Cancer Chemother Pharmacol (2006) 57: 65–72
DOI 10.1007/s00280-005-0006-7

ORIGINAL ARTICLE

V. Kirveliene · G. Grazeliene · D. Dabkeviciene · I. Micke
D. Kirvelis · B. Juodka · J. Didziapetriene

Schedule-dependent interaction between Doxorubicin and mTHPC-mediated photodynamic therapy in murine hepatoma in vitro and in vivo

Received: 26 July 2004 / Accepted: 2 March 2005 / Published online: 5 July 2005

Abstract *Purpose*: To evaluate cytotoxic and antitumor effects of a conventional anticancer drug Doxorubicin (Dox) and photodynamic therapy (PDT) mediated by a promising photosensitizer of second generation meta-tetra (3-hydroxyphenyl)-chlorin (mTHPC) in combination. *Methods*: Murine hepatoma MH-22A was used for investigation in vitro and in vivo. In vitro, the cells were incubated with 0.15 μg/ml mTHPC for 18 h and exposed to light from LED array ($\lambda = 660 \pm 20$ nm) at 0.6–2.4 kJ/m^2. 0.05–0.2 μg/ml Dox was administered either 24 h prior to or immediately after light exposure (Dox → PDT or PDT + Dox, respectively). The cytotoxicity was tested by staining with crystal violet. The character of the combined effect was assessed by multiple regression analysis. In vivo, the antitumor activity was estimated by monitoring the tumor volume over time, in mice transplanted subcutaneously with MH-22A and treated with Dox and/or PDT. For PDT, mice were exposed to light from diode laser ($\lambda = 650 \pm 2$ nm) at 12 kJ/m^2 following 24 h after administration of 0.15 mg/kg mTHPC. A 3 mg/kg Dox was administered either within 15 min prior to mTHPC or within 15 min after light exposure (Dox → PDT or PDT + Dox, respectively). *Results*: Both in vitro and in vivo, the combination of mTHPC-mediated PDT and Dox was evaluated to be more effective than each treatment alone. In vitro, the difference between cell viability curves after photodynamic treatment as a single modality and after combination of photodynamic treatment with Dox was statistically significant under most of the applied conditions ($P \leq 0.02$). In the case of PDT + Dox, the combination had an additive character, and the sequence Dox → PDT caused a sub-additive interaction. In vivo, both regimens of combination were more effective in inhibiting tumor growth than any single treatment ($P < 0.09$). The antitumor activity of PDT + Dox regimen was more prominent than that of Dox → PDT; however, significance of the difference was not high ($P = 0.08$). *Conclusions*: These results indicate that Dox potentiates therapeutic efficacy of mTHPC-mediated PDT and vice versa, and the degree of potentiation is influenced by the combination schedule: administration of Dox immediately after light exposure is preferable to administration of Dox at 24 h prior to light exposure.

Keywords Photodynamic therapy · Combination therapy · Doxorubicin mTHPC · Foscan

V. Kirveliene (✉) · D. Dabkeviciene · I. Micke · D. Kirvelis
B. Juodka
Department of Biochemistry and Biophysics,
Vilnius University, Ciurlionio 21,
LT 03101, Vilnius, Lithuania
E-mail: vida.kirveliene@gf.vu.lt
Tel.: +370-5-2398225
Fax: +370-5-2398231

G. Grazeliene · J. Didziapetriene
Institute of Oncology, Vilnius University,
Santariskiu 1, LT 08660, Vilnius, Lithuania

Introduction

Photodynamic therapy (PDT), as a novel mode of cancer treatment, is an object of intense investigation. PDT is based on the use of photosensitizing compounds that localize quite selectively in neoplastic/hyperplastic tissues and become cytotoxic when exposed to light [3, 10, 11]. Combination of PDT with conventional modes of cancer treatment, such as surgery [15], radiotherapy [18] or chemotherapy [14] became a subject of research at the very dawn of PDT. Newer photosensitizers with improved spectroscopic, photochemical and tissue-localizing properties and improved laser instrumentation have stimulated attempts to establish clinical protocols for incorporation of PDT into multimodality treatment.

The photosensitizer used in this study, *meta*-tetra (3-hydroxyphenyl) chlorin (mTHPC, drug trade name Foscan) [2] is one of the most potent photosensitizers currently available for clinical use [11]. Clinical evidence shows that mTHPC-PDT combination with surgery, i.e. surgical resection of tumor followed by intraoperative adjuvant mTHPC-PDT, seems to be a promising treatment option, which could improve the radicality of tumor resection and survival rate of patients with chest [1, 13, 26], head and neck [9] or brain [35] malignancies. Combination of mTHPC-PDT with radiotherapy demonstrated the efficacy of the clinical treatment for a wide range of head and neck tumors [9] and prostate cancer [23]. However, there are only few reports about mTHPC-PDT combination with chemotherapeutic drugs: in vitro studies revealed a significant effect of fluoropyrimidines [36] and mitomycin C [32] on viability of mTHPC-photosensitized cells.

The cytotoxic drug used in this study is a conventional anticancer drug Doxorubicin (Dox) of anthracyclines group [25]. The rationale behind the combination of PDT and Dox is the different modes of cytotoxic action. Light-activated photosensitizer generates reactive oxygen species that oxidize various biomolecules in close proximity to the site of localization of the photosensitizer [21]. The preferential sites of localization of mTHPC, the photosensitizer used in this study, are cellular membranes [4], especially endoplasmic reticulum and Golgi apparatus [31], and lipid peroxidation was shown to be an early response to mTHPC-photosensitization [17]. Meanwhile, Dox localizes to the cell nucleus [31], mainly, and the major molecular target that determines the clinical activity is DNA topoisomerase II [33]. From the clinical point of view, combination of PDT with Dox is likely to produce additive or even synergetic effect, since antitumor effect of PDT is local, while that of Dox is systemic. On the other hand, the major side-effect of PDT is phototoxicity, and that of Dox is cardiotoxicity, and the combination might help to soften the side-effects allowing the reduction of the administrated doses.

The ability of Dox to enhance effects of PDT in cell culture and transplantable mouse tumors has been studied by several groups using a number of photosensitizers, i.e. hematoporphyrin derivative [8, 22], Photofrin II [5], exogenous 5-aminolevulinic acid-induced endogenous porphyrins [7], aluminum disulfonated phthalocyanine [6]; Photohem [30], 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a [29] and mesochlorin e_6 [24]. In almost all cases, the combined treatment was more effective than either single treatment.

In this study, the cytotoxic and antitumor effects of the combined action of Dox and mTHPC-mediated PDT were evaluated. Two experimental systems, cell culture and transplanted mouse tumors of murine hepatoma MH-22A, were used. Two combination schedules, roughly, Dox prior to and simultaneously with PDT, were assessed.

Materials and methods

Materials

mTHPC (kindly provided by R. Bonnett, Queen Mary, University of London, UK) was dissolved in ethanol as 1 mg/ml stock solution and stored at −20°C in the dark. Adriamycin, Doxorubicin hydrochloride, (Dox, Ebewe Arzneimittel, Austria, 2 mg/ml) was stored at 4°C. All experiments in vitro were performed using dilutions of the stock solutions with cell incubation media. For experiments with mice, the stock solutions were diluted with water for injection to a final concentration of 0.15 mg/kg in 0.2 ml for mTHPC, and 3 mg/kg in 0.2 ml for Dox.

Methods

Cell culture

Culture flasks and plates were Primaria from BD Falcon. Fetal calf serum (FCS) was from Gibco BRL. Other tissue culture products were obtained from Sigma. MH-22A cells from murine hepatoma [13] were obtained from the Institute of Cytology, Sankt-Peterburg, Russia. The cells were cultured in monolayer in 25-cm^2 flasks in Dulbecco's minimal essential medium (DMEM) supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine, at 37°C in 5% CO_2atmosphere. Cells were subcultured by dispersal with 0.025% trypsin in 0.02% EDTA and replated at 1:3 dilutions twice a week.

Cell exposure and viability assessment

Cell exposure to chemicals and light were carried out in 24-well plates according to the experimental schedule shown in Fig. 1a. The cells were seeded out as a suspension in DMEM supplemented with FCS at a density of 3.5×10^5 cells/ml in 0.4 ml per well, and a drug was added after 30 h to the cell monolayer in log phase.

A factorial design was used for the experiments, where single durations of light exposure were coupled with several concentrations of Dox and vice versa, with three replicated independent wells. Both mTHPC and Dox were diluted at least 200-fold with cell medium from its stock solution. Serial dilutions of the stock solutions were used to construct the dose-response curves for Dox. An additional 0.4 ml of the medium was added to the wells that contained vehicle controls (ethanol or Dulbecco's phosphate buffer saline, DPBS) that comprised less than 0.5% of the total volume.

For photosensitized treatment, mTHPC was added to the cell cultivation medium to a final concentration of 0.15 μg/ml. When handling the samples containing mTHPC, precautions were taken to avoid irradiating the samples with room light by reducing the sources of

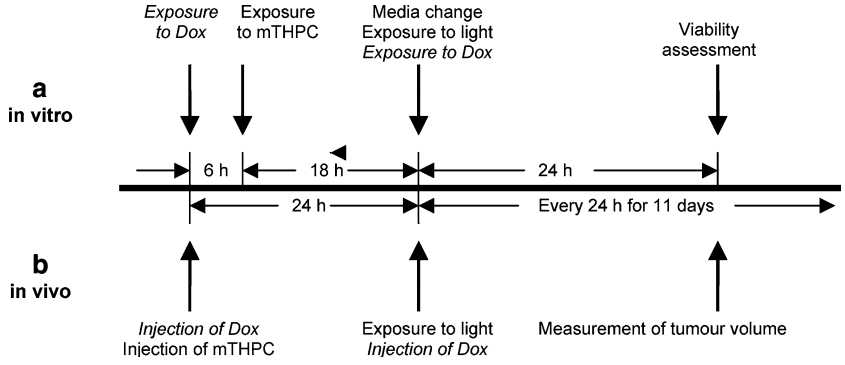

Fig. 1 Schedule for treatment of cells (**a**) and mice (**b**). **(a)** 0.15 μg/ml mTHPC was added to MH-22A cells and incubated in the dark for 18 h. Then the incubation medium was replaced with fresh one, and the cells were exposed to light at $\lambda = 660 \pm 20$ nm, 10 W/m^2, and the cells were incubated for 24 h post-exposure. Dox was added to the medium either 24 h prior to (Dox → PDT) or immediately after (PDT + Dox) light exposure; **(b)** mTHPC, 0.15 mg/kg, was injected i.p. to mice bearing subcutaneous hepatoma MH-22A. After 24 h, the mice were exposed to laser illumination. Dox, 3 mg/kg, was injected i.p. either prior to injection of mTHPC (Dox → PDT) or after light exposure (PDT + Dox). Over the next 11 days, tumor volume was measured every 24 h. The mice were observed till demise. *Text in italic* denotes the alternatives in the combined treatment

illumination to a minimum and by protecting the samples from light with aluminum sheets. After incubation for 18 h at 37°C in the dark, extracellular photosensitizer was removed by rinsing the cell monolayer three times with room-temperature DPBS, and DMEM containing FCS was added. The cells were exposed to light from LED array UNIMELA-1 ($\lambda = 660 \pm 20$ nm) (VU Laser Research Centre, Lithuania), the fluence rate at the level of the cells being 10 W/m^2, as measured using an irradiation power meter IMO (Russia). Dox was added to the cell incubation medium either 24 h prior to light exposure or immediately after light exposure. The cells were incubated for 24 h post-exposure until cell viability was estimated by staining the substratum-attached cells with crystal violet. Briefly, the cells were fixed with 96% ethanol for 10 min, 0.05% crystal violet (CV) solution in 20% ethanol was added for 30 min, the cells were rinsed, the remaining cell-attached dye was dissolved in 0.1% acetic acid solution in 50% ethanol, and the optical density at 585 nm was recorded [16].

Measurement of intracellular mTHPC concentration

The cells were harvested to 1.2 ml of 0.9% NaCl, and the mTHPC concentration was measured fluorometrically at 420 nm for excitation and 654 nm for emission with a Hitachi 850 spectrofluorometer, after addition of 17 μl of 8% Triton X-100 and 133 μl of methanol to the sample. For quantification, a minimal amount of a standard solution of the known mTHPC concentration, increasing the total fluorescence yield by about 50%, was added, and the fluorescence was recorded once more [19]. The protein content was determined by BCA method [28].

Fluorescence microscopy

Cells incubated with Dox or/and mTHPC were visualized with Olympus AX70 fluorescence microscope equipped with ×60, NA 1.25 oil immersion lens. The images were recorded with CCD camera Orca (Hamamatsu) and analyzed with MicroImage version 4.0 (Media Cybernetics) software. WG filter cube (510–550 nm for excitation and beyond 590 nm for emission) was used for visualization of Dox and the specially produced filter cube (400–410 nm for excitation and beyond 590 nm for emission) was used for visualization of mTHPC.

Mice

BDF_1 hybrid (C57 black/6 female×DBA/2 male) male mice (the facility of Immunology Institute, Lithuania) at 8–10 weeks of age and 22–25 g body weight were used throughout the study. The animals received care in accordance with the guidelines established by the Lithuanian Animal Care Committee, which approved the study.

Tumor inoculation and observation

Mice were injected subcutaneously with 0.2 ml of five times diluted MH-22A tumor mass suspension in right groin. PDT and/or drug administration was carried out 10 days after transplantation when tumor volume (TV) was 0.206 ± 0.028 cm^3. TV was determined by measuring the tumor diameters with vernier calipers and calculated according to the formula $TV = L \times W \times H \times \pi/6$, where L is length, W width and H height of the tumor. After tumor exposure to treatment, tumor growth was monitored each day for up to 11 days. The antitumor activity was evaluated by tumor growth inhibition (TGI) in treated (T) versus control (C) mice calculated according to the formulae $TGI = 100 - (TV_T/TV_C \times 100)$.

Tumor exposure to treatment

For evaluation of antitumor activity, mice were treated according to the experimental schedule shown in Fig. 1b. Mice were randomized into five groups, which consisted of 11 (group I) or 7 (groups II–V) mice: I, Control; II, Dox; III, PDT; IV, Dox → PDT; V, PDT + Dox. In group I, mice were not treated. In group II, 0.2 ml of Dox was injected i.p. at a dose of 3 mg/kg. In group III, PDT was performed as follows: 0.2 ml of mTHPC was injected i.p. at a dose 0.15 mg/kg, and after 24 h, tumors were exposed to light from the diode laser (λ, 650 ± 2 nm, fluence rate, 100 mW/cm^2) (Kaunas Technological University, Lithuania) for 20 min, reaching a dose of 12 kJ/m^2. In group IV, 3 mg/kg of Dox was injected i.p. within 15 min prior to injection of 0.15 mg/kg of mTHPC, and after 24 h, tumors were exposed to light as in group III. In group V, 0.15 mg/kg of mTHPC was injected i.p., after 24 h tumors were exposed to light as in group III, and 3 mg/kg of Dox was injected within 15 min.

Data analysis

The data of experiments in vitro were analyzed using two-way ANOVA, pairwise *t* test (the normal distribution criteria were fulfilled) and multiple regression analysis. For data analysis of the experiments in vivo, *t* test was performed with pairwise or multiple comparisons (Holm-Sidak method), when appropriate. SigmaStat 3.0.1 and Statistica 5.0 softwares were used for the statistical analysis.

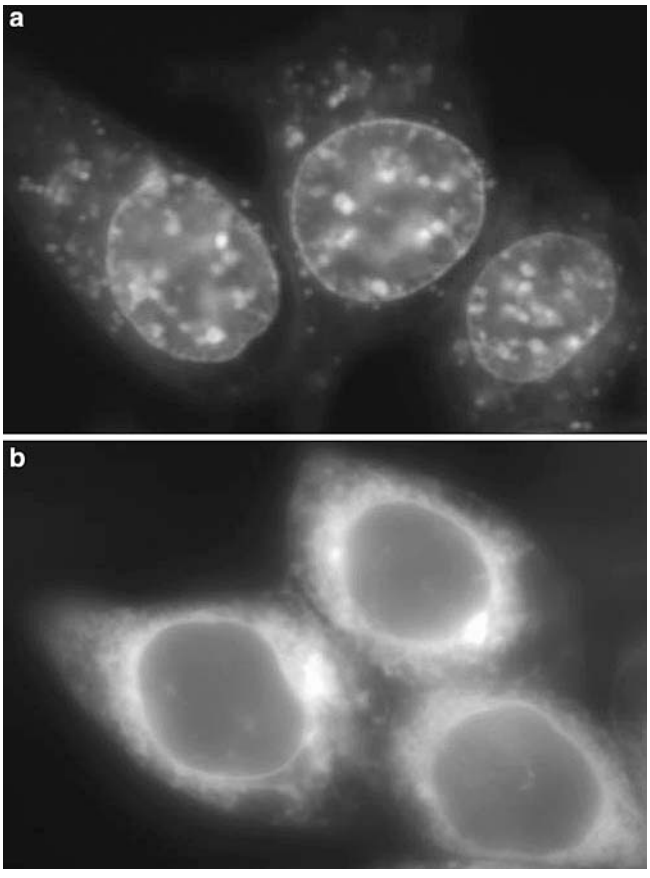

Fig. 2 Cellular localization of Dox and mTHPC fluorescence. MH-22A cells were incubated with mTHPC (0.4 μg/ml) or Dox (1 μg/ml) and examined with fluorescence microscope, as described in Materials and methods. **a** fluorescence of Dox following incubation for 4 h; **b** fluorescence of mTHPC following incubation for 18 h; magnification ×600

Results

Treatment in vitro

The effects of photodynamic treatment or/and Dox were characterized by investigating the dose-response relationship of cytotoxicity measured by staining with CV, since this test was shown to be the most sensitive assay for the evaluation of the effect of the combined action [20]. After a number of experiments aimed at evaluation of the optimal concentrations of Dox and mTHPC given alone, the following concentrations were selected: 0.05, 0.1and 0.2 μg/ml for Dox, and 0.15 μg/ml for mTHPC. Microscopic examination of cells loaded with Dox detected Dox fluorescence in cell nucleus, mainly (Fig. 2a). For sensitization to light, MH-22A cells were incubated with mTHPC for 18 h. Fluorescence microscopy of the cells at the end of incubation demonstrated cytoplasmic localization of mTHPC (Fig. 2b). Cytotoxic effect was induced by light exposure for 1, 2 or 4 min at a fluence of 0.6, 1.2 or 2.4 kJ/m^2, respectively. Neither cytotoxicity of the photosensitizer at the selected concentration without light exposure nor that of light without mTHPC-photosensitization were registered. For studies of the combined action, Dox was added to the cells either (1) 24 h prior to light exposure (Dox → PDT) or (2) immediately after light exposure (PDT + Dox). No photocytotoxicity of Dox due to light exposure was registered under the applied conditions. It should be noted, that we aimed to study the pattern of the combination, and not to achieve the full loss of cell viability.

Cytotoxic effects of single and combined treatments in vitro

Our data indicated that cytotoxicity of photodynamic treatment increased with increasing concentrations of mTHPC (not shown) and increasing durations of light exposure (Fig. 3). The same character of dose-cytotoxicity relationship was observed for the Dox-treatment in the range of the applied doses, as well (see Fig. 3, 0 min). In general, the addition of Dox to the photodynamically treated cells decreased the cell viability significantly. The difference between cell viability curves after photodynamic treatment as a single modality and after combination of photodynamic treatment with Dox

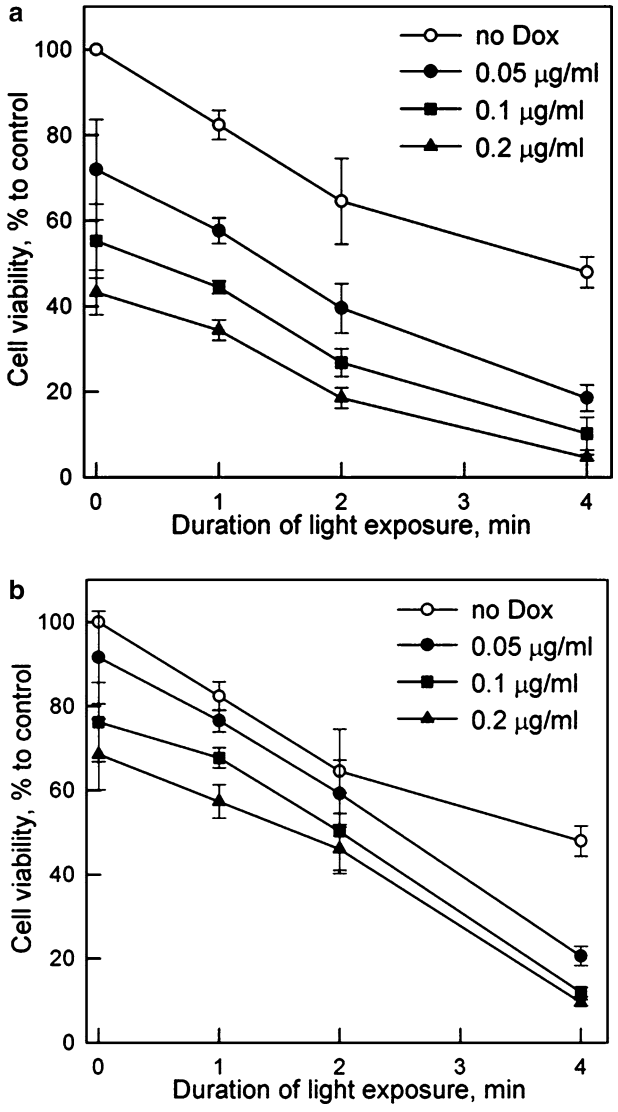

Fig. 3 Viability of MH-22A cells treated with Dox and PDT, alone or in combination. The cells were incubated with 0.15 μg/ml mTHPC or/and Dox, as shown in Fig.1, and exposed to light at 660 ± 20 nm and 10 W/m^2. Cell viability was evaluated by staining with crystal violet after following incubation for 24 h in the dark. **a** Dox was added 24 h before light exposure; **b** Dox was added immediately after light exposure; *bars* ± SD

was statistically significant under most of the applied conditions (*P*-values varied from 0.02 for 0.1 μg/ml Dox added after PDT to 0.0004 for 0.2 μg/ml Dox added 24 h prior to light exposure), except for the lowest concentration of Dox (0.05 μg/ml) under PDT + Dox regimen ($P = 0.1$). The most considerable difference in cell viability between photodynamically treated cells and cells exposed to the combined treatment exceeded 40% when 0.2 μg/ml Dox was added to the photosensitized cells 24 h prior to light exposure. The difference between cytotoxic effects of Dox and the combined treatment at 2.4 kJ/m^2 was approx. 40% in the case of the sequence Dox → PDT, and exceeded 60% when Dox was added after PDT. The most considerable decrease in cell viability (to the residual value of 4.6 ± 0.7%) was achieved by combination of 0.15 μg/ml mTHPC, light at 2.4 kJ/m^2 and 0.2 μg/ml Dox, added 24 h prior to light exposure.

Table 1 Significance of the cytotoxic effect of treatment factors

Treatment factor	*F* ratio		*P* value	
	Dox → PDT	PDT + Dox	Dox → PDT	PDT + Dox
Light exposure	258	288	< 0.001	< 0.001
Dox	280	272	< 0.001	< 0.001
Dox + light exposure	4.7	1.6	< 0.001	0.04

Differences in cytotoxicity between the two combination regimens in vitro

The contribution of combination of the treatment factors to the overall result was evaluated by the two-way analysis of variance. The analysis revealed that both concentration of Dox and duration of light exposure in the presence of mTHPC were significant determinants ($P < 0.001$) of the cytotoxic effect (Table 1). When Dox was added prior to light exposure, combination of light exposure with Dox did add a statistically significant value ($P < 0.001$) to the overall cytotoxic effect of the combined treatment. However, in the case of PDT + Dox, the added value of the combination was less significant ($P = 0.04$).

The multiple regression analysis producing the mathematical model of the process provided an insight into the details of the process. For the sequence Dox → PDT, the equation (1) was produced:

$$\mathrm{CV} = 100 - 22E - 340D + 78\mathrm{ED} \quad r = 0.9 \qquad (1)$$

where CV is cell viability (% control); *E*, duration of light exposure (min); *D*, concentration of Dox (μg/ml). In this case, the estimated regression coefficient at ED was significant, $P < 0.001$. The coefficient at ED corresponding to the combination of the treatment factors was positive by contrast to the negative estimated regression coefficients at *E* and *D* corresponding to the loss of cell viability induced by these factors. This implicated that the cooperativity of the combination had an antagonistic component, slightly decreasing the final result, which could be expected to let the combined effect be purely additive.

We compared the cellular uptake of mTHPC in the presence and absence of Dox. The accumulation of mTHPC in the cells concurrently incubated with Dox at a concentration of 0.7 μg/ml was significantly less ($P = 0.009$) than in the cells incubated in Dox-free media (Fig. 4). These data substantiated the presence of antagonistic component in cooperativity of Dox and mTHPC-PDT.

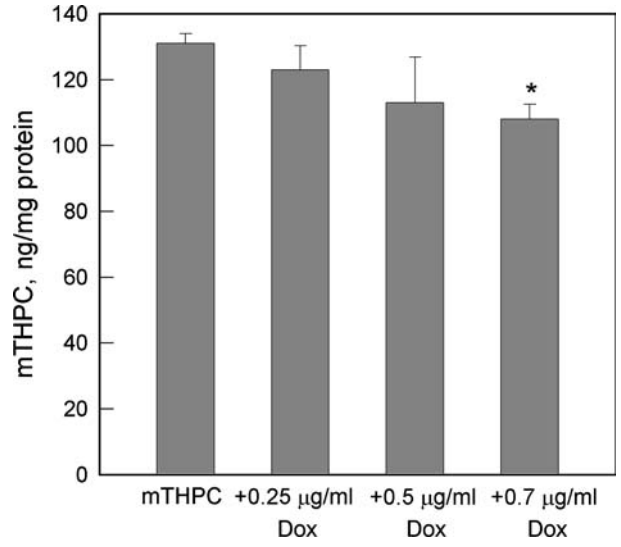

Fig. 4 Accumulation of mTHPC in MH-22A cells in presence or absence of Dox in incubation medium. The cells were incubated for 18 h with 0.3 μg/ml mTHPC in serum-free DMEM supplemented with Dox, when indicated; *bars* ±SD

For the sequence PDT + Dox, two equations (2 and 3) were found, which made the best fitting surfaces:

$$CV = 100 - 19E - 121D + 14ED \quad r = 0.98, \tag{2}$$

$$CV = 100 - 19E - 103D \quad r = 0.98, \tag{3}$$

where CV is cell viability (% to control); E, duration of light exposure (min); D, concentration of Dox (μg/ml). The estimated regression coefficient at ED in Eq. 2 was not statistically significant ($P > 0.05$), and, on the other hand, elimination of ED from the equation gave the Eq. 3, with the identical r value. Therefore, the term ED could be neglected, and the resulting absence of the equation terms corresponding to the combination of PDT with Dox would indicate that the overall loss of cell viability induced by the combined action resulted from the summing up the cytotoxicity of Dox and that of mTHPC-PDT. Thus, when Dox was added immediately after light exposure, the additivity of the combined effect of mTHPC-mediated PDT and Dox could be presumed.

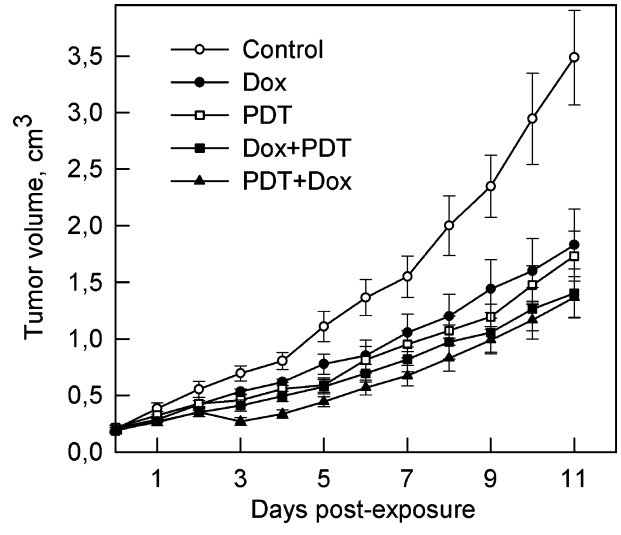

Fig. 5 Growth curves for subcutaneously transplanted hepatoma MH-22A in mice treated with Dox and PDT, alone or in combination, as shown in Fig. 1. Dox, 3 mg/kg of Dox was administered; PDT, 0.15 μg/ml of mTHPC was administered, after 24 h, tumors were exposed to light at 650 ± 2 nm and 12 kJ/m^2; Dox–PDT, 3 mg/kg of Dox was administered within 15 min prior to mTHPC; PDT + Dox, 3 mg/kg of Dox was administered within 15 min after light exposure (PDT + Dox); *bars* ±SE

Treatment in vivo

In MH-22A bearing mice, we investigated the effects of Dox at a dose of 3 mg/kg and mTHPC-mediated PDT at 0.15 mg/kg of mTHPC. Exposure to light was carried out at a dose of 12 kJ/m^2. For studies of the combined action in vivo, Dox was administered either (1) 24 h prior to light exposure (Dox → PDT) or (2) immediately after light exposure (PDT + Dox). Pilot experiments revealed the absence of antitumor activity of light without mTHPC or mTHPC without light.

Antitumor activity of single and combined treatments in vivo

Tumor volumes in all treated groups were significantly less ($P < 0.005$) than those in the control group, as shown in Fig. 4. Under applied treatment conditions, pretty close antitumor efficacy of mTHPC-PDT and Dox-treatment given alone was achieved (Table 2). Both regimens of combination of these two therapeutic modes were more effective in inhibiting tumor growth than any single treatment: TGI by Dox → PDT exceeded TGI by Dox significantly ($P = 0.003$) and that by PDT less significantly ($P = 0.09$); TGI by PDT + Dox significantly exceeded TGI by Dox and that by PDT ($P = 0.00006$ and 0.003, respectively) (Table 3). The antitumor activity of PDT + Dox regimen was higher than that of PDT → Dox; however, the significance of difference was not considerable ($P = 0.08$).

Discussion

The main objective of this study was to evaluate the potentiation of cytotoxic and antitumor efficacy of a

Table 2 Tumor growth inhibition in treated versus control mice ($P < 0.05$)

Group	TGI (%)
Dox	33.4 ± 6.1
PDT	38.6 ± 7.9
Dox → PDT	46.6 ± 6.3
PDT + Dox	54.5 ± 7.0

Table 3 *t* value and (*P*) for testing of mean equality of TGI

	Dox	PDT	Dox → PDT
PDT	1.16 (0.26)		
Dox → PDT	3.39 (0.003)	1.79 (0.09)	
PDT + Dox	5.07 (0.00006)	3.36 (0.003)	1.86 (0.08)

promising photosensitizer of second generation, mTHPC, by a conventional anticancer drug Dox and vice versa. Murine hepatoma MH-22A was selected for its ability to be easily cultivated both in vitro as cell culture and in vivo as transplantable mouse tumor. Furthermore, it was shown that mTHPC was retained in intrahepatic tumor and that mTHPC-mediated PDT was capable of inducing complete tumor remission of liver tumors [27].

Any combination implies a sequencing of the combining agents, and the sequence could be of significant relevance to the combination effect, as has already been shown for combination of Adriamycin with hematoporphyrin derivative (HPD) -mediated PDT, where the administration of Adriamycin after PDT was less effective than the administration with HPD or at the time of exposure to light [8]. Therefore, we chose to investigate two combination regimens: treatment with antitumor drug (1) 24 h prior to light exposure (Dox → PDT), and (2) nearly simultaneously with light exposure (PDT + Dox). When the effect was assessed after 24 h following light exposure, the sequence Dox → PDT produced higher effect due to the longer treatment with Dox. This circumstance is especially distinct in vitro, and could misrepresent the real outcome of the combination. Therefore, for the assessment of the difference between the combination regimens, the character of the combination effect was determined. The calculated additivity of the combination sequence PDT + Dox suggested a higher overall efficacy of this schedule against the sequence Dox → PDT, which displayed a sub-additive interaction.

Several assumptions could be useful for understanding the source of antagonistic component. The complexation of Dox with tetrapyrolic photosensitizer Photohem was registered in aqueous solutions [30]. On the other hand, Dox is known to activate cellular systems of drug resistance, preventing drug accumulation in the cell [22]. In both cases, concurrent cell incubation in the media containing Dox and mTHPC could inhibit the cellular uptake of mTHPC. This presumption was supported by our findings that uptake of mTHPC by the cells concurrently incubated with Dox was less than in the cells incubated in Dox-free media.

The validity of the in vitro approach and the conclusions it brought to was supported by the results of the investigation in vivo: in general, the higher antitumor activity against subcutaneously transplanted MH-22A treated with the combination of Dox and PDT corresponded with the higher cytotoxicity in vitro. The higher efficacy of tumor growth inhibition by the sequence PDT + Dox in comparison with Dox → PDT was apparent, especially having in the mind longer-lasting effect of Dox, which was added earlier in the case of Dox → PDT. However, statistical significance was not high.

In conclusion, our results indicate that combination of mTHPC-mediated PDT with Dox-treatment obviously exhibits increased therapeutic efficacy against murine hepatoma both in vitro and in vivo, and the simultaneous exposure to Dox and PDT is preferable to treatment with Dox at 24 h prior to PDT. It might be presumed that the combination of the two treatment modalities could be effective for tumors displaying low sensitivity to each drug given alone.

Acknowledgements We are indebted to Prof. R. Bonnett (Queen Mary, University of London, UK) for the generous gift of m-THPC and V. Piskarskiene for the excellent care of the cells. This work was partly supported by the European Commission, Program of Centers of Excellence, project CEBIOLA, ICA1-CT-2000–70027, and Lithuanian State Foundation for Science and Studies, Program Light in Biomedicine, P-14/01.

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