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Pyrrolopyrimidine derivatives and purine analogs as novel activators of Multidrug Resistance-associated Protein 1 (MRP1, ABCC1)

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Abstract. Multidrug resistance (MDR) is the main cause of diminished success in cancer chemotherapy. ABC transport proteins are considered to be one important factor of MDR. Besides P-glycoprotein (P-gp, ABCB1) and Breast Cancer Resistance Protein (BCRP, ABCG2), Multidrug Resistance-associated Protein 1 (MRP1, ABCC1) is associated with non-response to chemotherapy in different cancers. While considerable effort was spent in overcoming MDR during the last two decades, almost nothing is known with regard to activators of transport proteins. In this work we present certain pyrrolo[3,2-*d*]pyrimidine derivatives with variations at positions 4 and 5 and purine analogs as novel activators of MRP1-mediated transport of the MRP1 substrate calcein AM and the anticancer drug daunorubicin in low nanomolar

concentration range. Two different MRP1 overexpressing cell lines were used, the doxorubicin-selected human lung cancer cell line H69 AR and the transfected Madin-Darby Canine Kidney cell line MDCK II MRP1. No effect was observed in the sensitive counterparts H69 and MDCK II wild type (wt). Derivatives with higher molecular weight possessed also inhibitory properties at low micromolar concentrations, although most compounds were rather poor MRP1 inhibitors. Purine analogs derived from potent MRP1 inhibitors of the pyrrolopyrimidine class showed equal activating, but no inhibiting effects at all. All tested compounds were non-toxic and had only minor impact on P-gp or BCRP, showing no inhibition or activation.

Introduction. The overexpression of ABC transport proteins confers resistance to a broad range of cytotoxic agents. Besides P-gp and BCRP, MRP1 is associated with reduced drug retention in different cancers resulting in the phenomenon called MDR [1]. MRP1-mediated resistance is related to lung cancer [2] and other cancer types [3], affecting structurally diverse antineoplastic agents, like *Vinca* alkaloids, anthracyclines, alkylating agents, intercalators, or podophyllotoxines [4,5]. The export of drugs by MRP1 is mostly connected to co-binding and co-transportation of reduced glutathione (GSH) using the energy of ATP hydrolysis [5,6]. Inhibition of this transport protein has been studied extensively during the last two decades, providing a bulk of structurally diverse inhibitors [7,8]. Especially compounds with existing pharmacological use were found as MRP1 inhibitors, such as the calcium antagonist verapamil, the immunosuppressant cyclosporine A [9], the organic anion transporter inhibitor probenecid [10], or several NSAIDs [11], e.g. indomethacin [12]. Some inhibitors were evaluated in clinical trials against cancer and cancer-related diseases, but with limited success due to severe side effects amongst other reasons

[13,14,15,16,17]. Even compounds specifically designed to affect ABC transporters such as biricodar were clinically ineffective [18,19,20].

The development of new compounds inhibiting ABC transport proteins in general, and MRP1 in particular with high potency and selectivity with no serious side effects is desirable. But it is as much important to get a detailed understanding of the function of ABC transporters, as the current knowledge of the mechanistic aspects of these targets and the drug-protein interaction is still unsatisfactory. Activators of drug transport mediated by ABC transport proteins might help to elucidate the mode of action with respect to drug recognition, binding, transport and release.

The first study in this field was published by *Sharom* et al., who found that synthetic hydrophobic peptides with tyrosine C-terminus increased the ATP-dependent colchicine transport of P-gp accompanied by ATPase activation [21]. Since then, several authors reported on the activating characteristic of many structural diverse compounds with regard to P-gp, e.g. prazosin [22], (-)epicatechin [23], aged garlic extracts [24], the tyrosine kinase inhibitor erlotinib [25] and the imidazothiazole-derivative QB102 [26] as well as other benzimidazole and -thiazole derivatives [27].

In contrast to P-gp, however, almost nothing is known referred to transport activation and binding sites of MRP1 [28], although certain amino acids were found to be crucial for the binding and transport of endo- and xenobiotics [29,30], especially tryptophan [31,32], cysteine [33,34], and charged amino acids [35,36]. *Wesolowska* et al. showed in 2005 that phenothiazine maleates stimulated the MRP1-mediated transport of the fluorescence dyes BCPCF and BCECF [37]. The findings were confirmed with respect to pharmacologically used phenothiazines, like thiethylperazine or perphenazine [38], compounds which were known before as P-gp inhibitors [39]. The flavonoid genistein was found to stimulate the transport of

rhodamine 123 by MRP1 [40]. Analogs of GSH synthesized and evaluated by *Leslie* et al. proved to accelerate MRP1-mediated transport of [^{3}H]estrone 3-sulfate [41], a known substrate of this transport protein.

Activation of MRP1-mediated transport is often associated with enhanced efflux of GSH, since GSH is a co-substrate of this transporter [5,6]. The cardiovascular drug verapamil, a known P-gp and MRP1 inhibitor as well as a P-gp ATPase activator [42], was found to stimulate GSH transport [43,44], leading to decreased intracellular GSH levels and cell apoptosis [45,46]. This has also been shown for certain flavonoids by *Lorendeau* et al. [47]. *Loe* et al., who also reported on the stimulating effect of verapamil before [48], synthesized and evaluated dithiane derivatives of this cardiovascular drug. These compounds had a stimulating effect on GSH export by MRP1 [49]. Furthermore, *Brandmann* et al. showed that the protease inhibitors indinavir and nelfinavir stimulated MRP1-mediated GSH transport in human astrocytes [50], whereas *Perloff* et al. found that ritonavir increased the activity of MRP1 by inducing its expression in LS-180 cells [51].

An enhanced transport velocity can also be accompanied by an increase of the ATPase activity, as has been shown for several flavonoids by *Leslie* et al. and others [47,52,53]. Guggulsterone, a compound derived from the plant *Commiphora mukul.* and analyzed by *Nabekura* et al., increased MRP1 ATPase activity in high magnitude [54]. The same applies to the endogenous compound GSSG, as this was shown by *Mao* et al. [55].

In this study we provide two new classes of MRP1 activators, pyrrolopyrimidine derivatives and purine analogs. These compound classes were shown by us and others before to contain potent and selective inhibitors of MRP1 and P-gp [56,57,58]. Our aim was to analyze the transport increasing effect of these compounds in detail

with respect to MRP1 as well as to P-gp and BCRP. Using the MRP1 substrates calcein AM and daunorubicin, determination of the quality and quantity of transport activation of MRP1 was in focus of this study. The intrinsic toxicity of the tested compounds was evaluated using the MTT viability test utilizing different sensitive, selected and transfected cancer cell lines.

Results and Discussion.

Chemistry.

Scheme 1 A illustrates the preparation of compounds **6-11**. Ethoxymethylene-malononitrile was reacted by a nucleophilic substitution with different primary amines yielding the malononitril derivatives **1a-d**. These were cyclized by addition of ethyl bromoacetate providing intermediates **2a-d.** The amino side chain was derivatized by adding dimethyl formamide dimethyl acetale, yielding compounds **3a-d**. Exposure to gaseous ammonia gave the cyclized products **4a-d**. Aromatization using phosphoryl chloride provided the precursors **5a-d**. Microwave-assisted synthesis yielded compounds **6-11** by nucleophilic substitution of the aromatic ring system at position 4 with different primary amines. Scheme 1 B shows the synthesis of the purine analogs **12-15**. 6-chloropurine and 1*H*-piperazine derivatives were combined by nucleophilic substitution. The identity of all intermediates was confirmed using ^{1}H and ^{13}C NMR spectroscopy. The purity of the desired compounds **6-15** was determined using LC-MS analysis.

Biological Investigations.

Calculation of Flux Ratio and Activation Ratio of MRP1.

The following calculations were performed assuming first order kinetics for both active transport and passive diffusion in order to relate the resistance ratio of MRP1 expressing cells to the reduced intracellular concentration [59].

The influx or efflux velocity of a substrate, e.g. fluorescence dye or cytotoxic agent, is described as product of concentration and rate constant:

$$v = k \cdot c \qquad (1)$$

v = transport velocity

c = substrate concentration

k = velocity constant

Under steady state conditions the influx equals the efflux:

$$v_{(p,out)} = v_{(p,in)} + v_{(a,in)} \qquad (2)$$

(p,out)= passive transport from outside to inside

(p,in) = passive transport from inside to outside

(a,in) = active transport from inside to outside the cell mediated by a transport protein

Combination of equations 1 and 2 yields:

$$k_p \cdot c_{out} = (k_p + k_a) \cdot c_{in} \qquad (3)$$

Rearrangement gives the relative flux ratio (k_a/k_p) of active transport in relation to passive diffusion:

$$(k_a + k_p)/k_p = c_{out} / c_{in} \quad \rightarrow \quad k_a/k_p = (c_{out} / c_{in}) - 1$$

or $c_{in} / c_{out} = k_p /(k_a + k_p)$ → $c_{in} / c_{out} = k_{in} /(k_a + k_p)$

The effect of increased active transport in relation to passive diffusion on intracellular concentration is shown in Figure 1. The depicted relationship is in agreement with observations made for different anthracyclines with different lipophilicity and passive diffusion velocity [59]. Here, the cells were less resistant to more lipophilic compounds with a higher diffusion coefficient. The hyperbolic relationship makes it evident that low active transport velocity has initially a large effect on intracellular concentration, lowering it considerably for flux ratios of less than approximately two. However, the active transport velocity has to be increased strongly in order to further decrease the intracellular drug concentration. Thus, in a resistant cell a significant increase in active transport velocity is necessary for a noticeable further decrease of intracellular concentration. An infinite active transport rate would be needed to reach zero intracellular concentration, which is impossible. Even in the rare case of an active uptake of a drug, the same hyperbolic relationship between active efflux and intracellular concentration is observed (Figure 1). In such a case the numerator would be the sum of passive diffusion and active uptake rate.

In case of fluorescent probes, the fluorescence intensity can be used as surrogate for the concentration, as it was done extensively to characterize inhibitors of transport proteins in accumulation assays or inside-out membrane vesicles. The fluorescence intensity correlates in a linear manner with the substrate concentration, if no interfering effects like quenching occur. We have checked that our compounds do not influence the fluorescence of daunorubicin or calcein. Additionally we found a linear relationship between the extracellular substance concentration and measured fluorescence, for both, the active and inhibited transporter, as it is shown in Figure 2.

Therefore, no self-quenching occurs. The linear relationship also implies that other effects that could change the fluorescence intensity like protein binding or binding to DNA accompanied by fluorescence quenching in case of daunorubicin are proportional to the used concentration and not saturated under the assay conditions. Thus, such effects have no influence on the linear relationship between concentration and fluorescence intensity.

Theoretically, the decreased fluorescence in case of calcein AM could be caused by inhibition of intracellular hydrolases by the investigated compounds. However, taking into account that the compounds are used at nanomolar concentrations, this seems to be improbable. This conclusion is supported by the analogous results obtained for daunorubicin, where no hydrolases are involved in the measurement process. Thus, possible interfering processes do not change the linear relationship between concentration and measured fluorescence. Hence, the maximal fluorescence obtained when the transporter is completely inhibited is proportional to c_{out} and the fluorescence observed when the transporter is fully active corresponds to c_{in}. These two concentrations conform to the top and bottom values of calculated dose-response curves. The activation ratio was defined as flux ratio in presence of an activator divided by the flux ratio of the control in absence of any activator or inhibitor in order to make different experiments comparable:

$$\text{activation ratio} = \text{flux ratio (activator)} / \text{flux ratio (control)}$$

The activation ratio gives an X-fold increase (or decrease) of transport velocity and makes different data sets comparable to each other.

Calcein and Daunorubicin Accumulation Assays. We showed in our previous work that pyrrolopyrimidines are potent and selective inhibitors of MRP1, and correlated

their inhibitory potency with physicochemical parameters like molecular weight and lipophilicity [57]. In further experiments we found that the small, low-weight representatives **18**-**21** (Figure 3 A) enhanced MRP1-mediated transport of calcein AM and daunorubicin in low nanomolar concentration range (Figure S1 and S2), which led to a detailed screening of our compound library consisting of various structural classes. In this work we present the most potent activators in low nanomolar concentration range with none to modest inhibitory activity at low micromolar concentrations. We established compound 12 (SC12, Figure 3 B) as standard inhibitor of MRP1 in our previous work [57], which was also used in the present study.

The pyrrolopyrimidine derivatives **6**-**11** accelerated calcein AM and daunorubicin efflux as can be seen in Figure 4 A and B. The compounds were compared to each other at 10 nM, as this concentration was found to result in maximal activation for most compounds. A representative dose-response curve of compound **9** (Figure 3 C) in comparison to SC12 is shown in Figure 5 A. Figure 5 B depicts the concentration-dependent curve of the MRP1 activation ratio. Calculation of the activation ratio of the compounds yielded an increase of 68-98% in the daunorubicin and about 32-50% in the calcein accumulation assay, respectively (table 1), in comparison to basal pump velocity.

While H69 AR cells were used in the latter experiment, the former was conducted with MDCK II MRP1 cells. Comparable effects were observed for both substrates and both cell lines, showing that the effect of activation is not substrate or cell line specific. The sensitive cell lines H69 and MDCK II wt have been used to prove that the compounds have no nonspecific effects on the cells. As can be seen in Figure 5 A and B, daunorubicin accumulated in both sensitive cell lines to the same extent as

it was observed for fully inhibited MRP1 overexpressing cells. These results show that the activators do not interfere with the distribution of this fluorescence dye caused by changes in membrane fluidity or challenge of membrane integrity.

Lineweaver-Burk analysis of enzyme kinetic experiments showed a competitive interaction with daunorubicin (Figure 8 A). Although linearization according to Lineweaver-Burk gives direct information on the type of interaction, this method has been criticized as being notoriously susceptible to experimental errors due to the double reciprocal plot. Therefore we additionally analyzed the data using the linearization method according to Hanes-Woolf. The parallel lines seen in Figure 8 B are indicative of a competitive behavior. As a third method the direct linear plot according to Cornish-Bowden was used to investigate the type of interaction. This method has been claimed to be insensitive to outliers. As can be seen in Figure 8 C, K_m decreases while V_{max} remains constant. This clearly shows that the affinity for the substrate is increased. These results are in agreement with the behavior of a non-essential activator [60].

While small pyrrolopyrimidines with a molecular weight of less than 350 Da had no or only poor inhibitory power toward MRP1, compounds with higher molecular weight (**9**-**11**) showed moderate to good inhibition (table 1). This is in agreement with the already described observation that within this substance class, high molecular weight is preferred with respect to MRP1 inhibition [57]. Compound **9** was the most effective inhibitor with an IC_{50} value of 0.790 µM in the daunorubicin accumulation assay (Figure 5).

Screening of our compound library showed also purine analogs (Figure 3 D) to be equally stimulating with respect to calcein AM and daunorubicin efflux (Figures 4 A, B and 6 A, B), accelerating MRP1-mediated efflux of daunorubicin and calcein AM by

46-94% and 32-43%, respectively (table 1). While the substituents at position 4 were crucial partial structures of already described potent inhibitors of MRP1 (cmpds. 12-15 and 22-25 in ref. 57), these residues seem not to have any influence on MRP1 inhibition when linked to the purine scaffold. Therefore, the purine scaffold seems to abolish the inhibitory nature of the compounds. Figure S3 A and B show representative dose response curves of pyrrolopyrimidine derivatives and purine analogs in the calcein and daunorubicin accumulation assay.

Calcein Accumulation Assay to Screen for P-gp Activation and Inhibition. Due to the known functional and structural analogy of MRP1 and P-gp [1], we analyzed the capability of the compounds to activate or inhibit the latter transport protein (Figure 9 A). Except for compound **9**, which had poor P-gp inhibiting potency with an IC_{50} value of 14.0 µM, no activating or inhibiting effect of these compounds could be observed at 10 nM and 10 µM, respectively, compared to the standard inhibitor cyclosporine A at 10 µM (Figure 9 A, table 1).

Pheophorbide A Accumulation Assay to Screen for BCRP Activation and Inhibition. We screened our library for activation or inhibition with regard to BCRP-mediated transport of the chlorophyll-breakdown product and fluorescence dye pheophorbide A. Figure 9 B shows that except for compounds **6** and **9**, which had low inhibitory power toward BCRP with IC_{50} values of 20.6 µM and 20.4 µM, respectively, neither inhibition nor activation with regard to BCRP in comparison to the standard inhibitor Ko143 at 10 µM was observed (Figure 9 B, table 1). These findings indicate that the compounds are selectively activating MRP1.

MTT Assay to Determine Intrinsic Cytotoxicity of Selected Compounds. We also analyzed all compounds regarding their intrinsic cytotoxicity. Except for compound **9**, which possessed a GI_{50} value of 31.8 µM in MDCK II MRP1 cells, the compounds

proved to be non-toxic using MDCK II MRP1 cells and other cell lines (Figures S4-S8). Table 2 summarizes determined GI_{50} values of all compounds using different sensitive, selected and transfected cell lines. Figure 7 gives an overview of the viability data. At relevant concentrations of 1 µM and 10 µM, none of the compounds had a negative influence on cell growth or viability. Only compounds **6** and **9** reduced cell viability significantly at a concentration of 100 µM, a concentration ten times higher than used in the assays. Contradictory, compounds **8**, **10**, **11**, **12** and **15** had a tendency to enhance cell viability with increased concentration. This effect was significant for compounds **10** and **11** at 100 µM. This data points to no influence on the membrane integrity, since this would have a destructive effect on the cell viability within the assay time of 72 hours.

Conclusion.

In this work we provide further information on a rarely explored field of ABC transport proteins, namely compounds that activate the transporter-mediated efflux of xenobiotics. Since this area of transmembrane efflux systems is hardly known and the pool of already known activators of ABC transporters in general is very limited, the presented results might help to complement the knowledge about efflux proteins and contribute to elucidate the mode of action and function in general of these targets. Referring to MRP1, almost nothing is known about stimulators of drug transport [37,40].

While pyrrolopyrimidines with high lipophilicity and molecular weight have been presented before as potent and selective inhibitors of MRP1 [56,57], we showed that low-weight derivatives are able to stimulate MRP1-mediated transport of two known MRP1 substrates, calcein AM and the anticancer drug daunorubicin. The purine analogs **12-15** were equally effective with respect to MRP1 activation, increasing the

transport velocity to the same magnitude as the pyrrolopyrimidines **6**-**11**. We showed that the change in distribution of the MRP1 substrates calcein AM and daunorubicin is solely caused by the enhanced transport velocity of MRP1, since the compounds had no influence on sensitive cell lines H69 and MDCK II wild type, respectively. The substitution pattern at positions 4 and 5 of the pyrrolopyrimidines **6-11** had no influence on their activating effect. Molecular weight as a property played only a role for MRP1 inhibition, as has been shown previously [57]. While pyrrolopyrimidines with molecular weights of less than 350 Da (**6**-**8**) had no or only poor MRP1 inhibitory potency, compounds **9**-**11** showed moderate to good MRP1 inhibition. Compound **9** has a special nature, being not only a good activator of MRP1-mediated efflux in low nanomolar concentrations, but also inhibits the three major transport proteins involved in MDR, MRP1, P-gp and BCRP, at low micromolar concentrations, which makes it a rare representative of a triple inhibitor. This could be a starting point for future investigations to generate more potent compounds affecting the three major efflux pumps connected to MDR in cancer. The purine analogs **12**-**15** did not have any inhibitory power with regard to any tested transport protein.

Except for compound **9**, which showed slight intrinsic toxicity, all compounds were nontoxic in different cell lines, which also gives evidence that the observed distribution change of the used fluorescence probes by activation of MRP1 is not caused by cell barrier-related effect or other nonspecific factors. This assumption is supported by the viability data. Since none of the compounds showed destructive effects at relevant assay concentrations and most compounds had even a viability increasing effect on the cells, a distribution change caused by disturbing the membrane integrity is highly unlikely, as this would lead to the opposite effect.

The compounds are a useful tool in transporter science and might help to elucidate the mechanisms involved in the transport process. Since biochemistry and molecular modeling techniques are still up to elucidate the exact process of drug recognition, binding, transport and release as well as the relation between ATP hydrolysis and conformational changes required for transport, MRP1 activators can help to get an insight into the function of this transport protein. Finally, elucidation of its mechanism might help in future cancer therapy.

Experimental Section.

Chemistry. *Materials*. Chemicals 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. The reaction progress was followed by analytical thin layer chromatography 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) were used as eluent and an UV lamp at 254 nm was utilized for compound detection. Purification of all compounds was performed with column chromatography using 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). The identity of the intermediates was determined by ^{1}H spectroscopy. Additionally, ^{1}H and ^{13}C spectroscopy was used to characterize the desired compounds **6-15**, while the purity was determined by LC-MS analysis. The NMR spectra were recorded in DMSO-d_6 on

a Bruker Advance 500 MHz (500/126 MHz) and chemical shifts (δ) are expressed as ppm calibrated to the solvent signal (^{1}H NMR δ 2.50; ^{13}C NMR δ 39.5). ^{13}C signals were assigned employing distortion less enhancement by polarization transfer (DEPT) and attached proton test (APT) techniques. Spin multiplicities are depicted as singlet (s), doublet (d), doublet of doublets (dd), doublet of triplets (dt), triplet of doublets (td), triplet (t), quartet (q) and quintet (quint). LC-MS analysis was performed 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 the investigated compounds in biological testing was determined as ≥ 95%.

General procedure for the preparation of compounds **6-15**. The preparation of the herein presented intermediates **1a-d** - **5a-d** and their spectroscopic data has already been published by us before [57]. The desired title compounds were prepared as described in the literature [56,57] with minor modifications. Complete spectroscopic data are provided in the supplementary material.

Biological Investigation. *Chemicals.* The standard inhibitor SC12 was synthesized, purified and characterized as described before [56,57]. The reference compounds cyclosporine A and Ko143 ((3*S*,6*S*,12*aS*)-1,2,3,4,6,7,12,12*a*-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) were purchased from Tocris bioscience (Bristol, IO, USA). Calcein AM and pheophorbide A were supplied by Calbiochem (EMD Chemicals (San Diego, CA, USA), supply by Merck KGaA (Damstadt, Germany)) and Frontier Scientific Inc. (Logan, UT, USA), respectively. Daunorubicin was purchased from

Sigma (Oakville, ON, USA). All other chemicals were provided by Carl Roth GmbH (Karlsruhe, Germany), Merck KgaA (Darmstadt, Germany), Th. Greyer GmbH Co KG (Renningen, Germany) and Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The reference compounds were stored as 10 mM stock solution in DMSO at -20 °C. All dilutions 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-hydroxyethylpiperazin-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 small cell lung cancer cell line H69 AR overexpressing MRP1 (ATCC CRL-11351) and its sensitive counterpart H69 (NCL-H69, ATCC HTB-119) were provided by American Type Culture Collection and cultivated in RPMI-1640 medium (PAN Biotech GmbH, Aidenbach, Germany) complemented with 20% fetal bovine serum (FBS, Sigma Life Science, Steinheim, Germany), 2 mM L-Glutamine and 50 U/mL penicillin G and 50 µg/mL streptomycin (PAN Biotech GmbH, Aidenbach, Germany). The doxorubicin-selected human ovarian carcinoma cell line A2780 adr overexpressing P-gp was supplied by the European Collection of Animal Cell Culture (ECACC, No 931123120). Cultivation was performed in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine and 50 U/mL penicillin G and 50 µg/mL streptomycin. The MRP1 overexpressing cell line Madin-Darby Canine Kidney (MDCK II MRP1), the BCRP overexpressing cell line

MDCK II BCRP, both transfected with human wildtype cDNA C-terminally linked to the cDNA of green fluorescent protein (GFP), and their sensitive counterpart MDCK II wild type (wt) were a generous gift by Dr. A. Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands). These cell lines were cultivated in Dulbecco's modified Eagles 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 supplemented with 10% DMSO. The cultivation of cells was maintained at 37 °C under 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 ≥ 90% was reached, followed by subculturing or biological testing. The cell layer was rinsed three times with 5 mL of DBPS buffer. Harvesting was performed by exposure to a solution of 0.05% trypsin and 0.02% EDTA (PAN Biotech GmbH, Aidenbach, Germany), followed by an incubation period of 5-10 minutes. The detached cells were transferred into a 50 mL tube 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 Accumulation Assay. The assay was conducted as described earlier [57,61,62,63,64,65]. Twenty µL of the test compound in KHB at concentrations between 0.1 pM and 100 µM were added into clear F bottom 96-well microplates (Greiner bio one, Frickenhausen, Germany). After cell harvesting and counting as described above, 160 µL containing approximately 60,000 cells was seeded into

each well, followed by an incubation time of 20 minutes at 37 °C under 5% CO_2-humidified atmosphere. Twenty µL of a 3.125 µM solution of calcein AM (protected from light) was added. The 96 well plate was instantly measured with respect to 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. The standard inhibitor SC12 was used as reference as it was established before [57]. The linear part of the fluorescence-time curve was taken for the calculation of the slope between 0 and 30 minutes. Concentration-response curves were obtained by plotting the calculated slope values against the concentrations of the tested compound using the four-parameter logistic equation with variable Hill slope or the three-parameter logistic equation with fixed Hill slope, whichever was statistically preferred with GraphPad Prism version 5.03 for Windows (San Diego, CA, USA).

Calcein Accumulation Assay to Screen for P-gp Activation or Inhibition. The test compounds were screened for P-gp activation and inhibition as described earlier [57,61,62,63]. Dilution series, cell harvesting and counting was performed as stated above. 160 µL of a cell suspension containing approximately 30,000 cells per well was added to 20 µL of the test compound solution at concentrations between 1 nM and 100 µM followed by an incubation time of 30 minutes at 37 °C and under 5% CO_2-humidified atmosphere. 20 µL of a 3.125 µM solution of calcein AM (protected from light) was added. The 96 well plate was measured instantly with regard to fluorescence increase in constant time intervals of 60 seconds with microplate readers as stated above. The effect of 10 nM and 10 µM of the test compound was compared to the effect of 10 nM and 10 µM cyclosporine A and expressed as

percentage activation and inhibition in comparison to the standard compound, using GraphPad Prism as analyzation tool.

Daunorubicin Accumulation Assay. The daunorubicin accumulation assay was performed as described before [57,66]. Cell harvesting and preparation was conducted as stated above. The cell pellet was resuspended in fresh culture medium and 160 µL per well was seeded into colourless 96 well plates with approximately 60,000 cells per well. The dilution series of the test compounds between 1 pM and 100 µM were prepared in culture medium. The cells were preincubated with the compounds for 15 min, followed by addition of 20 µL of a daunorubicin solution (30 µM). After that the 96 well plate was incubated for 180 minutes at 37 °C and under 5% CO_2-humidified atmosphere to reach steady state conditions. The cells were resuspended before measurement to remove adherent cells from the bottom and to obtain a homogenous suspension. Fluorescence was measured by flow cytometry (FACSCalibur, Becton Dickinson Biosciences, Heidelberg, Germany). An argon laser with an excitation wavelength of 488 nm excited daunorubicin which could be detected in the FL3 channel (≥ 670 nm). Concentration response curves were generated by nonlinear regression based on the 4-parameter logistic equation with variable Hill slope or the three-parameter logistic equation with fixed Hill slope (GraphPad Prism) as stated above.

Pheophorbide A Accumulation Assay to Screen for BCRP Activation and Inhibition. The BCRP overexpressing cell line MDCK II BCRP was used to conduct this assay as described earlier [57,67,68,69]. Cells were harvested and prepared as described above. 20 µL of the test compounds in different concentrations between 1 nM and 100 µM were added to an U-shaped clear 96 well plate (Greiner, Frickenhausen, Germany). 160 µL of the cell suspension containing approximately 45,000 cells was

added to each well followed by an incubation time of 20 min at 37 °C under 5% CO_2-huminidified atmosphere. 20 µL of a pheophorbide A solution (5 µM, protected from light) were added to each well followed by an incubation of 120 min at 37 °C and under 5% CO_2-humidified atmosphere to reach steady state conditions. The cells were resuspended before starting measurement to detach adherent cells from the bottom of the plates and reach homogenous conditions. Fluorescence was measured by flow cytometry. An argon laser with an excitation wavelength of 488 nm excited pheophorbide A which could be detected in the FL3 channel (≥ 670 nm). BCRP expression was measured with GFP detection in the FL1 channel (530/15). The effect of the 10 nM and 10 µM concentration of the standard compound Ko143 was compared to the effect of 10 nM and 10 µM of the test compound, using GraphPad Prism as analyzation tool.

MTT Assay to Determine Intrinsic Cytotoxicity of Selected Compounds. The intrinsic toxicity of selected compounds was determined using a MTT viability assay was conducted as described in the literature [57,63] with minor modifications. 20 µL of different concentrations of the test compounds at 1 µM, 10 µM and 100 µM, respectively, were pipetted into 96-well tissue-culture treated plates (Starlab GmbH, Hamburg, Germany) followed by addition of 180 µL of a cell suspension (MDCK II MRP1, MDCK II BCRP or MDCK II wt: 3,000 cells per well; H69 AR: 20,000 cells per well; 2008/MRP1: 8,000 cells per well; A2780 adr: 8,000 cells per well). Culture medium without further supplements was used as negative control, while 10% DMSO was used as positive control, defining 100% and 0% viability, respectively. For an incubation time of 72 hours the microplate was kept at 37 °C and under CO_2-humidified atmosphere. Twenty µL of a 5 mg/mL solution of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) was added to the plate and incubated

for 1 hour. After removal of the supernatant 100 µL of DMSO was added to each well. Spectrophotometric measurement of the absorbance at 570 nm was performed using an Ex Multiscan microplate photometer (Thermo Fisher Scientific, Waltham, MA, USA) with a background correction at 690 nm. GraphpadPrism was used as analyzation tool.

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Abbreviations used.

ABC, ATP-binding cassette; ABCB1 *synonymous for* P-gp; ABCC1, *synonymous for* MRP1; ABCG2, *synonymous for* BCRP; APT, attached proton test; ATP, adenosine 5'-triphosphate; BCECF, 2'.7'-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein; BCPCF, BCRP, 3-carboxy-propyl)-5-(and-6-)-carboxyfluorescein; Breast Cancer Resistance Protein; calcein AM, calcein acetoxymethyl ester; δ chemical shift in ppm; DAD, diode array detector; DEPT, distortion less enhancement by polarization transfer; DMS, dimethyl sulfate; FACS, fluorescence activated cell sorting, GI_{50}, half-maximal growth inhibitory concentration; GSH, glutathione, reduced; GSSG, glutathione, oxidized; HEPES, 2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethansulfonic acid; log P calc., predicted partition coefficient of the neutral molecule; LTC_4, leukotriene C_4; LTD_4, leukotriene D_4; $[M+H]^+$, ionization from protonation; MDR, multidrug resistance; MRP1, Multidrug Resistance-associated Protein 1; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; NSCLC, non-small cell lung cancer; P-gp, permeability (P) glycoprotein; SC12, standard MRP1 inhibitor

compound 12; SCLC, small cell lung cancer; TKI, tyrosine kinase inhibitors; wt, wild type; *(Z), cis.*

Supplementary Material.

Molecular formula strings, complete spectroscopic data and additional biological data are provided.

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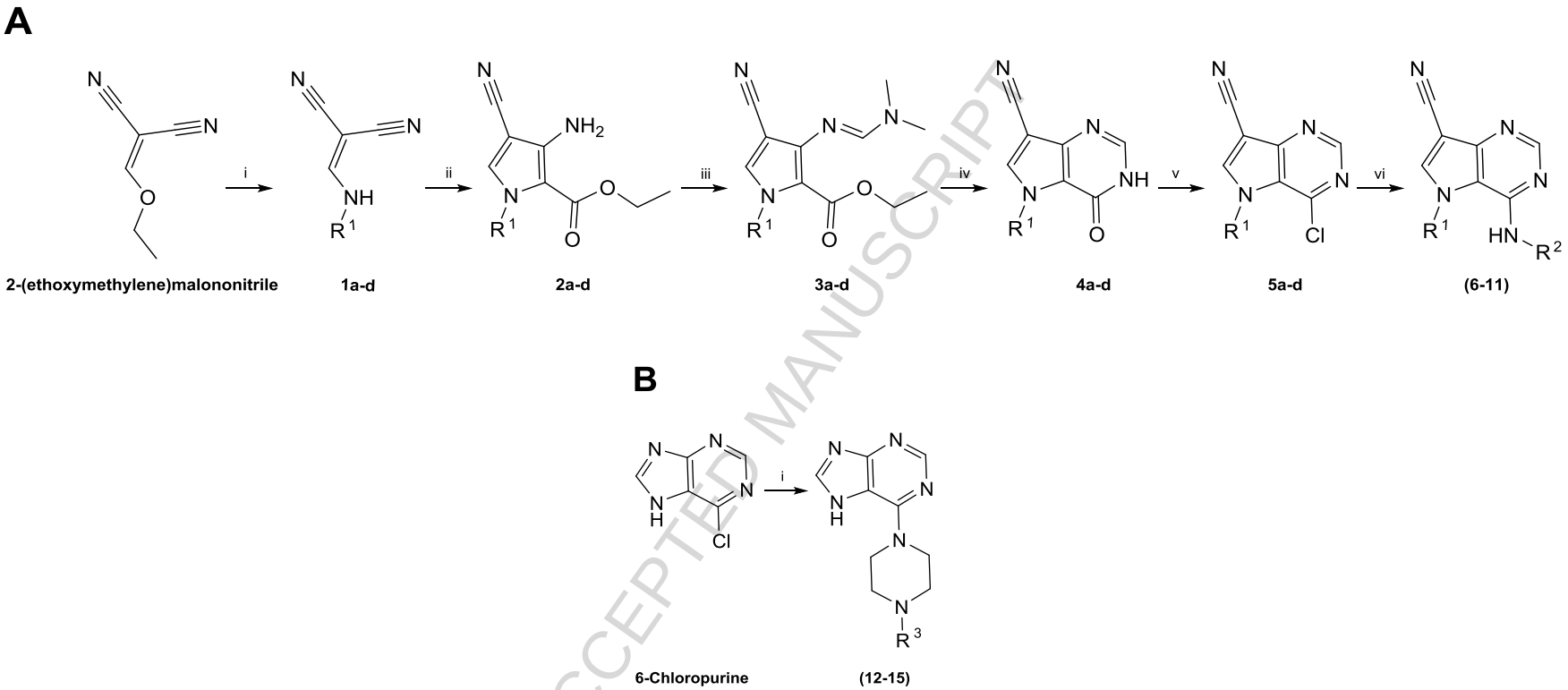
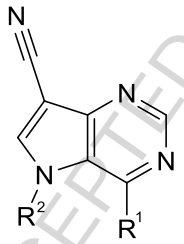


Scheme 1: Synthesis of (A) pyrrolopyrimidine with derivatives variations at position 4 and 5 and (B) purine analogs with variations at position 4. A: (i) H_2N-R^1, ethanol, room temperature, 1 h; (ii) ethyl bromoacetate, dimethyl formamide, 100 °C, 5 h; (iii) dimethyl formamide dimethyl acetale, dimethyl formamide, 100 °C, 5 h; (iv) ammonia, ethanol, reflux, 5 h; (v) Phosphoryl chloride, triethyl amine, reflux, 5 h; (vi) amine, triethyl amine, dimethyl formamide, 200 W, 110 °C, 1 h. R^1: a = methyl; b = phenyl; c = benzyl; d =

phenethyl. R^2: benzyl, phenethyl or phenylpropyl. B: (i) 1*H*-piperazine derivative, triethyl amine, diethyl formamide, 200 W 100 °C, 1 h. R^3: phenethyl, benzyl, diphenylmethyl or phenyl.

Table 1: Structures and biological activities of investigated pyrrolopyrimidines and purines. Activating effects were determined using the calcein accumulation assay and the H69 AR cell line as well as the daunorubicin accumulation assay and MDCK II MRP1 cells; Inhibition of daunorubicin efflux was measured using the MRP1 overexpressing cell line H69 AR as described before.[57] P-gp and BCRP inhibition was determined using A2780 adr and MDCK II BCRP cell lines, respectively, in a calcein and pheophorbide A accumulation assay, respectively. Shown is mean ± standard deviation (SD) of at least 3 independent experiments with duplicate measurements; n.t. = not tested due to lack of activity.



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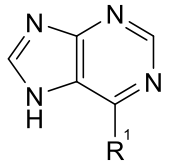

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Comp.	R^1	R^2	Molecular weight [Da]	MRP1 Calcein AM activation ratio	MRP1 Daunorubicin activation ratio	MRP1 Daunorubicin IC_{50} ± SD	P-gp Calcein AM IC_{50} ± SD	BCRP Calcein AM IC_{50} ± SD

				[% of basal activity at 10 nM]	[% of basal activity at 10 nM]	[μM]	[μM]	[μM]
6	Benzylamino	Methyl	263.3	151 ± 7	181 ± 14	17.1 ± 4.4	n.t.	20.6 ± 1.4
7	Benzylamino	Phenyl	325.4	132 ± 5	171 ± 14	4.02 ± 0.81	n.t.	n.t.
8	Benzylamino	Benzyl	339.4	143 ± 56	168 ± 16	7.43 ± 1.76	n.t.	n.t.
9	Phenylpropylamino	Benzyl	367.5	142 ± 10	193 ± 27	0.790 ± 0.141	14.0 ± 0.3	20.4 ± 1.6
10	Phenylethylamino	Phenylethyl	367.5	141 ± 9	178 ± 35	1.47 ± 0.45	n.t.	n.t.
11	Phenylpropylamino	Phenylethyl	381.5	144 ± 14	198 ± 25	0.827 ± 0.104	n.t.	n.t.
12	Phenylethylpiperazinyl	-	308.4	136 ± 5	182 ± 29	20.0 ± 0.8	n.t.	n.t.
13	Benzylpiperazinyl	-	294.2	143 ± 5	146 ± 8	23.9 ± 9.7	n.t.	n.t.
14	Diphenylmethylpiperazinyl	-	370.5	137 ± 3	194 ± 18	18.5 ± 6.6	n.t.	n.t.
15	Phenylpiperazinyl	-	280.3	132 ± 4	192 ± 23	32.5 ± 14.7	n.t.	n.t.

Table 2: Half-maximal growth inhibition values (GI_{50}) of pyrrolopyrimidine derivatives and purine analogs calculated using different MRP1, P-gp and BCRP overexpressing and parental cell lines. Measurements were conducted at least in triplicate. Values are expressed as mean ± SD.

Comp.	MDCK II MRP1 GI_{50} ± SD [µM]	MDCK II wt GI_{50} ± SD [µM]	H69 AR GI_{50} ± SD [µM]	2008 MRP1 GI_{50} ± SD [µM]	A2780 adr GI_{50} ± SD [µM]	MDCK II BCRP GI_{50} ± SD [µM]
6	> 100	> 100	> 100	85.0 ± 21.2	85.5 ± 1.4	82.8 ± 11.0
7	> 100	69.8 ± 25.1	64.4 ± 32.3	74.2 ± 36.5	70.7 ± 41.5	37.4 ± 14.8
8	> 100	> 100	> 100	> 100	85.3 ± 9.0	> 100
9	31.8 ± 3.0	29.7 ± 2.1	34.2 ± 7.6	35.4 ± 0.5	32.8 ± 8.6	18.5 ± 6.6
10	> 100	> 100	> 100	> 100	66.1 ± 8.7	> 100
11	> 100	> 100	> 100	> 100	> 100	> 100
12	> 100	> 100	> 100	> 100	79.4 ± 14.3	> 100
13	> 100	> 100	> 100	> 100	63.7 ± 5.6	85.6 ± 20.4
14	> 100	> 100	> 100	> 100	61.5 ± 18.5	79.6 ± 28.9
15	> 100	> 100	> 100	> 100	36.8 ± 1.1	> 100

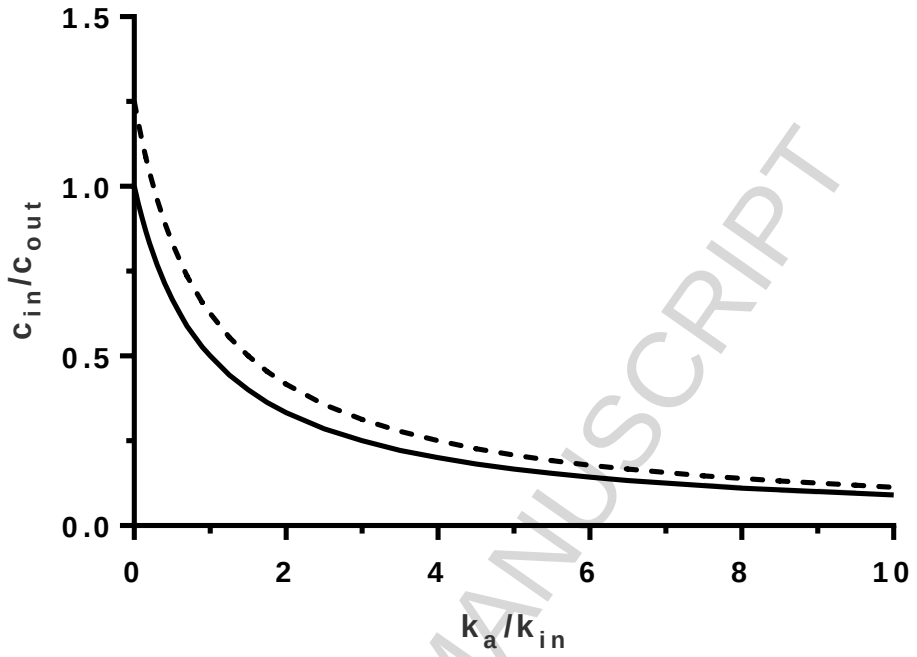


Figure 1: Relationship between decrease of intracellular concentration and active efflux with respect to uptake. The solid line represents the case of passive uptake only. The broken line was calculated assuming an active uptake at a rate one fourth of the passive diffusion rate.

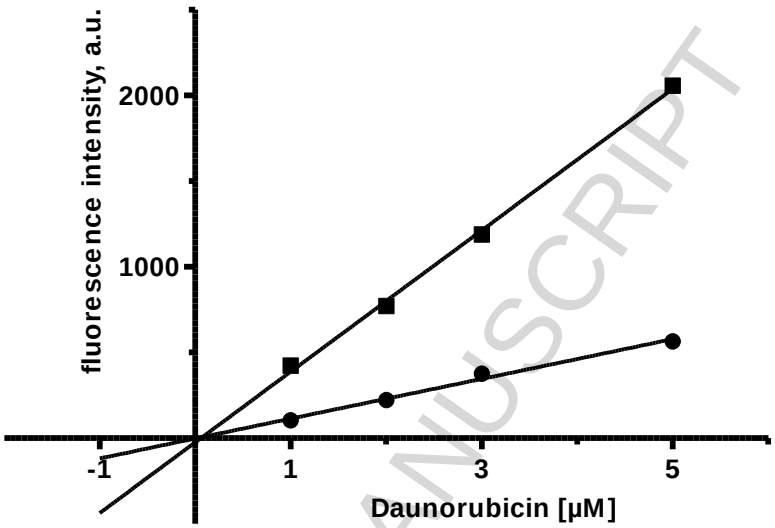


Figure 2: Plot of fluorescence values of SC12 (maximum inhibition at 10 µM, closed squares) and compound 6 (maximum activation at 10 nM, closed circles) at different concentrations of daunorubicin.

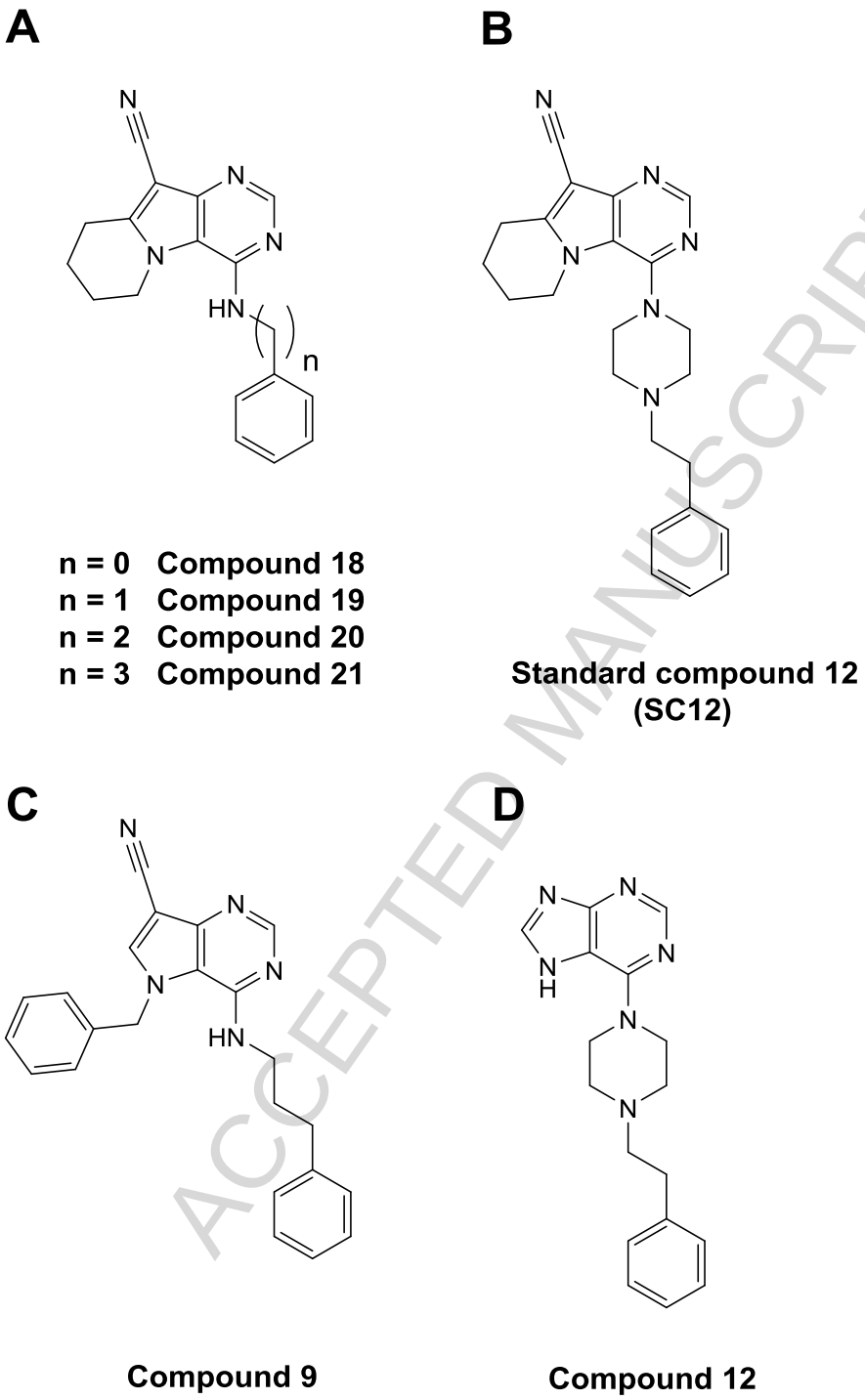


Figure 3. Depiction of already published and compounds presented in this study. While standard compound 12 (SC12, B) has been described before and was used as standard inhibitor [57], the inhibitors 18-21 (A) were found to have transport stimulating properties, too. Compounds **9** (C) and **12** (D) are representatives of

MRP1 transport stimulating compounds of the pyrrolopyrimidine and purine class, respectively.

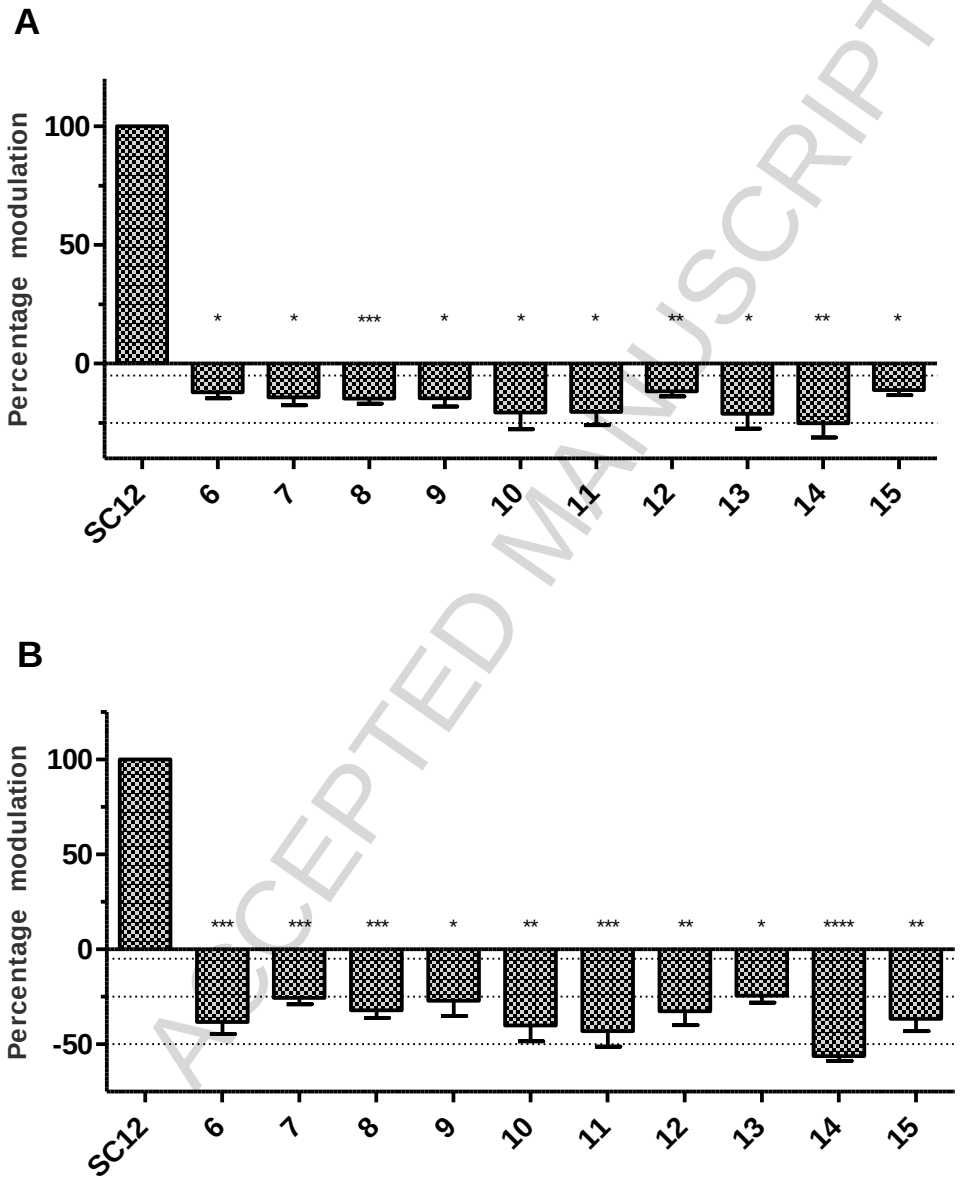


Figure 4: Activation of MRP1-mediated transport by compounds **6-15** at 10 nM. Basal transport velocity is defined as fluorescence increase without compound supplementation (0%). 100% is defined as full inhibition by SC12. Shown is mean ± SD of at least three independent experiments with duplicate measurements. A: Calcein accumulation assay using the MRP1 overexpressing cell line H69 AR. B: daunorubicin accumulation assay using the MRP1 overexpressing cell line MDCK II

MRP1. Significance was calculated using one sample t-tests and is given as *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$

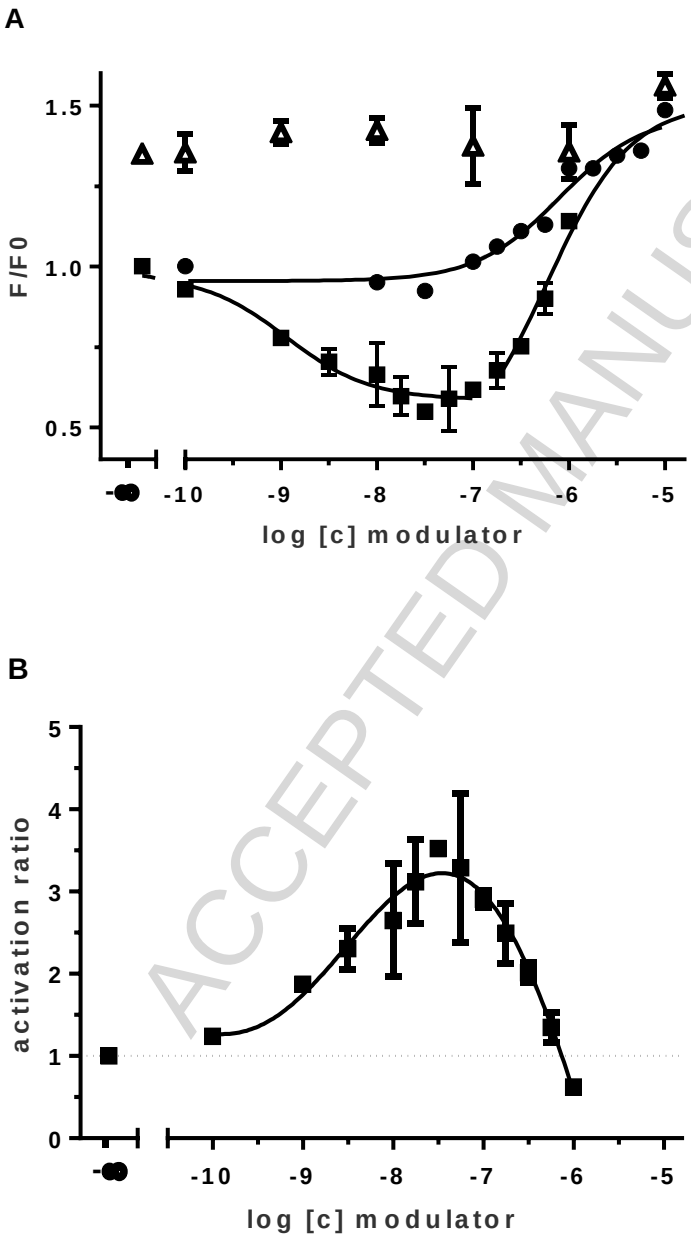


Figure 5: A: Depiction of compound **9** (closed squares) as activator of MRP1 in comparison to SC12 (closed circles) in the daunorubicin accumulation assay using the MRP1 overexpressing cell line MDCK II MRP1 and the sensitive counterpart MDCK II wild type (open triangle). Shown is a representative experiment out of three independent experiments with duplicate measurements. B: Calculation of activation

ratio derived from the dose-response curve of compound **9** out of the representative experiment in A.

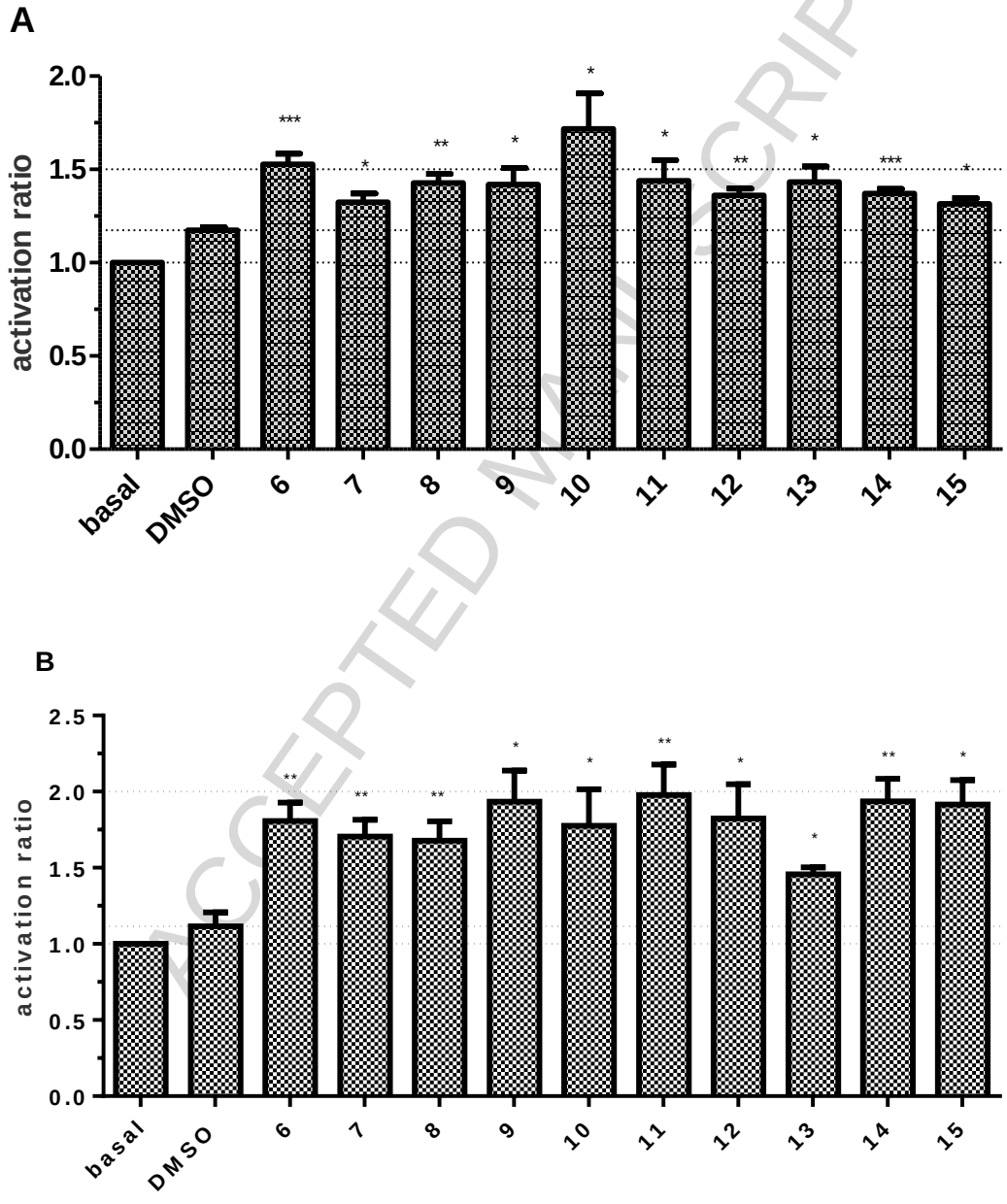


Figure 6: Acceleration of the MRP1 flux ratio of compounds **6-15** at 10 nM. Effects were compared to basal activity (1) and DMSO alone at the same concentration as it was used in the probes (1 µM). Shown is mean ± SD of at least three independent experiments with duplicate measurements. A: calcein accumulation assay using the

MRP1 overexpressing cell line H69 AR. B: daunorubicin accumulation assay using the MRP1 overexpressing cell line MDCK II MRP1. Significance was calculated using one sample t-tests and is given as *: p ≤ 0.05, **: p ≤ 0.01, ***: p ≤ 0.001, ****: p ≤ 0.0001, hypothetical value 1.17456 (DMSO, A) and 1.115 (DMSO, B).

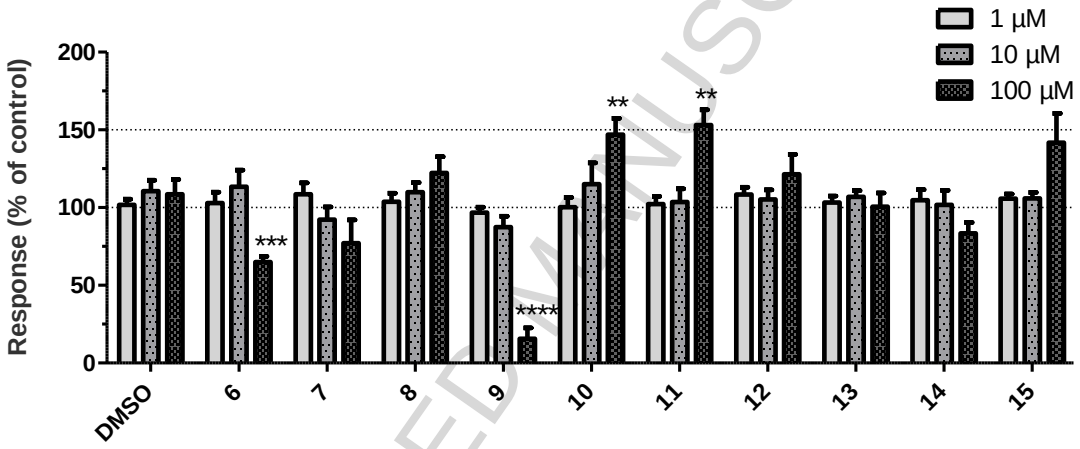
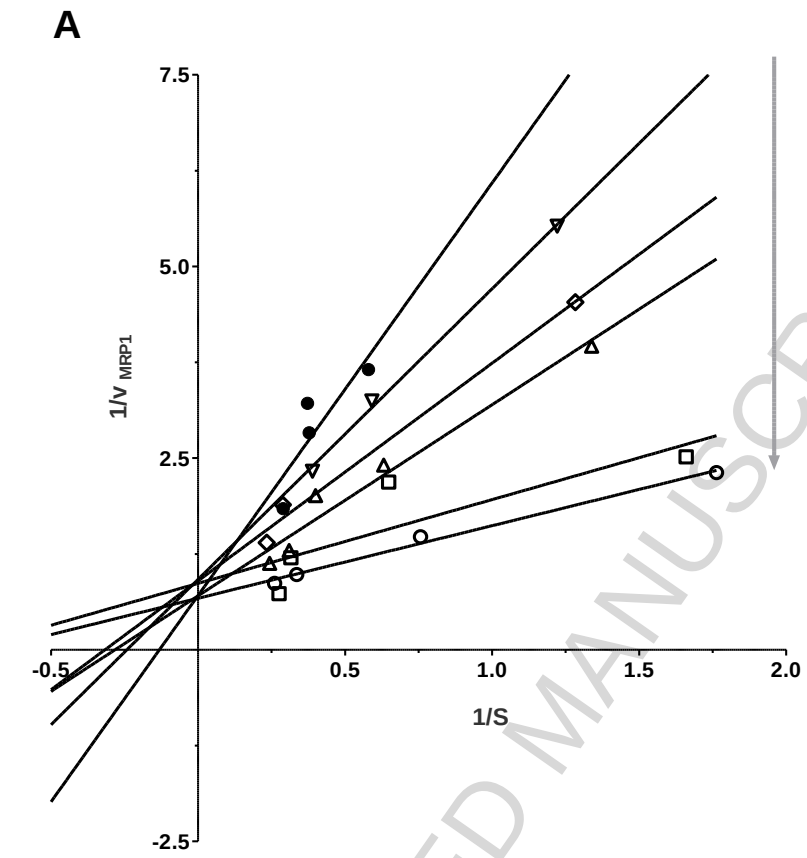
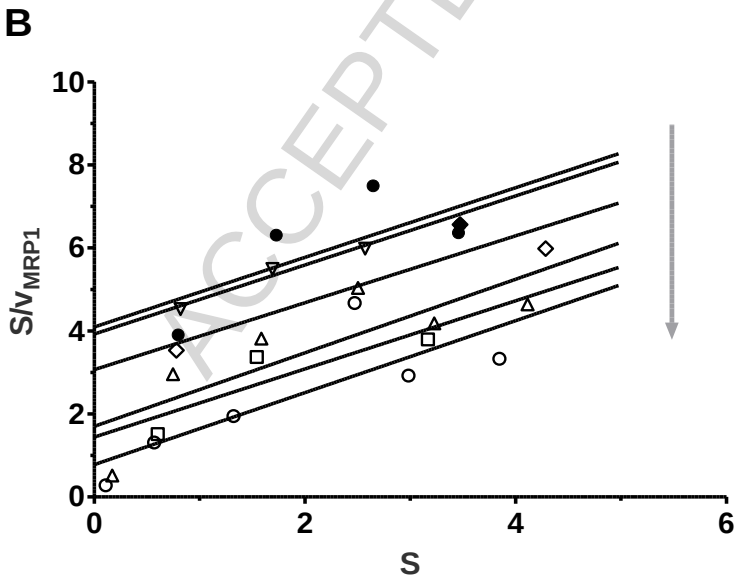


Figure 7: Summary of results of the MTT cytotoxicity assay using the MDCK II MRP1 overexpressing cell line. Compounds were used at concentrations of 1 µM, 10 µM and 100 µM. The DMSO reference represents the solvent content as present in the used probes at 1 µM, 10 µM and 100 µM. Cell culture medium was used as negative control, 10% DMSO as positive control, defining 100% and 0% cell viability, respectively. Shown is mean ± SD of at least 3 independent experiments with duplicate measurements. Significance was calculated using one sample t-tests and is given as *: p ≤ 0.05, **: p ≤ 0.01, ***: p ≤ 0.001, ****: p ≤ 0.0001. While compounds 6 and 9 significantly decreased cell viability at 100 µM, compounds 10 and 11 increased cell viability at this concentration.





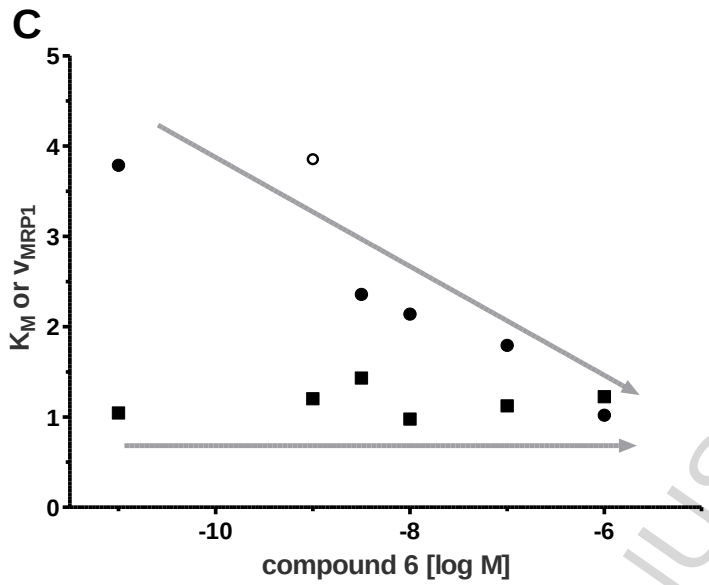


Figure 8: Kinetic data analysis of activity data obtained in the daunorubicin accumulation assay. (A): Lineweaver-Burk plot of compound **6**, which shows a competitive relation to daunorubicin as indicated by the point of intersection located on the Y-axis. The transport accelerating nature is shown by the decreasing slopes (grey arrow) of the lines: control (closed circle); Addition of compound **6**: downward triangle: 1.0 nM; rhombs: 3.2 nM; upward triangle: 10 nM; square: 100 nM; circle: 1 µM. (B): Hanes-Woolf plot. The parallel lines indicate a competitive relation. The transport accelerating feature is shown by the downward orientated lines (see grey arrow): control (closed circle); Addition of compound **6**: downward triangle: 1.0 nM; rhombs: 3.2 nM; upward triangle: 10 nM; square: 100 nM; circle: 1 µM. (C): Cornish-Bowden plot. While K_M decreases with increased daunorubicin concentrations, v_{MRP1} does not change. Closed circles: K_M; open circle: outlier; closed squares: v_{max}. Data was obtained using the MRP1 overexpressing cell line MDCK II MRP1. Shown is a representative experiment out of three independent experiments with duplicate measurements.

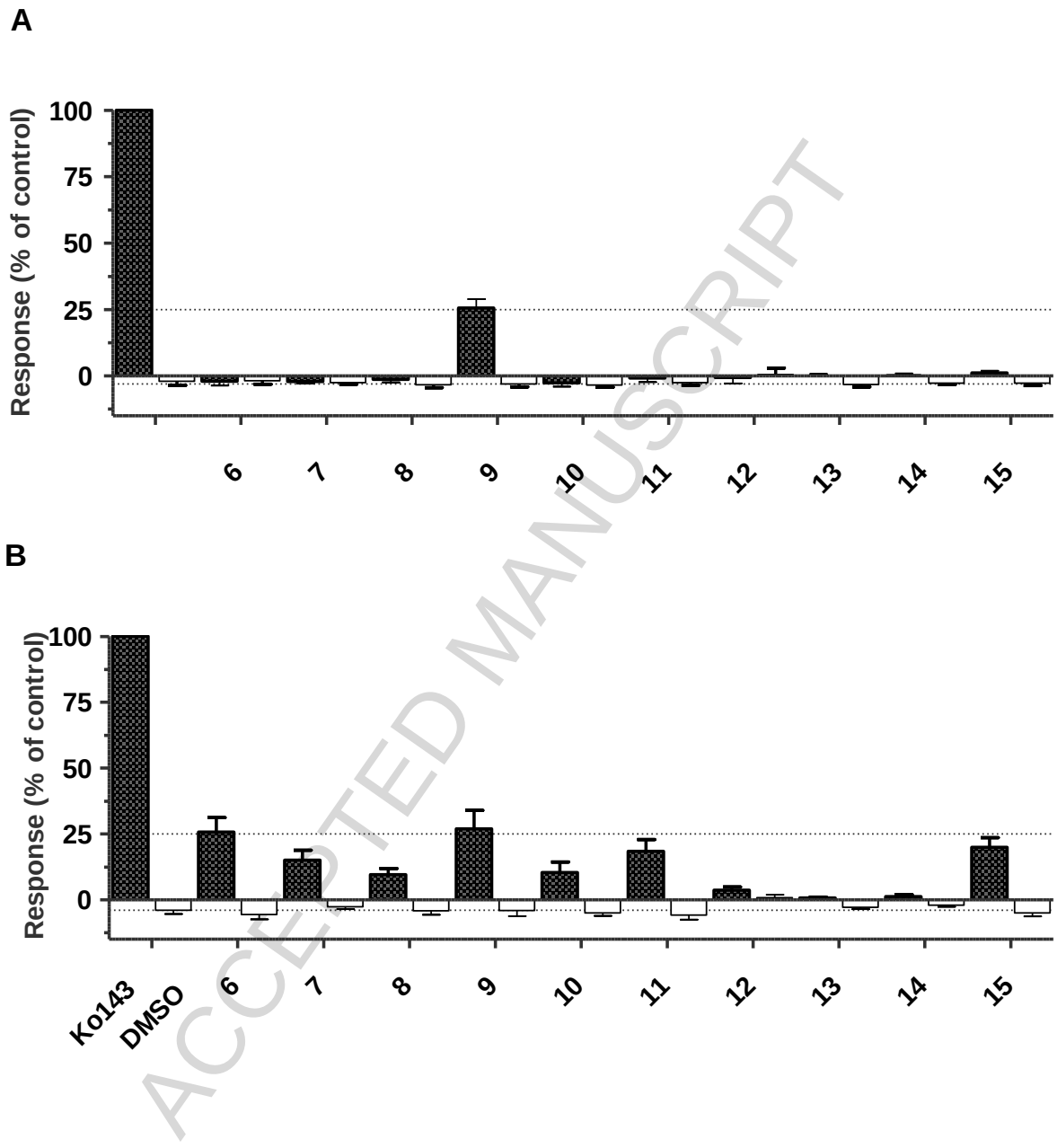
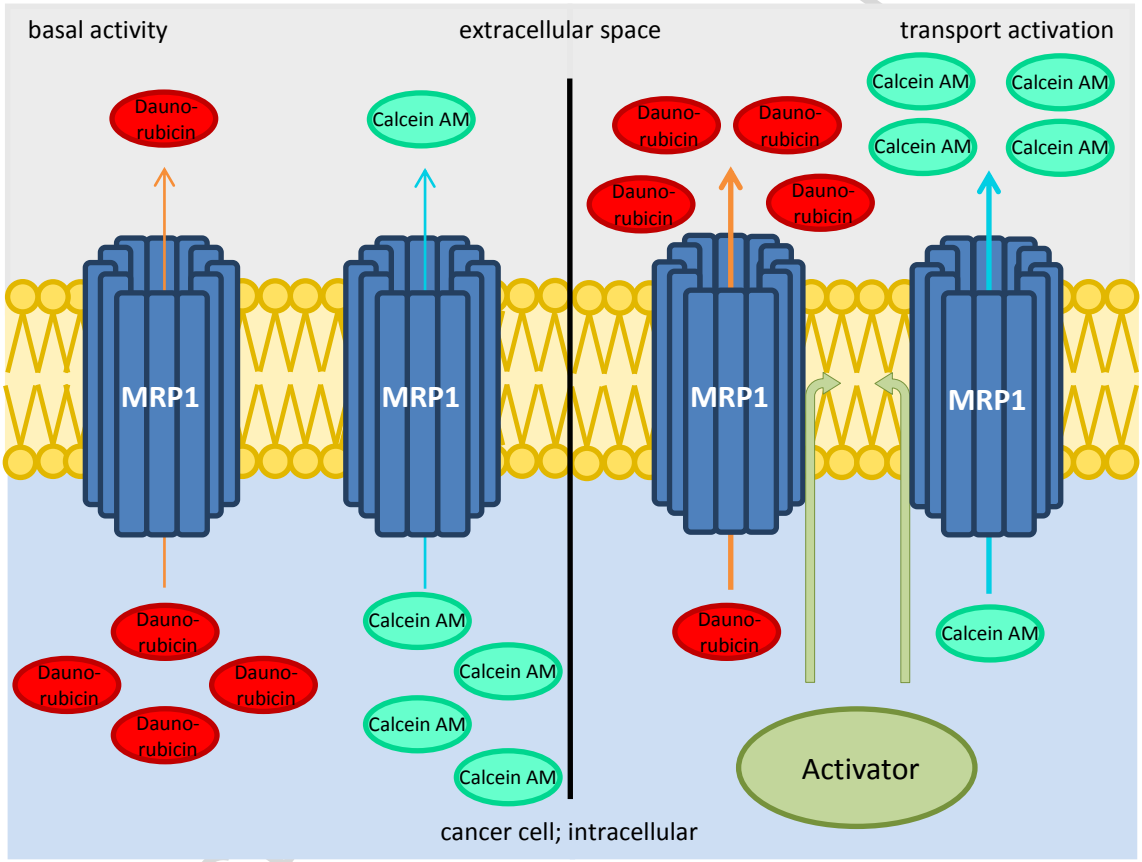


Figure 9: Summary of screening data. Shown are effects at 10 µM (dark grey) and at 10 nM (white) to analyze the transport inhibiting and activating properties in comparison to the standard inhibitor. Depicted is mean ± SD of at least 3 independent experiments with duplicate measurements. A: P-gp screening data obtained in the calcein accumulation assay using A2780 adr cells and cyclosporine A as standard inhibitor at 10 µM. Compound **9** has an inhibition level of 26% and an IC_{50} of 14.0 µM ± 0.3 µM. Data obtained using the P-gp overexpressing cell line

A2780 adr. B: BCRP screening data obtained in the pheophorbide A assay using transfected MDCK-II BCRP cells and Ko143 as standard inhibitor at 10 µM. DMSO was used at the same concentration as supplemented within the 10 nM probes of activators. Compound **6** has an inhibition level of 26% and IC_{50} of 20.6 µM ± 1.4 µM. Compound **9** has an inhibition level of 27% and an IC_{50} value of 20.4 µM ± 1.6 µM. Significance was calculated using one sample t-tests and is given as *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$. Hypothetical value -1.98 (DMSO, A) and -3.95 (DMSO, B).



Graphical abstract