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NOTE

Selective inhibition of P-gp transporter by goniothalamin derivatives sensitizes resistant cancer cells to chemotherapy

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Abstract

Overexpression of efflux transporters of the ATP-binding cassette (ABC) transporter family, primarily P-glycoprotein (P-gp), is a frequent cause of multidrug resistance in cancer and leads to failure of current chemotherapies. Thus, identification of selective P-gp inhibitors might provide a basis for the development of novel anticancer drug candidates. The natural product goniothalamin and 21 derivatives were characterized regarding their ability to inhibit ABC transporter function. Among the goniothalamins, selective inhibitors of P-gp were discovered. The two most potent inhibitors (*R*)-3 and (*S*)-3 displayed the ability to increase intracellular accumulation of doxorubicin, thereby sensitizing P-gp-overexpressing tumor cells to chemotherapy by decreasing doxorubicin IC_{50} value up to 15-fold. Molecular docking studies indicated these compounds to inhibit P-gp by acting as transporter substrates. In conclusion, our findings revealed a novel role of goniothalamin derivatives in reversing P-gp-mediated chemotherapy resistance.

Keywords Goniothalamin · Multidrug resistance · P-glycoprotein · Cancer chemotherapy · Resistance reversal

Abbreviations

ABC	ATP-binding cassette
BCRP	Breast cancer resistance protein
DMSO	Dimethylsulfoxide
MDR	Multidrug resistance
MRP1	Multidrug resistance-associated protein 1
P-gp	P-glycoprotein
SAR	Structure–activity relationship

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Introduction

Despite emerging progress in the development of novel anticancer therapies, multidrug resistance (MDR) remains a major challenge in tumor treatment [1, 2]. MDR describes a multifactorial phenomenon against a variety of marketed anticancer drugs [3]. The most common mechanisms of MDR encompass altered drug metabolism through the expression of phase II conjugating enzymes, including glutathione transferase P1 [4], or increased expression of efflux pumps of the ATP-binding cassette (ABC) transporter superfamily in the tumor, thereby extruding chemotherapeutic drugs [1]. To date, three ABC transporters have been most extensively studied [5]—P-glycoprotein (P-gp, ABCB1), multidrug resistance-associated protein 1 (MRP1, ABCC1) and breast cancer resistance protein (BCRP, ABCG2). P-gp, the best characterized transporter mediating MDR, is normally expressed in liver transport epithelium, the gastrointestinal tract and in the blood–brain barrier [6]. Among the ABC transporters, P-gp demonstrates the strongest

resistance to a variety of chemotherapeutics including anthracyclines, taxanes or tyrosine kinase inhibitors [7]. Besides P-gp, MRP1 and BCRP are in the focus of current research as mediators of chemotherapeutic resistance [8, 9].

The styryl-lactone goniothalamin can be found in species of the *Goniothalamus* genus, which is endemic in Southeast Asia [10–12]. Over past decades, the pharmacological effects of goniothalamin have been studied against pathogens such as *Trypanosoma* and *Plasmodium* and in various tumors. Amongst its anti-cancer activities, inhibition of inflammation [13], delay of tumor progression [14, 15], induction of apoptosis and cell cycle arrest [16, 17] were described. Interestingly, to date, goniothalamin has not been characterized regarding ABC transporter inhibition.

In the current study, we analyzed the cytotoxic efficacy of the natural occurring goniothalamin (*R*)-1 and its enantiomer (*S*)-1, as well as 21 derivatives (Fig. 1) [18] on a panel encompassing sensitive and corresponding resistant human cancer cells. Moreover, their inhibitory activity towards the ABC transporters P-gp, MRP1 and BCRP was characterized for the first time.

Experimental

Test compounds and chemicals

The chemical syntheses of goniothalamins were described previously [18]. Test compounds were dissolved in dimethyl sulfoxide (DMSO; Carl Roth, Karlsruhe, Germany). Calcein-AM, Hoechst 33342, PSC833, Ko143, MK-571 and doxorubicin were purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in DMSO, except for Hoechst 33342, which was dissolved in water. Hank's balanced salt solution was purchased from Biochrom (Berlin, Germany); hepes buffer was purchased from Lonza (Basel, Switzerland). Cell culture media and supplements were purchased from Thermo Fisher Scientific (Waltham, MA, USA), except for human recombinant insulin (Merck, Darmstadt, Germany).

	Compound	Structure	R
1	(*R*)-1	(*R*)-I	A
2	(*S*)-1	(*S*)-I	A
3	(*R*)-23c	(*R*)-II	A
4	(*S*)-23c	(*S*)-II	A
5	(*R*)-2	(*R*)-I	B
6	(*S*)-2	(*S*)-I	B
7	(*R*)-25	(*R*)-I	C
8	(*S*)-25	(*S*)-I	C
9	(*R*)-3	(*R*)-I	D
10	(*S*)-3	(*S*)-I	D
11	(*R*)-23e	(*R*)-II	D
12	(*S*)-23e	(*S*)-II	D
13	(*R*)-24	(*R*)-I	E
14	(*S*)-24	(*S*)-I	E
15	(*R*)-23a	(*R*)-II	E
16	(*S*)-23a	(*S*)-II	E
17	(*R,S,S*)-4	(*R*)-I	F
18	(*R,R,R*)-4	(*R*)-I	G
19	(*S,S,S*)-4	(*S*)-I	F
20	(*R,R,R*)-26	(*R*)-II	G
21	(*R,R*)-27	(*R*)-I	H
22	(*S,S,S*)-26	(*R*)-II	F
23	(*R,S,S*)-26	(*S*)-II	G

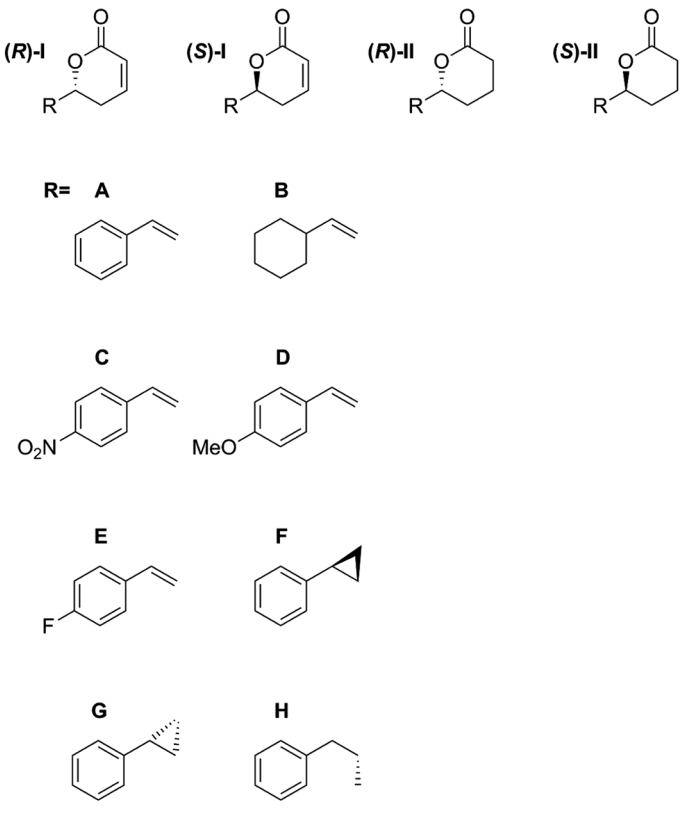


Fig. 1 Structures of natural compound goniothalamin (*R*)-1 and derivatives

Cell culture

P-gp-expressing HCT-15 colon adenocarcinoma cells (German Collection of Microorganisms and Cell Cultures [DSMZ], Braunschweig, Germany) were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin. For ABC transporter expression analyses see Supplementary Table S2. The multidrug-resistant MRP1-expressing small cell lung cancer cell line H69AR (American Type Culture Collection, Manassas, VA, USA) was cultured in RPMI 1640 (ATCC modification) containing 20% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. Multidrug-resistant BCRP-expressing MCF-7/MX breast cancer cells (generous gift from Dr. Erasmus Schneider, Wadsworth Center, New York State Department of Health, Albany, NY, USA) were cultured in DMEM containing 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin.

Cells were maintained in a humidified atmosphere at 37 °C and 5% CO_2 and subcultured at 80–90% confluency.

Cell viability assessment

To analyze the ability of identified inhibitors of P-gp to sensitize P-gp-expressing cancer cells to chemotherapy treatment, HCT-15 cells (3×10^3 cells/well) were either incubated with a serial dilution of doxorubicin alone or in combination with fixed concentrations of the test compounds. After 48 h, cell viability was determined with the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA) as described previously [18]. Cell seeding and addition of compounds were conducted with the CyBi-Well 96-channel simultaneous pipettor (Analytik Jena AG, Jena, Germany). Fold change of doxorubicin IC_{50} was calculated with the formula IC_{50}(doxorubicin)/IC_{50}(doxorubicin with modulator).

P-gp transport assay

Influence on the transport activity of P-gp was analyzed in P-gp-expressing HCT-15 cells by monitoring the intracellular accumulation of calcein.

Cells (5×10^4 cells/well) were seeded in black 96-well plates and incubated at 37 °C and 5% CO_2. After 24 h, the culture medium was replaced by Hank's balanced salt solution containing 10 mM hepes. The cells were pre-incubated with the test compounds, positive control (2.5 µM PSC833) or negative control (0.5% DMSO) in triplicate for 30 min and afterwards the P-gp substrate calcein-AM was added (0.5 µM). The fluorescence of calcein (excitation 485 nm, emission 520 nm) was measured over a period of 3 h with the Infinite M1000 pro microplate reader (Tecan Group AG, Männedorf, Switzerland) at 37 °C.

Inhibition of transport activity was analyzed by determining the slope of the fluorescence-time curve (0–30 min) using linear regression. Data were normalized to PSC833 (100% inhibition) and DMSO (0% inhibition).

MRP1 transport assay

As calcein-AM is also a substrate of MRP1, its transport was determined in MRP1-expressing H69AR cells (7.5×10^4 cells/well) using 20 µM MK-571 as a positive control.

Inhibition of transport activity was analyzed by determining the slope of the fluorescence-time curve (0–20 min) using linear regression. Data were normalized to MK-571 (100% inhibition) and DMSO (0% inhibition).

BCRP transport assay

Activity of the BCRP transporter was analyzed in BCRP-expressing MCF-7/MX cells (5×10^4 cells/well). Ko143 (1 µM) was used as a positive control and Hoechst 33342 (1 µM) as fluorescent BCRP substrate (excitation 355 nm, emission 460 nm).

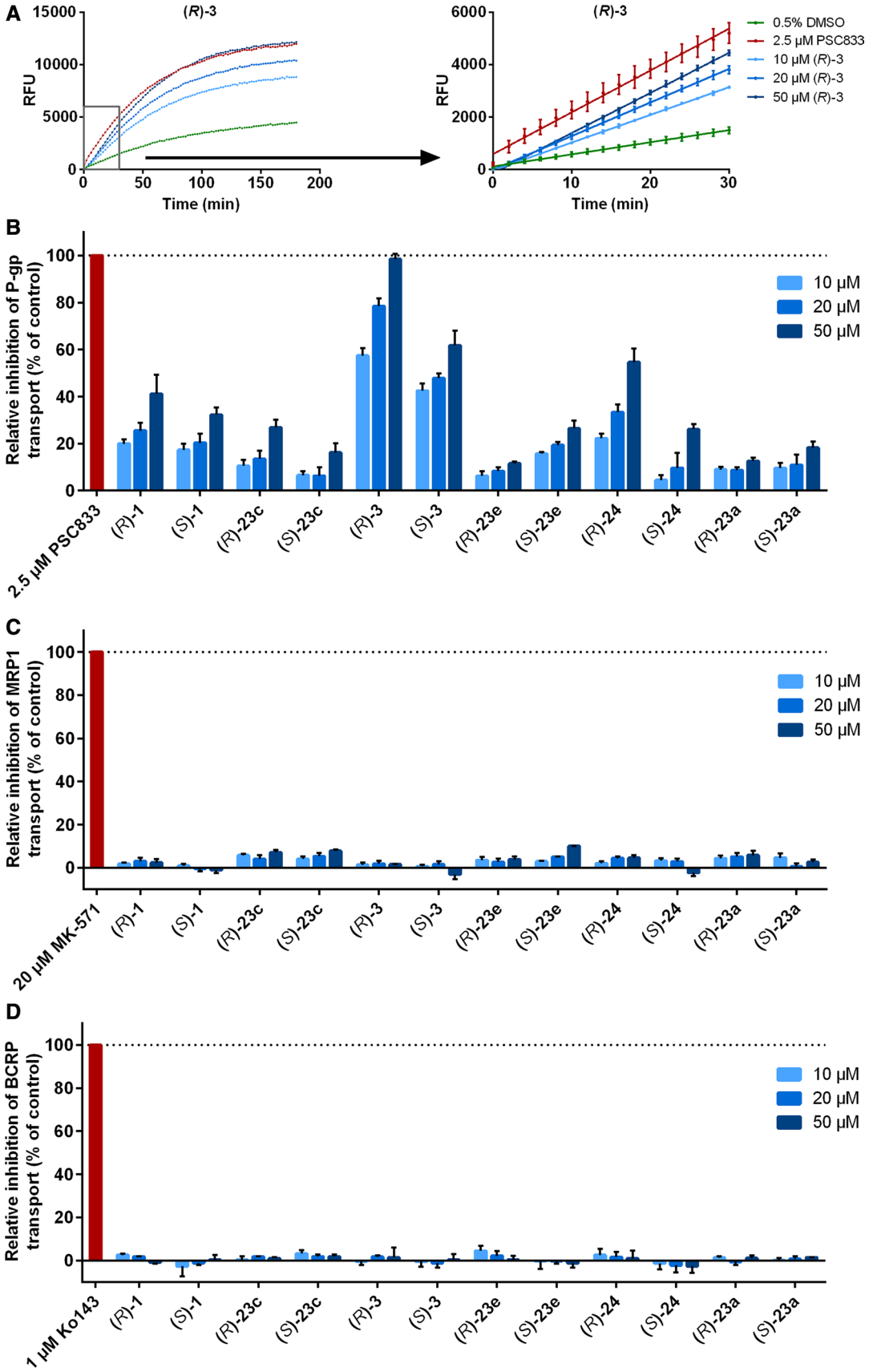
Inhibition of transport activity was analyzed by determining the plateau of the fluorescence-time curve using non-linear regression (one-phase exponential fit). Data were normalized to Ko143 (100% inhibition) and DMSO (0% inhibition).

Flow cytometry

Accumulation of doxorubicin in P-gp-expressing HCT-15 cells was analyzed by flow cytometry. Twenty-four hours after cell seeding, test compounds and controls were incubated for 30 min at 37 °C. PSC833 (2.5 µM) and DMSO (0.5%) were used as positive or negative controls. Afterwards, doxorubicin was added to a final concentration of 10 µM. After 3 h, the cells were collected and washed twice with ice-cold PBS. Doxorubicin fluorescence was measured with the CyFlow Cube 8 (Sysmex Corporation, Kobe, Japan) equipped with a 590/50 bandpass filter. Data were analyzed using FlowJo 10.3 (FlowJo LLC, Ashland, OR, USA). Live cells were gated based on forward/sideward scatter.

Molecular docking

Selected compounds were evaluated in terms of their docking pose and binding energy with AutoDock 4 [19] on the homology model of human P-gp prepared as described previously [20] by using mouse P-gp (PDB ID: 4M1 M) as the template structure. PSC833 was used as a positive control. For blind docking calculations, the whole protein


A
(R)-3
RFU
Time (min)
(R)-3
0.5% DMSO
2.5 μM PSC833
10 μM (R)-3
20 μM (R)-3
50 μM (R)-3
B
Relative inhibition of P-gp transport (% of control)
10 μM
20 μM
50 μM
2.5 μM PSC833
(R)-1
(S)-1
(R)-23c
(S)-23c
(R)-3
(S)-3
(R)-23e
(S)-23e
(R)-24
(S)-24
(R)-23a
(S)-23a
C
Relative inhibition of MRP1 transport (% of control)
20 μM MK-571
D
Relative inhibition of BCRP transport (% of control)
1 μM Ko143

◂**Fig. 2** Influence of goniothalamins on transport activity of ABC transporters. P-gp transport activity was analyzed in HCT-15 cells (A, B). Intracellular accumulation of the P-gp substrate calcein-AM was measured (**a**, left). For quantification, the slope of the linear part of the fluorescence-time curve (grey rectangle) was determined (**a**, right). Curves represent one exemplary measurement of the compound (*R*)-3. Data were normalized to PSC833 (100% inhibition) and DMSO (0% inhibition, **b**). MRP1 transport activity was analyzed in H69AR cells using calcein-AM and MK-571 as a positive control (**c**). BCRP transport activity was analyzed in MCF-7/MX cells using Hoechst 33342 and Ko143 as a positive control (**d**). Bars indicate the mean ± SEM of at least three independent experiments performed in triplicates

surface was covered and for defined docking, residues at the drug-binding pocket were covered [21]. Three independent docking calculations were conducted with 2,500,000 energy evaluations and 250 runs using Lamarckian Genetic Algorithm. Lowest binding energies and predicted inhibition constants were obtained from the docking log files. For visualization of the docking results, Visual Molecular Dynamics (VMD) was used. VMD software was developed with NIH support by the Theoretical and Computational Biophysics group at the Beckman Institute, University of Illinois, USA at Urbana-Champaign.

Data analysis and statistics

All experiments were performed in at least three independent replicates. GraphPad Prism v. 6.07 (GraphPad Software Inc., La Jolla, CA, USA) was used for data analyses. Results are presented as the mean ± SEM. For statistical analysis comparing different groups, one-way ANOVA followed by Dunnett's multiple comparisons test was performed. $P < 0.05$ was chosen to define a statistically significant difference.

Results and discussion

The cytotoxicity of natural occurring goniothalamin (*R*)-1 and 22 derivatives was analyzed in a panel of sensitive and multidrug-resistant cancer cell lines (Supplementary Table S1). Candidates with improved cytotoxic activity compared to goniothalamin (*R*)-1 on most cell lines investigated could be revealed and the structure–activity relationship (SAR) from our previous study could be confirmed [18].

To identify tumor cell lines with selectively high expression of P-gp, MRP1 or BCRP for further ABC transporter studies, a systematic screening of transporter expression by qPCR was conducted. Levels of respective mRNA in the cell lines were quantified in relation to the reference gene GAPDH (Supplementary Table S2). Based on our in-depth comparative analysis, the cell lines HCT-15, H69AR and MCF-7/MX were selected for further evaluation of the inhibitory effects of goniothalamin and derivatives on transporter activities as these cell lines showed distinct expression of either P-gp, MRP1 or BCRP, respectively (Supplementary Table S2).

The influence of the natural product goniothalamin on the transport activity of ABC transporters has not been described previously. To analyze influence on P-gp activity, intracellular calcein accumulation was determined (Fig. 2a). The slope of the linear part of the fluorescence-time curve and the relative inhibition rates were calculated by normalizing data of the tested compounds to the positive control PSC833 (100% inhibition) and the negative control DMSO (0% inhibition). Each compound was analyzed at three different concentrations (10, 20 and 50 µM) to detect dose-dependent changes in P-gp transport activity.

Goniothalamin (*R*)-1 dose-dependently inhibited P-gp transport activity (Fig. 2b). Its relative inhibition compared to the positive control PSC833 was between 19.9 ± 1.9% at 10 µM and 41.1 ± 8.2% at 50 µM (mean ± SEM). Interestingly, two non-cytotoxic derivatives with significantly improved P-gp inhibition compared to the natural product could be identified—(*R*)-3 and (*S*)-3. Highest inhibition rates were achieved by compound (*R*)-3 with relative values between 57.4 ± 3.1% (10 µM) and 98.5 ± 2.3% (50 µM). These rates were 2.4- to 3-fold higher compared to the natural product (*R*)-1. Inhibition of P-gp by (*S*)-3 was lower compared to the (*R*)-enantiomer with rates between 42.5 ± 3.1% at 10 µM and 61.8 ± 6.2% at 50 µM. Noteworthy, derivatives (*R*)-23e and (*S*)-23e with saturated lactone moiety, displayed significantly lower inhibition rates compared to the unsaturated compounds. The differences were around nine-fold between compounds (*R*)-3 and (*R*)-23e and around 2.5-fold between compounds (*S*)-3 and (S)-23e. Compound (*R*)-24 inhibited P-gp with similar rates compared to natural goniothalamin (*R*)-1. Relative inhibition varied between 22.2 ± 2.1% (10 µM) and 54.6 ± 5.8% (50 µM). Here, the (*S*)-enantiomer displayed lower inhibitory activity between 4.5 ± 2.1% (10 µM) and 26.1 ± 2.2% (50 µM). Compound (*R*)-23a with saturated lactone moiety exhibited significantly weaker inhibition of P-gp than compound (*R*)-24, while the inhibition of compound (*S*)-23e was comparable to compound (*S*)-24. With regard to the transport activity of MRP1 in H69AR cells and BCRP in MCF-7/MX cells, no modulation by the tested goniothalamins could be observed (Fig. 2c, d), proving selectivity for the inhibition capacity of goniothalamins towards P-gp.

Furthermore, intracellular accumulation of the chemotherapeutic drug doxorubicin, a proven P-gp substrate, was analyzed by flow cytometry. HCT-15 cells were incubated with different concentrations of the newly identified inhibitors, derivatives (*R*)-3 and (*S*)-3, as well as the structurally corresponding goniothalamins with saturated lactone moiety (*R*)-23e and (*S*)-23e, exhibiting significantly weaker inhibition of P-gp in the transporter screening assay (Fig. 2b).

PSC833 (2.5 µM) served as a positive control. Incubation with compounds (*R*)-3 and (*S*)-3 led to a dose-dependent increase in doxorubicin fluorescence intensity compared to cells treated with DMSO as a negative control (Fig. 3a, b). In line with the transporter inhibition data, compound (*R*)-3 showed stronger accumulation of doxorubicin in HCT-15 cells compared to compound (*S*)-3. In contrast, after incubation with derivatives (*R*)-23e and (*S*)-23e, doxorubicin accumulation in HCT-15 cells was not significantly different from DMSO-treated cells (Fig. 3c, d).

The goniothalamins (*R*)-3 and (*S*)-3 were identified as the most efficacious inhibitors of P-gp (Fig. 2). Doxorubicin, a clinically established chemotherapeutic drug represents a well-known P-gp substrate. Cytotoxicity of doxorubicin alone or in combination with different concentrations of selected goniothalamin derivatives was determined in HCT-15 cells selectively expressing P-gp (Supplementary Table S2). The IC_{50} value of doxorubicin on HCT-15 cells was 7.4 ± 0.8 µM (mean ± SEM; Table 1). However, co-treatment with goniothalamins (*R*)-3 and (*S*)-3 led to a dose-dependent decrease in IC_{50} (Fig. 4b, c; Table 1). The combination of doxorubicin with compound (*R*)-3 resulted in increased sensitivity towards doxorubicin treatment reflected by IC_{50} values between 2.23 ± 0.12 µM (10 µM (*R*)-3) and 0.47 ± 0.06 µM (50 µM (*R*)-3). In comparison to the treatment with doxorubicin alone, the shifts corresponded to a reduction of 3.3-fold (for 10 µM) and 15.7-fold (for 50 µM), respectively. The addition of the

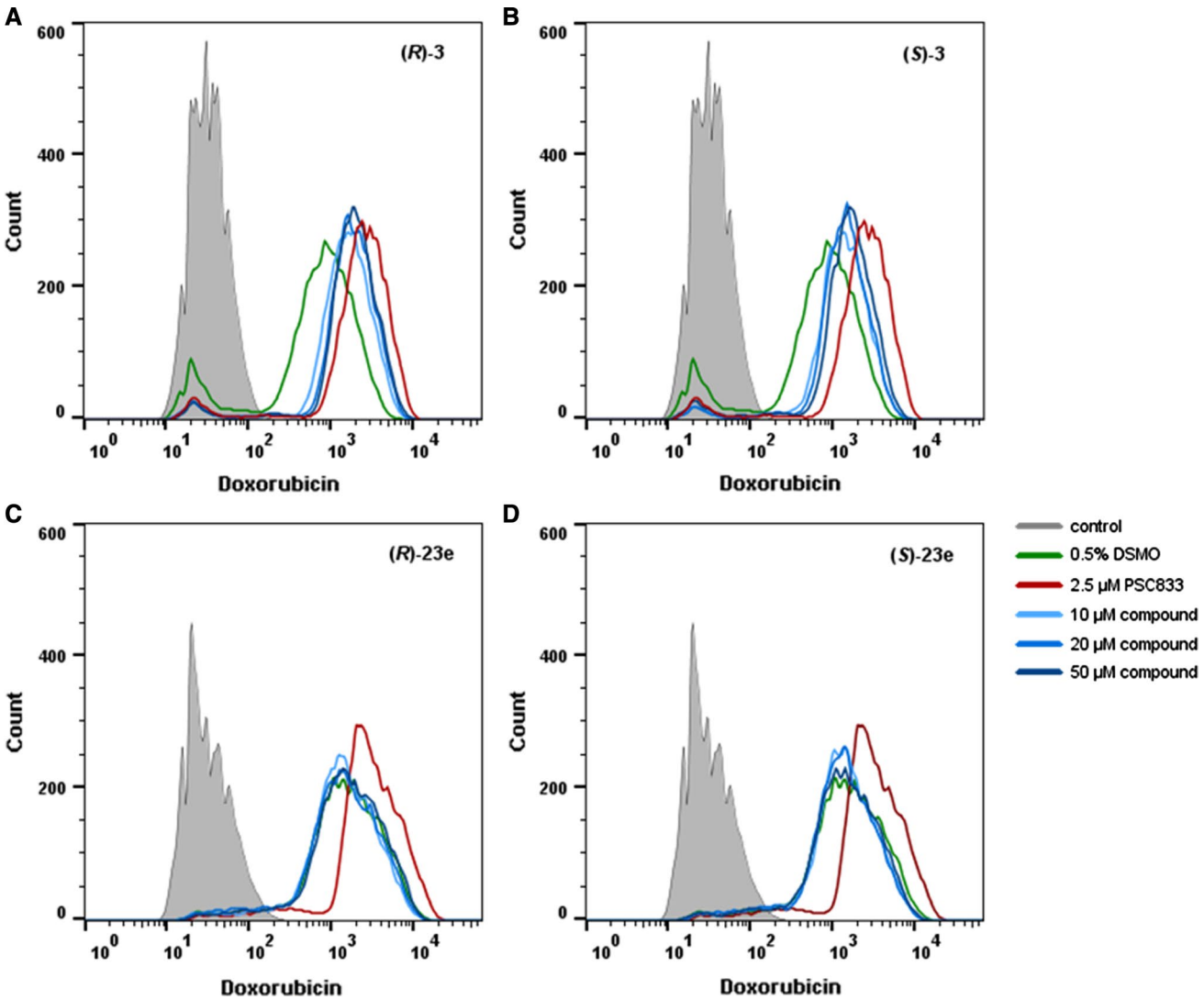


Fig. 3 Influence on intracellular doxorubicin accumulation. HCT-15 cells were treated with different concentrations of goniothalamins (*R*)-3 (**a**) (*S*)-3 (**b**) (*R*)-23e (**c**) or (*S*)-23e (**d**) and subsequently, doxorubicin. Intracellular doxorubicin fluorescence was analyzed by flow cytometry. PSC833 and DMSO served as positive or negative controls, respectively. Histograms are exemplary for three independent experiments

Table 1 Effects of selected goniothalamins on the sensitivity of HCT-15 cells to doxorubicin

Compound	IC_{50} (μM)	*P* values	Fold change
Doxorubicin	7.37 ± 0.83	–	1.00
+ 2.5 μM PSC833	0.36 ± 0.04****	< 0.0001	20.47
+ 10 μM (*R*)-3	2.23 ± 0.12**	0.0024	3.30
+ 20 μM (*R*)-3	1.63 ± 0.21***	0.0001	4.52
+ 50 μM (*R*)-3	0.47 ± 0.06****	< 0.0001	15.68
+ 10 μM (*S*)-3	2.79 ± 0.21**	0.0087	2.64
+ 20 μM (*S*)-3	2.64 ± 0.49**	0.0019	2.79
+ 50 μM (*S*)-3	1.73 ± 0.47***	0.0007	4.26
+ 10 μM (*R*)-23e	9.90 ± 1.04	0.3382	0.74
+ 20 μM (*R*)-23e	9.51 ± 1.60	0.5376	0.77
+ 50 μM (*R*)-23e	7.71 ± 1.87	0.9996	0.96
+ 10 μM (*S*)-23e	9.37 ± 1.79	0.6181	0.78
+ 20 μM (*S*)-23e	9.63 ± 1.62	0.4692	0.77
+ 50 μM (*S*)-23e	8.66 ± 1.62	0.9509	0.85

PSC833 was used as a positive control. Fold change of cytotoxicity was calculated by IC_{50}(doxorubicin)/IC_{50}(doxorubicin with modulator). Data represent the mean ± SEM of at least three independent replicates performed in quadruplicate

**$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$ compared to doxorubicin alone

control PSC833 resulted in a 20.5-fold decrease to an IC_{50} of 0.36 ± 0.04 μM (Fig. 4a).

The combination of doxorubicin with the weaker P-gp inhibitor compound (*S*)-3 reduced the doxorubicin IC_{50} value of 2.6- to 4.3-fold to 2.79 ± 0.21 μM (10 μM (*S*)-3) and 1.73 ± 0.47 μM (50 μM (*S*)-3) (Fig. 4c; Table 1). Treatment of the cells with the respective compounds alone had no effect on cell viability. Moreover, the combination of doxorubicin and goniothalamin derivatives displayed only minor P-gp-inhibiting activity, while compounds (*R*)-23e or (*S*)-23e had no significant influence on the cytotoxicity of doxorubicin in HCT-15 cells (Fig. 4d, e; Table 1).

Molecular docking of the P-gp-inhibiting goniothalamins (*R*)-3 and (*S*)-3 as well as the weak inhibitory goniothalamins (*R*)-23e and (*S*)-23e on the homology model of human P-gp was performed to gain insights into the possible binding mode.

Blind docking covering the whole protein surface revealed that the P-gp-inhibiting goniothalamin derivatives were able to interact with P-gp more strongly than PSC833. Unlike PSC833, the derivatives (*R*)-3 and (*S*)-3 bound in close proximity of the drug-binding pocket (Fig. 5a). Furthermore, compounds (*R*)-3 and (*S*)-3 bound slightly stronger than compounds (*R*)-23e and (*S*)-23e with lower predicted inhibition constants, confirming the P-gp inhibition data

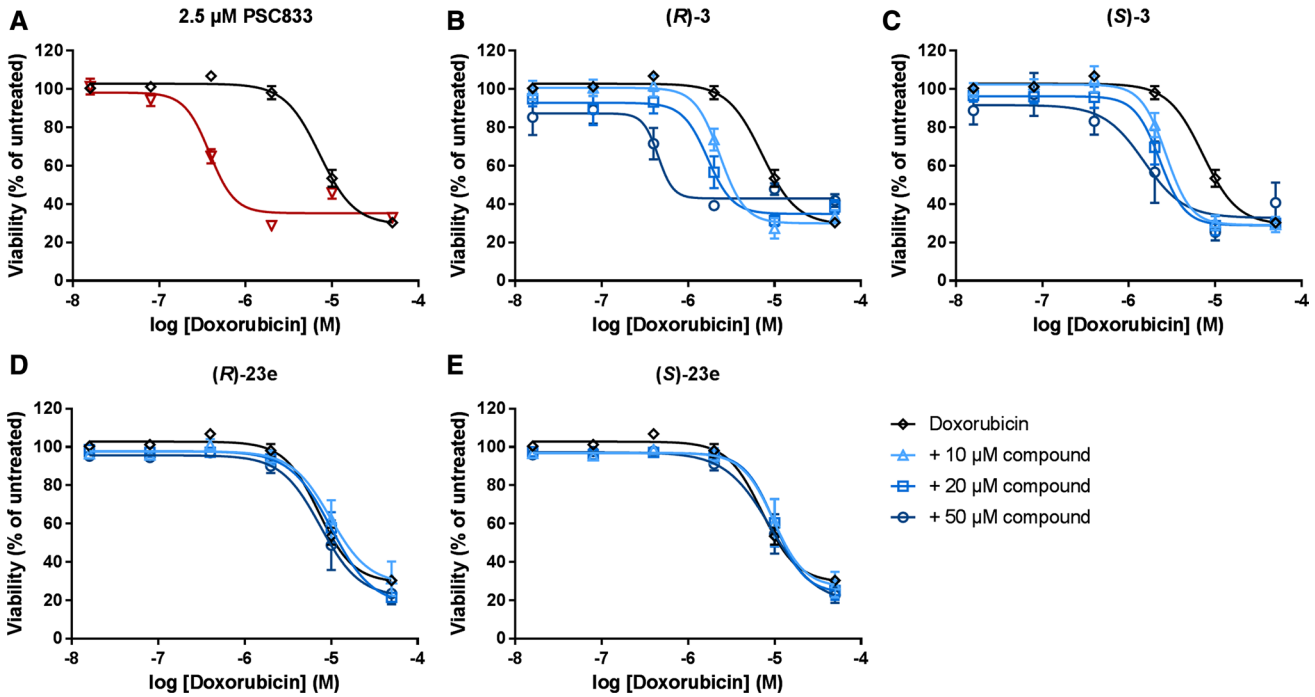


Fig. 4 Sensitization of HCT-15 cells to doxorubicin treatment (**a**–**e**). HCT-15 cells were incubated with either doxorubicin alone (◇), doxorubicin combined with 2.5 μM PSC833 (positive control; **a**), or doxorubicin in combination with 10 μM (△), 20 μM (□) or 50 μM (○) of the goniothalamins (*R*)-3 (**b**) (*S*)-3 (**c**), (*R*)-23e (**d**) and (*S*)-23e (**e**), respectively. Viability was determined after 48 h. Graphs represent the mean values ± SEM normalized to untreated cells of at least three independent experiments performed in quadruplicate

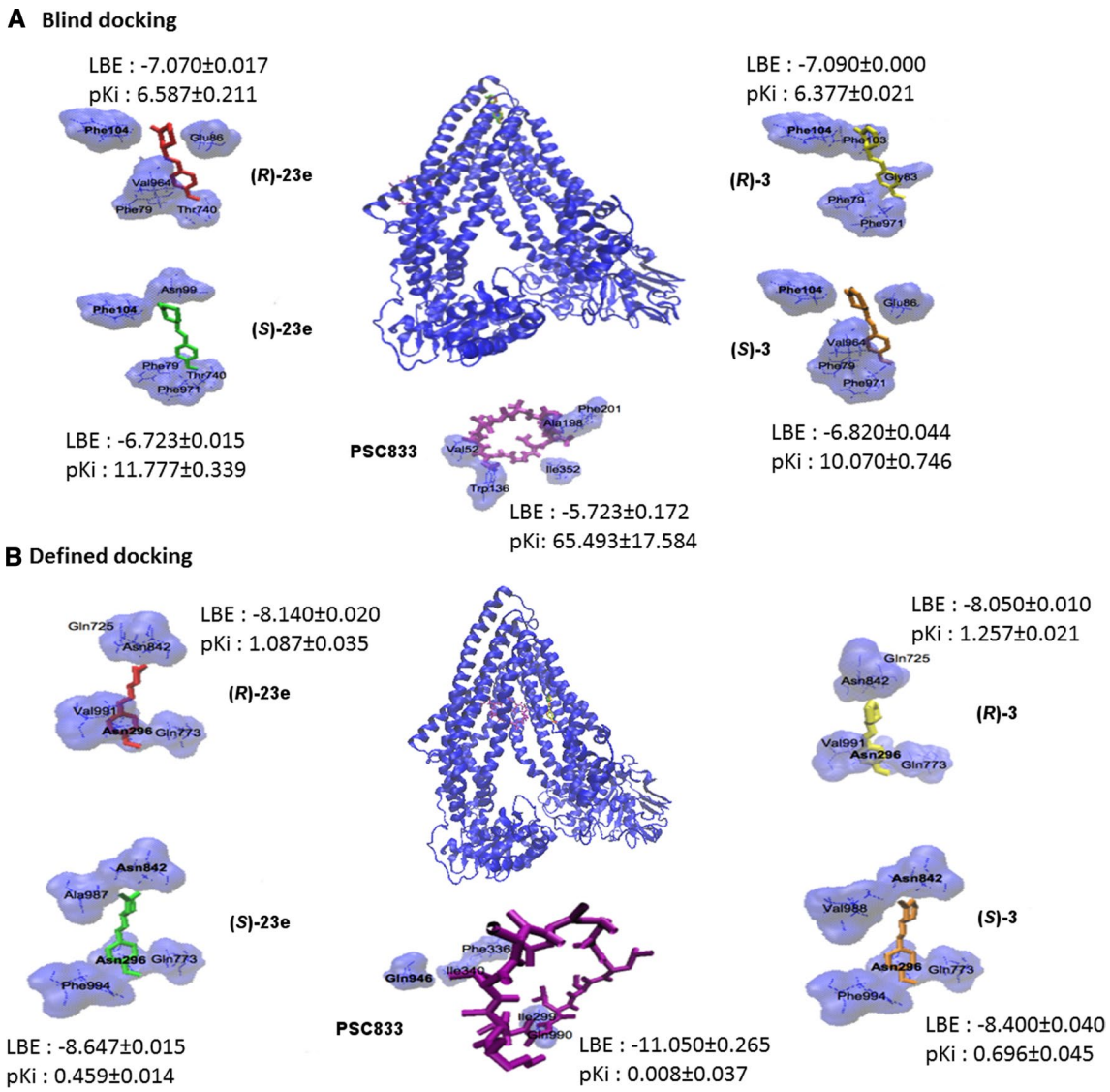


Fig. 5 Molecular docking of goniothalamins on homology-modeled human P-gp. Goniothalamins (*R*)-3, (*S*)-3, (*R*)-23e, (*S*)-23e and PSC833 were docked on the whole protein surface (blind docking, **a**) or the drug-binding site (defined docking, **b**). Lowest binding energies in kcal/mol (LBE) and predicted inhibition constant in μM (pKi) were determined as well as the interacting amino acids. Data represent the mean ± SD of three independent dockings with 2,500,000 energy evaluations and 250 runs each

Table 2 Molecular docking of selected goniothalamins on the whole protein surface of homology-modeled human P-gp

Compound	Lowest binding energy (kcal/mol)	Predicted inhibition constant (μM)	Interacting amino acids	Amino acids involved in hydrogen bonds
PSC833	− 5.723 ± 0.172	65.493 ± 17.584	6	–
(*R*)-3	− 7.090 ± 0.000	6.377 ± 0.021	6	Phe104
(S)-3	− 6.820 ± 0.044	10.070 ± 0.746	9	Phe104
(*R*)-23e	− 7.070 ± 0.017	6.587 ± 0.211	8	Phe104
(*S*)-23e	− 6.723 ± 0.339	11.777 ± 0.339	10	Phe104

Docking simulations were performed in three independent experiments with 2,500,000 energy evaluations and 250 runs each. Data represent the mean ± SD

Table 3 Molecular docking of selected goniothalamins on the drug-binding domain of homology-modeled human P-gp. Numbers in brackets denote shared amino acids between sites

Compound	Lowest binding energy (kcal/mol)	Predicted inhibition constant (μM)	Interacting amino acids	Amino acids involved in hydrogen bonds	Interacting amino acids in		
					M-site	R-site	H-site
PSC833	−11.050 ± 0.265	0.008 ± 0.037	13	Gln946	4 (3)	5 (2)	2 (1)
(*R*)-3	−8.050 ± 0.010	1.257 ± 0.021	10	Asn296	1 (1)	6 (1)	–
(S)-3	−8.400 ± 0.040	0.696 ± 0.045	10	Asn296, Asn842	–	5	–
(*R*)-23e	−8.140 ± 0.020	1.087 ± 0.035	11	Asn296	1 (1)	6 (1)	–
(*S*)-23e	−8.647 ± 0.015	0.459 ± 0.014	9	Asn296, Asn842	–	5	–

Docking simulations were performed in three independent experiments with 2,500,000 energy evaluations and 250 runs each. Data represent the mean ± SD

set presented in this study (Table 2). All tested derivatives formed hydrogen bonds with Phe104.

Results of the defined docking to the drug-binding domain suggested that compounds (*R*)-23e and (*S*)-23e bound slightly stronger than compounds (*R*)-3 and (*S*)-3, which is in contrast to the P-gp inhibition data (Fig. 5b). However, considering binding energies around − 8 kcal/mol (Table 3) indicated that the goniothalamin derivatives might inhibit P-gp drug efflux by strong interaction at the drug-binding pocket of P-gp. Allocation of the amino acids responsible for interaction to the three different drug-binding sites [22] revealed that the newly described goniothalamin derivatives mainly interact with the substrate-binding R-site (rhodamine 123 site).

Discussion

Overexpression of P-gp in tumor cells is the best characterized mechanism of cancer MDR [1, 23] and natural products have long been the focus of researchers to identify new anti-tumor agents and ABC transporter inhibitors [24]. To date, the pharmacological effects of goniothalamin against ABC transporters have not been studied. Our study demonstrates that the natural product (*R*)-1 moderately inhibited P-gp transport function in a dose-dependent manner, but not the transport mediated by MRP1 or BCRP. Interestingly, regarding the SAR of goniothalamins in inhibiting P-gp, we did not observe any differences between the (*R*)- and (*S*)-enantiomers of the natural product 1 or for the compounds with saturated lactone moiety 23c, 23e and 23a. This is in contrast to the SAR revealed for the cytotoxic effects (Supplementary Table S1). Comparing the enantiomers of the substituted derivatives 3 and 24, increased P-gp inhibition was detected for the (*R*)-enantiomers. Saturation of the lactone moiety did not affect P-gp inhibition compared to the unsaturated compounds 23c and 23a, whereas the activity of 23e was markedly reduced.

In accordance with the P-gp inhibition data set, both compounds (*R*)-3 and (*S*)-3 led to a dose-dependent increase of intracellular doxorubicin accumulation. In line, compound (*S*)-3 enhanced doxorubicin accumulation to a lesser extent than compound (*R*)-3. Both compounds dose-dependently sensitized colon carcinoma cells to doxorubicin treatment.

Molecular docking of compounds (*R*)-3 and (*S*)-3 to homology-modeled human P-gp demonstrated interaction close to the drug-binding domain. Applying defined docking to only the drug-binding domain of P-gp resulted in stronger interaction and lowest binding energies. Allocation of interacting amino acids to the three different drug-binding sites suggests that interaction mainly occurs at the R-site (rhodamine 123) [22], potentially explaining the increase of intracellular doxorubicin, as anthracyclines are presumed to bind to the R-site.

We hypothesize that goniothalamin derivatives (*R*)-3 and (*S*)-3 specifically inhibit P-gp by acting as alternative substrates, thereby leading to increased intracellular accumulation of chemotherapeutic drugs, which are transported by P-gp. Although the potency of the derivatives will have to be improved to submicromolar potencies, our results advocate further optimization of goniothalamin derivatives representing a versatile starting point for the development of novel drugs to overcome MDR in cancer therapy.

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Compliance with ethical standards

Conflict of interests The authors declare that they have no conflict of interest.

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