Cancer Letters 354 (2014) 290–298

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

Modulating cancer multidrug resistance by sertraline in combination with a nanomedicine

Velthe Drinberg [a,b,1], Rivka Bitcover [a,b,1], Wolf Rajchenbach [a,b], Dan Peer [a,b,*]

[a] Laboratory of NanoMedicine, Department of Cell Research and Immunology, George S. Wise Faculty of Life Sciences, Department of Materials Sciences and Engineering, Faculty of Engineering, Tel Aviv University, Tel Aviv 69978, Israel
[b] Center for Nanoscience and Nanotechnology, Tel Aviv University, Tel Aviv 69978, Israel

ARTICLE INFO

Article history:
Received 27 July 2014
Received in revised form 18 August 2014
Accepted 19 August 2014

Keywords:
Cancer MDR
SSRI
Sertraline
Nanomedicine

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.

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].

* Corresponding author. Tel.: + 1 3640 7925; fax: +972 3640 5926.
E-mail address: peer@tauex.tau.ac.il (D. Peer).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.canlet.2014.08.026
0304-3835/

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

each well was aspirated, and the cells were washed with PBS and incubated with efflux medium, PBS, PBS containing verapamil (15 μM), PBS containing fluoxetine (15 μM), and PBS containing sertraline (15 μM) for groups 1, 2, 3, and 4 respectively. At selected time points the medium from each well was collected and replaced with fresh efflux medium. Upon termination, the cells in each well were dissolved with 5% deoxycholate. Aliquots from all of the collected media and from each detergent-treated well were assayed for Doxorubicin in a fluorescence plate reader (Fluoroskan Ascent FL, TermoLabsystems, Vantaa, Finland) using appropriate calibration curves.

Preparation and characterization of pegylated liposomal doxorubicin

Pegylated liposomal doxorubicin was prepared according to a precise protocol provided to us by Prof. Chezy Barenholz (DOXIL® inventor), Hebrew University Jerusalem, Israel.

Briefly, hydrogenated Soy Phosphatidylcholine (HSPC), cholesterol (Chol), and methoxy polyethylene glycol 2000 – distearoyl phosphatidylethanolamine (mPEG-DSPE), were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). The lipids at a mole ratio of HSPC:Chol.:mPEG-DSPE 56.83:37.94:5.89 were dissolved in ethanol followed by injection to the ammonium sulfate 0.125M buffer pH 7.4 while stirring and heating to 65 °C for 30 min. The resulted multilamellar vesicles (MLV) were extruded through a LIPEX™ extrusion Device (Northern Lipids, Vancouver, Canada) operated at 65 °C and under a nitrogen pressure of 200–500 psi as previously reported [14,15]. The extrusion was carried out in stages using progressively smaller pore-size polycarbonate membranes (Whatman Inc, UK), with several cycles per pore-size, to achieve unilamellar vesicles in a final size range of ~ 100 nm in diameter. Lipid mass was quantified as previously reported [15]. The buffer was then exchanged to 10% w/v sucrose solution with a PD10 column sephadex TM G-25M (GE Healthcare). Particle size and distribution were determined by light scattering using a Malvern Nano ZS Zetasizer (Malvern Instruments Ltd., Worcestershire, UK).

DOX remote loading was performed at 60 °C for 30 min followed by Dox encapsulation assay as previously described [16]. The 10% w/v sucrose solution was then exchanged with PBS pH 7.4 containing 10 mM EDTA.

Lipid and DOX analysis

Each formulation was analyzed using reverse phase HPLC method equipped with Diode Array Detector (DAD) and Evaporative Light Scattering Detector (ELSD). The HPLC HP1100 series column Phenomenex Luna 100X 4.6 mm i.d 3 μ particle size the mobile phase composition was 70:20:10 IPA:PW:AcN in correlation.

DOX was assayed via its fluorescence excitation/emission 485/590 (nm) and a standard curve was made for each experiment performed.

Transmission electron microscopy (TEM) analysis

The ultrastructure of LNPs entrapping DOX was investigated using transmission electron microscope (TEM). Samples were adsorbed on formvar/carbon coated grids and negatively stained with 2% aquos uranyl acetate. Samples were examined using Jeol 1200EX TEM (Jeol, Japan).

Reversal of MDR in human ovarian adenocarcinoma: NCI-ADR/RES (NAR) xenografts

This animal protocol was approved by the Tel-Aviv Institutional Animal Care and Use Committee. The protocol was preformed essentially as previously described by our lab [12]. Briefly, athymic nude mice (6 weeks old) were housed in barrier facilities on a 12-hour light/dark cycle. Food and water were supplied *ad libitum*. On day zero 4×10^6 cells in 0.1 mL of Hank's Buffer were implanted subcutaneously by injection just above the right femoral joint. Two sets of experiments were preformed (testing DOX and Sertraline and separately, testing DOXIL® and Sertraline). For each experiment, when tumor volumes reached 125 mm^3 (day 0 of treatment), the mice were randomly separated into four groups.

DOX experiment: Saline and DOX (2 mg/kg body) were given every 3 days by intravenous administration for a total of 12 i.v. injections.

For the DOXIL® experiment: Saline and DOXIL® (2 mg/kg body) were given every 3 days by i.v. injection for total of 12 times. Sertraline (2 mg/kg body) was administrated in gavage in both types of experiments started on day 0 of treatment daily at 0.2 mL as long as the experiment was preformed. Tumor volume was calculated as $1/2(\text{width})^2 \times \text{length}$. The experiment was terminated upon reaching tumor volume of 1400–1800 mm^3.

Statistical analysis

In vitro data were expressed as means ± SD. Statistical analysis of the data was performed using the two-tail unequal variance Student's *t*-test, and $p < 0.05$ was considered statistically significant.

In vivo data were expressed as means ± SEM. Data were analyzed using Student's *t*-test. Differences between treatment groups were evaluated by one-way ANOVA with significance determined by Bonferroni adjusted t-test (GraphPad Prism software). Differences were considered as significant when $p < 0.05$.

Results

P-gp pumps are expressed and functional in ovarian adenocarcinoma resistant cells

NAR cells and its parental sensitive cell line, OVCAR-8, were assayed for pump expression and functionality. NAR expressed P-gp but not BCRP or MRP1 while OVCAR-8 did not express any of these pumps (Fig. 1A and B).

The efflux activity of the MDR pumps present in NAR cells was assayed using a fluorescent P-gp pump substrate, rhodamine 123 (Rh-123) [7]. The experiments were done as detailed in the methods section.

Decrease in Rh-123 intracellular levels as function of time was observed (Fig. 1C). NAR cells efflux Rh-123 within 210 min, demonstrating that the MDR pumps in NAR cells are functional in pumping out a pump substrate. As a control study, the same experiment was performed in OVCAR-8 cells showing no changes in the intracellular levels of Rh-123 (data not shown) over the same time points, strengthening the fact that indeed efflux pumps such as P-gp are predominantly involved in active drug efflux.

Sertraline modulates P-gp in ovarian adenocarcinoma resistant cells

Previous work published by us and others demonstrated that fluoxetine (Flx) acts as a chemosensitizer, moduating the function of P-gp pumps in P-gp-expressing cells [7,10,17]. Therefore, we screened other drugs from the SSRI family such as paroxetine and sertraline (Zoloft®; Ser.). In cytotoxicity experiments, paroxetine behaved as a P-gp modulator in several cell lines but when compared with Flx, this modulation was less effective (data not shown). The rationale for choosing sertraline (Ser) was that it also has halogen atoms as fluoxetine, and thus could also potentially modulate the P-gp extrusion pumps. The effects of Ser alone was tested over the dose range of 1–20 μM and was found to be non toxic to NAR and OVCAR-8 cells (data not shown). Consequently, we chose to work in the same dosing regime (15 μM) with Ser. as we used for Flx.

Cytotoxicity assays were preformed on NAR and OVCAR-8 cells confirming the resistant nature of NAR cells (Fig. 2A and B).

Free DOX (10 μM) modestly reduces cell viability in NAR cells (~85–90%) while the same DOX concentration potently reduces cell viability in OVCAR-8 cells (~5–7%) (compare Fig. 2A and B). This observation demonstrates that DOX does not reach an intracellular threshold that cumulates in cell death in NAR cells most likely due to active efflux of DOX whereas in OVCAR-8 enough DOX is accumulated in the cells to induce cell death. Addition of sertraline to DOX potently reduced cell viability in NAR cells (Fig. 2A). Verapamil (VP), a channel blocker and a well established MDR blocker was used as a positive control as well as fluoxetine (Flx) that was previously found to be an MDR blocker [7]. Addition of Ser to DOX induced more cell death ($p < 0.01$) than with other chemosensitizers such as VP or Flx. However, no additive effect was observed in OVCAR-8 cells when DOX treatment was combined with Ser, VP, or Flx (Fig. 2B), strengthening the hypothesis that Ser operates on P-gp efflux pumps. In order to gain insight into the mechanism by which sertraline affects cell death in NAR cells, we preformed DOX accumulation experiments as detailed in the experimental section.

We next examined the efflux of DOX from NAR and OVCAR-8 cells in the presence or absence of Ser (Fig. 2C and D).

One hour was sufficient to completely efflux DOX from NAR cells while the use of Ser inhibited DOX efflux and the drug retained in the cells long enough to induce cell death (Fig. 2A). VP and Flx also inhibited DOX efflux but to a smaller extent (Fig. 2C). In contrast, DOX efflux from OVCAR-8 cells was much slower and addition of any of the tested chemosensitizers (VP, Flx or Ser) did not increase the accumulation of DOX in the cells (Fig. 2D). The DOX

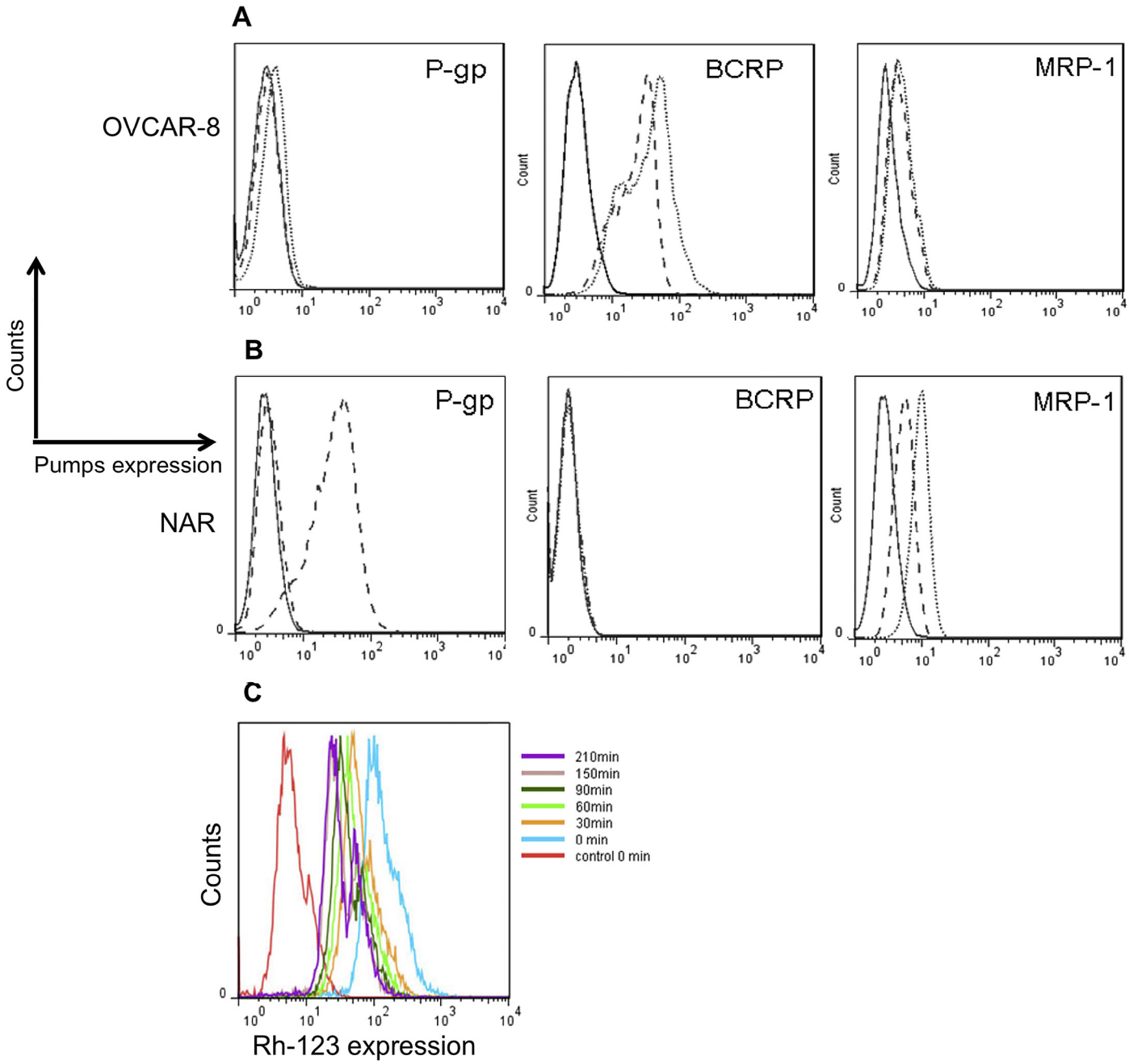

Fig. 1. P-gp is expressed and functional in resistant ovarian adenocarcinoma cells (NAR). Representative histograms of protein expression of various ABC transporters in drug sensitive human ovarian adenocarcinoma cell line, OVCAR-8 (A), and daughter drug resistant cells, NCI-Adr/Res; NAR cells (B) evaluated by flow cytometry using specific mAb as detailed in the experimental section. Solid line – no staining; dashed – matched isotype control mAb; dotted – specific pump protein (P-gp, BCRP or MRP-1). (C) Representative histograms showing efflux of Rh-123 over time in NAR cells. This assay demonstrates the functionality of the P-gp pumps in NAR cells. Control – 0 min – no staining, basal fluorescence level.

remained in the cells for about 12 hours. This drug accumulation cumulated in substantial cell death (Fig. 2B).

Sertraline reverse MDR in human ovarian adenocarcinoma xenograft mouse model

We have recently shown that NAR cells implanted in nude mice generate a highly vascularized human xenograft mouse model and maintain their resistant phenotype to DOX [12].

We next utilized this NAR xenograft model to test if Ser can also maintain its P-gp inhibitory effect *in vivo*.

Mice were given 2 mg/kg sertraline using gavage on a daily basis starting on the day of NAR cells inoculation as detailed in the experimental section.

In animals treated with saline and sertraline, tumors continued to grow rapidly with no significant changes between the two groups (Fig. 3A). Tumor volumes at day 33 were 24-fold larger than at the day of randomization in the untreated groups (day 0 in Fig. 3A). DOX treatment was able to decrease tumor progression at day 33 by 1.4-fold. In contrast, the combined treatment of doxorubicin and sertraline generated a regression in tumor progression. Moreover, the impact of the combined sertraline–DOX treatment was significantly better

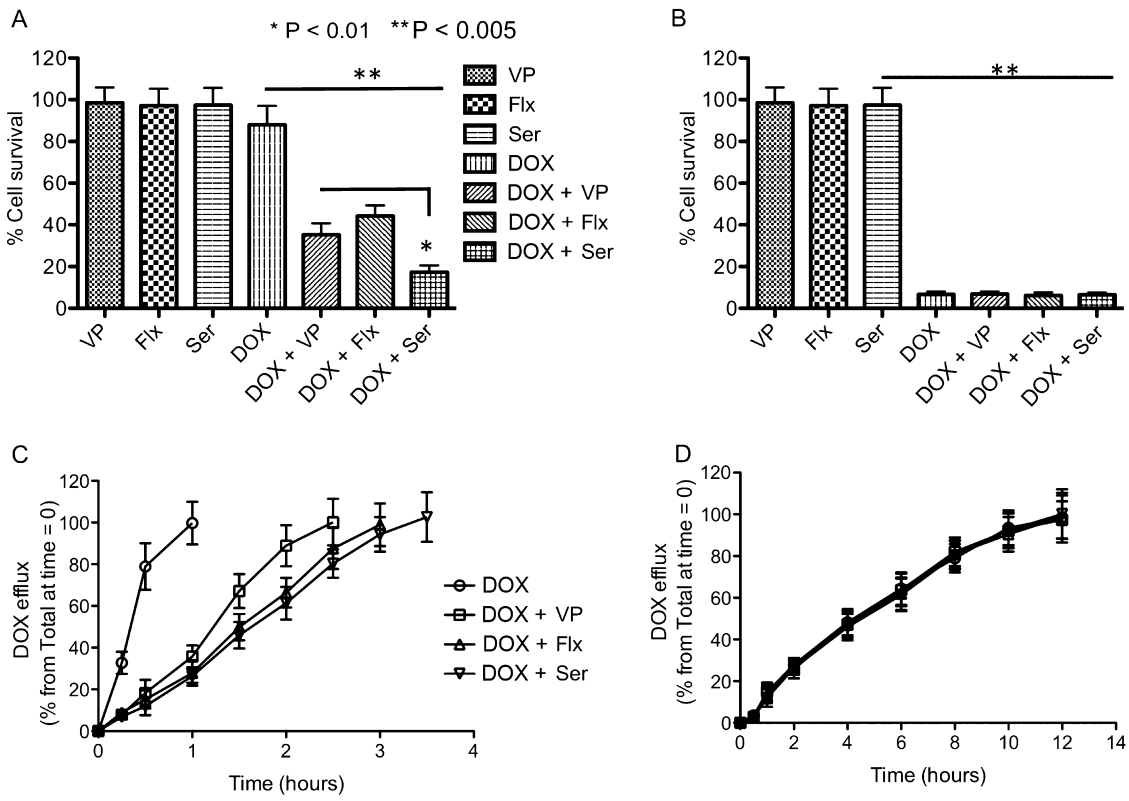

Fig. 2. Sertraline enhances cytotoxicity of DOX and reduces DOX efflux in NAR cells. (A) Cell viability assay shows that sertraline (Ser, 15 μM) enhances DOX cytotoxicity in drug resistant cells (NAR). Controls include verapamil (VP, 15 μM) and fluoxetine (Flx, 15 μM). (B) Same experimental settings as in A but with drug sensitive cells, OVCAR-8. (C) Sertraline slowed down the DOX efflux from NAR cells. (D) DOX efflux from OVCAR-8 cells is not changed with any type of chemosensitizers supporting the hypothesis that sertraline inhibits P-gp pump proteins.

than treatment by DOX alone. In this highly resistant DOX tumor model, the median survival of the saline, sertraline, DOX and a combined treatment of sertraline and DOX was 31.5, 33, 39 and 45, respectively (Fig. 3B). Increasing by 6 days the survival of DOX-treated mice by combining it with sertraline was found significant ($p = 0.013$) and suggests that sertraline can also be used as MDR modulator in the clinic, at least in P-gp expressing tumors.

A combination therapy of Sertraline and DOXIL® enhances the therapeutic response in a highly resistant ovarian adenocarcinoma xenograft mouse model

In order to test the hypothesis that a combination therapy with Sertraline that inhibits P-gp efflux pumps and a nanomedicine (such as DOXIL®) that increases the influx of the drug thus enhances the

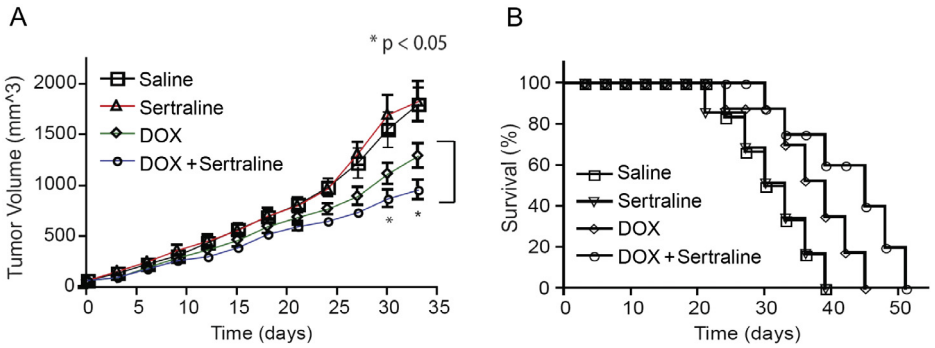

Fig. 3. Sertraline reverses MDR in human ovarian adenocarcinoma xenograft model. (A) Increase in tumor volume as function of time and treatment. DOX was injected I.V. (2 mg/kg) every second day and sertraline administrated in gavage (2 mg/kg) from treatment initiation on a daily basis until the end of the experiment. The points are the experimental data, each an average of seven animals per treatment group, and the error bars are the SEM. Statistical significance evaluations represented on the figure are by the asterisks symbol – comparisons between DOX alone and DOX and sertraline (*$p < 0.05$). There was no statistical significance between sertraline alone and saline. (B) Global survival rate was calculated by Kaplan–Meier method using the GraphPad Prism software.

A

Pegylated Liposomal Doxorubicin (Batch #)	Hydrodynamic Diameter (nm)	Zeta potential (mV)
1	90 ± 10	-8.4 ± 1.7
2	88 ± 14	-9.2 ± 2.5
3	93 ± 18	-9.0 ± 3.0

B

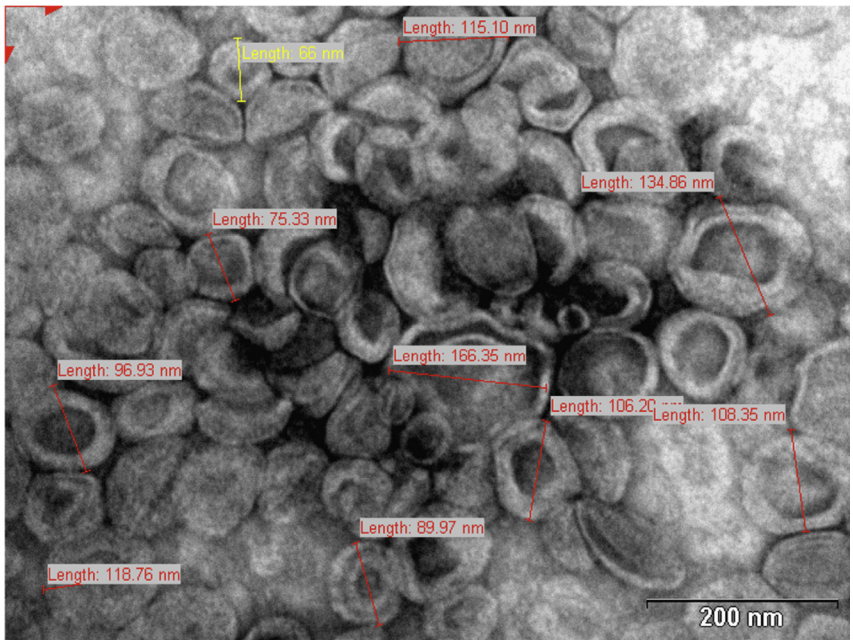

Fig. 4. Physicochemical and structural characterization of pegylated liposomal doxorubicin. (A) Size distribution and Zeta potential of three different batches of pegylated liposomal doxorubicin measured as detailed in the experimental section. Each result is an average ± SD of at least six independent measurements. Batch-to-batch variability was small, within the range reported for this particular batch. (B) Representative TEM analysis (from batch # 1) showing the typical ultrastructure of liposomes. Same results were obtained from batches # 2 and 3.

therapeutic response, we had to first prepare and characterize the nanomedicine.

Pegylated liposomal doxorubicin (DOXIL®) was prepared using a protocol provided by Prof. Yechezkel Barenholz, (DOXIL® inventor) [13] as detailed in the experimental section.

Three independent batches were prepared and characterized with excellent batch-to-batch reproducibility (Fig. 4A). Particles with mean diameter of ~ 90 nm and a mildly negative zeta potential were produced and the ultrastructure of these particles were investigated using transmission electron microscopy (TEM). Typical structure of liposomes was observed (Fig. 4B), which were in good agreement with previously published work [13,18,19].

We have recently demonstrated that DOXIL® cannot release its payload (DOX) in a cell culture system, NAR cells were used as a representative example [12]. Thus, we could only test our hypothesis *in vivo* in the NAR xenograft mouse model. A combination treatment reduced tumor volume by twofold compared to treatment with DOXIL® (Fig. 5A). This was also mirrored by extension survival by 20% from treatment with DOXIL and in combination treatment with sertraline (Fig. 5B).

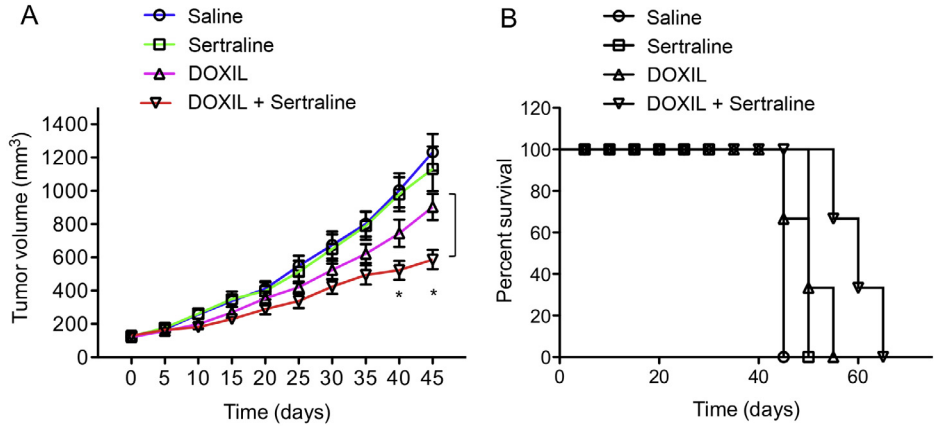

Fig. 5. Combination treatment of DOXIL with sertraline reverses MDR in human ovarian adenocarcinoma xenograft mouse model. (A) Increase in tumor volume as function of time and treatment. DOXIL was injected I.V. (2 mg/kg) every 3 days and sertraline administrated in gavage (2 mg/kg) from treatment initiation on a daily basis. The points are the experimental data each an average of seven animals per treatment group, and the error bars are the SEM. Statistical significance evaluations represented on the figure are by the asterisks symbol – comparisons between DOXIL alone and DOXIL and sertraline (*$p < 0.01$). There was no statistical significance between sertraline alone and saline. (B) Global survival rate was calculated by Kaplan–Meier method using the GraphPad Prism software.

Discussion

MDR is a major obstacle in treatment, whether the resistance is moderate or severe. Its currently known chemosensitizers (that unfortunately work *in vitro* but cannot be used *in vivo*) are divided into two groups: pump-specific and multi-pump. In the first group, a given chemosensitizer is specific to one type of MDR transporter; in the second, it can address more than one type. Well-known examples of the first group include: PSC833, XR9576, GF120918, LY335979 and OC144-093(ONT-093) for P-gp [20–24]; MK571 and probenecide for MRP-1 [25]; pheophorbide and FTC for BCRP [26,27]. Relevant examples of the second group include verapamil, CsA and MS-209 reported to address both P-gp and MRP1, and Biricodar (VX-710), reported to address P-gp, MRP1 and BCRP [20,28–31]. It is important to note that some of the above-mentioned chemosensitizers are limited to *in vitro* use, while others were also valid in animal studies, and a few have also progressed to early clinical trials. Yet, there is still no clinically approved chemosensitizer. Among the different reasons for clinical failure are severe drug–drug interactions that elongate the circulation time of the chemotherapy and increase adverse effects.

We previously found the fluoxetine (Prozac®) can inhibit P-gp [7,10] in several tumor-bearing mice. Fluoxetine was found to work mainly on P-gp (Bitcover R. and Peer D. 2010, personal communication). Thus, it was necessary to find a new drug from the SSRI family that may inhibit highly expressing P-gp and potentially other pumps MRP-1. Looking at the molecular structure of SSRI drugs it is clear that they all share a halogen group. According to a recently published molecular dynamics (MD) study we show that this group is responsible to the attachment of the drug to the protein [32]. Examining the suitable and available SSRI drugs, we decided to focus on sertraline.

As our tested biological system, we decided to center on human ovarian adenocarcinoma drug-resistant cells (NAR) as a highly resistant cell model, which maintain its resistant phenotype *in vivo* [12]. We first examined the expression of P-gp, MRP-1 and BCRP in NAR cells and in its parent sensitive cell line, OVCAR-8 (Fig. 1A and B). We found a substantial expression of P-gp only in NAR cells with low expression of MRP-1 in NAR cells but not in OVCAR-8 cells. Next, we examined the functionality of the P-gp pumps in the NAR cells (Fig. 1C). Rhodamine-123 (Rh-123), a known P-gp fluorescence substrate was used to determine the functionality of the P-gp efflux pump. It took 3.5 h for the cells to completely efflux the Rh-123, thus the P-gp pumps are pumping out this substrate which is only pumped out by P-gp and not by other pumps [5]. Next, combination treatment of sertraline with DOX enhanced cytotoxicity by threefold (Fig. 2A). Sertraline was also similar in trend but more potent than the *in vitro* benchmarks verapamil or with fluoxetine (Fig. 2A) whereas in drug sensitive cells (OVACR-8) no differences between treatment with DOX alone or with the chemosensitizers were observed (Fig. 2B). In order to determine sertraline's mechanism of action, efflux assay was preformed. The cells were loaded with DOX with or without sertraline (and other controls). Sertraline slowed down the efflux of DOX by threefold (Fig. 2C) whereas the other chemosensitizers were less potent in their activity. There were no changes observed in OVCAR-8 cells in DOX efflux with or without the chemosensitizer (Fig. 2D), strengthening that sertraline indeed inhibits P-gp pumps in the NAR cells. It is important to note that efflux of DOX was much faster then the Rh-123 and this is in good agreement with published data [7,33] supporting the hypothesis that the kinetics of efflux might be differential between different drugs.

MDR reversal *in vivo* is, obviously a pre-clinical requirement prior to clinical testing for any new chemosensitizer. A combination treatment between DOX and sertraline slows down tumor growth in NAR xenograft mouse model (Fig. 3A) and increased the survival (Fig. 3B) by 1.5-fold. Bearing in mind the dose-related problems with previous-

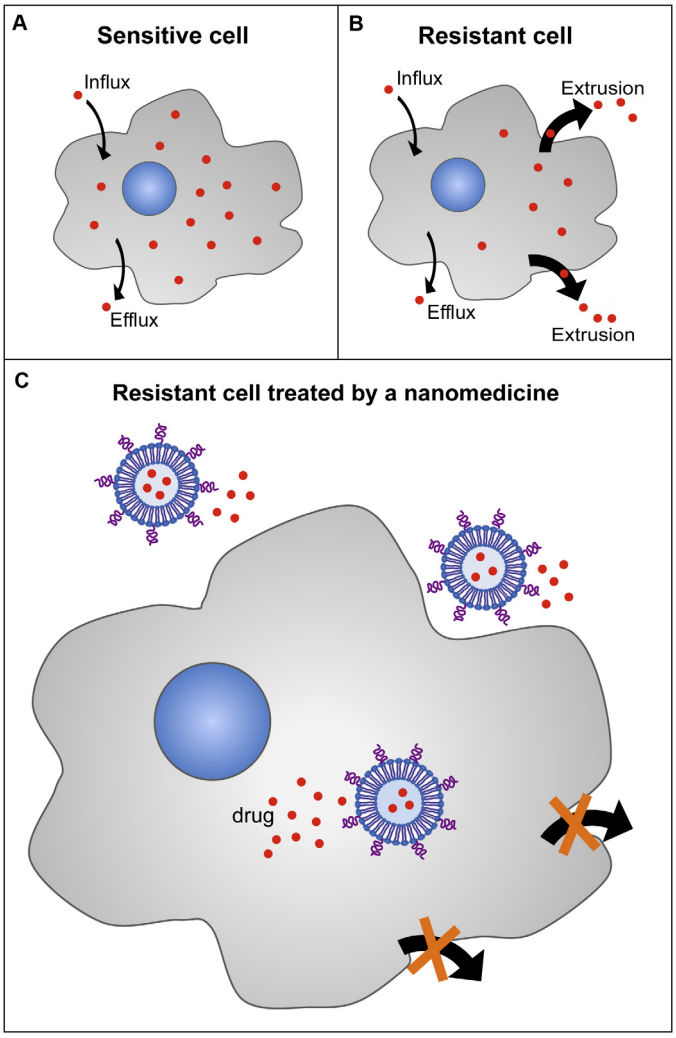

Fig. 6. Schematic illustration of the mechanisms by which combinational therapy could enhance the therapeutic response in highly resistant tumors. (A) A drug-sensitive tumor cell. Drug molecules (red dots) diffuse across the cell membrane. The influx of the drug is higher than the drug efflux due to the direction of the drug's electrochemical gradient, allowing sufficient drug accumulation inside the cell. The cell nucleus is labeled in blue. (B) A resistant tumor cell combines drug diffusion across the cell membrane with extrusion pumps that expel the drug out of the cell and thus reducing the drug accumulation inside the cell. (C) Combinational treatment with nanomedicines and chemosensitizers. By blocking the extrusion pumps, the drug diffused from the nanomedicine is allowed to accumulate inside the cell, comparable to the case of a drug-sensitive tumor cell. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

generation chemosensitizers, we deliberately set out to test, *in vivo*, a low sertraline dose: 2 mg/kg body/day compared (for example) to the ranges of 5 to 30, 2 to 8, and 200 to 300 mg/kg body/dose for OC144–093, XR9756 and MS-209 respectively [22,34–36].

For treatment of depression, sertraline is prescribed at the dose range of 50–100 mg/person/day [37]. Taking into account the metabolic differences between mouse and human, the sertraline dose applied here for MDR modulation corresponds to approximately 10 mg/person/day, which is well below the safety limits.

Our hypothesis was that increasing the drug influx using a nanomedicine such as DOXIL and at the same time decreasing the drug efflux by using a chemosensitizer such as sertraline would enhance the therapeutic response in highly resistant tumors (Fig. 6). In order to examine this hypothesis, we prepared pegylated liposomal doxorubicin (DOXIL®) according to a defined protocol provided by Prof. Barenholz, DOXIL® inventor. Liposomes were

characterized and three independent batches showed a remarkable batch-to-batch reproducibility (Fig. 4) and were in excellent agreement to published data [13,38]. Next, we tested in the same NAR xenograft mouse model a combination treatment with DOXIL® and sertraline. Combination therapy was found superior in regression of tumors (Fig. 5A) and increases the median survival by 20% over treatment with DOXIL® (Fig. 5B).

Comparing the treatment of DOXIL and Sertraline (Fig. 5A) to the treatment of DOX with Sertraline (Fig. 3A) at days 30 and 35 it becomes clear that the use of a nanomedicine has significant advantage in slowing down tumor growth ($p = 0.0017$, between these treatment groups). The ability of nanocarriers to bring more drug (DOX) in close proximity to the tumor and act as drug depots together with a chemosensitizer that blocks efflux pump provide a therapeutic benefit compared with free drug that will be washed out from the tumor vicinity.

Taking into account that both drugs (sertraline, Zoloft®) and DOXIL® are FDA-approved, it is hoped that the promise implied in the present study with respect to sertraline's ability to reverse MDR at low safe doses in combination with a nanomedicine such as DOXIL® could soon be materialized into the clinic.

Conclusion

In conclusion, sertraline met all three *in vitro* criteria for acting as a chemosensitizer. Sertraline also acted as a chemosensitizer *in vivo*, with relatively good *in vitro–in vivo* correlation showing capability of reversing MDR generated by P-gp pumps. Sertraline has additional advantages such as having been approved by the FDA and crossing the blood–brain barrier (BBB). These advantages make the sertraline a good candidate for brain tumor treatment in clinical trials.

Combination treatment with nanomedicine and a chemosensitizer increases cellular influx of the drug while inhibiting its efflux and overall enhancing the therapeutic response in a highly drug resistant ovarian tumor.

Conflict of interest

V.D., R.B. and W.R. declare no financial interest. D.P. declares financial interest in Quiet Therapeutics Ltd.

Acknowledgments

We wish to thank Prof. Y. (Chezy) Barenholz from the Hebrew University Jerusalem, Israel, DOXIL® inventor for all his help in the preparation and characterization of this drug.

We also thank Mrs. Varda Wexler for her help with the graphics and illustrations and the Peer laboratory members for helpful discussions. This work was supported in part by the grants from the Lewis Family Trust, the Israeli Centre of Research Excellence (I-CORE), Gene Regulation in Complex Human Disease, Center No 41/11; Israel Science Foundation (Award 181/10); FTA: Nanomedicine for Personalized Theranostics, and by The Leona M. and Harry B. Helmsley Nanotechnology Research Fund awarded to D.P.

References

[1] World Health Organization (WHO), *Cancer, Fact sheet N°297*, Updated February 2014.
[2] S.V. Ambudkar, C. Kimchi-Sarfaty, Z.E. Sauna, M.M. Gottesman, P-glycoprotein: from genomics to mechanism, Oncogene 22 (2003) 7468–7485.
[3] J.I. Fletcher, M. Haber, M.J. Henderson, M.D. Norris, ABC transporters in cancer: more than just drug efflux pumps, Nat. Rev. Cancer 10 (2010) 147–156.
[4] M.M. Gottesman, I. Pastan, Biochemistry of multidrug resistance mediated by the multidrug transporter, Annu. Rev. Biochem. 62 (1993) 385–427.
[5] D. Peer, R. Margalit, Fluoxetine and reversal of multidrug resistance, Cancer Lett. 237 (2006) 180–187.
[6] M.F. Ullah, Cancer multidrug resistance (MDR): a major impediment to effective chemotherapy, Asian Pac. J. Cancer Prev. 9 (2008) 1–6.
[7] D. Peer, Y. Dekel, D. Melikhov, R. Margalit, Fluoxetine inhibits multidrug resistance extrusion pumps and enhances responses to chemotherapy in syngeneic and in human xenograft mouse tumor models, Cancer Res. 64 (2004) 7562–7569.
[8] L.K. Bogart, G. Pourroy, C.J. Murphy, V. Puntes, T. Pellegrino, D. Rosenblum, et al., Nanoparticles for imaging, sensing, and therapeutic intervention, ACS Nano 8 (2014) 3107–3122.
[9] D. Rosenblum, D. Peer, Omics-based nanomedicine: The future of personalized oncology, Cancer Lett. 352 (2014) 126–136.
[10] M. Argov, R. Kashi, D. Peer, R. Margalit, Treatment of resistant human colon cancer xenografts by a fluoxetine–doxorubicin combination enhances therapeutic responses comparable to an aggressive bevacizumab regimen, Cancer Lett. 274 (2009) 118–125.
[11] J. Cookson, R. Duffett, Fluoxetine: therapeutic and undesirable effects, Hosp. Med. 59 (1998) 622–626.
[12] K. Cohen, R. Emmanuel, E. Kisin-Finfer, D. Shabat, D. Peer, Modulation of drug resistance in ovarian adenocarcinoma using chemotherapy entrapped in hyaluronan-grafted nanoparticle clusters, ACS Nano 8 (2014) 2183–2195.
[13] Y.C. Barenholz, Doxil® – the first FDA-approved nano-drug: lessons learned, J. Control. Release 160 (2012) 117–134.
[14] D. Peer, E.J. Park, Y. Morishita, C.V. Carman, M. Shimaoka, Systemic leukocyte-directed siRNA delivery revealing cyclin D1 as an anti-inflammatory target, Science 319 (2008) 627–630.
[15] S. Mizrahy, M. Goldsmith, S. Leviatan-Ben-Arye, E. Kisin-Finfer, O. Redy, S. Srinivasan, et al., Tumor targeting profiling of hyaluronan-coated lipid based-nanoparticles, Nanoscale 6 (2014) 3742–3752.
[16] S. Amselem, A. Gabizon, Y. Barenholz, Optimization and upscaling of doxorubicin-containing liposomes for clinical use, J. Pharm. Sci. 79 (1990) 1045–1052.
[17] Y. Zhang, T. Zhou, J. Duan, Z. Xiao, G. Li, F. Xu, Inhibition of P-glycoprotein and glutathione S-transferase-pi mediated resistance by fluoxetine in MCF-7/ADM cells, Biomed. Pharmacother. 67 (2013) 757–762.
[18] Y. Barenholz, D. Peer, Liposomes and other assemblies as drugs and nano-drugs: from basic and translational research to the clinics, J. Control. Release 160 (2012) 115–116.
[19] Y.C. Barenholz, D. Peer, Liposomes, lipid biophysics, and sphingolipid research: from basic to translation research, Chem. Phys. Lipids 165 (2012) 363–364.
[20] U.A. Germann, P.J. Ford, D. Shlyakhter, V.S. Mason, M.W. Harding, Chemosensitization and drug accumulation effects of VX-710, verapamil, cyclosporin A, MS-209 and GF120918 in multidrug resistant HL60/ADR cells expressing the multidrug resistance-associated protein MRP, Anticancer Drugs 8 (1997) 141–155.
[21] T. Litman, T.E. Druley, W.D. Stein, S.E. Bates, From MDR to MXR: new understanding of multidrug resistance systems, their properties and clinical significance, Cell. Mol. Life Sci. 58 (2001) 931–959.
[22] P. Mistry, A.J. Stewart, W. Dangerfield, S. Okiji, C. Liddle, D. Bootle, et al., In vitro and in vivo reversal of P-glycoprotein-mediated multidrug resistance by a novel potent modulator, XR9576, Cancer Res. 61 (2001) 749–758.
[23] M.J. Newman, J.C. Rodarte, K.D. Benbatoul, S.J. Romano, C. Zhang, S. Krane, et al., Discovery and characterization of OC144-093, a novel inhibitor of P-glycoprotein-mediated multidrug resistance, Cancer Res. 60 (2000) 2964–2972.
[24] R.L. Shepard, J. Cao, J.J. Starling, A.H. Dantzig, Modulation of P-glycoprotein but not MRP1- or BCRP-mediated drug resistance by LY335979, Int. J. Cancer 103 (2003) 121–125.
[25] D. Chauvier, G. Kegelaer, H. Morjani, M. Manfait, Reversal of multidrug resistance-associated protein-mediated daunorubicin resistance by camptothecin, J. Pharm. Sci. 91 (2002) 1765–1775.
[26] R.W. Robey, K. Steadman, O. Polgar, K. Morisaki, M. Blayney, P. Mistry, et al., Pheophorbide a is a specific probe for ABCG2 function and inhibition, Cancer Res. 64 (2004) 1242–1246.
[27] U. Stein, H. Lage, A. Jordan, W. Walther, S.E. Bates, T. Litman, et al., Impact of BCRP/MXR, MRP1 and MDR1/P-Glycoprotein on thermoresistant variants of atypical and classical multidrug resistant cancer cells, Int. J. Cancer 97 (2002) 751–760.
[28] T. Abe, K. Koike, T. Ohga, T. Kubo, M. Wada, K. Kohno, et al., Chemosensitisation of spontaneous multidrug resistance by a 1,4-dihydropyridine analogue and verapamil in human glioma cell lines overexpressing MRP or MDR1, Br. J. Cancer 72 (1995) 418–423.
[29] U.A. Germann, D. Shlyakhter, V.S. Mason, R.E. Zelle, J.P. Duffy, V. Galullo, et al., Cellular and biochemical characterization of VX-710 as a chemosensitizer: reversal of P-glycoprotein-mediated multidrug resistance in vitro, Anticancer Drugs 8 (1997) 125–140.
[30] H. Minderman, K.L. O'Loughlin, L. Pendyala, M.R. Baer, VX-710 (biricodar) increases drug retention and enhances chemosensitivity in resistant cells overexpressing P-glycoprotein, multidrug resistance protein, and breast cancer resistance protein, Clin Can Res 10 (2004) 1826–1834.
[31] L. Rieger, J. Rieger, S. Winter, J. Streffer, P. Esser, J. Dichgans, et al., Evidence for a constitutive, verapamil-sensitive, non-P-glycoprotein multidrug resistance phenotype in malignant glioma that is unaltered by radiochemotherapy in vivo, Acta Neuropathol. 99 (2000) 555–562.
[32] S. Amram, A. Ganoth, O. Tichon, D. Peer, E. Nachliel, M. Gutman, et al., Structural characterization of the drug translocation path of MRP1/ABCC1, Isr. J. Chem. (2014), doi:10.1002/ijch.201300132.

[33] F. Thiebaut, T. Tsuruo, H. Hamada, M.M. Gottesman, I. Pastan, M.C. Willingham, Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues, Proc. Natl Acad. Sci. U.S.A. 84 (1987) 7735–7738.
[34] L.G. Baggetto, M. Dong, J. Bernaud, L. Espinosa, D. Rigal, R. Bonvallet, et al., In vitro and in vivo reversal of cancer cell multidrug resistance by the semi-synthetic antibiotic tiamulin, Biochem. Pharmacol. 56 (1998) 1219–1228.
[35] E.S. Guns, T. Denyssevych, R. Dixon, M.B. Bally, L. Mayer, Drug interaction studies between paclitaxel (Taxol) and OC144-093 – a new modulator of MDR in cancer chemotherapy, Eur. J. Drug Metab. Pharmacokinet. 27 (2002) 119–126.
[36] M. Naito, T. Tsuruo, New multidrug-resistance-reversing drugs, MS-209 and SDZ PSC 833, Cancer Chemother. Pharmacol. 40 (Suppl.) (1997) S20–S24.
[37] M.F. Flament, R.M. Lane, R. Zhu, Z. Ying, Predictors of an acute antidepressant response to fluoxetine and sertraline, Int. Clin. Psychopharmacol. 14 (1999) 259–275.
[38] A.A. Gabizon, Pegylated liposomal doxorubicin: metamorphosis of an old drug into a new form of chemotherapy, Cancer Invest. 19 (2001) 424–436.