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SHORT COMMUNICATION

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Digitonin enhances the efficacy of carboplatin in liver tumour after intra-arterial administration

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Abstract Platinum-containing drugs enter the cell slowly and have a poor tissue penetration. Increasing the permeability of the cell membrane might increase the intracellular drug concentration. Digitonin, a detergent that increases cell permeability by binding to cholesterol molecules in the cell membrane, can increase cisplatin accumulation and reduce tumour growth in vitro. The aim of this study was to determine whether digitonin could increase the efficacy of carboplatin (CBDCA) in vivo. In LH rats, a hepatoma was implanted in the liver. At 7 days after implantation, digitonin (or saline in the control group) was infused via the hepatic artery and, 10 min later, CBDCA was injected. Biopsies from the tumour and liver parenchyma were obtained after 1 h. The concentration of platinum measured in the liver tumours was higher in the digitonin group than in the control groups. In the liver parenchyma the concentrations were of the same magnitude. Measured with the ^{133}Xe-clearance technique, digitonin did not alter the tumour blood flow. Digitonin enhanced the tumour-growth-retarding effect of CBDCA given intra-aterially at 5 mg/kg but not at 25 mg/kg. No increase in toxicity was observed for digitonin given together with CBDCA at 5 mg/kg. Systemic administration of CBDCA was not influenced by digitonin. These findings demonstrate that pretreatment with digitonin increases the tumour uptake of CBDCA and potentiates the cytotoxic effect of CBDCA.

Key words Carboplatin · Digitonin · Liver tumour · Tumour growth

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Introduction

One major reason for the therapeutic failure of cytotoxic drugs is that a sufficient intracellular concentration of the drug is not achieved. For platinum-containing drugs that enter the cell by passive diffusion this issue is of special importance [8]. Cell lines resistant to cisplatin (DDP) often show a reduction in DDP influx [1]. The intracellular entry of DDP is enhanced by several compounds (polymyxin B, benzalkonium chloride, cyanogen iodide, and deoxycholate) that compromise membrane integrity [8], whereas uptake is blocked by aldehydes and oubain [2]. Selective modulation of the permeability of the plasma membrane of the malignant cell might be a useful method of achieving an improved chemotherapeutic effect against tumours. Electroporation has been used to increase DDP delivery and cytotoxicity by increasing cell permeability in vitro [13]. Amphotericin B can increase DDP penetration and cytotoxicity in vitro by binding to cholesterol, but as this effect is seen only in the absence of human serum, it is probably not relevant to the clinical use of DDP [3].

Digitonin is a detergent that increases cell permeability by binding specifically to cholesterol in the cell membrane. A potential advantage of digitonin is that in low concentrations it has no effect on cholesterol-poor membranes such as the mitochondrial inner membrane and the endoplasmatic reticulum [15]. Digitonin has increased the uptake of a DDP analog into human ovarian carcinoma cells in vitro by a factor of 4 [10], and this effect was accompanied by increased platination of DNA. In vitro, DDP and digitonin have produced synergistic killing of malignant cells. Digitonin has enhanced the intracellular concentration of platinum in vivo in intraperitoneal B16 melanoma tumours in mice [11].

The aim of this study was to determine whether digitonin could increase the uptake of carboplatin (CBDCA) in tumour and whether digitonin influenced the effect of CBDCA on the tumour growth rate. As digitonin at high concentrations interferes with vital cell-membrane functions and significant plasma concentrations were antici-

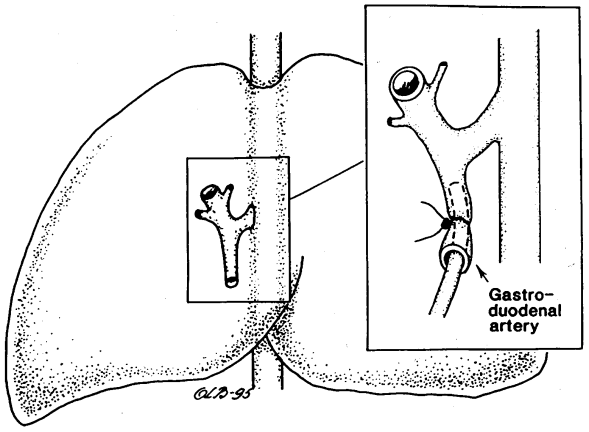

Fig. 1 The catheter was inserted via the gastroduodenal artery, with the tip being located in the proper hepatic artery

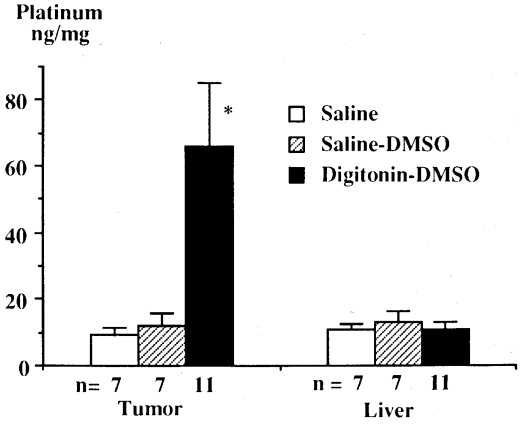

Fig. 2 Platinum content measured in hepatoma and in liver parenchyma at 1 h after intra-arterial infusion of CBDCA (25 mg/kg) with and without pretreatment with digitonin (0.2 ml 400 μ*M*). Data represent mean values ± SE. * $P < 0.05$ relative to the saline and DMSO-saline groups

pated to induce severe toxicity, a model of regional infusion into the hepatic artery in rats bearing experimental liver tumours was used. As the effect of regional treatment is dependent on the tumour blood flow, the effect on liver and tumour blood flow was also determined.

Materials and methods

Animal tumor system

Inbred rats of both sexes of the Lister-Hooded (LH) strain (B&K Universal, Sollentuna, Sweden) were kept on a normal day and night cycle and fed on standard pellets and water ad libitum. The syngeneic tumours were maintained by serial transplantation. All procedures were performed on animals under anesthesia (chloral hydrate 8%, 0,4 ml 100 g^{-1}). At 7 days before drug treatment, 1.0×10^6 cells of a 3-methyl-diaminobenzidine-induced syngeneic rat hepatoma from generation 484–525 were inoculated in the central liver lobe through a midline incision. Before measurements of the drug content and blood flow were made a catheter was inserted into the right carotid artery for continuous recording of blood pressure using a Statham transducer connected to a Grass polygraph 7C. The animals were initially randomly allocated to the different treatment schedules. The liver-tumour volume was estimated by measurement of the largest (a) and the smallest (b) perpendicular diameter [5]. The volume was calculated as $V = (a+b^2)/2$. At 7 days after tumour implantation a catheter (PE 10, inner diameter 0.28 mm, outer diameter 0.61 mm) was inserted into the hepatic artery proper via the gastroduodenal artery (Fig. 1) in the experiments in which regional drug infusion was used.[1]

Analysis of drug content

Digitonin (Sigma) was dissolved in dimethylsulfoxide (DMSO) and diluted with saline to yield a solution that contained 400 μ*M* digitonin and 40 μ*M* DMSO; 0.2 ml of the solution was infused into the hepatic artery. One group infused with 0.2 ml saline and one group treated with 0.2 ml DMSO-saline served as controls. At 10 min after the digitonin infusion, 25 mg/kg CBDCA (carboplatin, Paraplatin, 10 mg/ml; Bristol-Myers) was infused over 5 min. A 10-min interval between the infusions of the two drugs and the concentration of digitonin selected were chosen to yield a resemblance of the scheduled concentrations proven effective in vitro [11]. At 1 h after infusion the animals were killed and biopsies from the tumour and normal liver parenchyma were obtained. The biopsies were taken after 1 h, a period estimated to have permitted completion of tumour uptake from the first-pass component of total tumour exposure. The CBCDA content of tissues was measured as elemental platinum by atomic absorption spectroscopy using external standards [7] after digestion of 200 mg tissue in 0.5 ml hyamine hydroxide for 16 h at 55 °C. Samples were diluted to 5 ml with 0.15 *N* HCl, and 25 μl aliquots were used for analysis. Over the range of concentrations observed in this study the accuracy of the atomic absorption spectroscopy measurements of Pt in rat tissues ranged from 3% to 48%, the within-sample coefficient of variation was 3%, the between-day coefficient of variation ranged from 5% to 12%, and the recovery was 99±17%.

Tumor blood flow

^{133}Xe washout in the tumour and liver measured at 30–90 min after infusion into the hepatic artery of either 0.2 ml 400 μ*M* digitonin dissolved in DMSO-saline or DMSO-saline alone. ^{133}Xe at 0.4 MBq in 10–20 μl saline was injected with a fine needle into the right liver lobe or the center of the liver tumour. The washout of ^{133}Xe was registered continuously until the ^{133}Xe had completely disappeared [4]. In each animal, 15 min of washout measurements were made from four separate injections placed randomly in the tumour and normal liver parenchyma. The *k* value was calculated as the exponential rate constant and was equal to $k = ln2/t_{1/2}$. F (blood flow) was calculated as $F = k\times\lambda$. The partition coefficient (λ) was 0.57 in this tumour, which was determined by comparison of the solubility of xenon in the tumour and plasma.

Systemic treatment for determination of antitumour effect

At 7 days after tumour inoculation, CBCDA (25 mg/kg) or saline was infused into the tail vein, preceded by digitonin (1 ml 200 μ*M* digitonin) or by DMSO-saline as a control, daily for 3 days. The animals were killed on the 4th day, an autopsy was performed, and the tumour, liver, and spleen were weighed.

Regional treatment for determination of antitumour effect

Digitonin was dissolved in DMSO and diluted with saline to 400 μ*M*, and 0.2 ml was infused into the hepatic artery. A 0.2-ml dose of DMSO-saline was infused in the control group. At 10 min after the digitonin infusion, CBDCA (5 or 25 mg/kg) or saline was infused over

[1] This work was approved by the Ethics Committee for Animal Research of the University of Göteborg

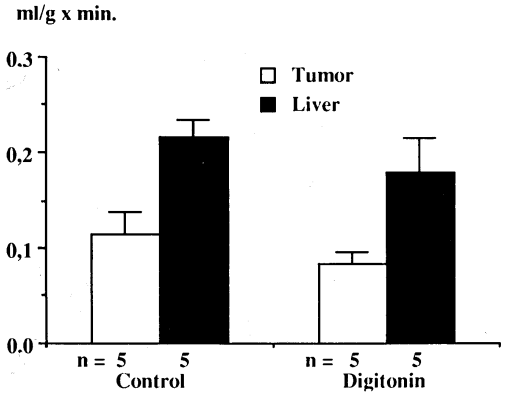

Fig. 3 Blood flow measured in tumour and liver parenchyma at approximately 1 h after intra-arterial infusion of digitonin (0.2 ml 400 μ*M*). Data represent mean values ± SE. Each *bar* represents the mean of 2 measurements undertaken at the same site

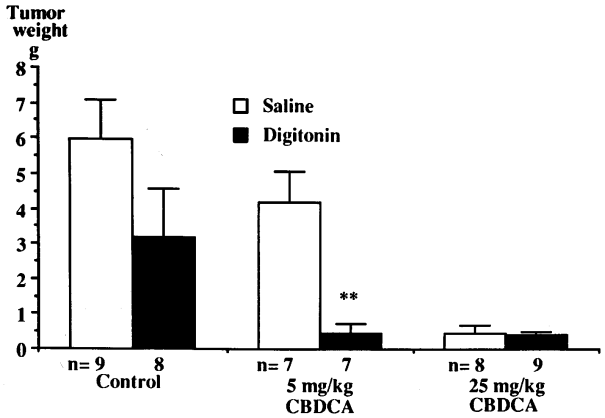

Fig. 4 Tumour weight determined in LH rats afflicted with hepatoma after intra-arterial treatment with digitonin (0.2 ml 400 μ*M*) and CBDCA (5 and 25 mg/kg). Data represent mean values ± SE. ** $P < 0.01$ relative to the control group

5 min. After the infusion the catheter was withdrawn and the gastroduodenal artery was ligated. The tumour volume was measured after 4 and 7 days. The animals were killed after 7 days and the tumour, liver, and spleen were weighed. Blood samples were taken at 1, 4, and 7 days after the experimental procedure and samples were analyzed for ASAT and ALAT (Reflotron, Boehringer Mannheim, Germany).

Statistical analysis

The results are given as mean values ± SE. One-factor analysis of variance (ANOVA) followed by Fischer's PLSD test were used (or Student's *t*-test when relevant). Differences were considered to be significant when $P < 0.05$.

Results

After pretreatment with digitonin the platinum content in the tumour as measured at 1 h after CBDCA infusion was 66 ± 19 ng/mg. This was more than 5 times higher ($P < 0.05$) than the values obtained after pretreatment with saline alone (12 ± 4 ng/mg) or with DMSO-saline

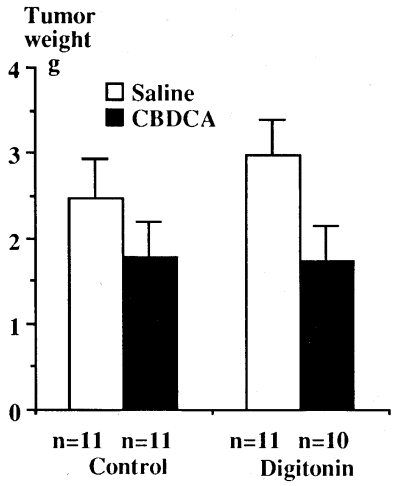

Fig. 5 Tumour weight determined in LH rats afflicted with hepatoma after 3 days of i.v. treatment with digitonin (1 ml 200 μ*M*) and CBDCA (25 mg/kg). Data represent mean values ± SE

(10 ± 2 ng/mg; Fig. 2). The platinum content measured in the liver parenchyma was 11 ± 2 ng/mg in the digitonin-treated group, which was in the same range as the values found in the group given saline alone (13 ± 3 ng/mg) and in the DMSO-saline group (11 ± 2 ng/mg). No difference in mean arterial blood pressure was found between the groups during the experiments. To avoid poor mixing with the hepatic blood flow and the risk of uneven drug distribution in the liver [12], a rapid infusion rate (0.2 ml/min) was chosen. This is less than 20% of the estimated blood flow in the hepatic artery (1.2–1.5 ml/min). After the injection of blue dye at this flow rate, no retrograde flow into the aorta was observed. The blood flow in the tumour (tumour volume 165 ± 43 mm^3) was 0.17 ± 0.03 ml g^{-1} min^{-1}, which was lower than the blood flow in the liver parenchyma (0.33 ± 0.03 ml g^{-1} min^{-1}, $P < 0.001$). Administration of digitonin in the liver artery did not induce any significant change in tumour blood flow (Fig. 3).

At 7 days after regional treatment there was a high correlation between tumour volume and tumour weight ($r = 0.97$). At a CBDCA dose of 5 mg/kg, regional infusion of digitonin prior to regional CBDCA infusion produced a significantly greater growth-retarding effect on the tumour than did regional CBDCA preceded by DMSO-saline alone. Indeed, at this dose of CBDCA, tumours that had been pretreated with digitonin had only 12% of the weight of tumors treated with CBDCA alone (Fig. 4). In animals treated with CBDCA at 25 mg/kg it was not possible to detect any additional effect of digitonin on tumour weight. In animals given systemic treatment with i.v. CBDCA at 25 mg/kg for 3 days it was not possible to detect any effect of systemic pretreatment with digitonin (Fig. 5). However, CBDCA given alone at this dose also had no significant effect on tumour growth rate as compared with untreated controls. There was no increased mortality in the groups subjected to treatment with digitonin and/or CBDCA as compared with untreated controls. The body weight loss

Table 1 Body weight loss observed during the 7 days after regional drug infusion and ALAT and ASAT values determined at 1, 4, and 7 days after drug infusion. Data represent mean values ± SE

	CBDCA	Body weight loss (%)	ALAT Day 1 (μkat/l)	ALAT Day 4 (μkat/l)	ALAT Day 8 (μkat/l)	ASAT Day 1 (μkat/l)	ASAT Day 4 (μkat/l)	ASAT Day 7 (μkat/l)
Digitonin	25 mg/kg	11.3±2.0*	9.0±1.3	7.8±3.1**	5.1±1.9**	15.7±2.2	14.0±3.8**	14.4±3.6**
	5 mg/kg	4.8±1.1	8.0±2.3	1.9±0.7	0.7±0.1	14.0±2.5	3.2±0.7	2.1±0.4
	Control	5.2±1.7	7.9±1.4	0.6±0.1	1.9±1.1	16.0±2.0	2.3±0.3	4.0±1.1
Saline	25 mg/kg	10.2±1.4*	5.9±1.4	2.6±0.6	1.2±0.3	14.4±2.2	7.6±4.2	7.2±4.4
	5 mg/kg	4.0±0.7	4.0±1.6	0.9±0.1	1.0±0.3	10.6±2.4	2.2±0.3	4.6±0.9
	Control	7.8±0.7	5.9±1.4	1.3±0.5	0.8±0.1	13.6±2.6	4.2±0.8	7.5±2.6

* $P < 0.05$ relative to untreated controls; ** $P < 0.05$ relative to the corresponding saline group

was significantly higher in the two groups given regional CBDCA at 25 mg/kg (Table 1).

Digitonin treatment did not influence body weight loss. No difference was found in liver and spleen weight between the groups. ALAT and ASAT values were measured at 1, 4, and 7 days after treatment; ALAT and ASAT levels were elevated in all groups on the day after the operative procedure, but no difference between the groups was apparent. Digitonin given together with CBDCA at 5 mg/kg did not elevate the ALAT and ASAT levels, but digitonin infused together with CBDCA at 25 mg/kg elevated the ALAT and ASAT levels significantly at both 4 and 7 days after treatment as compared with untreated controls.

Discussion

As compared with many other cytotoxic agents, the platinum-containing drugs enter cells relatively slowly [9]. In the case of human ovarian carcinoma cells, steady-state equilibrium is not reached for 24–48 h [11]. This is a major limitation to the use of these drugs when they are given by the i.v. route, since the half-life of free DDP is approximately 30 min and that of free CBDCA is 15–20 min [14]. The slow entry of drug is even more of a problem when DDP and CBDCA are given by the intra-arterial route, since the relative advantage of an intra-arterial infusion is limited to the extra drug exposure that occurs during the first passage of drug through the tumour [6]. In principle, anything that selectively permeabilizes the plasma membrane to DDP or CBDCA should increase drug uptake and, therefore, cell kill, and this should be particularly true in the setting of intra-arterial drug administration, where extremely high, albeit transient, drug concentrations can be attained.

The same pharmacological principles that make intra-arterial infusion of a cytotoxic agent attractive as a strategy for attaining selectivity also make intra-arterial (i.a.) infusion of a permeabilizing agent attractive. The relative advantage of i.a. infusion over i.v. infusion of the same dose (R_t) is a function of tumour blood flow (Q), plasma clearance (CL_p), and the extraction ratio of the drug by the target region (Et as given in Eq. 1):

$$Rt = \frac{CL_p}{Q(1 - E_t)}. \quad (1)$$

The greater the systemic clearance, the lower the total exposure to the systemic circuit once the drug has passed through the tumour bed. The smaller the tumour blood flow, the smaller the extent of dilution of the drug during injection into the tumour bed. The primary concern regarding the administration of most membrane-permeabilizing agents is their potential for serious systemic toxicity. However, even small doses delivered i.a. may produce concentrations in the tumour bed that are sufficient for permeabilization, the dilution into the total blood pool combined with rapid plasma clearance being sufficient to limit the risk of nonspecific toxicity to other tissues.

Permeabilization of the plasma membrane with detergent or electroporation can increase the uptake of DDP and CBDCA [8, 11, 13, 16], and we have previously shown in vitro that this can be accomplished with appropriate concentrations of digitonin and that the effect of digitonin is synergistic with that of DDP [10]. The experiments reported in this paper extend this concept to the in vivo setting. Administration i.a. of a small dose of digitonin a few minutes prior to the i.a. administration of CBDCA resulted in a selective 6-fold increase in the uptake of CBDCA, measured as elemental platinum, in the tumour but caused no increase in the uptake of CBDCA in the normal hepatic parenchyma.

Most importantly, the regional administration of digitonin and CBDCA via the hepatic artery at an efficient dose did not increase the toxicity of CBDCA to either the liver, as reflected by ALAT and ASAT levels, or the whole animal, as evidenced by change in body weight. However, with increasing CBDCA dose a local toxicity in the liver is observed.

The mechanism by which digitonin was capable of increasing CBDCA uptake in the hepatoma but not in normal hepatic parenchyma is not currently understood. Injection of digitonin via the hepatic artery did not alter either tumour or normal parenchymal blood flow signifi-

cantly; thus, one could argue that the selective effect is not likely to be due to differential delivery of CBDCA. However, the possibility that digitonin produced differential changes in blood flow at the capillary level has not been excluded. Likewise, although is apparent that digitonin altered the access of CBDCA to the tumour, it is possible that this resulted from an effect on capillary permeability rather than on the permeability of the cancer-cell membrane itself.

The finding that digitonin did not increase CBDCA uptake or efficacy when the latter was given at the very high dose of 25 mg/kg and the observation that digitonin failed to increase CBDCA efficacy when both drugs were given i.v. provide confirmation of the pharmacological principles on which this approach is based. Under conditions whereby the tumour-bed concentration of CBDCA is high enough that factors other than permeability limit drug uptake, one would expect no additional effect of digitonin. Likewise, under circumstances whereby the concentration of digitonin in the tumour bed never reaches a level sufficient to alter permeability, one would expect little effect.

There is little information on the animal or human toxicology of digitonin and no information on its pharmacology. However, even if digitonin enhances the efficacy of CBDCA, it is possible that digitonin is not the optimal detergent for use by the i.a. route and that other agents have less general toxicity and more favorable clearance characteristics.

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