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Research paper

Effect of Cremophor EL on the pharmacokinetics, antitumor activity and toxicity of doxorubicin in mice

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Cremophor EL (CR) is a solubilizing agent and a modulator of P-glycoprotein (P-gp)-mediated anticancer multidrug resistance. The present study was undertaken to evaluate whether doxorubicin (Dox) pharmacokinetics, therapeutic activity and cardiotoxicity in Swiss albino mice is modified when combined with CR treatment. CR (2.5 ml/kg, i.p) given simultaneously with Dox (20 mg/kg, i.p.) increased Dox levels in plasma, heart, liver and kidneys of healthy mice. Using an Ehrlich ascites carcinoma (EAC)-bearing mice experimental model, CR (2.5 ml/kg) improved the survival and antitumor activity of Dox. The enhanced antitumor activity of Dox was related to a significant increase in EAC tumor cellular Dox content by CR. Furthermore, CR (1 μg/ml) potentiated the *in vitro* cytotoxicity of Dox in cultured EAC cells. In healthy mice, Dox-induced mortality was markedly reduced by simultaneous treatment with CR. CR enhanced DOX-induced increase in plasma lactate dehydrogenase, creatine phosphokinase (CPK) and CPK-MB isozyme activities, as well as the cardiac malondialdehyde level. CR also increased Dox-induced focal necrotic myocardial lesions. These findings suggest that CR increased DOX antitumor activity and cardiotoxicity as a result of enhancing its bioavailability, and decreased Dox-induced mortality in mice by a mechanism not yet defined. [© 1998 Lippincott Williams & Wilkins.]

Key words: Carcinoma, cardiotoxicity, Cremophor EL, cytotoxicity, doxorubicin, pharmacokinetics.

Introduction

The anthracycline antibiotic doxorubicin (Dox) is effective against a wide range of human neoplasms.[1,2] The drug produces a range of toxic effects such as alopecia, stomatitis, gastroenteritis and myelosuppression, but the dose-limiting toxicity is cardiotoxicity.[3] An approach to potentiate the clinical value of Dox is to enhance its therapeutic index by using combined administration with other agents.

Cremophor EL (CR), a polyethoxylated castor oil derivative used to solubilize drugs, has been found to reverse P-glycoprotein (P-gp)-mediated anticancer multidrug resistance (MDR).[4–6] It may also possess cytotoxic activity.[5,7] Moreover, CR was reported to increase the bioavailability of both Dox and its major metabolite, doxorubicinol, when given i.v. to mice.[8] However, one major problem which could be encountered in attempting to increase the efficacy of Dox against human tumors that express high levels of P-gp, by reformulation of Dox to include CR, is that the toxicity of Dox may be increased. This is suggested by the observation that P-gp expression is not only confined to tumor cells, but is also present in normal tissues in several species including humans.[9–11] The present experiment was, therefore, designed to examine the effect of CR on the tissue distribution, antitumor activity, cardiotoxicity, morbidity and/or mortality of Dox in mice.

Materials and methods

Chemicals

Doxorubicin hydrochloride (Dox) was obtained from Farmitalia Carlo Erba (Milan, Italy). CR, reduced glutathione (GSH), thiobarbituric acid (TBA), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), Trypan blue dye and RPMI 1640 media were obtained from Sigma (St Louis, MO). Silver nitrate and *n*-butanol (spectral grade) were purchased from Merck (Darmstadt, Germany). The diagnostic reagent kits for determination of lactate dehydrogenase (LDH) and creatine phosphokinase (CPK) were purchased from Stanbio (San Antonio, TX).

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Animals

Female Swiss albino mice weighing 22-25 g were obtained from the Breeding Unit of King Saud University (Riyadh, KSA). Mice were housed in a constant (22°C) temperature environment with alternating 12 h light and dark cycles with the light on at 7.00 a.m., and fed Purina chow (Grain Soils and Flower Mills Organization, Riyadh, KSA) and water *ad libitum*. The principles of laboratory animal care (NIH publication no. 85-93, revised 1985) were followed.

Cell culture

The murine Ehrlich ascites carcinoma (EAC) cell line was kindly supplied by courtesy of Dr AM Osman (Cairo, Egypt) and was maintained in our laboratory by weekly i.p. passage in female Swiss albino mice.[12]

Pharmacokinetic studies

Dox at a dose of 20 mg/kg, either alone or combined with CR (2.5 ml/kg), was administered i.p to healthy female Swiss albino mice, in a volume of 10 ml/kg body weight. At 2, 4, 8, 24 and 48 h after drug administration, four mice in each treatment group were bled from the retero-orbital sinus into heparinized tubes under light ether anesthesia and were killed by cervical dislocation. The liver, spleen, kidney, lungs and heart were rapidly excised, rinsed in ice-cold saline solution and stored at −20°C. Plasma and tissues were analyzed for Dox according to the spectrofluorometric method of Formelli *et al.*[13] as modified by Rahman *et al.*[14] Plasma (0.25 ml) was diluted to 1 ml with distilled water, followed by the addition of 0.2 ml $AgNO_3$ (33% w/v). Tissues were homogenized in 1 ml distilled water in a polytron homogenizer. To each tube, 0.2 ml $AgNO_3$ was added. The tubes were vortexed vigorously followed by the addition of 5 ml *n*-butanol saturated with water. Each tube was vortexed again for 1 min and then centrifuged at 5000 r.p.m. for 10 min. The fluorescence of the butanol extract was read in a Perkin-Elmer spectrofluorometer at 470 nm excitation and 585 nm emission. Control plasma and tissues obtained from mice given CR were treated in the same way as other samples and read in the spectrofluorometer to correct for any endogenous fluorescence. The fluorescence measured in these experiments represents both unchanged Dox and its fluorescent metabolites,[15] and thus drug levels have been expressed as μg Dox equivalents per ml plasma or g wet tissue weight.

Dox content of tumor cells

EAC cells were inoculated i.p. into female Swiss albino mice (10^7 cells/mouse). On day 7 after tumor inoculation Dox was injected i.v. in a single dose (20 mg/kg) alone or mixed with CR (2.5 ml/kg, i.v.). The ascitic fluid was removed at 2 and 4 h after administration, and tumor cells were counted, washed and centrifuged. The pellets were extracted and assayed for Dox as previously mentioned.

In vitro *cytotoxicity assay.* The growth inhibition method was used to determine the cytotoxicity of Dox and/or CR in EAC cells cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine and penicillin/streptomycin. In brief, 10^6 EAC cells, in exponential growth, were incubated with varying concentrations of Dox and/or CR for 24 h at 37°C. Cells were then counted in a hemocytometer and viability was determined by the Trypan blue exclusion method.[16]

In vivo *antitumor activity.* Female Swiss albino mice were injected i.p. on day 0 with 2.5×10^6 viable EAC cells. On day 1, mice were randomly divided into groups of 10 mice. Four groups, were given Dox (2.5 or 5 mg/kg, i.p) alone or combined with CR (2.5 ml/kg, i.p). Two control groups received CR or saline. Survival was recorded every day up to 60 days.

Toxicity studies

Cardiotoxicity. The acute cardiotoxic effect of Dox (20 mg/kg, i.p) alone or in the presence of CR (2.5 ml/kg i.p) was evaluated in healthy female Swiss albino mice (nine animals/group). At 24 h after drug administration, six mice/group were anesthetized with ether and bled from the retero-orbital sinus into heparinized tubes, and then killed by cervical dislocation. Hearts were quickly removed, extensively washed with ice-cold 0.15 M KCl and 20% (w/v) homogenates were prepared in ice-cold 0.15 M KCl solution. Plasma LDH, total CPK and CPK-MB isozyme levels were measured according to the methods of Buhl and Jackson,[17] Swanson and Wilkinson,[18] and Kachmar and Moss,[19] respectively. Total SH group reactivity was determined in heart homogenate by DTNB reaction[20] and was taken as a good approximation for GSH cardiac content. Measurement of lipid peroxidation by determination of myocardial malondialdehyde (MDA) content in the

whole heart homogenate was performed by the TBA method.[21] Three hearts from each group were routinely fixed in 10% neutral buffered formalin, and then embedded in paraffin and cut with a microtome set at a thickness of 5 μm. The sections were stained with hematoxylin & eosin and examined by light microscopy.

LD_{50}. For the comparison of the lethal toxicity of Dox alone and in combination with CR, healthy female mice received varying doses of Dox (5-50 mg/kg, i.p.) alone or mixed with CR (2.5 ml/kg). The mice were observed for 7 days after drug administration and the percent mortality was recorded. The LD_{50} was calculated using the method of Litchfield and Wilcoxon.[22]

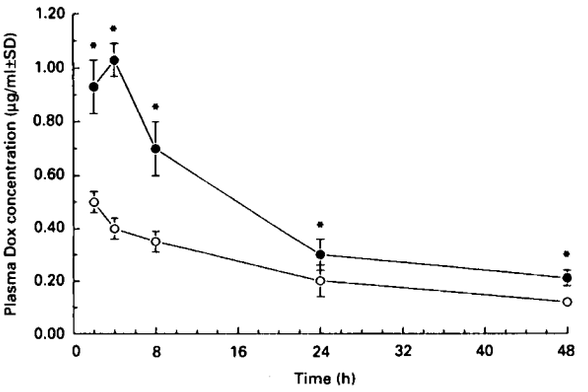

Figure 1. Plasma concentration–time relationship for Dox (open circles) and Dox+CR (closed circles). Female Swiss albino mice were injected i.p. with Dox (20 mg/kg) alone or mixed with CR (2.5 ml/kg) and plasma levels of Dox equivalent were determined by fluorescence upto 48 h. Each point represents a mean ± SD (μg Dox/ml plasma) of four animals. *Significantly different from Dox-treatment alone at $p \leq 0.01$.

Statistical methods

The statistical significance of difference between means was calculated by Student's *t*-test. The Mann-Whitney *U*-test was used to compare group median survival. Differences in end-point survivals was analysed by χ^2 test.

Results

Pharmacokinetic distribution of Dox

The plasma concentration of Dox (and its major metabolite) was significantly higher at all time points in mice that received Dox+CR compared with those given Dox alone (Figure 1). The disposition of Dox in liver, spleen, kidneys and lungs of mice given Dox (20 mg/kg, i.p) alone or combined with CR (2.5 ml/kg, i.p) is shown in Table 1. Tissue Dox levels were about 100-fold that of plasma. At 2 and 4 h, the levels were not significantly different in all tissues following administration of Dox alone or combined with CR. By 8 h, however, the drug levels in liver and kidneys were significantly ($p < 0.05$) higher in the group treated with Dox+CR, and remained so in the liver and kidneys up to 48 h. Dox levels fell off rapidly in liver, kidneys and lungs, in contrast to those in spleen. The concentration of Dox in spleen following Dox administration was 31 μg/g at 2 h and 35 μg/g at 48 h (Table 1).

The highest cardiac concentration of Dox (17.5 μg/g) was seen at 2 h following Dox+CR treatment. The corresponding value was 15 μg/g when Dox was given alone (Figure 2). Both concentrations decreased in time during the observation period of 48 h and was significantly higher in the group given Dox+CR at all time points.

Table 1. Pharmacokinetic distribution of Dox after i.p. administration of 20 mg/kg of Dox alone or combined with CR (2.5 ml/kg)

Time (h)	Liver		Spleen		Kidneys		Lungs	
	Dox	Dox+CR	Dox	Dox+CR	Dox	Dox+CR	Dox	Dox+CR
2	65±15	71±14	31±4.4	38±6.0	45±7	41±7.8	32±4.2	34±6
4	45±6.8	55±9	35±6.2	41±7	37±6.0	37.1±6.2	33±6	33±4
8	21±3.8	40±16[a]	33±5.8	40±10	20±3	27±2.0[a]	27±5	25±2
24	10±1.6	15±2.4[a]	33±6.0	41±14	8.5±1.8	13±2.0[a]	17±4	16±4
48	7±1.8	10±2[a]	35±6.0	43±10	7.5±1.2	10±14[a]	12±1.8	14±4

Values are means ± SD of four mice (μg Dox equivalents/g tissue)
[a]Significantly different from the corresponding Dox-treated mice at $p < 0.05$.

Dox tumor content

Dox levels (mean ± SD) in carcinoma cells of mice 2 and 4 h after i.v. administration of Dox (20 mg/kg) in combination with CR (2.5 ml/kg) (28 ± 4 and 38 ± 4.2 ng/10^6) were significantly higher ($p < 0.01$) than those given Dox (20 mg/kg) alone (20 ± 3 and 33 ± 4 ng/106).

Antitumor activity

In vitro *cytotoxicity.* CR enhanced the cytotoxicity of Dox against cultured EAC cells (Figure 3). The concentration of CR employed, 1 μg CR/ml medium, was equivalent to 1% of the IC_{50} for CR as a single agent in these cells (data not shown).

In vivo *antitumor activity (effect on survival).* When CR (2.5 ml/kg) was given simultaneously with Dox, improvement of survival of EAC-bearing mice was observed (Table 2). CR significantly ($p < 0.01$) increased survival of mice treated with Dox 5 mg/kg up to 40 days; however, there was no increase in 60 day survivors. On the other hand, although CR combination with Dox (2.5 mg/kg) resulted in the same median (36 days), there was 1-fold increase in long-term (60 days) survivors. Moreover, CR combined with low-dose (2.5 mg/kg) Dox resulted in 60% survival at 60 days after drug treatment compared to only 20% survival for high-dose (5 mg/kg) Dox. CR alone showed a non-significant increase in mice survival.

Cardiotoxicity

Cardiac GSH levels 24 h after treatment with Dox and/or CR were not changed compared to control values. On the other hand, cardiac MDA was significantly elevated (31 and 57%) in Dox-treated and Dox+CR groups, respectively. Cardiac MDA level (percent of control) in the Dox+CR group was significantly higher than that of the Dox group (158 ± 19 versus 120 ± 11 %).

Plasma LDH, CPK and CPK-MB activities were significantly increased in the Dox-treated mice by 106, 180 and 387% relative to the control group, respectively. CR alone showed no significant effect on the enzyme levels. CR, however, significantly increased Dox-induced elevation in the enzyme activities (Table 3).

Histopathological examination of heart tissues from Dox-treated mice showed focal necrotic myocardial fibers. The myocardial lesions were significantly higher in Dox+CR-treated mice with wavy necrosis and extensive vacuolization of cells and interstitial edema (not shown). The control and CR-treated mice showed, however, no pathological changes.

Lethal toxicity

CR (2.5 ml/kg, i.p), given mixed with Dox at different toxic dose levels, increased the LD_{50} (95% CI) of Dox in healthy mice from 9.73 (6.24–15.18) to 22.70 (16.45–31.33) mg/kg ($p < 0.05$).

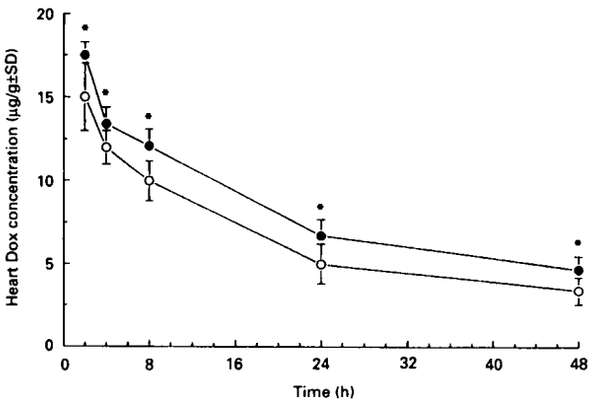

Figure 2. Elimination of Dox in heart of mice treated i.p. with Dox (20 mg/kg) alone (open circles) or combined with CR (2.5 ml/kg) (closed circles). Each point represents the mean ± SD cardiac levels (μg Dox/g tissue) of four animals. *Significantly different from Dox treatment alone at $p \leq 0.05$.

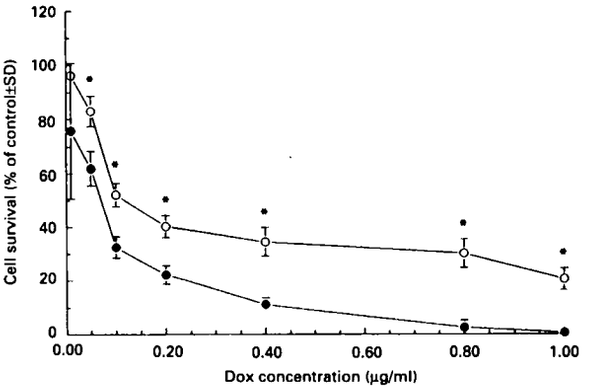

Figure 3. Cytotoxicity of Dox alone and in combination with CR in cultured EAC cells. Cells were exposed to Dox (0.025–1.0 μg/ml, open circles) alone and in the presence of CR (1 μg/ml, closed circles) for 24 h and cytotoxicity was determined by growth inhibition assay using the Trypan blue exclusion method. Each value is the mean ± SD of three experiments carried out in duplicate.

Table 2. Effect of CR on antitumor activity of Dox in EAC-bearing mice treated 1 day after tumor transplantation

Dose (mg/kg)	Mean ± SD (days)	Median (range)	T/C (%)	60 day survivors (%)
–	15.1 ± 6.8	17 (5–23)	–	0
CR 2.5	17.1 ± 5.2	19 (6–23)	113	0
Dox 2.5	33.4 ± 8.6[c]	36 (19–45)	221	30
Dox 2.5+CR	37.5 ± 16[c]	36 (19–59)	275	60
Dox 5	19.6 ± 13	18 (5–39)[a]	130	20
Dox 5+CR	40.6 ± 17[c,d]	49 (5–54)[b]	269	20

[a]Three mice died on day 5 from Dox toxicity.
[b]One mouse died on day 5 from Dox toxicity.
[c]Significantly different from control EAC-bearing mice at $p < 0.05$
[d]Significantly different from the revelant Dox-treated group at $p < 0.05$.
Mean=Mean survival time, calculated on survival time excluding 60 day survivors.

Table 3. Plasma levels of LDH, CPK and CPK-MB isozyme activities 24 h after treatment of mice with Dox (20 mg/kg, i.p.) without and with CR (2.5 ml/kg, i.p.)

	Plasma enzyme activities (U/l)		
	LDH	CPK	CPK-MB
Control	670 ± 77	216 ± 32	61 ± 13
CR	710 ± 97	240 ± 37	70 ± 17
Dox	1377 ± 237[a]	605 ± 187[a]	297 ± 77[a]
Dox+CR	1833 ± 425[a,b]	1134 ± 375[a,b]	550 ± 125[a,b]

The values are means ± SD of six mice.
[a]Significantly different from control group at $p \leq 0.05$.
[b]Significantly different from Dox group at $p \leq 0.05$.

Discussion

The present study shows that CR alters the pharmacokinetics of Dox in mice. Dox concentrations were significantly increased in plasma, heart, liver and kidney. This finding is in full agreement with the report of Webster *et al.*[8] that administration of CR just before Dox increases the bioavailability of Dox and its major metabolite, Doxorubicinol, in mice. There is a possibility that CR has inhibited P-gp-mediated biliary excretion of Dox. P-gp is not only expressed in resistant tumor cells, but also in many normal tissues,[9,11,23] where it probably helps defend against potentially toxic xenobiotics. P-gp has been demonstrated in secretory epithelial cells in the kidneys, adrenals, liver and small intestine,[24,25] and there is evidence that P-gp in the biliary canalicular membrane contributes to the biliary excretion of Dox.[26] There is another suggestion that the increased plasma concentration of Dox may be due to displacement of plasma protein bound Dox by CR. Dox binds mainly to albumin[27] and CR is known to associate in plasma preferentially with low-density lipoproteins (LDL).[28] This possible interaction may find support from the earlier finding that CR alters the biodistribution of paclitaxel by decreasing its affinity to albumin and increasing its association with LDL.[29]

CR may have increased the apparent absorption of Dox into plasma; this could be due to a prolonged Dox plasma residence time which may explain why CR addition resulted in a higher LD_{50} Dox dose. However, this possibility requires further investigation. In the presence of CR, the Dox tissue/plasma concentration ratio decreases (data not shown), whereas the plasma concentration goes up. It could be concluded that the higher tissue level of Dox in the presence of CR may be solely due to the increased Dox plasma level.

The enhancement of Dox bioavailability was evident by the increased *in vitro* cytotoxicity, increased tumoral Dox content and increased survival of EAC-bearing mice, especially at the low dose of Dox. Of interest is that CR combination with Dox (2.5 mg/kg) increased the animal survival time to more than that of the Dox double dose (5 mg/kg). Consistent with our data, Woodcock *et al.*[32] have reported that CR co-administered with Dox into mice bearing P388 transplantable tumor significantly increased the survival time of mice compared with Dox treatment alone. Moreover, Nygren *et al.*[7] demonstrated that much of the cytotoxic activity of paclitaxel (Taxol) in primary cultures of tumor cells from patients was partly mediated by its solvent, CR. However, the mechanism of CR enhancement of Dox efficacy in EAC experimental tumor remains to be elucidated.

A protective effect of CR for Dox-induced acute mortality of EAC-bearing mice was observed during the first few days of the survival study. This important observation prompted us to undertake a LD_{50} study. CR seems to protect mice against the lethal effects of Dox as evidenced by the significant increase of Dox LD_{50} in the presence of CR.

For any practical application of CR in combination with Dox, however, it was essential to examine

whether CR modified the cardiotoxicity usually associated with Dox treatment. In this regard, the conclusion reached was that concurrent treatment with CR significantly increased the Dox-induced cardiac damage as indicated by increased activities of plasma LDH and CPK enzyme levels as compared to those of Dox treatment alone. This finding was parallel to the significant elevation in cardiac lipid peroxidation. Our data are in agreement with the early reports that Dox promotes the production of free radicals[31] and these toxic species are known to cause myocardial dysfunction.[32] On the other hand, no decrease in thiol concentration was observed in response to Dox. The effect of Dox on GSH appears to occur earlier and may rebound to control values by the time of tissue sampling.[33]

The enhanced cardiotoxicity of Dox is likely to be simply a direct consequence of increased Dox heart content. This finding is in harmony with the enhanced bioavailability and antitumor activity of Dox; however, there is an apparent inconsistency with the observed protective effect of CR on Dox-induced acute death in mice. Other toxicities of Dox were not investigated in the present study. CR might have improved survival after Dox treatment by limiting Dox-induced leukopenia and thrombocytopenia; however, such an effect remains to be investigated.

In conclusion the results shown in the present study demonstrate that CR enhances Dox availability, tissue distribution, antitumor activity and cardiotoxicity in mice. In addition the lethal effect of Dox was shown to be ameliorated by the presence of CR. However, the antitumor superiority of the combination of CR and Dox over Dox alone in EAC-bearing mice is limited by the increased cardiotoxicity. Further experimental studies are required to clarify the benefit/toxic ratio of Dox+CR combination.

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