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Fluoxetine Inhibits Multidrug Resistance Extrusion Pumps and Enhances Responses to Chemotherapy in Syngeneic and in Human Xenograft Mouse Tumor Models

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ABSTRACT

Multidrug resistance (MDR) operated by extrusion pumps such as P-glycoprotein and multidrug-resistance-associated-proteins, is a major reason for poor responses and failures in cancer chemotherapy. MDR modulators (chemosensitizers) were found among drugs approved for noncancer indications and their derivatives. Yet toxicity, adverse effects, and poor solubility at doses required for MDR reversal prevent their clinical application. Among newly designed chemosensitizers, some still suffer from toxicity and adverse effects, whereas others progressed to clinical trials. Diversities among tumors and among MDR pumps indicate a need for several clinically approved MDR modulators. Here we report for the first time that fluoxetine (Prozac), the well-known antidepressant, is a highly effective chemosensitizer. *In vitro*, fluoxetine enhanced (10- to 100-fold) cytotoxicity of anticancer drugs (doxorubicin, mitomycin C, vinblastine, and paclitaxel) in drug-resistant but not in drug-sensitive cells (5 and 3 lines, respectively). Fluoxetine increased drug accumulation within MDR-cells and inhibited drug efflux from those cells. *In vivo*, fluoxetine enhanced doxorubicin accumulation within tumors (12-fold) with unaltered pharmacokinetics. In four resistant mouse tumor models of both syngeneic and human xenograft, combination treatment of fluoxetine and doxorubicin generated substantial ($P < 0.001$) improvements in tumor responses and in survivals (2- to 3-fold). Moreover, fluoxetine reversed MDR at doses that are well below its human safety limits, free of the severe dose-related toxicity, adverse effects, and poor solubility that are obstacles to other chemosensitizers. This low-dose range, together with the findings reported here, indicate that fluoxetine has a high potential to join the arsenal of MDR reversal agents that may reach the clinic.

INTRODUCTION

Chemotherapy frequently fails cancer patients due to inherent or acquired multidrug resistance (MDR; refs. 1–3). In the dominant mechanism, intracellular levels of cytotoxic drugs are reduced below lethal thresholds by active extrusion of the cytotoxic drug(s) from the tumor cell, operated by ATP-dependent pumps such as P-glycoprotein and multidrug resistance-associated protein (1–7). Inhibition of the extrusion is a main approach to overturn MDR (1–9).

The search for effective inhibitors (also named chemosensitizers, MDR modulators, and MDR reversal agents) is now into the third generation. First-generation candidates were drugs already approved for other noncancerous indications, such as verapamil, cyclosporine A, and progesterone (8–13). Unfortunately, although successful *in vitro*, patients could not benefit from these chemosensitizers, because their clinically relevant doses exceed safety limits resulting in unacceptable adverse effects, frequent poor solubility, and toxicity (8–13). Chemical derivatization of first-generation molecules and combinatorial chemistry lead to second- and third-generation chemosensitizers, such as VX-710, PSC833, XR9051, XR9576, MS-209, GF120918, R101933, LY335979, and OC144–093 (ONT-093; refs. 14–32), some of which are in clinical trials (25, 27, 28, 30–32). Several of the latter, whereas more potent and less toxic than first-generation compounds, may still be prone to adverse effects, poor solubility, and unfavorable changes in pharmacokinetics of the anticancer drugs. Moreover, given tumor diversity, it is rational to assume that more than one chemosensitizer will be needed in the clinic.

Here we report for the first time that fluoxetine (Prozac), the well-known antidepressant (33), acts as a highly effective chemosensitizer, joining the arsenal of MDR modulators with prospects of reaching the clinic. Seemingly back to first-generation candidates in terms of using drugs approved for noncancerous indications, we suggest it should be viewed as a fourth-generation chemosensitizer. Unlike first-generation molecules, fluoxetine modulates MDR at low doses, free of the severe dose-related drawbacks experienced with chemosensitizers of previous generations and free of solubility limitations.

This report presents results of *in vitro* and *in vivo* studies. Activities and mechanism of chemosensitization by fluoxetine were studied in eight cell lines, covering drug-sensitive and drug-resistant cells (inherent and acquired MDR) Verapamil and Cyclosporin A were included as *in vitro* benchmarks, the rationale for this choice stemming from their acknowledged *in vitro* activities as MDR reversal agents and their availability. The *in vivo* studies were conducted in three syngeneic and one human xenograft mouse tumor models, exploring pharmacokinetics, biodistribution, and therapeutic responses.

MATERIALS AND METHODS

Reagents and Cell Cultures. Paclitaxel, rhodamine-123 (Rh-123), verapamil, cyclosporine A, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Trypan-blue were from Sigma Chemical Co. (St. Louis, MO). Doxorubicin and Vinblastine were a kind gift from TEVA Pharmaceutical Ltd. (Natania, Israel). Mitomycin C was a kind gift from Dexon Ltd. (Or Akiva, Israel). Fluoxetine was a kind gift from Unipharm (Ramat Gan, Israel). Cisplatin was from TEVA Pharmaceutical Ltd. Materials for cell cultures (specified under Materials and Methods) were from Biological Industries (Beit Haemek, Israel). [^{3}H]Vinblastine sulfate (specific activity 17 Ci/mmol) was from Amersham (Buckinghamshire, England). [^{3}H]paclitaxel (specific activity 20 Ci/mmol) was from American Radiolabeled Chemicals Inc., (St. Louis, MO).

Cell monolayers or suspensions were grown in 100 × 20-mm dishes (culture plates and dishes were from Corning Glass, Corning, New York). B16F10.9 and HT29 cells were cultured in DMEM at 37°C in 5% CO_2 supplemented with 10% fetal calf serum, penicillin (10,000 units/mL), streptomycin (10 mg/mL), and L-glutamine (200 mmol/L). C-26, P388, and D122 cells were similarly cultured, except the medium was RPMI 1640. MCF-7 and MCF-7/ADR cells were maintained as monolayer cultures in MEM containing 10% fetal bovine serum, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, and 1.5 g/L of sodium bicarbonate. P388/ADR and MCF-7/ADR were grown in the presence of 0.25 μg Doxorubicin/mL and 0.5 μg Doxorubicin/mL, respectively. Cells were free of *Mycoplasma* contamination. determined by a *Mycoplasma* ELISA test (Boehringer Mannheim GmbH, Mannheim, Germany) performed every 3 months. Cell viability was determined by the following: (1) the MTT method, recording the absorbencies in a plate reader, at two wavelength 550 and 650 nm, (2) the Trypan-blue method, using a hemocytometer, and (3) total cell protein by the Bradford method.

Fluorescence-Activated Cell Sorter Analysis. Suspensions of P388/ADR or C-26 (1×10^6 cells/mL) in RPMI 1640 were incubated at 37°C for 30 minutes with 5 μmol/L Rh-123 with and without a chemosensitizer, selected from verapamil (15 μmol/L), cyclosporine A (15 μmol/L), and fluoxetine (5 μmol/L). Intracellular fluorescence was determined by Fluorescence Activated Cell Sorter

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(FACSort, Becton Dickinson, Franklin Lakes, NJ). Excitation and emission were at 485 nm and 547 nm, respectively. Sample sizes were 10^4 cells.

Drug Efflux. C-26 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 confluency. The wells, divided into three groups, were incubated for 10 hours with serum-supplemented growth medium containing 0.1 μg/ml Doxorubicin and the following additions: group 1: none, group 2: 15 μmol/L verapamil, and group 3: 15 μmol/L fluoxetine. Upon end of incubation, the medium from each well was aspirated, and the cells washed with PBS and incubated with efflux medium, PBS, PBS+ verapamil (15 μmol/L), and PBS+fluoxetine (15 μmol/L), for groups 1, 2, and 3, 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. The protocol for Doxorubicin efflux from P388/ADR cells was similar to that of the C-26 cells, except for the following: (1) cell seeding was of $1*10^6$ cells/mL into six-well plates, and (2) the cells were divided into four groups, to accommodate testing three chemosensitizers, verapamil, cyclosporine A, and fluoxetine, each at a concentration of 5 μmol/L. Efflux of paclitaxel from MCF-7/ADR cells was done similar to that of Doxorubicin.

***In vitro* Cytotoxicity.** Twenty-four hours before an experiment, cells of a given line were seeded onto 96-multiwell plates at a density of 1×10^4 cells/mL. Upon initiation the regular media was replaced by treatment media, consisting of drug-containing regular growth media with or without a chemosensitizer. Doxorubicin, mitomycin C, vinblastine, and paclitaxel were the test drugs, each tested with fluoxetine and one or more of the benchmark chemosensitizers. Four hours after administration, the media from each well was removed and the cells washed and fed with drug-free chemosensitizer-free fresh serum-supplemented media. The experiments were terminated 20 hours later (24 hours from start), and the quantity of viable cells was determined by the MTT method. Cells treated with paclitaxel (with and without chemosensitizer) were incubated with the treatment media for 48 hours, at the end of which the MTT assay was performed.

Duration of MDR Reversal. The experiments followed Dantzig *et al.* (21) with minor changes. Briefly, 1×10^6 cells/mL of the MCF-7/ADR and of the C-26 lines were incubated for 24 hours with fluoxetine (5 μmol/L) or verapamil (15 μmol/L), before being washed zero or three times with serum-supplemented growth media. Incubation continued for selected periods (from 0 to 24 hours), after which Doxorubicin was added. Twenty four hours later, the cells were detached, washed twice, and replated at the concentration of 2.5×10^5 cells/mL. Three days later the experiment was terminated, and cell viability was determined using the MTT method. This type of experiment was repeated over many Doxorubicin doses to allow for determination of IC_{50}. Control experiments were conducted under similar conditions but without chemosensitizer.

Mouse Tumor Models, Treatment Protocols, and Measured Parameters. Animals were obtained from the animal breeding center, Tel Aviv University (Tel Aviv, Israel) and were maintained and treated according to NIH guidelines. All of the animal protocols were approved by the Tel-Aviv Institutional Animal Care and Use Committee.

In all of the syngeneic mouse tumor models, fluoxetine was administered p.o. by inclusion in the drinking water, at the dose of 0.04 mg/kg body/day, from tumor inoculation and on. Drug administration was by injection of 100 μL of Doxorubicin-buffered (PBS) solution to the lateral tail vein using 26-gauge needles. Doxorubicin doses and administration regimen were model-specific, as detailed below.

Intraperitoneal Ascites Model. The protocol was adapted from previous methods (6, 23). P388/WT and (separately) P388/ADR murine leukemia cells were propagated in the peritoneum of 6–8 weeks old BDF_1 female mice by weekly transfer of 0.5 mL of peritoneal fluid containing 5×10^5 cells. In each experiment the animals were divided into four treatment groups ($n = 5$): saline, fluoxetine, Doxorubicin, and Doxorubicin+fluoxetine. Doxorubicin dose was 3 mg/kg body. Injections were on days 1, 5, and 9 from tumor inoculation. Survival of all of the animals was monitored continuously.

Lung Metastatic Disease. The protocols were adapted from methods described previously (34). B16F10.9 cells (5×10^5/50 μL PBS) were administered i.v. into 10- to 12-week-old male C57BL/6 mice. Treatment groups, animals/group, and Doxorubicin administration regimen were as listed in the section above, except the Doxorubicin dose was 10 mg/kg body. An additional group of untreated tumor-free mice served as control. Two independent experiments were run, one to evaluate survival and the other to evaluate lung metastatic burden. In the latter, terminated 21 days after tumor injection, the lungs of all of the animals were removed, weighted, and fixed in Bouin's solution. Increase in lung weight was calculated using the formula (34): Lung weight increase (%) = 100 $\times$ (tumor lung weight $-$ normal lung weight)/normal lung weight. Surface metastases were counted, using a dissecting microscope, by a pathologist blinded to the experimental groups involved. For survival evaluation animals were monitored daily, and the experiment was terminated on day 75.

Pharmacokinetics and Drug Biodistribution in Lung Tumors. Animal species, tumor cell line, and tumor inoculation were essentially as described in the section above. Two independent experiments were run, one for pharmacokinetics and one for biodistribution. In each run, the animals were divided into two groups ($n = 5$), one treated with Doxorubicin and the other with Doxorubicin+fluoxetine. A single dose of Doxorubicin (10 mg/kg body) was injected at day 10 from tumor inoculation. Blood samples for pharmacokinetics were drawn up to 6 hours after drug injection. For drug biodistribution, animals were sacrificed 6 hours after drug injection, lungs and selected organs removed, viewed by the pathologist, weighed, and processed to extract and assay the drug (by fluorescence, as detailed under drug efflux).

Solid Tumor Model. The protocol was adapted from methods described previously (34). C-26 cells (5×10^5/30 μL Hank's Buffer) were injected into the right-hind foot pad of 6- to 8-week-old female BALB/c mice. The number of treatment groups and animals/group were similar to those listed in Intraperitoneal Ascites Model section above. Doxorubicin dose was 10 mg/kg body, and treatments were given on days 5, 12, 19, and 26 after tumor inoculation. Tumor size was measured, using an electronic caliper, every other day for the next 29 days, and the tumor volume was calculated according to the formula: tumor volume = $1/2(\text{width})^2 \times$ length. Animal survival was monitored continuously.

Table 1 *Fluoxetine effects on the cytotoxicity of chemotherapeutic drugs, in drug-sensitive and in drug-resistant cells*

	Mitomycin C			Doxorubicin			Vinblastine		
	IC_{50}* (μM)			IC_{50} (μM)			IC_{50} (μM)		
Cell line	Drug	Drug + fluoxetine	RF†	Drug	Drug + fluoxetine	RF	Drug	Drug + fluoxetine	RF
MCF-7	30	28	1.1	0.35	0.33	0.9	0.28	0.30	1.1
P388/WT	45	42	1.1	4.7	4.8	1.1	4.2	4.4	1.0
HT29	30	33	0.9	3.6	3.3	0.9	3.5	3.3	1.1
B16F10.9	209	3.6	58	12	0.78	29	13	0.45	15
D122	209	3.3	63	19	0.90	21	21	1.0	21
C-26	105	2.5	42	25	0.84	36	9.1	0.25	30
MCF-7/ADR	200	4.5	44	19	0.29	69	22	0.32	66
P388/ADR	224	5.4	42	31	0.55	27	38	1.4	56

* The IC_{50} value was determined for each cell line after exposure to a series of drug concentrations with/without 15 μM fluoxetine, using the transient protocol. SD values were $<5\%$ of the IC_{50} value ($n = 5$).

† RF represents fold-change in drug sensitivity.

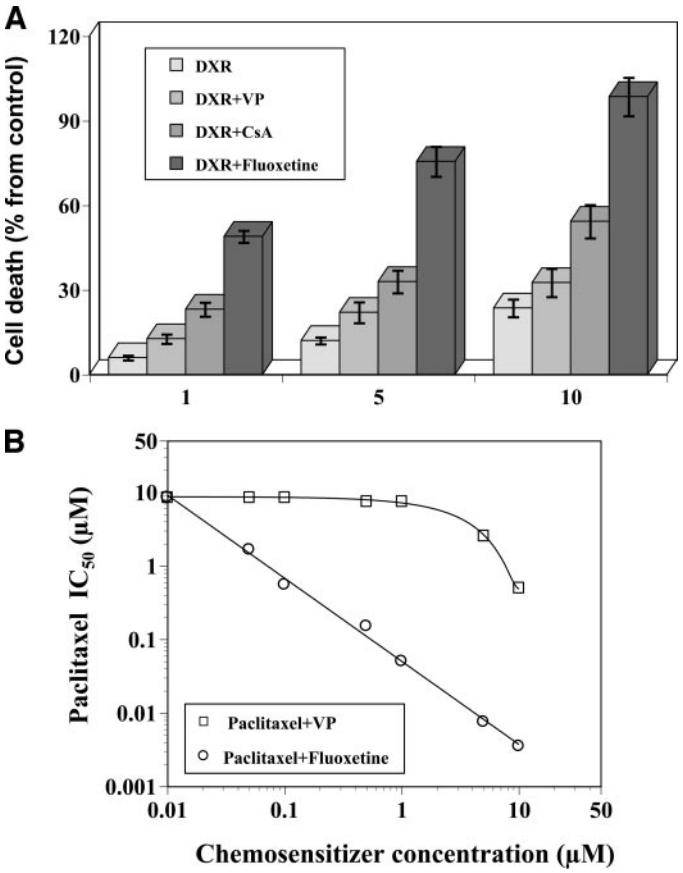

Fig. 1. *In vitro* cytotoxicity of chemotherapeutic drugs in MDR-cells, with/without chemosensitizers. *A*, doxorubicin (*DXR*) cytotoxicity in P388/ADR cells, presented as percentage of dead cells (from untreated control). Verapamil (*VP*), Cyclosporin A (*CsA*), and fluoxetine were each 5 μmol/L. Each *bar* is an average of 32 to 64 wells; *bars* $\pm$SD. The set of four bars for each drug dose are (*left* to *right*): DXR, DXR+VP, DXR+CsA, and DXR+fluoxetine, respectively. *B*, paclitaxel IC_{50} in MCF-7/ADR cells, as function of chemosensitizer species and concentration. □, VP; ○, fluoxetine. *Points* are experimental, the *solid curves* nontheoretical, drawn to emphasize the data trends.

Reversal of MDR in Human Breast Carcinoma (MCF-7/ADR) Xenografts. The protocol was adapted from methods described previously (35). 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 2.5×10^6 cells in 0.1 mL of PBS were implanted s.c by injection just above the right femoral joint. When tumor volumes reached 75 mm^3 (day 0 of treatment), the mice were randomly separated into four groups, similar to those of the syngeneic mice. Saline and Doxorubicin were given every 2 days by i.p. injection. Doxorubicin dose was 4 mg/kg body, and fluoxetine administration (similar in all respects to that given to the syngeneic mice) was started on day zero of treatment. Tumor volume was calculated as (length $\times$ width) [(length + width)/2]. The experiment was terminated upon reaching tumor volumes of 2,500 mm^3.

RESULTS

In vitro Cytotoxicity

The eight cell lines used in this study fall into three categories: (1) drug-sensitive: HT29 (human colon carcinoma), P388/WT (murine leukemia), and MCF-7 (human breast carcinoma); (2) inherently resistant (P-glycoprotein): C-26 (Colon carcinoma ref. 26), B16F10.9 (highly aggressive mouse melanoma, ref. 36), and D122 (highly aggressive mouse Lewis lung carcinoma, refs. 37, 38); and (3) acquired-resistant (P-glycoprotein): P388/ADR (derived from P388/WT) and MCF-7/ADR (derived from MCF-7). The effects of fluoxetine alone were tested over the dose range of 2 to 50 μmol/L and found to be nontoxic up to 40 μmol/L. Consequently, in all of the subsequent *in vitro* experiments fluoxetine doses were well below toxicity limits. Each cell line was tested with three drugs: vinblastine, mitomycin C, and doxorubicin.

Cytotoxicity trends were quite similar among the three drugs tested, with obvious quantitative differences from one drug to the other (Table 1). As expected, and confirming the MDR nature of the resistant lines, IC_{50} values of drug-sensitive cells treated with drug alone were significantly lower than those of drug-resistant cells, irrespective of whether the resistance was acquired or inherent (Table 1 rows 1–3 *versus* rows 4–7). The three drug-sensitive lines were unaffected by the combination treatment of fluoxetine + chemotherapeutic drug (Table 1, rows 1–3). Drug-resistant cells were, on the other hand, quite affected by the combination treatment, the addition of fluoxetine resulting in 15- to 69-fold increases in drug sensitivity (Table 1, RF columns). We found similar trends when the cytotoxicity experiments were repeated with other fluoxetine doses, within the range of 2 to 30 μmol/L, and/or using longer exposures to the treatment media. Comparing, for the acquired-MDR cell lines, the parent *versus* the resensitized cells, shows mixed trends: P388/ADR cells were resensitized to higher levels than the parent cells, 7.8-, 8.7-, and 3.1-fold, for mitomycin C, doxorubicin, and vinblastine, respectively. Higher resensitization of MCF-7/ADR cells was seen with mitomycin C (6-fold) but not with the other two drugs. These mixed trends indicate that in the transition from a sensitive to a resistant culture, more than the resistance characteristics may have changed. Two major approaches provide MDR acquisition: the selective growth used in this study and MDR1 gene transfection. The former may be more active in changing culture characteristics but is better in modeling acquired-MDR in real life. To verify that fluoxetine resensitized P-glycoprotein-generated MDR, we repeated studies of the type reported in Table 1, testing cisplatin. IC_{50} values of cisplatin in MCF-7 cells were 35 and 40 ng/mL, with and without fluoxetine, respectively,

Table 2 *Persistence of MDR reversal in MCF-7/ADR and C-26 cells*

	Treatment protocol		Doxorubicin IC_{50} (μM)	
Chemosensitizer	Chemosensitizer washout*	Time span between washout and doxorubicin addition (hours)†	C-26 cells	MCF-7/ADR cells
None	None	0	>19	>30
15 μM Verapamil	None	0	9.1	11.5
	Yes	0	>19	>30
5 μM Fluoxetine	None	0	0.29	0.84
	Yes	0	0.62	1.20
	Yes	6	1.25	1.45
	Yes	12	1.60	1.85
	Yes	24	19	26

* Chemosensitizer washout: None indicates no washout. Yes indicates washout was performed according to the details described under Materials and Methods.

† Zero hours indicates doxorubicin was added immediately after the prior step (whether washout was or was not performed) and 6,12, and 24 hours indicate the time span between termination of the washout (during which the system was incubated with serum-supplemented growth media alone) and doxorubicin addition.

and those for MCF-7/ADR cells were 60 and 55 ng/mL, with and without fluoxetine, respectively.

Fluoxetine chemosensitization was similar in trend to that of the two benchmarks, verapamil and cyclosporine A, but at equal doses, fluoxetine was more active, as shown for doxorubicin (Fig. 1*A*) and for paclitaxel (Fig. 1*B*). The effect of fluoxetine persisted much longer compared with verapamil (Table 2). Combined treatment of verapamil + doxorubicin generated 2- to 3-fold reduction in IC_{50} values compared with doxorubicin alone (Table 2), but responses reverted to control values upon verapamil washout before drug administration (Table 2). Results of similar experiments with fluoxetine were quite different (Table 2): a 3-fold lower fluoxetine dose (5 *versus* 15 μmol/L) generated a 40- to 70-fold decrease in doxorubicin IC_{50}s, and it took a 24-hour gap between fluoxetine washout and cell exposure to doxorubicin for return to control IC_{50}s.

In vitro Studies on the Mechanism of MDR Reversal by Fluoxetine

Insight into the mechanism(s) by which fluoxetine modulates MDR was pursued through the effects of fluoxetine on drug efflux from and on drug accumulation within MDR-cells.

The efflux of doxorubicin or paclitaxel from MDR-cells was quite fast (Fig. 2) for both acquired and inherent (P388/ADR and MCF-7/ADR) and inherent (C-26 line) MDR. One to 2 hours sufficed for complete depletion (Fig. 2). Fluoxetine and the benchmark chemosensitizers verapamil and cyclosporine A slowed down the efflux and, at equimolar doses, fluoxetine was the most effective of the three (Fig. 2). Similar trends were found for mitomycin C efflux from C-26 cells (data not shown).

Accumulation of Rh-123, a well-established P-glycoprotein substrate, was rather poor in both inherent and acquired MDR cells (Fig. 3). Adding 15 μmol/L verapamil or cyclosporine A to the incubation medium generated mild increases in intracellular Rh-123 accumulation, whereas adding 5 μmol/L fluoxetine generated a substantial, 2 to 3 orders of magnitude, increase in intracellular accumulation (Fig. 3).

In vivo Studies

Pharmacokinetics and Biodistribution in C57BL/6 Mice Bearing B16F10.9 Lung Tumors. Fluoxetine did not alter the plasma pharmacokinetics of i.v. administered doxorubicin (Fig. 4*A*) nor did it affect drug accumulation in spleen, kidneys, and liver (Fig. 4*B*), organs found by pathology to be tumor-free. On the other hand, doxorubicin accumulation in the tumor-bearing lungs was 12-fold ($P < 0.01$) higher in the animals treated with doxorubicin + fluoxetine compared with those treated with the drug alone (Fig. 4*B*).

Lung Metastatic Burden and Survival of C57BL/6 Mice Bearing B16F10.9 Lung Metastatic Disease. The lung metastatic burden was evaluated by two measures: the increase in lung weight compared with a control group of healthy animals (*i.e.*, no tumor inoculation and no treatment), and the number of lung metastasis. Good correlation was obtained between both measures for each of the four treatment groups. Upon termination of the experiment, day 21, the lungs of the healthy mice weighed 0.21($\pm$0.03) g. Animals receiving saline or fluoxetine had the highest increase in lung weight and the largest number of lung metastasis (Fig. 5*A*). Doxorubicin was better than saline or fluoxetine, but the animals still had a high metastatic burden (Fig. 5*A*). Only the combination of doxorubicin + fluoxetine generated a significant reduction in lung metastatic burden (Fig. 5*A*). The effects of each treatment group on the metastatic disease were mirrored by the survival data (Fig. 5*B*): all animals treated with saline, fluoxetine, or doxorubicin did not survive beyond day 25, whereas animals treated with doxorubicin and fluoxetine were long survivors, the increase in life span >3-fold.

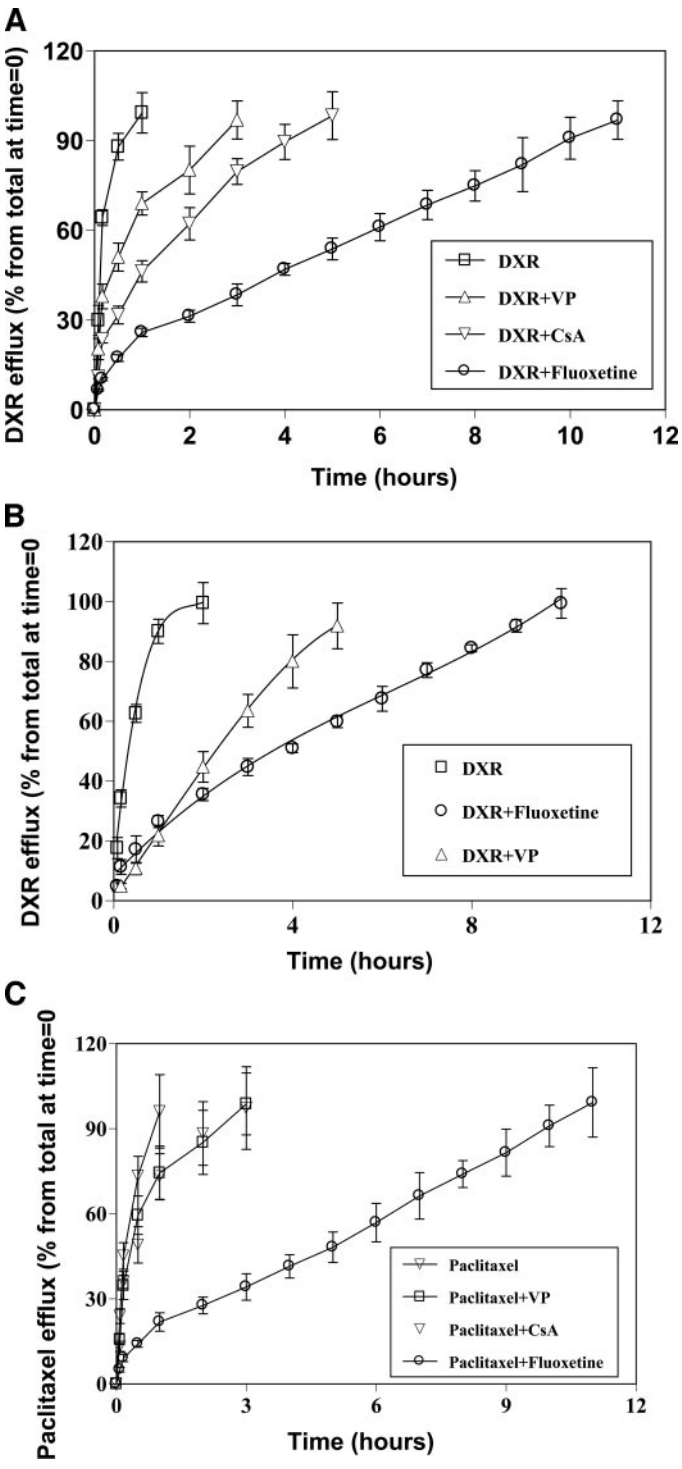

Fig. 2. Chemosensitizer effects on drug efflux from MDR-cells. *A*, doxorubicin (*DXR*) efflux from P388/ADR cells. Verapamil (*VP*), Cyclosporin A (*CsA*), and fluoxetine doses were each 5 μmol/L. □, DXR alone; △, DXR+VP; ▽, DXR + CsA; ○, DXR + fluoxetine. *Points* are experimental, each an average of 10 determinations; *bars*, ±SD. The *solid curves* are nontheoretical drawn to emphasize the data trends. *B*, same as *A*, except the cells were C-26, and the chemosensitizer concentrations were 15 μmol/L. *C*, same as *A*, except the cells were MCF-7/ADR, chemosensitizer concentrations were 15 μmol/L, and the anticancer drug was paclitaxel.

Survival of BDF_1 Mice Bearing Drug-Sensitive (P388/WT) or Drug-Resistant (P388/ADR) Intraperitoneal Ascites. Over a period of 20 days animals inoculated with P388/ADR cells showed the

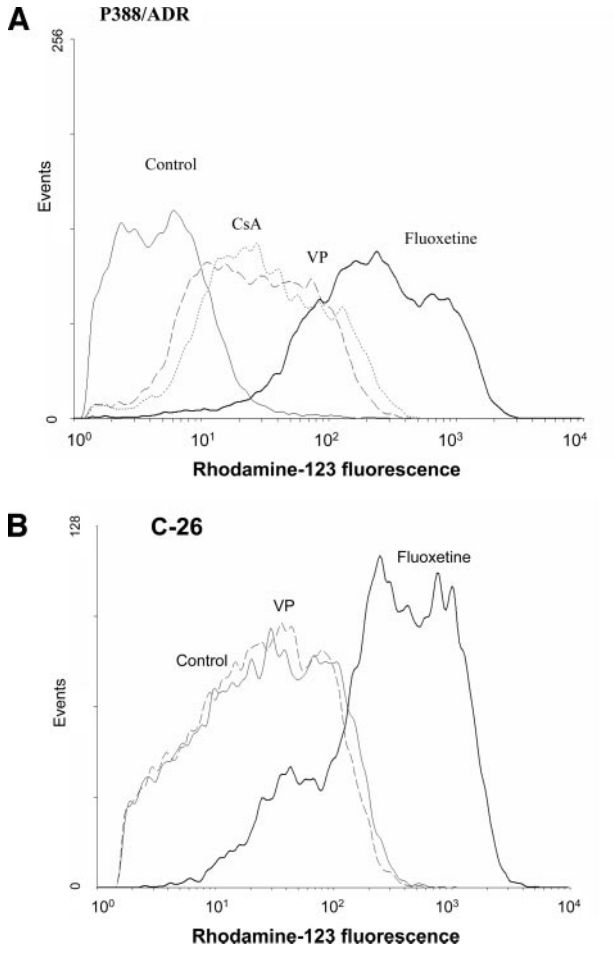

Fig. 3. Chemosensitizer effects on intracellular accumulation of Rhodamine-123 in MDR-cells. Fluorescence-activated cell sorter analysis of Rhodamine-123 accumulation inside MDR-cells. Cells were incubated with Rhodamine-123 alone (*control*) or with Rhodamine-123 +chemosensitizer. Rh-123 concentration (all systems) was 5 μmol/L. *A*, P388/ADR cells; *B*, C-26 cells. Verapamil (*VP*), Cyclosporin A (*CsA*), and fluoxetine concentrations were 15 μmol/L, 15 μmol/L, and 5 μmol/L, respectively.

following changes in body weight: mild increase (11%) when treated with saline or fluoxetine, 20% loss when treated with doxorubicin, and a milder loss of 13% when treated with doxorubicin + fluoxetine. Similar trends and body loss values were observed for the animals inoculated with the parent line P388/WT. Survival of animals implanted with either type of cells (sensitive or MDR) was unaffected by treatment with saline or with fluoxetine, and their rather poor survival reflects the aggressive nature of these tumors (Fig. 6). Treatment with doxorubicin increased survival of animals implanted with the drug-sensitive cells, but adding fluoxetine to the doxorubicin made no change compared with those receiving doxorubicin alone (Fig. 6). Responses of the animals bearing the MDR ascites tumor were quite different: poor survival upon treatment with saline, fluoxetine, or doxorubicin and a 2.5-fold increase in life span upon treatment with doxorubicin + fluoxetine (Fig. 6).

Tumor Progression and Survival of BALB/c Mice Bearing C-26 Solid Tumors. The increase in tumor volume with time was fast and exponential for animals treated with saline, fluoxetine, or doxorubicin, and tumor responses were quite similar among these three treatments (Fig. 7*A*). Tumor growth rate and tumor volumes were both significantly smaller ($P < 0.001$) in the animals treated with doxorubicin and fluoxetine than in the other three groups (Fig. 7*A*). These responses were mirrored by the effects on animal survival (Fig. 7*B*). All of the animals treated with saline, doxorubicin, or fluoxetine, died between days 35 and 45, whereas animals treated with doxorubicin + fluoxetine were long survivors, with a 2- to 3-fold increase in life span (Fig. 7*B*).

Therapeutic Responses in Nude Mice Bearing MCF-7/ADR Xenografts

On the day of treatment initiation, tumor volumes in all of the treatment groups were 70 to 75 mm^3. In animals treated with saline, fluoxetine, or doxorubicin, tumors continued to grow rapidly with no significant distinction between the three groups (Fig. 8*A*). Response of animals treated with doxorubicin and fluoxetine were strikingly different (Fig. 8*A*): tumor growth was arrested upon the first treatment and thereafter, with time and completion of full treatment course, there was complete tumor regression. All of the animals in this group were tumor-free on day 28. These results are mirrored by the survival data (Fig. 8*B*): all animals treated by saline, fluoxetine, or drug were dead by day 38, with only slight differences between the three groups, whereas all of the animals treated with doxorubicin + fluoxetine were alive on day 48 when the experiment was terminated.

DISCUSSION

The choices we made for the *in vitro* studies were based on MDR variables observed in the clinic: resistance toward more than one drug and a series of cell lines that include both drug-sensitive and drug-

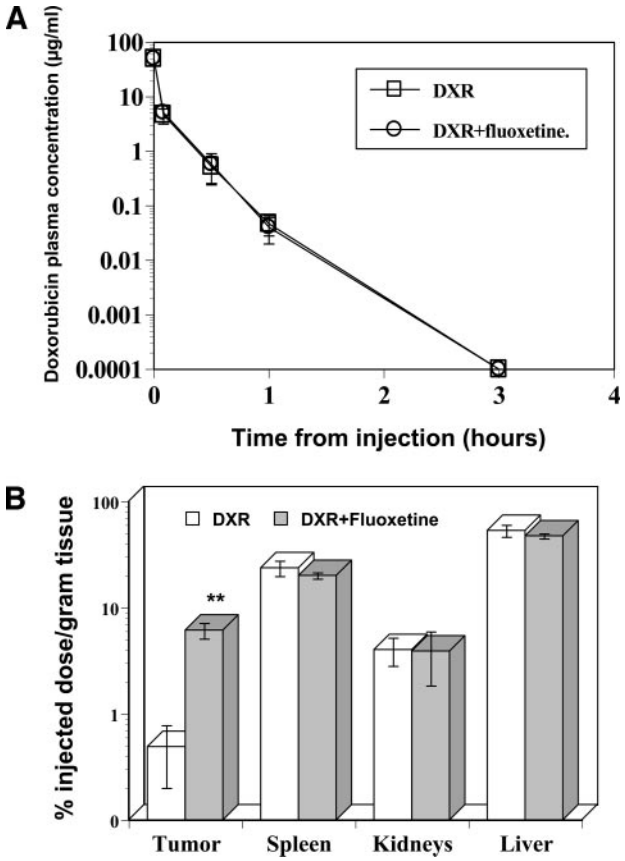

Fig. 4. Fluoxetine effects on doxorubicin (*DXR*) pharmacokinetics and biodistribution in C57BL/6 mice bearing B16F10.9 lung tumors. DXR and fluoxetine doses were 10 mg/kg body and 0.04 mg/kg body/day (see Materials and Methods for additional details). *A*, DXR plasma concentration, in animals treated with DXR (□) or with DXR + fluoxetine (○). *Points* are experimental, each an average from 5; *bars*, ±SD. The *curves* are nontheoretical, drawn to emphasize the data trends. *B*, DXR biodistribution in selected organs. *Light-shaded bars*, DXR alone; *dark-shaded bars*, DXR+fluoxetine. Each *bar* is an average from 5 animals; *bars*, ±SD. **, $P < 0.01$ compared with drug alone.

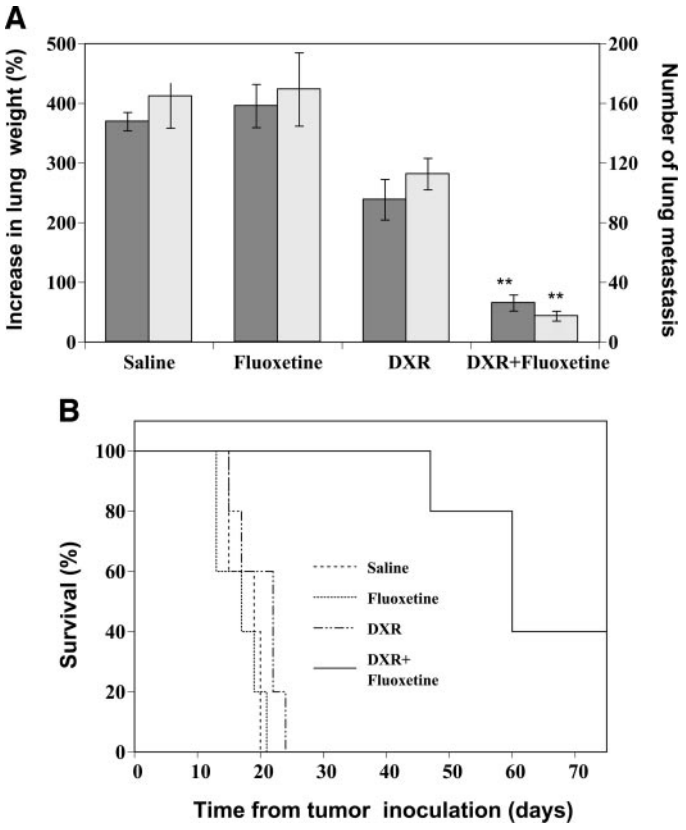

Fig. 5. Fluoxetine effects on tumor response and survival of doxorubicin-treated C57BL/6 mice, bearing B16F10.9 lung metastasis. Doxorubicin (*DXR*) and fluoxetine doses were as listed for Fig. 4 above. *A*, lung metastatic burden. *Light-shaded bars*, percentage of increase in lung weight (over lungs of healthy mice). *Dark-shaded bars*, number of lung metastasis. Each bar is an average from 5; bars, ±SD. **, $P < 0.01$ compared with drug alone and to saline. *B*, survival ($n = 5$). Each *line* connects the symbols representing the daily survival state of the group, the symbols themselves were omitted to avoid a cluttered figure. *Line codes* are listed on the figure.

resistant systems, the latter exhibiting either inherent or acquired resistance. A chemosensitizer is expected to overturn the poor response of drug-resistant cells to anticancer drugs and at the same time to have little or no effect on drug-sensitive cells (23, 26, 29, 39). Fluoxetine, tested in *vitro* over the matrix of selected variables, met both expectations. In drug-sensitive cells cytotoxicity generated by a given drug was unaffected by the presence of fluoxetine in the treatment media (Table 1; Fig. 1*B*). In drug-resistant cells, acquired and inherent, combination treatment of fluoxetine with an anticancer drug (be it mitomycin C, doxorubicin, vinblastine, or paclitaxel) enhanced cytotoxicity by 2 to 3 orders of magnitude (Table 1; Fig. 1*B*), similar to trends observed with other chemosensitizers (23, 26, 29, 37). Fluoxetine was also similar in trend but more potent than the *in vitro* benchmarks verapamil and cyclosporine A (Table 2; Fig. 1). The ability of fluoxetine to enhance cytotoxicity for several drugs that differ from one another in mechanism of action and in cellular localization of the site of drug action (5) carries the implication that fluoxetine exerts its beneficial effect in pathways or processes that are independent of the particular mechanism(s) by which each drug causes cell demise.

Bearing in mind the dose-related problems with previous-generation chemosensitizers, we deliberately set out to test, *in vivo*, a low fluoxetine dose: 0.04 mg/kg body/day compared (for example) to the ranges of 1.6, 5 to 30, 2 to 8, and 200 to 300 mg/kg body/dose for Tiamulin, OC144–093, XR9756, and MS-209, respectively (23, 26, 29, 39). One of the advantages of fluoxetine was the ability to administer it through the drinking water, which was less stressful and friendlier than direct (usually forced) oral administrations or injections (i.v. and i.p.; refs. 23, 26, 29, 39).

A MDR reversal agent should be able to truly increase, *in vivo*, accumulation of an anticancer drug at the tumor and there alone, without altering the pharmacokinetics of the drug. We found fluoxetine to pass both tests. Similar to trends found with OC144–093 (23) and XR9576 (26), and unlike some other chemosensitizers, the pharmacokinetics of doxorubicin were the same, with and without fluoxetine (Fig. 4*A*). Fluoxetine generated a substantial 12-fold increase of doxorubicin accumulation in the tumor and at the same time did not affect the accumulation of the drug in other healthy organs (Fig. 4*B*).

The impact of fluoxetine on tumor responses and on survival was tested *in vivo*, using five of the cell lines tested *in vitro*: MCF-7/ADR, B16F10.9, C-26, P388/WT, and P388/ADR. We found common and encouraging trends, as well as *in vitro–in vivo* correlations, among the results from all of the animal systems, despite the diversities in animal species, tumor models, and measures of tumor response. In lung metastatic disease, the doxorubicin + fluoxetine treatment reduced the metastatic burden 4- to 7-fold better than doxorubicin alone, with a corresponding increase in life span (Fig. 5). In peritoneal ascites, the doxorubicin + fluoxetine treatment did not affect the responses of mice bearing the drug-sensitive tumor P388/WT but had a significant and positive impact on mice bearing the drug-resistant tumor P388/ADR (Fig. 6). These results (for the P388/ADR line) are on a par or

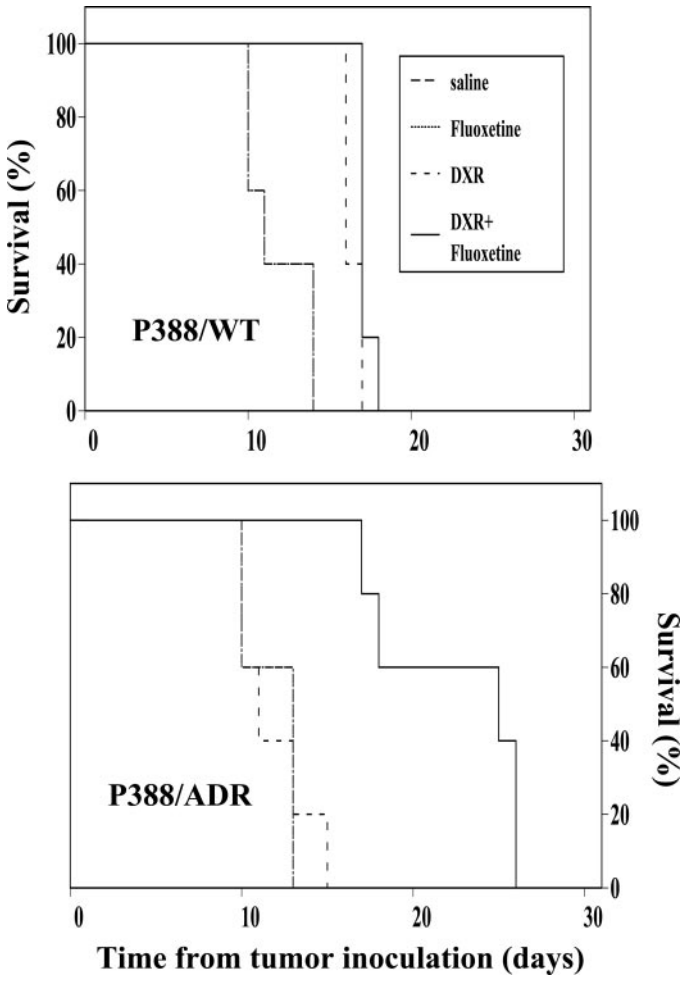

Fig. 6. Fluoxetine effects on survival of doxorubicin (*DXR*)-treated BDF_1 mice bearing P388/WT or P388/ADR ascites tumors. DXR dose was 3 mg/kg body, fluoxetine dose as listed for Fig. 4 above. In both types of tumors, survivals of animals treated with saline or with fluoxetine alone coincide, the different patterns for each data set masking each other. All other details are as listed for Fig. 5*B* above.

higher than those achieved (albeit using doses 10- to 100-fold higher than fluoxetine) with MS-209, Tiamulin, and OC144–093 (23, 29, 30). In solid tumors, the doxorubicin + fluoxetine combination generated a 6- to 7-fold reduction in tumor progression (Fig. 7*A*), with a corresponding increase in life span (Fig. 7*B*). The effects on tumor size (Fig. 7*A*) seem better than reported (same tumor model and drug but different protocol) for XR9576 (26). In human xenografts (MCF-7/ADR) complete tumor regression and significantly longer survival were achieved solely by the doxorubicin + fluoxetine treatment (Fig. 8). Other studies showed MDR reversal of human xenografts in nude mice by OC144–093 and XR9576, with significant arrest or slowdown of tumor progression (23, 26). The differences in animal models, in protocols, and in drugs preclude direct comparisons of those encouraging findings with our results, yet the similarity in trends is clear.

Two independent types of experiments, measuring the impact of chemosensitizer on drug efflux from and on drug accumulation within MDR-cells, can shed light on the mechanism(s) by which a reversal agent modulates MDR (7, 23, 26, 29, 37). Fluoxetine had no effect on drug efflux (paclitaxel and doxorubicin) from drug-sensitive cells such as MCF-7 or P388/WT (data not shown). It did, however, inhibit the efflux of these drugs in the corresponding acquired MDR cells, as well as from the inherent MDR cells (Fig. 2). By both qualitative and quantitative tests fluoxetine induced significant increases in drug accumulation within MDR-cells (Fig. 3). In both efflux and accumulation, fluoxetine was qualitatively similar to and quantitatively better than the established benchmarks verapamil and cyclosporine A (Figs. 2 and 3), clearly indicating that a major mechanism by which fluoxetine modulates MDR is inhibition of extrusion pumps.

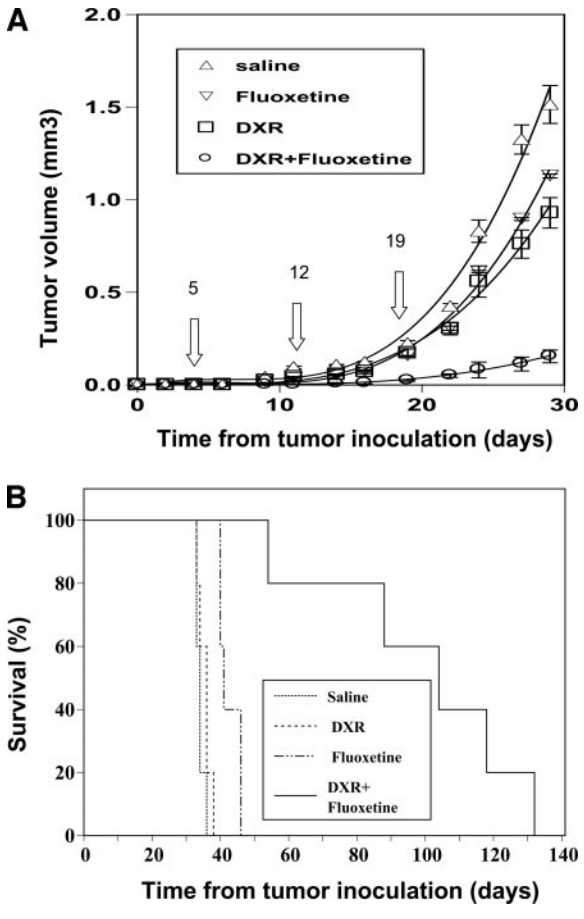

Fig. 7. Fluoxetine effects on tumor response and on survival of DXR-treated BALB/c mice bearing a C-26 solid tumor in the right-hind foot pad. Fluoxetine and DXR doses are as listed for Fig. 4 above. *A*, increase in tumor volume with time. △, saline; ▽, fluoxetine; □, DXR; ○, DXR+fluoxetine. *Points* are experimental, each an average of 5 animals; bars, ±SE. The *solid curves* are nontheoretical drawn to emphasize the data trends. The *arrows* and *numbers* above them indicate treatment days. *B*, survival ($n = 5$). All details similar to those listed for Fig. 5*B* above.

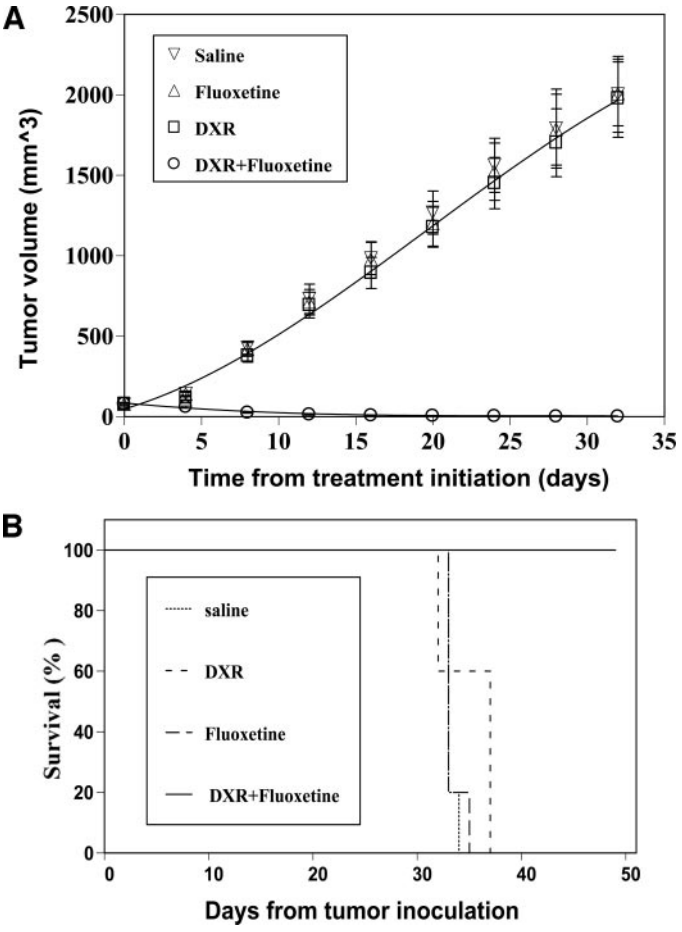

Fig. 8. Fluoxetine effects on tumor response and on survival of DXR-treated athymic nude mice bearing MCF-7/ADR solid tumors. *A*, changes in tumor volume with time. *Symbols* are as listed in Fig. 7*A*. *Points* are experimental, each an average of 5 animals; bars, ±SE. *Solid curves* are nontheoretical drawn to emphasize the data trends. *B*, survival ($n = 5$). All of the details similar to those listed for Fig. 5*B* above.

The complexities of cancer diseases and of extrusion pump-mediated MDR will probably require an arsenal of more than one clinically approved chemosensitizer. We find it encouraging that several third-generation chemosensitizers, among them OC144–093 (25), XR9576 (27, 30), MS-209 (28), and VX-710 (31, 32), are in clinical trials. Looking at structural features, fluoxetine, verapamil, OC144–093, and other under-development chemosensitizers, share a similarity in having a "head" consisting of two aromatic rings with a spacer between them. Yet fluoxetine, verapamil, and OC144–093 differ from one another in the size and nature of the spacer and in the substituents on the head rings. It will require in-depth theoretical structural analysis of all of these molecules to understand the relationships among structural similarities and dissimilarities to chemosensitization. Clearly, additional mechanistic and *in vivo* studies are needed to understand and substantiate the activities of fluoxetine as a chemosensitizer. Yet the low-dose range at which fluoxetine was found to modulate MDR in mice and its ability to reverse MDR in human-originating cells (*in vitro* and *in vivo*) hold a promise that fluoxetine may reverse MDR in the clinic at doses well below the safe and approved range of 20 to 80 mg/day prescribed for psychiatric therapy (33).

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REFERENCES

1. Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. Nature Rev Cancer 2002;2:48–58.
2. Tan B, Piwnica-Worms D, Ratner L. Multidrug resistance transporters and modulation. Curr Opin Oncol 2000;12:450–8.
3. Sonneveld P, Wiemer E. Inhibitors of multidrug resistance. Curr Opin Oncol 1997; 9:543–8.
4. Stein WD. Kinetics of the multidrug transporter (P-glycoprotein) and its reversal. Physiol Rev 1997;77:545–90.
5. Barrows LR. Antineoplastic and Immunoactive Drugs in: Affonso RG, editor. Remington:The Science and Practice of Pharmacy. Easton PA: Mack Publishing Co; 1995. p. 1236–62.
6. Izquierdo MA. Relationship of LRP-human major vault protein to in vitro and clinical resistance to anticancer drugs. Cytotechnology 1996;19:191–7.
7. Tsimberidou AM, Paterakis G, Androutsos G, Anagnostopoulos N, Galanopoulos A, Kalmantis T, et al. Evaluation of the clinical relevance of the expression and function of P-glycoprotein, multidrug resistance protein and lung resistance protein in patients with primary acute myelogenous leukemia. Leuk Res 2002;26:143–54.
8. Tsuruo T, Lida H, Nojri M, Tsukagoshi S, Sakurai Y. Overcoming of vincristine resistance in P388 leukemia in vivo and in vitro through enhanced cytotoxicity of vincristine and vinblastine by verapamil. Cancer Res 1981;41:1967–72.
9. Yung BY, Chang FJ, Bor AM. Modulation of the reversibility of Actinomycin D cytotoxicity in HeLa cells by verapamil. Cancer Lett 1991;60:221–7.
10. Foxwell BMJ. Mackie A, Ling V, Ryffel B. Identification of the multidrug resistance-related P-glycoprotein as a cyclosporin binding protein. Molecul Pharmcol 1989;36: 543–6.
11. Twentyman PR, Fox NE, White DJ. Cyclosporin A and its analogues as modifiers of adriamycin and vincristine resistance in a multi-drug resistant human lung cancer cell line. Br J Cancer 1987;56:55–7.
12. Naito M, Yusa K, Tsuruo T. Steroid hormones inhibit binding of Vinca alkaloid to multidrug resistance related P-glycoprotein. Biochem Biophys Res Comm 1989;158: 1066–71.
13. Yang CP, DePinoho SG, Greenberger LM, Arceci JR, Horwitz SB. Progesterone interacts with P-glycoprotein in multidrug-resistant cells and in the endometrium of gravid uterus. J Biol Chem 1989;264:782–8.
14. Ganapath R, Grabowski D. Enhancement of sensitivity to adriamycin in resistant P388 leukemia by the calmodulin inhibitor trifluoperazine. Cancer Res 1983;43: 3696–9.
15. Germann UA, Shlyakhter D, Mason VS, Zelle RE, Duffy JP, Galullo V, et al. Cellular and biochemical characterization of VX-710 as a chemosensitizer: reversal of P-glycoprotein-mediated multidrug resistance in vitro. Anticancer Drugs 1997;8: 125–40.
16. Atadja P, Watanabe T, Xu H, Cohen D. PSC-833, a frontier in modulation of P-glycoprotein mediated multidrug resistance. Cancer Metastasis Rev 1998;17:163–8.
17. Dale IL, Tuffley W, Callaghan R, et al. Reversal of P-glycoprotein-mediated multidrug resistance by XR9051, a novel diketopiperazine derivative. Br J Cancer 1998; 8:885–92.
18. Sikic BI, Fisher GA, Lum BL, Halsey J, Beketic-Oreskovic L, Chen G. Modulation and prevention of multidrug resistance by inhibitors of P-glycoprotein. Cancer Chemother Pharmacol 1997;40:Suppl S13–19.
19. Rowinsky EK, Smith L, Wang YM, et al. Phase I and pharmacokinetic study of paclitaxel in combination with biricodar, a novel agent that reverses multidrug resistance conferred by overexpression of both MDR1 and MRP. J Clin Oncol 1998;6:2964–76.
20. Hyafil F, Vergely C, Du Vignaud P, Grand-Perret T. In vitro and in vivo reversal of multidrug resistance by GF120918, an acridonecarboxamide derivative. Cancer Res 1993;53:4595–602.
21. Dantzig AH, Shepard RL, Cao J, et al. Reversal of P-glycoprotein-mediated multidrug resistance by a potent cyclopropyl dibenzosuberane modulator, LY335979. Cancer Res 1996;56:4171–79.
22. Starling JJ, Shepard RL, Cao J, et al. Pharmacological characterization of LY335979: a potent cyclopropyl dibenzosuberane modulator of P-glycoprotein. Adv Enzyme Regul 1997;37:335–47.
23. Newman MJ, Rodarte JC, Benbatoul KD, et al. Discovery and characterization of OC144–093, a novel inhibitor of P-glycoprotein-mediated multidrug resistance. Cancer Res 2000;60:2964–72.
24. Guns ES, Denyssevych T, Dixon R, Bally MB, Mayer L. Drug interaction studies between paclitaxel (Taxol) and OC144–093 - a new modulator of MDR in cancer chemotherapy. Eur J Drug Metab Pharmacokinet 2002;27:119–26.
25. Zhang C, Sarshar S, Moran EJ, et al. 2,4,5- Trisubstituted imidazoles: novel nontoxic modulators of P-glycoprotein mediated multidrug resistance. Part 2 Bioorg Med Chem Lett 2000;10:2603–5.
26. Mistry P, Stewart AJ, Dangerfield W, et al. In vitro and in vivo reversal of P-glycoprotein-mediated multidrug resistance by a novel potent modulator, XR9576. Cancer Res 2001;61:749–58.
27. Stewart A. Steiner J, Mellows G, Laguda B, Norris D, Bevan P. Phase I trial of XR9576 in healthy volunteers demonstrates modulation of P-glycoprotein in CD56+ lymphocytes after oral and intravenous administration. Clin Cancer Res 2000;6: 4186–91.
28. Naito M, Matsuba Y, Sato S, Hirata H, Tsuruo T. MS-209, a quinoline-type reversal agent, potentiates antitumor efficacy of docetaxel in multidrug-resistant solid tumor xenograft models. Clin Cancer Res 2002;8:582–8.
29. Baggetto LG, Dong M, Bernaud J, Espinosa L, Rigal D, Bonvallet R, Marthinet E. In vitro and in vivo reversal of cancer cell multidrug resistance by the semi-synthetic antibiotic tiamulin Biochem Pharmacol 1998;56:1219–28.
30. Agrawal M, Abraham J, Balis FM, Edgerly M, Stein WD, Bates S, et al. Increased ^{99m}Tc-Sestamibi accumulation in normal liver and drug-resistant tumors after the administration of the glycoprotein inhibitor, XR9576. Clin Cancer Res 2003;9: 650–6.
31. Seiden MV, Swenerton KD, Matulonis U, et al. A phase II study of the MDR inhibitor biricodar (INCEL, VX-710) and paclitaxel in women with advanced ovarian cancer refractory to paclitaxel therapy. Gynecol Oncol 2002;86:302–10.
32. Toppmeyer D, Seidman AD, Pollak M, et al. Safety and efficacy of the multidrug resistance inhibitor Incel (biricodar; VX-710) in combination with paclitaxel for advanced breast cancer refractory to paclitaxel. Clin Cancer Res 2002;8:670–8.
33. Cookson J, Duffett R. Fluoxetine: therapeutic and undesirable effects. Hosp Med 1998;59:622–6.
34. Peer D, Margalit R. Loading mitomycin C inside long circulating hyaluronan targeted nano-liposomes increases its antitumor activity in three mice tumor models. Inter J Cancer 2004;108:780–9.
35. Fu LW, Zhang YM, Liang YJ, Yang XP, Pan QC. The multidrug resistance of tumour cells was reversed by tetrandrine in vitro and in xenografts derived from human breast adenocarcinoma MCF-7/adr cells. Eur J Cancer 2002;38:418–26.
36. Yoon SS, Fidler IJ, Beltran PJ, Bucana CD, Wang YF, Fan D. Intratumoral heterogeneity for and epigenetic modulation of mdr-1 expression in murine melanoma. Melanoma Res 1997;7:275–87.
37. Bruno NA, Carver LA, Slate DL. Isolation and characterization of doxorubicin-resistant Lewis lung carcinoma variants. Cancer Commun 1990;2:151–8.
38. Michowitz M, Dayan-Avidan G, Bar-Shira-Maymon B, Donin N, Leibovici J. Drug resistance and its counteraction by cyclosporin A in function of metastatic potential in the Lewis lung carcinoma system. Cell Mol Biol 1994;40:551–60.
39. Naito M, Tsuruo T. New multidrug-resistance-reversing drugs, MS-209 and SDZ PCS 833. Cancer Chemother Pharmacol 1997;40:Suppl S20–4.