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Original Articles

Triapine-mediated ABCB1 induction via PKC induces widespread therapy unresponsiveness but is not underlying acquired triapine resistance

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ABSTRACT

Although triapine is promising for treatment of advanced leukemia, it failed against solid tumors due to widely unknown reasons. To address this issue, a new triapine-resistant cell line (SW480/tria) was generated by drug selection and investigated in this study. Notably, SW480/tria cells displayed broad cross-resistance against several known ABCB1 substrates due to high ABCB1 levels (induced by promoter hypomethylation). However, ABCB1 inhibition did not re-sensitize SW480/tria cells to triapine and subsequent analysis revealed that triapine is only a weak ABCB1 substrate without significant interaction with the ABCB1 transport function. Interestingly, in chemo-naive, parental SW480 cells short-time (24 h) treatment with triapine stimulated ABCB1 expression. These effects were based on activation of protein kinase C (PKC), a known response to cellular stress. In accordance, SW480/tria cells were characterized by elevated levels of PKC. Together, this led to the conclusion that increased ABCB1 expression is not the major mechanism of triapine resistance in SW480/tria cells. In contrast, increased ABCB1 expression was found to be a consequence of triapine stress-induced PKC activation. These data are especially of importance when considering the choice of chemotherapeutics for combination with triapine.

Introduction

It is well-known that tumor cells require enhanced iron levels due to their higher proliferative activity [1]. One reason is the constant need of deoxyribonucleotides (dNTPs) generated by the iron-dependent enzyme ribonucleotide reductase (RR). Consequently, enhanced levels of this enzyme are frequently reported for many cancer types, which makes RR an attractive target for cancer treatment [2]. RR inhibition leads to dNTP pool depletion and subsequent cell cycle arrest. To take advantage of this RR-mediated iron dependency, several iron chelators have been developed for anticancer therapy during the last decades [1]. Of these, the most promising representatives belong to the class of thiosemicarbazones (TSC) and 3-aminopyridine-2-carboxaldehyde thiosemicarbazone, also called triapine or 3-AP, is the best developed TSC. Noteworthy, while this drug showed promising activity against advanced leukemia [2,3] in clinical phase I and II trials, neither an increase in patients' survival nor response rate (single treatment or combination therapy) could be observed in solid tumors [4–8]. The reason for this lack of efficacy of triapine in solid tumors is currently relatively unknown.

In general, the occurrence of acquired or intrinsic drug resistance is one of the most important reasons for the failure of systemic cancer therapy [9,10]. Moreover, relapsing tumors are frequently not only resistant against first line treatment, but also against several other chemotherapeutic agents, a phenomenon called multidrug resistance (MDR) [10]. Key players in MDR are members of the

Abbreviations: ABCB1, P-glycoprotein; ABCC1, multidrug resistance-associated protein; AP-1, activator protein 1; CSA, cyclosporine A; DFO, desferrioxamine; DNMT1, DNA methyltransferase 1; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; IRP1, iron-responsive element-binding protein 1; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MDR, multidrug resistance; MGMT, O6-methylguanine DNA methyltransferase; MMR, mismatch repair; Nrf2, nuclear factor erythroid-2 related factor 2; rOCT1, organic cation transporter 1; PI3K, phosphatidylinositide 3-kinase; PKC, protein kinase C; ROS, reactive oxygen species; RR, ribonucleotide reductase; TSC, thiosemicarbazone.

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ATP-binding cassette (ABC) transporter family, like ABCB1 (P-glycoprotein, P-gp) [9] and ABCC1 (multidrug resistance protein 1, MRP1) [10]. ABC transporter-mediated drug resistance is often based on profound upregulation of protein expression, which is frequently associated with amplification of the ABC transporter gene, especially in the case of acquired resistance by drug selection [11,12].

Notably, the physiological function of these transporters is to protect the healthy tissue (e.g. colon, liver or kidney) against exo- and endotoxins [10]. Also cancer cells from these origins often exhibit intrinsic low-level ABC transporter expression and short-term drug/toxin exposure is known to occasionally induce transient expression of ABCB1 [13]. The regulatory mechanisms underlying this rapid protective response are multi-faceted and complex. However, activation of protein kinase C (PKC) is one of the most prominent mechanisms [14]. Thus, enhanced PKC expression has been directly linked to increased ABCB1 phosphorylation, expression, and activity [15]. PKC family members belong to the group of serine/threonine kinases and take part in numerous cellular processes like proliferation, apoptosis and migration [16]. At least ten PKC isoforms are known which are divided into three groups: classical PKCs (α, β I/II, γ), novel PKCs (δ, ε, η, θ, μ) and atypical PKCs (ζ, λ) [17]. Especially PKC alpha is involved in regulation of ABCB1 expression as described e.g. in ovarian cancer cells [18] and colon carcinoma cells [14], but also the PKC beta isoforms seem to play an important role in this process [18].

In the present study, the first triapine-resistant cancer cell model was developed to gain more insights into the mechanisms underlying treatment failure of triapine in solid cancers. As triapine was reported as an ABCB1 substrate and upregulation of this transporter is associated with triapine-resistance in a murine leukemia model [19], our study focused on the role of ABCB1 in chemoresistance of our new triapine-resistant cell model.

Materials and methods

Drugs and chemicals

Triapine and pyridine-2-carbaldehyde thiosemicarbazone (KP1553) were synthesized at the Institute of Inorganic Chemistry of the University of Vienna [20]. Verapamil was purchased from Abbott (IL, USA), cyclosporine A (CSA) from Sandoz (Basel, Switzerland), bisindolylmaleimide I from Cayman Chemicals (Michigan, USA), enzastaurin from Eli Lilly (Indiana, USA), and JNK inhibitor II from Calbiochem (California, USA). For analytical measurements, formic acid (98–100%, Merck, Darmstadt, Germany), ammonium formate (>99%, Fluka, Vienna, Austria) and sodium chloride (99.99%, Merck KGaA) of Suprapur® quality were used. Acetonitrile and water (Fluka) were purchased of LC MS grade. All other compounds were supplied by Sigma–Aldrich.

Cell culture

The human cancer cell lines and their respective drug-resistant sublines used in this study are given in Supplementary Table S1 [21]. All cell lines were grown in RPMI-1640 supplemented with 10% FCS with the exception of SW480 cells, which were grown in MEM with 10% FCS. The ABC-transporter expression was confirmed by Western blotting.

Selection of SW480/tria cells

A detailed description of the selection process of SW480/tria is given in the Supplementary material and methods.

Cell viability assay

To determine cell viability, 2×10^4 cells/ml were plated on 96-well plates (100 μl/well) and allowed to recover for 24 h. Then, cells were exposed to the test drugs for the indicated concentrations for 72 h. Anticancer activity was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based vitality assay (EZ4U; Biomedica, Vienna, Austria) following the manufacturer's recommendations [21]. Cell viability was calculated using the Graph Pad Prism software (using a point-to-point function) and was expressed as IC_{50} values calculated from full dose–response curves (drug concentrations inducing a 50% reduction of cell number in comparison to untreated control cells cultured in parallel).

Western Blot analysis

Total protein lysates of membrane-enriched extracts were prepared, separated by SDS-PAGE, and transferred onto a polyvinylidene difluoride membrane for Western blotting as described previously [21]. Primary antibodies used are given in Supplementary Table S2. Secondary, horseradish peroxidase-labeled antibodies from Santa Cruz Biotechnology were used in working dilutions of 1:10 000.

Rhodamine 123 (Rh123) accumulation assay

Accumulation of the fluorescent ABCB1 substrate Rh123 was determined in HL60 and SW480 cells and their respective ABCB1-overexpressing sublines HL60/vinc and SW480/tria as previously published [21].

Cellular drug uptake

For detection of the intracellular triapine levels a novel HPLC method has been established. A detailed description is given in the Supplementary material and methods.

ABCB1-ATPase assay

ATPase assay kit MDR1-Sf9 was purchased from Solvo Biotechnologies (Szeged, Hungary). The assay was done according to the manufacturer's protocol. ATPase activity was calculated from OD values (620 nm) reflecting the amount of inorganic phosphate (Pi) liberated/mg ABCB1 membrane protein/minute which is proportional to its transport activity [22].

RNA isolation and real-time PCR

Total RNA was isolated with Trizol reagent. mRNA was transcribed into cDNA and real-time polymerase chain reaction (PCR) was performed as described [23] using following primers: ABCB1 sense: 5′-CCCATCATTGCAATAGCAGG-3′ and ABCB1 antisense: 5′-GTTCAAACTTCTGCTCCTGA-3′. β-actin primer sequence: β-actin sense: 5′-GGATGCAGAAGGAGATCACTG-3′ and β-actin antisense: 5′-CGATCCACACGGAGTACTTG-3′. β-actin served as a control.

Determination of promoter methylation levels and pyrosequencing

To gain more insights into the epigenetic regulation of ABCB1 and ABCC1 expression promoter methylation levels were determined and pyrosequencing analysis was performed as described in detail in the Supplementary material and methods.

Array comparative genomic hybridization (array CGH, aCGH)

aCGH analyses were done as previously published [24] using 4x44K oligonucleotide-based microarrays (Agilent). Labeling and hybridization procedures were performed according to protocols provided by Agilent. For direct comparison of SW480 and SW480/tria, indirect aCGH was performed: SW480 (instead of normal human reference DNA) was labeled with Cy3 and SW480/tria cells with Cy5.

PKC knock-down by siRNA

siRNAs were transfected with Lipofectamine 2000 (Invitrogen) using 100 nM of multiple PKC siRNA (α, β, γ, δ, ε, η, μ, and ζ; Santa Cruz Biotechnology) or scrambled siRNA (Dharmacon) following the manufacturer's recommendations. Specificity of downregulation was proven at the protein level by Western blot following 48 h of transfection (Supplementary Fig. S1).

Results

Selection of triapine-resistant SW480 cells and characterization of cross-resistance

SW480/tria cells were generated by continuous exposure of SW480 cells to increasing concentrations of triapine (starting point 0.05 μM; end point 20 μM) over a period of one year. At the end of selection, SW480/tria cells showed a >56-fold resistance compared to parental SW480 cells. In addition, the triapine-resistant cell line exhibited a broad cross-resistance against several other chemotherapeutics as shown in Table 1. Notably, reduced sensitivity against several ABCB1 substrates like vincristine, taxol, adriamycin, and etoposide was observed. In addition, 2-formylpyridine thiosemicarbazone KP1553 was tested and the triapine-resistant subline exhibited more than 4-fold cross-resistance to this derivative.