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Reversal of the human and murine multidrug-resistance phenotype with megestrol acetate

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Abstract. MA is an orally active PG derivative with an excellent safety profile that is used primarily for the treatment of carcinomas of the breast and endometrium. We investigated the potential application of MA as an MDR-reversal agent using cell culture and human tumor xenograft models. The reversing activity of MA in vitro was compared with that of PG and VER in two human MDR cell lines, the colon carcinoma HCT-116/VM46 and the breast carcinoma MCF-7/ADR, and in a murine cell line, J774.2. At concentrations as low as 3 μ*M*, MA was capable of partially restoring sensitivity to Act D in the HCT-116/VM46 cells and sensitivity to DOX in the MCF-7/ADR cells. Although less effective than VER, MA was about 2.5 times more potent than PG in reversing MDR at equimolar concentrations. Increased accumulation of DOX in drug-resistant cells that were treated simultaneously with MA was observed by flow cytometry. In vivo, using established human colon and breast carcinoma xenografts implanted s.c. in athymic mice, the combined therapy with MA and DOX resulted in enhanced antitumor activity relative to that of DOX alone in the MDR sublines. These results suggest that MA may be a promising clinical MDR-reversing agent.

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Abbreviations: Act D, actinomycin D; DOX, doxorubicin; PG, progesterone; MA, megestrol acetate; VER, verapamil; VBL, vinblastine; MDR, multidrug resistance; P-gp, P-glycoprotein; MAP, medroxyprogesterone acetate; MTD, maximum tolerated dose; q4dx3, every 4 days for 3 treatments; TVDD, tumor volume-doubling time; IC_{50}, drug concentration producing 50% cell kill in treated cultures as compared with controls; PBS, phosphate-buffered saline; VM-26, teniposide

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Introduction

Chemotherapy is successful for the treatment of several neoplasias, yet many patients continue to perish due to intrinsic or acquired chemoresistance of tumor cells. Drug resistance in human malignancies may result from multiple mechanisms, among which MDR is of particular concern, since it involves a broad spectrum of lipophilic chemotherapeutic drugs currently in use. The most common mechanism involved in this phenotype is overexpression of the *mdr*-1 gene, leading to increased levels of a pleiotropic membrane transport protein termed P-glycoprotein (P-gp). P-gp is an energy-dependent efflux pump responsible for reducing intracellular drug concentration in resistant cells [7, 12, 18, 19]. In addition, increased levels of P-gp have been detected in several types of refractory tumor samples from untreated or relapsed patients, indicating a possible role of this transport protein in clinical drug resistance [2, 5, 29].

A promising approach to circumvent MDR is to utilize nontoxic compounds that competitively bind to P-gp, thereby antagonizing its drug-efflux activity [3, 17]. A variety of compounds, including calcium channel blockers, calmodulin inhibitors, noncytotoxic anthracyclines, vinca alkaloid analogs, and cyclosporins, are capable of enhancing drug accumulation in vitro [15, 31]. Among these, only a few of the most potent sensitizers such as VER [11, 26], trifluoperazine [25], cyclosporin A [33], and bepridil [20] have been tested in clinical trials. Positive results with VER were reported in multiple myeloma [11] and malignant lymphoma [26], whereas other studies were less successful. The limited success in these MDR-reversal attempts may be attributed to two main factors: (1) lack of direct correlation between *mdr*-1 expression in tumors and clinical response and (2) unattainable effective plasma concentrations of the reversing agent due to severe adverse reactions in patients. It is therefore warranted to search for additional chemosensitizers with a larger therapeutic index and true clinical value.

We have previously demonstrated that a number of steroidal hormones interact with P-gp [34]. Among these,

PG, which is very hydrophobic, had the strongest ability to compete with [^{3}H]-azidopine photoaffinity labeling of P-gp and was capable of inhibiting VBL binding and efflux in murine MDR J7.V1-1 cells. A similar potentiation of DOX toxicity in resistant human leukemia K526 cells was also observed [27].

MA, a synthetic derivative of PG, is active against endometrial, ovarian, and breast cancer in patients. Numerous clinical trials indicate that MA is well tolerated in a wide dose range; doses as high as 1,600 mg/day have been given without producing serious toxicities [1, 23, 28]. Like PG, MA has a strong inhibitory effect on the [^{3}H]-azidopine photoaffinity labeling of P-gp [36] and has been shown to sensitize human neuroblastic cells to vincristine in vitro [14]. This excellent safety profile makes MA particularly attractive as a potential nontoxic MDR-reversing agent.

We investigated the ability of MA to increase DOX and Act D accumulation in human MDR breast and colon carcinoma cell lines and in a murine cell line. MA was also evaluated in the corresponding s.c. tumor xenografts in athymic mice. Our results demonstrate that MA is capable of partially reversing MDR both in vitro and in vivo and suggest that MA may be a promising adjuvant drug that could have an impact on current cancer combination therapy.

Materials and methods

Drugs. DOX, VER, VBL, Act D, and PG were purchased from Sigma Chemical Co. (St. Louis, Mo.). MA (17α-acetoxy-6-methylpregna-4,6-diene-3,20-dione) was obtained from Bristol-Myers Squibb Co. (Wallingford, Conn.). DOX (obtained from Bristol-Myers Squibb) was dissolved in distilled water; all other drugs were dissolved in dimethylsulfoxide (Sigma) and further diluted with culture medium to a final nontoxic concentration (<0.1%) of the solvent in cultures. For in vivo treatments, DOX was dissolved in normal saline and given i.v.; MA was suspended in various vehicles as denoted.

Cell lines and culture. The mouse macrophage-like J7.V1-1 MDR cell line, obtained by stepwise selection of J774.2 cells with VBL, exhibits 1000-fold resistance to VBL and overproduces the *mdr*-1b gene product [35]. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 20% horse serum. HCT-116/VM46 cells were selected from human colon carcinoma HCT-116 cells for resistance to VM-26 [22]. MCF-7/ADR cells, provided by Dr. B. Teicher (Dana Farber Research Institute, Boston, Mass.), were selected from human breast carcinoma MCF-7 cells for resistance to DOX. Both human cell lines exhibit the MDR phenotype and overexpress *mdr*-1 mRNA [13, 22]. Cells were grown at 37° C in a humidified atmosphere containing 5% CO_2 in McCoy's 5A medium (Gibco) and 10% fetal bovine serum in the absence of the drug used for their selection.

Cytotoxicity assay. Human carcinoma cells plated in 96-well plates (5×10^3 cells/well) were grown for 24 h at 37° C, then exposed to appropriate concentrations of DOX or Act D. PG, MA, and VER were added at concentrations ranging from 0.08 to 40 m*M*. After 48 h, cells were washed, fixed, and stained with crystal violet. Absorbance was read using a microtiter plate reader (Molecular Devices) at 595 nm. The IC_{50} value was determined after correction for the growth inhibition exerted by the modifier alone (<15%). The effect on J7.V1-1 cells was measured as previously described [34].

Flow cytometry. DOX retention in MDR cells was tested according to Bruno and Slate [8]. Cells (10^6 cells/ml in PBS) were incubated at 37° C for 2.5 h with 2 mg DOX/ml and with modulators at the stated concentrations, washed with PBS, and resuspended in culture medium.

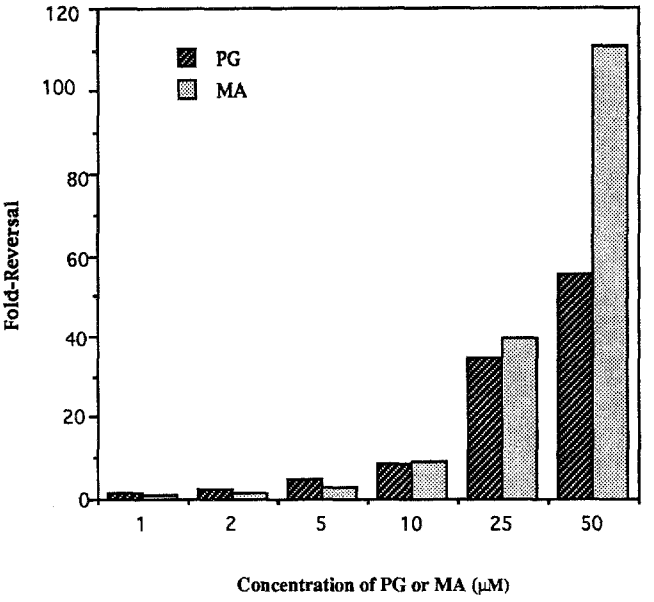

Fig. 1. Reversal effect of PG and MA on VBL resistance in murine MDR J7.V1-1 cells. Cells were plated in 35-mm tissue-culture dishes and incubated with increasing concentrations of VBL (0–0.3 m*M*) in the absence or presence of MA or PG. IC_{50} values were determined from dose-response curves after 72 h of continuous exposure. The IC_{50} for PG and MA is ~90 μ*M* in J7.xV1-1 cells in the absence of VBL. Fold-reversal is obtained by dividing the IC_{50} for VBL measured in the absence of steroids by the IC_{50} for VBL measured in the presence of steroids.

DOX fluorescence was measured by flow cytometry using a FACStar Plus flow cytometer (Becton Dickinson, Mountain View, Calif.) equipped with 488-nm laser light. Forward-angle light scatter and right-angle light scatter were used to define the cell population to be analyzed for DOX fluorescence (FL2, emission at ≥570 nm). After gating on live cells, the FACStar Plus or LYSYS software package (Becton Dickson) was used to generate FL2 histograms. Data for 5×10^3 cells were calculated per sample.

Human xenograft models. The HCT-116, HCT-116/VM46, and MCF-7/ADR tumor lines were maintained by serial passage in athymic mice (BALB/c nu/nu; Harlan Sprague-Dawley, Indianapolis, Ind.). The MCF-7 human breast line is estrogen-dependent and was maintained by serial passage in athymic mice supplemented with β-estradiol (0.72 mg, 60-day release; Innovative Research). Tumors were measured in two perpendicular directions weekly or biweekly using calipers. Tumor volume was calculated according to Begg [4]. Antitumor activity is expressed in terms of tumor volume-doubling delays (TVDD) as TVDD = (T-C)/TVDT, where T-C is defined as the median time (days) required for treated tumors to reach 500 mm^3 in size minus the median time required for control tumors to reach the same size. An active result was defined as 3 TVDDs, which corresponds to roughly 1 log of gross cell kill. Treatment was started when tumors had reached 75–125 mm^3 in size (8–10 mice/group).

Results

In vitro effects of MA on MDR cells

In murine J7.V-1-1 cells that overexpress the *mdr*-1b gene product and are about 1000 times more resistant to VBL (IC_{50}, 1.66 m*M*) than the parental J774.2 cells, both MA and PG produced dose-dependent sensitizing responses to VBL (Fig. 1). At low concentrations (up to 25 μ*M*), MA and PG had comparable activity. At higher concentrations

Table 1. Inhibition of cell proliferation by drugs alone in human carcinoma cells

Cell line	IC_{50} of drugs				
	Act D (n*M*)	ADR (μ*M*)	PG (μ*M*)	MA (μ*M*)	VER (μ*M*)
HCT116	0.4	0.26	23.5	40.0	40.0
HCT116/VM46	8.4	3.4	22.5	44.5	44.5
MCF-7	–	0.4	58.0	>160.0	60.0
MCF-7/ADR	–	60.0	40.0	>160.0	>160.0

Cells were exposed continuously to different concentrations of drug for 48 h. All values were derived from at least 3 separate experiments with an SD of <10%. IC_{50}, Drug concentration producing 50% inhibition of cell growth in treated cultures as compared with controls

Table 2. Reversal of ActD resistance by PG, MA, and VER in human colon HCT-116/VM46 carcinoma cells

Cell line	Compound	Conc. (μ*M*)	ActD IC_{50} (ng/ml)	Resistance factor
HCT 116	None		0.5	1.0
HCT116/VM46	None		10.5	21.0
	PG	3.0	4.2	8.4
	PG	5.0	2.0	4.0
	PG	8.6	0.9	1.8
	MA	3.0	1.8	3.6
	MA	5.0	0.8	1.6
	MA	8.6	0.5	1.0
	VER	3.0	0.6	1.2
	VER	5.0	0.5	1.0

Cells were treated with various concentrations of ActD in the absence or presence of PG, MA, or VER at the indicated concentrations for 48 h of continuous drug exposure. At these concentrations, chemosensitizers alone produced ≤15% inhibition of cell growth. The resistance factor is defined as the IC_{50} of resistant cells with or without chemosensitizers divided by the IC_{50} of the parental cells

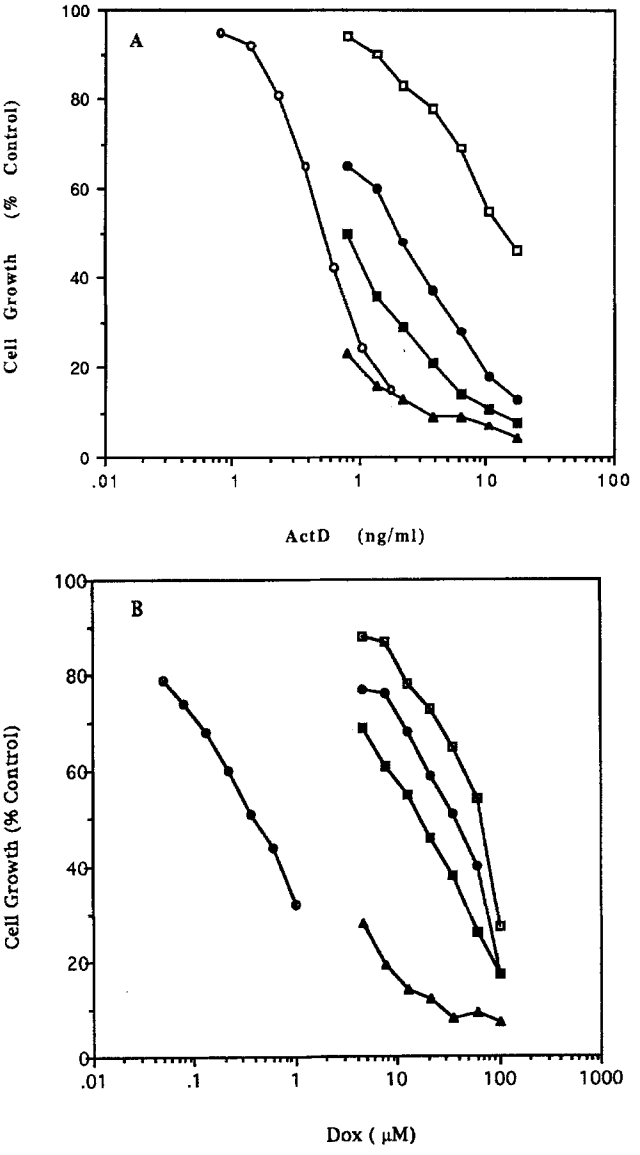

Fig. 2. A Effect of MA, PG, and VER on ActD resistance in human colon carcinoma HCT-116 cells. HCT-116 (-○-) and HCT-116/VM46 (-□-) cells were treated with ActD at the indicated concentrations. HCT116/VM46 cells were incubated with increasing amounts of ActD in the presence of 5 μ*M* PG (-●-) and MA (-■-) for 48 h. VER (-▲-) was included as a positive control at the same concentration. **B** Effect of MA, PG, and VER on DOX resistance in human breast carcinoma MCF-7 cells. MCF-7 (-○-) and MCF-7/ADR (-□-) cells were treated with DOX at the indicated concentrations. MCF-7/ADR cells were incubated with increasing amounts of DOX in the presence of 12 μ*M* PG (-●-) and MA (-■-) for 48 h. VER (-▲-) was included as a positive control at the same concentration

(50 μ*M*), however, MA was approximately twice as effective as PG.

The effect on the human *mdr*-1 gene product was investigated in colon carcinoma HCT-116 and breast carcinoma MCF-7, and in their corresponding MDR counterparts, HCT-116/VM46 and MCF-7/ADR. Relative to the parental cell line, the HCT-116/VM46 cells showed 13- and 21-fold resistance to DOX and Act D, respectively, and the MCF-7/ADR cells exhibited at least 150-fold resistance to DOX (Table 1). MA and VER were relatively nontoxic (IC_{50}, ≥40 m*M*), and PG was slightly more toxic than the other two agents. To examine MDR reversal, cells were exposed for 48 h to the cytotoxic drugs in the presence or absence of modulating agents. No drug-enhancing response was observed in the parental cell lines (data not shown).

In the resistant lines, all three modulators overcame resistance in a dose-dependent manner, and although MA was less active than VER, it consistently showed reversing effects superior (2- to 2.5-fold) to those of PG in both cell lines. In the moderately resistant HCT-116/VM46 cells, MA, VER, and PG could completely restore sensitivity to Act D at 5, 3, and 8 μ*M*, respectively (Table 2). However, in the highly resistant cell line MCF-7/ADR, both MA and PG were significantly less effective than VER and could only partially reverse the DOX resistance at nontoxic concentrations (Table 3).

Figure 2A and 2B show the comparative sensitizing effects of MA, PG, and VER at equimolar concentrations on Act D and DOX cytotoxicities in HCT-116/VM46 and MCF-7/ADR cells, respectively. At any concentration tested, each reversal agent was capable of increasing the sensitivity of the resistant cells.

Table 3. Reversal of DOX resistance by PG, MA, and VER in human breast MCF-7/ADR carcinoma cells

Cell line	Compound	Conc. (μM)	DOX IC_{50} (μM)	Resistance factor
MCF-7	None		0.4	1.0
MCF-7/ADR	None		60.0	150.0
	PG	7.5	42.0	105.0
	PG	12.0	36.0	90.0
	MA	3.0	44.0	110.0
	MA	7.5	20.0	50.0
	MA	12.0	18.0	45.0
	MA	21.0	10.0	25.0
	VER	3.0	60.0	150.0
	VER	7.5	2.0	5.0
	VER	12.0	1.3	3.3

Cells were treated with various concentrations of DOX in the absence or presence of PG, MA, or VER at the indicated concentrations for 48 h of continuous drug exposure. At these concentrations, chemosensitizers alone produced ≤15% inhibition of cell growth. IC_{50} values, obtained graphically from dose-response curves, represent the mean of 3 independent experiments, and the SD is within 20% of each value. The resistance factor is defined as the IC_{50} of resistant cells with or without chemosensitizers divided by the IC_{50} of the parental cells

The relative intracellular amount of DOX was evaluated by flow cytometry, and the DOX accumulation in resistant cells in the presence of MA is illustrated in Fig. 3. MA enhanced DOX accumulation in both of the resistant cell lines HCT-116/VM46 and MCF-7/ADR.

Antitumor effects of DOX and combined therapy with MA and DOX on human tumor xenografts

The antitumor activity of DOX, of MA, and of the combined DOX and MA therapy is shown in Table 4. Optimal antitumor activity against established HCT-116 tumor xenografts was observed when DOX was given i.v. q4dx3 at the MTD of 8 mg/kg. With this treatment regimen, DOX caused 3.7 TVDDs against drug-sensitive HCT-116 tumor xenografts but was not active (1.9 TVDD) against drug-resistant HCT-116/VM46 tumor xenografts. MA given s.c. at 50 mg/kg was not active. However, when DOX (8 mg/kg, i.v.) was given in combination with MA (50 mg/kg, s.c.), the combined DOX-MA therapy produced an antitumor activity equivalent to 3.7 TVDDs in established drug-resistant HCT-116/VM46 tumor xenografts.

Optimal antitumor activity against established drug-sensitive MCF-7 tumor xenografts was observed when DOX was given i.v. at 6 mg/kg q4dx3 (which is the MTD of DOX in estrogen-supplemented animals). With this treatment regimen, antitumor activity of 3.3 TVDDs was typically observed.

In contrast to MCF-7 tumor xenografts, the DOX-resistant counterparts do not require supplemental estrogen for growth in vivo. In animals implanted with the MCF-7/ADR tumor line, the MTD of DOX was 8 mg/kg. In studies using the MCF-7/ADR xenografts, MA (50 mg/kg) injected i.p. daily for 9 days to maintain circulating levels and DOX given i.v. at the MTD q4dx3 were not active as single agents. However, when an i.p. dose of 50 mg/kg MA was combined with an i.v. dose of 8 mg/kg DOX, significant antitumor activity (equivalent to 3.5 TVDDs) was observed in the drug-resistant MCF-7/ADR tumors.

Discussion

Although the role of P-gp in clinical MDR has not yet been firmly established, increasing evidence indicates P-gp plays an important role in clinical drug resistance in leukemias [21], lymphomas [11], neuroblastomas [6, 10], and childhood soft-tissue sarcomas [9]. Although a wide variety of compounds, particularly VER, are capable of restoring drug sensitivity in MDR cells in vitro, to date the use of modulators of MDR in patients has not provided clear benefit. This may result from the inability to administer safely the dose necessary to achieve efficacy. Other sensitizing agents less toxic and more potent than VER are currently under clinical investigation.

A number of steroids, including dexamethasone, testosterone, and, in particular, PG, have been identified as possible chemosensitizers on the basis of their ability to compete with azidopine photolabeling of P-gp [34]. In photoaffinity-labeling studies corticosterone has been shown to label P-gp and to be a substrate for P-gp [38]. Similarly, MA and another semisynthetic progestin, MAP, can increase [^{3}H]-VBL accumulation in MDR murine cells [36], but although MAP exhibited activity comparable with that of MA in murine cells, it was much less active in human carcinoma cells (data not shown). As these steroids are extremely well tolerated at high doses, their potential use to overcome drug resistance should be considered.

Table 4. Antitumor activity of DOX and combined DOX and MA therapy on MDR human tumor xenografts

Tumor line	Dose (mg/kg)			TVDD		
	DOX[a]	MA	DOX[a]+MA	DOX	MA	DOX+MA
HCT116	8	ND	ND	3.7	ND	ND
HCT/VM46	8	50[b]	8+50[b]	1.9	0	3.7
MCF7	6	ND	ND	3.3	ND	ND
MCF7/ADR	8	50[c]	8+50[c]	1.8	0	3.5

ND, Not determined

[a] DOX was given i.v. every 4 days for 3 injections

[b] MA was given s.c. every 4 days for 3 injections

[c] MA was given i.p. every day for 9 injections

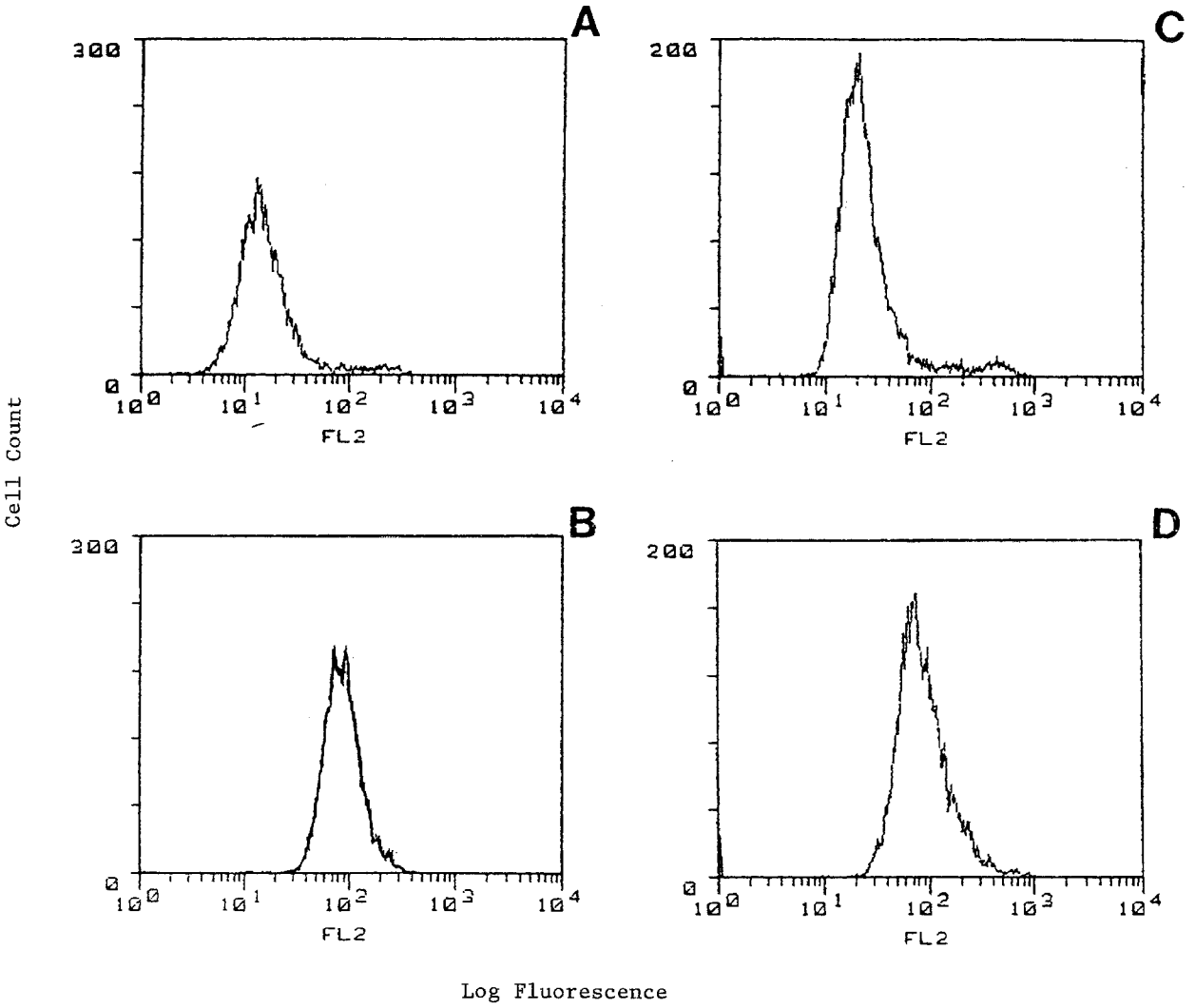

Fig. 3A–D. Intracellular DOX accumulation in MDR cells. Effect of MA on the accumulation of DOX in MDR HCT-116/VM46 and MCF-7/ADR cell lines. Cells were exposed to 2 mg DOX/ml in the absence or presence of MA at the stated concentrations for 2.5 h. DOX fluorescence was measured by a FACStar Plus flow cytometer. **A** HCT116/VM46 + DOX. **B** HCT116/VM46 + DOX + 50 μ*M* MA. **C** MCF-7/ADR + DOX. **D** MCF-7/ADR + DOX + 25 μ*M* MA

It has previously been shown that PG interacts preferentially with the murine *mdr*-1b gene product in J7.V1-1 cells as compared with the murine *mdr*-1a product in J7.V3-1 cells [35]. As the human *mdr*-1 gene product is more homologous to the murine *mdr*-1a gene product [19], it was of interest to confirm the previously reported effect of MA on human MDR cells [14] and to extend these observations to in vivo human tumor xenograft models.

In the present study we investigated Act D and DOX resistance reversal by MA in rodent and human tumor cell lines. In rodent J7.VI MDR cells, MA is slightly less effective than PG at low doses (<10 μ*M*) and becomes significantly more potent at higher concentrations (50 μ*M*). In human MDR cells, on the other hand, even at low concentrations (≤ 12 μ*M*), MA is 2–2.5 times more effective than PG in enhancing drug sensitivity, and at 8.6 μ*M* it can completely abolish drug efflux in HCT-116/VM46 cells, which have a relatively low (about 10-fold) level of drug resistance. However, MA is much less efficient in the highly resistant cell line MCF-7/ADR, where other factors such as elevated levels of glutathione and glutathione-S-transferase may also play some role [13, 28]. This observation should not curtail the potential application of MA as a chemosensitizer, since clinical tumor samples, unlike highly resistant cell lines developed in the laboratory, have only low levels of P-gp and are devoid of *mdr*-1 gene amplification, a common phenomenon in highly resistant cultured cells [12].

Our animal studies further confirm the modulating effect of MA in vivo. MA itself, given at doses of up to 100 mg/kg, showed no activity against established HCT/VM46 or MCF-7/ADR tumors. However, when MA was given in combination with DOX, an enhancement in antitumor effect was observed over that seen with DOX alone. In fact, the efficacy of the combined therapy against drug-resistant tumor xenografts was similar to that of DOX alone against the sensitive parental tumor line.

The mechanism of interaction of MA and PG with P-gp needs to be further investigated. In fact, unlike most chemosensitizers that are transported by P-gp, including VER [37], PG does not seem to be a substrate for P-gp [35].

MA has some pharmacological advantages over other chemosensitizers. MA can be given orally and has excellent bioavailability, since it is well absorbed and plasma concentrations can build up rapidly. At standard doses (160 mg/day), MA results in blood levels 5–10 times higher than those obtained with MAP [24]. Administration of 800 mg/day MA, a dose well below the tolerated dose of 1600 mg/day [1, 28] results in a mean serum concentration of 600 ng/ml, although there is wide variability among different patients (152–1389 ng/ml) [30]. Furthermore, MA has a rather extended elimination half-life of 30–40 h [16]. On the basis of these observations, we suggest that MA should be clinically evaluated either alone or in combination with other modulators as a MDR-reversing agent.

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