CANCER GENOMICS & PROTEOMICS 7: 191-206 (2010)

Inhibition of P-glycoprotein at the Blood–Brain Barrier by Phytochemicals Derived from Traditional Chinese Medicine

ANNE MAHRINGER[1], SHIRIN KARAMUSTAFA[2], DANIEL KLOTZ[2], STEFAN KAHL[3], V. BADIREENATH KONKIMALLA[2], YIFEN WANG[4], JUNSONG WANG[4], HAI-YANG LIU[4], HERBERT BOECHZELT[5], XIAOJIANG HAO[4], RUDOLF BAUER[3], GERT FRICKER[1] and THOMAS EFFERTH[2]

[1]*Institute of Pharmacy and Molecular Biotechnology, University of Heidelberg, Heidelberg, Germany;*
[2]*Pharmaceutical Biology (C015), German Cancer Research Center, Heidelberg, Germany;*
[3]*Institute of Pharmaceutical Sciences, University of Graz, Graz, Austria;*
[4]*State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming, Institute of Botany, Chinese Academy of Sciences, Kunming, P.R. China;*
[5]*Joanneum Research, Graz, Austria*

Abstract. *The blood–brain barrier (BBB) is a key determinant for drug transport through brain vessels. It restricts the pharmacological efficacy in numerous neurological diseases, including brain tumors. A major functional constituent of BBB is P-glycoprotein, which is also a major obstacle for effective chemotherapy of brain tumors. An appealing strategy is to selectively modulate BBB function using P-glycoprotein inhibitors. We assessed 57 chemically defined compounds derived from medicinal plants used in traditional Chinese medicine for their potential to inhibit P-glycoprotein. Nine phytochemicals inhibited P-glycoprotein in porcine brain capillary endothelial cells (PBCECs) and multidrug-resistant CEM/ADR5000 cells as shown by a calcein fluorescence assay. The cytotoxicity of the 57 phytochemicals was measured by a growth inhibition assay. Seven compounds inhibiting P-glycoprotein at lower doses were cytotoxic to drug-sensitive parental CCRF-CEM cells at higher doses. Of them, five were not cross-resistant to CEM/ADR5000 cells (baicalein, bufalin, glybomine B, deoxyserofendic acid, and shogaol). Bufalin was chosen as a lead compound. Of a further six bufalin-related compounds, scillarenin showed improved features in comparison to bufalin. It was cytotoxic to cancer cells at a nanomolar range. COMPARE and hierarchical cluster analyses of microarray-based mRNA expression were used to investigate determinants of sensitivity or resistance of the bufalin-related compounds downstream of P-glycoprotein. CEM/ADR5000 cells were not cross-resistant, but were collaterally sensitive towards scillarenin. Finally, scillarenin inhibited P-glycoprotein in PBCECs. Taken together, these data show that scillarenin is a potential novel candidate for P-glycoprotein inhibition at BBB, and, thereby, may improve the efficacy of therapy regimens in treating brain diseases.*

The blood–brain barrier (BBB) provides the precise homeostatic environment required for high neural activity and proper synaptic transmission. The vascular barrier protects the brain from bacteria, fluctuations in toxic metabolites, and xenobiotics (1). The BBB is formed by endothelial cells that line microvessels in the brain. Tight junctions, low pinocytotic activity and a lack of fenestrations are major features of this barrier, forcing drug compounds to enter the brain *via* transcellular passage (2). One major gatekeeper at the BBB is P-glycoprotein, which prevents the entry of lipophilic drugs into the brain (3). P-Glycoprotein is highly expressed at the luminal membrane of brain capillary endothelial cells (4). Hence, P-glycoprotein represents a major obstacle to the effective treatment of common CNS diseases such as brain tumors (5, 6).

This transporter is also localized in other barrier-forming tissues, *e.g.* intestine, kidney, bile canaliculi, and placenta (7). Additionally, P-glycoprotein transports a broad spectrum of structurally and functionally unrelated drugs such as *Vinca* alkaloids (*e.g.* vinblastine, vincristine), paclitaxel, epipodophyllotoxins (*e.g.* etoposide) and anthracyclines (*e.g.* doxorubicin) and works in concert with other transporters, such as multidrug-resistance proteins (MRPs) and breast cancer-resistance protein (BCRP), and organic anion/cationic

Correspondence to: Professor Dr. Thomas Efferth, Chair, Department of Pharmaceutical Biology, Institute of Pharmacy and Biochemistry, University of Mainz, Staudinger Weg 5, 55128 Mainz, Germany. Tel: +49 61313924322, Fax: +49 61313923752, e-mail: efferth@uni-mainz.de

Key Words: Bufadienolides, calcein assay, P-glycoprotein, multidrug resistance, natural products, phytochemicals, pharmacogenomics.

transporters (OA/CTs) (8). Other substrates include calcium channel blockers (*e.g.* verapamil), antiarrhythmics (*e.g.* quinidine), steroids (*e.g.* dexamethasone) and anti-parasitics (*e.g.* ivermectin), antidepressants and antiepileptic drugs to mention but a few (9, 10). Consequently, P-glycoprotein not only affects drug absorption, distribution and secretion, but also protects the body from harmful xenobiotics.

Several strategies have been developed to increase CNS delivery of drugs. Drugs may be delivered either by intracerebroventricular injection or matrix implantation directly into the cerebrospinal fluid, although diffusion and clearance oppose penetration into the surrounding tissue (11). The intranasal pathway of drug administration or the method of reversible BBB disruption by osmotically (mannitol) or biochemically active agents represent alternatives. In contrast to these invasive methods, pharmaceutical strategies involve lipidization of drugs or their inclusion into liposomes or nanoparticles (12). Endogenous transporter uptake systems such as LAT-1 mediate brain uptake of L-dopamine, melphalan, and baclofen. Moreover, drug uptake can be improved by conjugation to monoclonal antibodies against the transferrin receptor, which facilitates receptor-mediated transcytosis (13). Finally, efflux transporters such as P-glycoprotein present an interesting target for CNS delivery. Their selective inhibition reduces drug efflux and increases brain concentrations to pharmacologically effective levels. Thus, the development of specific inhibitors is of major interest. Co-administration of transport inhibitors together with the actual chemotherapeutic may enhance drug penetration into the brain (14). First-generation compounds such as verapamil and cyclosporin A as well as the second-generation modulators dexverapamil and valspodar (PSC-833), demonstrated significant advances in mice (15), but failed in patients due to side-effects and concomitant inhibition of cytochrome P-450 monooxygenases (CYPs) (16). Clinical trials with third-generation drugs such as elacridar (GF120918) (17), biricodar, and zosuquidar (18) are ongoing, but do not seem to be very promising yet. Hence, there is a continued urgent need for the improvement of drug therapy by crossing the BBB using P-glycoprotein inhibitors.

During recent years, the industrial reservoirs of chemical lead structures have begun to dwindle and increasing attention has been paid to natural products from traditional Asian medicines (19, 20) to find new chemical structures for cancer therapy.

More than a decade ago, we initiated a research program on molecular pharmacology and pharmacogenomics of natural products derived from traditional Chinese medicine (TCM). This project turned out to be fertile ground for the identification and characterization of compounds with activity towards tumor cells and viruses. TCM commands a unique position among traditional medicines because an enormous variety of drugs of plant origin has been identified and followed for more than 5,000 years traditionally. An elaborate system that contained many written documents and textbooks has been handed down for millennia. This might imply that inactive plants and recipes vanished over the centuries and that the *materia medica* of modern TCM is enriched with bioactive plants. This may significantly improve prospects for identifying novel active constituents from TCM (21). Based on this rationale, we started a systematic investigation of P-glycoprotein inhibitors that are capable of crossing the BBB.

Materials and Methods

Test compounds. The collection and botanical identification of medicinal plants mainly from the Yunnan Province, China, has been described (22). The finely ground plant material was successively extracted with solvents of increasing polarity (petroleum ether or *n*-hexane, ethyl acetate, and methanol). Organic solvent extracts were performed in a Soxhlet apparatus, whereas water extracts were prepared as decoctions according to the traditional medicinal preparation. The aim was to divide plant constituents into fractions of different polarity for extraction. Bioactivity guided-isolation of phytochemicals by chromatographic methods was carried out as described elsewhere (23, 24). The chemical structures were elucidated by spectrometric methods and crystal structure analysis (25). Twenty-three out of 57 compounds were biologically active in the present investigation. Their chemical structures are shown in Figure 1. Six bufadienolides and bufatrienolides (acetylmarinobufogenin, bufotalin-3-acetate, proscillaridin, resibufogenin, scillaren A, and scillarenin) were obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute (Bethesda, Maryland, USA) (Figure 1). Natural products (biochanin A, diosmetin, and hesperitin) used as control inhibitors of P-glycoprotein function were obtained from commercial sources (Sigma-Aldrich, Taufkirchen, Germany). Verapamil was obtained from Abbott Laboratories (Ludwigshafen, Germany) and PSC-833 from Novartis (Basel, Switzerland).

Isolation of porcine brain capillaries and capillary endothelial cells (PBCECs). Porcine brain capillaries were isolated as previously described (26), and PBCECs were isolated from porcine brains following a recently described protocol (27).

P-Glycoprotein transport activity. Calcein-acetoxymethylester (calcein-AM) is immediately extruded by P-glycoprotein. Inhibition of P-glycoprotein reduces calcein-AM efflux and cytosolic esterases rapidly cleave ester bonds, leading to the formation of highly fluorescent organic anion calcein, which is intracellularly trapped. Consequently, the intracellular accumulation of calcein detected by its fluorescence measures P-glycoprotein inhibition and permeation of the BBB. The assay with PBCECs was performed as described elsewhere (28). P-Glycoprotein-expressing human CEM/ADR5000 leukemia cells and their drug-sensitive parental cell line, CCRF-CEM were used as a model for P-glycoprotein, since CEM/ADR5000 cells selectively express P-glycoprotein, but not other transporters of the ABC transporter family (29). Each concentration of test compound was measured in duplicates (n=2), and experiments were performed at least in triplets.

For flow cytometry-based calcein-AM assay, cell suspensions at a density of 2.5×10^7 cells/ml were used. Intracellular fluorescence was measured using a fluorescence-activated cell sorting system

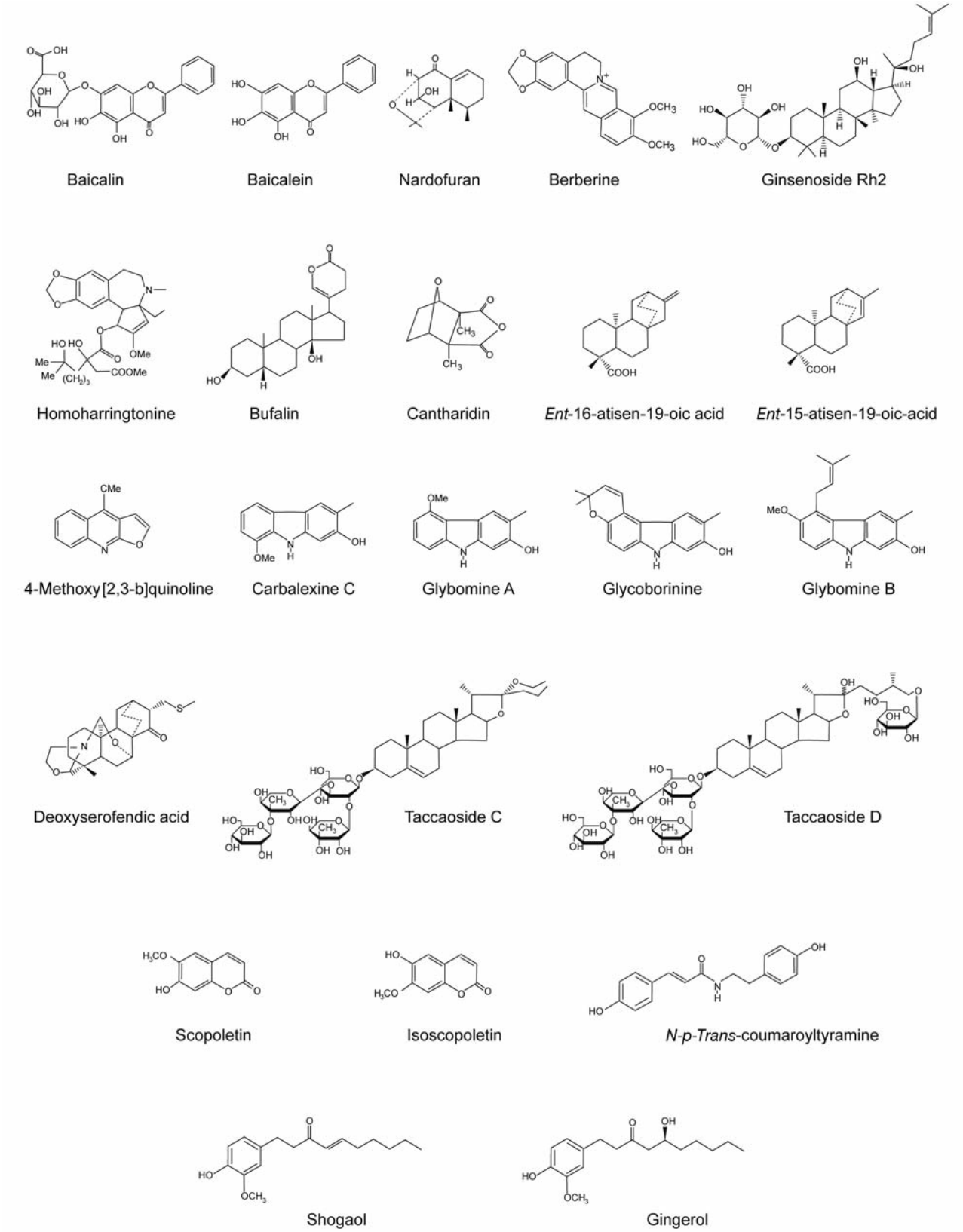

Figure 1. *Chemical structures of natural products derived from traditional Chinese medicine which interacted with P-glycoprotein either as substrate or as inhibitor.*

Table I. *Inhibition of P-glycoprotein at the blood–brain barrier (BBB) and cytotoxicity towards cancer cells by phytochemicals derived from traditional Chinese medicine (TCM). BBB inhibition was determined in PBCECs by the calcein assay and cytotoxicity in drug-sensitive parental CCRF-CEM and multidrug-resistant CEM/ADR5000 cells.*

	BBB inhibition			Cytotoxicity in cancer cells		
	Molecular weight	Calcein assay PBCEC		IC_{50} (μM)		Degree of resistance
		EC_{max} (%)	EC_{50} (μM)	CCRF-CEM	CEM/ADR5000	
TCM compounds						
Baicalin	446	Inactive		54.6 (±12.3)	51.8 (±5.9)	0.95 (±0.2)
Baicalein	270	82.8 (±39.0)	55.7 (±43.0)	50 (±8.8)	54.6 (±4.1)	1.1 (±0.2)
Nardofuran	250	Inactive		97.5 (±7.6)	212.5 (±35.3)	2.2 (±0.4)
Berberine	372	Inactive		26 (±3.3)	158 (±9.7)	6.1 (±0.9)
Ginsenoside Rh2	622	Inactive		75.1 (±22.3)	81.7 (±14.9)	1.1 (±0.4)
Homoharringtonine	546	Inactive		$5.8 (\pm1.5) \times10^{-3}$	$10.5 (\pm1.7) \times10^{-3}$	1.8 (±0.4)
Bufalin	387	274.0 (±119.9)	21.2 (±17.0)	$21.4 (\pm3.7) \times10^{-3}$	$11.25 (\pm1.2) \times10^{-3}$	0.53 (±0.1)
Cantharidin	196	Inactive		19.6 (±2.6)	17.7 (±3.1)	0.9 (±0.2)
Ent-16-atisen-19-oic acid	316	219.9 (±41.4)	16.8 (±9.4)	>30	N.D.	N.D.
Ent-15-atisen-19-oic acid	316	130.7 (±22.5)	13.6 (±8.9)	1.1 (±0.7)	2.9 (±1.7)	2.6 (±2.3)
4-Methoxy[2,3-b]quinoline	199	377.5 (±86.7)	63.3 (±42.4)	234.5 (±63.3)	339.7 (±49.2)	1.4 (±0.1)
Carbalexine C	227	Inactive		12.3 (±2.9)	21.6 (±4.0)	1.8 (±0.4)
Glybomines A	227	229.6 (±35.8)	60.0 (±19.5)	24.2 (±6.6)	194.3 (±15.4)	8.0 (±2.3)
Glycoborinine	279	Inactive		9.9 (±0.8)	7.6 (±1.2)	0.77 (±0.1)
Glybomine B	295	219.8 (±26.7)	32.9 (±9.5)	22.0 (±6.3)	12.2 (±3.6)	0.6 (±0.3)
Deoxyserofendic acid	403	449.6 (±258.7)	151.7 (±128.6)	6.0 (±0.6)	2.4 (±0.4)	0.4 (±0.1)
Taccaoside C	1030	Inactive		1.3 (±0.3)	1.8 (±0.9)	1.4 (±0.8)
Taccaoside D	1210	Inactive		1.7 (±0.2)	2.5 (±0.3)	1.5 (±0.3)
Scopoletin	192	Inactive		15.8 (±5.4)	19.0 (±7.2)	1.2 (±0.6)
Isoscopoletin	192	Inactive		25 (±8.1)	6.3 (±6.0)	0.25 (±0.25)
N-p-Trans-coumaroyltyramine	283	Inactive		12.7 (±3.5)	25.7 (±3.6)	2.0
Shogaol	276	0.1-10μM	250-300	2.1 (±0.1)	2.2 (±0.006)	1.0 (±0.1)
Gingerol	294	Inactive		6.2 (±0.4)	5.4 (±2.1)	0.9 (±0.4)
Control drugs						
Biochanin A	284	210.7 (±12.7)	9.9 (±1.8)	16.1 (±4.0)	14.9 (±2.7)	0.9 (±0.3)
Diosmetin	300	340.5 (±68.9)	16.3 (±8.2)	0.2 (±0.1)	3.5 (±1.3)	17.5 (±10.8)
Hesperitin	302	297.0 (±76.2)	66.6 (±26.5)	9.1 (±5.4)	1.8 (±0.6)	0.2 (±0.1)
Verapamil	491	350.5 (±24.4)	3.2 (±1.0)	>50	>50	N.D.
PSC-833	1215	758.1 (±24.4)	2.0 (±1.7)	>10	>10	N.D.

N.D., Not determined.

(FACS: Calibur flow cytometer, Becton–Dickinson, Franklin Lakes, NJ, USA) with λ (excitation)=488 nm and a 530/30 band pass filter to collect emitted fluorescence. Dead cells were excluded using propidium iodide staining. About twenty thousand cells were analyzed using CellQuest software (Becton–Dickinson).

All fluorescence values were corrected by subtracting the background fluorescence. Calcein–AM auto-hydrolysis was measured in all control samples (n=6) without cells. The increase in intracellular fluorescence caused by a test compound was referred to a fluorescence control level that was set to 100% and the final values are represented as a percentage of the control.

P-Glycoprotein-specific transport functions in isolated brain capillaries were also studied using the fluorescent substrate NBD-cyclosporin A (NBD-CsA) (30).

Cell growth inhibition assay. Drug-sensitive parental CEM/CCRF and multidrug-resistant P-glycoprotein-expressing CEM/ADR5000 cells (29, 31) were incubated with test substances as described elsewhere (32). The degree of cross-resistance of a substance was calculated by dividing the 50% inhibition concentration (IC_{50}) of CEM/ADR5000 by the IC_{50} of CCRF-CEM.

Electron donor group pattern formation. The determination of spatial distances between the electron donor groups has previously been described as a method for the recognition of interactions of chemical compounds with P-glycoprotein (33, 34). Two types of electron donor patterns have been suggested to predict interactions with P-glycoprotein (as substrate or inhibitor) (type 1) or induction of P-glycoprotein expression (type 2). The first type consists of two electron donor (hydrogen bond acceptor) groups, which are separated by one, two, or three carbon atoms. The spatial distance formed by the two electron donor groups is 2.5±0.3Å (denoted as type 1 electron donor unit). A second type of pattern is formed by three electron donor groups separated by 2.5±0.3Å by each other with a spatial separation of the outer two donor groups of 4.6±0.6Å or by two electron donor groups with a spatial separation of 4.6±0.6Å (type 2 electron donor units). The chemical structures for test compounds were sketched using HyperChem Release 2 program for Windows (Autodesk, Inc.).

Western blot analysis. Brain, brain capillaries, CEM/ADR5000 and CEM/CCRF cells were subjected to Western blotting as described elsewhere (35). P-Glycoprotein was detected by the monoclonal

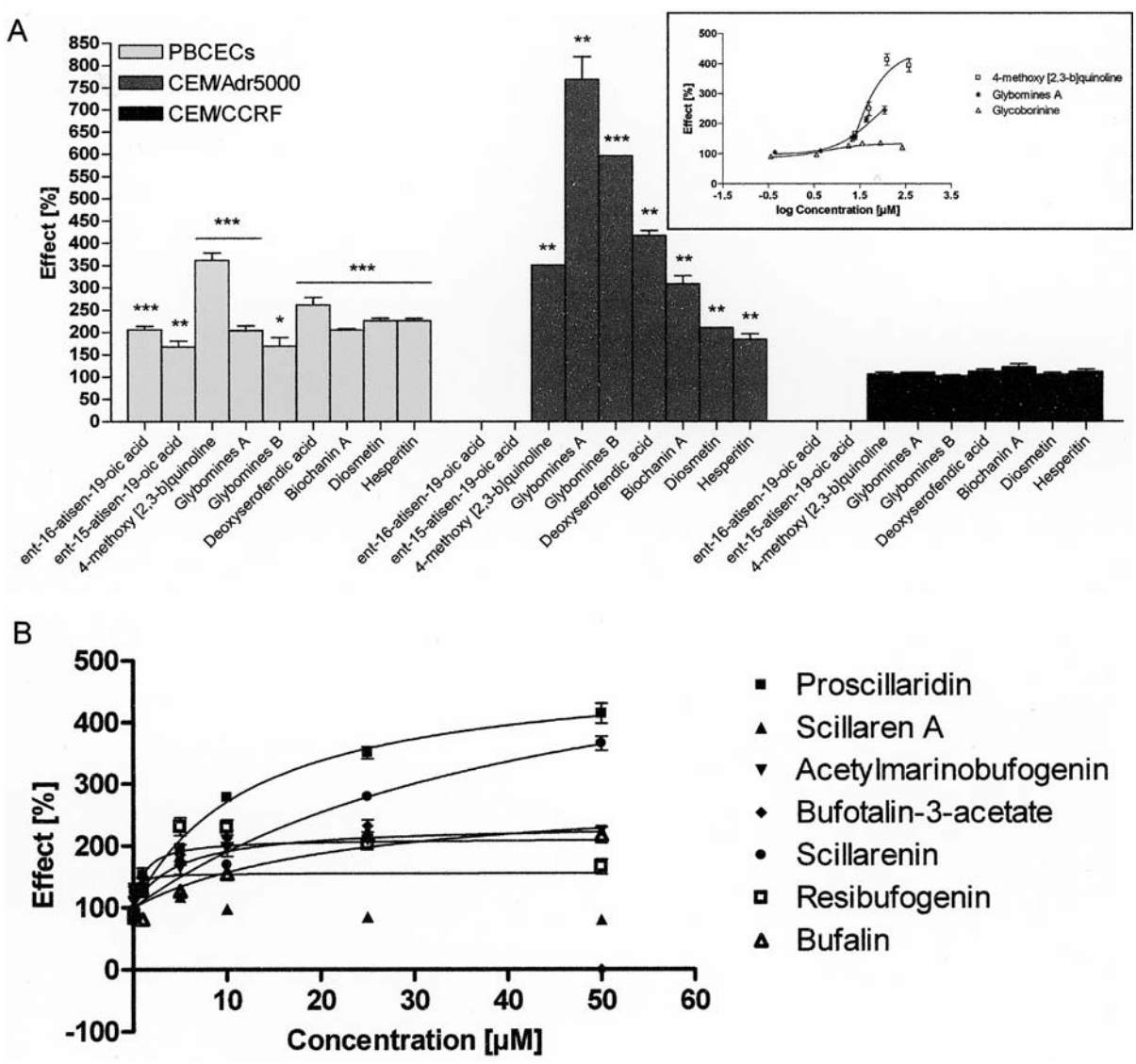

Figure 2. *Inhibition of P-glycoprotein by phytochemicals from traditional Chinese medicine and derivatives thereof using a calcein assay. A: Concentration-dependent increase in intracellular fluorescence in P-glycoprotein expressing brain capillary endothelial cells sub-cultured as monolayer, P-glycoprotein-expressing CEM/ADR5000 cells and P-glycoprotein-negative CCRF-CEM cells. Intracellular calcein fluorescence in PBCECs was determined in a fluorescence plate reader (two independent experiments with n=6), whereas fluorescence accumulation in CEM/ADR5000 and CCRF-CEM cells was determined by flow cytometry (2 experiments with n=2). The bar diagram shows intracellular fluorescence (E_{max}) values at a concentration of 5 µg/ml for PBCECs and 50 µg/ml for CCRF-CEM and CEM/ADR5000 cells. The inset exemplarily shows four dose–response curves used for E_{max} calculation. ***p<0.001; **p<0.01; *p<0.05. B: Inhibition of P-glycoprotein by bufadienolides and bufatrienolides showing a concentration-dependent increase in intracellular fluorescence in freshly isolated PBCECs two independent experiments (n=6).*

mouse antibody, clone C219, against P-glycoprotein (Alexis Biochemicals, Grünberg, Germany) at a dilution of 1:100. Na^+/K^+-ATPase served as negative control. A monoclonal antibody (Axxora, Grünberg, Germany) against the α–1 subunit was used (dilution 1:250).

Statistics. All values are presented as mean±SEM. Control and treatment groups were compared by either Student's *t*-test or one-way analysis of variance, followed by a Dunnett's *post hoc* test. Differences were considered significant at $p<0.05$. Dose-response curves were made using Prism software (GraphPad Software, San Diego, CA, USA). The 50% effective concentration (EC_{50}) of compounds tested in the calcein-AM assay was obtained by using an E_{max} model (36).

COMPARE analysis of microarray-based mRNA expression data of the tumor cell line panel of the National Cancer Institute (NCI, Bethesda, MA, USA) has been previously described (http://dtp.nci.nih.gov) (37, 38).

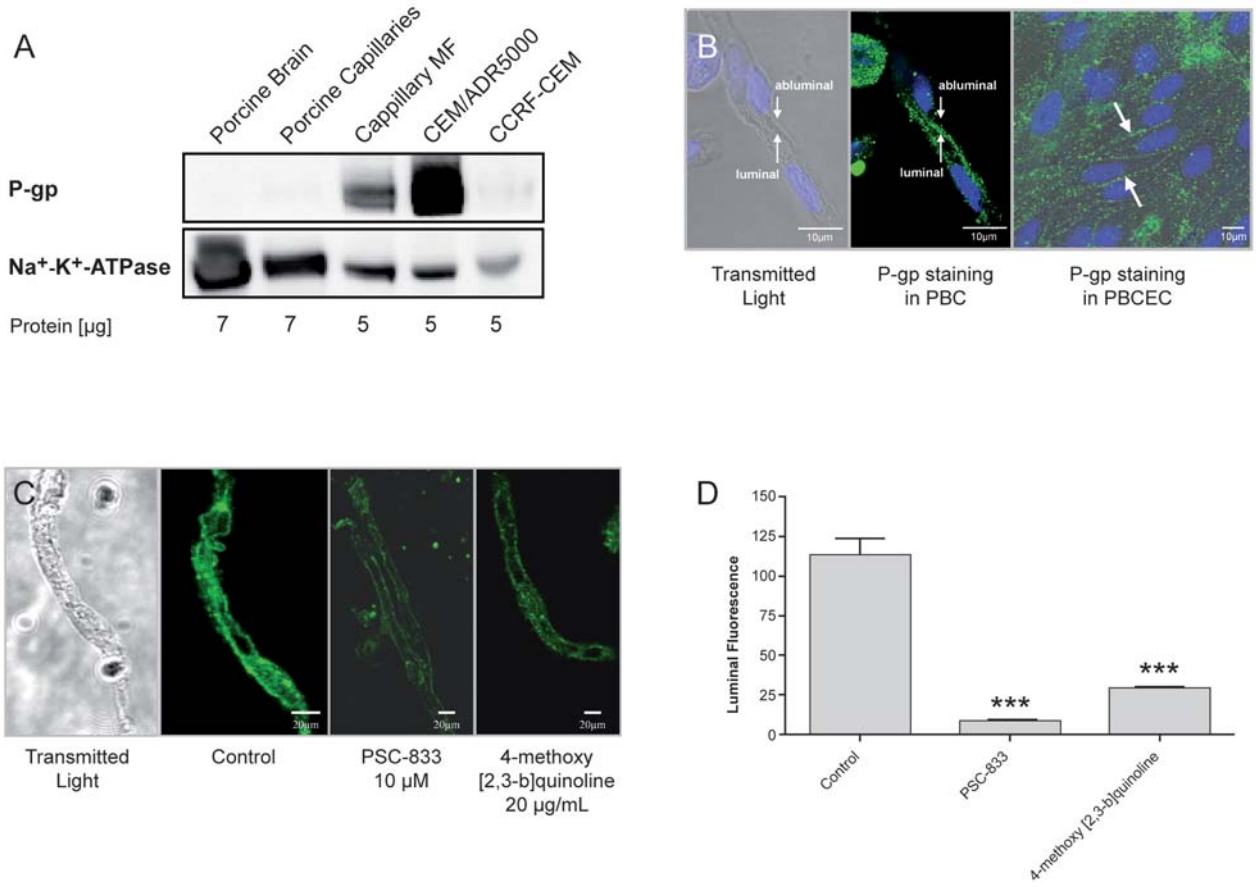

Figure 3. *Transport of NBD-cyclosporin A into brain capillaries and expression of P-glycoprotein. A: Detection of P-glycoprotein by Western blotting (upper row). The expression of Na^+/K^+-ATPase in the lower row was detected as an example of an ion transporter which is unrelated to drug transport. The amount of loaded protein per lane is indicated. MF: membrane fraction. B: Immunostaining for P-glycoprotein in porcine brain capillaries shows luminal localization of the efflux transporter. In cultivated PBCECs, P-glycoprotein is located on the membrane, but was also expressed in vesicle-like structures in the cytoplasm. The left image shows a transmitted light image of a porcine capillary discriminating between luminal and basolateral endothelial membrane side. C: Freshly isolated brain capillaries were incubated with NBC-CsA and left untreated (control) or treated for 30 min with PSC-833 (10 µM) or 4-methoxy [2,3-b]quinoline (100 µM). Images are representative for two separate capillary isolations. D: Quantification of NBD-CsA fluorescence. ***Significant at p<0.001 (two independent experiments with n=7-10 capillaries).*

Hierarchical cluster analyses applying the complete-linkage method were carried out with the WinSTAT program (Kalmia, Cambridge, MA, USA) as described previously (39-43).

Results

As a starting point, well-known P-glycoprotein inhibitors, verapamil and PSC-833, were chosen as positive controls, both of which strongly inhibited calcein efflux in PBCEC monolayers (Table I, Figure 2A). The EC_{50} values were 3.2 (±1.0) and 2.0 (±1.7) µM respectively, with an intracellular fluorescence (E_{max}) that reached 350.5 (±24.4) and 758.1 (±24.4)%, respectively, in comparison to the untreated controls.

As natural products, we used flavonoids, which are also known P-glycoprotein inhibitors (44). Biochanin A, diosmetin, and hesperitin displayed an intermediate to strong inhibition of calcein efflux in P-glycoprotein-expressing CEM/ADR5000 cells and PBCECs, but not in P-glycoprotein-negative CCRF-CEM cells (Figure 2A, Table I).

We then analyzed 57 chemically defined compounds derived from medicinal plants used in TCM. Nine phytochemicals considerably increased calcein fluorescence in PBCECs (Table I), baicalein, bufalin, *ent*-16-atisen-19-oic acid, *ent*-15-atisen-19-oic acid, 4-methoxy[2,3-b]quinoline, glybomine A, glybomine B, deoxyserofendic acid, and shogaol. The chemical structures of these active compounds are depicted in Figure 1. Their EC_{50} values were in the range of 13.6 (±8.9) to 151.7 (±128.6) µM. Intracellular calcein fluorescence (E_{max}) increased from 82.8 (±39.0) to 449.6 (±258.7) % of control levels (fluorescence in absence of P-

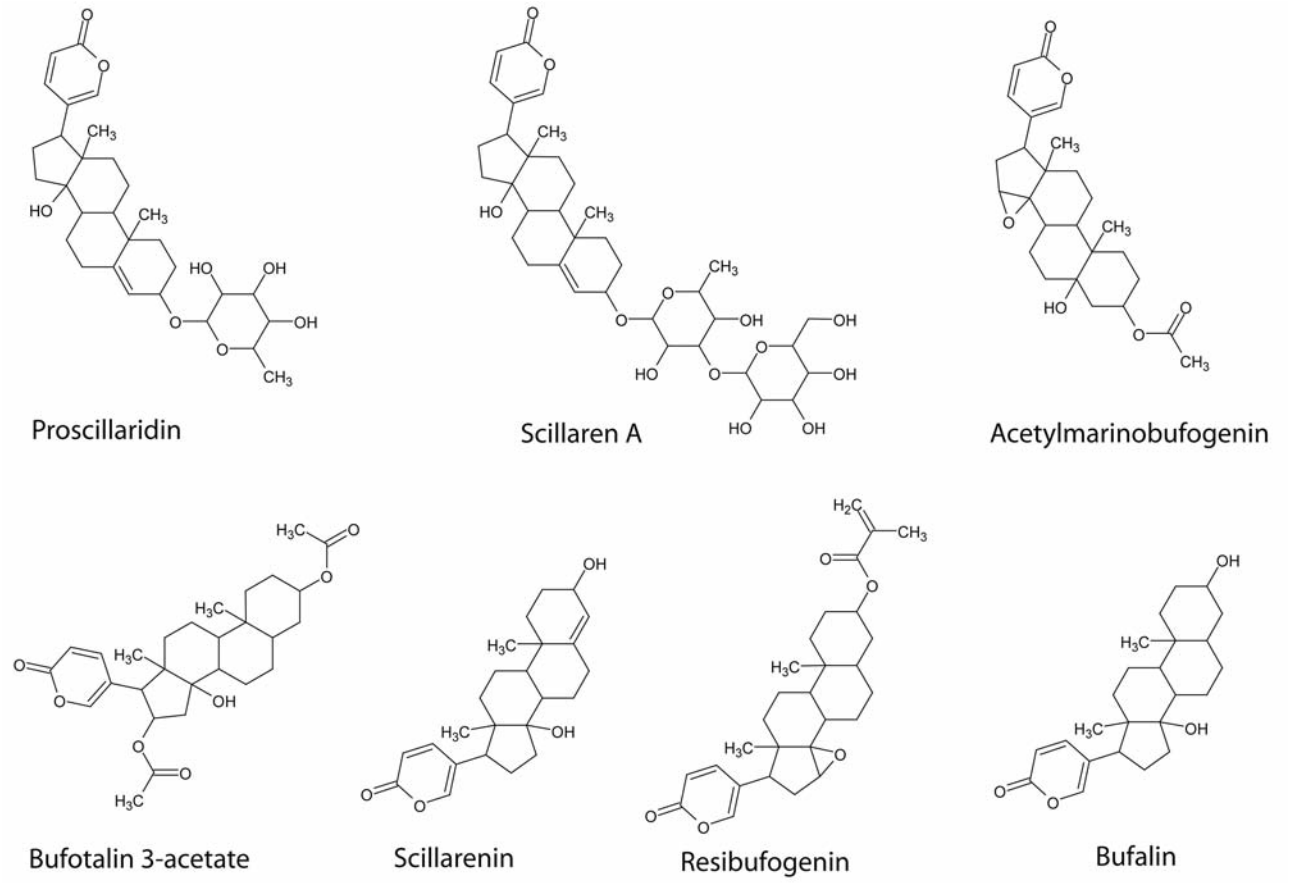

Figure 4. *Chemical structures of bufadienolides and bufatrienolides*.

glycoprotein inhibitors), suggesting high affinity to porcine P-glycoprotein. EC_{50} and E_{max} values were calculated from concentration kinetics of intracellular calcein fluorescence (for illustration see inset in Figure 2A).

The E_{max} values of intracellular calcein fluorescence of PBCECs, CEM/ADR5000, and CCRF-CEM cells are shown in Figure 2A. The E_{max} values tended to be higher in CEM/ADR5000 cells than in PBCECs, indicating higher modulatory activity. The E_{max} values of CCRF-CEM cells were not increased as compared to untreated controls.

Western blots were performed to examine whether the interaction of the test compounds with calcein transport was associated with P-glycoprotein expression. P-Glycoprotein was not expressed in porcine brain tissue and porcine capillaries, but in capillary membrane fractions (Figure 3A), implicating P-glycoprotein enrichment at the capillary membrane within brain tissue, but not in pericytes, astrocytes, or neurons. CEM/ADR5000 cells, which served as control cells, showed strong overexpression of P-glycoprotein. This was not observed in drug-sensitive CCRF-CEM parental cells. The expression of Na^+/K^+-ATPase was taken as an example of an ion transporter which is unrelated to calcein transport and served as negative control. Immunostaining revealed a luminal localization of P-glycoprotein in the membrane of freshly isolated porcine capillaries, which is consistent with its function as an efflux transporter (Figure 3B). The image of Figure 3B presents the plasma membrane localization of P-glycoprotein in cultivated PBCECs. The scattered, cytoplasmic localization indicated storage of the transporter in vesicles.

Confocal living cell microscopy was used for confirmation of the results shown in Figure 2A. Brain capillaries with luminally localized P-glycoprotein effluxed a fluorescent P-glycoprotein substrate, NBD-cyclosporin A (NBD-CSA) back into the capillary lumen (green) (Figure 3C). For control, PSC-833 was analyzed. Indeed, PSC-833 resulted in an almost empty lumen. NBD-CSA accumulated only in endothelial cells, but was no longer secreted into the capillary lumen due to effective P-glycoprotein inhibition. Likewise, one of the TCM test compounds, 4-methoxy [2,3,-b]quinoline, inhibited P-glycoprotein transport function. The effects of NBD-CsA and 4-methoxy[2,3,-b]quinoline were statistically significant (Figure 3D).

Table II. *Inhibition of P-glycoprotein at the BBB and cytotoxicity towards cancer cells by bufadienolides and bufatrienolides. For details see Table I.*

Substance	Name	BBB Inhibition			Cytotocixity in cancer cells		
		Molecular Weight	Calcein Assay EC_{max} (%)	(PBCEC) EC_{50} (μM)	IC_{50} (nM) CCRF-CEM	IC_{50} (nM) CEM/ADR5000	Degree of Resistance
1	Proscillaridin	531	12.8± 2.1	390±23.9	6.7±9.7	13.6±5.5	2.0
2	Scillaren A	693	Inactive	Inactive	0.042±0.059	0.87±0.45	20.7
3	Acetylmarinobufogenin	443	4.6±1.3	133.4±9.5	938.0±65.7	1090.0±33.0	1.2
4	Bufotalin-3-acetate	487	0.3±0.9	56.0±18.7	24.2±2.5	30.0±2.6	1.2
5	Scillarenin	385	46.4±11.6	510.0±73.2	5.9±1.4	1.7± 1.0	0.28
6	Resibufogenin	453	1.1±0.76	11.4±11.4	15.9±1.26 $\times 10^3$	9.8±1.7 $\times 10^3$	0.62

Next, we investigated the cytotoxicity of all 57 phytochemicals. Whereas concentrations necessary to modulate P-glycoprotein function were not cytotoxic in PBCECs, inhibition of cancer cell growth was observed. The IC_{50} values were first determined in parental drug-sensitive CCRF-CEM cells. Of 57 compounds tested, 22 strongly inhibited the growth of CCRF-CEM cells. IC_{50} values for CCRF-CEM cells were in the range from 5.8 (±1.5) nM (homoharringtonine) to 234.5 (±63.3) μM (4-methoxy[2,3-b]quinoline). One compound, *ent*-16-atisen-19-oic acid, which inhibited P-glycoprotein in the calcein assay did not reveal growth-inhibitory activity (Table I). None of the other phytochemicals affected cell growth. We then determined the IC_{50} values in CEM/ADR5000 cells to analyze cross-resistance. As a cut-off value, the degree of drug resistance (IC_{50} of CEM/ADR5000 divided by IC_{50} of CCRF-CEM) was set as 1.2. Higher values were considered as being indicative of cross-resistance; values of 0.9 to 1.2 reflected no cross-resistance, and IC_{50} values below 0.9 indicated collateral sensitivity of CEM/ADR5000 cells to the corresponding phytochemical.

Taking all results into account, five phytochemicals (baicalein, bufalin, glybomine B, deoxyserofendic acid, shogaol) inhibiting P-glycoprotein in PBCECs displayed cytotoxic effects towards CCRF-CEM cells, and CEM/ADR5000 cells were not cross-resistant to them. Furthermore, two compounds were not or were only weakly cytotoxic, but inhibited P-glycoprotein (*ent*-16-atisen-19-oic acid and 4-methoxy[2,3-b]quinoline).

Next, we addressed the question of whether interacting properties of natural products with P-glycoprotein either as substrates exhibiting cross-resistance of CEM/ADR5000 cells or as inhibitors in the calcein assay can be predicted on the basis of their chemical structure. The compounds listed in Table I were subjected to a pattern recognition approach (33). According to this approach, type 1 patterns are indicative of interaction with P-glycoprotein activity either as substrate or inhibitor and type 2 patterns of induction of P-glycoprotein expression. As shown in Table V, 16 out of 23 compounds revealed a type 1 electron donor pattern indicative of interaction with P-glycoprotein. Three phytochemicals (nardofuran, homoharringtonine, and deoxyserofendic acid) showed both type 1 and type 2 electron donor units and six compounds only type 2 patterns (cantharidin, 4-methoxy[2,3-b]quinolone, carbalexine C, glybomine A, glycoborinine, glybomine B). One compound derived from TCM displayed neither type 1 nor type 2. The three control phytochemicals (biochanin A, diosmetin, hesperitin) with known P-glycoprotein interaction revealed type 1 and 2 patterns. Verapamil had a type 1 and PSC-833 type 2 pattern. We analyzed the correlation of all phytochemicals with type 1 electron donor units (16 TCM compounds and three control natural products) and did not find a significant relationship ($p>0.05$; Fisher exact test), indicating that type 1 patterns in our set of natural products did not predict interaction with P-glycoprotein. The influence of our compounds exhibiting type 2 patterns on up-regulation on P-glycoprotein expression was not further investigated.

Since bufalin inhibited P-glycoprotein and CEM/ADR5000 cells were not cross-resistant to it, we further analyzed six bufadienolides and bufatrienolides with related chemical structures (Figure 4) to identify compounds with improved features concerning P-glycoprotein inhibition without cross-resistance. Inhibition of P-glycoprotein at the BBB was analyzed by calcein assays in cultivated PBCECs. The results are shown in Figure 2B and Table II. Scillarenin revealed the highest EC_{50} (46.4±11.6 μM) and E_{max} values (510.9±73.2).

Furthermore, we determined cross-resistance of these compounds. CEM/ADR5000 cells are known to reveal high degrees of resistance to established anticancer drugs such as doxorubicin (<1000-fold), vincristine (<400-fold), paclitaxel (<200-fold) and others (45). As shown in Table II, a 20.7-fold cross-resistance of CEM/ADR5000 cells was observed towards scillaren A, while proscillaridin, acetylmarinobufogenin, and bufotalin-3-acetate revealed only low degrees of cross-resistance (1.2- to 2.0-fold). Collateral sensitivity was observed towards scillarenin and resibufogenin, *i.e.* otherwise drug-resistant CEM/ADR5000 cells were even more sensitive to this compound than were the parental drug-sensitive CCRF-CEM cells.

Table III. *Correlation of log_{10} IC_{50} values for resibufogenin to microarray-based mRNA expressions identified by COMPARE analyses in 47 NCI cell lines.*

	Compare			
Genes symbol	Genbank #	coefficient	Name	Function
none	N68924	0.645	Unknown	Unknown
NXT2	AL031387	0.619	Nuclear transport factor 2-like export factor 2	Nuclear transport?
RPL17	X53777	0.608	Ribosomal protein L17	Structural constituent of ribosome and signal transducer
NCR2	NM004828	0.604	Natural cytotoxicity triggering receptor 2	Transmembrane receptor
SHB	AL138752	–0.601	SHB (Src homology 2 domain containing) Adaptor protein B	Unknown

Since the brain represents an organ to which tumors of other origin frequently metastasize, we were also interested to investigate the activity of these six compounds on cell lines of different tumor types (tumors of brain, colon, breast, ovary, kidney, lung, prostate, melanoma or leukemia). For this reason, we took advantage of the database of the Developmental Therapeutics Program of the National Cancer Center (NCI), USA (http://dtp.nci.nih.gov). All six compounds showed considerable cytotoxicity to the panel of NCI tumor cell lines (data not shown). We correlated the IC_{50} values of these six compounds with the microarray-based expression of 11 different clones of the P-glycoprotein-coding *MDR1/ABCB1* gene, the P-glycoprotein expression-regulating miR-451 and miR-27a prec microRNAs, and the copy number of the *MDR1* gene at chromosomal locus 7q21, as well as with the accumulation of rhodamine 123 (R123) as a functional measure for P-glycoprotein. Interestingly, except for one significant correlation (resibufogenin and miR27a prec expression, R=0.502, $p<0.001$), mRNA and microRNA expression, or *MDR1* DNA copy number or R123 accumulation did not correlate at a level of R>0.5 and $p<0.05$) with IC_{50} values for these six compounds (Table VI), indicating that the cytotoxicity of these bufadienolides and -trienolides may not be hampered by multidrug resistance. Doxorubicin was used as a control drug, and significant relationships between the IC_{50} values and all of the mentioned parameters were found.

Since P-glycoprotein did not hamper the activity of bufadienolides and -trienolides, the question arises as to whether other determinants of sensitivity or resistance towards these compounds may exist in cancer cells. Therefore, we performed COMPARE analyses of the IC_{50} values for all six bufadienolides and bufatrienolides and the transcriptome-wide mRNA expression of the NCI cell lines to produce scale indices of correlation coefficients. We first performed a standard COMPARE analysis in which cell lines that were most inhibited by the compounds (lowest IC_{50} values) were correlated with the lowest mRNA expression levels of genes. These genes may be considered as possible candidate genes determining cellular resistance to drugs. Afterwards, reverse COMPARE analyses were performed, which correlated the most inhibited cell lines with the highest gene expression levels. This approach provided genes that might determine cellular sensitivity towards cytotoxic compounds. Only correlations between mRNA expression levels and IC_{50} values of R>0.6 or R<–0.6 were taken into consideration. Five genes were found to be correlated with cellular response to resibufogenin (Table III). All other correlations to bufadienolides and –trienolides did not meet the correlation criteria of R>0.6 or R<–0.6.

Table IV. *Separation of clusters of 47 NCI cell lines obtained by hierarchical cluster analysis shown in Figure 5 in comparison to drug sensitivity. The median $Log_{10}IC_{50}$ value (M) for each compound was used as a cut-off to separate tumor cell lines as being "sensitive" or "resistant".*

	Resibufogenin	
	Sensitive	Resistant
	<–5.311	>–5.311
Cluster 1	6	19
Cluster 2	18	4

Fisher exact test ($p=8.56\times10^{-5}$).

The genes obtained by COMPARE analyses for resibufogenin were subjected to hierarchical cluster analysis to obtain a dendrogram, where the cell lines are arranged according to their expression profile of these genes. The dendrogram for resibufogenin was divided into two major cluster branches (Figure 5). The median log_{10} IC_{50} value for resibufogenin was used as cut-off threshold to define cell lines as being sensitive or resistant. As can be seen in Table IV, the distribution of sensitive and resistant cell lines was significantly different between the branches of the dendrograms ($p=8.56\times10^{-5}$), indicating that cellular response to resibufogenin was indeed predictable by these genes.

Table V. *Electron donor patterns observed in phytopchemicals derived from traditional Chinese medicine. Type 1 unit denotes a hydrogen bonding acceptor group (electron donor group) with a spatial separation of 2.5 ± 0.3 Å. Type II unit is formed by either three electron donor groups with a spatial separation of the outer two electron donor groups of 4.6 ± 0.6 Å.*

	Type	Electron donor pattern	Bond length (2.2-2.8 Å)				Bond length (4.0-5.2 Å)				Comments
TCM compounds											
Baicalin	1	y	2.7	2.7	2.2	2.4	4.8				
Baicalein	1	y	2.8	2.7	2.7		4.8				
Nardofuran	1 and 2	y	2.7				4.8				
Berberine	1	y	2.2	2.8							
Ginsenoside Rh1	------	n	------								Sugar moiety shows type 1
Homoharringtonine	1 and 2	y	2.2				4.8	4.9			
Bufalin	1	y	2.3								Resonance structure
Cantharidin	2	y					4.5	4.5	4.5		
Ent.-16-atisen-19-oic acid	1	y	2.3								Resonance structure
Ent.-15-atisen-19-oic acid	1	y	2.2								Resonance structure
4-Methoxy[2,3-b]quinoline	2	y					4.2				
Carbalexine C	2	y					4.9				
Glybomine A	2	y					4.6	4.8			
Glycoborinine	2	y					4.9				
Glyomine B	2	y					4.8				
Deoxyserofendic acid	1 and 2	y	2.3				4.4	4.7			
Taccaoside C	1	y	2.3								Sugar moiety shows type 1
Taccaoside D	1	y	2.4								Sugar moiety shows type 1
Scopoletin	1	y	2.8								
Isoscopoletin	1	y	2.8								
N-*p-Trans*-coumaroyltyramine	1	y	2.3								Resonance
Shogaol	1	y	2.7								
Gingerol	1	y	2.8								
Control drugs											
Biochanin A	1 and 2	y	2.8				4.8	4.8			
Diosmetin	1 and 2	y	2.7	2.8			4.8	5.0	4.8		
Hesperitin	1 and 2	y	2.7	2.7			4.0	4.8	4.8	5.0	
Verapamil	1	y	2.7	2.8							
PSC-833	2	y					4.9	4.5	4.5	4.7	

Discussion

In the present investigation, we focused on phytochemical compounds capable of inhibiting P-glycoprotein at the BBB. P-Glycoprotein encoded by the *MDR1* (*ABCB1*) gene is a member of the ATP-binding cassette (ABC) transporter family and plays a key role as efflux transporter for maintaining brain homeostasis at the BBB. It limits the entry of xenobiotics, including a large number of drugs, into the CNS (46, 47). Since P-glycoprotein is responsible for the poor clinical outcome of many neurological diseases, it has emerged as a main therapeutic target that should be considered for drug design and development. Ideally, future CNS and anticancer drugs exhibit two characteristic features. Firstly, they show pharmacological activity towards their target of interest, and secondly, candidates are either non-substrates of P-glycoprotein or at best, inhibit efflux activity non-competitively, leading to beneficial drug regimens. Therefore, we searched for cytotoxic compounds which also inhibit P-glycoprotein. The ideal compound is highly cytotoxic towards cancer cells, does not reveal cross-resistance in multidrug-resistant cells, and inhibits P-glycoprotein in multidrug-resistant cancer cells and at the BBB (so-called "three-in-one drugs"). Five out of 57 phytochemicals tested fulfilled these criteria (baicalein, bufalin, glybomine B, deoxyserofendic acid, and shogaol). Among them, bufalin was the only compound which was active in the nanomolar range (IC_{50} in CCRF-CEM cells: 21.4±3.7 nM).

Bufalin has been shown to inhibit P-glycoprotein (48). Its inhibitory function at the BBB is a novel finding and is shown in the current investigation for the first time. Our strategy was to test whether the pharmacological features of bufalin could be improved by using derivatives. Identifying lead compounds for derivatization represents a classical approach in natural product

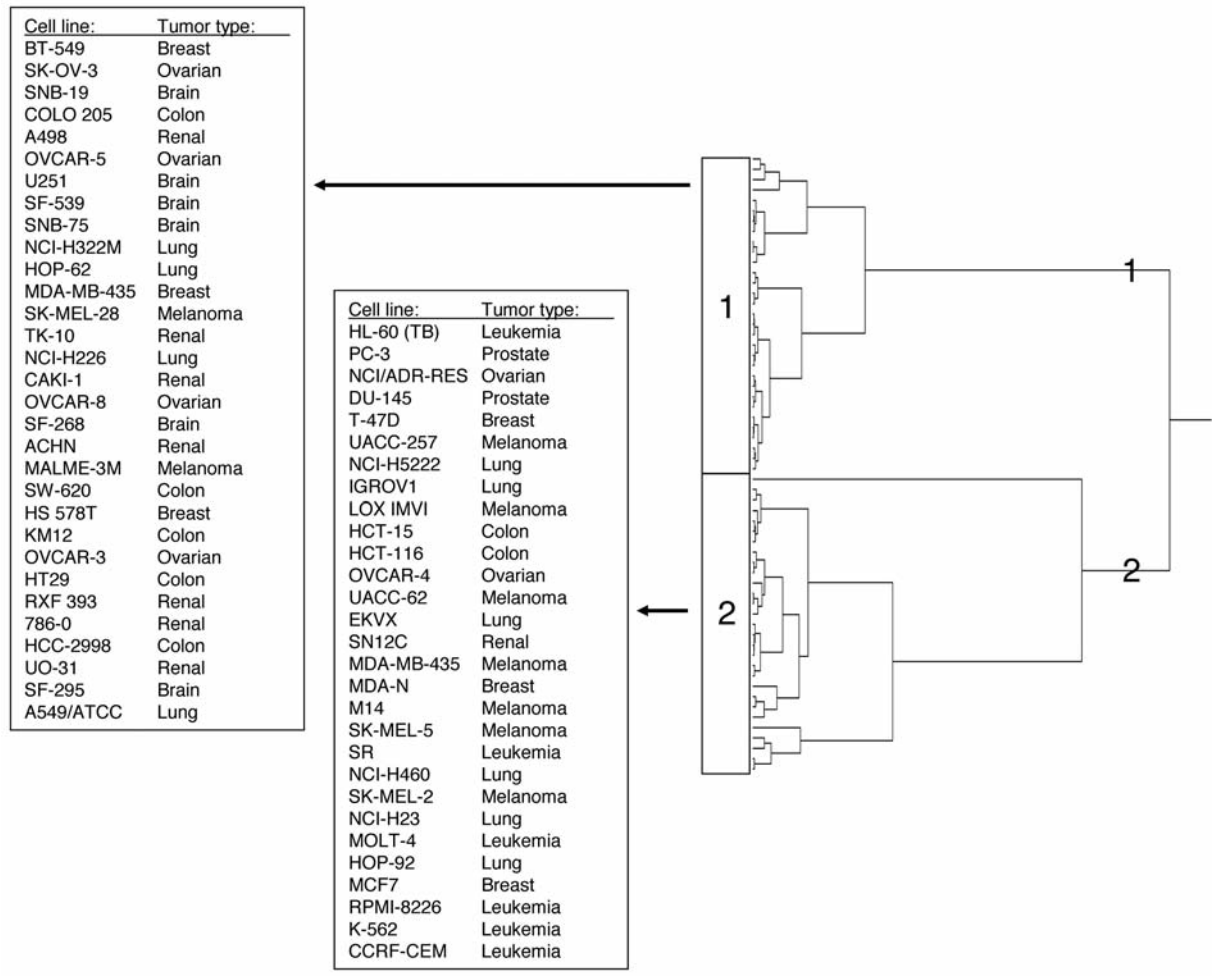

Figure 5. *Dendrogram of hierarchical cluster analysis (complete linkage method) obtained from mRNA expression of genes correlating with* $Log_{10}IC_{50}$ *values for resibufogenin. The dendrogram shows the clustering of cell lines of the NCI's screening panel.*

research. Indeed, we were able to identify one compound, scillarenin, which combines three favorable features. Firstly, it was cytotoxic to cancer cells in a nanomolar range. Secondly, P-glycoprotein-expressing multidrug-resistant CEM/ADR5000 cells were not cross-resistant, but were collateral sensitive towards scillarenin, and, thirdly, this compound was a strong inhibitor of P-glycoprotein in multidrug-resistant cancer cells and at the BBB. Hence, scillarenin may represent a novel candidate for improving the efficacy of cancer combination therapy regimens. Further analyses are warranted to characterize this compound in more detail.

A typical, but unusual feature of P-glycoprotein is that it translocates a wide variety of chemically diverse compounds. While many speculations on the mechanism of action of P-glycoprotein have been made, the only common property of P-glycoprotein substrates is their relative hydrophobic, amphiphilic nature (49, 50).

An interesting approach has been introduced by Seelig and colleagues (33, 34). They screened the structures of a hundred chemically diverse compounds, which had been previously tested as P-glycoprotein substrates, to find potential structural elements responsible for substrate-P-glycoprotein interaction. They observed that the basic elements are electron donor units formed by electron donor groups which must be arranged in distinct spatial patterns. Type I units comprise two electron donor groups with a spatial separation of 2.5±0.3Å. Type II units consist of two electron donors with a spatial separation of 4.6±0.6Å or three electron donor groups with a spatial separation of the outer two groups of 4.6±0.6Å. All molecules that contain at least one type I or one type II unit are predicted to be P-glycoprotein substrates.

Applying this approach to our set of control compounds (verapamil, PSC-833, four flavonoids) indeed revealed that all of these P-glycoprotein inhibitors fit these criteria. We

Table VI. *Correlation of bufadienolides and -trienolides with DNA copy number, mRNA expression and microRNA expression of the MDR1/ABCB1 gene as well as with P-glycoprotein function in a rhodamine 123 accumulation assay. Doxorubicin is a well-known substrate of P-glycoprotein and was used as control drug.*

Name		AMB	BA	Pros	Res	SciA	Sci	Dox
DNA copy number of *MDR1/ABCB1*,	R-value	–0.04937	0.03443	–0.11118	0.07432	–0.09319	0.20783	0.52179
Chromosome 7q21, Pattern ID: CG2550	P-value	0.37808	0.41428	0.2095	0.31995	0.26662	0.09329	2.20E-05
DNA copy number of *MDR1/ABCB1*,	R-value	–0.0339	–0.01222	–0.02638	0.04112	–0.09761	–0.04437	–1.39E-04
Chromosome 7q21, Pattern ID: CG2551	P-value	0.41456	0.46898	0.42417	0.39672	0.25933	0.38876	0.4996
MDR1/ABCB1 mRNA expression,	R-value	–0.0478	–0.01175	–0.04975	0.08388	–0.08009	0.1903	0.56276
GenBank: M14758, Pattern ID GC33586	P-value	0.37482	0.46875	0.3541	0.28753	0.28818	0.10005	1.75E-06
MDR1/ABCB1 mRNA expression,	R-value	–0.04195	–0.00315	–0.05854	0.08506	–0.07993	0.19648	0.55924
GenBank: M14758, Pattern ID: GC33587	P-value	0.38972	0.49161	0.32979	0.28485	0.28856	0.09279	2.07E-06
MDR1/ABCB1 mRNA expression,	R-value	–0.04131	–0.01544	–0.07433	0.08114	–0.08485	0.18903	0.56949
GenBank: M14758, Pattern ID: GC89510	P-value	0.39136	0.45896	0.28622	0.29384	0.27691	0.10158	1.02E-06
MDR1/ABCB1 mRNA expression,	R-value	0.01704	0.01544	–0.03884	0.07693	–0.00686	0.2097	0.62059
GenBank: M14758, Pattern ID: GC89511	P-value	0.45474	0.45896	0.38412	0.30363	0.48093	0.07856	6.14E-08
MDR1/ABCB1 mRNA expression,	R-value	–0.06226	0.02997	–0.07278	0.12767	–0.10101	0.16964	0.5197
GenBank: AA994037, Pattern ID: GC53715	P-value	0.33879	0.42074	0.29025	0.19621	0.2403	0.12715	1.04E-05
MDR1/ABCB1 mRNA expression,	R-value	0.01397	0.08768	0.0232	0.16809	–0.1452	0.3064	0.54564
GenBank: AA887211, Pattern ID: GC150426	P-value	0.46285	0.27891	0.43015	0.12936	0.15465	0.01809	3.26E-06
MDR1/ABCB1 mRNA expression,	R-value	–0.06429	–0.02487	–0.06927	0.05598	–0.07317	0.20165	0.5752
GenBank: AA887211, Pattern ID: GC194059	P-value	0.33559	0.43483	0.30267	0.35585	0.30491	0.08949	1.16E-06
MDR1/ABCB1 mRNA expression,	R-value	–0.04666	–0.01077	–0.07574	0.07998	–0.08856	0.19475	0.56835
GenBank: AF016535, Pattern ID: GC152001	P-value	0.37772	0.47133	0.28257	0.29651	0.26827	0.09478	1.08E-06
MDR1/ABCB1 mRNA expression,	R-value	–0.01914	–0.00631	–0.05923	0.08563	–0.0565	0.19854	0.58366
GenBank: AF016535, Pattern ID: GC152002	P-value	0.44917	0.48318	0.32651	0.28354	0.34685	0.09047	4.92E-07
MDR1/ABCB1 mRNA expression,	R-value	–0.04092	–0.01734	–0.06582	0.07972	–0.06587	0.19662	0.57172
GenBank: AF016535, Pattern ID: GC227857	P-value	0.39356	0.45445	0.31175	0.29921	0.323	0.09515	1.38E-06
MDR1/ABCB1 mRNA expression,	R-value	–0.03016	–0.01504	–0.06189	0.08352	–0.06446	0.19165	0.57504
GenBank: AF016535, Pattern ID: GC227858	P-value	0.42114	0.46048	0.32221	0.29052	0.32655	0.10098	1.17E-06
Mature-hsa-miR-451 (Probe 1)	R-value	0.07577	0.05128	–0.08716	0.01982	0.02517	–0.02085	0.18034
microRNA expression, Pattern ID: MT16523	P-value	0.30636	0.36604	0.2539	0.44738	0.4304	0.44468	0.08396
Mature-hsa-miR-451 (Probe 2)	R-value	0.05481	0.03553	–0.04854	–0.07296	–0.04863	–0.06646	–0.15403
microRNA expression, Pattern ID: MT16524	P-value	0.35719	0.40627	0.3563	0.31299	0.36734	0.32856	0.11998
Stemloop-hsa-miR-45m microRNA expression,	R-value	0.23284	0.12538	0.1308	0.50201	0.14466	0.36099	0.44246
Pattern ID: MT16114	P-value	0.05762	0.20051	0.15958	1.62E-04	0.15557	0.00633	2.00E-04
hsa-miR-27a, microRNA expression,	R-value	–0.10412	–0.12214	–0.09402	–0.19496	–0.07022	–0.14019	0.0905
Pattern ID: MT3074	P-value	0.24304	0.20671	0.23936	0.09455	0.31218	0.17362	0.24772
hsa-miR-27aN, microRNA expression,	R-value	–0.07868	–0.0167	–0.03837	–0.25214	–0.13511	–0.09631	0.02565
Pattern ID: MT3075	P-value	0.29954	0.45562	0.38644	0.04365	0.17224	0.25978	0.42354
Rhodamine exclusion (MDR activity),	R-value	–0.00859	–0.107	–0.02564	0.04174	–0.00191	0.08441	0.46528
Pattern ID: MT215	P-value	0.47541	0.2206	0.4203	0.38218	0.49468	0.27195	1.02E-04

AMB: Acetylmarinobufogenin; BA: bufotalin-3-acetate; Pros: proscillaridin; Res: resibufogenin; ScA: scillaren A; Sci: scillarenin; Dox: doxorubicin.

subsequently tried to predict the capability of natural products derived from TCM to inhibit P-glycoprotein and compared these *in silico* results with the experimental data. We did not, however, find a significant relationship between the features of the chemical structure and the ability to inhibit P-glycoprotein. Nevertheless, compounds not predictable by this *in silico* method were experimentally identified for their P-glycoprotein inhibitory activity. Three reasons may explain this discrepancy.

The binding of small molecules to P-glycoprotein is a matter of a long and inconclusive discussion. While initially one binding domain and subsequently two binding sites have been proposed for P-glycoprotein (3), more recent investigations suggested multiple different binding sites (51, 52). An alternative model hypothesized that P-glycoprotein extrude diverse drugs by an induced-fit mechanism (53). Recently, homology models for P-glycoprotein based on the crystal structure of the bacterial ABC transporter from *Staphylococcus aureus* Sav1866 have been described (54). Three main membrane-related binding regions in P-glycoprotein were outlined. Binding region 1 is located at the interface between the membrane and the cytosol and two

other binding regions are located in the transmembrane parts of the protein. The regions contain multiple binding pockets. Hence, it is possible that drugs, depending on their structural properties, may bind to either more hydrophobic or more hydrophilic pockets, or even to more than one pocket simultaneously. Additionally, a large binding pocket resides in the protein cavity, which may represent an "escaping" site, where the compounds that bind to any of these regions are released from the protein. Site-directed mutagenesis experiments fit to these putative binding sites of the homology models (55), but a final proof can only be delivered by drug-protein crystal structures. Considering this complex and unresolved situation, the substrate specificity of P-glycoprotein may be much broader than estimated thus far.

Furthermore, the calcein-AM assay might not reflect binding to all binding domains, or compounds may act by other mechanisms than *via* P-glycoprotein. Passive diffusion through the BBB is the primary process of translocation from blood stream to brain for most therapeutic compounds. On a molecular level, the principal diffusion barrier consists of the lipid bilayer of the brain endothelial cells. Structural criteria of drugs and features of the lipid bilayer determine the ability of drugs to cross BBB by passive diffusion (56). Therefore, it is reasonable to take parameters of passive diffusion into account to explain the crossing of the BBB by phytochemicals. Ramu *et al*. provided evidence that drugs passively diffuse into cells, and that the rate of this diffusion into drug-resistant cells is considerably lower than that found in drug-sensitive cells (57). The rates of drug entry fully explained the multidrug resistance phenotype independent of P-glycoprotein. It is therefore, possible that modulatory agents alter the membrane fluidity, hence, overcoming drug resistance. Further investigations to dissect drug-modulatory effects caused by inhibition of P-glycoprotein and by altering the membrane fluidity or integrity are warranted.

Another question is how to deal with the problem that P-glycoprotein in other normal tissues may also be affected by P-glycoprotein inhibitors. Efforts to improve cancer chemotherapy by MDR inhibitors such as verapamil or PSC-833 were not successful in clinical phase III studies (58, 59) due to increased neurotoxic side-effects. Novel MDR inhibitors may overcome these problems. However, none of them reached clinical trials yet. Concerning BBB, a possibility might be to achieve high local concentrations of MDR inhibitors by local rather than by systemic application, *e.g*. by intracerebroventricular application.

The response of tumor cells to cytotxic compounds is mostly determined by multiple other factors in addition to P-glycoprotein or other ABC transporters. For this reason, we performed COMPARE and hierarchical cluster analyses of microarray-based mRNA expression values of the 60 NCI cell lines in an effort to gain deeper insight into the multi-factorial nature of cellular response to bufadienolides and bufatrienolides. Most microarray-based mRNA expressions correlated with the IC_{50} values of the bufadienolides and bufatrienolides with correlation coefficients below R=0.6 and above R=–0.6, indicating only weak associations. However, for resibufogenin, we identified several genes *e.g*. *NXT2, RPL17, NCR2*, and *SHB*, whose expression met the criteria (R>0.6; R<–0.6). Although none of these genes have been described as being related to drug sensitivity or resistance yet, the results obtained in this study suggest that these genes may contribute to resibufogenin resistance. Further studies are warranted to clarify their causative relevance for cellular drug response.

References

1 Loscher W and Potschka H: Drug resistance in brain diseases and the role of drug efflux transporters. Nat Rev Neurosci *6*: 591-602, 2005.

2 Ballabh P, Braun A and Nedergaard M: The blood–brain barrier: an overview: structure, regulation, and clinical implications. Neurobiol Dis *16*: 1-13, 2004

3 Schinkel AH, Wagenaar E, Mol CA and van Deemter L: P-glycoprotein in the blood–brain barrier of mice influences the brain penetration and pharmacological activity of many drugs. J Clin Invest 97: 2517-2524, 1996.

4 Miller DS: Confocal imaging of xenobiotic transport across the blood-brain barrier. J Exp Zoolog A Comp Exp Biol *300*: 84-90, 2003.

5 Bart J, Groen HJ, Hendrikse NH, van der Graaf WT, Vaalburg W and de Vries EG: The blood–brain barrier and oncology: new insights into function and modulation. Cancer Treat Res *26*: 449-462, 2000.

6 Hermann DM, Kilic E, Spudich A, Kramer SD, Wunderli-Allenspach H and Bassetti CL: Role of drug efflux carriers in the healthy and diseased brain. Ann Neurol *60*: 489-498, 2006.

7 Fromm MF: Importance of P-glycoprotein at blood–tissue barriers. Trends Pharmacol Sci *25*: 423-429, 2004.

8 Gottesman MM, Fojo T and Bates SE: Multidrug resistance in cancer: role of ATP-dependent transporters. Nat Rev Cancer *2*: 48-58, 2002.

9 Ford JM, Yang JM and Hait WN: P-Glycoprotein-mediated multidrug resistance: experimental and clinical strategies for its reversal. Cancer Treat Res *87*: 3-38, 1996.

10 Schinkel AH and Jonker JW: Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. Adv Drug Deliv Rev *55*: 3-29, 2003.

11 Takara K, Sakaeda T and Okumura K: An update on overcoming MDR1-mediated multidrug resistance in cancer chemotherapy. Curr Pharm Des *12*: 273-286, 2006.

12 Kreuter J: Nanoparticles - a historical perspective. Int J Pharm *331*: 1-10, 2007.

13 Bickel U, Yoshikawa T and Pardridge WM: Delivery of peptides and proteins through the blood–brain barrier. Adv Drug Deliv Rev *46*: 247-279, 2001.

14 Nobili S, Landini I, Giglioni B and Mini E: Pharmacological strategies for overcoming multidrug resistance. Curr Drug Targets *7*: 861-879, 2006.

15 Fellner S, Bauer B, Miller DS, Schaffrik M, Fankhänel M, Spruss T, Bernhardt G, Graeff C, Färber L, Gschaidmeier H, Buschauer A and Fricker G: Transport of paclitaxel (Taxol) across the blood–brain barrier *in vitro* and *in vivo*. J Clin Invest *110*: 1309-1318, 2002.

16 Fischer V, Rodriguez-Gascon A, Heitz F, Tynes R, Hauck C, Cohen D and Vickers AE: The multidrug resistance modulator valspodar (PSC 833) is metabolized by human cytochrome P450 3A. Implications for drug–drug interactions and pharmacological activity of the main metabolite. Drug Metab Dispos *26*: 802-811, 1998.

17 Kruijtzer CM, Beijnen JH, Rosing H, ten Bokkel Huinink WW, Schot M, Jewell RC, Paul EM and Schellens JH: Increased oral bioavailability of topotecan in combination with the breast cancer resistance protein and P-glycoprotein inhibitor GF120918. J Clin Oncol *20*: 2943-2950, 2002.

18 Fracasso PM, Goldstein LJ, de Alwis DP, Rader JS, Arquette MA, Goodner SA, Wright LP, Fears CL, Gazak RJ, Andre VA, Burgess MF, Slapak CA and Schellens JH: Phase I study of docetaxel in combination with the P-glycoprotein inhibitor, zosuquidar, in resistant malignancies. Clin Cancer Res *10*: 7220-7228, 2004.

19 Efferth T, Fu YJ, Zu YG, Schwarz G, Konkimalla VS and Wink M: Molecular target-guided tumor therapy with natural products derived from traditional Chinese medicine. Curr Med Chem *14*: 2024-2032, 2007.

20 Efferth T, Miyachi H and Bartsch H: Pharmacogenomics of a traditional Japanese herbal medicine (Kampo) for cancer therapy. Cancer Genomics Proteomics *4*: 81-91, 2007.

21 Efferth T, Li PC, Konkimalla VS and Kaina B: From traditional Chinese medicine to rational cancer therapy. Trends Mol Med *13*: 353-361, 2007.

22 Efferth T, Kahl S, Paulus K, Adams M, Rauh R, Boechzelt H, Hao X, Kaina B and Bauer R: Phytochemistry and pharmacogenomics of natural products derived from traditional Chinese medicine and Chinese *materia medica* with activity against tumor cells. Mol Cancer Ther *7*: 152-161, 2008.

23 Wang J, Di Y, Yang X, Li S, Wang Y and Hao X: Hydroquinone diglycoside acyl esters from the stems of *Glycosmis pentaphylla*. Phytochemistry *67*: 486-491, 2006.

24 Wang YF, Lai GF, Efferth T, Cao JX and Luo SD: New glycosides from *Tetracentron sinense* and their cytotoxic activity. Chem Biodivers *3*: 1023-1030, 2006.

25 Hu M, Chen J and Lin H: Metabolism of flavonoids *via* enteric recycling: mechanistic studies of disposition of apigenin in the Caco-2 cell culture model. J Pharmacol Exp Ther *307*: 314-321, 2003.

26 Fricker G and Miller DS: Modulation of drug transporters at the blood–brain barrier. Pharmacology *70*: 169-176, 2004.

27 Huwyler J, Drewe J, Klusemann C and Fricker G: Evidence for P-glycoprotein-modulated penetration of morphine-6-glucuronide into brain capillary endothelium. Br J Pharmacol *118*: 1879-1885, 1996.

28 Bauer B, Miller DS and Fricker G: Compound profiling for P-glycoprotein at the blood–brain barrier using a microplate screening system. Pharm Res *20*: 1170-1176, 2003.

29 Gillet JP, Efferth T, Steinbach D, Hamels J, de Longueville F, Bertholet V and Remacle J: Microarray-based detection of multidrug resistance in human tumor cells by expression profiling of ATP-binding cassette transporter genes. Cancer Res *64*: 8987-8993, 2004.

30 Schramm U, Fricker G, Wenger R and Miller DS: P-glycoprotein-mediated secretion of a fluorescent cyclosporin analogue by teleost renal proximal tubules. Am J Physiol *268*: F46-F52, 1995.

31 Kimmig A, Gekeler V, Neumann M, Frese G, Handgretinger R, Kardos G, Diddens H and Niethammer D: Susceptibility of multidrug-resistant human leukemia cell lines to human interleukin 2-activated killer cells. Cancer Res *50*: 6793-6799, 1990.

32 Efferth T, Davey M, Olbrich A, Rücker G, Gebhart E and Davey R: Activity of drugs from traditional Chinese medicine towards sensitive and MDR1- or MRP1-overexpressing multidrug-resistant human CCRF-CEM leukemia cells. Blood Cells Mol Dis *28*: 160-168, 2002.

33 Seelig A: A general pattern for substrate recognition by P-glycoprotein. Eur J Biochem *251*: 252-261, 1998.

34 Seelig A and Landwojtowicz E: Structure–activity relationship of P-glycoprotein substrates and modifiers. Eur J Pharm Sci *12*: 31-40, 2000.

35 Bubik M, Ott M, Mahringer A and Fricker G: Rapid assessment of P-glycoprotein–drug interactions at the blood–brain barrier. Anal Biochem *358*: 51-58, 2006.

36 Eneroth A, Astrom E, Hoogstraate J, Schrenk D, Conrad S, Kauffmann HM and Gjellan K: Evaluation of a vincristine resistant Caco-2 cell line for use in a calcein AM extrusion screening assay for P-glycoprotein interaction. Eur J Pharm Sci *12*: 205-214, 2001.

37 Scherf U, Ross DT, Waltham M, Smith LH, Lee JK, Tanabe L, Kohn KW, Reinhold WC, Myers TG, Andrews DT, Scudiero DA, Eisen MB, Sausville EA, Pommier Y, Botstein D, Brown PO and Weinstein JN: A gene expression database for the molecular pharmacology of cancer. Nat Genet *24*: 236-244, 2000.

38 Wosikowski K, Schuurhuis D, Johnson K, Paull KD, Myers TG, Weinstein JN and Bates SE: Identification of epidermal growth factor receptor and erbB2 pathway inhibitors by correlation with gene expression patterns. J Natl Cancer Inst *89*: 1505-1515, 1997.

39 Efferth T and Volm M: Protein expression profiles indicative for drug resistance of kidney carcinoma. Cancer Genomics Proteomics *1*: 17-22, 2004.

40 Volm M, Koomägi R and Efferth T: Prediction of drug sensitivity and resistance of cancer by protein expression profiling. Cancer Genomics Proteomics *1*: 157-166, 2004.

41 Efferth T, Koomägi R, Mattern J and Volm M: Lung cancer protein expression profiles of smokers and non-smokers. Cancer Genomics Proteomics *1*: 249-254, 2004.

42 Efferth T and Kaina B: Microarray-based prediction of cytotoxicity of tumor cells to arsenic trioxide. Cancer Genomics Proteomics *1*: 363-370, 2004.

43 Efferth T, Chen Z, Kaina B and Wang G: Molecular determinants of response of tumor cells to berberine. Cancer Genomics Proteomics *2*: 115-124, 2005.

44 Morris ME and Zhang S: Flavonoid-drug interactions: effects of flavonoids on ABC transporters. Life Sci *78*: 2116-2130, 2006.

45 Efferth T, Konkimalla VB, Wang YF, Sauerbrey A, Meinhardt S, Zintl F, Mattern J and Volm M: Prediction of broad-spectrum resistance of tumors towards anticancer drugs. Clin Cancer Res *14*: 2405-2412, 2008.

46 Weiss J, Dormann SM, Martin-Facklam M, Kerpen CJ, Ketabi-Kiyanvash N and Haefeli WE: Inhibition of P-glycoprotein by newer antidepressants. J Pharmacol Exp Ther *305*: 197-204, 2003.

47 Kemper EM, Boogerd W, Thuis I, Beijnen JH and van Tellingen O: Modulation of the blood–brain barrier in oncology: therapeutic opportunities for the treatment of brain tumours? Cancer Treat Rev *30*: 415-423, 2004.
48 Lee G, Dallas S, Hong M and Bendayan R: Drug transporters in the central nervous system: brain barriers and brain parenchyma considerations. Pharmacol Rev *53*: 569-596, 2001.
49 Gottesman MM and Pastan I: Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu Rev Biochem *62*: 385-427, 1993.
50 Rautio J, Humphreys JE and Webster LO: *In vitro* P-glycoprotein inhibition assays for assessment of clinical drug interaction potential of new drug candidates: a recommendation for probe substrates. Drug Metab Dispos *34*: 786-792, 2006.
51 Ayesh S, Shao YM and Stein WD: Co-operative, competitive and non-competitive interactions between modulators of P-glycoprotein. Biochim Biophys Acta *1316*: 8-18, 1996.
52 Borgnia MJ, Eytan GD and Assaraf YG: Competition of hydrophobic peptides, cytotoxic drugs, and chemosensitizers on a common P-glycoprotein pharmacophore as revealed by its ATPase activity. J Biol Chem *271*: 3163-3171, 1996.
53 Safa AR: Identification and characterization of the binding sites of P-glycoprotein for multidrug resistance-related drugs and modulators. Curr Med Chem Anticancer Agents *4*: 1-17, 2004.
54 Globisch C, Pajeva IK and Wiese M: Identification of putative binding sites of P-glycoprotein based on its homology model. Chem Med Chem *3*: 280-295, 2008.
55 Shapiro AB and Ling V: Extraction of Hoechst 33342 from the cytoplasmic leaflet of the plasma membrane by P-glycoprotein. Eur J Biochem *250*: 122-129, 1997.
56 Loo TW, Bartlett MC and Clarke DM: Val133 and Cys137 in transmembrane segment 2 are close to Arg935 and Gly939 in transmembrane segment 11 of human P-glycoprotein. J Biol Chem *279*: 18232-18238, 2004.
57 Ramu A, Pollard HB and Rosario LM: Doxorubicin resistance in P388 leukemia–evidence for reduced drug influx. Int J Cancer 44: 539-547, 1989.
58 Dalton WS, Crowley JJ, Salmon SS, Grogan TM, Laufman LR, Weiss GR and Bonnet JD: A phase II randomized study of oral verapamil as a chemosensitizer to reverse drug resistance in patients with refractory myeloma. A Southwest Oncology Group study. Cancer *75*: 815-820, 1995.
59 Baer MR, George SL and Dodge RK: Phase 3 study of the multidrug-resistance modulator PSC-833 in previously untreated patients 60 years of age and older with acute myeloid leukemia: Cancer and Leukemia Group B Study 9720. Blood *100*: 1224-1232, 2002.

Received April 6, 2009
Revised May 25, 2010
Accepted May 28, 2010