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Augmentation of vincristine cytotoxicity by megestrol acetate

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Abstract *Purpose:* To determine the effect of a semisynthetic progesterone, megestrol acetate (MA), on the cytotoxicity of various chemotherapeutic agents including vincristine, doxorubicin, actinomycin-D, taxol, vinblastine and colchicine in cell lines with or without P-gp expression. *Methods:* Three cell lines with high P-gp expression (two colon cancer and one leukemia), and a control cell line with no P-gp expression were exposed to chemotherapeutic agents in the presence or absence of MA and drug sensitivity was determined using the MTT colorimetric assay. P-gp-170 expression was detected by flow cytometry using JSB-1 monoclonal antibody and the functionality of MDR expression was tested by rhodamine-123 uptake studies. In vitro drug accumulation studies were performed using [^{3}H]-vincristine. The results were subjected to paired *t*-test analysis and 95% confidence intervals were determined in cytotoxicity tests. *Results:* MA augmented the cytotoxicity of vincristine, but not doxorubicin, actinomycin-D, taxol, vinblastine or colchicine in the three P-gp-expressing cell lines, whereas verapamil augmented the cytotoxicity of doxorubicin and vincristine. MA did not augment the cytotoxicity of vincristine in the P-gp-negative HUT-102 cell line. *Conclusion:* MA augmented vincristine cytotoxicity in P-gp-expressing cell lines. However, this phenomenon did not occur with the other classic MDR drugs. Therefore, the augmentation of vincristine cytotoxicity by MA can be explained either by involvement of a different mechanism that coexists with the mdr-1 phenotype or by the presence of a different affinity or binding site on the P-gp molecule for MA compared to that for the other classic MDR drugs and verapamil.

Key words Vincristine · Megestrol acetate · Cytotoxicity · Multidrug resistance

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Introduction

Resistance to chemotherapeutic agents is a major cause of treatment failure in cancer. One of the most important types of drug resistance is multidrug resistance (MDR) which is associated with overexpression of the mdr-1 gene product, a 170-kDa membrane glycoprotein known as P-glycoprotein (P-gp) [4]. P-gp functions as an ATP-dependent efflux pump and causes reduced steady-state accumulation of structurally unrelated natural drugs such as vinca alkaloids, doxorubicin, colchicine and actinomycin D [1, 2, 10]. P-gp-mediated MDR can be reversed by diverse hydrophobic compounds, including verapamil, cyclosporins, progesterone, and others [8, 11, 14, 16]. Unfortunately, many of these compounds cannot be administered to patients at doses sufficient to provide levels needed for MDR reversal in vitro owing to unacceptable toxicity [7].

It has been suggested that megestrol acetate (MA), a synthetic derivative of progesterone, interacts with P-gp in vitro and reverses MDR to vincristine in several human cell lines at concentrations that may be achievable in vivo [3]. To explore the MDR-reversing properties of MA further and to develop laboratory data in support of a clinical trial employing high-dose MA as an MDR-reversing agent, we studied the effects of MA on the in vitro cytotoxicity of vincristine, vinblastine, doxorubicin, taxol, actinomycin-D and colchicine and the MDR-unrelated drug cisplatin in a panel of cell lines with and without MDR expression.

Materials and methods

Drugs and chemicals

Vincristine, vinblastine, doxorubicin, actinomycin-D, colchicine, taxol and cisplatin were obtained from Sigma (St. Louis, Mo.), and prepared as aqueous stock solutions. MA was obtained from Sigma and prepared as a stock solution in ethanol. ^{3}H-Vincristine was obtained from Amersham (Arlington Heights, Ill.). The JSB-1 monoclonal antibody (mAb) was purchased from Boehringer Mannheim (Germany). Sodium dodecyl sulfate (SDS), 3-(4,5-dimehtylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) and rhodamine-123 were obtained from Sigma, and dimethyl formamide was purchased from Fisher (Pittsburgh, Pa.). Cell culture medium RPMI-1640 and fetal bovine serum were obtained from GIBCO (Grand Island, N.Y.).

Cell lines and culture conditions

Human colon cancer cell lines (HT-29 and CACO-2) and the human T-cell leukemia cell line (HUT-102) were obtained from ATCC Laboratories (Rockville, Md.). The human pre-B-cell leukemia line NALM-6 was kindly donated by Dr. M.S. Coleman (University of Kentucky). Cells were grown in RPMI-1640 medium supplemented with fetal bovine serum, 2 m*M* glutamine, 10^5 U/l penicillin and 100 mg/l streptomycin. Cells were maintained in an atmosphere containing 5% CO_2 at 37 °C. Estrogen and progesterone receptor status were determined by immunohistochemical analysis. The characteristics of the cell lines are shown in Table 1.

Determination of P-gp expression

Expression of P-gp was determined by flow cytometry using JSB-1 mAb (anti-pgp-170, Boehringer Mannheim). Cells were washed with 10 m*M* Hepes, pH 7.4, 150 m*M* NaCl and 4% heat-inactivated newborn bovine serum at 4 °C and resuspended in phosphate-buffered saline (PBS) before the analysis. JSB-1 mAb was prepared at optimal dilution (50 μg/ml) in phosphate-buffered saline/2% fetal calf serum (PBS/FCS) with 2% normal rabbit serum as the final concentration, and 20 μl of this solution was added to each cell pellet consisting of 2×10^5 cells in a volume of 80 μl and the mixture was resuspended and incubated for 45 min on ice. After the cells had been washed with PBS/FCS, 20 μl of FITC-conjugated antimouse-IgG_1 polyclonal antibody was added to each tube, and the mixtures mixed well and incubated for 30 min on ice. Immunofluorescence analysis was performed under 488 nm argon laser excitation (FacScan, Becton Dickinson, San José, Calif.). Internal controls for the P-gp analysis consisted of the same cell line incubated with FITC-conjugated antimouse IgG_1 polyclonal antibody without the JSB-1 mAb. Positivity for P-gp expression was determined as greater than 15% fluorescent staining of cells with the JSB-1 mAb FITC-conjugated antimouse IgG_1 polyclonal antibody complex compared with the internal control with FITC-conjugated antimouse IgG_1 polyclonal antibody alone.

Cytotoxicity assay

Drug sensitivity was determined using the MTT colorimetric assay. Exponentially growing cells were plated in 96-well microtiter plates as 50-μl aliquots of culture medium. Stock solutions of drugs were diluted to different concentrations in culture medium and added to achieve a final volume of 100 μl. Cells were exposed to MA 60 min before the cytotoxic agents were added. Since MA has to be dissolved in 100% ethanol, conditions were adjusted to keep the final concentration of ethanol below 0.5%. Cells were treated in the presence or absence of MA and allowed to grow for an additional 4 days. Microplates were then processed by adding 50 μl MTT (3 mg/ml in PBS) to obtain a final concentration of 1 mg/ml and incubating for 4 h at 37 °C. Formazan crystals were then dissolved in lysing buffer (20% SDS in 50% dimethylformamide with 2.5% acetic acid, adjusted to pH 4.7 by adding *N* HCl). After an overnight incubation at 37 °C, optical densities (OD) were measured at 540 nm using a ThermoMax Microplate Reader (Molecular Devices).

Drug accumulation studies

Cells at a density of 5×10^5/ml were incubated in 5-mm dishes in the presence of 300 n*M* [^{3}H]-vincristine, pretreated with 5 or 50 μ*M* MA or not pretreated for 60 min. Following incubation for 15 to 60 min in culture medium at 37 °C, drug accumulation was terminated by washing the cells twice with ice-cold PBS. The cells were harvested and the radioactivity was measured by liquid scintillation counting using a Beckman LS 7500 device.

Rhodamine-123 uptake studies

Briefly, cells at a concentration of 1×10^6/ml were suspended in RPMI-1640 without serum and preincubated for 60 min at 37 °C in the presence or absence of 5 μ*M* MA. Rhodamine-123 was then added to the cells at a concentration of 200 ng/ml. Accumulation was terminated at different time-points by two washes in ice-cold medium and cells were kept at 4 °C. Fluorescence intensity was measured using flow cytometric analysis. Rhodamine-123 uptake was considered positive for cell lines which retained more than 10% of rhodamine-123 dye at 30 min compared with 0-min uptake by flow cytometric evaluation. If the difference in rhodamine-123 uptake was less than 10% between these two samples, the uptake was considered negative. Efflux studies and determination of the activity of P-gp were carried out by the method of Ludescher et al. [5].

Statistical analysis

The significance of the differences between the cytotoxic effects of vincristine in the presence and absence of MA was determined using the paired *t*-test at specific concentrations of vincristine as well as by nonoverlapping 95% confidence intervals.

Results

Effect of MA on the cytotoxicity of vincristine

We examined the impact of MA on the cytotoxicity of vincristine in cell lines HT-29, CACO-2, NALM-6, and HUT-102. As shown in Table 1, the cell lines had different amounts of P-gp expression ranging from 8% in HUT-102, to 83% in NALM-6, as shown by flow cytometric analysis. To determine functional activity of P-gp, we used the flow cytometric measurement of cellular rhodamine-123 uptake/efflux. Efflux was positive in the cell lines HT-29, CACO-2 and NALM-6 and negative in the cell line HUT-102. To rule out any potential interaction, we determined progesterone receptor expression which was negative in all cell lines by immunohistochemistry.

MA enhanced vincristine cytotoxicity in MDR-expressing cell lines HT-29, CACO-2, and NALM-6, but had no effect on cytotoxicity in the MDR-negative cell line HUT-102 (Fig. 1). Potentiation of vincristine cytotoxicity by MA ranged from 1.4-fold to 5-fold and did not correlate with the percentage of MDR expression by flow cytometric analysis (Table 1). In the cell line NALM-6, in which 83% of the cells were positive for P-gp expression by flow cytometry, MA enhanced vincristine cytotoxicity by over 2-fold, whereas in HT-29 cell line the enhancement was 5-fold despite 46% of the cells staining positive for P-gp. MA did not enhance the

Table 1 Cell line characteristics in terms of sensitivity to vincristine (VCR) and vincistine + megestrol acetate (VCR + MA) exposure, pgp-170 expression, rhodamine-123 efflux, and progesterone receptor (PR) expression

Cell line	VCR IC_{50}(n*M*)	VCR + MA(5 μ*M*) IC_{50}(n*M*)	Pgp-170 FACS (%)	Rh-123 efflux	PR receptor
NALM-6	5.2	2.3	83	Negative	Negative
CaCO-2	7.0	5.0	86	Negative	Negative
HT-29	30	6.0	46	Negative	Negative
HUT-102	250	248	8	Positive	Negative

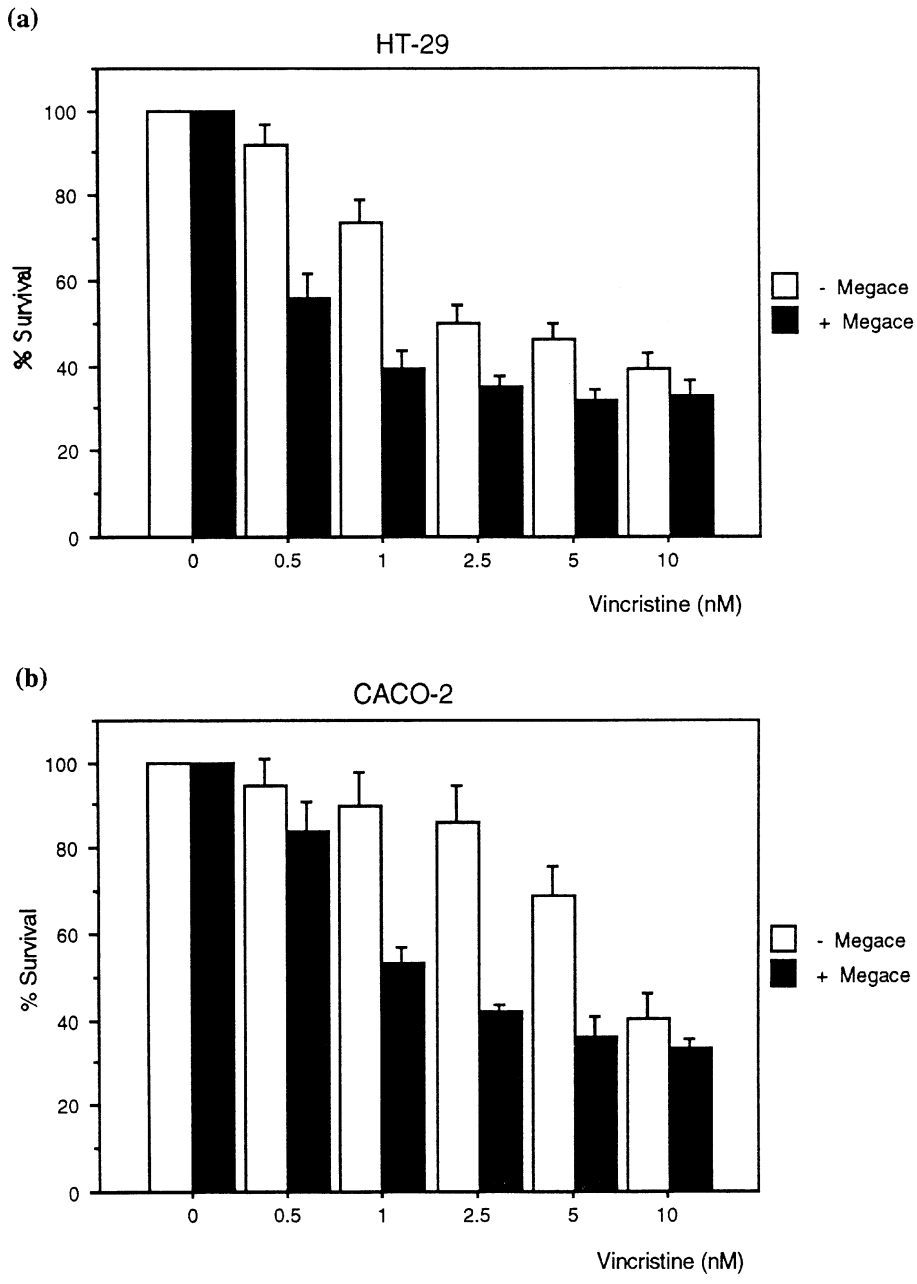
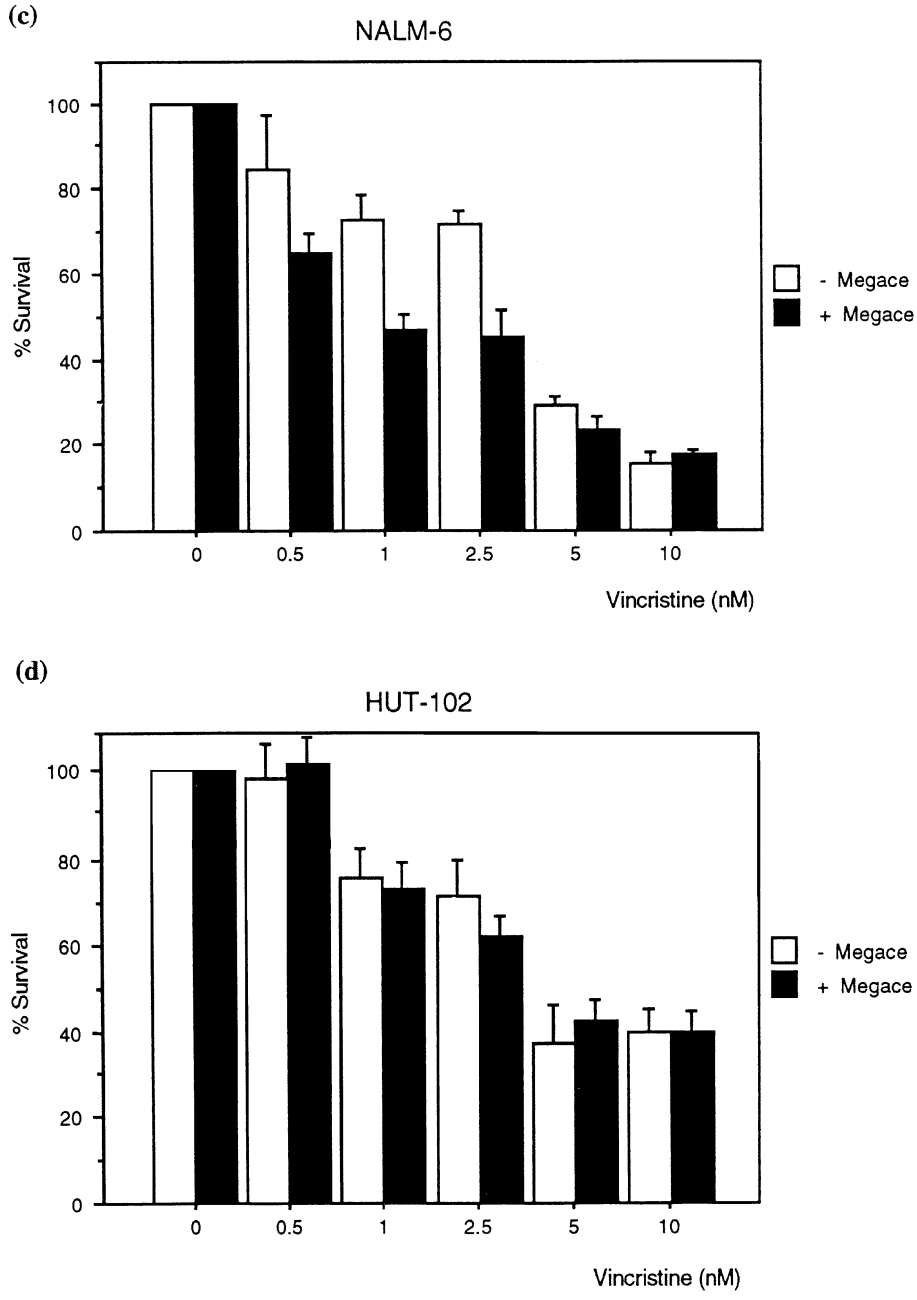


Fig. 1a–d Augmentation of vincristine cytotoxicity by MA against HT-29, CACO-2 and NALM-6 cell lines (**a**, **b** and **c**). The cytotoxic effect of vincristine on the pgp-170-negative HUT-102 cell line (**d**) was not affected by the addition of MA. Adherent cell lines (HT-29 and CACO-2) were incubated with cytotoxic agents for 96 h. Suspension cell lines (NALM-6 and HUT-102) were incubated with cytotoxic agents for 48 h. MA was used at a concentration 5 μ*M*. The values shown were derived from the mean OD value of six microplate wells per drug concentration. *Error bars* reflect 95% confidence intervals. The P-values at 1 and 2.5 n*M* vincristine concentrations in **a**, **b** and **c** are less than 0.01 (paired *t*-test)

Fig. 1 (Contd.)



cytotoxicity of the other MDR drugs doxorubicin, vinblastine, taxol, actinomycin-D and colchicine in any cell line (Figs. 2, 3). In contrast, verapamil augmented cytotoxicity of both vincristine and doxorubicin in MDR-expressing cell lines (Fig. 3). MA failed to enhance the cytotoxic activity of the non-MDR drug, cisplatin (data not shown).

Effect of MA on [^{3}H]-vincristine accumulation

We measured the effects of MA on the intracellular accumulation of vincristine using [^{3}H]-vincristine. MA enhanced vincristine accumulation in the MDR-expressing cell line HT-29 at concentrations of 5 and 50 μM by 1.6–2.35-fold at 1 h (Fig. 4). This effect was more pronounced at high MA concentrations.

Comparison of the effects of verapamil and MA on the cytotoxicity of vincristine and doxorubicin

To determine whether we could obtain similar results using verapamil as the classic MDR modulator, we determined the cytotoxicity of vincristine and doxorubicin in the cell line HT-29 (Fig. 3) in the

presence of verapamil or MA. In contrast to MA, verapamil augmented the cytotoxicity of both vincristine and doxorubicin.

Discussion

MDR is an important problem in the treatment of cancer. Over the past decade, numerous hydrophobic compounds have been shown to reverse MDR in vitro. However, attempts to clinically reverse MDR have been complicated by direct toxicity related to these compounds or by decreased elimination of cytotoxic drugs as a result of interference with their metabolism [7, 9]. Furthermore, the majority of the modulators have to be administered intravenously over days to achieve the levels shown in vitro to modulate MDR. Despite some limited success in clinical reversal of MDR, it is generally thought that further research is needed to define less toxic and more efficacious chemosensitizers. MA is a promising drug as an MDR-reversing agent. It can be administered orally and is extremely well tolerated at doses as high as 1600 mg/day [13]. At a dose of 800 mg/day, peak plasma concentrations of 0.5–3.3 μM can be achieved (Bristol Myers, data on file) and at doses of 1600–2400 mg/day plasma concentrations of 3–13 μM have been recorded (S. Tansan, unpublished data). A maximally tolerated dose (MTD) has not been defined. It has been previously shown that MA interacts with pgp-170 in vitro and reverses vincristine resistance in several cell lines at a concentration of 5 μM and enhances the intracellular accumulation of vincristine [3].

In the present study we investigated the role of MA on the cytotoxicity of a panel of MDR-related drugs in cell lines with or without MDR expression. In MDR-expressing cell lines MA enhanced the cytotoxicity of vincristine (1.4- to 5-fold) but had no effect on the cytotoxicity of vinblastine, doxorubicin, actinomycin D, colchicine or taxol. In contrast, verapamil was able

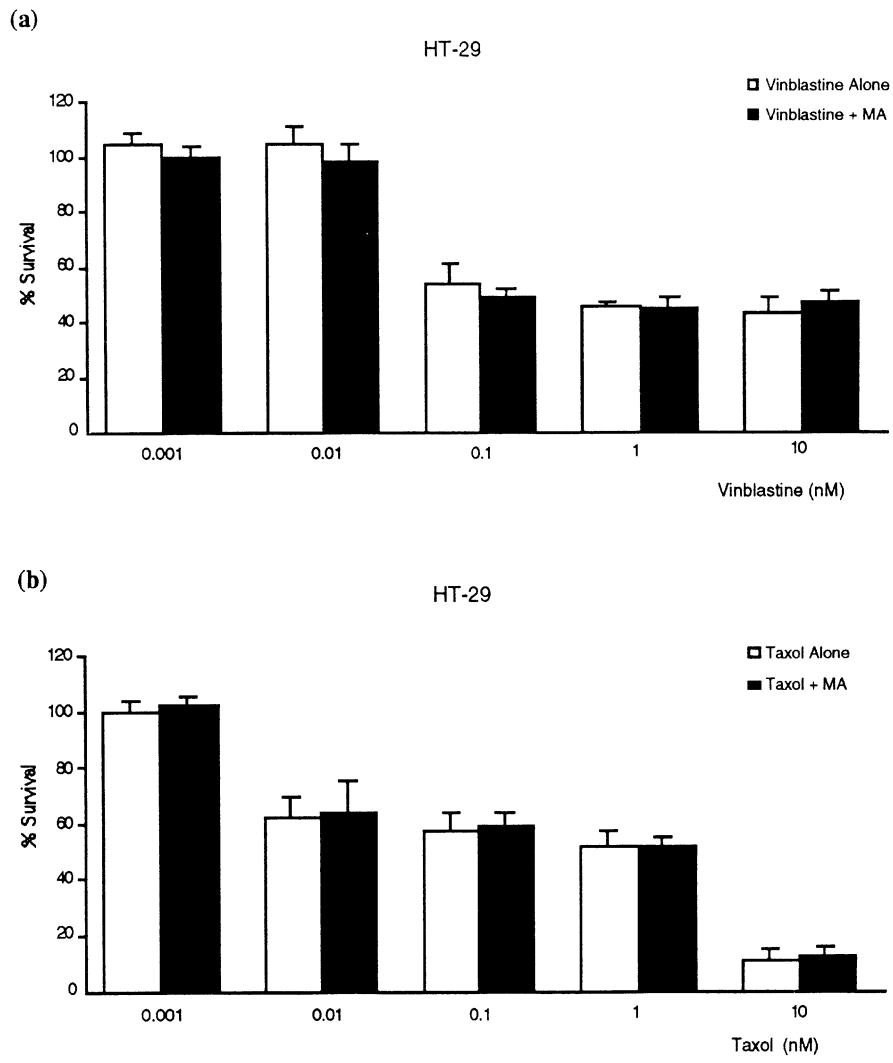
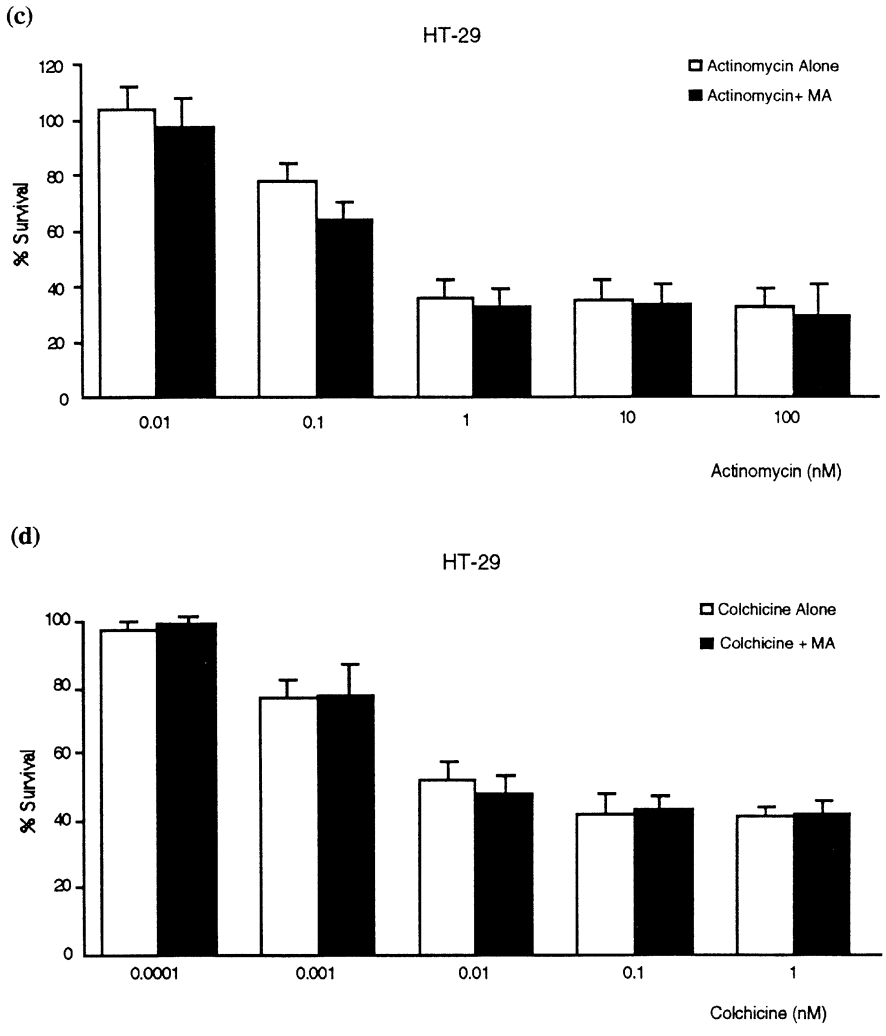


Fig. 2a–d Cytotoxic effect of vinblastine (**a**), taxol (**b**), actinomycin-D (**c**) and colchicine (**d**) on HT-29 colon carcinoma cells in the presence or absence of 5 μM MA. Assay conditions are the same as described for Fig. 1. *Error bars* reflect 95% confidence intervals

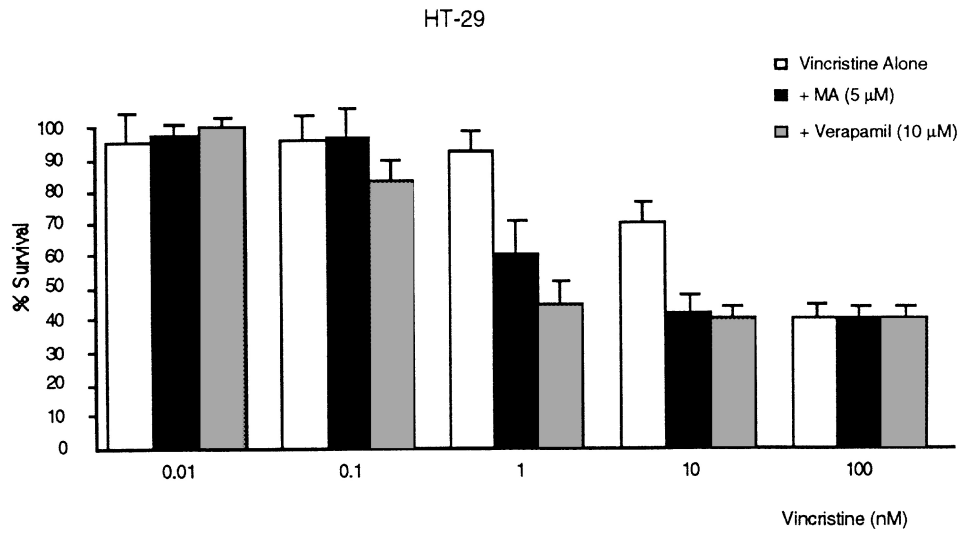
Fig. 2 (Contd.)



to enhance the cytotoxicity of both vincristine and doxorubicin. The enhancement of vincristine cytotoxicity appeared to be due to increased accumulation in MDR-expressing cell lines. The reason for selective enhancement of vincristine cytotoxicity and the apparent lack of enhancement of cytotoxicity of other MDR drugs is unclear. MDR-reversing agents may fall into several classes. The exact mechanisms of MDR reversal for each of these agents is not well defined. Most MDR-reversing agents are transported by pgp-170 and have been shown to compete with azidopine for binding to this membrane protein [12, 16]. On the other hand, MA has been shown to enhance the binding of azidopine to pgp-170 [3]. P-gp is a large molecule which may exhibit specific binding sites for different compounds as has been previously suggested [17]. Therefore, it is plausible that MA may reverse MDR for vincristine only, while verapamil may have a broader MDR reversal profile.

More recently it has been suggested that MA can reverse resistance against doxorubicin and actinomycin D in cell lines that overexpress MDR [15]. In three human cell lines with various levels of MDR expression, we were unable to demonstrate this phenomenon. The MDR-reversing abilities of various agents may depend on the cell line or the level of MDR expression. For example, MA may reverse resistance to other MDR drugs as well as vincristine in highly resistant cell lines with significant MDR overexpression, but not in cell lines that do not exhibit significant MDR expression. Another explanation for the lack of augmentation of cytotoxicity by MA in cell lines expressing the MDR phenotype treated with classical MDR drugs other than vincristine is the possible presence of a different affinity or binding site for MA on the P-gp molecule [6].

It appears that there is still a significant gap in our understanding of the MDR phenomenon. There is an ongoing need to investigate the mechanisms of interaction between pgp-170 and various MDR-reversal agents in cell lines with different levels of MDR overexpression. Until more definitive data become available, it may be more appropriate to refrain from generalized statements about the class of MDR drugs when only



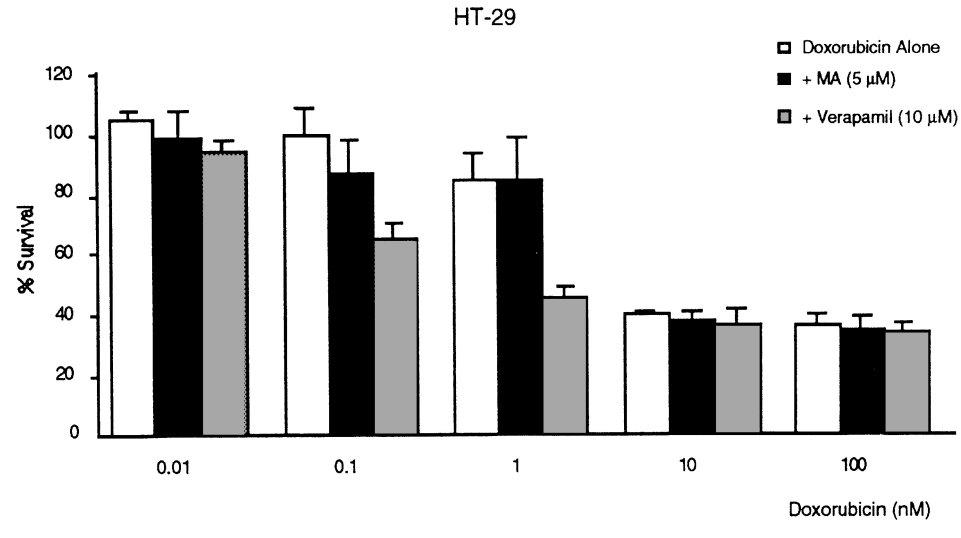


Fig. 3 Enhancement of vincristine and doxorubicin cytotoxicity on HT-29 cell line by verapamil (10 μ*M*). MA failed to augment doxorubicin-induced cell kill. MA was used at a concentration of 5 μ*M*. Assay conditions are the same as described for Fig. 1. *Error bars* reflect 95% confidence intervals

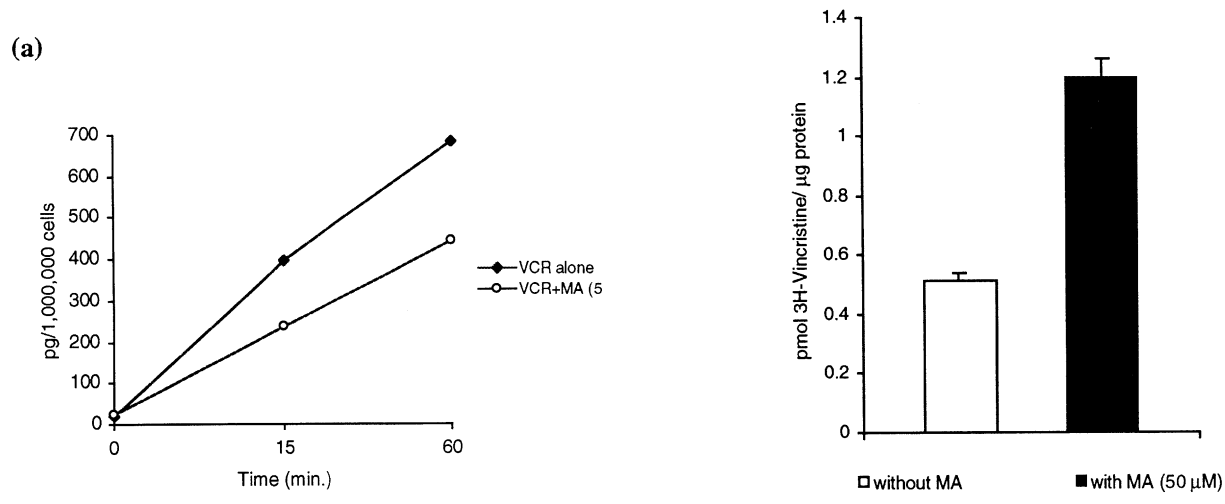


Fig. 4a, b Increased radioactive vincristine accumulation by pretreatment with MA at concentrations of 5 μ*M* (**a**) and 50 μ*M* (**b**). Cells (10^5) were incubated in culture medium containing 10 n*M* ^{3}H-vincristine for 1 h at 37 °C. Accumulation was terminated by washing cells in ice-cold PBS. Cells were digested in 1 *N* NaOH, and radioactivity was measured using a scintillation counter. Radioactivity was normalized with the amount of protein. Protein was determined using the Bio-Rad Assay. Values shown are means of three experiments with *error bars* reflecting 95% confidence intervals.

one particular drug or cell line has been tested. Meanwhile, clinical trials using megestrol in combination with vincristine in MDR-expressing malignancies are warranted.

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