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Original Paper

In Vitro Modulation of Doxorubicin and Docetaxel Antitumoral Activity by Methyl-β-cyclodextrin

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Methyl-β-cyclodextrin (MEBCD) was investigated for its effect on the antitumoral activity of various antineoplastic agents (doxorubicin (DOX), docetaxel (DXL), 5-fluorouracil (5-FU) and cisplatin (CDDP)) in three different human parental sensitive cancer cell lines (K562 S, MCF7 S and A2780 S) and their multidrug resistant variant sublines (K562 R, MCF7 R and A2780 R). At non-cytotoxic concentrations, MEBCD was able to increase significantly DOX and DXL cytotoxic activity in all the cell lines tested. The sensitisation ratios (IC_{50} drug control/IC_{50} drug-MEBCD treated) ranged from 3.1 to 14.3. Moreover, intracellular DOX accumulation, determined by high-performance liquid chromatography, was also increased when cells were treated with MEBCD combined with DOX (approximately 2–3-fold). The effects of MEBCD in resistant sublines were greater than in their parental sensitive cell lines. Other experiments demonstrated that the action of the MEBCD was independent of DOX. These data provided a basis for the potential therapeutic application of MEBCD in cancer therapy.

Key words: cyclodextrins, antineoplastic agents, tumour cells cultured, doxorubicin, cytotoxic activity, intracellular concentrations
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INTRODUCTION

CHEMOTHERAPY IS an important therapeutic modality in a variety of malignancies. However, two major obstacles, development of acquired resistance and toxicity, limit its use. To overcome these shortcomings, investigators have explored new approaches by modifying the antineoplastic drug's chemical structure or circumventing multidrug resistance by several processes. This may involve identifying and exploiting recently discovered molecular features of cancer cells. One possible new target may be the cell membrane that is the potential site for methylated-β-cyclodextrin (MEBCD) activity. Cyclodextrins (CDs) are oligosaccharide cyclic torus-shaped molecules which have the ability to form molecular inclusion compounds with a wide range of molecules, such as drugs. Three different native CDs are known: α-CD, β-CD and γ-CD (6, 7 and 8 D-glucose units, respectively). The internal structure of the molecule is a hydrophobic cavity, while the outer surface is hydrophilic (Figure 1), enabling improvement in the solubilisation of various drugs. These molecules differ mainly in their cavity size and in their solubility. The solubility of CDs can be improved through derivatisation of the hydroxyl groups, such as methylation, hydroxyethylation, hydroxypropylation and acetylation [1–4]. Indeed, whereas substituted CDs are commonly used for the optimisation of biopharmaceutical properties of drugs, such as solubility, delivery, membrane permeability and bioavailability, new interesting issues in CD research, such as the interaction with the lipid and the cholesterol of biological membranes, have been recently addressed. Several authors [5–10] have shown that methylated-β-CDs can alter the cholesterol content of the cell membrane, which play structural roles, thus modifying membrane fluidity and permeability between external and internal environments. Moreover, an enhancement of transport of many molecules has been observed in the presence of MEBCD [11–16]. Thus, in a previous study, we showed potentiation of the

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antitumoral activity of doxorubicin (DOX) in HL-60 cancer cell lines [12].

The aim of the present study was to determine the role of CDs associated with various antineoplastic agents on the cellular proliferation of three different human parental sensitive cancer cell lines and their multidrug resistant variant sublines. To examine the mechanism of action of MEBCD, intracellular kinetics of DOX were performed in liquid and solid tumour cell lines. In addition, the intracellular accumulation of MEBCD was investigated.

MATERIALS AND METHODS

Drugs and chemicals

Doxorubicin hydrochloride (DOX) (Pharmacia, St. Quentin-Yvelines, France), 5-fluorouracil (5-FU) (Roche, Neuilly-sur-seine, France), cisplatin (CDDP), daunorubicin and docetaxel (DXL), (Rhône Poulenc Rorer, Neuilly-sur-seine, France), β-cyclodextrin (BCD), methyl-β-cyclodextrin (MEBCD), γ-cyclodextrin (GCD), tetrazolium dye (MTT) and phosphate-buffered saline (PBS) (Sigma, St. Quentin Fallavier, France), RPMI 1640 medium and fetal calf serum (Polylabo, Paris, France) were used in this study. All other reagents were of analytical grade and were obtained from Carlo Erba (Milan, Italy) or Prolabo (Paris, France).

Cell culture and culture conditions

The human chronic myeloid leukaemia cell line, K562, and the human breast adenocarcinoma cell line, MCF 7, were obtained from the American Type Culture Collection (Rockville, Maryland, U.S.A.). The human ovarian adenocarcinoma cell line, A2780, was a kind gift from Dr. Canal, Centre Claudius Régaud, Toulouse, France. The doxorubicin-resistant sublines K562 R, MCF7 R and A 2780 R were established by continuous exposure of the parent cells to gradually increasing concentrations of doxorubicin and were maintained in medium supplemented with 0.1 μg doxorubicin/ml medium. MDR phenotype expression of K562 R, MCF7 R and A2780 R cell lines was assessed by an immunohistochemical method using two P-glycoprotein specific murine monoclonal antibodies C219 (Centocor, Malvern, Pennsylvania, U.S.A.) and JSB1 (Tebu, Le Perray-en-Yvelines, France). Exponentially growing cells were used for all experiments and all cells were free of mycoplasma. Cells were maintained in culture flasks at 37°C in a humidified atmosphere containing 5% CO_2, in RPMI-1640 medium supplemented with 10% fetal calf serum, antibiotics and glutamine. The viability of the cells was assessed by their ability to exclude 0.5% trypan blue dye. Cell density in culture flasks was determined by a Coulter counter (Model Z1, Hialeah, Florida, U.S.A.).

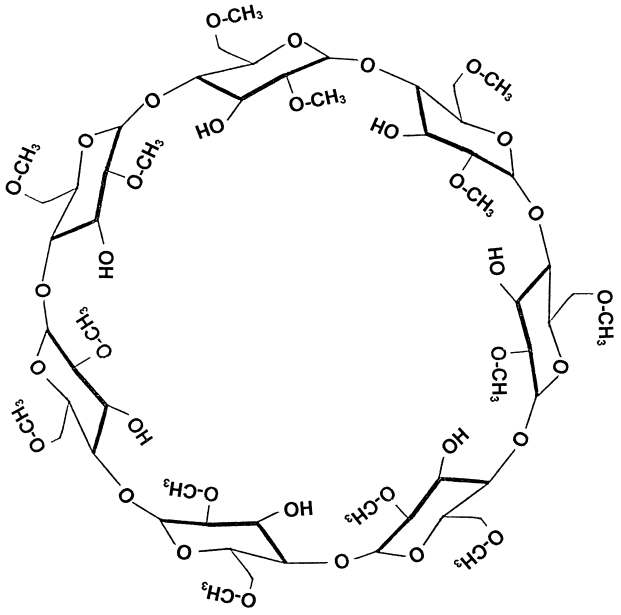

Figure 1. Structural formulae of methyl-β-cyclodextrin (MEBCD).

Cytotoxic assays

In order to determine the effect of various CDs on the cytotoxicity of DOX, CDDP, 5-FU and DXL, preconfluent cells from stock cultures of all parental sensitive and DOX-resistant cell lines were treated as follows. Cell lines derived from solid tumours MCF7 S, MCF7 R, A2780 S and A2780 R were detached with trypsin–EDTA (0.25–0.02%, w/v) in PBS, washed twice with PBS and resuspended in complete culture medium to obtain a single-cell suspension. Cells were counted and then seeded at a final density of 6×10^3 cells/well in 96-well microtitre plates in a final volume of 100 μl. The cells were then allowed to attach for 24 h at 37°C. For leukaemia-derived cell lines K562 S and K562 R, cells were simply resuspended in complete medium, counted and seeded at a density of 6×10^3 cells/well. 5-FU, CDDP, and DOX were reconstituted in purified water, while DXL was solubilised in a water/ethanol mixture (90:10; v/v). Cytotoxic drugs were diluted in culture medium and were added in various concentrations to wells, all cells were incubated in the presence or absence of CDs for 96 h. Stock solutions of GCD and MEBCD (0.1 M) were prepared in purified water while BCD was dissolved in absolute ethanol (0.5 M). Final concentrations of BCD and GCD (1000 μM), and MEBCD (100, 200, 500 and 1000 μM), were obtained by dilution of stock solutions with culture medium. These concentrations were chosen because they had no significant effect on cell viability during 96-h exposure (more than 95% of cell proliferation compared to controls; data not shown). The cytotoxicity of drugs in the CD-treated and CD-free cultures was quantified by the MTT assay [17–19]. Metabolic reduction of the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] leads to formation of MTT-formazan. MTT (20 μl of 5 mg/ml in sterile PBS) was added to each well and plates were incubated for 4 h at 37°C. Blue formazan crystals formed were dissolved in a mixture of isopropanol and hydrochloric acid 1 M (96-4; v/v). The plates were then gently agitated for 10 min and the absorbance measured at 570 nm on a microculture plate reader (Dynatech MR5000, France). The IC_{50} values were defined as the concentration of drug resulting in 50% survival of the treated cells compared with controls and were calculated using a program implemented on EXCEL 5.0 software. For each assay, three different experiments were performed in triplicate.

Determination of intracellular DOX kinetics

To assay intracellular DOX accumulation over time, cell DOX concentrations were determined by a high-performance liquid chromatography (HPLC) method with fluorescence detection as described previously [20]. Samples consisting of

1 ml of K562 S, MCF7 S, K562 R and MCF7 R cells suspensions (10^6/ml) were treated with DOX in the presence or absence of MEBCD (DOX concentration: 0.1 μmol for 10^6 cells; MEBCD concentrations: 2, 5, 10 μmol for 10^6 cells) and then incubated for different periods (30 min, 1, 2, 3, 4 and 6 h). After treatment, cells were centrifuged at 1000*g* and washed twice with cold PBS (pH 7.3). After a final centrifugation at 1000*g*, the supernatant was removed and daunorubicin was added as an internal standard. Cells were lysed by 2 ml of a chloroform–methanol mixture (4:1; v/v). After vigorous shaking and centrifugation, the organic phase was evaporated to dryness under nitrogen stream. The residue was reconstituted with 200 μl of mobile phase and 100 μl were injected into the column. Two experiments were carried out in triplicate and performed at laboratory temperature.

The chromatographic system consisted of a Shimadzu LC9-A (Tokyo, Japan) solvent pump, a 20 μl sample loop, a guard column, a Spherisorb ODS column (5 μm, 250 by 4.6 mm i.d.) and a Shimadzu RF 535 fluorimetric detector. The excitation and emission wavelengths were 478 and 590 nm, respectively.

Elution was performed using a mobile phase consisting of 0.05 M phosphate buffer, acetonitrile, tetrahydrofuran and triethylamine (59.8:35:5:0.2; v/v), pH 10.5, at a flow rate of 1 ml/min. Linear detection response was obtained for concentrations ranging from 5 to 2000 ng/ml in cell homogenate. The intra- and interday precision was lower than 10%. The limit of quantitation was 2 ng/ml.

Optimal schedule of cell treatment

To determine the role of chronology in the treatment of cells by either DOX or MEBCD, various schedules were performed in K562 S and K562 R cell lines. Aliquots containing a cell suspension in RPMI at a density of 1×10^6 cells/ml were treated for different durations, either simultaneously or successively, with DOX (0.1 μmol in 1 ml of RPMI containing 10^6 cells) and MEBCD (10 μmol for 10^6 cells). K562 S and K562 R intracellular DOX concentrations were then determined as described above.

Intracellular MEBCD determination

To verify the hypothetical cellular penetration of MEBCD, intracellular concentrations were determined by HPLC with fluorescence detection after *in situ* complexation with 1-naphthol [21]. Samples consisting of 50 ml of K562 S and K562 R cell suspensions (10^6 cells/ml) were treated with MEBCD in the presence or absence of DOX (MEBCD concentration: 10 μmol for 10^6 cells; DOX concentration: 0.1 μmol for 10^6 cells) and then incubated for different periods (1 and 4 h). After treatment, cells were centrifuged at 1500*g* for 10 min and washed twice with cold PBS (pH 7.3). After a final centrifugation at 1500*g* for 10 min, the supernatant was removed and daunorubicin was added as an internal standard. Cells were lysed by 5 ml of a chloroform–ethanol mixture (4:1; v/v). After vigorous shaking and centrifugation, the organic phase was evaporated to dryness under a nitrogen stream. The residue was reconstituted with 250 μl of mobile phase and filtered through a 0.45 μm filter (Millipore, Bedford, Massachusetts, U.S.A.) and then injected into the column. Experiments were carried out in triplicate ($n = 3$).

The chromatographic system consisted of a Shimadzu LC9-A (Tokyo, Japan) solvent pump, a 50 μl sample loop, a guard column, a stainless-steel analytical column (300 by

Table 1. *Effect of MEBCD on drug toxicity in various drug-sensitive and drug-resistant cancer cell lines*

Anticancer drug	Cell line	IC_{50}(M) ± S.D. ($n = 3$) Control	MEBCD-treated	Sensitisation ratio*	*P* values†
Doxorubicin	K562 S	$0.091 \pm 0.012\ 10^{-6}$	$0.03 \pm 0.006\ 10^{-6}$	3.3	<0.01
	K562 R	$481 \pm 0.7\ 10^{-6}$	$1.0 \pm 0.5\ 10^{-6}$	4.8	<0.01
	MCF7 S	$0.29 \pm 0.06\ 10^{-6}$	$0.093 \pm 0.006\ 10^{-6}$	3.1	<0.001
	MCF7 R	$31 \pm 5\ 10^{-6}$	$4.3 \pm 0.2\ 10^{-6}$	5.8	<0.01
	A2780 S	$0.78 \pm 0.12\ 10^{-6}$	$0.25 \pm 0.04\ 10^{-6}$	3.1	<0.01
	A2780 R	$5.6 \pm 0.3\ 10^{-6}$	1.6 ± 0.2 10-6	3.5	<0.001
Docetaxel	K562 S	$0.064 \pm 0.009\ 10^{-7}$	$0.013 \pm 0.003\ 10^{-7}$	4.9	<0.01
	K562 R	$0.28 \pm 0.04\ 10^{-7}$	$0.041 \pm 0.004\ 10^{-7}$	6.8	<0.01
	MCF7 S	$0.55 \pm 0.16\ 10^{-7}$	$0.11 \pm 0.05\ 10^{-7}$	5.0	<0.02
	MCF7 R	$89 \pm 21\ 10^{-7}$	$6.2 \pm 0.7\ 10^{-7}$	14.3	<0.01
	A2780 S	$0.37 \pm 0.07\ 10^{-7}$	$0.079 \pm 0.003\ 10^{-7}$	4.7	<0.01
	A2780 R	$42 \pm 8\ 10^{-7}$	$3.1 \pm 0.6\ 10^{-7}$	13.5	<0.01
5-Fluorouracil	K562 S	$0.87 \pm 0.11\ 10^{-5}$	$0.79 \pm 0.11\ 10^{-5}$	1.1	N.S.
	K562 R	$0.77 \pm 0.08\ 10^{-5}$	$0.72 \pm 0.08\ 10^{-5}$	1.1	N.S.
	MCF7 S	$7.3 \pm 0.9\ 10^{-5}$	$7.7 \pm 0.7\ 10^{-5}$	0.9	N.S.
	MCF7 R	$8.1 \pm 0.5\ 10^{-5}$	$7.5 \pm 0.9\ 10^{-5}$	1.1	N.S.
	A2780 S	$11 \pm 3\ 10^{-5}$	$9.0 \pm 0.6\ 10^{-5}$	0.9	N.S.
	A2780 R	$8.4 \pm 0.6\ 10^{-5}$	11 ± 10^{-5}	0.8	N.S.
Cisplatin	K562 S	$2.3 \pm 0.6\ 10^{-6}$	$1.6 \pm 0.2\ 10^{-6}$	1.6	N.S.
	K562 R	$1.8 \pm 0.2\ 10^{-6}$	$1.9 \pm 0.4\ 10^{-6}$	1.0	N.S.
	MCF7 S	$1.5 \pm 0.4\ 10^{-6}$	$1.1 \pm 0.3\ 10^{-6}$	1.4	N.S.
	MCF7 R	$0.25 \pm 0.06\ 10^{-6}$	$0.24 \pm 0.03\ 10^{-6}$	1.0	N.S.
	A2780 S	$3.1 \pm 0.7\ 10^{-6}$	$2.6 \pm 0.4\ 10^{-6}$	1.2	N.S.
	A2780 R	$2.8 \pm 0.6\ 10^{-6}$	$3.0 \pm 0.8\ 10^{-6}$	0.9	N.S.

*IC_{50} (untreated, control)/IC_{50} (MEBCD treated). †Student's *t*-test (N.S = not significant)—control versus MEBCD-treated.

7.5 mm I.D.) packed with exclusion gel TSK 3000 SW and a Shimadzu RF 535 fluorescence detector. The excitation and emission wavelengths were 290 and 360 nm, respectively. A Shimadzu C-R6A Chromatopac was used for integrating and recording chromatograms.

The eluent phase was constituted by a mixture of water and methanol (98-2, v/v) containing 10^{-4} M of 1-naphthol as a fluorophore. The flow rate was 1 ml/min (pressure 3 MPa). The linear detection response was obtained for concentrations ranging from 1 to 100 μM in cell homogenate. The intra- and interday precision were lower than 11%. The limit of quantitation was 0.5 μM.

Statistical analysis

Results were expressed as mean values ± standard deviations (S.D.). Student's *t*-test was used to compare the five concentrations of DOX and MEBCD–DOX inhibiting 50% of cell proliferation. A P value < 0.05 was considered significant.

RESULTS

Potentiation of DOX and DXL cytotoxicity by MEBCD

As previously described in HL-60 cells [12], concentrations of MEBCD < 1000 μM are not cytotoxic. In the present study, the effect of various CDs (BCD, GCD and MEBCD) at 1000 μM on the cytotoxicity of DOX was studied in K562 S and K562 R cell lines. Only MEBCD was able to improve significantly the cytotoxicity of DOX (data not shown). Other CDs used proved to be completely inactive. Therefore, to determine the relationship between MEBCD concentration and effect on DOX cytotoxicity, four non-cytotoxic concentrations of MEBCD (100, 200, 500 and 1000 μM) were tested. Only the concentrations of 500 and 1000 μM improved DOX antitumoral activity, whilst concentrations of 100 and 200 μM were inactive (data not shown).

Further experiments were performed with a MEBCD concentration of 1000 μM (for all cell lines, the cytotoxicity of MEBCD alone ranged from 94 ± 4.5% to 104 ± 7.8%). Higher concentrations tested appeared to be cytotoxic for all the cell lines employed (IC_{50} ranged from 1430 ± 124 μM to 1615±178 μM). Increments of concentrations of three other antineoplastic agents (DXL, 5-FU and CDDP) were tested in six cancer cell lines, in the presence or absence of MEBCD. Corresponding IC_{50} values are reported in Table 1. The data clearly show that in each cell line, DOX and DXL IC_{50} values were significantly decreased by co-treatment with MEBCD. For these two drugs, the sensitisation ratios (IC_{50} DOX treated/IC_{50} DOX–MEBCD treated) ranged from 3.1 to 14.3. Moreover, it was noticeable that in most cases, variant multidrug resistant sublines seemed to be more affected by the activity of MEBCD compared with their respective parental sensitive cell line, particularly for DXL. In contrast, no increase in cytotoxic activity was observed when cells were treated with 5-FU or CDDP in combination with MEBCD.

Enhancement of DOX intracellular accumulation

To explain the potentiation effect of MEBCD on DOX and DXL activity in all the cell lines studied, K562 S, K562 R, MCF7 S and MCF7 R intracellular accumulation of DOX, during exposure to DOX with or without MEBCD, was determined by HPLC. Results are presented in Figures 2 and 3. In K562 S cell line (Figure 2a), concentrations of MEBCD (5 and 10 μmol in 1 ml of RPMI containing 10^6 cells) resulted in a substantial enhancement of intracellular accumulation of DOX after 30 min of incubation. Indeed, after 1 h of exposure, cellular DOX accumulation was approximately 2-fold higher with MEBCD (10 μmol in 1 ml of cell suspension) than without MEBCD. Moreover, this phenomenon seemed to be durable since a similar ratio was observed after 6 h. In K562 R cell line (Figure 2b), the DOX intracellular concentration was approximately 2.5–3.5-fold higher in cells co-treated with DOX and MEBCD at 5 and 10 μmol for 10^6 cells of RPMI containing 10^6 cells compared with cells treated with DOX alone. However, no enhancement of DOX intracellular accumulation was observed with MEBCD at 1 μmol for 10^6 cells of cell suspension. In order to confirm this increase in DOX accumulation, assays on MCF7 S and MCF7 R cell lines were performed (Figure 3). Similarly, after 1 h drug exposure, mean intracellular DOX concentrations in MCF7 S and MCF7 R cells were significantly enhanced in cells treated with DOX and MEBCD–DOX compared with DOX alone. As shown in the K562 cell line, intracellular DOX enhancement caused by MEBCD was less obvious in the parental sensitive cell line than in its multidrug resistant subline (mean enhancement factors after 4 h of exposure: 1.7 and 2.7 for MCF7 S and MCF7 R cell lines, respectively).

Cellular effect of different drug treatment schedules

Determinations of K562 S and K562 R intracellular DOX

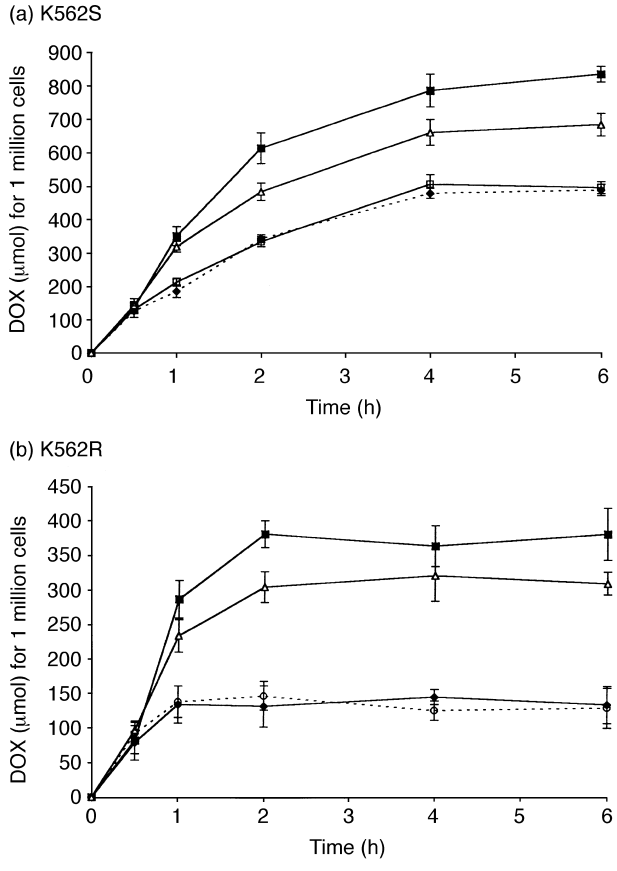

Figure 2. (a) and (b) Intracellular DOX accumulation in K562 S (a) and K562 R (b) cells, during treatment with DOX alone (◆) at 0.1 μmol in 1 ml of RPMI containing 10^6 cells or in combination with MEBCD at 10 (■), 5 (△) and 2 (□) μmol in 1 ml of RPMI containing 10^6 cells. Intracellular concentrations are expressed in nmol for 10^6 cells. Data are the mean of two experiments carried out in duplicate. Error bars represent S.D.

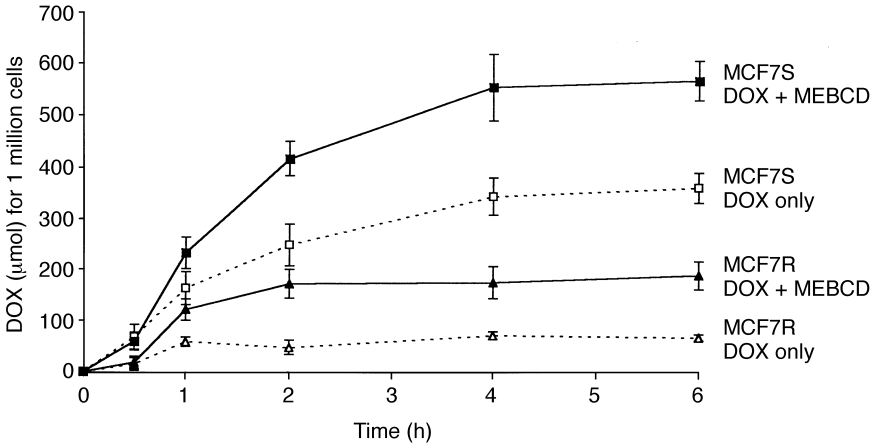

Figure 3. Intracellular DOX accumulation in MCF7 S and MCF7 R cells, during treatment with DOX alone at 0.1 μmol for 10^6 cells (□ and △) or in association with MEBCD at 10 μmol for 10^6 cells (■ and ▲). Intracellular concentrations are expressed in nmol for 10^6 cells. Data are the mean of two experiments carried out in duplicate. Error bars represent S.D.

concentrations after different schedules of treatment are illustrated in Figure 4. The optimal DOX accumulation was obtained when DOX and MEBCD were added simultaneously in the supernatant for 2 h (schedule 6 in Figure 4). However, the most remarkable aspect of these experiments is the fact that, after treating cells with MEBCD for 1 h, discarding the supernatant and then adding DOX for a further 1 h (schedule 3), intracellular DOX accumulation was drastically increased compared with the opposite schedule (schedule 2). Moreover, the effect of schedule 3 on intracellular DOX concentration was greater than simultaneous treatment with MEBCD and DOX for 1 h and very close to that of simultaneous treatment with MEBCD and DOX for 2 h. Similar results were obtained for both K562 S and K562 R cell lines.

Intracellular MEBCD determination

Intracellular MEBCD accumulation was investigated in K562 S and K562 R cells previously treated with MEBCD in the presence or absence of DOX. Whatever the drugs and the cell line employed, results of analysis on 50×10^6 cell samples showed no detectable MEBCD after cell lysis. At the retention time of MEBCD, chromatograms obtained after treatment with MEBCD at 10 μmol for 10^6 cells were identical to those obtained for blank cell lysate (data not shown).

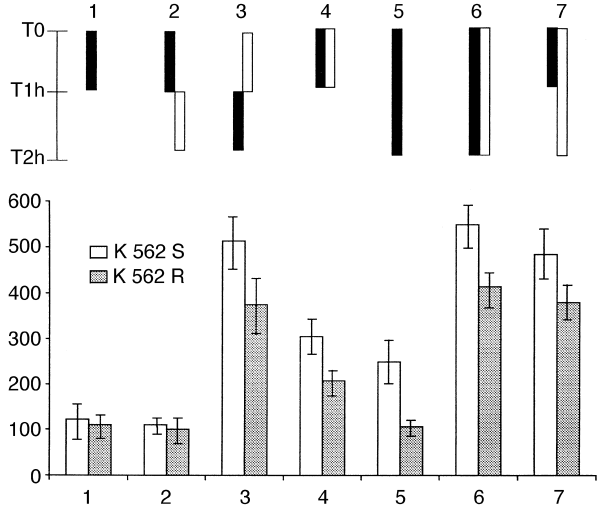

Figure 4. Intracellular DOX accumulation in K562 S (□) and K562 R (▨) cells. Schedules of treatment with DOX at 0.1 μmol for 10^6 cells (solid bars) and MEBCD at 10 μmol for 10^6 cells (open bars) are shown above the histogram. Intracellular DOX concentrations in nmol for 10^6 cells. Data are the mean of two experiments carried out in duplicate. Error bars represent S.D.

DISCUSSION

In cancer chemotherapy, there is a need to develop new therapeutic concepts to overcome current problems of drug resistance, high toxicity and lack of target specificity for many useful anticancer agents. One method to overcome drug resistance is to use modifiers (i.e. liposomes, nanoparticles, flavenoids, tunicamycin [22–26]), but another is to identify novel target sites that should not be affected by known resistant mechanisms.

Our results showed that after 96 h of exposure, at fixed non-cytotoxic concentrations of 1000 and 500 μM, MEBCD was able to potentiate significantly the growth inhibitory activity of DOX and DXL in the six cell lines used. The corresponding IC_{50} sensitisation ratios ranged from 3.1 to 14.3, indicating that the association of MEBCD with DOX or DXL could be more active than drugs alone. This phenomenon was not observed with 5-FU and CDDP, which could be explained by the lower molecular weight of 5-FU and CDDP compared with DOX and DXL; their different physicochemical properties; their different mechanisms of cellular transport uptake; and their affinity for the hydrophobic cavity of MEBCD; indeed, as previously described by Beckers and associates [27], the BCD cavity is not wide enough to form inclusion complexes with DOX, while 5-FU and CDDP are able to be incorporated in a BCD derivative cavity [28–30]. In all cell lines used, results of cytotoxic assays indicate that MEBCD increases the sensitivity to anticancer agents of resistant sublines to a greater extent than their respective parental controls, suggesting that the effect of MEBCD could be related to Pgp, a plasma membrane phosphoglycoprotein overexpressed in multidrug resistant cells [31]. Therefore, a relative selectivity on chemoresistant tumour cells could be expected.

Although the cholesterol content of the membranes in the cells under study either before or after treatment with

MEBCD was not measured, such a determination has already been performed by Kilsdonk and associates [7]. Moreover, it has been reported that cholesterol influences the membrane permeability of the phospholipid bilayer accompanied by a change in its fluidity. MEBCD recognises this lipid molecule in the membrane by virtue of the cavity size. The lowering of the cholesterol content of the membrane by MEBCD may induce perturbation of the lipid layer in the membrane, leading to an increased permeability in the transcellular route [7, 9–11, 14]. Different experiments have been carried out using methylated CDs to increase biological and cellular membranes' permeability to several molecules, such as calcitonin, insulin and PEG 4000 [11, 14–16]. Such a phenomenon has not been reported for non-methylated CDs [14–16]. In the present study, no significant increase of DOX cytotoxicity was found using BCD and GCD.

In order to elucidate the potentiation mechanism of the DOX cytotoxic activity by MEBCD observed in this study, we determined the intracellular accumulation kinetics of DOX when cells were treated with either MEBCD and DOX or DOX alone. In K562 S, K562 R, MCF7 S and MCF7 R cells, after 1 h of drug exposure, intracellular DOX concentrations were significantly increased when cells were treated with MEBCD and DOX compared with cells treated with DOX alone. Moreover, the effect of MEBCD was found to be dose-dependent in the range of 1–10 μmol for 10^6 cells. Thus, we confirmed that the MEBCD's ability to increase the inhibitory activity of DOX could be due, at least in part, to an increase of cellular drug incorporation. We also observed that in resistant sublines, this effect was higher than in their parental sensitive cell lines, confirming results obtained in the cytotoxic assays. In this context, Leibovici and associates [32] demonstrated that Pgp efficiency was related to the cholesterol content of the cell membrane. Consequently, we think that alteration in Pgp function and cell membrane permeabilisation, both due to a membrane cholesterol captation could be the main mechanisms implicated in the enhancement of DOX cellular uptake in multidrug resistant cells. Moreover, preliminary results carried out with verapamil, a known Pgp inhibitor, show that the increase of cellular DOX accumulation in the presence of MEBCD was at least equal to that obtained through co-incubation of cells with verapamil (data not shown).

Different schedules of treatment were tested. The optimal DOX accumulation was obtained when DOX and MEBCD were added simultaneously in the supernatant for 2 h. However, a similar DOX accumulation was obtained after treating cells with MEBCD, discarding the supernatant and then adding DOX. When the treatment of cells with DOX was followed by MEBCD, the results were similar to those observed in cells treated with DOX alone. These results suggest that the effect of MEBCD and DOX on cell membrane is independent, and that MEBCD could induce an increase in membrane permeability to further facilitate cellular DOX penetration. The non-detectable amount of MEBCD in cell lysate confirms that the proposed site for MEBCD activity is the cell membrane.

In conclusion, our data provided a biological basis for the potential therapeutic application of MEBCD in cancer therapy in combination with other conventional cytotoxic drugs. Although the mechanism by which MEBCD increases the *in vitro* cytotoxicity of DOX and DXL is still to be fully clarified, our results indicate that MEBCD could act by improving the permeability of the cell membrane to these two drugs, consequently enhancing intracellular drug concentration, leading to enhancement of their cytoplasmic and nuclear effects. Most anticancer agents have significant toxicity. The *in vivo* toxicity of CDs seems to be relatively low, but is correlated with the degree of methylation [33, 34]. The choice of MEBCD could be justified by a balance between toxicity, hydrosolubility and lipophilicity. The less toxic MEBCDs could permit the reduction of doses of common antitumour agents, so decreasing toxicity but without reducing efficacy. Moreover, the other remarkable aspect is the higher effect of MEBCD in multidrug resistant cell lines. The drugs for which MEBCD improved cytotoxicity were those recognised by Pgp, so we think that there is a relationship between drug entry and a form of circumvention of multidrug resistance. *In vivo* studies are needed to confirm these *in vitro* results.

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