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In vitro Evaluation of the Association of Thermosensitive Liposome-encapsulated Doxorubicin with Hyperthermia

Jean-Louis Merlin

Doxorubicin was encapsulated in small unilamellar thermosensitive liposomes which were strictly defined in terms of size distribution and size stability: more than 95% of vesicles with a maximal diameter of 50 nm stable for a minimum of 24 hours. In addition, the preparation procedure was optimised to achieve the highest differential thermal stability defined as the difference of release between 37° and 43°C exposures in serum-containing medium (dipalmitoylphosphatidylocholine/distearoylphosphatidlylcholine/cholesterol mixture in 5:4:2 molar ratio). The cytotoxicity of thermosensitive-liposome encapsulated doxorubicin was then evaluated in combination with 43°C hyperthermia on HelaS3 human tumour cells using colony-forming assays. Results confirmed that hyperthermia potentiates the cytotoxic effects of doxorubicin. Liposome encapsulation was found to further enhance these effects when 0.05 μmol/l doxorubicin concentration was used.
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INTRODUCTION

SELECTIVE LOCALISATION of antitumour drugs is of great interest to achieve better control of cancers. Doxorubicin is reported to interact with non-malignant tissues, principally cardiac, which reduces its therapeutic efficiency when given systemically because of cumulative dose-limiting cardiotoxicity [1].

Liposome-encapsulated drugs appeared to represent an interesting alternative to reduce the cumulative cardiotoxicity [2–12]. In addition, nephrotoxicity [9, 10, 12] as well as other signs of toxicity, such as alopecia [7, 12], weight loss [7, 9, 12] and dermal necrosis due to extravasation [11, 13] were reported to be drastically reduced or even eliminated by employing liposome-encapsulated doxorubicin. More recently, phase I and II clinical studies were reported with liposome-encapsulated doxorubicin [14, 15] with a suggestion of a reduction of cardiotoxicity. On the other hand, many studies reported that hyperthermic treatment could potentiate the cytotoxic activity of doxorubicin [16–20]. The combination of liposome-mediated drug delivery and hyperthermic treatment appeared a very attractive approach since synergistic applications have already been demonstrated on tumour models with encapsulated methotrexate [21–23], cisplatin [24] or bleomycin [25, 26], hyperthermia playing the double role of treating the tumour and triggering the local efflux of the drug from the liposomes. To our knowledge, no experimentation has been published with doxorubicin encapsulated in thermosensitive liposomes. Our preliminary studies [27] reported that optimised thermosensitive liposomes could be prepared from a lipid mixture composed of dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC) and cholesterol in a 5:4:2 molar ratio in order to reach high differential thermal stability between physiological (37°C) and hyperthermic (43°C) temperatures in serum-containing media. In this paper, we evaluate the cytotoxicity of such liposomes containing doxorubicin combined with hyperthermia on the colony-forming ability of HelaS3 tumour cells.

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MATERIAL AND METHODS

Liposome preparation

DPPC, DSPC and cholesterol were obtained from Sigma as products of higher purity (99%) and were used without further purification. Doxorubicin was obtained from Laboratoires Roger Bellon (Neuilly, France). Sephadex G75 gel was obtained from Pharmacia. All other chemicals used were reagent grade and purchased from Prolabo (Paris). Liposomes were prepared according to Bangham's procedure [28] with slight modifications [27].

Cell culture conditions

All culture media and additives were purchased from Gibco. Culture materials were purchased from Falcon (Meylan, France). Stock culture of HelaS3 cells was maintained in MEM supplemented with 10% fetal calf serum (FCS), penicillin and streptomycin at 37°C in a 95% air/5% CO_2 atmosphere. Exponentially growing cells were harvested by enzymatic disaggregation then assayed for cytotoxicity.

Evaluation of cytotoxicity

200 viable cells were incorporated in culture medium and plated into 35 mm dishes. Aliquots of free, liposome-encapsulated doxorubicin, doxorubicin in empty liposome suspensions and empty liposomes were diluted in culture medium then incubated for 1 h at 37°C. The dishes were then washed twice with PBS in order to remove the non-internalised drug.

When hyperthermia was reached, the dishes were incubated in a water bath then washed twice with PBS before being assayed for colony formation. The temperature was controlled

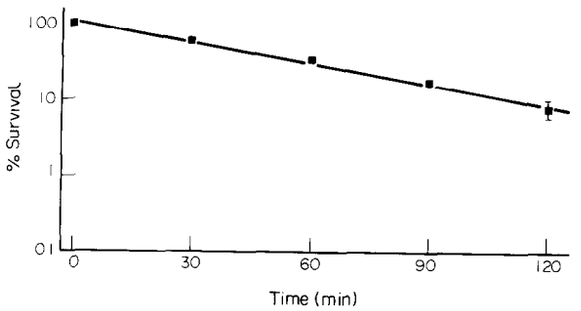


Fig. 1. Thermosensitivity of HelaS3 cells at 43°C: triplicate values (S.D.).

by immersion of a thermocouple in the culture medium and regulated to be 43°C (within 0.1°C).

Triplicate dishes were incubated for 12 days at 37°C, then colonies exceeding 100 μm in diameter (> 50 cells) were scored after being fixed by methanol and stained with Giemsa. When combinations of treatments was used, results were compared to an expected additive effect which was estimated according to Steel and Peckham [29] from the respective survival rates obtained with each treatment used alone. Results were statistically analysed using Student's *t* test.

RESULTS

Thermosensitivity of HelaS3 cells

HelaS3 cells were tested for thermosensitivity at 43°C for 30–120 minutes' incubation time. Results, expressed as percentage of survival between control (37°C) and experimental dishes showed a exponential decrease in survival with the duration of the hyperthermic treatment (Fig. 1). A 30 minute incubation was selected in order to achieve a moderate cytotoxicity level [68 (S.D.5) % survival rate] usable in combination with doxorubicin.

Sensitivity to doxorubicin

Doxorubicin sensitivity of HelaS3 cells was evaluated with drug concentrations ranging between 0 and 0.4 μmol/l. The dose-response curve (Fig. 2) allowed to select three doses leading to moderate cytotoxic effects for combination with hyperthermic treatment. These doses were 0.05, 0.1 and 0.2 μmol/l, giving respective survival rates of 82 (S.D. 2) 67 (2) and 24 (1)% at

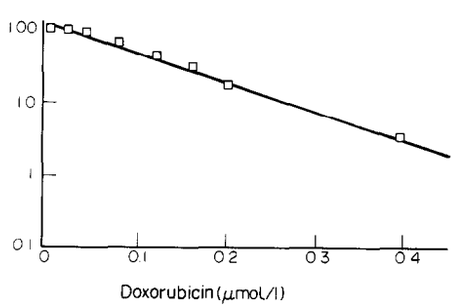
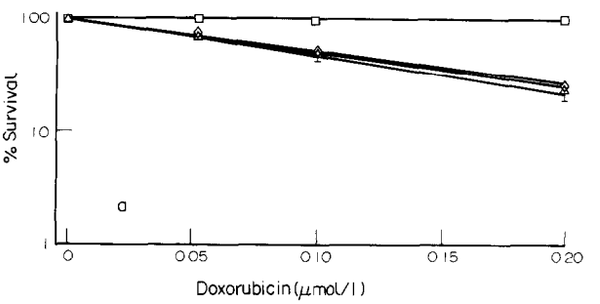


Fig. 2. Cytotoxicity of free doxorubicin on HelaS3 cells at 37°C: triplicate values (S.D.)



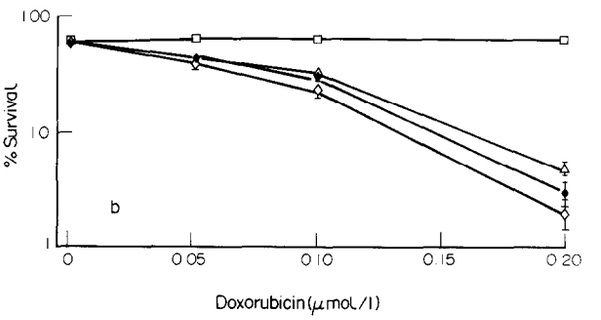


Fig. 3. Evaluation of liposome-encapsulated doxorubicin on HelaS3 cells at 37°C (a) or at 43°C (b). -□- = empty liposomes, -◆- = free doxorubicin, -△- = empty liposomes + free doxorubicin, -◇- = doxorubicin liposomes.

37°C. Encapsulated doxorubicin, as well as empty liposomes alone or associated with free doxorubicin, were evaluated at the same doses (Fig. 3a). Empty liposomes did not exhibit any particular cytotoxicity when administered alone to the cultured cells.

When associated with free doxorubicin, the cytotoxicity level was comparable to the level reached with free doxorubicin alone. In addition, no significant difference was observed between free, empty liposome-associated and liposome-encapsulated doxorubicin.

Combination and hyperthermia

Similar experiments were then performed in combination with 30 minute 43°C hyperthermia. Results (Fig. 3b) showed a

Table 1. Free or encapsulated doxorubicin with hyperthermia

	Doxorubicin (μmol/l)			
	Control	0.05	0.1	0.2
Free	68	45 (56)*	29 (45)*	3 (16)*
Liposome-encapsulated	66	37 (55)*	23 (44)*	2 (17)*

The expected additive effects, calculated from the respective survival rates obtained with each treatment used alone, are shown in parentheses. Control experiments were PBS or empty liposomes in PBS. Significant potentiations (*, $P < 0.001$) were found when hyperthermia was combined with doxorubicin. Liposome encapsulation was found to further potentiate the cytotoxicity for 0.05 μmol/l doxorubicin under hyperthermic conditions ($P < 0.02$).

decrease in survival in all cases. No significant differences were found between free and liposome-encapsulated doxorubicin except at 0.05 μmol/l, where doxorubicin liposomes induced a significantly higher cytotoxicity ($P < 0.02$). In both cases, the combination of doxorubicin with hyperthermia exerted a synergistic effect on cytotoxicity, since the experimental survival rates were always significantly ($P < 0.001$) lower than the expected effects (Table 1)—calculated by adding logarithms of each respective surviving fraction as described previously [29]—since the logarithms of surviving fractions were linear with dose (Figs 1 and 2).

DISCUSSION

The ability of liposomal encapsulation of doxorubicin to enhance its cytotoxicity while reducing its other toxicities is now well-documented [2–13]. Since 1986, several phase I and II studies have been reported, using liposome-encapsulated doxorubicin hepatocellular carcinomas [30], or, more recently in advanced breast cancer [14, 15]. All clinical results showed that the antitumour activity of liposome-encapsulated doxorubicin was conserved and suggested that it was less cardiotoxic than free doxorubicin at cumulative doses up to 880 mg/m^2.

On the other hand, hyperthermia was shown to enhance the cytotoxic effects of doxorubicin [16–20]. Since selective thermally-induced release from liposomes has already been achieved with methotrexate [20–23], cisplatin [24] or bleomycin [25, 26], the mediation of doxorubicin delivery associated with a selective delivery, and a local treatment of tumours which could potentiate the drug effects, appeared a very interesting challenge. Experimental conditions required thermosensitive vesicles which exhibit high release patterns of the encapsulated compound in hyperthermic conditions, as well as simultaneous high stability at 37°C, even in serum-containing biological fluids. Moreover, small liposome suspensions have been shown to have greater stability [31, 32], to be sterilisable by 0.2 μm filtration and to have increased antitumour activity [33]. In our experiments, we used liposomes made of DPPC/DSPC/cholesterol in 5:4:2 molar ratio which we previously reported as a lipid mixture yielding high differential stability results between 37 and 43°C with exposure times ranging between 30 and 60 minutes [27]. In addition, the preparation procedure was optimised to use SUV-liposome suspensions, which we defined as containing more than 95% of vesicles with a maximal diameter of 50 nm, and being stable in size for at least 24 hours during the time of experimentation.

30 minute exposure time was selected in order to reach a moderate cytotoxicity level (near 70% of survival) on cultured tumour cells at 43°C and to potentiate the cytotoxicity of doxorubicin, which needs a minimal 43°C temperature to appear [16]. Our experimental results first confirmed the potentiation of the cytotoxicity of doxorubicin by hyperthermia which was reported in the literature [16–20], and that liposome encapsulation did not impair the antitumour activity of doxorubicin. Furthermore, thermosensitive liposome encapsulation with hyperthermic exposure generated a significant ($P < 0.02$) increase in the cytotoxicity with a 0.05 μmol/l concentration of doxorubicin. Similar statistical results (with a maximal $P = 0.05$) were not found with higher concentrations (0.1 and 0.2 μmol/l), even when the mean values were always lower for the doxorubicin liposome series. These findings appeared very encouraging and should be further investigated in order to investigate the local antitumour effects of this combination.

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Synergy between Preactivated Photofrin-II and Tamoxifen in Killing Retrofibroma, Pseudomyxoma and Breast Cancer Cells

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Exposure of photoactive compounds to light prior to their use in biological systems (preactivation) results in the generation of tumour cell specific metastable cytotoxic species that are no longer dependent on the light energy. Thus, preactivation renders the photoactive compounds suitable for systemic use. We have examined the *in vitro* effect of preactivated photofrin-II and tamoxifen in retroperitoneal fibroma, pseudomyxoma and male breast carcinoma cell lines. These cells were found to be non-responsive to tamoxifen and were negative for oestrogen receptors. Incubation of these cells with 0.5 μg/ml preactivated photofrin-II and tamoxifen ($< 10^{-6}$ mol/l) resulted in a significantly enhanced ($P < 0.001$) inhibition of DNA synthesis compared with either agent alone. This synergistic effect between tamoxifen and preactivated photofrin-II was determined by multiple drug effect analysis. Treatment of cells with preactivated photofrin-II did not cause the increased expression of oestrogen receptors. These observations suggest that a combination of antihormonal drugs with preactivated compounds may be of clinical value.
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INTRODUCTION

TAMOXIFEN IS one of a number of non-steroidal compounds known for its anti-oestrogenic and antifertility properties in laboratory animals [1]. The antitumour activity of tamoxifen was reported in a preliminary report [2]. However, clinical experience has now established tamoxifen as the antihormonal agent of choice. In recent years, it has been used successfully in the treatment of oestrogen positive breast cancer [3, 4], but the mechanism of its antitumour activity is not clear. It has been reported that tamoxifen mediates its antitumour activity via oestrogen [5–7] through one of its reactive metabolites, hydroxytamoxifen, which is formed *in vivo* [8]. The effect of tamoxifen in oestrogen receptor positive cell lines is mediated via oestrogen receptors (ER) requiring low concentrations of tamoxifen, while the antitumour effects observed in oestrogen receptor negative cell lines are thought to be non-receptor-mediated non-specific cytotoxic mechanisms [5, 9–14]. Tamoxifen is a tumoristatic agent but not a tumoricidal agent, since if therapy is stopped, regrowth of tumour occurs [15].

Recently, three cell lines were established in our laboratory from tumour biopsy specimens obtained from patients with retroperitoneal fibrosis, pseudomyxoma and male breast carcinoma. The case histories and tamoxifen-mediated clinical

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