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Antiproliferative Activity of Thermosensitive Liposome-encapsulated Doxorubicin Combined with 43°C Hyperthermia in Sensitive and Multidrug-resistant MCF-7 Cells

Jean-Louis Merlin, Sophie Marchal, Carole Ramacci, Dominique Notter and Claude Vigneron

Thermosensitive liposome-encapsulated doxorubicin (TLED) was compared to free doxorubicin, at 37°C or combined with 43°C hyperthermia, in sensitive and multidrug-resistant MCF-7 human tumour cells using clonogenic assays. In the resistant subline, TLED was found to partly circumvent multidrug resistance (MDR). The reversal was comparable to that obtained when verapamil was added to free doxorubicin. When hyperthermic treatment was applied, no difference in thermosensitivity was found between sensitive and resistant cells. The combination of hyperthermia with free doxorubicin did not reverse MDR. Hyperthermia and TLED yielded additive effects in the resistant cells while potentiation was observed in the sensitive cells. These results confirmed the usefulness of the liposome encapsulation of doxorubicin in reversing MDR. The possibility of obtaining additive cytotoxicity using TLED combined with hyperthermia may represent an alternative way of intensification of doxorubicin cytotoxicity concomitant with the circumvention of MDR without using MDR reversing agents, which often generate limiting toxic side-effects.
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INTRODUCTION

BECAUSE OF its implication in the failure of some chemotherapeutic treatments, drug resistance has been largely studied in the last two decades [1–4] and is now known to occur through different mechanisms, including the increase in activity of enzymes such as glutathione-S-transferase [5, 6], glutathione peroxidase [7, 8], superoxide dismutase [7], as well as the alteration of topoisomerase II activity [9, 10] or hexose phosphate metabolism [11]. Beside all these mechanisms of resistance, the so-called multidrug resistance (MDR) phenotype involves the overproduction of a 170 kD transmembrane glycoprotein called P-glycoprotein (Pgp) which works as a drug extruding pump and which is associated with the overexpression of *mdr*1 gene [4]. The MDR phenotype is found to induce resistance to several unrelated compounds including anthracyclines, vinca alkaloids, epipodophylotoxins and dactinomycin, as well as mitoxantrone and taxol derivatives [4]. Pgp is detected

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not only in resistant cells but also in normal cells of secreting organs such as the colon, kidney, pancreas and liver [4], suggesting a role of Pgp in the normal secretion of biological compounds.

Clinically, it is obviously most important to aim to detect MDR in tumour samples, in order to prevent chemotherapeutic treatment failures [12] in proposing drugs which are not subject to MDR or in using substances able to circumvent the resistance.

Many drugs have proved capable of reversing MDR including calcium channel blockers like verapamil, diltiazem or nicardipidine [13, 14], calmodulin inhibitors like quinine, quinidine or reserpine [15], as well as other molecules such as tamoxifen and cyclosporin A or its non-immunosuppressive derivatives [16, 17]. Although *in vitro* or *in vivo* preclinical studies yielded encouraging results, the clinical reversal of resistance by such agents has been impaired mainly because of the suprapharmacological concentrations which were needed to achieve significant reversal of resistance [18] and because of the toxic side-effects that arose using high doses of the reversing compounds. New molecules such as the triazinoamino-piperidine S9788 [19, 20] are actually proposed and studied to circumvent MDR with a better efficiency to side-effects ratio.

An alternative way to circumvent MDR has been investigated using liposomes [21–26] already evaluated in phase I and II clinical trials [27–29] and reported to decrease the cardiotoxicity [27] of doxorubicin.

As we recently reported that thermosensitive doxorubicin liposomes could be prepared [30] which potentiate the *in vitro* antitumour activity of doxorubicin when used in combination with 43°C hyperthermia [31], this study was designed to evaluate the potential usefulness of this combination on MCF-7 human breast adenocarcinoma cells expressing the MDR phenotype.

MATERIALS AND METHODS

Liposome preparation

Doxorubicin was obtained from Laboratoires Roger Bellon (Neuilly, France), and the lipids were purchased from Sigma (St Louis, Missouri, U.S.A.) as products of the highest available purity (99%).

Liposomes were prepared according to the procedure we previously reported [31]. Briefly, dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidyl choline (DSPC) and cholesterol (CHOL) were mixed (5:4:2 molar ratio) in chloroform and the organic solvent was evaporated using a rotary evaporator. The dried lipid film was then rehydrated at 50°C with 1 ml pH 7.4 phosphate-buffered saline (PBS) containing 2 μmol doxorubicin to reach a final lipid concentration of 35 μmol/l. The suspension was sonicated for 15 min using a 20 kHz, 500 W probe sonicator (Sonics and Materials, Plainview, New Jersey, U.S.A.) then gel filtered on Sephadex G75 (Pharmacia, Uppsala, Sweden) to separate the non-encapsulated doxorubicin and sterilised by filtration through 0.22 μm polycarbonate membrane (Nuclepore, Cambridge, Massachusetts, U.S.A.). Size analysis was performed by photon correlation spectroscopy on Coulter N4 (Coultronics, Margency, France) and it was ensured that the liposome suspension contained more than 95% of vesicles with a diameter less than 50 nm. Doxorubicin concentrations were determined spectrometrically at 480 nm after addition of sodium deoxycholate.

The release kinetics at 37 and 43°C reported in our previous paper [31] showed that doxorubicin was maximally released out of the liposomes within 30 min at 43°C while the vesicles remained quite stable at 37°C with a differential thermal stability (percentage of doxorubicin released at 43°C minus percentage released at 37°C) approximating 40%.

Cell culture conditions

All culture media and additives were purchased from Biochrom (Berlin, F.R.G.) and culture materials from Falcon (Meylan, France). Stock cultures of the MCF-7 breast adenocarcinoma cell line and its resistant subline MCF-7^R were obtained from Dr J. Robert (Fondation Bergonié, Bordeaux, France) with permission of Dr K.H. Cowan (NCI, Bethesda, Maryland, U.S.A.) who originated the resistant subline [32]. Both cell lines were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum at 37°C in 95% air/5% CO_2 atmosphere.

Clonogenic assays

Aliquots of free doxorubicin or thermosensitive liposome-encapsulated doxorubicin (TLED) were diluted in culture medium and added in the dishes containing monolayered cells which were incubated for 2 h at 37°C. The dishes were then washed twice with pH 7.2, 150 mmol/l NaCl PBS in order to remove the non-internalised drug.

Hyperthermia was applied after drug exposure by incubating the dishes for 30 min in a water bath. The dishes were washed twice with PBS before being assayed for colony formation. The temperature was monitored by immersing a thermocouple in the culture medium and regulating it to 43 ± 0.1°C.

Clonogenic assays were performed using a double layer soft agar technique [33] adapted from Hamburger and Salmon [34]. One thousand cells were incorporated in 0.3% agar containing supplemented RPMI 1640 culture medium and plated onto 0.5% agar-coated 35-mm dishes. Triplicated dishes were incubated for 14 days at 37°C, then colonies exceeding 100 μm in diameter (> 50 cells) were scored. When combinations of treatments were used, the results were analysed according to Steel and Peckham [35] after drafting of isobolograms (iso-effect plots) from the dose–response curves obtained with each treatment used alone. This allows the definition of an envelope of theoretical additivity. If the results obtained with the combination of treatment lies within this envelop, the effect is additive. Above, the effect is antagonistic (subadditive); below it is synergistic (supra-additive).

In MCF-7^R cells, verapamil (Laboratories Biosedra, Malakoff) was used at 15 μmol/l as the standard reversing agent and the reversing activity was evaluated as % cell kill in resistant cell line/% cell kill in sensitive cell line with free doxorubicin under comparable thermal conditions. Results were statistically analysed using Student's *t*-test.

RESULTS

Thermosensitivity

Both cell lines (MCF-7 and MCF-7^R) were first tested for thermosensitivity at 43°C for 30 to 120 min. Results, expressed as percentage of surviving cells in 43°C-treated dishes, revealed an exponential decrease in survival with the duration of hyperthermia (Fig. 1) with no significant difference between MCF-7 and MCF-7^R cells.

Thirty-minute incubation was selected to be used in combination with doxorubicin, yielding 48% (standard error 6) and 37% (5) survival rates, respectively, for MCF-7 and MCF-7^R cells.

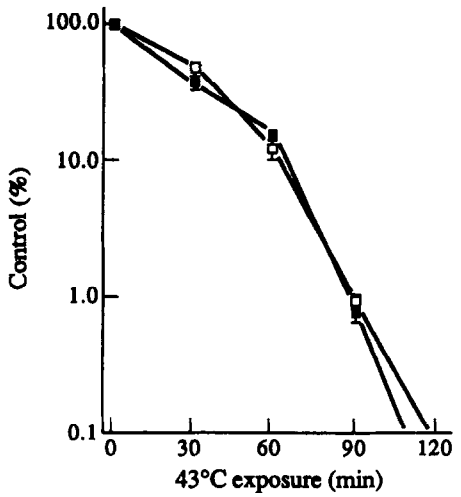

Fig. 1. Thermosensitivity of parental sensitive (□) and multidrug-resistant (■) MCF-7 cells exposed to 43°C hyperthermia. Results are mean percentages of survival obtained from three colony-forming assays performed in triplicate, bars are standard errors. Thirty-minute exposure was selected to be combined with thermosensitive liposome-encapsulated doxorubicin.

Sensitivity to doxorubicin

The sensitivity to doxorubicin was then evaluated for the two cell lines and it was confirmed that MCF-7^R cells were highly resistant to doxorubicin. From the dose–response curve of MCF-7 cells exposed to concentrations ranging between 0.01 and 1 μmol/l (Fig. 2), three doses were selected to be combined with hyperthermic treatment: 0.05, 0.15 and 0.35 μmol/l giving, respectively, 79 (6), 48 (3) and 22% (2) survival at 37°C in MCF-7 cells, while no cytotoxic activity was noted within this range of concentrations in MCF-7^R.

Verapamil (15 μmol/l), as well as empty liposomes, was not found to induce any cytotoxicity when administered alone to the cultured cells (data not shown).

No significant difference was observed between free doxorubicin and TLED for MCF-7 cells (Table 1). On the other hand, TLED appeared to significantly ($P<0.05$) circumvent the resistance of the MCF-7^R cells for both 0.15 and 0.35 μmol/l concentrations, reaching levels of cytotoxicity which were comparable to those achieved with free doxorubicin combined with 15 μmol/l verapamil.

The addition of verapamil did not significantly ($P>0.05$) enhance the reversing activity of TLED (Table 2).

The experiments described above were reproduced in order to test the effect of doxorubicin exposure combined with 30-min 43°C hyperthermia and the results showed a decrease in survival in all cases (Table 1). Hyperthermia was found to significantly

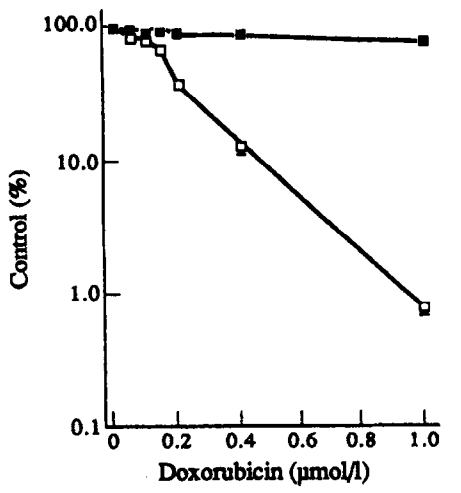

Fig. 2. Dose–response curve of parental sensitive (□) and multidrug-resistant (■) MCF-7 cells exposed to free doxorubicin at 37°C. Results are mean percentages of survival obtained from three colony-forming assays performed in triplicate, bars are standard errors. Doses leading to approximately 80, 50 and 20% survival were selected to be used in combination with 43°C hyperthermia.

Table 1. Cytotoxicity of the combination of free or thermosensitive liposome-encapsulated doxorubicin with 43°C hyperthermia on parental sensitive or multidrug-resistant MCF-7 cells evaluated by colony-forming assays

		Doxorubicin (μmol/l)			
		Control	0.05	0.15	0.35
Sensitive MCF-7 cells					
37°C	Free DXR	100	79	48	22
	TLED	98	86	52	24
43°C	Free DXR	43	16 (22–27)	10 (16–18)	1 (4–4)
	TLED	46	18 (21–28)	9 (15–18)	1 (4–5)
Multidrug-resistant MCF-7 cells					
37°C	Free DXR	100	104	95	99
	Free DXR + VPL	102	84	71	41
	TLED	104	82	68	38
	TLED + VPL	98	76	63	31
43°C	Free DXR	38	37	36	39
	Free DXR + VPL	42	27 (30–35)	24 (22–27)	12 (10–15)
	TLED	40	29 (32–32)	21 (22–24	8 (10–14)
	TLED + VPL	42	28 (27–30)	23 (19–20)	10 (7–9)

Results are mean percentages of survival obtained from at least three experiments in triplicate, S.E. are not presented but were always less than 15%. The figures in parentheses are the limits of the envelope of additivity of the two cytotoxic treatments as determined from isobolograms. DXR, doxorubicin; TLED, thermosensitive liposome-encapsulated doxorubicin; VPL, verapamil.

Table 2. Evaluation of the reversing activity of verapamil (VPL) or/and thermosensitive liposome-encapsulated doxorubicin (TLED) in multidrug-resistant MCF-7 cells

		Doxorubicin (μmol/l)		
		0.05	0.15	0.35
37°C	VPL	76 (9)	56 (7)	75 (7)
	TLED	86 (9)	62 (9)	79 (10)
	TLED + VPL	114 (10)	71 (9)	88 (8)
43°C	VPL	87 (9)	84 (8)	89 (9)
	TLED	85 (7)	88 (10)	93 (11)
	TLED + VPL	86 (11)	86 (7)	91 (9)

Results are mean percentages (S.E.) of reversing activity calculated for each experiment as % cell kill in resistant cell line /% cell kill in sensitive cell line with free doxorubicin under comparable thermal conditions.

potentiate (supra-additive effect) the cytotoxicity of free doxorubicin in the parental cell line (Table 1) exposed to 0.05 and 0.15 μmol/l doxorubicin ($P<0.01$). For 0.35 μmol/l, no significant ($P>0.05$) potentiation was found but there was a trend ($P<0.10$). No significant difference ($P>0.05$) was found between free doxorubicin and TLED. No significant effect ($P>0.05$) of hyperthermia was noted on the sensitivity of the resistant cells to free doxorubicin.

No synergistic but additive effects were observed with the combination of TLED and hyperthermia on MCF-7^R cells. In all these cases, addition of verapamil did not induce any further reversal of MDR.

On another hand, the results obtained with TLED, alone or combined with verapamil, confirmed the ability of liposome encapsulation to reverse MDR (Table 2). The addition of verapamil to TLED did not induce any significant additional circumvention of MDR ($P>0.05$), no difference was evidenced between 37 and 43°C, further indicating that hyperthermia alone did not reverse the MDR phenotype.

DISCUSSION

Drug resistance is becoming a major field of research in oncology since its contribution in chemotherapy failure has been established. Among the several mechanisms of resistance which have been reported [1–11], MDR has been largely studied through the detection of the *mdr*1 gene and Pgp, as well as drug accumulation and conventional cytotoxicity studies.

A lot of drugs have now been reported to reverse the MDR phenotype [13–20] but many of them failed to be safely usable in humans because of their dramatic toxicity when administered at the high doses which are often needed to achieve significant levels of reversal [18].

Colloidal drug delivery systems such as liposomes [21–26] or nanospheres [27] encapsulating doxorubicin were also found to circumvent MDR. More recently, liposome-encapsulated taxol further confirmed the ability of colloidal drug delivery systems to represent alternative non-toxic ways to reverse MDR.

On another hand, because hyperthermia was reported to enhance the cytotoxicity of doxorubicin when the temperature was over 41.5°C [36–39], we recently reported the preparation of small unilamellar vesicle liposomes [31] whose formulation was optimised to achieve a maximal temperature-induced release of the encapsulated compound when exposed at 43°C for 30 min in serum-containing culture media. The formulation we used (DPPC/DSPC/CHOL 5:4:2 molar ratio) contained cholesterol thus differing from those which were already reported in the literature encapsulating methotrexate [40–42], cisplatin [33, 34, 45, 46] or bleomycin [48] and which were always only composed of pure synthethic phospholipid mixtures. According to Kirby [49], in the absence of cholesterol these vesicles should very rapidly destabilise in the presence of serum, as we demonstrated [31] with the DPPC/DSPC mixture originally proposed [42] and used in many further studies [40–44]. Our model proved to be quite unaffected by serum [31] and was validated through *in vitro* assays in HeLaS3 human cervix carcinoma cells [32] where it further enhanced the potentiation of the cytotoxicity of doxorubicin by hyperthermia. In this paper, we first reported results obtained in MCF-7 human breast adenocarcinoma cells exposed to free doxorubicin or TLED at 37 and 43°C which are in agreement with those we previously obtained in HeLaS3 cells [32], i.e. potentiation of doxorubicin by 43°C hyperthermia and comparable activity of TLED and free doxorubicin.

Then, our data confirmed the ability of TLED to circumvent the multidrug resistance of the MCF-7^R subline selected for its high level of resistance to doxorubicin (approximately 200-fold), as already reported in the literature concerning "conventional liposomes" encapsulating either doxorubicin [21–24, 27], taxol [25] or vinblastine [26].

The combination of TLED with hyperthermia resulted in additive cytotoxicity and these results slightly differed from those obtained in sensitive cells where hyperthermia significantly potentiated doxorubicin, only because of a higher interexperimental variability of the data, thus leading to non-significant differences as compared to the theoretical additivity value calculated from Steel and Peckham [35].

When verapamil was added to TLED and combined with hyperthermia, no significant higher reversal effect was observed, contrasting with the results previously reported [21] but this could be explained by the complete reversing activities which were obtained with either TLED or verapamil alone (Table 2). This appeared interesting though encapsulated doxorubicin could represent an alternative to the use of verapamil in overcoming drug resistance without the occurence of extra side-effects.

As far as the circumvention of MDR by liposome encapsulation of the drugs is concerned, several explanations were proposed, including bypass of drug interaction with Pgp [39], alteration of membrane permeability through modifications of the packing density and motion of lipids within the cell membrane [26] after fusion of the liposomal lipids in the cell membrane. Further *in vitro* studies are needed to confirm the present results using three dimensional cultures which maintain tissue architecture [50] and that could be extended to patient specimens, as well as *in vivo* studies with pharmacological considerations. On the other hand, attention could be focused on determining Pgp-mediated resistance in the tumours of the patients entered into clinical studies of liposome-encapsulated drugs.

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