Cancer Chemother Pharmacol (1997) 40: 489–494

ORIGINAL ARTICLE

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Methyl-β-cyclodextrin in HL-60 parental and multidrug-resistant cancer cell lines: effect on the cytotoxic activity and intracellular accumulation of doxorubicin

Received: 13 July 1996 / Accepted: 25 March 1997

Abstract The purpose of this work was to determine the role of methyl-β-cyclodextrin (MEBCD) in combination with doxorubicin (DOX) on the cellular proliferation of a sensitive parental and a multidrug-resistant human cancer cell line (HL-60 S and HL-60 R) and to study the effect of MEBCD on DOX intracellular accumulation. The cytotoxicity of DOX at five concentrations (50–50,000 n*M*) was evaluated with or without the coadministration of four fixed noncytotoxic concentrations of MEBCD (100, 200, 500, and 1,000 μ*M*). Intracellular DOX concentrations were determined by a high-performance liquid chromatography (HPLC) method with fluorescence detection. MEBCD applied at 500 and 1000 μ*M* in combination with doxorubicin (DOX) significantly potentiated the activity of DOX used alone on both sensitive and multidrug-resistant cell lines; 50% growth-inhibitory (IC_{50}) ratios (IC_{50} MEBCD-DOX/IC_{50} DOX) were about 3:4 and 1.6:4 for HL-60 S and HL-60 R, respectively. Moreover, intracellular DOX accumulation, determined by HPLC during 6 h of drug exposure, was about 2–4 times higher for cells treated with MEBCD in combination with DOX than in those treated with DOX alone. Similar results were obtained using other paired MCF 7 sensitive and resistant cell lines. Correlation between these results and an MEBCD-cell membrane interaction was discussed. These initial data provide a basis for the potential therapeutic application of MEBCD in cancer therapy.

Key words Methyl-β-cyclodextrin · HL-60 · Parental and multidrug-resistant human cancer cell lines · Doxorubicin · Cytotoxic activity · Intracellular concentration

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Introduction

Cyclodextrins (CDs) are oligosaccharide cyclic torus-shaped molecules that have the ability to form molecular inclusion compounds with a wide range of molecules. They have been used for more than 12 years in different applications such as protection against various types of degradation, solubilization in water, and extraction from a medium. Different studies have shown that the use of agents capable of forming inclusion structures with drugs can optimize some biopharmaceuticals in terms of the solubility, delivery, membrane permeability, and bioavailability of the therapeutic component. The recent development of new "host-guest" systems opened some interesting issues in several industrial and application fields [1, 3, 10, 13, 15, 18, 22, 24, 26].

Three different native CDs are known: α-CD, β-CD, and γ-CD (6, 7, and 8 D-glucose units, respectively). The inside of the molecule forms a hydrophobic cavity, whereas the outer surface is hydrophilic, enabling improvement of the solubilization of various drugs. These molecules differ mainly in their cavity size and in their solubility. A way to improve the solubility of CDs is available through derivatization of the hydroxyl groups via processes such as methylation, hydroxyethylation, hydroxypropylation, and acetylation [1, 22–24].

Several authors [6, 24] have shown that methylated CDs are more lipophilic than are non-substituted CDs and can interact with some lipid components of the cell membrane, which play structural roles, providing a matrix for functional membrane proteins and maintaining a permeability barrier between the external and internal environments. Moreover, an enhancement of the transport of many molecules was observed in the presence of β-CD, and increasing its degree of methylation seemed to improve the intracellular accumulation of molecules.

In cancer chemotherapy there is a need to develop new drugs. This may involve the identification and exploitation of novel molecular features of cancer cells. One possible new target may be the cell membrane, the potential site for methylated CDs' activity [6, 23]. Co-incubation of cells with doxorubicin (DOX), a known DNA-intercalating antitumoral agent [5, 16] and CDs, which interact with the cell membrane, might be an interesting concept to develop. The aim of the work described herein was to determine the effect of methyl-β-CD (MEBCD) combined with doxorubicin (MEBCD-DOX) on the cellular proliferation of HL-60 S and HL-60 R cells and to study the effect of MEBCD on DOX intracellular accumulation.

Materials and methods

Drugs and other chemicals

Doxorubicin hydrochloride was purchased from Pharmacia Laboratories (St. Quentin-Yvelines, France) and daunorubicin was purchased from Bellon Laboratories (Neuilly-sur-seine, France). Methyl-β-cyclodextrin (mol.wt. 1,285 g mol^{-1}; Fig. 1), tetrazolium dye (MTT), and phosphate-buffered saline (PBS) were obtained from Sigma (St. Quentin Fallavier, France). RPMI 1640 medium and fetal calf serum were obtained from Polylabo (Paris, France). Reagents used for the analytical part of this study were of high-performance liquid chromatography (HPLC) grade.

Cell culture

The human promyelocytic leukemia cell line HL-60 and the human breast carcinoma MCF 7 cell line were obtained from the American Type Culture Collection (Rockville, Md., USA). The doxorubicin-resistant sublines HL-60 R and MCF 7 R were established by the continuous exposure of cells to gradually increasing concentrations of daunorubicin and were maintained in medium supplemented with daunorubicin at 0.1 μg/ml. The multidrug resistance (MDR) phenotype expression of the HL-60 R and MCF 7 R cell lines was assessed by an immunohistochemistry method using the two P-glycoprotein-specific murine monoclonal antibodies C219 (Centocor, Malvern, Pa., USA) and JSB1 (Tebu, Le Perray-en-Yvelines, France). Cultures were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, antibiotics, and glutamine at 37 °C in a humidified atmosphere containing 5% CO_2. The viability of the cells was assessed by their ability to exclude 0.5% trypan blue dye. The cell density in culture flasks was determined by a Coulter counter (Model Z1, Hialeah, Fla., USA).

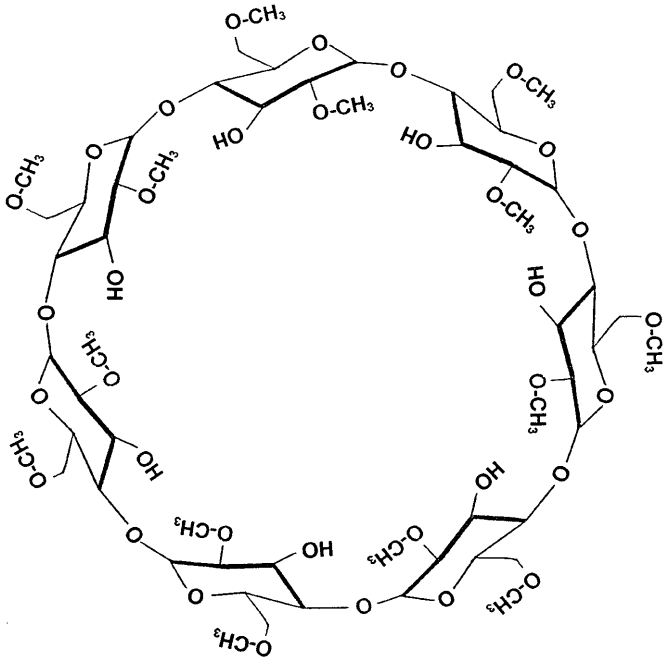

Fig. 1 Structure of methyl-β-cyclodextrin (MEBCD)

Cytotoxicity assays

Only exponentially growing, viable cells were used for the studies. In all experiments, parental sensitive and DOX-resistant HL-60 cells were seeded at a final density of 6,000 cells/well in 96-well microtiter plates and were treated with drugs (MEBCD, DOX, and MEBCD-DOX). First, the cytotoxicity of MEBCD at concentrations of 100, 200, 500, and 1,000 μ*M* was studied, and then the cytotoxicity of DOX at five concentrations (5, 50, 500, 5,000, and 50,000 n*M*) was evaluated in the presence or absence of concentrations of MEBCD (100, 200, 500, and 1,000 μM) for 96 h at 37 °C. After incubation, 20 μl of MTT solution in PBS (5 mg/ml) was added to each well and the wells were then exposed to 37 °C for 4 h. This colorimetric assay is based on the ability of live and metabolically unimpaired tumor-cell targets to reduce MTT to a blue formazan product [2, 21]. Then, 100 μl of a mixture of isopropanol and 1 *M* hydrochloric acid (96:4, v/v) was added to each well. Complete and homogeneous solubilization of the formazan crystals produced was achieved after 10 min of vigorous shaking. The absorbance was measured on a microculture plate reader (Dynatech MR5000, France) at 570 nm. The IC_{50} values were defined as the concentration of drug resulting in 50% survival of the treated cells as 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

In parallel, cell counting was performed as follows: in 24-well microtiter plates, HL-60 S and HL 60-R cells were seeded (20,000 cells/well) and were treated with DOX at various concentrations (10, 25, 50, 100, 200, 500, 1,000, 2,000, 5,000, 10,000, 20,000, and 50,000 n*M*) in the presence or absence of MEBCD at 1,000 μ*M*. After 96 h of incubation at 37 °C the number of cells (size ≥8 μm) contained in each well was determined by the electronic counter. The IC_{50} values were defined as described above. Each assay was performed in triplicate.

Determination of intracellular DOX concentrations

Intracellular DOX concentrations were determined by an HPLC method with fluorimetric detection as described elsewhere [17]. Samples containing 10^6 HL-60 sensitive and DOX-resistant cells in 1 ml of RPMI medium were treated with MEBCD-DOX or DOX alone (DOX concentration 0.1 μmol/ml; MEBCD concentrations 2, 5, and 10 μmol/ml). The volumes added were always smaller than or equal to 1% of the total volume of the samples such that the integrity of the medium was maintained. Cells were then incubated for different periods (30 min, 1 h, 2 h, 3 h, 4 h, and 6 h).

Samples (1 ml) containing 10^6 cells of MCF 7 S and MCF 7 R in suspension in RPMI medium were treated with DOX at 0.1 μmol/ml in the presence or absence of MEBCD at 10 μmol/ml and were then incubated for 5 h at 37 °C. After treatment, HL-60 and MCF 7 cell samples were centrifuged at 1,000 *g* for 10 min and the pellets containing 10^6 cells were washed twice with cold PBS (pH 7.3). After a final centrifugation at 1,000 *g* for 10 min the supernatant was removed and daunorubicin was added as an

internal standard. Cells (10^6) 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 a nitrogen stream. The residue was reconstituted with 200 µl of mobile phase and injected into the column.

The chromatography 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 × 4.6 mm inside diameter), and a Shimadzu RF 535 fluorimetric detector. The excitation and emission wavelengths were 478 and 590 nm, respectively. A Shimadzu C-R6A Chromatopac was used for the integration and recording of chromatograms. Two experiments were carried out in duplicate and performed at laboratory temperature.

The elution step was performed using a mobile phase consisting of 0.05 *M* phosphate buffer, acetonitrile, tetrahydrofuran, and triethylamine (59.8:35:5:0.2, by vol.) at pH 10.5 and a flow rate of 1 ml min^{-1}. A linear detection response was obtained for concentrations ranging from 2.5 to 1,000 nmol in 1 ml of cell homogenate. The intra- and interday variation was lower than 10%. The limit of quantitation was 1 nmol in 1 ml of cell homogenate. Results are expressed in the text as the amount of DOX for 10^6 cells.

Statistical analysis

Results were expressed as mean values ± SD. Student's *t*-test was used to compare cell proliferation-inhibiting effect of the five DOX and MEBCD-DOX concentrations and the corresponding IC_{50} values. A threshold value of $P < 0.05$ was considered as significant.

Results

Sensitivity to MEBCD coadministration with DOX

After 96 h of exposure the effect of MEBCD applied at 100, 200, 500, and 1,000 µ*M* in combination with DOX on cell proliferation was assessed and compared with DOX antitumoral activity. The results are shown in Fig. 2. MEBCD at 500 and 1,000 µ*M* significantly potentiated the growth-inhibitory action of DOX at some of the five DOX concentrations studied, whereas no significant increase in cytotoxic effect was observed for MEBCD at 100 and 200 µ*M*. In no experiment was a cytotoxic effect found for MEBCD applied alone at concentrations ranging from 100 to 1,000 µ*M* (mean percentage of cell survival 97% ± 5%). Similar results were obtained on both sensitive and resistant cell lines.

Mean IC_{50} values are reported in Table 1. For MEBCD concentrations of 500 and 1000 µ*M* the

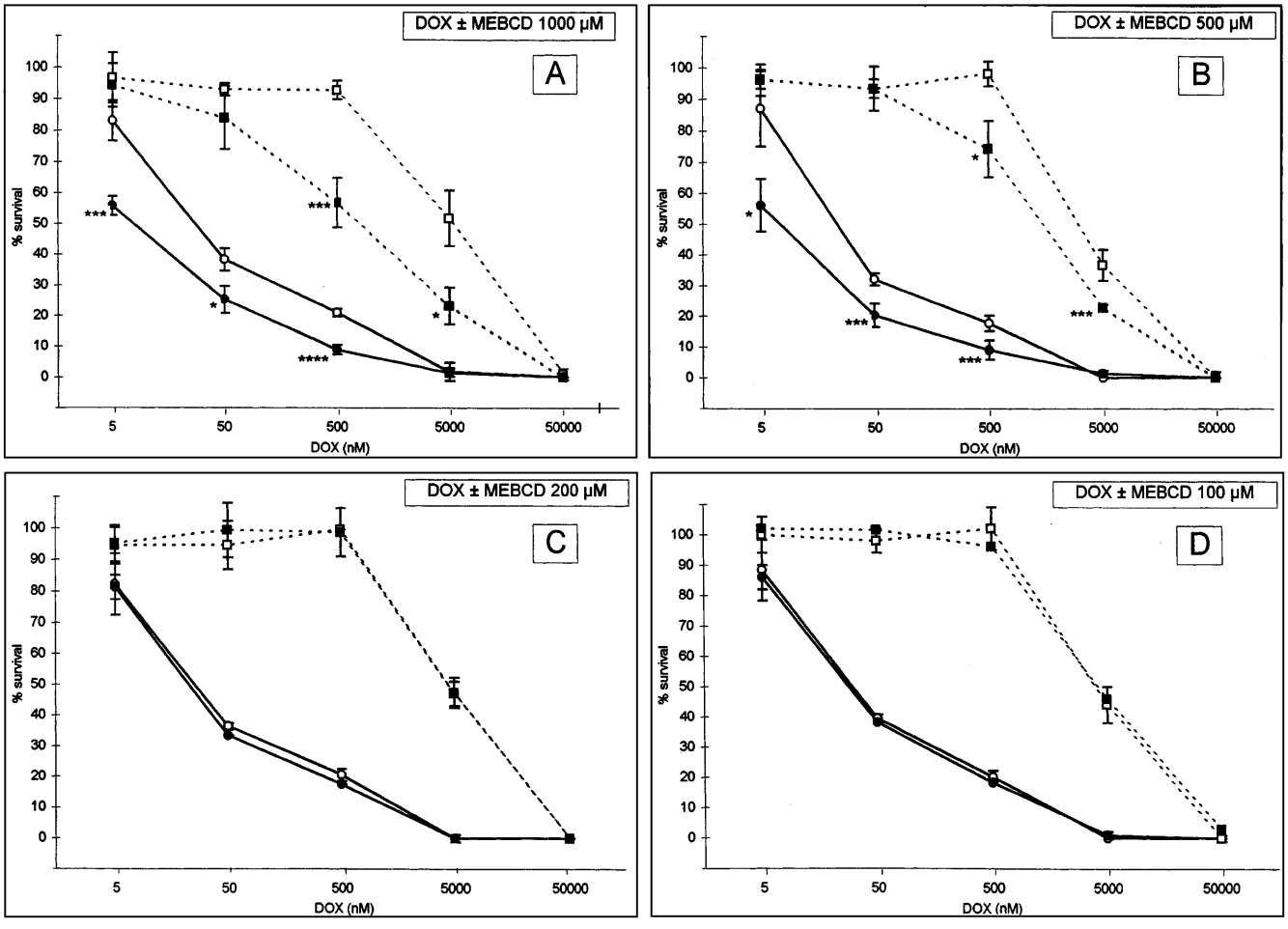

Fig. 2A–D Survival of HL-60 S (*circles*) and HL-60 R (*squares*) cells treated with DOX alone (○, □) or in combination with **A** 1,000, **B** 500, **C** 200, and **D** 100 µ*M* MEBCD (●, ■). Each *point* represents the mean value for 3 different experiments performed in triplicate; *error bars* represent SDs. $^{*}P < 0.05$; $^{**}P < 0.02$; $^{***}P < 0.01$; $^{****}P < 0.001$ (Student's *t*-test)

Table 1 Effect of various concentrations of MEBCD on DOX IC_{50} values obtained in HL-60 S and HL-60 R cells by MTT assay (*NS* Not significant)

Cell line	MEBCD (μM)	Mean IC_{50}values ± SD (nM)		*t*-Test	IC_{50} DOX/IC_{50} MEBCD-DOX
		DOX	MEBCD-DOX		
HL-60 S	1,000	38 ± 3.28	12.82 ± 4.09	$P < 0.001$	2.96
	500	34.7 ± 4.3	11.29 ± 9.54	$P < 0.01$	3.07
	200	38.6 ± 0.65	38.45 ± 1.32	NS	1.00
	100	36.0 ± 2.2	36.2 ± 1.1	NS	0.99
HL-60 R	1,000	5,959 ± 1,481	1,323 ± 501	$P < 0.02$	4.02
	500	4,033 ± 301	2,528 ± 333	$P < 0.01$	1.64
	200	5,398 ± 1,138	5,340 ± 1,222	NS	1.01
	100	5,562 ± 696	5,480 ± 895	NS	1.01

corresponding IC_{50} ratios (IC_{50} DOX / IC_{50} MEBCD-DOX) were about 3 and 1.6–4 for HL-60 S and HL-60 R cells, respectively.

Cell-counting assays performed in triplicate with 12 increments of DOX concentrations (10–50,000 nM) in the presence or absence of MEBCD (1,000 μM, the concentration producing optimal enhancement of the DOX cytotoxic effect in MTT assays) resulted in similar observations. In the HL-60 S cell line, IC_{50} values were 28 ± 4.5 and 11 ± 3.7 nM for DOX and MEBCD-DOX, respectively (sensitization ratio 2.54, $P < 0.01$). In the HL 60 R cell line, IC_{50} values were 3,842 ± 352 and 1,691 ± 367 nM for DOX and MEBCD-DOX, respectively (sensitization ratio 2.27, $P < 0.01$). In both cell lines, cell proliferation in MEBCD-treated controls was always higher than 95%.

Enhancement of intracellular DOX accumulation

For elucidation of the synergistic effect of MEBCD on DOX activity in HL-60 cells the intracellular accumulation of DOX during exposure to MEBCD-DOX and DOX was determined by HPLC. The results are presented in Fig. 3. Increasing concentrations of MEBCD (1–10 μmol in 1 ml of RPMI containing 10^6 cells) substantially increased HL-60 S intracellular concentrations of DOX after 30 min of incubation. Indeed, after 1 h of exposure the cellular DOX accumulation was about 2 times 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 the HL-60 R cell line the DOX intracellular concentration was 3–4 times higher when MEBCD was used at concentrations of 5 and 10 μmol/ml of RPMI containing 10^6 cells than when DOX was applied alone. However, no enhancement of DOX intracellular accumulation was observed for MEBCD at 1 μmol/ml of cell suspension. For confirmation of this increase in DOX accumulation, assays on MCF 7 S and MCF 7 R cell lines were performed. After 5 h of drug exposure (the time at which the intracellular steady-state DOX concentration occurred in HL-60 cell lines), mean intracellular DOX concentrations measured in MCF 7 S cells were 331 ± 36 and 564 ± 71 nmol for 10^6 cells after treatment with DOX and MEBCD-DOX, respectively (enhancement factor 1.7). In MCF 7 R cells these concentrations were 69.5 ± 7 and 188.25 ± 28 nmol for 10^6 cells, respectively (enhancement factor 2.7).

Discussion

In cancer chemotherapy there is a need for the development of new drugs to overcome current problems of drug resistance, high-grade toxicity, and lack of target specificity for many useful anticancer agents. One way to overcome drug resistance is to use modifiers, but another is to identify novel target sites that should not be affected by known resistance mechanisms. This way involves the identification and exploitation of novel molecular features of cancer cells. The aim of this study was to investigate the potential anticancer activity of MEBCD applied alone or in combination with DOX, a well-known ubiquitous antitumoral agent, in both HL-60 human leukemia parental sensitive and multidrug-resistant cell lines.

Our results showed for the first time that after 96 h of exposure at fixed noncytotoxic concentrations of 500 and 1,000 μM, MEBCD was capable of potentiating significantly the growth-inhibitory activity of DOX in both parental and multidrug-resistant HL-60 cell lines. For these two active concentrations, the corresponding IC_{50} ratios (IC_{50} DOX/IC_{50} MEBCD-DOX) were about 3 and 1.6–4 for HL-60 S and HL-60 R cells, respectively, indicating that MEBCD-DOX should be more active than DOX alone. Similar results were obtained using MTT and cell-counting assays. However, two in vitro studies using other CDs in cancer research (one using an organic synthesized compound of γ-CD conjugated with DOX [25] and the other using a platinum(II) derivative complex of β-cyclodextrin [8]) demonstrated no significant enhancement of cytotoxic activity.

To try to elucidate a part of the implicated mechanism, we determined the intracellular accumulation of DOX when cells were treated with either MEBCD-DOX or DOX alone. After 1–6 h of exposure we found a 2-fold increase in intracellular DOX accumulation when sensitive HL-60 cells were treated with MEBCD-DOX as compared with DOX alone (MEBCD concentrations 5 and 10 μmol in 1 ml of RPMI containing 10^6 cells). In HL-60 R cells this ratio reached 4 when MEBCD was

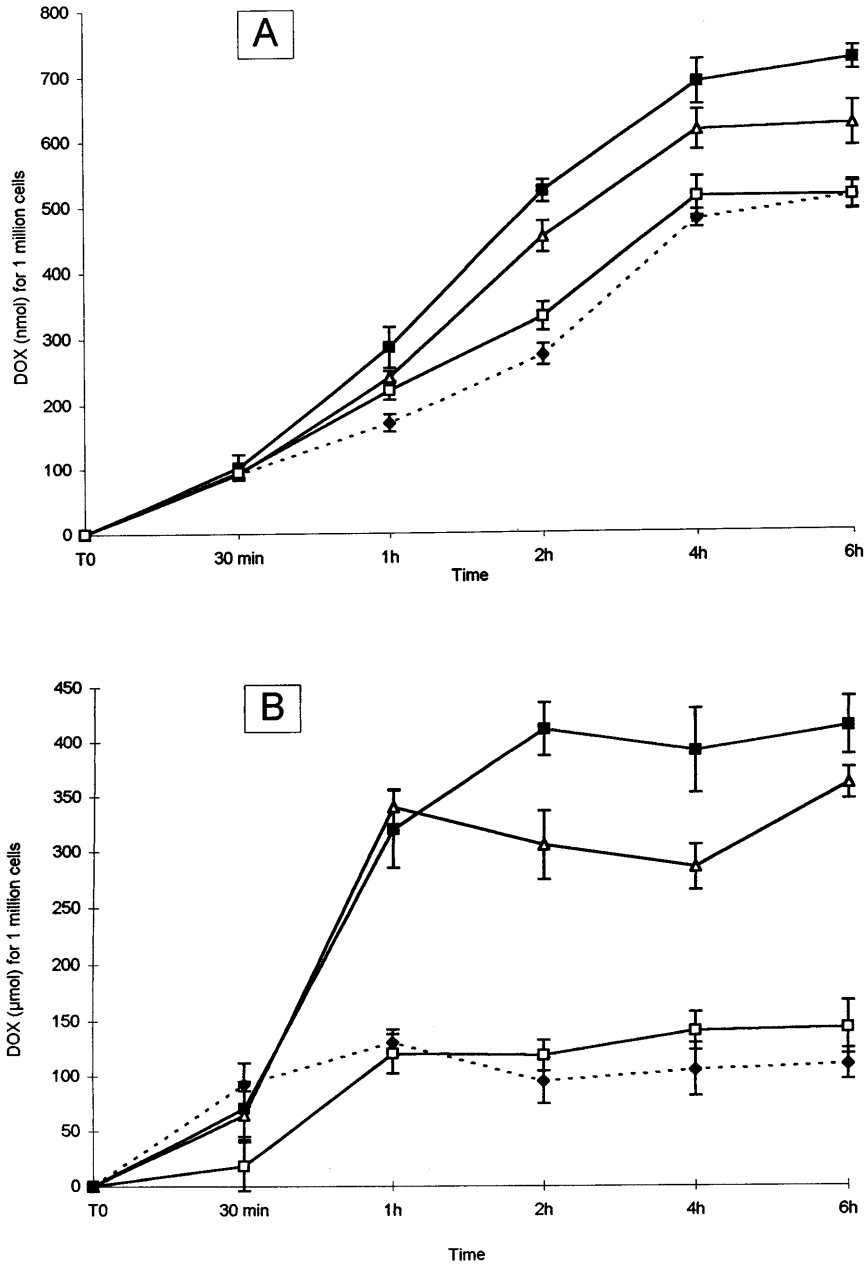

Fig. 3A,B Intracellular DOX accumulation in **A** HL-60 S and **B** HL-60 R cells as determined during treatment with DOX alone at 0.1 µmol in 1 ml of RPMI containing 10^6 cells (◆) or in combination with ME-BCD at 10 (■), 5 (△), and 2 (□) µmol in 1 ml of RPMI containing 10^6 cells. Intracellular concentrations are expressed in nmol for 10^6 cells. Data represent the mean values for 2 experiments carried out in duplicate; *error bars* represent SDs

used at 10 µmol for 10^6 cells. Thus, we confirmed that the effect of MEBCD on the higher cell-growth-inhibitory activity of DOX shown previously could be due at least in part to an increase in cellular drug incorporation. It is noteworthy that after 6 h of MEBCD-DOX exposure the intracellular DOX concentration was always about 2 times higher than that attained with DOX alone. Moreover, the increase in intracellular DOX accumulation due to MEBCD (at 5 and 10 µmol for 10^6 cells) observed in the HL-60 R cell line seemed to be more important than that seen in the sensitive parental cell line, indicating that DOX efflux due to the activity of membranous P-glycoprotein expressed in multidrug-resistant cells could be partially reduced by combination of the former drug with MEBCD. Similar results were obtained in the MCF 7 R cell line, where enhancement of DOX cellular uptake reached by the drug's combination with MEBCD was higher than that observed in the sensitive parental cell line. This phenomenon could be partially explained by the relationship between P-glycoprotein functionality and the cell-membrane cholesterol content [14], which could lead to a greater sensitivity of multidrug-resistant cells. Therefore, a relative selectivity for resistant tumor cells could be expected.

Since methylated β-CDs have been shown to enhance in vitro cell-membrane permeability [6, 24], it would be reasonable to think that it could be one of the mechanisms resulting in an increase in drug transmembrane transport and, hence, in a greater cytotoxic effect. A change in membrane fluidity had previously been

achieved with multidrug-resistance modulators such as antiestrogens [7] which can interact with membrane phospholipids, altering the lipid packing density and the diffusion rate of various drugs.

In vivo studies on CD drug-encapsulating complexes' behavior after intravenous injection [4, 10, 15] demonstrated that complexes were almost immediately dissociated owing to the competition between CD-cavity and plasmatic components. As the cavity size of β-CD is not wide enough to include any part of the DOX molecule [3], MEBCD should never incorporate DOX and, hence should act alone, in contrast to drug carriers such as liposomes or nanoparticles [11, 13, 19]. To test this point, studies on the influence of the scheduling of MEBCD and DOX in HL-60 S and HL-60-R cell lines are actually being carried out in our laboratory. Preliminary results show that after preincubation of cells with MEBCD followed by washing and treatment with DOX an increase in cytotoxic activity is observed that is similar to that seen in cells treated simultaneously with MEBCD and DOX.

DOX, like almost all anticancer agents, presents a large and problematic toxicity. The in vivo toxicity of CDs seems to be relatively low but has been correlated with the degree of methylation [4, 6]. Hence, the choice of MEBCD could be justified by a careful balance between toxicity, hydrosolubility, and lipophilicity. Reduction of the doses of common antitumoral agents so as to decrease toxicity without compromising efficacy, in combination with new, less toxic molecules such as quercetin [20] or MEBCD, could offer new perspectives in cancer treatment. In vivo assays are actually being performed to confirm our in vitro results in mice. In conclusion, our data provide a biological basis for the potential therapeutic application of MEBCD in cancer therapy, either alone or in combination with other conventional cytotoxic drugs.

Acknowledgement This study was supported by the Ligue Nationale contre le Cancer-comité de l' Ardèche.

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