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Megestrol acetate reverses multidrug resistance and interacts with P-glycoprotein*

Gini F. Fleming, Jacqueline M. Amato, Michael Agresti, and Ahmad R. Safa

Department of Medicine, Section of Hematology/Oncology, University of Chicago, Chicago Ill., USA

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Summary. We evaluated the multidrug resistance (MDR)-modulating effects of progesterone (PRG) and an orally active, structurally related compound, megestrol acetate (MA), in several MDR human cell lines. At 100 μM, both steroids inhibited the binding of a *Vinca* alkaloid photoaffinity analog to P-glycoprotein (P-gp) in MDR human neuroblastic SH-SY5Y/VCR cells [which show >1500-fold resistance to vincristine (VCR) in the tetrazolium dye (MTT) assay]. However, 100 μM MA markedly enhanced the binding of [^{3}H]-azidopine to P-gp in both SH-SY5Y/VCR cells and the MDR human epidermoid KB-GSV2 cell line (which displays 250-fold resistance to VCR in the MTT assay). PRG had little effect on the binding of [^{3}H]-azidopine to P-gp. MA at low doses was more effective than PRG in sensitizing cells to VCR and enhancing their accumulation of [^{3}H]-VCR. The highly resistant SH-SY5Y/VCR subline exhibited significant collateral sensitivity to both steroids. These data suggest that MA may be a clinically useful modulator of MDR.

Introduction

Multidrug resistance (MDR) is a phenomenon in which cells selected for resistance to a single compound display broad cross-resistance to a variety of structurally unrelated cytotoxic agents that are generally of natural-product origin [11]. P-glycoprotein (P-gp) is an integral membrane protein that is overexpressed in MDR cell lines as well as in some human tumors [13, 17] and is believed to confer resistance by actively pumping cytotoxic agents out of the cell [27]. A wide variety of structurally unrelated agents have been shown to reverse MDR and increase cytotoxic drug accumulation in MDR cell lines [12]. It has been suggested that inhibition of the binding of [^{3}H]-azidopine to P-gp might serve as a rapid screening test for the ability to reverse MDR [29].

Recent reports have suggested that progesterone (PRG) inhibits [^{3}H]-azidopine binding to P-gp and reverses MDR in a murine macrophage-like cell line [29, 30], specifically binds to P-gp in a human lymphoblastic leukemia cell line [19], and decreases the binding of both [^{3}H]-vincristine ([^{3}H]-VCR) and a photoactive *Vinca* analog to P-gp in a human myelogenous leukemia cell line [16]. Moreover, previous work in our laboratory showed that PRG and promegestone were among the most effective of a large group of steroids tested in inhibiting the binding of a photoactive analog of verapamil, *N*-(*p*-azido[3,5-^{125}I]-salicyl) aminomethyl verapamil [23] to P-gp in MDR DC-3F/VCRd-5L Chinese hamster lung cells (manuscript in preparation).

Megestrol acetate (MA; Fig. 1) is an orally active synthetic congener of PRG that is commonly used clinically and can safely be given in very large amounts [1]. A dose of 800 mg/day in humans has been reported to give a plasma concentration of 2 μM [28]; at least 1600 mg/day is tolerated well, and the maximum tolerated dose has not yet been determined. We therefore investigated the ability of MA to reverse MDR in some human cell lines and examined its effect on the binding of [^{3}H]-azidopine and the photoactive analog of vinblastine, [^{125}I]-NASV, to P-gp in these cell lines.

Materials and methods

Materials. [^{3}H]-VCR (6.67 Ci/mmol) and [^{3}H]-azidopine (52 Ci/mmol) were purchased from Amersham International. The photoactive analog *N*-(*p*-azido[3-^{125}I]-salicyl)-*N*-β-aminoethylvindesine ([^{125}I]-NASV) was synthesized as previously described [20, 21]. VCR and vinblastine (VBL) were kindly donated by Eli Lilly & Co. MA and PRG were purchased from Sigma. All other chemicals were of reagent grade and were obtained commercially.

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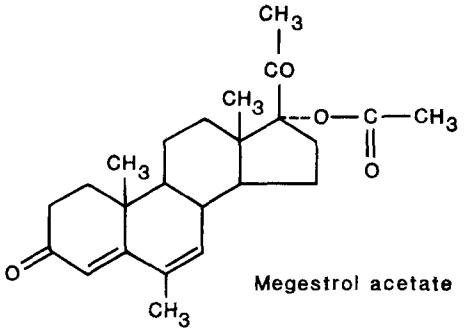
Offprint requests to: Ahmad R. Safa, Section of Hematology/Oncology, University of Chicago Medical Center, 5841 S. Maryland Ave., MC2115 Chicago, IL 60637, USA



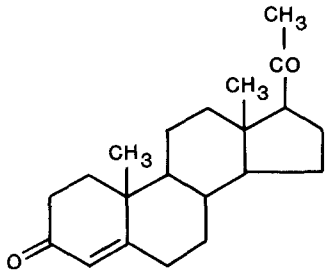


Fig. 1. Structures of megestrol acetate and progesterone

Cell culture. The SH-SY5Y human neuroblastic cell line and its VCR-resistant derivative SH-SY5Y/VCR [4, 5], as well as the DC-3F Chinese hamster lung cell line and its MDR variant DC-3F/VCR-d5L were generously supplied by Dr. J. L. Biedler [18, 22]. SH-SY5Y/VCR cells were maintained in medium containing 5 μg VCR/ml, and DC-3F/d5L cells, in medium containing 50 μg VCR/ml. KB-3-1 is a human epidermoid cell line, and KB-8-5 and KB-V1 are MDR sublines selected for resistance to colchicine and VBL, respectively [2, 25]. KB-8-5 cells were maintained in medium containing 10 ng colchicine/ml, and KB-V1 cells were maintained in medium containing 1 μg VBL/ml. KB-GSV2 cells, a generous gift from Dr. I. Roninson, were generated by transfecting KB-3-1 cells with the P-gp gene [10]; they were maintained in medium containing 10 ng VBL/ml. All cells were grown in media free of cytotoxic agents for 3–7 days prior to the experiments.

Membrane preparations. Cell-membrane vesicles were prepared by nitrogen cavitation and the differential centrifugation procedure described by Lever [15]. Protein concentrations were determined by the Bradford assay [7] using the Bio-Rad kit.

In vitro drug sensitivity. Growth-inhibition assays were performed using a modified 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium (MTT) assay [8, 14]. Briefly, cells were plated in 50-μl aliquots of growth medium in 96-well microtiter plates and incubated at 37° C overnight. Initial cell numbers were chosen so as to keep cells in the log phase of growth throughout the experiment and to assure that optical density measurements would be on the linear portion of the curves relating optical density to cell number for each cell line. Drugs diluted in medium were added to achieve a final volume of 100 μl; chemosensitizers were added 30–60 min before the cytotoxic agents. Each assay was performed in triplicate. Drugs were dissolved in dimethylsulfoxide (DMSO) before being diluted in medium; conditions were adjusted to keep the final concentration of DMSO less than or equal to 0.1%. Cells were then maintained at 37° C another 72 h, at which time 25 μl MTT (5 mg/ml in phosphate-buffered saline, PBS) was added to each well for an additional 4-h incubation. Formazen crystals were dissolved by adding 100 μl lysing buffer (20% sodium dodecyl sulfate, SDS, in 50% dimethylformamide with 1% acetic acid; pH adjusted to 4.6–4.8) and allowed to equilibrate for 6–18 h. The optical density of each well was determined by absorbance spectrometry at a wavelength of 570 nm using a Dynatech MR 700 microplate reader.

Table 1. Sensitization of sensitive and MDR human cell lines to VCR by MA and PRG

Modulator	VCR IC_{50} (μM)				
	SH-SY5Y	KB-3-1	KB-8-5	KB-GSV2	KB-V1
None	0.014	0.00035	0.038	0.087	2.1
MA 1 μM	0.01	0.00023	0.038	0.07	1.2
MA 2 μM	0.007	0.00022	0.025	0.03	0.53
MA 5 μM	0.0029	0.00016	0.0078	0.013	0.18

Data represent mean values for 1–3 experiments performed in triplicate. IC_{50}, Concentration inhibiting cell growth by 50%

Drug-accumulation studies. [^{3}H]-VCR accumulation was performed essentially as previously described [24]. KB-3-1 and KB-GSV2 cells were plated at 10^5 cells/well and allowed to attach overnight in 24-well plates. The medium was then carefully aspirated and replaced with fresh medium containing 300–400 nM [^{3}H]-VCR in the presence or absence of various concentrations of steroids. Experiments were terminated by rapidly washing the cells twice with ice-cold PBS. Radioactivity associated with trypsinized cells was measured by scintillation counting.

Thin-layer chromatographic analysis. Steroids were dissolved in methanol, spotted on Silica Gel GHLF plates, and run vertically in a solvent system consisting of chloroform: methanol: water (20 : 5 : 1, by vol.). Relative hydrophobicity was estimated by determining the mobility of each steroid in this system. Compounds were detected by UV irradiation and the R_f values were measured.

Photoaffinity labeling. Cells (5–10 × 10^5/assay) or plasma membranes (25 μg protein/assay) were photolabeled with 0.5 μM [^{3}H]-azidopine or 0.04 μM [^{125}I]-NASV (200 Ci/mmol) in the absence or presence of modulating agent by irradiation at 302 or 366 nM for 20 min using two self-filtering lamps as previously described [20–22]. Proteins were separated on NaDodSO$_4$/5%–15% polyacrylamide gels. After autoradiography and fluorography, quantitation of labeled P-gp was carried out using a model 1650 Bio-Rad scanning densitometer.

Results

Effects of MA and PRG on the cytotoxicity of VCR

The ability of MA to reverse VCR resistance in KB-3-1 cells and the VCR-resistant derivative lines KB-8-5, KB-V1, and KB-GSV2 was examined. We observed dose-dependent reversal of VCR resistance by nontoxic levels of MA in all four cell lines as shown in Table 1. The degree of sensitization appeared to be greater in the more resistant KB-V1 line. The parent KB-3-1 cell line was sensitized 2-fold by 5 μM MA. We could not use PRG to reverse MDR in KB cells because low levels of this hormone were toxic to all four KB cell lines (Table 2). PRG concentrations below 1 μM had no effect on VCR toxicity (data not shown).

To compare the effects of MA and PRG on sensitization to VCR, we used the SH-SY5Y human neuroblastoma parent cell line. This cell line contains an amount of *mdr1* mRNA similar to that of KB-8-5 cells [3], and concentrations of 1–5 μM MA or PRG alone produced no measurable growth inhibition. MA (5-fold sensitization to VCR at 5 μM) was approximately twice as potent a sensitizer as PRG (2-fold sensitization at 5 μM).

Table 2. Sensitivity of cell lines to MA and PRG

Cell line	IC_{50} (μM)	
	MA	PRG
KB-3-1	27	3.4
KB-8-5	ND.	5.8
KB-GSV2	25	2.2
KB-V1	17	1.5
SH-SY5Y	71	53
SH-SY5Y/VCR	15	12.5
DC-3F	14	16
DC-3F/VCRd-5L	6.5	4.6

IC_{50}, Concentration inhibiting cell growth by 50%; ND, not done

Neither MA nor PRG could be used to sensitize the highly resistant SH-SY5Y/VCR and DC-3F/VCR-d5L cell lines because, as can be seen in Table 2, these cells were 4–5 times more sensitive to both MA and PRG than were the parent cell lines, and even low levels of either steroid produced significant toxicity. SH-SY5Y/VCR cells are known to be hypersensitive to some other MDR-modulating agents, including verapamil [6].

Effects of MA and PRG on [^{3}H]-VCR accumulation

The effect of various doses of MA and PRG on [^{3}H]-VCR accumulation was measured. Figure 2a shows the VCR accumulation of KB-3-1 and KB-GSV2 cells over time in the absence of modulating agent. As shown in Fig. 2b, neither MA nor PRG had a great effect on [^{3}H]-VCR accumulation by KB-3-1 cells. At 50 and 100 μM of either steroid, [^{3}H]-VCR accumulation was increased by over 13 times in KB-GSV2 cells. At lower, clinically relevant concentrations, MA was much more effective than PRG.

Lipophilicity of MA and PRG

To determine whether the differences in MDR-reversing ability were a function of differences in lipophilicity, we performed thin-layer chromatography. There was no significant difference between the two compounds (R_f 0.85 for MA vs 0.86 for PRG.

Effect of MA and PRG on [^{3}H]-azidopine and [^{125}I]-NASV photoaffinity labeling of P-gp

As shown in Fig. 3, MA enhanced the binding of [^{3}H]-azidopine to P-gp in KB-GSV2 cells. Binding (expressed as a percentage of control values) was 154% at 1 μM MA, 200% at 10 μM MA, 204% at 100 μM MA, and 233% at 500 μM MA. PRG had little effect on the binding of [^{3}H]-azidopine to P-gp in KB-GSV2 cells, enhancing binding by less than 30% at similar concentrations. Similar results were obtained in SH-SY5Y/VCR cells, in which the binding of [^{3}H]-azidopine to P-gp was 253% at 10 μM MA, 218% at 100 μM, and 172% at 1000 μM MA (Fig. 4a). On the other hand, [^{125}I]-NASV labeling of P-gp in SH-SY5Y/VCR cells was inhibited in a dose-dependent manner by both PRG (50% inhibition at 45 μM) and MA (50% inhibition at 25 μM; Fig. 4b). Studies using KB-GSV2 membrane vesicles also showed that MA could inhibit the binding of [^{125}I]-NASV to P-gp (26% binding at 100 μM MA).

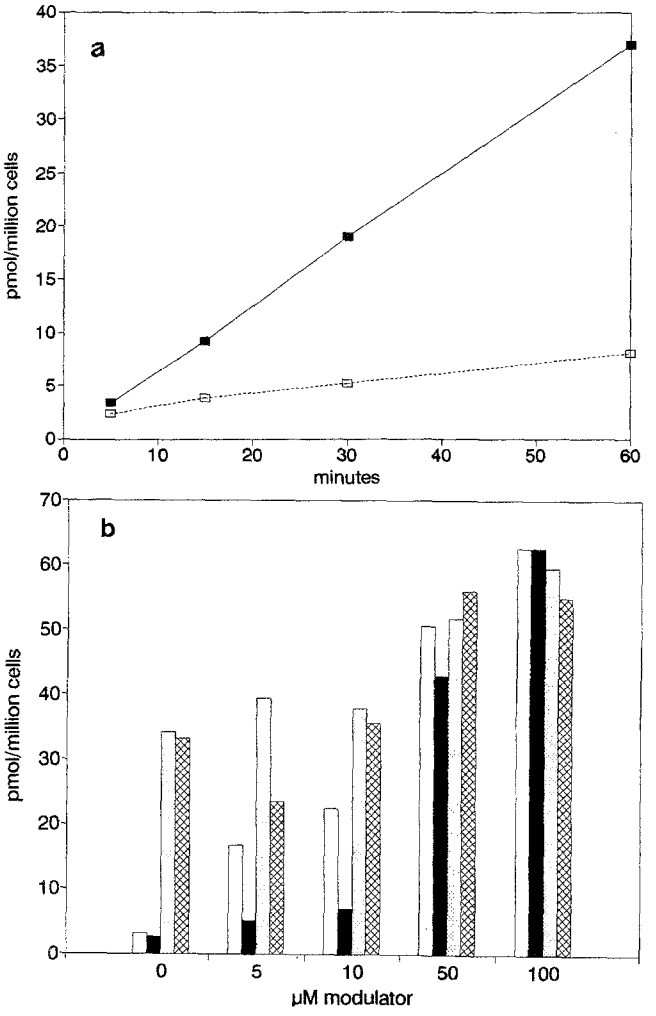


Fig. 2. A [^{3}H]-VCR accumulation by KB-3-1 (■) and KB-GSV2 (□) cells. Cells were incubated with 400 nM [^{3}H]-VCR in growth medium for various periods and the radioactivity associated with the cells was determined. SEM for triplicate wells, <10%. **B** Representative experiment showing [^{3}H]-VCR accumulation after 60 min incubation with 331 nM [^{3}H]-VCR in sensitive and MDR cells in the presence and absence of modulator. *Open bars*, KB-GSV2 cells incubated with various concentrations of MA; *solid bars*, KB-GSV2 cells incubated with various concentrations of PRG; *dotted bars*, KB-3-1 cells incubated with MA; *cross-hatched bars*, KB-3-1 cells incubated with PRG. SEM for triplicate wells, <10%

Discussion

It has been suggested that the mechanism by which MDR-modulating agents sensitize cells involves their direct or indirect interaction with cytotoxic-drug-binding site(s) on P-gp [22, 23]. However, little is known about the numbers or characteristics of or the variations in P-gp drug-binding sites. Rodent cell lines contain two classes of P-gp that are

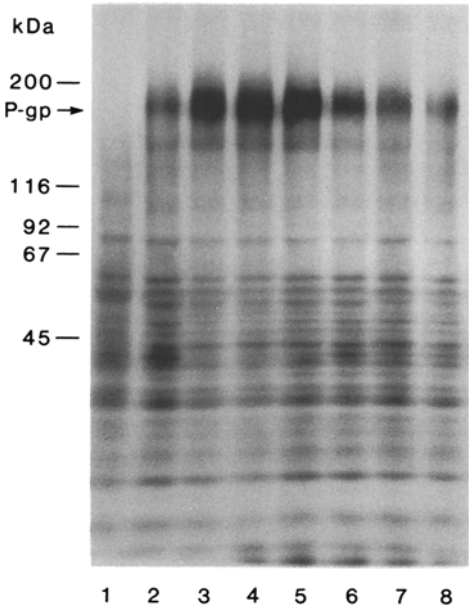


Fig. 3. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)/fluorography of [^{3}H]-azidopine-photolabeled KB-3-1 (*lane 1*) and KB-GSV2 cells (*lanes 2–8*) in the absence (*lanes 1, 2*) or presence of 500, 100, 10, or 1 μM MA (*lanes 3–6*) or 500 or 100 μM PRG (*lanes 7, 8*)

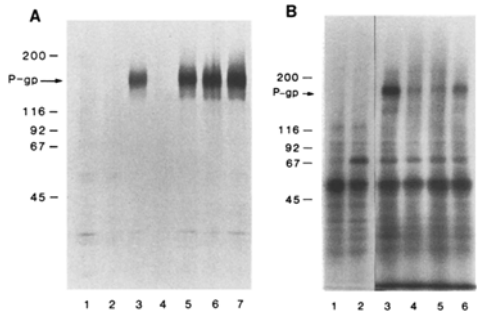


Fig. 4. A SDS-PAGE autoradiography/fluorography of [^{3}H]-azidopine-photolabeled SH-SY5Y (*lanes 1, 2*) and SH-SH5Y/VCR cells (*lanes 3–7*) in the absence (*lanes 1, 3, 5–7*) or presence (*lanes 2, 4*) of 50 μM cold azidopine and in the absence (*lanes 1–4*) or presence of 1000 (*lane 5*), 100 (*lane 6*), or 10 μM MA (*lane 7*). **B** SDS-PAGE autoradiography of [^{125}I]-NASV-photolabeled SH-SY5Y (*lanes 1, 2*) and SH-SH5Y/VCR cells (*lanes 3–6*) in the absence (*lanes 1, 3*) or presence of 500 (*lanes 2, 4*), 100 (*lane 5*), or 10 μM MA (*lane 6*)

related to drug resistance: one is encoded by the *mdr*1a gene and the other, by the *mdr*1b gene. Humans are believed to express only one gene (*mdr* 1) that is responsible for encoding MDR, and it shares a greater sequence homology with *mdr*1a than with *mdr*1b [9]. It is known that there are differences in drug interactions between the different classes of P-gp in rodent cell lines. Yang et al. [30] noted that in murine J7.V1-1 cells (which overexpress the *mdr*1b gene), PRG markedly inhibited azidopine binding, inhibited VBL efflux, and increased VBL cytotoxicity, whereas in J7.V3-1 cells (which overexpress the *mdr*1a gene), these effects were seen only to a much more modest degree. These authors have suggested that inhibition of azidopine binding to P-gp might be used as a rapid screening test for selection of compounds that reverse MDR. However, in our study, MA, which proved to be an effective MDR-reversing agent, did not inhibit but rather strongly enhanced azidopine binding to P-gp in KB-GSV2 and SH-SY5Y/VCR cells. β-Estradiol was reported to enhance the binding of azidopine to J7.V-1 cells by 39%, but it had no effect on VBL cytotoxicity in that cell line [29].

Recent work in our laboratory has shown that there are at least two kinetically distinguishable drug-binding sites on P-gp [26]: one for *Vinca* alkaloids, verapamil, and cyclosporin A, and a second for azidopine. It may be that interaction of modulating agents with the first binding site is more important in reversing MDR than interaction with the second. On the basis of the present findings, we conclude that (1) not all agents that reverse MDR are capable of inhibiting azidopine photoaffinity labeling of P-gp, and (2) MA interacts with P-gp and reverses MDR in several human cell lines at concentrations that may be achievable in vivo.

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