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Cytotoxicity of the indole alkaloid reserpine from *Rauwolfia serpentina* against drug-resistant tumor cells

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ABSTRACT

Background: The antihypertensive reserpine is an indole alkaloid from *Rauwolfia serpentina* and exerts also profound activity against cancer cells *in vitro* and *in vivo*. The present investigation was undertaken to investigate possible modes of action to explain its activity toward drug-resistant tumor cells.
Material and methods: Sensitive and drug-resistant tumor cell lines overexpressing P-glycoprotein (*ABCB1/MDR1*), breast cancer resistance protein (*ABCG2/BCRP*), mutation-activated epidermal growth factor receptor (*EGFR*), wild-type and p53-knockout cells as well as the NCI panel of cell lines from different tumor origin were analyzed. Reserpine's cytotoxicity was investigated by resazurin and sulforhodamine assays, flow cytometry, and COMPARE and hierarchical cluster analyses of transcriptome-wide microarray-based RNA expressions.
Results: P-glycoprotein- or BCRP overexpressing tumor cells did not reveal cross-resistance to reserpine. EGFR-overexpressing cells were collateral sensitive and p53- Knockout cells cross-resistant to this drug compared to their wild-type parental cell lines. Reserpine increased the uptake of doxorubicin in P-glycoprotein-overexpressing cells, indicating that reserpine inhibited the efflux function of P-glycoprotein. Using molecular docking, we found that reserpine bound with even higher binding energy to P-glycoprotein and EGFR than the control drugs verapamil (P-glycoprotein inhibitor) and erlotinib (EGFR inhibitor). COMPARE and cluster analyses of microarray data showed that the mRNA expression of a panel of genes predicted the sensitivity or resistance of the NCI tumor cell line panel with statistical significance. The genes belonged to diverse pathways and biological functions, *e.g.* cell survival and apoptosis, EGFR activation, regulation of angiogenesis, cell mobility, cell adhesion, immunological functions, mTOR signaling, and Wnt signaling.
Conclusion: The lack of cross-resistance to most resistance mechanisms and the collateral sensitivity in EGFR-transfectants compared to wild-type cells speak for a promising role of reserpine in cancer chemotherapy. Reserpine deserves further consideration for cancer therapy in the clinical setting.

Introduction

Reserpine is an indole alkaloid derived from the roots of *Rauwolfia serpentina* and serves as potent antihypertensive drug (Panda et al. 2012) It represents an established second line treatment against hypertension (Milne and Pinkney-Atkinson 2004; Pillay 2009).

Reserpine mediates the depletion of neurotransmitters from postganglionic nerve endings, which consequently lower arterial pressure and total peripheral resistance ultimately leading to decreased heart rates and cardiac output. Its administration together with diuretics effectively lowers arterial pressure and significantly reduces morbidity and mortality related to hypertension (Bakris and Frohlich 1989).

Early studies started at the mid-1950s reported anti-tumor effect of reserpine *in vivo* independent from its cardiovascular action. Experimental studies in mice bearing advanced leukemia, reserpine increased animals' life span by three-fold (Burton et al. 1956). Other studies reported the anti-tumor activity of reserpine in different mouse sarcomas *in vivo* (Belkin and Hardy 1957; Nelson et al. 1981).

Since the 1980s, attention was paid toward phenomena related to chemotherapy failure (Efferth 2001; Gillet et al. 2007; Eichhorn and Efferth 2012) and the ATP binding cassette (ABC) transporter, P-glycoprotein (ABCB1), which confers multidrug resistance (MDR) by energy-dependent drug efflux process (Hall et al. 2009). Drugs such as verapamil, quinidine, tamoxifen, progesterone, rapamycin,

Abbreviations: ABC, ATP-binding cassette; BCRP, Breast cancer resistance protein; EGFR, Epidermal growth factor receptor; MAPK, Mitogen-activated protein kinase; MDR, Multidrug resistance; MSH2, MutS homologue 2, mismatch repair enzyme; mTOR, Mammalian target of rapamycin; NCI, National Cancer Institute; PI3K, Phosphoinositid-3-kinase; STAT3, Signal transducer and activator of transcription 3.

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cyclosporins and others were found to inhibit P-glycoprotein and to overcome MDR (Arceci 1993). Interestingly, reserpine was also reported as P-glycoprotein inhibitor. It suppressed photolabeling of P-glycoprotein with a vinblastine analogue in MDR cell lines (Akiyama et al. 1988). Reserpine and other indole alkaloids enhanced the sensitivity of MDR cancer cells toward cytotoxic agents (Beck et al. 1988; Zamora et al. 1988).

In this study, we investigated the role of reserpine in different cancer cell lines in an approach to understand possible molecular modes of action. Since MDR is multifactorial in nature and other mechanisms in addition to P-glycoprotein also contribute to unresponsiveness of tumors, we also investigated several other mechanisms. The ABC-transporter BCRP/ABCG2, the mutated tumor suppressor gene p53 and the activated epidermal growth factor receptor (EGFR) all mediate drug resistance (El-Deiry 1997, 2003; Gillet et al. 2007). Therefore, we investigated, whether or not cell lines expressing these genes reveal cross-resistance to reserpine. The intention was to prove, whether reserpine could be used to bypass drug-resistance and to eradicate otherwise unresponsive tumors by reserpine.

Material and methods

Cell culture

Drug-sensitive CCRF-CEM and multidrug-resistant CEM/ADR5000 leukemia cell lines were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 1% penicillin (100 U/ml)-streptomycin (100 μg/ml) (PIS) antibiotic (Invitrogen) and incubated in humidified 5% CO_2 atmosphere at 37 °C.

Breast cancer cells transduced with control vector (MDA-MB-231-pcDNA3) or with a cDNA for the breast cancer resistance protein BCRP (MDA-MB-231BCRP clone 23), human wild-type HCT116 ($p53^{+/+}$) colon cancer cells as well as knockout clones HCT116 ($p53^{-/-}$) derived by homologous recombination, non-transduced human U87MG glioblastoma multiforme cells and U87MG cells transduced with an expression vector harboring an epidermal growth factor receptor (*EGFR*) gene with a genomic deletion of exons 2 through 7 (U87MG.ΔEGFR) were all maintained in DMEM medium, supplemented with 10% FBS and 1% penicillin-streptomycin and incubated under standard conditions as described for leukemia cell lines.

The resistance of the different resistant cell lines has been maintained by using 5000 ng/ml doxorubicin for CEM/ADR5000, 400 μg/ml geneticin for U87MG.ΔEGFR and HCT116 ($p53^{-/-}$) and 800 ng/ml of the same compound for MDA-MB-231 BCRP clone 23.

The glioblastoma cell lines were kindly provided by Dr. W. K. Cavenee (Ludwig Institute for Cancer Research, San Diego, CA). Transfected breast cancer cell lines were a generous gift from Dr. B. Vogelstein and H. Hermeking (Howard Hughes Medical Institute, Baltimore, MD). The leukemia cells were kindly provided by Dr. J. Beck (Department of Pediatrics, University of Greifswald, Greifswald, Germany).

Cytotoxicity assay

The cytotoxicity of reserpine has been investigated using the resazurin reduction assay (Borra et al. 2009). Resazurin is an indicator dye, which is reduced in viable cells to highly fluorescent resorufin, in contrast to non-viable cells, which lost their metabolic capability and are not able reduce resazurin. In total 96-well cell culture plate (Thermo Scientific, Germany) were seeded with 20,000 cells/well in a total volume of 100 μL, and then treated with different concentrations of reserpine diluted in 100 μL medium. Adherently growing cells were allowed to attach overnight and treated after 24 h. The cells were incubated with reserpine for 72 h. Then, 0.01% of resazurin (Sigma-Aldrich, Germany) diluted in double distilled water (ddH_2O) was added (20 μL/well) and incubated for another 4 h. Infinite M2000 Pro™ plate reader (Tecan, Germany) was used to measure the fluorescence using an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Experiments were performed three times with at least with six replicates per experiment. The 50% inhibition concentrations (IC_{50}) were calculated from dose response curves of each cell using Microsoft Excel 2013 software.

Doxorubicin uptake assay

Flow cytometry has been used to measure the retention of doxorubicin. Doxorubicin is substrate of P-glycoprotein and its inherent fluorescence was used to assess the efflux activity of this drug transporter. CCRF-CEM and CEM/ADR5000 cells were seeded in phenol red–free RPMI 1640 medium in a concentration of 5×10^5 cells/well in 12 well plates at a total volume 500 μL. Then, cells were treated with medium containing 10 μM doxorubicin with and without reserpine (15 μM). Cells were incubated at 37 °C in an atmosphere containing 5% CO_2 for 3 h, which is the time required for maximum doxorubicin uptake (Krishan and Hamelik, 2005). Finally, cells were washed to remove free doxorubicin. Cells were measured on LSR-Fortessa FACS analyzer (Becton-Dickinson, Germany) equipped with argon blue laser, the excitation and emission wavelength of doxorubicin were 488 nm and 610/20 nm, respectively. Only living cells, identified by DAPI to stain dead cells were considered for the analyses. Data were processed by Flowjo software. Three controls were taken, unstained cells to determine auto-fluorescence, cells treated with doxorubicin alone to measure the efflux efficacy, and in combination with verapamil as positive control for a P-glycoprotein inhibitor.

Molecular docking

AutoDock4 (Hetenyi and Van Der Spoel 2002) was used for molecular docking calculations of reserpine. A homology model human ABCB1 based on the crystal structure of murine P-glycoprotein was previously constructed by us (Zeino 2014; Tajima et al. 2014) and used in the present investigation for binding site determination of reserpine. Verapamil was included in our analyses as control drug for a well-known P-glycoprotein inhibitor.

Molecular docking of reserpine to EGFR was done on the ATP-binding site of EGFR kinase domain. The crystal structure of EGFR was retrieved from The Protein Data BANK (Database code PDB1M17; http://www.rcsb.org/pdb/home/home.do). Erlotinib which is an irreversible tyrosine kinase inhibitor of EGRF was taken as control drug for docking. The 3D structure of reserpine, verapamil and erlotinib were downloaded from PubChem (pubchem.ncbi.nlm.nih.gov).

Drug binding residues of ABCB1 were identified as His61, Gly64, Leu65, Met69, Ser222, Leu304, Ile306, Tyr307, Phe336, Leu339, Ile340, Ala342, Phe343, Gln725, Phe728, Phe732, Leu762, Thr837, Ile868, Gly872, Phe942, Thr945, Tyr953, Leu975, Phe978, Ser979, Val982, Gly984, Ala985, Met986, Gly989, Gln990, and Ser993 (Aller et al. 2009). At the other hand, residues identified as binding sites for EGFR include Leu620, Leu694, Phe699, Val702, Ala719, Lys721, Met742, Leu764, Thr766, Gln767, Met769, Pro770, Cys773, Thr830 and Asp831 (Yadav et al. 2014).

Crude BDP structures of the receptors proteins were refined, energy minimized and polar hydrogens were added and saved as pdbqt files. Then, the grid map parameters were set to cover the defined residues. Numbers of runs and energy evaluations were reset to 250 and 25,000,000, respectively. Docking calculations were performed using Lamarckian Genetic Algorithm. For image visualization of docking results, Visual Molecular Dynamics (VMD) software was used.