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In vitro cytotoxicity of paclitaxel/β-cyclodextrin complexes for HIPEC

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ABSTRACT

Hyperthermic intraperitoneal chemotherapy (HIPEC) is a promising strategy in the treatment of peritoneal carcinomatosis. To perform HIPEC, a tensioactive- and solvent-free paclitaxel formulation consisting of water-soluble paclitaxel/randomly methylated-β-cyclodextrin (Pac/RAMEB) complexes was developed previously. Using MTT and SRB assays the cytotoxic activity of this formulation versus Taxol®, was evaluated as well as the cytotoxicity of the different formulation excipients (RAMEB and Cremophor EL®). The possible synergistic effect of heat and paclitaxel-based chemotherapy during HIPEC was also evaluated in vitro. The cytotoxicity assays revealed differences in viability between Cremophor EL® and RAMEB treated cells of 40 and 50% for the CaCo-2 human and the CC531s rat colon cancer line, respectively, in favour of RAMEB. Despite the higher cytotoxicity of Cremophor EL®, Pac/RAMEB complexes and Taxol® were equipotent. Using the MTT and SRB assays the average difference in viability between both cell lines was below 10% and IC50 values showed no significant difference. Hyperthermia after drug administration (41 °C during 1 h) had no effect on cell viability. These results indicated that it was possible to reformulate paclitaxel with a less cytotoxic vehicle while maintaining the cytotoxic activity of the formulation and that there is no synergism between paclitaxel and heat for in vitro cytotoxicity.

1. Introduction

Peritoneal carcinomatosis is characterised by metastatic implants on the peritoneal surface of patients suffering in most cases from intra-abdominal cancer, i.e. stomach, colon, pancreas or ovarium cancer. For example, after colon cancer resection approximately 25–35% of the patients will develop peritoneal carcinomatosis (Minsky et al., 1988). Due to the ascites, which frequently accompanies peritoneal cancer, the patients have a very low quality of life combined with a high morbidity and mortality (McQuellon et al., 2001). Peritoneal carcinomatosis remains difficult to cure because even extended surgery can never completely remove all cancer cells embedded in the peritoneum. The median survival of these patients is approximately one year when treated intravenously with 5-fluorouracil-based chemotherapy (5-FU) and palliative surgery (Machover, 1997). Hyperthermic intraperitoneal chemotherapy (HIPEC) after debulking surgery is a strategy in the treatment of this type of cancer. This treatment does not only allow the use of a higher dosage of the drug because of the peritoneum–plasma barrier, it also combines the tumoricidal properties of hyperthermia and various chemotherapeutic drugs. Research has shown a synergism between hyperthermia and some cytotoxic drugs, including doxorubicin (Jacquet et al., 1998), gemcitabin (Pestieau et al., 1998), cisplatin (Bartlett et al., 1998), mitomycin C (Teicher et al., 1981) and immunomodulators like TNF (Bartlett et al., 1997). Randomized clinical trials also supported the use of HIPEC in the treatment and prevention of peritoneal carcinomatosis following resection of pT3 or pT4 gastric cancer (Ceelen et al., 2000).

Paclitaxel (Pac), one of the most potent cancer drugs of recent years, is a good candidate for HIPEC due to the limited absorption from the peritoneal cavity, its high first-pass effect in the liver and its activity in ovarian cancer (Witkamp et al., 2001). However, a major problem of paclitaxel is its low aqueous solubility. This problem is currently overcome by dissolving paclitaxel in a Cremophor EL®/ethanol-mixture (1/1, v/v) which is commercially available as Taxol®. Since the Cremophor EL® fraction in this formulation can cause hypersensitivity reactions, patients have to be pre-treated with corticosteroids and antihistamines when receiving an intravenous administration of Taxol® (Gelderblom et al., 2001; van Zuylen et al., 2001). Considering that high doses of Cremophor EL® and ethanol would be administered during a

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HIPEC procedure (due to the large volume of perfusion liquid used (1.5 l/m^2 body surface area) and the high intraperitoneal paclitaxel concentration required), there was a need for a solvent- and tensioactive-free paclitaxel formulation suitable for HIPEC. Therefore a paclitaxel formulation was developed using randomly methylated-β-cyclodextrins (RAMEB), resulting in a new solvent- and tensioactive-free formulation consisting of Pac/RAMEB complexes (Bouquet et al., 2007). The absence of ethanol is noteworthy because a previous study still used ethanol in their formulation (Szente et al., 1999). The current paper investigates the in vitro efficiency of this newly developed formulation versus Taxol® using a human (CaCo-2) and a rat colon (CC531s) cancer cell line. In addition the cytotoxicity of the excipients used in both formulations is compared and the possible synergism between paclitaxel and heat during application of HIPEC is determined.

2. Materials and methods

2.1. Materials

Randomly methylated-β-cyclodextrin (RAMEB) with a degree of substitution (DS) of 13 was purchased from Cyclolab (Budapest, Hungary), paclitaxel (Pac) from Acros Organics (Geel, Belgium), Taxol® from Bristol-Myers Squibb (Brussels, Belgium) and Cremophor EL® from Alpha Pharma (Waregem, Belgium).

2.2. Preparation of the inclusion complexes

The required amount of paclitaxel was dissolved in absolute ethanol (Merck, Overijse, Belgium), in a ratio of 1/60 (w/w). Randomly methylated-β-cyclodextrin (RAMEB) was added to the solution to obtain Pac/RAMEB ratios varying from 1/60, 1/40 to 1/20 (mol/mol) and the solution was placed in an ultrasonic bath (Sonis 3 GT, Iskra-Pio, Šentjernej, Slovenia) for 5 min. Next, phosphate buffered saline (PBS, 0.01 M, pH 7.4) (1 PBS tablet (Sigma, Bornem, Belgium) in 200 ml demineralised water) was added in a ratio of 1/2 (w/w) versus the RAMEB fraction. Next, the solution was placed in an ultrasonic bath for 1 min and afterwards stirred with a magnetic stirrer for 5 min. After evaporation of most of the solvent under reduced pressure, the solution was frozen at −70 °C using solid carbon dioxide and freeze dried for 24 h at −50 °C and 1 mbar. After freeze drying, a white amorphous powder was obtained (Alcaro et al., 2002).

2.3. Determination of the administered paclitaxel concentrations

The HPLC-system consisted of a pump (L-6000, Merck-Hitachi, Tokyo, Japan), an integrator (D-2000, Merck-Hitachi), an injector (Vici, Valco Instruments, Houston, USA) with a 25 μl loop and a UV/vis detector (UV 2000, Spectra-systems, Darmstadt, Germany). Detection was performed at a wavelength of 227 nm. Chromatographic separation was achieved with a guard column (Lichrospher® 100-RP-18, 4 mm × 4 mm (5 μm), Merck, Darmstadt, Germany) and an analytical column (Lichrospher® 100-RP-18, 125 mm × 4 mm (5 μm), Merck). Before use, the mobile phase consisting of acetonitrile (Biosolve, Valkenswaard, The Netherlands) and 0.1% (v/v) phosphoric acid in water (Acros Organics) (42:58, v/v) was degassed by ultrasonication under vacuum. A calibration curve was validated for a concentration ranging from 1 to 100 μg paclitaxel/ml.

2.4. Cell culture

The human colon adenocarcinoma cell line, CaCo-2, was obtained from the Institut National de la Santé et de la Recherche Médicale (Unité 55 Hopital St-Antoine, Paris, France). The rat colon cancer cell line, CC531s, was obtained from the Laboratory of Experimental Oncology (University Antwerp, Belgium). Both cell lines were maintained in T 25 culture flasks (Sarstedt, Newton, NC, USA) at 37 °C in a humidified atmosphere containing 10% CO_2. Dulbecco's modified medium (DMEM; Invitrogen, Merelbeke, Belgium), was supplemented with 10% foetal bovine serum (FBS; Invitrogen), 100 IU/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen) and 2.5 μg/ml fungizone (Bristol Myers Squibb, Brussels, Belgium), was used as growth medium for both cell lines. For subculturing, cells were rinsed with Moscona's solution (8.0 g NaCl; 0.3 g KCl; 0.05 g $Na_2HPO_4.H_2O$; 0.025 g KH_2PO_4; 1.0 g $NaHCO_3$ and 2.0 g dextrose in 950 ml distilled water) and were finally detached with trypsin/EDTA (Invitrogen). The viability of the cells was determined by their ability to exclude 0.4% trypan blue dye (Sigma). All cells were tested free of mycoplasma with DAPI staining.

2.5. Cytotoxicity assays

The cytotoxicity of Taxol® and the three Pac/RAMEB formulations (1/20, 1/40 and 1/60 mol/mol ratio or 1/30, 1/60 and 1/90, w/w ratio) was tested at paclitaxel concentrations of 0.01, 0.1, 1, 5 and 10 μg/ml (corresponding to 0.012, 0.12, 1.17, 5.86 and 11.72 μM, respectively). In the case of Taxol® these paclitaxel concentrations corresponded to a Cremophor EL® concentration ranging from 0.880 to 880 μg/ml. In the case of Pac/RAMEB complexes the paclitaxel concentration corresponded to a cyclodextrin concentrations varying between 0.3 and 900 μg/ml depending on the molar ratios. The cytotoxicity of RAMEB and Cremophor EL was also tested separately (without drug) using 10 concentrations: 0, 0.01, 0.1, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 mg/ml.

To evaluate the cytotoxicity, cells were seeded in 96-well plates (Sarstedt) at a concentration of 15×10^3 and 60×10^3 cells/ml for the CC531s and CaCo-2 cell line, respectively. After 24 h, 20 μl medium was replaced by 20 μl drug (or excipient) solution to obtain the required drug (or excipient) concentration. Eight wells per concentration were used and all experiments (MTT and SRB) were repeated ($n = 2$). After drug administration, the plates were incubated for 1 h at normothermic (37 °C) or hyperthermic (41 °C) conditions. Next the cells were incubated for 96 h at 37 °C and 10% CO_2. Hereafter, the cytotoxicity of the formulations was determined via MTT and SRB assays.

2.5.1. MTT assay

One hundred microlitres medium was replaced by 100 μl MTT-reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-difenyl-tetrazolium bromide, at a concentration of 1 mg/ml in PBS-D^+). The reagent was mixed and incubated in dark for 2 h at 37 °C. Afterwards all medium was removed and 200 μl DMSO (Acros Organics) was added to dissolve the formed formazan. After incubation of the plates for 1 h at 37 °C and mixing, the optical density was measured at 490 nm using an ELISA reader (Molecular Devices, Sunnyvale, USA).

2.5.2. SRB assay

The SRB test was initiated by fixing the cells via addition of 50 μl of 50% trichloracetic acid (Sigma) to the incubation medium and incubating the plates for 1 h at 2 °C. Afterwards, the wells were rinsed five times with water and dried. The cells were stained with 200 μl SRB (0.4% in 1% acetic acid) (Sigma) for 30 min and rinsed 4 times in 1% glacial acetic acid (Novolab, Geraardsbergen, Belgium). After drying the 96 well plate, 200 μl 10 mM Tris buffer (pH 10.5) was added per well to release the dye. After mixing, the optical density was measured at 490 nm with an ELISA reader (Molecular Devices).

For both assays, cell viability of each well was expressed using the following equation:

$$\text{Cell viability}\,(\%) = \left(\frac{\text{Absorbance}_{\text{test cell}}}{\text{Absorbance}_{\text{control cells}}}\right) \times 100$$

2.6. Statistical analysis

For each individual experiment the 50% inhibitory drug concentration (IC50) was determined using probit analysis. To determine the difference in cytotoxicity between RAMEB and Cremophor EL® the mean cell viability per excipient concentration was determined (i.e. average of 8 wells). Next, the mean cell viability of RAMEB at a specific concentration was subtracted from the mean cell viability of Cremophor EL® at the same concentration. This was done at all 9 concentrations of the formulation excipients (excluding the blank), thus creating a new set of 9 variables. Of this group ($n = 9$) the overall average and its 95% confidence interval was determined. When the cytotoxicity experiments were repeated ($n = 2$, performed on different days) the average difference in viability of all experiments was $6.6 \pm 5.1\%$ and $6.8 \pm 6.3\%$ for the CC531s and CaCo-2 cell line, respectively. As this percentage was caused by (biological) variation of the experiment (i.e. the MTT and SRB test), a 10% difference in cytotoxicity was set as cut-off value to determine the equivalence in cytotoxicity between RAMEB and Cremophor EL®. When the difference of the overall average in cell viability between both excipients was less than 10% (absolute value), their cytotoxic activity was considered equipotent. The same protocol was followed when comparing normo- versus hyperthermic conditions. In this case the viability at normothermic conditions was subtracted from the viability at hyperthermic conditions. The equivalence of the cytotoxic activity of the paclitaxel/RAMEB complex and Taxol® formulation was analysed using a similar procedure: 5 concentrations (excluding the blank) were used to determine the difference in overall cytotoxicity of Taxol versus paclitaxel/RAMEB complexes. Repeated experiments revealed a biological variation of $6.4 \pm 6.1\%$ and $8.12 \pm 6.7\%$ for the CC531s and CaCo-2 cell line, respectively. Hence, a 10% difference was again chosen as cut-off value to determine equipotency of both formulations. All other statistical analyses were performed using a one-way-ANOVA test (two-sided) and p-values of less than 5% were considered statistically significant. Statistical Program for Social Scientists (SPSS 14.0) was used to analyse the results.

3. Results

3.1. Cytotoxicity of Cremophor EL® and RAMEB

In a first series of experiments, the cytotoxicity of the formulation excipients was investigated, in a concentration that ranged from 0.01 to 3.5 mg/ml. The viability graphs (Fig. 1) showed that the cell viability of RAMEB and Cremophor EL® was comparable at the lowest concentrations (0.01 and 0.1 mg/ml) but at approximately 0.5 mg/ml a sharp drop in viability was observed in case of Cremophor EL®, reaching a minimum viability at 1.5 mg/ml. At hyper- and normothermic conditions, the viability graphs of RAMEB indicated a viability of more than 50% for both cell lines and both staining methods at the highest concentration (3.5 mg/ml) tested (Fig. 1B–D), except for the SRB staining of the CaCo-2 cell line with a viability of 50% for a concentration of 3 mg/ml (Fig. 1A). This was confirmed by the determination of the IC50 values. Compared to RAMEB, the IC50 values for Cremophor EL® at hyper- and normothermic conditions were much lower for both cell lines. The IC50 values of the SRB tests for Cremophor EL® were between 0.1 and

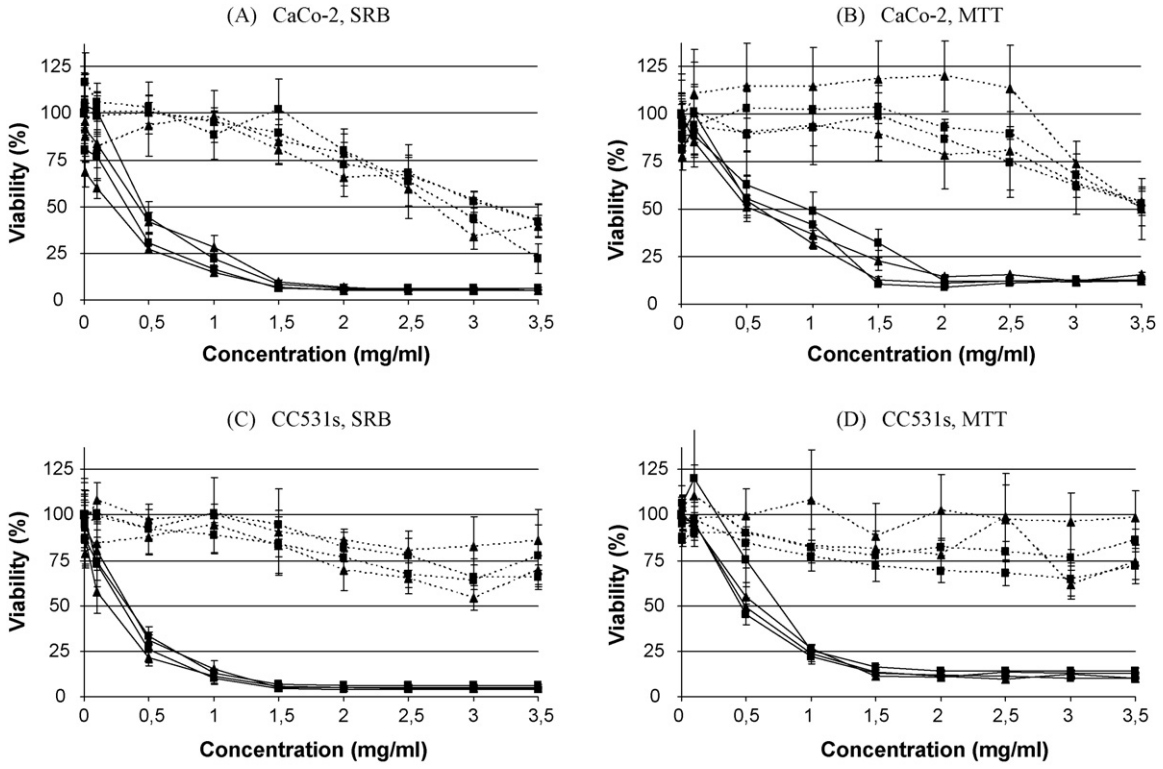

Fig. 1. Viability graphics (determined via SRB and MTT cytotoxic assays) of the CaCo-2 and CC531s cell line after addition of different concentrations of Cremophor EL and RAMEB at normothermic (37 °C) and hyperthermic (41 °C) conditions ($n = 8$ wells per concentration, error bars represent SD). (–▲–) Cremophor EL® 37 °C, (–■–) Cremophor EL® 41 °C, (- -▲- -) RAMEB 37 °C and (- -■- -) RAMEB 41 °C. The replicate experiments ($n = 2$) of the SRB and MTT test are presented as individual data sets, not as mean value.

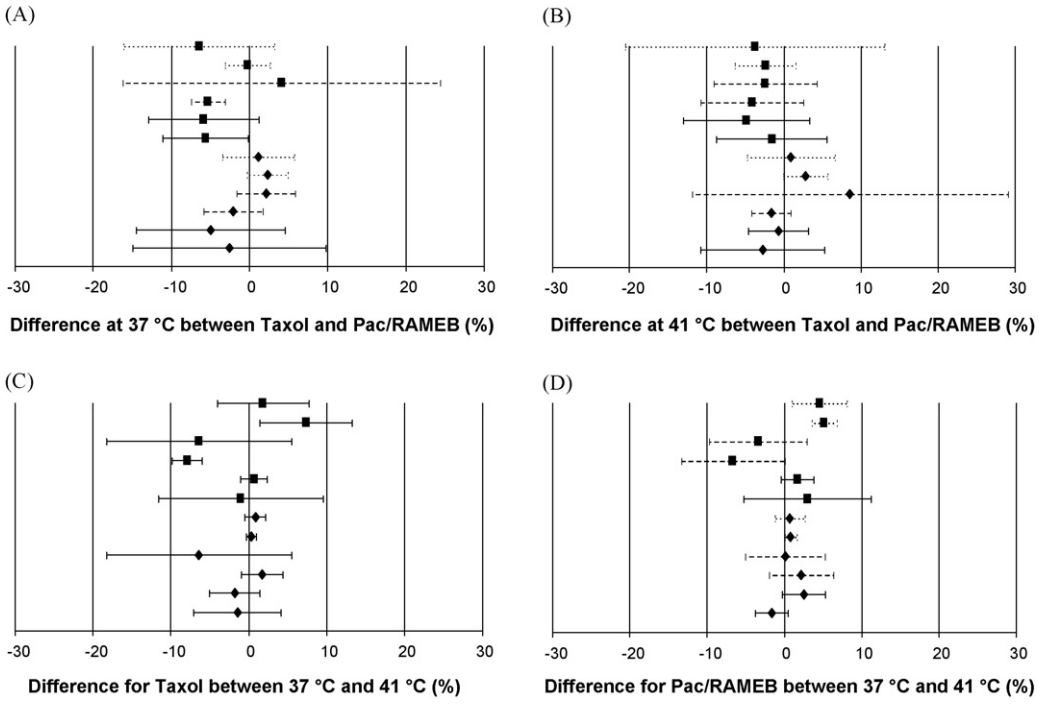

Fig. 2. Mean (n = 5 concentrations and 8 wells per concentration) and 95% confidence intervals of the difference in cell viability (determined via SRB (♦) and MTT (■) cytotoxicity assays, n = 2) between Taxol® and Pac/RAMEB at normo- (A) and hyperthermic conditions (B) and between normo- and hyperthermic conditions for Taxol® (C) and Pac/RAMEB (D) for the CC531s cell line. (—) 1/60 (mol/mol) ratio, (- - - -) 1/40 (mol/mol) ratio and (···) 1/20 (mol/mol) ratio.

0.5 mg/ml for both cell lines. The MTT test resulted in IC50 values between 0.5 and 1 mg/ml and between 0.3 and 0.7 mg/ml for the CaCo-2 and CC531s cell line, respectively. No statistical analysis was performed between RAMEB and Cremophor EL® as the majority of IC50 values for RAMEB were above the highest concentration tested and could therefore not be included in the statistical test. The large differences in cytotoxicity were further confirmed by determining the equivalence of both excipients. Here, the minimum difference in cytotoxicity between both excipients at 37 °C for both assays was 50.0 and 42.2% for the CC531s and CaCo-2 cell line, respectively. Hyperthermia for 1 h at 41 °C did not increase the cytotoxicity of either excipient. In case of CaCo-2 cells the largest difference in cytotoxicity between both temperatures was −7.4% for the SRB test, whereas these differences ranged between −2.8 and 1.2% for the MTT test. Similar results were found when investigating the effect of heat using the CC531s cell line: the absolute differences between RAMEB and Cremophor EL® were smaller than 10% (except for one MTT experiment of RAMEB: 15.7%). Heat was unable to create differences larger than the biological variation of the experiment.

3.2. Cytotoxicity of Taxol® and paclitaxel/RAMEB complexes

In a next step the cytotoxic activity of the Pac/RAMEB formulations and Taxol® was evaluated. The procedure, which was used to evaluate the cytotoxicity of the formulations on the cancer cell lines, was based on the HIPEC procedure intended during in future in vivo work. Three molar Pac/RAMEB ratios (1/20, 1/40 and 1/60) were tested because a difference in stability as a function of the Pac/RAMEB ratio was revealed in a previous study (Bouquet et al., 2007) which might affect the cytotoxicity of these inclusion complexes. In addition, the highest ratio (1/90, w/w) was included as this ratio is similar to the Pac/Cremophor EL® ratio used in Taxol® (1/88, w/w).

3.2.1. CC531s cell line

The viability data of the CC531s cell line revealed differences all below 10% in cytotoxic activity between Taxol® and the 3 different Pac/RAMEB formulations at 37 °C (Fig. 2A): the average difference in viability varied between −5.0 and 2.3% for the SRB test (Table 1A) and between −6.5 and 4.0% for the MTT test (Table 1B).

Table 1
Minimum (Min.) and maximum (Max.) mean (n = 5 concentrations and 8 wells per concentration) differences in cell viability (determined via MTT and SRB cytotoxicity assays, n = 2) between Pac/RAMEB (all three ratios) and Taxol® at a temperature (T) of 37 °C and 41 °C.

Cell line	Test	T (°C)	Min. difference (%)	Confidence interval (%)	Max. difference (%)	Confidence interval (%)
CC531s						
A	SRB	37	−5.0	[−14.5; 4.5]	2.3	[−0.4; 4.9]
B	MTT		−6.5	[−16.1; 3.2]	4.0	[−16.3; 24.4]
C	SRB	41	−2.7	[−10.8; 5.3]	8.6	[−11.9; 29.1]
D	MTT		−4.8	[−13.0; 3.4]	−1.6	[−8.7; 5.6]
CaCo-2						
E	SRB	37	−9.2	[−21.2; 2.9]	3.3	[0.5; 6.1]
F	MTT		−8.5	[−11.6; −5.5]	3.3	[−3.1; 9.7]
G	SRB	41	−10.0	[−14.0; −6.1]	3.7	[−2.2; 9.5]
H	MTT		−7.6	[−20.2; 4.9]	7.1	[−2.5; 16.6]

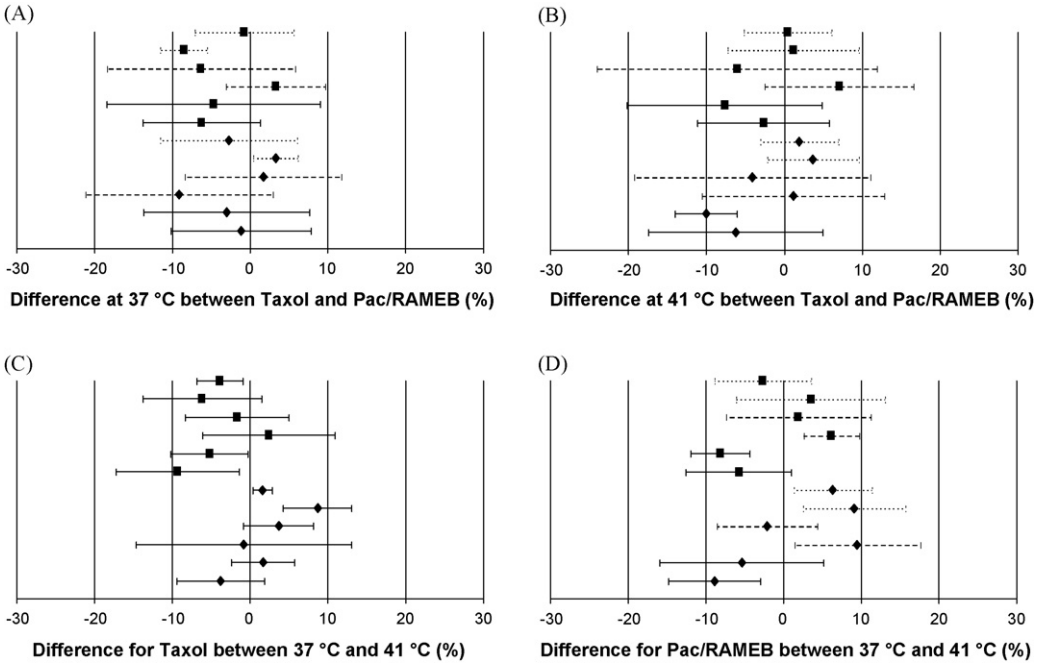

Fig. 3. Mean ($n = 5$ concentrations and 8 wells per concentration) and 95% confidence intervals of the difference in cell viability (determined via SRB (♦) and MTT (■) cytotoxicity assays, $n = 2$) between Taxol® and Pac/RAMEB at normo- (A) and hyperthermic conditions (B) and between normo- and hyperthermic conditions for Taxol® (C) and Pac/RAMEB (D) for the CaCo-2 cell line. (—) 1/60 (mol/mol) ratio, (- - - -) 1/40 (mol/mol) ratio and (···) 1/20 (mol/mol) ratio.

At 41 °C, the differences in cell viability between Taxol® and the Pac/RAMEB complexes remained below 10% (Table 1C and D). At both temperatures, the two different types of formulation were equivalent (Fig. 2A and B). Heat was unable to significantly increase or decrease the differences in cell viability between Taxol® and the Pac/RAMEB complexes (p-value of 0.376 and 0.963 for the SRB and MTT test, respectively). This showed that the two types of formulation were similarly affected by heat. The effect of the addition of heat was also studied for each formulation individually. For Taxol®, the differences in cell viability (Fig. 2C) between the different temperatures (37 °C vs. 41 °C) were small: all below 10% and on average: $-0.9 \pm 5.6\%$ and $-1.1 \pm 2.9\%$ for the MTT and SRB test, respectively. For Pac/RAMEB (not considering the different ratios) the average difference in viability (Fig. 2D) between normothermic and hyperthermic treatment was $0.7 \pm 4.7\%$ and $0.8 \pm 1.5\%$ for the MTT and SRB test, respectively. These observations showed that heat did not improve the activity of both formulations.

The IC50 values obtained after evaluation of both types of formulation at 37 °C did not show a difference: all values were smaller or equal than the lowest paclitaxel concentration (0.01 μg/ml) tested, except for two MTT tests of Taxol® (IC50: 0.03 and 0.05 μg/ml) and for one MTT test of the complexes (IC50: 0.06 μg/ml). The IC50 values at 41 °C were similar to the values obtained at 37 °C: most IC50 values (±80%) were smaller than the lowest concentration tested (0.01 μg/ml) and could not be taken into account for statistical analysis to compare between the two temperatures.

3.2.2. CaCo-2 cell line

The results (Fig. 3) of the CaCo-2 cell line were similar to the CC531s cell line. All of the Pac/RAMEB formulations were equivalent to Taxol® at both temperatures (Fig. 3A and B). The difference in viability at 37 °C ranged between −9.2 and 3.3% for SRB (Table 1E) and between −8.5 and 3.3% for MTT (Table 1F). The difference in viability at 41 °C for the SRB ranged from −10.0 to 3.7% (Table 1G) and for the MTT from −7.6 to 7.1% (Table 1H). The differences between Taxol® and Pac/RAMEB were not significantly different when comparing between both temperatures (p-value of 0.885 and 0.383 for SRB and MTT test, respectively). When investigating the influence of temperature on either the activity of Taxol® or the Pac/RAMEB formulations, the differences were small. For Taxol® (Fig. 3C), the SRB test showed an average difference of $2.4 \pm 3.5\%$ and the MTT test a difference of $-4.0 \pm 4.0\%$. For the inclusion complexes (Fig. 3D) the average difference was $1.4 \pm 7.9\%$ and $-0.9 \pm 5.6\%$ for the SRB and MTT test, respectively.

The IC50 values of the MTT at 37 °C (Table 2) ranged from 2.76 to 8.59 μg/ml and from 3.12 to 10.64 μg/ml for Taxol® and Pac/RAMEB formulations, respectively. There was no significant difference between these groups ($p = 0.958$). At 41 °C, the values for the MTT ranged from 4.77 to 7.72 μg/ml and from 4.29 to 12.90 μg/ml for Taxol® and Pac/RAMEB formulations, respectively (Table 2). There was also no significant difference ($p = 0.179$) between these IC50 values. When comparing the data of Taxol® at 37 °C versus the data at 41 °C to evaluate the influence of heat, no significant difference between both groups ($p = 0.672$) could be detected for MTT. The IC50 values of the MTT test also did not differ significantly ($p = 0.227$) between both temperatures for the Pac/RAMEB formulations. Considering the SRB test, the IC50 values were smaller compared to the MTT test (some even below the lowest concentra-

Table 2
Minimum and maximum IC50 values (determined via MTT and SRB cytotoxic assays) for the Pac/RAMEB (all three ratios) and Taxol® at 37 °C and 41 °C for the CaCo-2 cell line.

Formulation	T (°C)	Test	Minimum IC50 (μg/ml)	Maximum IC50 (μg/ml)
Taxol®	37	MTT	2.76	8.59
		SRB	<0.01	0.57
	41	MTT	4.77	7.72
		SRB	<0.01	0.35
Pac/RAMEB	37	MTT	3.12	10.64
		SRB	0.03	0.42
	41	MTT	4.29	12.90
		SRB	<0.01	0.58

tion tested, i.e. <0.01 μg/ml). At 37 °C the SRB test had a maximum IC50 value of 0.57 and 0.42 μg/ml for Taxol® and the Pac/RAMEB formulations, respectively (Table 2). There was no statistical significant difference between both formulations ($p = 0.450$). Similar results were seen at 41 °C: no significant difference between both formulations ($p = 0.906$) with maximum IC50 values of 0.35 and 0.58 μg/ml for Taxol® and Pac/RAMEB, respectively. In function of temperature, no significant differences between the IC50 values were detected for Taxol® and the Pac/RAMEB formulations (p-values of 0.573 and 0.791 respectively).

4. Discussion

Paclitaxel is an essential cytotoxic agent used in the management of different types of cancer. However, the adverse effects observed after administration of Taxol® are a considerable drawback for the use of paclitaxel. It has become clear that Cremophor EL® (essential in the formulation in order to solubilise paclitaxel) is not a physiological inert compound as numerous pharmacokinetic and pharmacodynamic effects of Cemophor EL® have been reported in literature (Gelderblom et al., 2001; van Zuylen et al., 2001). Due to these adverse effects, the development of new paclitaxel formulations, containing a suitable carrier to improve the aqueous solubility, is essential.

A paclitaxel formulation intended for a HIPEC procedure in the treatment of peritoneal cancer of colorectal origin was developed using RAMEB (Bouquet et al., 2007). The difference with a similar approach, used in the past (Szente et al., 1999), was that our formulation did not contain ethanol. Ethanol was excluded because of the synergism with volatile anaesthetics, which may lead to increased fatalities in a future in vivo model. RAMEB significantly improved the solubility of paclitaxel and the in vitro stability of the formulation was sufficient to achieve suitable paclitaxel concentrations (Bouquet et al., 2007). In this study we evaluated the cytotoxicity of this Pac/RAMEB formulation and compared the cytotoxic activity of RAMEB versus Cremophor EL®.

Based on the results of the MTT and SRB assays it is obvious that Cremophor EL® is a stronger cytotoxic agent in comparison to RAMEB with average differences in viability above 40%. This was also reflected in the IC50 values. The values for RAMEB were in most cases above 3.5 mg/ml (highest concentration tested), whereas the IC50 values for Cremophor EL® were all below or equal to 1 mg/ml. This confirmed a study (Szente et al., 1999), which concluded that methylated-beta-cyclodextrins did not exhibit cytotoxicity on PC3 cancer cell lines in contrast to Cremophor EL®. However, it has been reported that a concentration of 1–2 mg/ml methylated-beta-cyclodextrins showed cytotoxic effects on human breast and ovarian adenocarcinoma cell lines (Grosse et al., 1998) which is in contrast to our data.

The presented data clearly illustrated that Cremophor EL® is more cytotoxic than RAMEB, although in literature there have been conflicting reports about the cytotoxic activity of Cremophor EL®. Liebmann et al. (1994) observed that a Cremophor EL® concentration of 1.35 mg/ml was able to block a portion of the cells in the G1-phase, making it impossible for paclitaxel to exert its effect during mitosis and because of this effect, lower paclitaxel concentrations (0.043 μg/ml or 50 nM) were as effective as higher concentrations. In contrast, Cordes and Plasswilm (1998) reported a cytotoxic effect of the diluent used in Taxol® and Fjällskog et al. (1994) discovered that paclitaxel in Cremophor EL®/ethanol was more potent against doxorubicin-resistant cell lines than paclitaxel dissolved in ethanol alone, suggesting an additional cytotoxic effect of Cremophor EL®. The latter was confirmed by Reinecke et al. (1997), who observed a higher cytotoxicity of Taxol® than paclitaxel dissolved in DMSO and suggested that this was caused by additional growth inhibitory effect of Cremophor EL®. Although it is difficult to compare our data with other literature reports because of differences in cell lines and administered concentrations, our data showed a clear cytotoxic effect of Cremophor EL® on both cell lines at a concentration of approximately 1 mg/ml whereas RAMEB is much less cytotoxic with IC50 values above 3.5 mg/ml. Exposure to heat did not affect cytotoxicity.

When comparing Taxol® versus the Pac/RAMEB complexes, there was no relevant difference between both formulations since the differences in viability were below 10%. Although the formulation excipients have a large difference in cytoxicity, both types of paclitaxel formulations were equipotent, even for the Pac/RAMEB formulation with the lowest ratio (1/20, mol/mol). This shows that the different in vitro stability observed for the different molar ratios reported in previous research (Bouquet et al., 2007) did not have a negative effect on the cytotoxicity of the inclusion complexes: all three ratios yielded similar results in cytotoxicity. For both cell lines, this similarity in cytotoxicity of both Taxol® and Pac/RAMEB was confirmed by the IC50 values. This is in accordance with other researchers who did not observe a loss in activity of paclitaxel formulated in β-cyclodextrins (Szente et al., 1999). Our research has proven that although the formulation excipients are very different in cytotoxicity, the Pac/RAMEB formulation is as cytotoxic as Taxol®. We hope that these promising in vitro results can be confirmed in vivo: producing a paclitaxel formulation for intraperitoneal chemotherapy and more specific for HIPEC, that is equipotent to Taxol® but with less toxicity. If this new formulation proves to be effective, it could also be studied for a peritoneal cancer of other origin in the future; ovarian cancer being the most relevant.

After investigating the cytotoxicity of the excipients and both formulations, one question remained unanswered: does hyperthermia have a beneficial effect on the cytotoxicity of the investigated paclitaxel formulations? Because paclitaxel and hyperthermia both act on the microtubuli, it is questioned if they have a synergistic or antagonistic effect. Our results showed no enhancement of the cytotoxic activity of all investigated paclitaxel formulations at higher temperature using the rat CC531s and the human CaCo-2 cell line, indicating no synergistic effect of heat nor a loss of activity due to instability of the formulation at higher temperatures. Rietbroek et al. (1997) also reported a lack of thermal enhancement for paclitaxel and docetaxel. This was confirmed for docetaxel (Dumontet et al., 1998). In contrast, Cividalli et al. (1999) reported an effect between paclitaxel and hyperthermia in a mouse tumour model and Othman et al. (2001) confirmed that there was an hyperthermic enhancement of the apoptotic and antiproliferative activity of paclitaxel. More recently, results were published in which no enhancement between hyperthermia and paclitaxel was seen, but there was an enhancement for docetaxel (Faheez et al., 2003). A possible explanation for these contradictory results is the numerous factors involved in this process. Recent research illustrated that, depending on the cell line, the applied temperature and the number of days after treatment, differences in results were observed (Michalakis et al., 2007). At present, there is no consensus on hyperthermia and taxanes, but our data suggested that there was no thermal enhancement of the cytotoxic activity of paclitaxel, independent of the type of formulation, on the CaCo-2 and CC531s cell line.

5. Conclusion

A newly developed Pac/RAMEB formulation has shown a similar cytotoxic activity as Taxol® against a rat (CC531s) and a human (CaCo-2) colon cancer cell line, as was confirmed via two cyto-

toxicity assays (MMT and SRB). For the Pac/RAMEB formulation there was no difference in cytotoxicity between the different ratios of RAMEB used. Importantly, a clear difference in cytotoxicity between Cremophor EL and RAMEB was detected; the latter being the less toxic. There was no effect of applying hyperthermia (41 °C during 1 h) on the survival rate of the cells.

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