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Original Articles

Modulating cancer multidrug resistance by sertraline in combination with a nanomedicine

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ABSTRACT

Inherent and acquired multiple drug resistance (MDR) to chemotherapeutic drugs is a major obstacle in cancer treatment. The ATP Binding Cassettes (ABC) transporter super family that act as extrusion pumps such as P-glycoprotein and multidrug-resistance-associated-proteins have prominent roles in cancer MDR. One of the most efficient strategies to modulate this active drug efflux from the cells is to physically block the pump proteins and thus change the balance between drug influx and efflux toward an accumulation of drug inside the cell, which eventually cumulates into cell death. MDR modulators (also known as chemosensitizers) were found among drugs approved for non-cancer indications. Yet, toxicity, adverse effects, and poor solubility at doses required for MDR reversal prevent their clinical application. Previous reports have shown that drugs belonging to the selective serotonin reuptake inhibitors (SSRI) family, which are clinically used as antidepressants, can act as effective chemosensitizers both *in vitro* and *in vivo* in tumor bearing mouse models. Here, we set out to explore whether sertraline (Zoloft®), a molecule belonging to the SSRI family, can be used as an MDR modulator. Combining sertraline with another FDA approved drug, Doxil® (pegylated liposomal doxorubicin), is expected to enhance the effect of chemotherapy while potentially reducing adverse effects. Our findings reveal that sertraline acts as a pump modulator in cellular models of MDR. In addition, in an aggressive and highly resistant human ovarian xenograft mouse model the use of sertraline in combination with Doxil® generated substantial reduction in tumor progression, with extension of the median survival of tumor-bearing mice. Taken together, our results show that sertraline could act as a clinically relevant cancer MDR inhibitor. Moreover, combining two FDA approved drugs, DOXIL®, which favor the influx of chemotherapy inside the malignant cell with sertraline, which blocks the extrusion pumps, could readily be available for clinical translation in the battle against resistant tumors.

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Introduction

Cancer is a leading cause of death worldwide, causing 8.2 million deaths in 2012 according to the World Health Organization (WHO). The WHO expects that the annual cancer cases will rise from 14 million in 2012 to 22 million within the next two decades [1].

Chemotherapy remains the principal therapeutic modality in cancer treatment at all stages, but its efficacy remains suboptimal. A major factor in therapeutic failure for cancer involves the development of drug resistance to a variety of structurally unrelated anticancer drugs, also known as multiple drug resistance (MDR). In the clinic, MDR occurs in over 50% of patients, whose cancer relapses, accounting in large part for the high mortality associated with cancer [2–6].

Tumors may intrinsically be resistant to drug treatment. This phenomenon often occurs in tumors originating from epithelial cells such as renal or adrenal tumors, which naturally express high levels of efflux pumps as part of their cellular clearance machinery. Acquired resistance, on the other hand, arises following therapy, and tumors normally present with the MDR phenotype subsequent to various genetic changes [3,4].

In the dominant MDR mechanism intracellular levels of cytotoxic drugs are reduced below lethal thresholds by active extrusion of cytotoxic drugs from the tumor cell. This phenomenon is attributed to over-expression of ATP-dependent extrusion pumps from the ABC protein super family, such as: P-gp (MDR1; ABCB1), MRP-1 (ABCC1), and BRCP (MXR; ABCP; ABCG2). These proteins, although sharing relative modest homology, transport a wide variety of structurally and functionally diverse substrates [3,4].

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Blocking the flow of chemotherapeutic drugs out of MDR cells by pump inhibition has been for long the mainstay approach to resistance reversal [5,7].

Yet, the first two generations of chemosensitizers, drawn from drugs approved for other indications and their derivatives, did not progress to become established clinical modalities, mainly due to adverse effects and toxicity. Moreover, given tumor heterogeneity, it is rational to assume that more than one chemosensitizer will be needed in the clinic [5,8,9].

In an earlier study [7], we reported that fluoxetine (Prozac®), the well-known antidepressant, a member of the selective serotonin reuptake inhibitors (SSRI) family, acts as a highly effective chemosensitizer on P-gp expressing cells [10,11]. These previous findings led us to explore whether other members from this family such as sertraline (Zoloft®) could also modulate resistance in cells overexpressing efflux pumps.

In this study, we have chosen the human ovarian adenocarcinoma cell line NCI/ADR-Res (NAR) and its parent line (OVCAR-8) as our model system for MDR. In ovarian cancer, MDR is considered a major cause of chemotherapy failure and might be particularly involved in the secondary treatment failure frequently observed in the clinic [12].

We hypothesize that in order to eradicate highly resistant tumors the use of an effictive chemosensiziter that blocks efflux pumps will not be sufficient and a combinational therapy with a nano-scale drug carrier that can increase the influx of the drug into the cell while utilizing the chemosensitizer to block the efflux of the drug might be much more effective in eradicating these tumors. To utilize this approach, we used DOXIL®, the first FDA approved nanodrug [13], in combination with Zoloft®. Combining these two FDA approved drugs enhanced the therapeutic efficacy in a highly resistant human ovarian tumor. This study may pave the way for utilizing known FDA approved drugs in novel combinational therapy to treat highly resistant tumors.

Materials and methods

Reagents, chemicals and mAbs for flow cytometry

Verapamil (VP), XTT, GSH and Trypan-blue were from Sigma Chemical Co. (St. Louis, MO, USA). Doxorubicin (DOX) was a kind gift from TEVA Pharmaceutical Ltd (Netanya, Israel). Sertraline was a kind gift from Unipharm (Ramat Gan, Israel). Materials for cell cultures including XTT survival kit and Mycoplasma test kit were from Biological Industries, Ltd (Beit Haemek, Israel).

Fixation and permeabilization kit for flow cytometry was from IntraStain (Dako, Denmark).

Monoclonal antibodies (mAbs) for flow cytometry were purchased from ABCAM (Cambridge, UK):

1. Mouse anti-human P-Glycoprotein (clone 4E3), which recognizes an external epitope of the protein.
2. Mouse anti-human and rat MRP-1 (clone MRPm5), which recognizes a cytoplasmic epitope of the protein.
3. Mouse anti-human and mouse BCRP (clone BXP-53), which recognizes a cytoplasmic epitope of the protein.

Matched isotype control mAbs purchased from Exbio Praha (Czech Republic) were as follows:

Mouse IG2a isotype control for BCRP (clone BXP-53), P-Glycoprotein (clone 4E3) and MRP-1 (clone MRPm5):

2nd mAb: FITC conjugated goat anti mouse IgG secondary antibody was purchased from ABCAM.

Cell culture and maintenance

Human ovarian adenocarcinoma cells (OVCAR-8) were purchased from the ATCC and cultured in 100 × 20 mm dishes (culture plates and dishes were from Corning Glass Works, Corning, NY, USA) in RPMI 1640 medium at 37 °C in 5% CO_2 supplemented with 10% fetal calf serum (FCS), penicillin (1000 units/mL), Streptomicine (10 mg/mL) and L-Glutamine (200 mM).

Human ovarian adenocarcinoma cells NCI-ADR/RES (NAR), which is a sub-line of OVCAR-8 expressing P-glycoprotein [12] were grown in RPMI 1640 medium at 37 °C in 5% CO_2 supplemented with 10% fetal calf serum (FCS), penicillin (1000 units/mL), Streptomicine (10 mg/mL) and L-Glutamine (200 mM).

Cells were free of Mycoplasma contamination as determined by a *Mycoplasma* ELISA test (Biological Industries).

Quantitative analysis of drugs

Excitation and emission were at 485 nm and 530 nm, respectively for Rhodamine 123 and 485 nm and 573 nm for DOX. Lipid analysis was preformed as previously reported [14].

Pump expression analysis using flow cytometry

Assaying P-gp expression

P-gp expression assay was done as we reported previously [10]. Briefly, 5×10^5 cells were suspended in FACS buffer (PBS with 1% FBS) with 10 μg/mL anti-human P-gp clone 4E3 or its matched isotype control for 30 min on ice, then washed three times with cold FACS buffer and incubated with 2nd mAb (goat anti-mouse) 5 μg/mL stock diluted 1:400 for 30 min on ice following three washes with cold FACS buffer and immediate acquisition (of at least 10,000 cells) by FACSCalibur (Becton Dickinson) and analysis using Flowjo™ software.

Assaying MRP1 expression

A total of 5×10^5 cells were permeabilized using IntraStain Fixation and permeabilization kit for flow cytometry (Dako Cytomation) with Buffer A for 15 min at RT followed by three washes with FACS buffer. Then, Buffer B containing 10 μg/mL of anti-human MRP1 (clone MRPm5) or matched isotype control (mouse IgG2a respectively) for 20 min at RT flowed by three washing with cold FACS buffer and incubation with 2nd goat anti-mouse 5 μg/mL stock diluted 1:400 for 30 min on ice. Finally three washings were performed using cold FACS buffer and the cells were subjected to analysis via FACSCalibur.

Assaying BCRP expression

BCRP expression was done as we reported previously [10]. Briefly, 5×10^5 cells were suspended in FACS buffer with 10 μg/mL anti-human BCRP (clone 5D3) or its match isotype control (mouse immunoglobulin IgG2b) for 30 min on ice followed by three washings with cold FACS buffer. Then 2nd mAb goat anti-mouse 5 μg/mL stock diluted 1:400 was incubated with the cells for 30 min on ice. Finally three washings were performed using cold FACS buffer and the cells were subjected to analysis via FACSCalibur.

Pump functionality efflux assays

Extrusion functionality of the pumps expressed was assayed with fluorescent pump substrate, Rhodamine 123.

A total of 5×10^5 cells were used for this assay. Cells were washed and re-suspended in Phenol red free DMEM medium containing Rhodamine 123 (1 μM) then incubated for 1 h in 37 °C and 5% CO_2; washed twice and re-suspended in Phenol red-free DMEM medium. Flow cytometry analysis was performed at time 0, 30 min, 60 min, 90 min, 150 min and 210 min.

Cytotoxicity assay

A total of 3×10^3 cells/well were seeded onto 96 multi-well plates; 24 hours later the medium was replaced by a treatment medium that consisted of a medium with DOX alone at the concentration of 10 μM or DOX with chemosensitizer, selected from Verapamil 15 μM, Sertraline 10 μM or a medium containing the chemosensitizers alone at the same concentrations mentioned above without DOX. Four hours post administration the media from each well was aspirated, the cells washed and fed with drug-free, chemosensitizer-free, serum-supplemented cell culture media. The experiments were terminated 72 hours later. Quantization of cell viability per well by XTT was done as previously reported [12]. Two to five hours after incubation of the XTT reagent on cells, the absorbance of the samples (450 nm) against a background control (630 nm) was measured using Microplate Photometer Synergy HT (BioTec).

Drug efflux assay

NAR or OVCAR-8 cells were seeded onto 24-multiwell culture plates at densities of 5×10^4 to 5×10^5 cells/mL, and the experiments were initiated upon cell confluency. The wells, divided into four groups, were incubated for 10 hours with serum-supplemented growth medium containing 10 μM Dox and the following additions: group 1: none, group 2: 15 μM verapamil (VP), group 3: 15 μM fluoxetine (Flx) and group 4: 15 μM Sertraline (Ser). Upon end of incubation, the medium from

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