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Treatment of resistant human colon cancer xenografts by a fluoxetine–doxorubicin combination enhances therapeutic responses comparable to an aggressive bevacizumab regimen

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ABSTRACT

Pre-clinical studies of multidrug resistance (MDR) usually address severe resistance, yet moderate MDR is already clinically-impeding. The purpose of this study was to characterize moderate drug resistance in human colon cancer, and it's modulation by fluoxetine. *In vitro* fluoxetine enhanced doxorubicin's cytotoxicity (10-fold), increased doxorubicin's intracellular accumulation (32%) and decreased efflux of intracellular doxorubicin (70%). *In vivo*, mild treatment with a doxorubicin–fluoxetine combination slowed-down tumor progression significantly ($p < 0.001$ vs. doxorubicin alone), comparable to aggressive treatment with bevacizumab. Collectively, our results suggest that combinations of fluoxetine with chemotherapeutic drugs (P-glycoprotein substrates) are worthy of further pursuit for moderate MDR in the clinic.

1. Introduction

Surgery followed by drug therapy is standard treatment for colorectal cancer – the third most common form of cancer and the second leading cause of cancer-related death in the Western world [1,2]. Recently, a novel modality utilizing drugs that target tumor vasculature, such as bevacizumab (avastin), was added to the veteran modality of conventional chemotherapeutic drugs [3–10]. Frequently-administered combinations include: fluorouracil, doxorubicin and mitomycin C or methotrexate; etoposide, doxorubicin and cisplatin; docetaxel, cisplatin with/without fluorouracil [6–10]. The veteran modality is prone, however, to multiple drug resistance (MDR) operated by the ABC transporters ABCB1 (P-glycoprotein, Pgp), members of the ABCC family (multidrug resistance-associated proteins, MRP) and ABCG2 (breast cancer resistance protein, BCRP) [11–19]. Doxorubicin, mitomycin C and docetaxel are known substrates of Pgp and doxorubicin is also a substrate of MRP1 [12,13,15–17].

Drug resistance operated by the ABC transporters is an influx–efflux imbalance. The MDR transporters actively pump their substrates out of the cell, reducing intracellular drug doses below lethal thresholds [11,13,15]. A major approach to correct this imbalance is by pump inhibition, utilizing chemosensitizers that would be administered together with the anti-cancer drugs [11,13,15,17,20,21]. Currently, several third-generation chemosensitizers, mostly against Pgp, are in clinical trials [17,22]. However, few trials are currently conducted, and the debate on this strategy is still going on. In the chemosensitizer arena, most pre-clinical studies have focused, *in vitro* and

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Abbreviations: ABC, ATP binding cassette; BCRP, breast cancer resistance protein; BSA, bovine serum albumin; FBS, fetal bovine serum; HBS, hepes buffered saline; MDR, multiple drug resistance; MRP, multidrug resistance-associated protein; PBS, phosphate buffered saline; Pgp, P-glycoprotein. SD, standard deviation; SEM, standard error of the mean.

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in vivo, on systems exhibiting high levels of drug resistance which is technically advantageous [23,24]. For patients, moderate resistance is already a severe therapeutic impediment, that is often further aggravated after exposure to chemotherapy [25–27]. A recommended strategic approach is to apply a combination of chemotherapy and chemosensitizer at the beginning of cancer treatment when resistance is still low or moderate [27]. To gain more insight into modulating clinically-relevant resistance levels, there is an obvious need to also examine chemosensitization in pre-clinical moderate-resistance systems. Such examination should include functional and mechanistic aspects, assessing whether the level of resistance affects patterns and quantitative measures of resistance modulation.

In this manuscript, we focused on pre-clinical studies in the HCT-15 cell line derived from human colorectal adenocarcinoma, a system postulated to model moderate resistance in colorectal cancer. HCT-15 is reported to be an inherent MDR line expressing moderate levels of Pgp [28–30]. In one study, it was also reported to moderately-express MRP [31]. Consequently, we first affirmed that the cells we studied were a Pgp-alone line.

The purpose of the *in vitro* functional and mechanistic studies was 2-fold: (i) To characterize MDR in this cell line selecting doxorubicin, a frequent component of chemotherapy combinations for colorectal cancer and a Pgp substrate, as the test drug [6,8–10,15–17,20]. (ii) To modulate this resistance by chemosensitization, investigating fluoxetine (Prozac), previously shown by us to act as a chemosensitizer for highly-resistant Pgp-expressing cancer cells, as the test chemosensitizer [20,21]. The purpose of the *in vivo* studies conducted in an established animal model of HCT-15 xenografts [31–33], was also 2-fold: (i) To determine whether the functional results obtain *in vitro* come into expression *in vivo*, which is (obviously) a critical pre-clinical step. (ii) To compare the two treatment modalities discussed above for colorectal cancer, i.e. chemotherapy and bevacizumab, under conditions where resistance to chemotherapy may be significantly reduced. As will be shown, modulating doxorubicin resistance by fluoxetine makes this combination a modern-day therapeutic option worthy of consideration for colon cancer.

2. Materials and methods

2.1. Materials

Doxorubicin was from Teva Pharmaceutical Inc. (Netanya, Israel), bevacizumab and fluoxetine were a kind gift from Teva Pharmaceutical Inc. (Netanya, Israel). Bovine serum albumin (BSA), sodium azide, sodium chloride and hepes were from Sigma (St. Louis, USA). Materials for cell culture, phosphate buffered saline (PBS) and XTT kit were from Biological industries (Beit Haemek, Israel).

2.1.1. Monoclonal antibodies

(i) Dako (Glostrup, Denmark): mouse anti-human Pgp clone 4E3, that recognizes an external epitope of Pgp, and IgG2a (isotype control). (ii) Chemicon (Billerica, USA): mouse anti-human Anti-MRP clone MRPm6, that recognizes a cytoplasmic epitope of MRP. (iii) eBioscience (San Diego, USA): IgG1 isotype control for MRPm6, mouse anti-human Anti-BCRP clone 5D3, that recognizes an external epitope of BCRP, and IgG2b (isotype control). (iv) IQ Products (Groningen, Netherlands): rabbit anti-mouse IgG F(ab)′2-FITC, the secondary antibody for flow cytometry. (v) Molecular probes Inc. (Eugene, USA): goat anti-mouse IgG F(ab)′2-Alexa 488, the secondary antibody for confocal laser scanning microscopy. All other reagents were of analytical grade.

2.1.2. Cell cultures

HCT-15 cells were from ATCC (ATCC No. CCL-225). The cells were cultured in RPMI 1640 medium at 37 °C in 5% CO_2, supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 20 mM hepes buffer, 10,000 U/mL penicillin, 10 mg/mL streptomycin and 1250 U/mL nystatin. Cells were free of mycoplasma contamination, determined by an EZ-PCR Mycoplasma Test Kit performed every 3 months.

2.2. In vitro cytotoxicity

Cells (4×10^3/well) were seeded onto 96-multiwell plates, and the experiments were initiated 24 h later, upon sub-confluency. The regular serum-supplemented media was replaced by treatment media, similar to the regular except additions of: bevacizumab or doxorubicin with/without fluoxetine, at selected concentrations. Treatment media was removed 24 h later, followed by washing with RPMI 1640, and 24 h incubation with regular serum-supplemented media. Upon termination (48 h from start), cell viability was determined by the XTT method. Bevacizumab was also tested by 48 h exposure to treatment media.

2.3. Flow cytometry analysis of pump expression

Specific pre-treatments for each ABC pump protein will be listed below. At least 10,000 events were determined for each test sample using a Becton Dickinson FACSort (CA, USA) and analyzed using the CellQuest Pro™ software. Excitation was by a single 15 mW argon-ion laser beam (488 nm). Emission was collected through a 530 nm band pass filter.

2.3.1. Determination of Pgp expression

The experiments were done essentially according to [34]. Briefly, 1×10^6 cells were suspended in PBS containing 2% BSA and 10 μg/mL monoclonal antibody clone 4E3 or isotype control (mouse immunoglobulin IgG2a). The reaction mixture was subjected to the following processes: 30 min incubation at room temperature, washing with PBS + 2% BSA, 40 min incubation (on ice in the dark) with the secondary antibody (50 μL stock diluted 1:10), washing with PBS + 2% BSA, suspension in PBS + 0.1% sodium azide, and storage at 4 °C until the flow cytometry analysis.

2.3.2. Determination of MRP expression

The experiments were done essentially according to [35,36]. Briefly, 1×10^6 cells were subjected to the following processes: permeabilization in methanol (−20 °C) for 30 min, washing in growth medium containing 5% FBS

and 0.1% sodium azide, 1 h incubation at 4 °C in the same medium containing 10 μg/mL of monoclonal antibody MRPm6, or isotype control (mouse immunoglobulin IgG1). From this point on procedures were similar to those listed above for Pgp, except the wash solution was growth medium containing 5% FBS and 0.1% sodium azide.

2.3.3. Determination of BCRP expression

The experiments were done essentially according to [37]. Briefly, 1×10^6 cells were suspended in PBS containing 1% FBS and 10 μg/mL monoclonal antibody clone 5D3 or alternatively mouse immunoglobulin IgG2b isotype control. The reaction mixture was incubated for 30 min on ice. The procedures from this point and on were similar to those listed above for Pgp except the wash solution was PBS + 1% FBS.

2.4. Doxorubicin accumulation and efflux

The experiments were done essentially according to [38]. Briefly, test systems were 1×10^6 cells suspended 10 μM doxorubicin buffered by hepes buffered saline (HBS) with or without 25 μM fluoxetine. Control was a similar cell suspension in HBS. All systems were incubated for 4 h at 37 °C, at the end of incubation each system was divided into two parts, for accumulation and efflux. For accumulation, the cells were washed twice with HBS or HBS with fluoxetine, suspended in HBS (with/without fluoxetine) with 0.1% sodium azide and kept at 4 °C until assayed. For efflux, the cells were suspended in HBS (with/without 25 μM fluoxetine) and incubated for 1 h at 37 °C, at the end of which the cells were processed as described above for the accumulation-designated cells. Intracellular doxorubicin concentrations were measured and the geometric means calculated using a Becton Dickinson FACSort (CA, USA) and the CellQuest Pro™ software. A single 15 mW argon-ion laser beam (488 nm) was used for excitation, and the emission was collected through a 630 nm band pass filter.

2.5. Intracellular doxorubicin distribution

Intracellular doxorubicin distribution was studied by confocal laser scanning microscopy. Cells grown on glass coverslips were incubated upon sub-confluency for 2 h with 10 μM doxorubicin with/without fluoxetine (10 μM or 25 μM), washed and examined using Zeiss LSM 510 confocal microscope (Carl Zeiss AG, Oberkochen, Germany) using 40× with NA 1.2 water immersion objective. Argon laser line 488 nm was employed for excitation, and emission was captured through a 505 nm long pass filter.

2.6. In vivo studies

The experiments were done at Teva Pharmaceutical Inc. and the animal protocol was approved by the Teva Pharmaceutical Inc. Animal Care and Use Committee.

Athymic 5- to 8-week-old nude female mice (Foxn1nu, Harlan animal breeding center, Rehovot, Israel) were inoculated by subcutaneous injection of 1×10^6 HCT-15 cells/mouse in 0.1 mL PBS. When tumors reached the average size of 110(±4) mm^3 (day 13), the mice were randomized into five treatment groups ($n = 8$): (1) no treatment (2) fluoxetine alone (3) doxorubicin alone (4) doxorubicin and fluoxetine and (5) bevacizumab. Fluoxetine (groups 2 and 4) was given by gavage, 0.2 ml at the dose of 1 mg/kg body, daily administration for 6 consecutive days, for 3 weeks. Doxorubicin (groups 3 and 4) was administered by i.v. injection 0.2 ml per 20 g (v/w) at the dose of 2 mg/kg body, on days 15, 22 and 29 from tumor inoculation, starting two days post fluoxetine administration. Bevacizumab (group 5), at the dose of 5 mg/kg body was given by i.v. injection of 0.2 ml per 20 gr (v/w), five times a week for 3 weeks, starting at day 13 from tumor inoculation. Mice in group 1 (no treatment) were injected with PBS as a control to the doxorubicin injections (same volume and schedule), and given water by gavage as a control for fluoxetine (same volume and schedule). Tumor size was measured, using an electronic caliper every week, and the tumor volume was calculated according to the formula: tumor volume = $\pi(\text{width}/2)^2 \times \text{length}$, Animal survival was monitored continuously, and the animals were weighed weekly.

2.7. Statistics

Data were expressed as means ± SD (Figs. 2 and 3) and means ± SEM (Fig. 5). 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. The actual p values calculated are listed in the figure legends.

3. Results

3.1. Expression of ABC transporters

Although HCT-15 cells are known to be an inherent-Pgp cell line [28–31], due to the dynamic nature of cells in culture, we found it imperative to affirm prior to any MDR-related studies, that the specific cell batches we studied expressed Pgp. Since in one case, presence of MRP in these cells was reported as well, we also tested for the other two major transporter types – MRP and BCRP – known to be involved in MDR. Typical results of flow cytometry studies, using antibodies specific to each of these ABC transporters, are shown in Fig. 1. As clearly seen, the cells expressed Pgp (Fig. 1A), whereas MRP (Fig. 1B) and BCRP (Fig. 1C) were not detected. These findings affirm that the cells we studied were strictly a Pgp line.

3.2. Cytotoxicity

To assess the level of HCT-15 resistance and to evaluate the ability of fluoxetine to modulate its reversal, we studied responses of these cells to treatments by doxorubicin alone or with fluoxetine. Exposed to doxorubicin alone over the dose range of 0.1–30 μM (see typical results in Fig. 2A) exhibited the expected mild resistance to doxorubicin, with an IC_{50} of 4.7(±0.3) μM (Fig. 2B). Combination treatment with 10 μM fluoxetine (Fig. 2B) generated a statistically significant 10-fold reduction in IC_{50}. At this dose, fluoxetine alone did not affect cell viability (data not shown). To verify that, as expected, *in vitro* bevacizumab had low or no effect on cell viability, cells were treated (for 24 or 48 h) with bevacizumab over the dose range of 0.1–10 μM. Over most of the concentration range bevacizumab had negligible effect on cell viability, and even at the 10 μM dose (which, for this drug, is rather high), its effect on cell viability was small, especially compared to doxorubicin (Fig. 2C). Extending the bevacizumab exposure time from 24 to 48 h did not make any significant difference (data not shown). These findings fit with the known mechanism of bevacizumab's anti-tumor activity as an anti-angiogenic factor that binds to secreted VEGF [3–5]. Such activity obviously comes into effect *in vivo* and not *in vitro*.

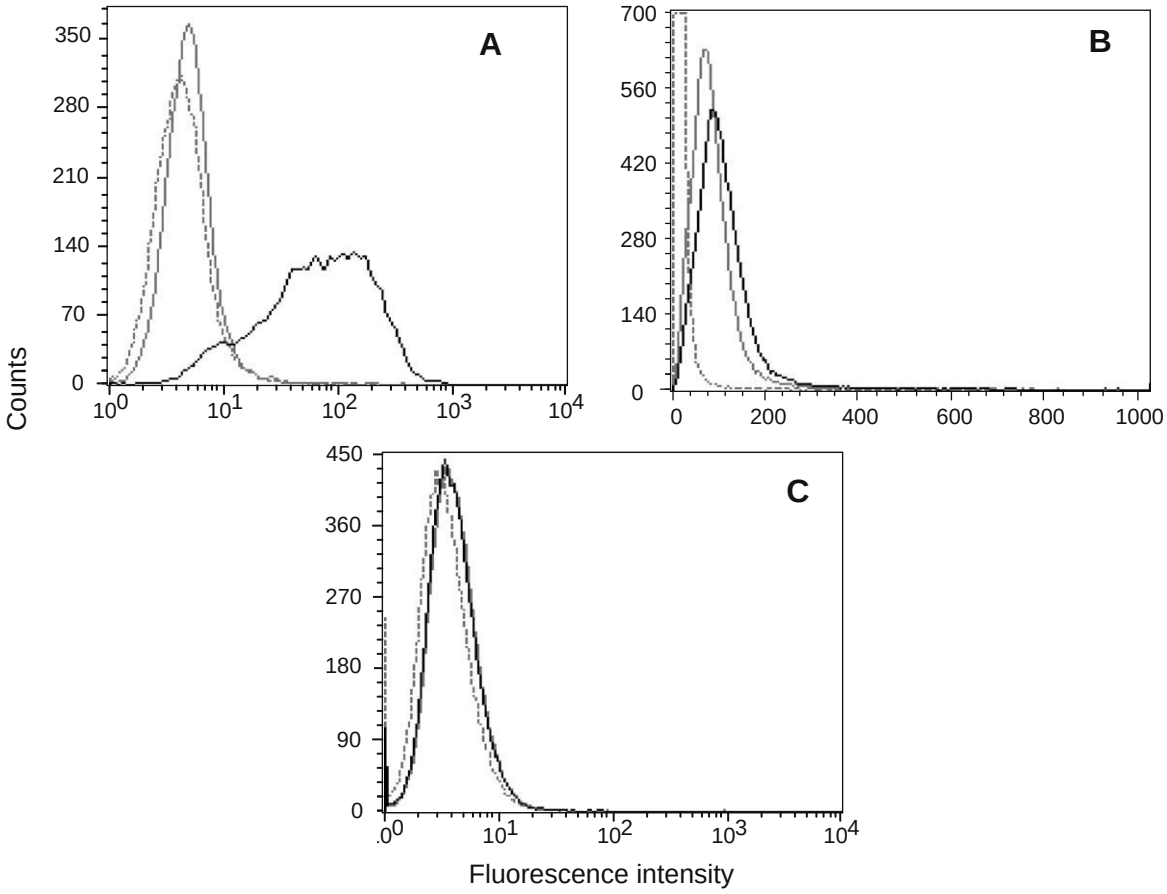

Fig. 1. Expression of ABC transporters in HCT-15 cells, evaluated by flow cytometry. No staining – dotted gray line; isotype control – solid gray line; transporter-specific antibody – solid black line. (A) Pgp, (B) MRP, (C) BCRP.

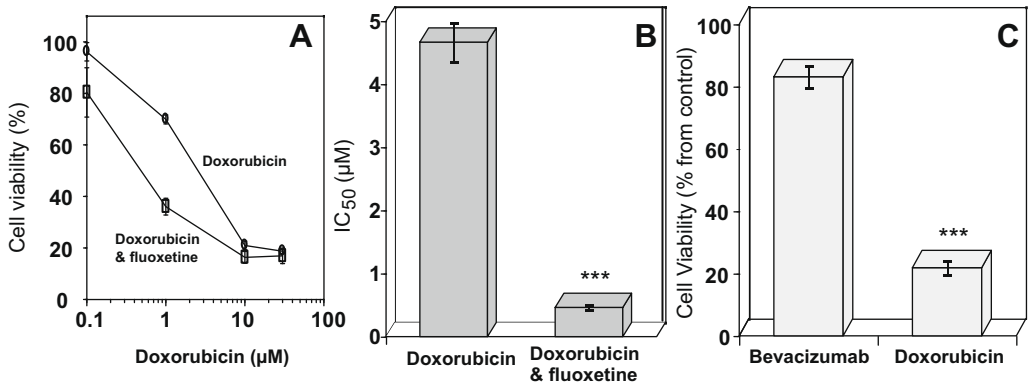

Fig. 2. Drug effects on viability of HCT-15 cells. (A) Cell viability (% from untreated control) as function of increase in doxorubicin concentration, treatment with doxorubicin alone (open circles) and doxorubicin with 10 μM fluoxetine (open rectangles). The symbols are the experimental data points each an average of 6 repeats, the error bars (seen within the symbols) are the standard deviations (SD), and the solid curves are non-theoretical drawn to emphasize the patterns of the data. (B) IC_{50} values of doxorubicin alone, and with 10 μM fluoxetine. Each bar is an average of three separate experiments, 6 repeats in each, and the error bars are the SD. (C) Effects of 10 μM bevacizumab and (separately) of 10 μM doxorubicin, on cell viability (% from untreated control). Each bar is an average of three separate experiments, 6 repeats in each, and the error bars are the SD. Statistical significance evaluations represented on the figure by asterisks are comparisons of doxorubicin and fluoxetine vs. doxorubicin alone ($^{***}p < 0.003$) and of doxorubicin vs. bevacizumab ($^{***}p < 0.0002$).

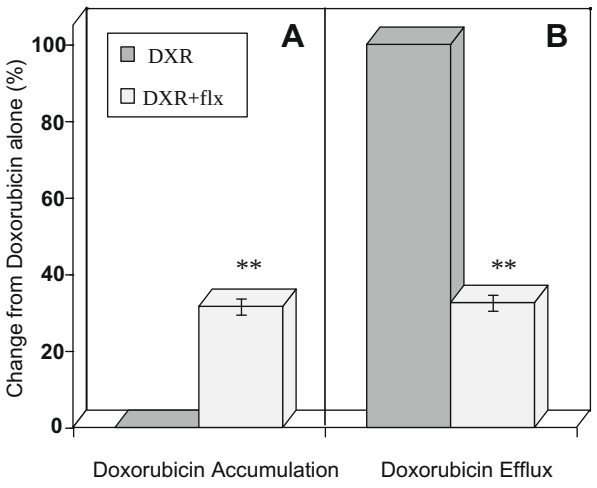

Fig. 3. Changes in doxorubicin intracellular levels as function of time and treatment, for HCT-15 cells. (A) Fluoxetine-induced increase in intracellular doxorubicin accumulation. Incubations were for 4 h, with 10 μM doxorubicin and 25 μM fluoxetine. (B) Fluoxetine-induced decrease in efflux of intracellular doxorubicin, after 1 h of incubation in drug-free media. Dark-shaded bars are doxorubicin alone, light-shaded bars are doxorubicin and fluoxetine. Each bar is an average of two independent experimental runs, 10,000 cells measured in each run. Statistical significance evaluations represented on the figure by asterisks are comparisons of doxorubicin and fluoxetine vs. doxorubicin alone for efflux ($^{**}p < 0.02$) and accumulation ($^{**}p < 0.04$).

3.3. Doxorubicin accumulation within, and efflux from, HCT-15 cells

Insights into the mechanism by which fluoxetine modulates MDR were gained by studying the effects of fluoxetine on intracellular doxorubicin accumulation and on efflux of intracellular doxorubicin. Incubating the cells for 4 h with 10 μM doxorubicin and 25 μM fluoxetine, induced a significant increase of 32% in intracellular doxorubicin concentration (Fig. 3A). Presence of fluoxetine in the cell suspension media induced a significant decrease of 70% in the efflux of intracellular doxorubicin (Fig. 3B).

3.4. Intracellular doxorubicin distribution

Doxorubicin accumulation and cellular drug localization were studied by confocal microscopy. Comparing cells incubated with doxorubicin alone (Fig. 4A) to cells incubated with the same doxorubicin dose but with the addition of fluoxetine (Fig. 4B and 4C) shows two distinct beneficial differences induced by this chemosensitizer: Doxorubicin intracellular accumulation is low in the absence of fluoxetine whereas in the presence of fluoxetine the accumulation is markedly increased (Fig. 4A vs. B and C). In the absence of fluoxetine the modest amount of drug that gained entry into the cells is mostly in the cytoplasm including adjacent to the nuclear envelope (Fig. 4A). In contrast, in the presence of fluoxetine and for both doses used, the pattern of intracellular drug distribution changes and it is almost exclusively in the nucleus (Fig. 4B and C).

We wish to emphasize that, as required for a chemosensitizer, in all these *in vitro* experiments, the fluoxetine dose was well below its toxicity limits and had no measurable effect on cell viability. In addition, although the doxorubicin dose was 10 μM, during the relatively short time spans the cells were exposed to this dose, it had no measurable effect on cell viability.

3.5. In vivo studies

The effects of treatment on changes in tumor volume were performed as described in Section 2. All treatments were deemed safe, as changes in animal weights along the entire time span of the experiment did not exceed 5% of initial weight. In untreated animals and in animals treated with fluoxetine alone, the tumors progressed exponentially. Tumor volumes at day 20 were 12-fold larger than at the day of randomization (day 0 in Fig. 5A, which was day 13 from tumor initiation). The impact of the three doxorubicin doses was rather small, tumor volumes at day 20 from treatment initiation were 10-fold larger than at the day of randomization with no statistical significance compared to untreated animals (Fig. 5A). In contrast, the combined treatment of doxorubicin and fluoxetine generated a highly-significant slow-down in tumor progression: tumor volumes increasing only 4-fold from the day of randomization (Fig. 5A). Moreover, the impact of the combined fluoxetine–doxorubicin treatment was significantly better than treatment by doxorubicin alone. The results of this combination treatment are comparable to those obtained when treatment was by bevacizumab alone (Fig. 5B). Albeit with bevacizumab it required 15 injections and a cumulative dose of 75 mg/kg body to provide the same level of response as 3 doses of doxorubicin and a cumulative dose of 6 mg/kg body – contingent on combination of this treatment with fluoxetine.

4. Discussion

For cancer patients MDR is a major impediment, whether the resistance is moderate or severe. Pre-clinical investigations into the resistance and its modulation are frequently performed utilizing cells that express very high levels of resistance. In the present study we deliberately focused on moderate resistance. The selected HCT-15 line, originating from human colorectal cancer, is classified as expressing Pgp [28–30] and in one reported to also express

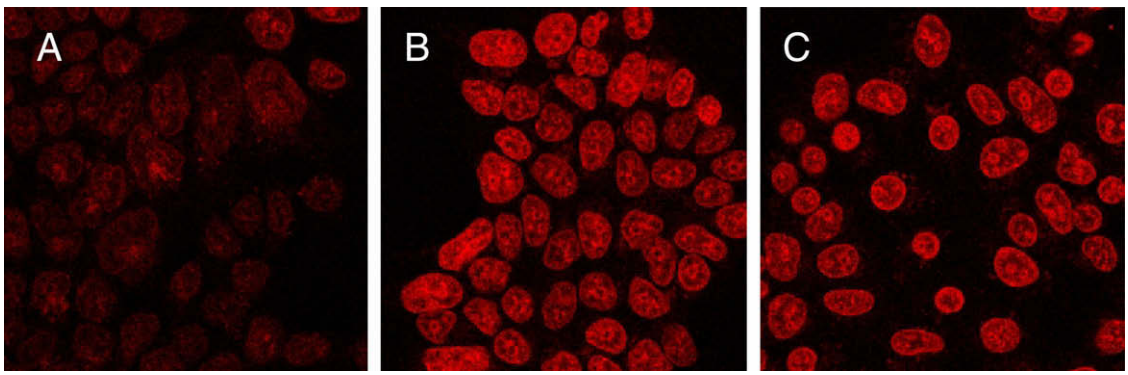

Fig. 4. Confocal laser scanning microscopy images of fluoxetine's impact on intracellular doxorubicin accumulation and distribution, for the Pgp-expressing HCT-15 cells. Representative data from one of three independent experiments (that were quite similar to one another). (A) Cells incubated for 2 h with 10 μM doxorubicin. (B) Cells incubated with doxorubicin under the same conditions listed under (A), with the addition of 10 μM fluoxetine to the incubation media. (C) Similar to B, but with 25 μM fluoxetine.

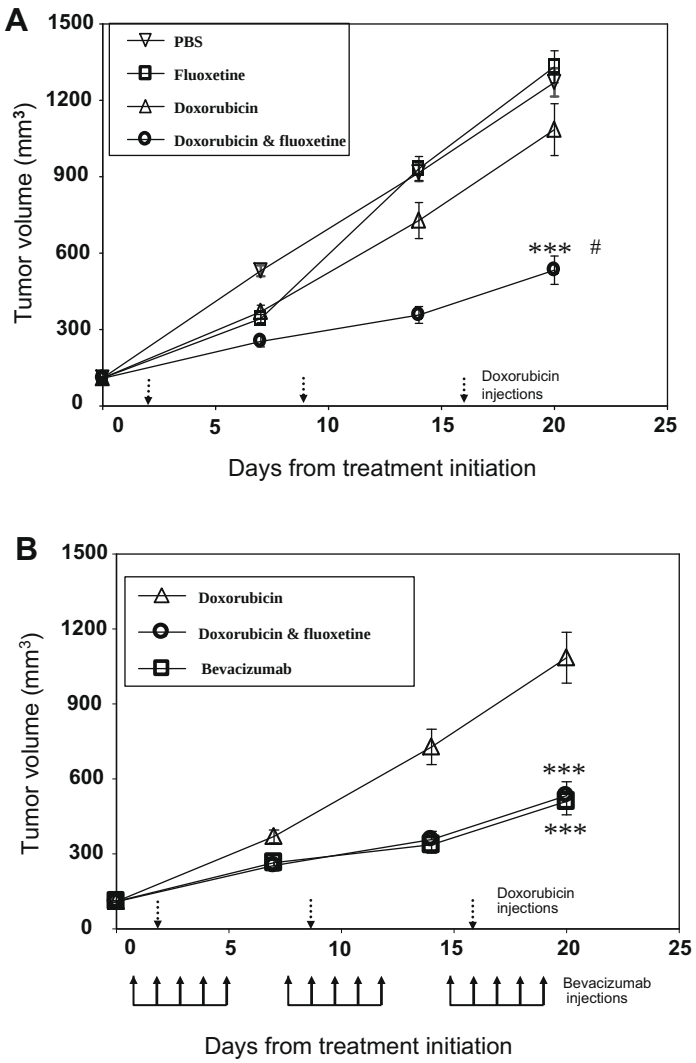

Fig. 5. *In vivo* therapeutic responses of HCT-15-inoculated nude mice. (A) Increase in tumor volume as function of time and treatment. PBS and doxorubicin (2 mg/kg body) were injected i.v., at days 2, 9 and 16 from treatment initiation as indicated by the dotted arrows on the figure. Fluoxetine (1 mg/kg body) was given by gavage, 6 days a week, for the duration of the experiment. Symbols are listed on the figure. (B) Similar to (A), except comparing doxorubicin with and without fluoxetine, to treatment by bevacizumab. The doxorubicin with/without fluoxetine and the arrows indicating the days of drug injection are re-drawn from (A). Bevacizumab (5 mg/kg body) was injected i.v. 5 days a week, for three weeks, as indicated by the solid arrows on the figure. The points are the experimental data each an average of eight animals, and the error bars are the SEM. The solid lines are non-theoretical, drawn to emphasize the trends in the data. Statistical significance evaluations represented on the figure are: (i) by the asterisks symbol – comparisons of the following treatments to no treatment: doxorubicin and fluoxetine vs. PBS ($^{***}p < 0.0003$) and bevacizumab vs. PBS ($^{***}p < 0.00004$). (ii) By the hash symbol – doxorubicin and fluoxetine vs. doxorubicin alone ($^{\#}p < 0.0006$). There was no statistical significance between doxorubicin alone and PBS ($p < 0.2$) and between fluoxetine alone and PBS ($p < 0.6$).

MRP [31]. We tested for all the three major types of ABC transporters and found that, similar to most previous reports, HCT-15 cells to express Pgp alone (Fig. 1). Together with those previous findings, the present case demonstrates that the changes established cell lines can undergo, make it important to determine anew the make-up of ABC transporters in an investigated MDR cell line. Previous reports demonstrated the moderate MDR nature of the HCT-15 cells: Upon a 72-h exposure to doxorubicin, reported IC_{50} values were 0.1–0.5 μM [30,33,39]. Bearing in mind that, for the same drug and cell line, the longer the cells are exposed to the drug, the lower the IC_{50}, the value we obtained (4.7(±0.3) μM) for a 24 h exposure to the drug (Fig. 2) fits quite well with the previous findings.

A chemosensitizer is expected to improve the response of drug-resistant cells to anti-cancer drugs and at the same time to have no effect on cell viability. In the HCT-15 cells, we first found fluoxetine to act as a chemosensitizer *in vitro*: combined treatment of doxorubicin and fluoxetine under conditions similar to those applied for the drug alone, reduced the IC_{50} value of doxorubicin by an order of magnitude, down to 0.46(±0.04) μM. This is in line with findings of others for this cell line, using different chemosensitizers [30,40]. Moderate resistance carries the

implication that drug accumulation and efflux with/without a chemosensitizer will also be moderate, running the risk of being below detection limits. This requires combinations of incubation times with drug and chemosensitizer doses that are high enough to be above detection limits, yet low enough that the cells will not suffer any detectable damage. The doses we applied fit both sides of this requirement. Incubating the cells for 4 h with a combination of fluoxetine and doxorubicin sufficed to increase intracellular drug accumulation by 32% over than of drug alone (Fig. 3A). As expected for the short efflux duration and for a moderate MDR cell line, the presence of a chemosensitizer sufficed to generate a significant inhibition (70%) of drug efflux from the cell (Fig. 3B). These "two sides of the coin" indicate that fluoxetine is also able to modulate moderate MDR, and that a major mechanism by which fluoxetine exerts its effect is the inhibition of extrusion pumps. Insights into the molecular nature of the fluoxetine–Pgp interactions will be the subject of future studies. Further mechanistic support can be drawn from the confocal microscopy studies of intracellular drug accumulation where fluoxetine not only increased doxorubicin intracellular accumulation, but also its nuclear uptake within the cells. Similar observations for doxorubicin in cells overexpressing LRP were reported for cyclosporin A and (separately) for sirolimus [41]. We find this fluoxetine effect quite encouraging, in view of the nucleus being a major cellular compartment in which doxorubicin exerts its molecular therapeutic effect.

The moderate resistance of doxorubicin is also evident *in vivo*, in human xenografts of the HCT-15 line: Three injections of doxorubicin, to a cumulative dose of 6 mg/kg body had little effect on tumor progression (Fig. 5). These results are in keeping with other reports, taking into account the differences in treatment regiments [31–33]. The combination of doxorubicin and fluoxetine generated a significant slow-down of tumor progression (Fig. 5A). This combination, showing the ability of fluoxetine to modulate resistance *in vivo*, was as effective as treatment with bevacizumab (Fig. 5B). To obtain a significant impact of bevacizumab in this model we chose intense regimen and dosing, which were well-tolerated by the animals and were not toxic. It required, however, 15 injections of bevacizumab providing a cumulative dose of 75 mg/kg body, to generate the same level of response obtained by only 3 injections of doxorubicin providing a cumulative dose of only 6 mg/ml. The latter, however, was dependent on combining the doxorubicin treatment with almost daily administration of a low fluoxetine dose (1 mg/kg body) at the patient-friendly oral route of administration. In a similar HCT-15 *in vivo* study, the chemosensitizers PSC833 and (separately) cyclosporin A were applied for MDR modulation, but at a significantly higher dose of 50 mg/kg body 4 h prior to the injection of 8 mg/kg body doxorubicin [33].

For treatment of depression, fluoxetine is prescribed at the dose range of 20–80 mg/person/day [42]. Taking into account the metabolic differences between mouse and human, the fluoxetine dose applied here for MDR modulation, corresponds to 5 mg/person/day, which is well below the safety limits. It is hoped that the promise implied in the present study with respect to fluoxetine's ability to reverse MDR at low safe doses, will materialize in the clinic. As discussed in the Introduction, the chemotherapy modality, frequently used for treatment of colorectal cancer, includes not only doxorubicin, but additional drugs that are substrates of the MDR pumps. Adding fluoxetine to such chemotherapeutic combinations may improve therapeutic responses to this veteran modality. Clinical trials underway explore the combinations of bevacizumab with chemotherapeutic drugs, for colorectal as well as for other types of cancer [43]. We offer that there is merit in contemplating and testing the addition of fluoxetine – also an approved drug – to such combinations.

Conflicts of interest statement

None declared.

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