRP1 (ATCC CRL-11351) and its sensitive counterpart H69 (NCL-H69, ATCC HTB-119) were provided by American Type Culture Collection. These cell lines were cultivated in RPMI-1640 medium (PAN Biotech GmbH, Aidenbach, Germany) complemented with either 10% (A2780/ADR and A2780) or 20% (H69AR and H69) fetal bovine serum (FBS, Sigma Life Science, Steinheim, Germany), 2 mM L-Glutamine, 50 U/mL penicillin G and 50 µg/mL streptomycin (PAN Biotech GmbH, Aidenbach, Germany). The BCRP overexpressing cell line Madin-Darby Canine Kidney (MDCK) II BCRP transfected with human wildtype cDNA and C-terminally linked to the cDNA of green fluorescent protein (GFP), as well as the sensitive counterpart MDCK II wild type (wt), were a generous gift by Dr. A. Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Cultivation was performed using Dulbecco's modified Eagle's medium (DMEM, Sigma Life Science, Steinheim, Germany) complemented with 10% FBS, 2 mM L-glutamine, 50 U/mL penicillin G and 50 µg/mL streptomycin. The cell lines were stored under liquid nitrogen complemented with 10% DMSO. Cell cultivation was conducted at 37 °C in 5% CO_2-humidified atmosphere in a cell incubator (Münchener Medizin Mechanik GmbH, Planegg, Germany) using T75 or T175 cell culture flasks (Greiner bio one,

Frickenhausen, Germany) until a confluence of over 90% was reached, followed by subculturing or biological testing. The cell layer was rinsed three times with 5 mL of phosphate buffered saline solution (PBS, PAN Biotech GmbH, Aidenbach, Germany) before addition of a solution of 0.05% trypsin and 0.02% EDTA (PAN Biotech GmbH, Aidenbach, Germany) for harvesting, followed by an incubation period of 5-10 minutes. After surface detachment, the cells were transferred into a 50 mL tube (Greiner bio one, Frickenhausen, Germany) for centrifugation at 266 x *g* and 4 °C for 5 minutes. The supernatant was replaced by fresh medium to prepare the cell pellet for cell density counting using a CASY1 model TT (Schärfe System GmbH, Reutlingen, Germany) equipped with a 150 µm capillary. The necessary amount of cells was taken to perform subculturing or biological testing.

Calcein AM Assay to Investigate Target Compounds for P-gp Inhibition. Before further investigation, the target compounds were screened for P-gp inhibition via calcein AM assay as described earlier[43,60,72,73,74,75,76] with minor modifications.[37,63] 20 µL of a solution of KHB and the target compound at concentrations between 100 nM and 100 µM were added into clear F bottom 96-well microplates (Greiner bio one, Frickenhausen, Germany). The cells were harvested and counted as described above. 160 µL containing approximately 30,000 cells were seeded into each well and incubated with the test compounds for 30 minutes in 5% CO_2-humidified atmosphere at 37 °C. 20 µL of a 3.125 µM solution of calcein AM (protected from light) were added followed by instant measurement of the fluorescence increase in constant time intervals of 60 seconds with a Fluostar Optima or Fluostar Polarstar microplate reader (BMG-Labtech, Software versions 2.00R2, 2.20 and 4.11-0, respectively) tempered at 37 °C. Using an excitation wave length of 485 nm and an emission wave length of 520 nm. Cyclosporine A was used as standard inhibitor. The slope of the linear part of the fluorescence-time curve was calculated between minutes 5 and 30 and plotted against

the logarithmic concentration of the test compounds. The effect value of the 10 µM concentration was compared to the effect of 10 µM cyclosporine A and expressed as percentage inhibition in comparison to the standard compound. Compounds with an inhibition level below 25% were not further characterized. For compounds with an inhibition level above 25% full dose-response curves were generated to determine the IC_{50} value using the four-parameter logistic equation with variable Hill slope or the three-parameter model with fixed Hill slope (=1), whatever model was statistically preferred, with GraphPad Prism version 5.03 for Windows (San Diego, CA, USA). Compounds that did not reach 75% of the maximal inhibition value of cyclosporine A (I_{max}) were not considered for testing in the MDR reversal assays or interaction type experiments.

Daunorubicin Assay to Investigate Target Compounds for MRP1 Inhibition. Before further investigation, the target compounds were screened for MRP1 inhibition in a daunorubicin assay as described earlier[77] with minor modifications.[37,63] H69AR cells were prepared as described above. The obtained cell pellet was resuspended in fresh culture medium and 160 µL were seeded into colourless 96 well plates with a density of approximately 60,000 cells per well. 20 µL of the target compound at concentrations between 100 nM and 100 µM were prepared in cell culture medium without further supplements and preincubated with the cell suspension for 15 min. Then 20 µL of a 30 µM daunorubicin solution was added to each well. Steady state conditions were obtained after 180 minutes under 5% CO_2-humidified atmosphere and 37 °C. Before starting measurement the cells were resuspended to remove adherent cells from the bottom to get homogenous conditions. Fluorescence was measured by flow cytometry (FACS calibur, Becton Dickinson Biosciences, Heidelberg, Germany). Dauorubicin was excited with an argon laser at an excitation wavelength of 488 nm, and

fluorescence was detected in the FL3 channel (≥ 670 nm). The effect of 10 μM of the test compound was compared to the effect of 10 μM **75** and expressed as percentage inhibition in comparison to the standard compound. Compounds with an inhibition level below 25% were not further characterized. For compounds with an inhibition level above 25% full dose-response curves were generated to determine the IC_{50} value using the four-parameter logistic equation with variable Hill slope or the three-parameter model with fixed Hill slope (=1), whatever model was statistically preferred, with GraphPad Prism. Compounds that did not reach 75% of the maximal inhibition value of **75** (I_{max}) were not considered for testing in the MDR reversal assays or interaction type experiments.

Pheophorbide A Assay to Investigate Target Compounds for BCRP Inhibition. Before further investigation, the target compounds were screened for BCRP inhibition in a pheophorbide A assay as described earlier[73,78] with minor modifications.[37,63] The BCRP overexpressing cell line MDCK II BCRP was used to conduct this assay and prepared as described above. 20 μL of various target compounds at different concentrations between 100 nM and 100 μM were added to a U-shaped clear 96 well plate (Greiner, Frickenhausen, Germany). Then 160 μL of the cell suspension containing approximately 45,000 cells were added to each well and preincubated for 20 min in 5% CO_2 huminidified atmosphere at 37 °C. 20 μL of a 5 μM pheophorbide A solution (protected from light) was added to each well and the microplate was maintained under 5% CO_2 and 37 °C for an incubation time of 120 min to reach steady state conditions. Before starting measurement the cells were resuspended to remove adherent cells from the bottom and to get a homogeneous suspension. Fluorescence was measured by flow cytometry as stated above. Pheophorbide A was excited by an argon laser with an excitation wavelength of 488 nm, and its fluorescence was detected in the FL3 channel (≥ 670 nm). BCRP expression was measured with GFP detection

in the FL1 channel (530/15). The effect of 10 µM of the test compound was compared to the effect of 10 µM **24** and expressed as percentage inhibition in comparison to the standard compound. Compounds with an inhibition level below 25% were not further characterized. For compounds with an inhibition level above 25% full dose-response curves were generated to determine the IC_{50} value using the four-parameter logistic equation with variable Hill slope or the three-parameter model with fixed Hill slope (=1), whatever model was statistically preferred, using GraphPad Prism. Compound that did not reach 75% of the maximal inhibition value of **24** (I_{max}) were not considered for testing in MDR reversal assays or interaction type experiments.

*MTT-viability Assay to Determine Intrinsic Cytotoxicity of Broad-spectrum Inhibitor **55**.* The MTT-viability assay was conducted as described in the literature.[74,79,80] with minor modifications.[37,63] 20 µL of different dilutions (up to 1000 µM) of the test compound **55** was given into a 96-well tissue-culture treated plate (Starlab GmbH, Hamburg, Germany) followed by addition of 180 µL of a cell suspension (A2780/ADR and A2780: 8,000 cells per well; H69AR and H69: 20,000 cells per well; MDCK II BCRP and MDCK II: 3,000 cells per well). Culture medium was used as negative control (100% viability), while the positive control was represented by the effect of 10% DMSO (0% viability). The microplate was kept at 37 °C and under CO_2-humidified atmosphere for 72 hours. After addition of 20 µL of a MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) solution (5 mg/mL) a second incubation time of 1 hour followed. The supernatant was removed and 100 µL of DMSO added per well. An Ex Multiscan microplate photometer (Thermo Fisher Scientific, Waltham, MA, USA) with a background correction at 690 nm was used for the spectrophotometric absorbance measurement at 570 nm. The resultant values were expressed as percentage viability in comparison to the above stated controls.

*MDR Reversal Assay to Determine Capability of Compound **55** to Restore Sensitivity of ABC Transporters.* The compounds with highest potencies (IC_{50}) and I_{max} values in the screenings of more than 75% were analyzed with regard to the capability to restore the sensitivity of the three ABC transport proteins. This applied only for compound **55**. The preparation of this experiment is very similar to the MTT-viability assay. 20 µL of a 1 µM, 10 µM and 100 µM concentrated solution of the test compound were added to a 96 well plate before adding 160 µL of the cell suspension (A2780/ADR and A2780: 8,000 cells per well; H69AR and H69: 20,000 cells per well; MDCK II BCRP and MDCK II: 3,000 cells per well). A dilution series between 100 nM and 100 µM of daunorubicin (P-gp or MRP1) or SN-38 (BCRP) was added to yield a final concentration range between 10 nM and 10 µM. After an incubation period of 72 hours cell vibility was determined as stated above. The effect values were plotted against logarithmic concentrations of the test compounds and dose-response curves with variable Hill slope were calculated with nonlinear regression using GraphPad Prism.

Associated Content

Supporting Information

SMILES and the corresponding biological data (csv).

Author Information

Corresponding Author

* Phone: +49-228-735213. E-mail: mwiese@uni-bonn.de

ORCID

Michael Wiese 0000-0002-5851-5336

Abbreviations Used

ABC transporter, ATP-binding cassette transporter; ABCB1, synonymous for P-gp; ABCC1, synonymous for MRP1; ABCG2, synonymous for BCRP; ATP, adenosine 5'-triphosphate; BCRP, Breast Cancer Resistance Protein; calcein AM, calcein acetoxymethyl ester; DMF, dimethyl formamide; DMF-DMA, dimethyl formamide dimethyl acetale; DMS, dimethyl sulfate; EC_{50}, half-maximal effect-concentration; GI_{50}, half-maximal growth inhibition; HEPES, 2-(4-(2-hydroxyethyl)-1-piperazinyl)-ethansulfonic acid; IC_{50}, half-maximal inhibitory concentration; KHB, Krebs-HEPES buffer; MDR, multidrug resistance; MRP1, Multidrug Resistance-associated Protein 1; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromid; P-gp, Permeability glycoprotein; SEM, standard error of the mean.

References

(1) Juliano, R. L.; Ling, V. A Surface Glycoprotein Modulating Drug Permeability in Chinese Hamster Ovry Cell Mutants. *Biochim. Biophys. Acta* **1976**, *455*, 152-162.

(2) Leonard, G. D.; Fojo, T.; Bates, S. E. The Role of ABC Transporters in Clinical Practise. *Oncologist* **2003**, *8*, 411-424.

(3) Nooter, K.; Westerman, A. M.; Flens, M. J.; Zaman, G. J. R.; Scheper, R. J.; van Wingerden, K. E.; Burger, H.; Oostrum, R.; Boersma, T.; Sonnenveld, P.; Gratama, J. W.; Kok, T.; Eggermont, A. M. M.; Bosman, F. T.; Stoter, G. Expression of the Multirdrug Resistance-associated Protein (*MRP*) Gene in Human Cancers. *Clin. Cancer Res.* **1995**, *1*, 1301-1310.

(4) Diestra, J. E.; Scheffer, G. L.; Catala, I.; Maliepaard, M.; Schellens, J. H. M.; Scheper, R. J.; Germa-Lluch, J. R.; Izquierdo, M. A. Frequent Expression of the Multi-drug Resistance-associated Protein BCRP/MXR/ABCP/ABCG2 in Human Tumours Detected by the BXP-21 Monoclonal Antibody in Paraffin-embedded Material. *J. Pathol.* **2002**, *198*, 213-219.

(5) Cole, S. P. C.; Bhardwaj, J. H.; Gerlach, J. H.; Mackie, J. E.; Grant, C. E.; Almquist, K. C.; Stewart, A. J.; Kurz, E. U.; Duncan, A. M. V.; Deeley, R. G. Overexpression of a Transporter Gene in a Multidrug-Resistant Human Lung Cancer Cell Line. *Science* **1992**, *258*, 1650-1654.

(6) Doyle, L. A.; Yang, W. Y.; Abruzzo, L. V.; Krogmann, T.; Gao, Y.; Rishi, A. K.; Ross, D. D. A Multidrug Resistance Transporter from Human MCF-7 Breast Cancer Cells. *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95*, 15665-15670.

(7) Grant, C. E.; Valdimarsson, G.; Hipfner, D. R.; Almquist, K. C.; Cole, S. P. C.; Deeley, R. G. Overexpression of Multidrug Resistance-associated Protein (MRP) Increases Resistance to Natural Product Drugs. *Cancer Res.* **1994**, *54*, 357-361.

(8) Cole, S. P. C.; Sparks, K. E.; Fraser, K.; Loe, D. W.; Grant, C. E.; Wilson, G. M.; Deeley, R. G. Pharmacological Characterization of Multidrug Resistant MRP-Transfected Human Tumor Cells. *Cancer Res.* **1994**, *54*, 5902-5910.

(9) Eckford, P. D. W.; Sharom, F. J. ABC Efflux Pump-Based Resistance to Chemotherapy Drugs. *Chem. Rev.* **2009**, *109*, 2989-3011.

(10) Shukla, S.; Wu, C.-P.; Ambudkar, S. V. Development of inhibitors of ATP-binding Cassette Drug Transporters – Present Status and Challenges. *Drug Metab. Toxicol.* **2008**, *4*, 205-223.

(11) Norman, B. H. Inhibitors of MRP1-mediated Multidrug Resistance. *Drugs Future* **1998**, *23*, 1001-1013.

(12) Boumendjel, A.; Baubichon-Cortay, H.; Trompier, D.; Perrotton, T.; Di Pietro, A. Anticancer Multidrug Resistance Mediated by MRP1: Recent Advances in the Discovery of Reversal Agents. *Med. Res. Rev.* **2005**, *25*, 453-472.

(13) Cole, S. P. Targeting Multidrug Resistance Protein 1 (MRP1, ABCC1): Past, Present, and Future. *Annu. Rev. Pharmacol. Toxicol.* **2014**, *54*, 95-117.

(14) Wiese, M. BCRP/ABCG2 Inhibitors: a Patent Review (2009 – Present). *Expert Opin. Ther. Pat.* **2015**, *25*, 11, 1229-1237.

(15) Juvale, K.; Wiese, M. Design of Inhibitors of BCRP/ABCG2. *Future Med. Chem.* **2015**, *7*, 1521-1527.

(16) Schwarz, T.; Montanari, F.; Cseke, A.; Wlcek, K.; Visvader, L.; Palme, S.; Chiba, P.; Kuchler, K.; Urban, E.; Ecker, G. F. Subtle Structural Differences Trigger Inhibitory Activity of Propafenone Analogues at the Two Polyspecific ABC Transporters: P-Glycoprotein (P-gp) and Breast Cancer Resistance Protein (BCRP). *Chem. Med. Chem.* **2016**, *11*, 1380-1394.

(17) Montanari, F.; Zdrazil, B.; Digles, D. ; Ecker, G. Selectivity Profiling of BCRP versus P-gp Inhibition: from Automated Collection of Polypharmacology Data to Multi-Label Learning. *J. Cheminf.* **2016**, *8*, 1-13.

(18) Pajeva, I. K.; GLobisch, C.; Wiese, M. Combined Pharmacophore Modelling, Docking, and 3D QSAR Studies of ABCB1 and ABCC1 Transporter Inhibitors. *Chem. Med. Chem.* **2009**, *4*, 1883-1896.

(19) Gallus, J.; Juvale, K.; Wiese, M. Characterization of 3-methoxy Flavones for their Interaction with ABCG2 as Suggested by ATPase Activity. *Biochim. Biophys. Acta* **2014**, *1838*, 2929-2938.

(20) Kraege, S.; Stefan, K.; Juvale, K.; Ross, T.; Willmes, T.; Wiese, M. The Combination of Quinazoline and Chalcone Moieties Leads to Novel Potent Heterodimeric Modulators of Breast Cancer Resistance Protein (BCRP/ABCG2). *Eur. J. Med. Chem.* **2016**, *117*, 212-229.

(21) Köhler, S.; Silbermann, K.; Wiese. Phenyltetrazolyl-phenylamides: Substituent Impact on Modulation Capability and Selectivity toward the Efflux Protein ABCG2 and Investigation of Interaction with the Transporter. *Eur. J. Med. Chem.* **2016**, *124*, 881-895.

(22) Tsuruo, T.; Iida, H.; Tsukagoshi, S. ; Sakurai, Y. Overcoming of Vincristine Resistance in P388 Leukemia *in Vivo* and *in Vitro* through Enhanced Cytotoxicity of Vincristine and Vinblastine by Verapamil. *Cancer Res.* **1981**, *41*, 1967-1972.

(23) Slovak, M. L.; Hoeltge, G. A.; Dalton, W. S.; Trent, J. M. Pharmacological and Biological Evidence for Differing Mechanisms of Doxorubicin Resistance in Two Human Tumor Cell Lines. *Cancer Res.* **1988**, *48*, 2793-2797.

(24) Cornwell, M. M.; Pastan, I.; Gottesmann, M. M. Certain Calcium Channel Blockers Bind Specifically to Multidrug-resistant Human KB Carcinoma Membrane Vesicles and Inhibit Drug Binding to P-glycoprotein. *J. Biol. Chem.* **1987**, *48*, 2793-2797.

(25) Abe, T.; Koike, K.; Ohga, T.; Kubo, T.; Wada, M.; Kohno, K.; Hidaka, K.; Kuwano, M. Chemosensitisation of Spontaneous Multidrug Resistance by a 1,4-Dihydropyridine Analog and Verapamil in Human Glioma Cell Lines Overexpressing *MRP1* or *MDR1*. *Br. J. Cancer* **1995**, *72*, 418-423.

(26) Twentyman, P. R.; Fox, N. E.; White, D. J. G. Cyclosporin A and its Analogues as Modifiers of Adriamycin and Vincristine Resistance in a Multidrug-resistant Human Lung Cancer Cell Line. *Br. J. Cancer* **1987**, *56*, 55-57.

(27) Barrand, M. A.; Rhodes, T.; Center, M. S.; Twentyman, P. R. Chemosensitisation and Drug Accumulation Effects of Cyclosporin A, PSC-833 and Verapamil in Human MDR Large Cell Lung Cancer Cells Expressing a 190k Membrane Protein Distinct from P-glycoprotein. *Eur. J. Cancer* **1993**, *29A*, 408-415.

(28) Narasaki, F.; Oka, M.; Fukuda, M.; Nakano, R.; Ikeda, K.; Takatani, H.; Terashi, K.;Soda, H.; Yano, O.;Nakamura, T.; Doyle, L. A.; Tsuruo, T.; Kohno, S. A Novel Quinolone Derivative, MS-209, Overcomes Drug Resistance of Human Lung Cancer Cells Expressing the Multidrug Resistance-associated Protein (MRP) Gene. *Cancer Chemother. Pharmacol.* **1997**, *40*, 425-432.

(29) Sato, W.; Fukuzawa, N.;Nakanashi, O.; Baba, Makoto, Suzuki, T.; Yano, O.; Naito, M.; Tsuruo, T. Reversal of Multidrug Resistance by a Novel Quinolone Derivative, MS-209. *Cancer Chemother. Pharmacol.* **1995**, *35*, 271-277.

(30) Baba, M.; Nakanashi, O.; Sato, W.; Saito, A.; Miyama, Y.; Yano, O.; Shimada, A.; Fukazawa, N.; Naito, M.; Tsuruo, T. Relationship between Multidrug Resistant Gene Expression and Multidrug Resistant-reversing Effect of MS-209 in Various Tumor Cells. *Cancer Chemother. Pharmacol.* **1995**, *36*, 361-367.

(31) Norman, B. H.; Gruber, J. M.; Hollinshead, S. P.; Wilson, J. W.; Starling, J. J.; Law, K. L.; Self, T. D.; Tabas, L. B.; Williams, D. C.; Paul, D. C.; Wagner, M. M.;

Dantzig, A. H. Tricyclic Isoxazoles are Novel Inhibitors of the Multidrug Resistance Protein (MRP1). *Bioorg. Med. Chem. Lett.* **2002**, *12*, 883-886.

(32) Dantzig, A. H.; Shepard, R. L.; Pratt, S. E.; Tabas, L. B.; Lander, P. A.; Ma, L.; Paul, D. C.; Williams, D. C.; Peng, S.-B.; Slapak, C. A.; Godinot, N.; Perry III.; W. L. Evaluation of the Binding of the Tricyclic Isoxazole Photoaffinity Label LY475776 to Multidrug Resistance Associated Protein 1 (MRP1) Orthologs and Several ATP-Binding Cassette (ABC) Drug Transporters. *Biochem. Pharmacol.* **2004**, *67*, 1111-1121.

(33) Norman, B. H.; Lander, P. A.; Gruber, J. M.; Kroin, J. S.; Cohen, J. D.; Jungheim, L. N.; Starling, J. J.; Law, Kevin L.; Self, Tracy D.; Tabas, L. B.; Williams, D. C.; Paul, D. C.; Dantzig, A. H. Cyclohexyl-linked Tricyclic Isoxazoles are Potent and Selective Modulators of the Multidrug Resistance Protein (MRP1). *Bioorg. Med. Chem. Lett.* **2005**, *15*, 5526-5530.

(34) Wang, S.; Ryder, H.; Pretswell, I.; Depledge, P.; Milton, J.; Hancox, T. C.; Dale, I.; Dangerfield, W.; Charlton, P.; Faint, R.; Dodd, R.; Hassan, S. Studies on Quinazolinones as Dual Inhibitors of Pgp and MRP1 in Multidrug Resistance. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 571-574.

(35) Wang, S.; Folkes, A.; Chuckowree, I.; Cockcroft, X.; Sohal, S.; Miller, W.; Milton, J.; Wren, S. P.; Vicker, N.; Depledge, P.; Scott, J.; Smith, L.; Jones, H.; Mistry, P.; Faint, R.; Thompson, D.; Cocks, S. Studies on Pyrrolopyrimidines as Selective Inhibitors of Multidrug-resistance-associated Protein in Multidrug Resistance. *J. Med. Chem.* **2004**, *47*, 1329-1338.

(36) Wang, S.; Wan, N. C.; Harrison, J.; Miller, W.; Chuckowree, I.; Sohal, S.; Hancox, T. C.; Baker, S.; Folkes, A.; Wilson, F.; Thompson, D.; Cocks, S.; Farmer, H.; Boyce, A.; Freathy, C.; Broadbridgem J.; Scott, John, Depledge, P.; Faint, R.; Mistry, P.; Charlton, P. Design and Synthesis of New Templates Derived from Pyrrolopyrimidine

as Selective Multidrug-resistance-associated Protein Inhibitors in Multidrug Resistance. *J. Med. Chem.* **2004**, *47*, 1339-1350.

(37) Schmitt, S. M.; Stefan, K.; Wiese, M. Pyrrolopyrimidine Derivatives as Novel Inhibitors of Multidrug Resistance-associated Protein 1 (MRP1, ABCC1), *J. Med. Chem.* **2016**, *59*, 3018-3033.

(38) Maliepaard, M.; van Gastelen, M. A.; Tohgo, A.; Hausheer, F. H.; van Waardenburg, R. C. A. M.; de Jong, L. A.; Pluim, D.; Bijnen, J. H.; Schellens, J. H. M. Circumvention of Breast Cancer Resistance Protein (BCRP)-mediated Resistance to Camptothecins *in Vitro* Using Non-Substrate Drugs or the BCRP Inhibitor GF120918. *Clin. Cancer Res.* **2001**, *7*, 935-941.

(39) Robey, R. W.; Steadman, K.; Polgar, O.; Morisaki, K.; Blayney, M.; Mistry, P.; Bates, S. E. Pheophorbide A Is a Specific Probe for ABCG2 Function and Inhibition. *Cancer Res.* **2004**, *64*, 1242-1246.

(40) Pick, A.; Müller, H.; Wiese, M. Structure-activity Relationships of New Inhibitors of Breast Cancer Resistance Protein (ABCG2). *Bioorg. Med. Chem.* **2008**, *16*, 8224-8236.

(41) Köhler, S. C.; Wiese, M. HM30181 Derivatives as Novel Potent and Selective Inhibitors of the Breast Cancer Resistance Protein (BCRP/ABCG2). *J. Med. Chem.* **2015**, *58*, 3910-3921.

(42) Spindler, A.; Stefan, K.; Wiese, M. Synthesis and Investigation of Tetrahydro-*β*-carboline Derivatives as Inhibitors of the Breast Cancer Resistance Protein (ABCG2). *J. Med. Chem.* **2016**, *59*, 6121-6135.

(43) Pick, A.; Müller, H.; Mayer, R.; Haensch, B.; Pajeva, I. K.; Weigt, M.; Boenisch, H.; Müller, C. E.; Wiese, M. Structure-activity Relashionships of Flavonoids as Inhibitors of Breast Cancer Resistance Protein (BCRP). *Bioorg. Med. Chem.* **2011**, *19*, 2090-2102.

(44) Gu, X.; Ren, Z.; Tang, X.; Peng, H.; Ma, Y.; Lai, Y.; Peng, S.; Zhang, Y. Synthesis and Biological Evaluation of Bifendate-chalcone Hybrids as a New Class of Potential P-glycoprotein Inhibitors. *Bioorg. Med. Chem.* **2012**, *20*, 2540-2548.

(45) Gu, X.; Ren, Z.; Peng, H.; Peng, S.; Zhang, Y. Bifendate-chalcone Hybrids: A New Class of Potential Dual Inhibitors of P-glycoprotein and Breast Cancer Resistance Protein. *Biochem. Biophys. Res. Comm.* **2014**, *455*, 318-322.

(46) Gu, X.; Tang, X.; Zhao, Q. ; Peng, H. ; Peng, S.; Zhang, Y. Discovery of Alkoxyl Biphenyl Derivatives Bearing dibenzo[*c,e*]azepine Scaffold as Potential Dual Inhibitors of P-glycoprotein and Breast Cancer Resistance Protein. *Bioorg. Med. Chem. Lett.* **2014**, *24*, 3419-3421.

(47) Juvale, K.; Pape, V. F. S.; Wiese, M.; Investigation of Chalcones and Benzochalcones as Inhibitors of Breast Cancer Resistance Protein. *Bioorg. Med. Chem*. ***2012****,* 20*, 346-355.*

(48) Juvale, K.; Wiese, M. 4-Substituted-2-phenylquinazolines as Inhibitors of BCRP. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 6766-6769.

(49) Singh, M. S.; Juvale, K.; Wiese, M.; Lamprecht, A. Evaluation of Dual P-gp-BCRP iInhibitors as Nanoparticle Formulation. *Eur. J. Pharm. Sci.* **2015**, *77*, 1-8.

(50) Krapf, M. K.; Gallus, J.; Wiese, M. Synthesis and Biological Evaluation of 4-Anilino-2-pyridylquinazolines and –pyrimidines as Inhibitors of Breast Cancer Resistance Protein (ABCG2). *J. Med. Chem.* **2017**, *60*, 4474-4495.

(51) Kraege, S.; Köhler, S. C.; Wiese, M. Acryloylphenylcarboxamides: A New Class of Breast Cancer Resistance Protein (ABCG2) Modulators. *Chem. Med. Chem.* **2016**, *11*, 21, 2422-2435.

(52) Kraege, S.; Stefan, K.; Köhler, S. C.; Wiese, M. Optimization of Acryloylphenylcarboxamides as Inhibitors of ABCG2 and Comparison with Acryloylphenylcarboxylates. *Chem. Med. Chem.* **2016**, *11*, 21, 2547-2558.

(53) Häcker, H.-G.; Leyers, S.; Wiendlocha, J.; Gütschow, M.; Wiese, M. Aromativ 2-(Thio)ureidocarboxylic Acids As a New Family of Modulators of Multidrug Resistance-Associated Protein 1: Synthesis, Biological Evaluation, and Structure-Activity Relationships. *J. Med. Chem.* **2009**, *52*, 4586-4595.

(54) Chow, L. M. C.; Chan, T. H.; Chan, K. F.; Wong, I. L. K.; Law, M. C. Alkyne-, Azide- and Triazole-containing Flavonoids as Modulators for Multidrug Resistance in Cancers. U.S. Patent WO 2013127361 A1, September 6, 2013.

(55) Li, S.; Lei, Y.; Jia, Y.; Li, N.; Wink, M.; Ma, Y. Piperine, a Piperidine Alkaloid from *Piper nigrum* re-sensitizes P-gp, MRP1 and BCRP Dependent Multidrug Resistant Cancer Cells. *Phytomedicine* **2011**, *19*, 83-87.

(56) Rowinski, E. K.; Smith, L.; Wang, Y.-M.; Chaturvedi, P.; Villalona, M.; Campbell, E.; Aylesworth, C.; Echardt, S. G.; Hammond, L.; Kraynak, M.; Drengler, R.; Stephenson, J.; Harding Jr.; M. W.; Von Hoff, D. D. Phase I and Pharmacokinetic Study of Paclitaxel in Combination With Biricodar, a Novel Agent That Reverses Multidrug Resistance Conferred by Overexpression of Both, MDR1 and MRP1. *J. Clin. Oncol.* **1998**, *16*, 9, 2964-2976.

(57) Peck, R. A.; Hewett, J.; Harding, M. W.; Wang, Y.-M.; Chaturvedi, P. R.; Bhatnagar, A.; Ziessman, H.; Atkins, F.; Hawkins, M. J. Phase I and Pharmacokinetic Study of the Novel MDR1 and MRP1 Inhibitor Biricodar Administered Alone and in Combination With Doxirubicin. *J. Clin. Oncol.* **2001**, *19*, 12, 3130-3141.

(58) Minderman, H.; O'Loughlin, K. L.; Pendyala, L.; Baer, M. R. VX-710 (Biricodar) Increases Drug Retention and Enhances Chemosensitivity in Resistant Cells Overexpressing P-glycoprotein, Multidrug Resistance Protein, and Breast Cancer Resistance Protein. *Clin. Cancer Res.* **2004**, *10*, 1826-1834.

(59) Weidner, L. D.; Zoghbi, S. S.; Lu, S.; Shukla, S.; Ambudkar, S. V.; Pike, V. W.; Mulder, J.; Gottesmann, M. M.; Innis, R. B.; Hall, M. D. The Inhibitor Ko143 Is Not Specific for ABCG2. *J. Pharmacol. Exp. Ther.* **2015**, *354*, 384-393.

(60) Pick, A.; Klinkhammer, W.; Wiese, M. Specific Inhibitors of the Breast Cancer Resistance Protein (BCRP). *Chem. Med. Chem.* **2010**, *5*, 1498-1505.

(61) Juvale, K.; Gallus, J.; Wiese, M. Investigation of Quinazolines as Inhibitors of Breast Cancer Resistance Protein (ABCG2). *Bioorg. Med. Chem.* **2013**, *21*, 7858-7873.

(62) Krapf, M.; Wiese, M. Synthesis and Biological Evaluation of 4-Anilino-quinazolines and -quinolines as Inhibitors of Breast Cancer Resistance Protein (ABCG2). *J. Med. Chem.* **2016**, *59*, 5449-5461.

(63) Schmitt, S. M.; Stefan, K.; Wiese, M. Pyrrolopyrimidine Derivatives and Purine Analogs as Novel Activators of Multidrug Resistance-associated Protein 1 (MRP1, ABCC1). *Biochim. Biophys. Acta* **2017**, *1859*, 69-79.

(64) De Coen, L. M.; Heugebaert, T. S. A. ; Garcia, D. ; Stevens, C. V. Synthetic Entries to and Biological Activity of Pyrrolopyrimidines. *Chem. Rev.* **2016**, *116*, 80-139.

(65) Chuckowree, I.; Folkes, A.; Hancox, T.; Miller, W.; Milton, J.; Sohal, S.; Wang, S.; Wren, S. Pyrroloyrmidine Derivatives Useful as Modulators of Multidrug Resistance. U.S. Patent WO 2004065389 A1, August 5, 2004.

(66) Moore, J. L.; Taylor, S. M. T.; Soloshonok, V. A.; An Efficient and Operationally Convenient General Synthesis of Tertiary Amines by Direct Alkylation of Secondary Amines with Alkyl Halides in the Presence of Huenig's Base. *ARKIVOC* **2006**, *6*, 287-292.

(67) Wang, E.-J.; Casciano, C. N.; Clement, R. P.; Johnson, W. W. *In Vitro* Flow Cytometry Method to Quantitatively Assess Inhibitors of P-glycoprotein. *Drug Metab. Dispos.* **2000**, *28*, 522-528.

(68) Wang, E.J.; Casciano, C. N.; Clement, R. P.; Johnson, W. W. Cooperativity in the Inhibition of P-Glycoprotein-Mediated Daunorubicin Transport: Evidence for Half-of-the-Sites Reactivity. *Arch. Biochem. Biophys.* **2000**, *383*, 1, 91-98.

(69) Vanhoefer, U.; Cao, S.; Minderman, H.; Toth, K.; Scheper, R. J.; Slovak, M. L.; Rustum, Y. M. PAK-104P, a Pyridine Analogue, Reverses Paclitaxel and Doxorubicin Resistance in Cell Lines and Nude Mice Bearing Xenografts That Overexpress the Multidrug Resistance Protein. *Clin. Cancer Res.* **1996**, *2*, 369-377.

(70) Gekeler, V.; Ise, W.; Sanders, K. H.; Ulrich, W.-R.; Beck, J. The Leukotriene LTD4 Receptor Antagonist MK571 Specifically Modulates MRP Associated Multidrug Resistance. *Biochem. Biophys. Res. Comm.* **1995**, *208*, 1, 345-352.

(71) Kühnle, M.; Egger, M.; Müller, C.; Mahringer, A.; Bernhardt, G.; Fricker, G.; König, B.; Buschauer, A. Potent and Selective Inhibitors of Breast Cancer Resistance Protein (ABCG2) Derived from the *p*-Glycoprotein (ABCB1) Modulator Tariquidar. *J. Med. Chem.* **2009**, *52*, 1190-1197.

(72) Müller, H.; Pajeva, I. K.; Globisch, C.; Wiese, M. Functional Assay and Structure-activity Relationships of New Third-generation P-glycoprotein Inhibitors. *Bioorg. Med. Chem.* **2008**, *16*, 2456-2470.

(73) Pick, A.; Müller, H.; Wiese, M. Novel Lead for Potent Inhibitors of Breast Cancer Resistance Protein (BCRP). *Bioorg. Med. Chem. Lett.* **2010**, *20*, 180-183.

(74) Juvale, K.; Stefan, K.; Wiese, M. Synthesis and Biological Evaluation of Flavones and Benzoflavones as Inhibitors of BCRP/ABCG2. *Eur. J. Med. Chem.* **2013**, *67*, 115-126.

(75) Marighetti, F.; Steggemann, K.; Hanl, M.; Wiese, M. Synthesis and Quantitative Structure-activity Relationships of Selective BCRP-inhibitors. *ChemMedChem* **2013**, *8*, 125-135.

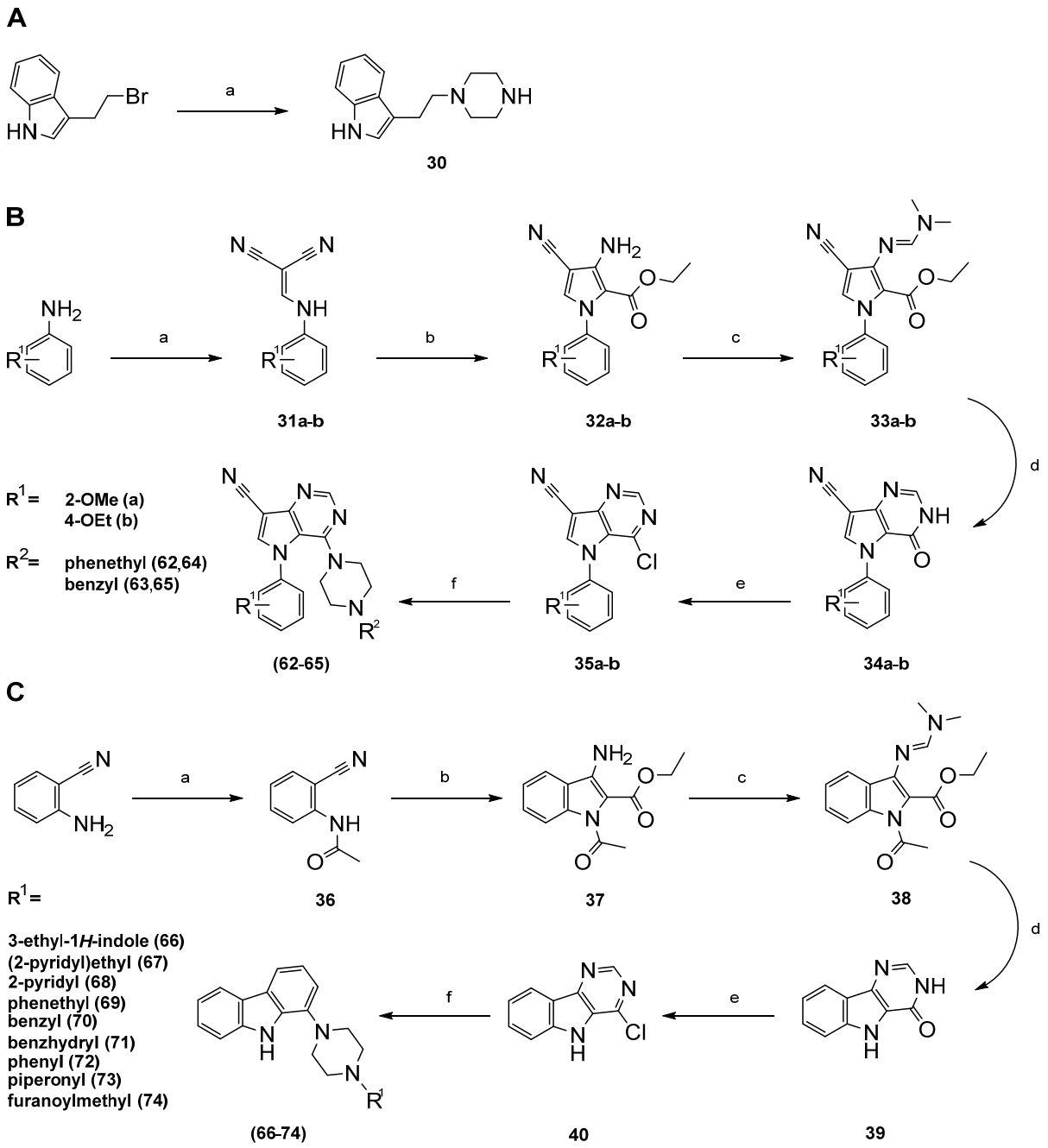
(76) Marighetti, F.; Steggemann, K.; Karbaum, M.; Wiese, M. Scaffold Identification of a New Class of Potent and Selective BCRP-inhibitors. *ChemMedChem* **2015**, *10*, 742-751.

(77) Jekerle, V.; Klinkhammer, W.; Scollard, D. A.; Breitbach, K.; Reilly, R. M.; Piquette-Miller, M.; Wiese, M. *In Vitro* and *in Vivo* Evaluation of WK-X-34, a Novel Inhibitor of P-glycoprotein and BCRP, Using Radio Imaging Techniques. *Int. J. Cancer* **2006**, *119*, 414-422.

(78) Pick, A.; Wiese, M. Tyrosine Kinase Inhibitors Influence ABCG2 Expression in EGFR-positive MDCK BCRP Cells via the PI3K/Akt Signaling Pathway. *ChemMedChem* **2012**, *7*, 650-662.

(79) Müller, H.; Klinkhammer, W.; Globisch, C.; Kassack, M. U.; Pajeva, I. K.; Wiese, M. New Functional Assay of P-glycoprotein Using Hoechst 33342. *Bioorg. Med. Chem.* **2007**, *15*, 7470-7479.

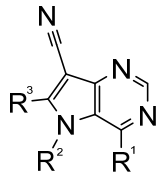
(80) Müller, H.; Kassack, M. U.; Wiese, M. Comparison of the Usefulness of the MTT, ATP, and Calcein Assays to Predict the Potency of Cytotoxic Agents in Various Human Cancer Cell Lines. *J. Biomol. Screen.* **2004**, *9*, 506-515.



Scheme 1: Synthesis of intermediates **30**, **31a-b** to **35a-b**, **36-40** and target compounds **62-65** as well as **66-74**. (A) Preparation of 3-(2-(piperazine-1-yl)ethyl)-1*H*-indole precursor (**30**); (a) MeCN, rt, 24 h. (B) Preparation of compounds **62-65**. (a) (ethoxymethylene)malononitrile, EtOH, rt, 1 h; (b) ethyl bromoacetate, DMF, 100 °C, 5 h; (c) DMF-DMA, DMF, 100 °C, 5 h; (d) NH_3, EtOH, reflux, 5 h; (e) $POCl_3$, TEA, reflux, 5 h; (f) phenethylpiperazine or benzylpiperazine, TEA, DMF, 200 W, 110 °C, 1 h. (C)

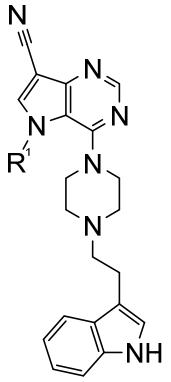
Preparation of compounds **66**-**74**. (a) (ethoxymethylene)malononitrile, EtOH, rt, 1 h; (b) ethyl bromoacetate, DMF, 100 °C, 5 h; (c) DMF-DMA, DMF, 100 °C, 5 h; (d) NH_3, EtOH, reflux, 5 h; (e) $POCl_3$, TEA, reflux, 5 h; (f) phenethylpiperazine or benzylpiperazine, TEA, DMF, 200 W, 110 °C, 1 h.

Table 1: Summary of calcein AM, daunorubicin and pheophorbide A assay results for 7,8-cyclisized 9-deazapurines (subclass (i)) with nitrogen containing heterocyclic residues at position 6 that reached more than 25% inhibition at 10 µM in the screening assays. Shown is mean ± SEM of at least three independent experiments of duplicate measurements. n. t. = not teste, due to low effect in screening or lack of solubility (**47**).



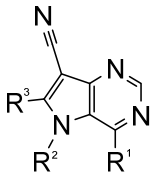
comp.	R^1	R^2	R^3	P-gp calcein AM IC_{50} ± SEM [µM]	MRP1 daunorubicin IC_{50} ± SEM [µM]	BCRP pheophorbide A IC_{50} ± SEM [µM]
41	4-(2-(1*H*-indole-3-yl)ethyl)piperazine-1-yl	cyclohexyl		5.62 ± 0.41	0.344 ± 0.018	3.41 ± 0.20
42	4-(2-(1*H*-indole-3-yl)ethyl)piperazine-1-yl	cycloheptyl		1.71 ± 0.14	0.289 ± 0.008	n.t.
43	(2-(1*H*-indole-3-yl)ethyl)amino	cyclohexyl		n.t.	0.957 ± 0.019	n.t.
44	4-(2-(pyridin-4-yl)ethyl)piperazine-1-yl	cyclohexyl		n.t.	0.672 ± 0.034	27.8 ± 4.3
45	4-(2-(pyridin-4-yl)ethyl)piperazine-1-yl	cycloheptyl		n.t.	1.14 ± 0.07	12.4 ± 0.4
46	4-(pyridin-2-yl)piperazine-1-yl	cyclohexyl		n.t.	1.02 ± 0.02	4.86 ± 0.36
47	4-(pyridin-2-yl)piperazine-1-yl	cycloheptyl		n.t.	n.t.	n.t.

Table 2: Summary of calcein AM, daunorubicin and pheophorbide A assay results of 9-deazapurines with (2-(1*H*-indole-3-yl)ethyl)piperazine-1-yl residue at position 6 and aliphatic or aliphatic-aromatic variations at position 7 (subclass (ii)) that reached more than 25% inhibition at 10 µM in the screening assays. Shown is mean ± SEM of at least three independent experiments of duplicate measurements. n. t. = not tested due to low effect in screening.



comp.	R^1	P-gp calcein AM IC_{50} ± SEM [µM]	MRP1 daunorubicin IC_{50} ± SEM [µM]	BCRP pheophorbide A IC_{50} ± SEM [µM]
48	methyl	n.t.	0.337 ± 0.020	n.t.
49	ethyl	n.t.	0.855 ± 0.063	13.8 ± 1.0
50	propyl	n.t.	0.468 ± 0.012	n.t.
51	cyclopropyl	3.85 ± 0.36	0.316 ± 0.009	18.9 ± 1.7
52	phenyl	1.28 ± 0.14	0.328 ± 0.022	n.t.
53	benzyl	4.48 ± 0.25	0.405 ± 0.028	4.07 ± 0.36
54	phenethyl	1.64 ± 0.04	0.524 ± 0.013	1.39 ± 0.08
55	phenylpropyl	1.46 ± 0.10	0.501 ± 0.025	1.69 ± 0.07

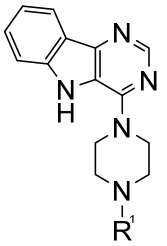
Table 3: Summary of calcein AM, daunorubicin and pheophorbide A assay results of 7,8-cyclisized 9-deazapurines with oxygen containing heterocyclic residues at position 6 and 7 (subclass (iii)) that reached more than 25% inhibition at 10 µM in the screening assays. Shown is mean ± SEM of at least three independent experiments of duplicate measurements. n. t. = not tested due to low effect in screening.



comp.	R^1	R^2	R^3	P-gp calcein AM IC_{50} ± SEM [µM]	MRP1 daunorubicin IC_{50} ± SEM [µM]	BCRP pheophorbide A IC_{50} ± SEM [µM]
56	4-(benzo[*d*][1,3]dioxol-5-ylmethyl)piperazine-1-yl	cyclohexyl		13.9 ± 1.4	0.322 ± 0.019	14.6 ± 1.8
57	4-(benzo[*d*][1,3]dioxol-5-ylmethyl)piperazine-1-yl	cycloheptyl		5.71 ± 0.60	1.02 ± 0.04	23.7 ± 4.0
58	4-(benzo[*d*][1,3]dioxol-5-ylmethyl)piperazine-1-yl	phenyl	H	n.t.	0.751 ± 0.126	n.t.
59	4-(benzo[*d*][1,3]dioxol-5-ylmethyl)piperazine-1-yl	phenethyl	H	n.t.	1.80 ± 0.16	n.t.
60	4-(furan-2-carbonyl)piperazine-1-yl	cyclohexyl		n.t.	0.883 ± 0.060	29.5 ± 1.4
61	4-(furan-2-carbonyl)piperazine-1-yl	cycloheptyl		n.t.	1.01 ± 0.04	12.9 ± 0.8
62	4-phenethylpiperazine-1-yl	2-methoxyphenyl	H	n.t.	0.638 ± 0.026	n.t.
63	4-benzylpiperazine-1-yl	2-methoxyphenyl	H	19.1 ± 0.7	0.327 ± 0.028	37.9 ± 3.6

64	4-phenethylpiperazine-1-yl	4-ethoxyphenyl	H	n.t.	1.32 ± 0.10	n.t.
65	4-benzylpiperazine-1-yl	4-ethoxyphenyl	H	7.29 ± 0.44	0.859 ± 0.067	n.t.

Table 4: Summary of calcein AM, daunorubicin and pheophorbide A assay results of 8,9-annulated 9-deazapurines with variations at position 6 (subclass (iv)) that reached more than 25% inhibition at 10 µM in the screening assays. Shown is mean ± SEM of at least 3 independent experiments of duplicate measurements. n. t. = not tested due to low effect in screening.



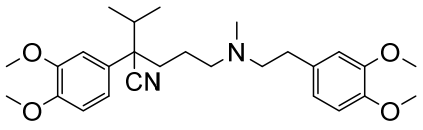
Comp.	R^1	P-gp calcein AM IC_{50} ± SEM [µM]	MRP1 daunorubicin IC_{50} ± SEM [µM]	BCRP pheophorbide A IC_{50} ± SEM [µM]
66	2-(1*H*-indole-3-yl)ethyl	3.50 ± 0.36	0.921 ± 0.012	6.21 ± 0.30
67	2-(pyridin-2-yl)ethyl	10.7 ± 0.7	0.345 ± 0.012	11.6 ± 0.7
68	pyridin-2-yl	5.00 ± 0.40	0.495 ± 0.037	1.81 ± 0.10
69	phenethyl	1.11 ± 0.04	0.405 ± 0.019	0.839 ± 0.054
70	benzyl	9.49 ± 0.67	0.363 ± 0.014	6.08 ± 0.39
71	benzhydryl	2.33 ± 0.17	0.409 ± 0.030	1.38 ± 0.12
72	phenyl	n.t.	0.146 ± 0.004	1.10 ± 0.08
73	piperonyl	3.92 ± 0.23	0.717 ± 0.030	7.93 ± 0.51
74	furanoyl	n.t.	0.169 ± 0.005	6.06 ± 0.30

Table 5: MTT-viability assay with respect to compound **55** at varying concentrations in all used cell lines. Shown is mean of GI_{50} ± SEM of at least three independent experiments of duplicate measurements. Additionally, the therapeutic ratio has been determined (right column).

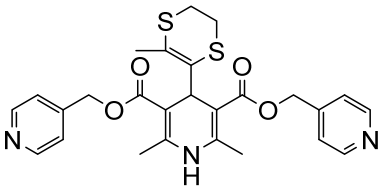
cell line	GI_{50} ± SEM [μM]	therapeutic ratio
A2780/ADR	13.7 ± 0.3	9.4
A2780	6.36 ± 0.11	
H69AR	152 ± 20	303
H69	55.4 ± 4.8	
MDCK II BCRP	23.2 ± 1.0	14
MDCK II wt	37.2 ± 0.1	

Table 6: Shift of half-maximal growth inhibition values (GI_{50}) of daunorubicin (P-gp and MRP1) and SN-38 (BCRP) in presence of compound **55** at 0.1 μM, 1 μM and 10 μM. Shown is also the resistance factor, the potentiation factor and the percentage of maximal sensitization at 10 μM. Shown is mean ± SEM of at least 3 independent experiments of duplicate measurements.

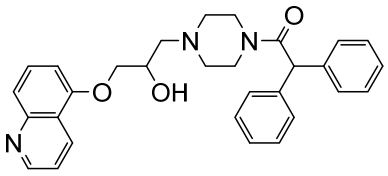
ABC transporter	GI_{50} (no comp) ± SEM [μM]	GI_{50} (0.1 μM) ± SEM [μM]	GI_{50} (1 μM) ± SEM [μM]	GI_{50} (10 μM) ± SEM [μM]	GI_{50} sensitive cell line ± SEM [μM]	maximal sensitization (10 μM) [%]
P-gp	0.575 ± 0.005	0.286 ± 0.004	0.058 ± 0.001	0.044 ± 0.002	0.010 ± 0.001	
rf	54	27	5.5	4.1	1	24
pf	-	2.0	9.8	13	54	
MRP1	3.84 ± 0.60	2.10 ± 0.29	1.17 ± 0.003	0.445 ± 0.003	0.361 ± 0.016	
rf	11	5.8	3.2	1.2	1	81
pf	-	1.8	3.3	8.6	11	
BCRP	1.71 ± 0.16	0.916 ± 0.054	0.211 ± 0.002	0.061± 0.007	0.086 ± 0.004	
rf	20	11	2.5	0.7	1	140
pf	-	1.9	8.1	28	20	



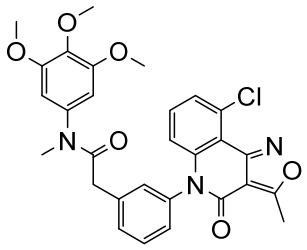
1: verapamil



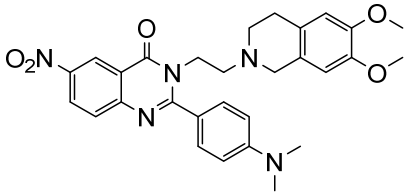
2: NIK-250



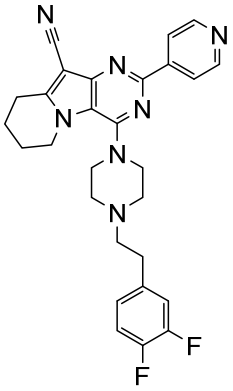
3: MS-209



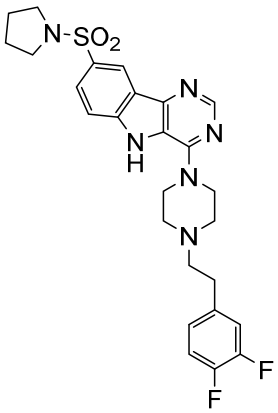
4: tricyclic isoxazole derivative No. 13



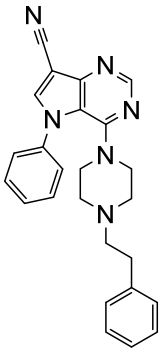
5: quinazolinone derivative No. 40



6: pyrrolopyrimidine derivative No. 47

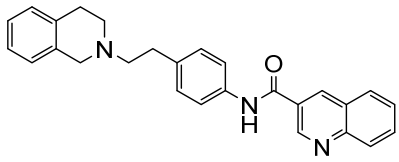


7: indolopyrimidine derivative No. 49

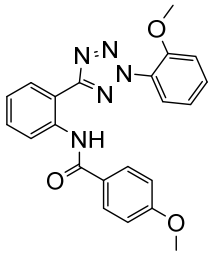


8: pyrrolopyrimidine derivative No. 31

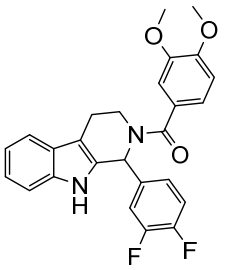
Figure 1: Depiction of published dual inhibitors of P-gp and MRP1.



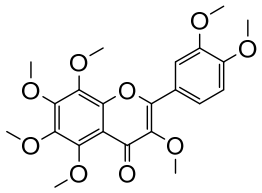
9: tariquidar analogue No. 16



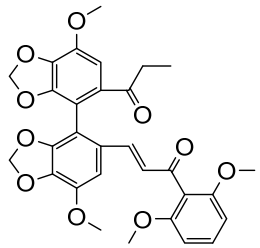
10: tetrazolic HM30181 derivative No. 23



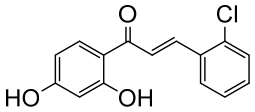
11: tetrahydro-beta-carboline derivative No. 44



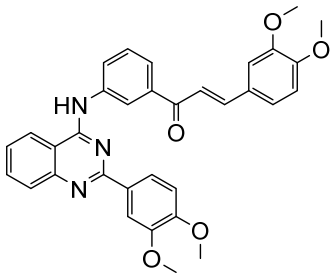
12: flavonoid derivative No. 21



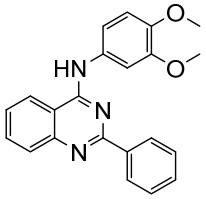
13: bifendate-chalcone 8f



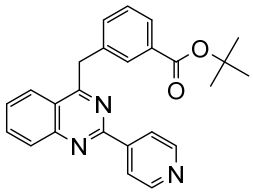
14: chalcone No. 10



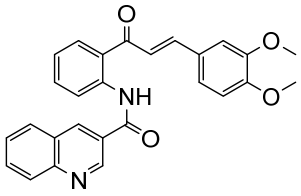
15: quinazoline-chalcone No. 24



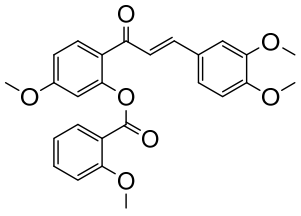
16: 4-anilinoquinazoline No. 2



17: 4-anilino-2-pyridylquinazoline No. 39

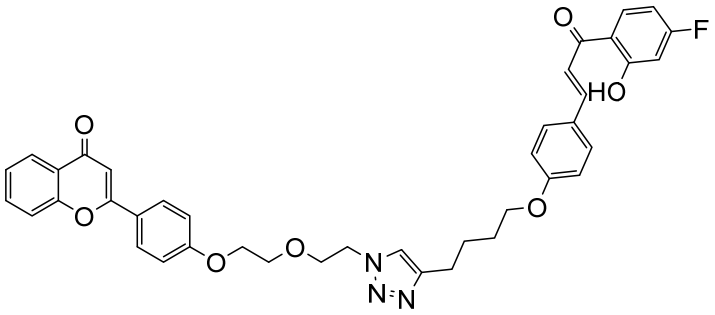


18: acryloylphenylcarboxamid derivative No. 33

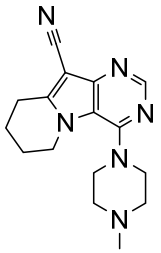


19: acryloylphenylcarboxylate derivative No. 29

Figure 2: Depiction of published dual inhibitors of P-gp and BCRP.

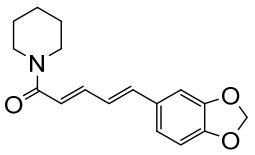


20: triazole containing flavonoid No. AclOAzl

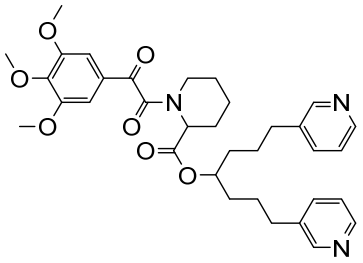


21: pyrrolopyrimidine derivative No. 16

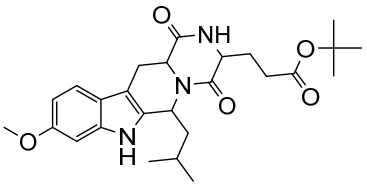
Figure 3: Depiction of published dual inhibitors of MRP1 and BCRP.



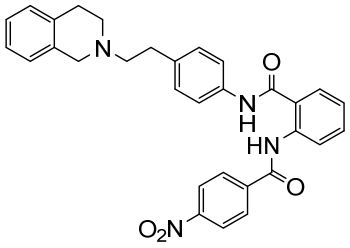
22: piperine



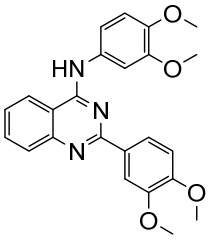
23: biricodar (VX-710)



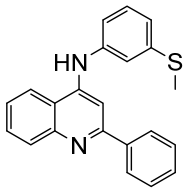
24: Ko143



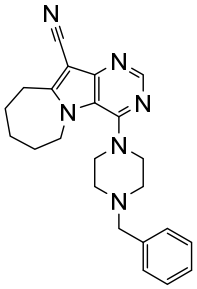
25: tariquidar analog No. 14



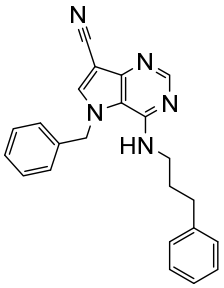
26: quinazoline derivative No. 29



27: 4-anilinoquinoline derivative 29



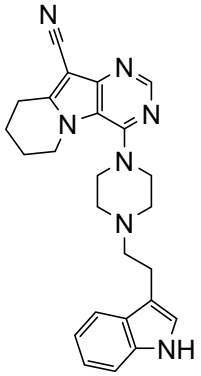
28: pyrrolopyrimidine derivative No. 23



29: pyrrolopyrimidine derivative No. 9

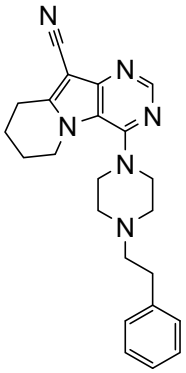
Figure 4: Depiction of published triple inhibitors of P-gp, MRP1 and BCRP.

A



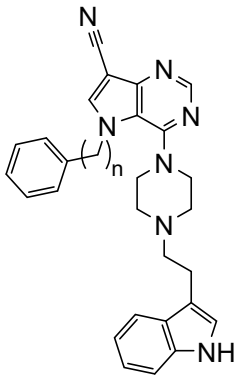
41 (ref. 35: comp. 29)

B



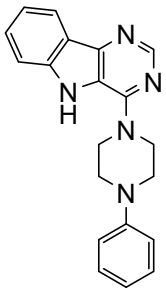
75: standard compound 12 (SC12)

C



n = 0 compound **53**
n = 1 compound **54**
n = 2 compound **55**

D



compound **72**

Figure 5: Depiction of compounds of this work.

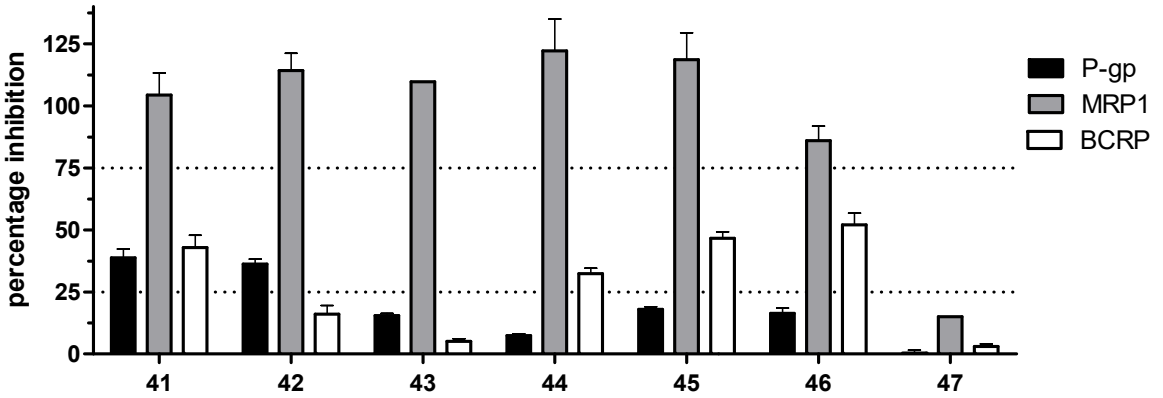


Figure 6: Summary of screenings of subclass (i). The target compounds **41**-**47** were used at 10 µM. IC_{50} values were calculated for compounds over 25% (dashed lower line) inhibition (table 1). Compounds that reached more than 75% (dashed upper line) in all three assays were evaluated in the MDR reversal-efficacy assay. Shown is mean ± SEM of at least 3 independent experiments of duplicate measurements. Black bars: P-gp screening obtained by the calcein AM assay. Normalization was performed using cyclosporine A at 10 µM as reference for 100% inhibition and KHB as 0%. Grey bars: MRP1 screening obtained by the daunorubicin assay. Normalization was performed using **75** at 10 µM as reference for 100% inhibition and KHB as 0%. White bars: BCRP screening obtained by the pheophorbide A assay. Normalization was performed using **24** at 10 µM as reference for 100% inhibition and KHB as 0%.

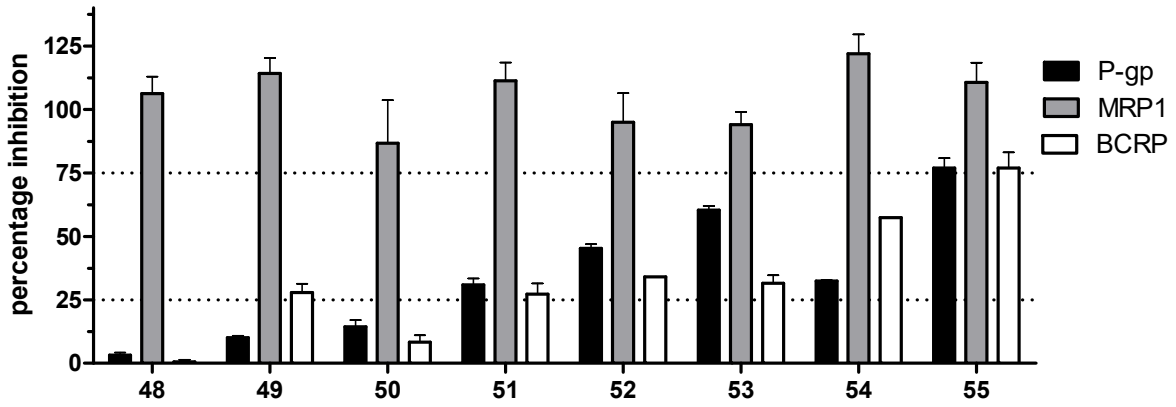


Figure 7: Summary of screenings of subclass (ii). The target compounds **48**-**55** were used at 10 µM. IC_{50} values were calculated for compounds over 25% (dashed lower line) inhibition (table 1). Compounds that reached more than 75% (dashed upper line) in all three assays were evaluated in the MDR reversal-efficacy assay. Shown is mean ± SEM of at least 3 independent experiments of duplicate measurements. Black bars: P-gp screening obtained by the calcein AM assay. Normalization was performed using cyclosporine A at 10 µM as reference for 100% inhibition and KHB as 0%. Grey bars: MRP1 screening obtained by the daunorubicin assay. Normalization was performed using **75** at 10 µM as reference for 100% inhibition and KHB as 0%. White bars: BCRP screening obtained by the pheophorbide A assay. Normalization was performed using **24** at 10 µM as reference for 100% inhibition and KHB as 0%.

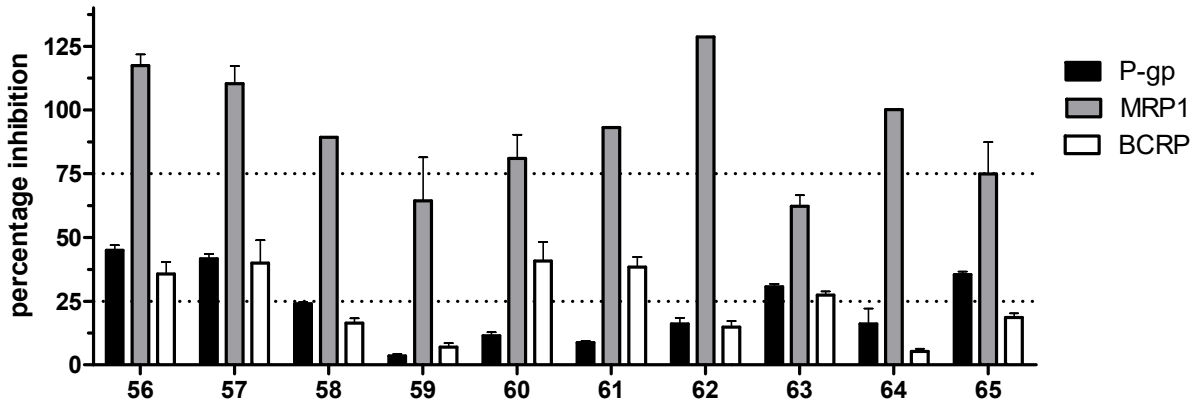


Figure 8: Summary of screenings of subclass (iii). The target compounds **56**-**65** were used at 10 µM. IC_{50} values were calculated for compounds over 25% (dashed lower line) inhibition (table 1). Compounds that reached more than 75% (dashed upper line) in all three assays were evaluated in the MDR reversal-efficacy assay. Shown is mean ± SEM of at least 3 independent experiments of duplicate measurements. Black bars: P-gp screening obtained by the calcein AM assay. Normalization was performed using cyclosporine A at 10 µM as reference for 100% inhibition and KHB as 0%. Grey bars: MRP1 screening obtained by the daunorubicin assay. Normalization was performed using **75** at 10 µM as reference for 100% inhibition and KHB as 0%. White bars: BCRP screening obtained by the pheophorbide A assay. Normalization was performed using **24** at 10 µM as reference for 100% inhibition and KHB as 0%.

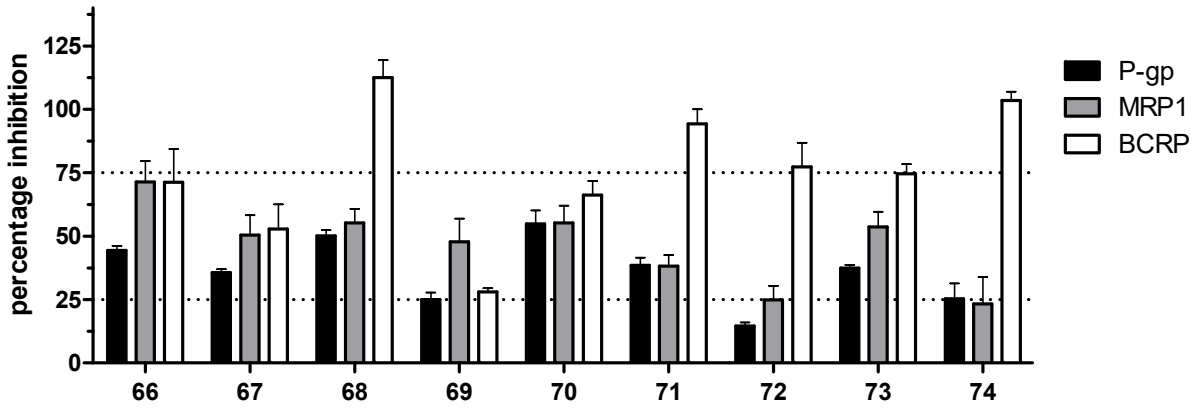
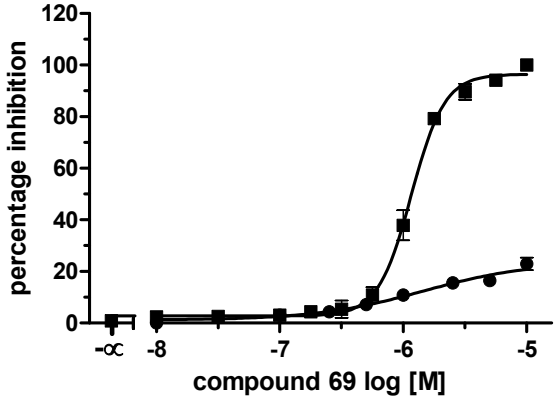
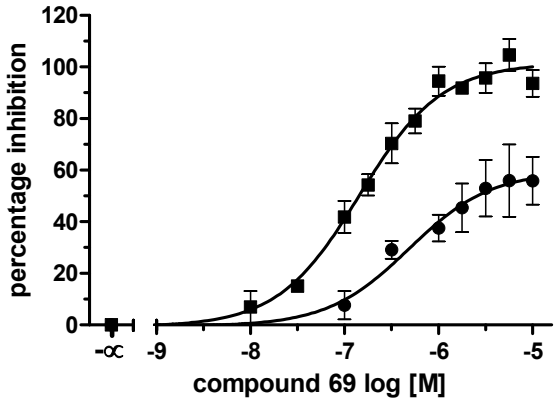


Figure 9: Summary of screenings of subclass (iv). The target compounds **66**-**74** were used at 10 µM. IC_{50} values were calculated for compounds over 25% (dashed lower line) inhibition (table 1). Compounds that reached more than 75% (dashed upper line) in all three assays were evaluated in the MDR reversal-efficacy assay. Shown is mean ± SEM of at least 3 independent experiments of duplicate measurements. Black bars: P-gp screening obtained by the calcein AM assay. Normalization was performed using cyclosporine A at 10 µM as reference for 100% inhibition and KHB as 0%. Grey bars: MRP1 screening obtained by the daunorubicin assay. Normalization was performed using **75** at 10 µM as reference for 100% inhibition and KHB as 0%. White bars: BCRP screening obtained by the pheophorbide A assay. Normalization was performed using **24** at 10 µM as reference for 100% inhibition and KHB as 0%.

A



B



C

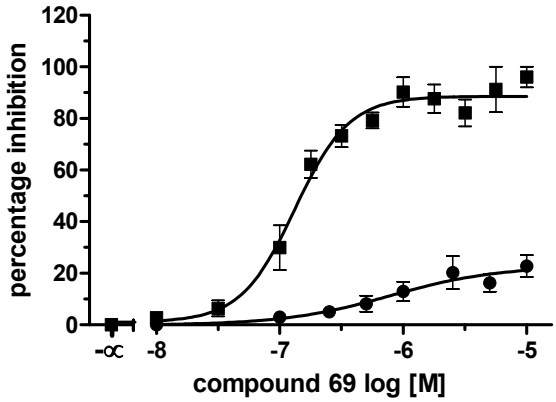
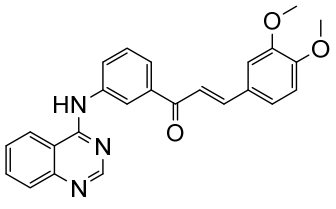
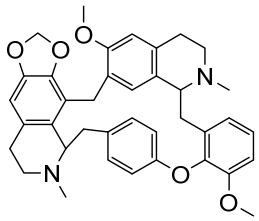


Figure 10: Compound 69 (closed circles) as representative of the 8,9-annulated 9-deazapurines evaluated in the calcein AM assay using A2780/ADR cells (A),

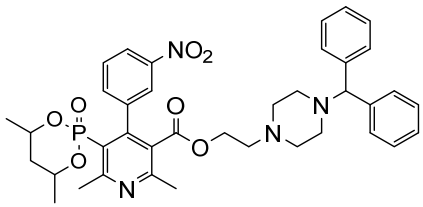
daunorubicin assay using H69AR cells (B), and pheophorbide A assay using MDCK II BCRP cells (C) in comparison to the corresponding standard inhibitors (closed squares) cyclosporine A (A), **75** (B), and **24** (C).



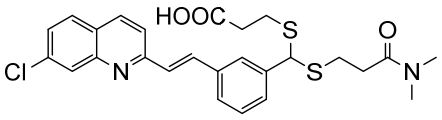
76: quinazoline-chalcone no. 17



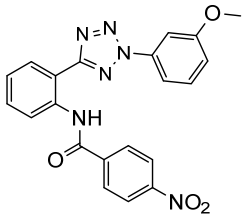
77: cepharanthine



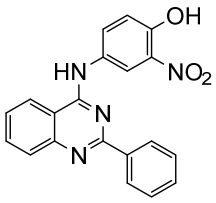
78: PAK-104 P



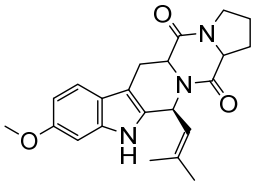
79: MK571 (verlukast)



80: tetrazolic HM30181 derivative no. 10



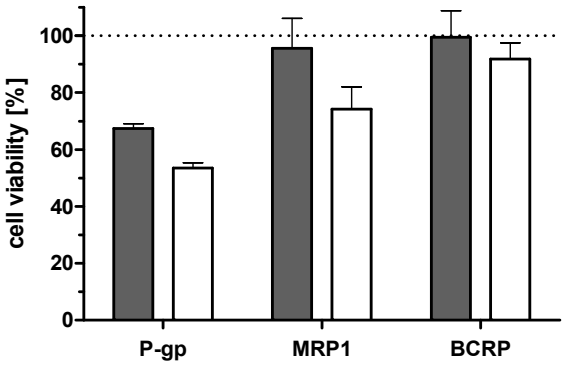
81:4-anilino-quinazoline no. 4



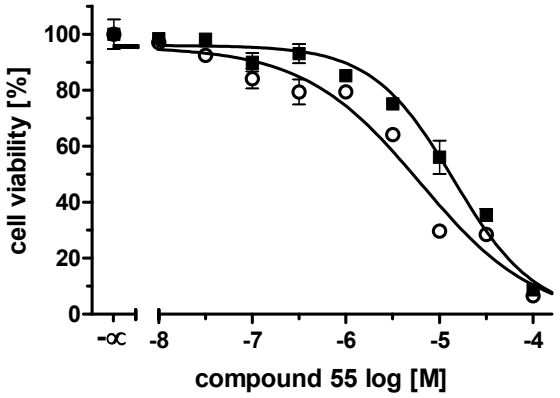
82: fumitremorgin C

Figure 11: Depiction of published partial inhibitors of ABC transport proteins. The quinazoline-chalcone no. 17 (**76**) reported by *Kraege* and *Stefan* et al. had good affinity toward P-gp with an IC_{50} of 0.42 µmol · L^{-1}, but with a maximum inhibition level of only 66%. The tetrazolic HM30181 derivative (**80**) reported by *Köhler* et al. possessed an IC_{50} value of 78.9 nmol · L^{-1} against BCRP, but with only 58% inhibition as compared to the standard compound **24**. With respect to the same transport protein, *Krapf* et al. reported on the aniline-quinazoline no. 4 (**81**), which possessed an IC_{50} value of 80 nmol · L^{-1} but with 79% inhibition. Fumitremorgin C (**82**) is the natural compound from which standard BCRP inhibitor **24** is derived from. Compared to the former, the latter achieves only a maximal inhibition of approximately 82%.

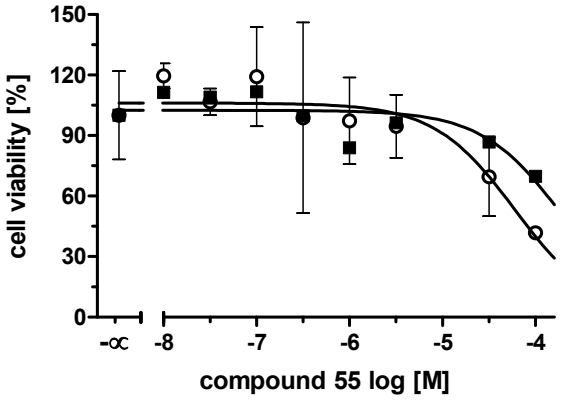
A


cell viability [%]
100
80
60
40
20
0
P-gp
MRP1
BCRP

B


cell viability [%]
100
80
60
40
20
0
-∝
-8
-7
-6
-5
-4
compound 55 log [M]

C



D

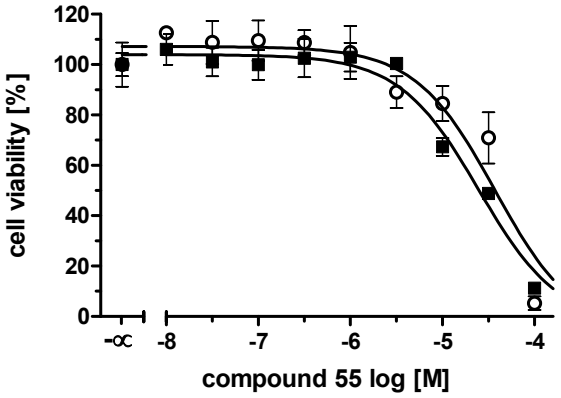
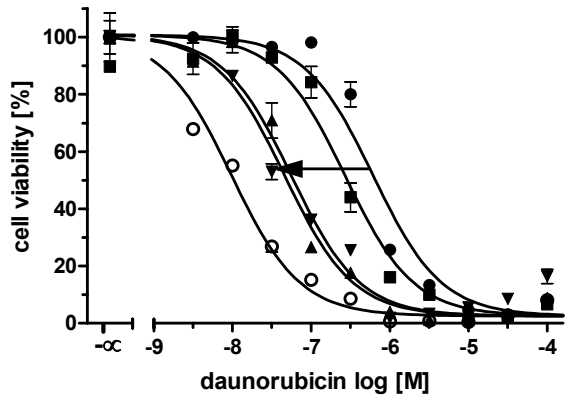


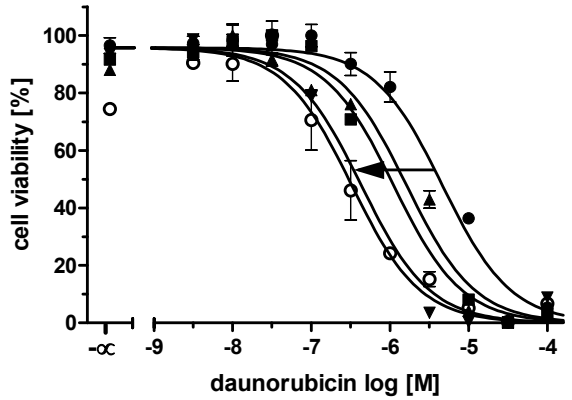
Figure 12. (A): Summary of results of the MTT-viability assay at 10 μM compound concentration for the resistant (grey) and parental cell lines (white). Pure cell culture medium without was used as negative control, 10% DMSO as positive control, defining 100% and 0% cell viability, respectively. Shown is mean ± SEM of at least three independent experiments of duplicate measurements. (B-D): Concentration-effect curve of compound **55** using different cell lines: A2780/ADR (B; closed squares) and

A2780 (B; open circles); H69AR (C; closed squares) and H69 (C; open circles) and MDCK II BCRP (D; closed squares) and MDCK II (D; open circles).

A



B



C

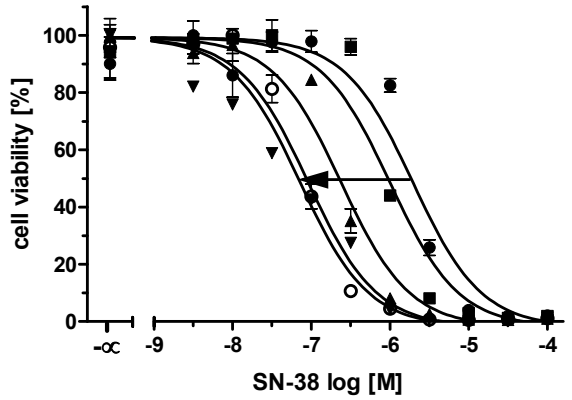


Figure 13: Sensitization of P-gp (A), MRP1 (B) and BCRP (C) overexpressing cells by compound **55** at 0.1 μM (closed square), 1 μM (upward closed triangle) and 10 μM (downward closed triangle). These calculated concentration-effect curves of daunorubicin and SN-38 between 10 nM and 100 μM were compared to the ABC

transporter overexpressing cells without supplementation of compound **55** (resistant cell line; closed circles) and the sensitive cell lines A2780 (A), H69 (B) and MDCK II (C) expressed as open circles. Shown are representative experiments out of three independent experiments with duplicate measurements.

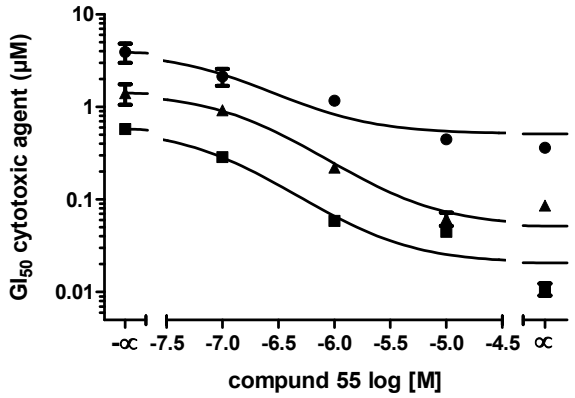
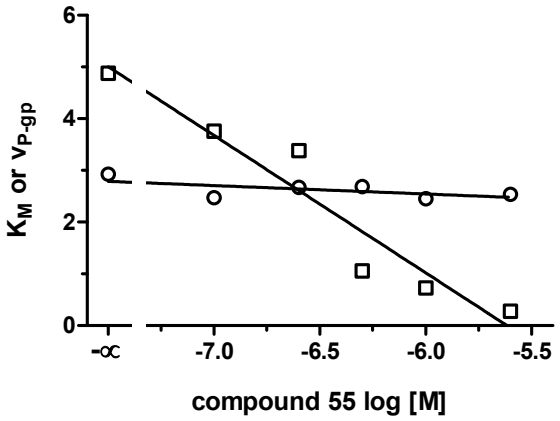
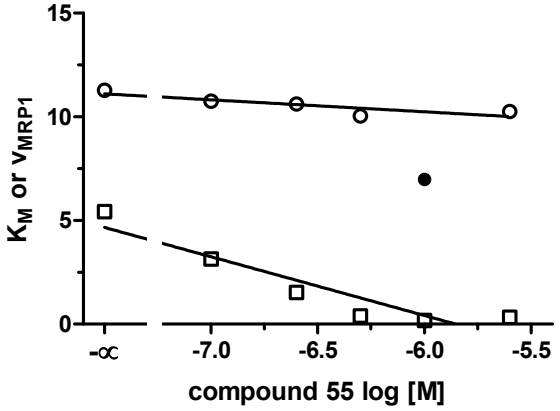


Figure 14: Plot of GI_{50} values in logarithmic form from table 7 and the concentrations of compound **55** referred to P-gp (closed circles), MRP1 (closed squares) and BCRP (closed upward triangles). The resultant EC_{50} value represents the concentration at which the resistance of the corresponding cancer cell line is half-maximal.

A


K_M or $v_{P\text{-}gp}$
6
4
2
0
-∝
-7.0
-6.5
-6.0
-5.5
compound 55 log [M]

B


K_M or v_{MRP1}
15
10
5
0
-∝
-7.0
-6.5
-6.0
-5.5
compound 55 log [M]

C

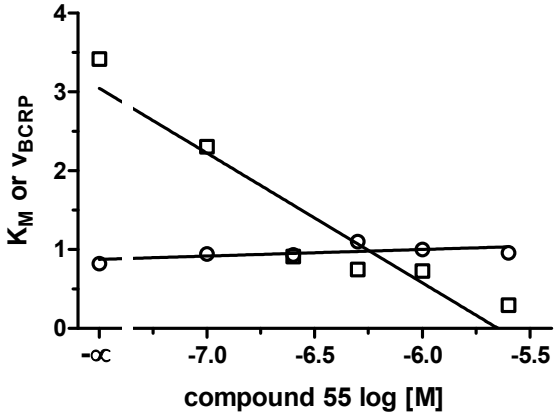

K_M or v_{BCRP}
4
3
2
1
0
-∝
-7.0
-6.5
-6.0
-5.5
compound 55 log [M]

Figure 15: Cornish-Bowden analysis of compound **55**. (A) P-gp-mediated transport calcein AM; (B): MRP1-mediated transport of daunorubicin; (C): BCRP-mediated transport of pheophorbide A. All three plots show a non-competitive inhibition of the ABC transporter-mediated transport of the corresponding substrate as indicated by the constant K_M value and decreasing transport velocity of the corresponding transporter (v_{max}). Compound **55** was used at 0.1, 0.25, 0.5, 1.0 and 2.5 μM; (A): Calcein AM was used at 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 μM; Daunorubicin was used at 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 μM; closed circle: outlier; (C): Pheophorbide A was used at 0.2, 0.4, 0.5, 0.75, 1.0 and 1.5 μM.

Topic of content graphic.