Development of a combination drug-eluting bead: towards enhanced efficacy for locoregional tumour therapies

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Drug-eluting beads (DEBs) are becoming a mainstay locoregional therapy for hepatic malignancies but are currently loaded with single drugs alone. Here, we wished to prepare DEB containing different drug combinations, to screen their efficacy using an in-vitro cell culture assay and to include any promising combinations that demonstrate additive efficacy in an in-vivo model of locoregional tumour treatment.
A modified in-vitro assay was used based upon the use of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) with either HepG2 liver cancer or PSN1 pancreatic cancer cell lines. The comparative cytotoxicity of DEB combinations prepared containing doxorubicin, irinotecan, topotecan and rapamycin was evaluated. Those combinations that demonstrated an additive cytotoxicity effect were investigated *in vivo* using a nude mouse xenograft model of pancreatic cancer. Although many of the DEB combinations showed either no effect or a slight antagonistic effect, the combination of doxorubicin and rapamycin DEBs demonstrated synergistic activity. On the basis of these findings, a method was developed to prepare a doxorubicin/rapamycin dual-loaded DEB, which was shown to possess the same drug-loading capacities, drug elution properties and HepG2 cell cytotoxicity synergy as the single drug-loaded DEB combination. Evaluation of this dual-loaded combination DEB versus the respective single drug-loaded DEBs in a mouse xenograft model of pancreatic cancer showed an equivalent tumour volume reduction as the doxorubicin DEB, but with less toxicity than the rapamycin DEB. The doxorubicin/rapamycin combination DEB offers great potential for enhanced efficacy in the locoregional treatment of malignant tumours. *Anti-Cancer Drugs* 23:355–369

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Introduction

Drug-eluting beads (DEBs) are controlled-release microspherical devices that are used for sustained locoregional delivery of chemotherapeutic agents to various tumour types [1–4]. DEBs are finding increasing use in the treatment of hepatic malignancies by intrahepatic arterial administration by a procedure known broadly as transarterial chemoembolization [5]. This procedure is in itself a combination therapy, which induces oxygen and nutrient deprivation to the tumour by occluding the feeding blood vessels, while providing for a concomitant sustained delivery of a high local dose of chemotherapy. DEBs loaded with doxorubicin are administered primarily to patients suffering from hepatocellular carcinoma (primary cancer originating in the liver) [6–9], whereas those suffering from metastatic cancer to the liver, such as colorectal cancer metastases , may be treated with DEBs loaded with irinotecan [10–14]. It is also possible to administer DEBs by directly injecting them at the disease site, for instance in preclinical animal models of peritoneal carcinomatosis [4], glioblastoma [2,15] and pancreatic cancer [3,16].

A number of recently initiated clinical studies using DEBs are investigating the potential benefits of the combination of this locoregional approach with some of the newer chemotherapeutics, including oral agents such as sorafenib (Nexavar; Bayer Healthcare/Onyx), sunitinib (Sutent; Pfizer) and everolimus (Afinitor; Novartis) to treat hepatocellular carcinoma, or intravenous systemic regimens including bevacizumab (Avastin; Genentech) or cetuximab (Erbitux; ImClone/Bristol-Myers Squibb) to treat colorectal cancer metastases. These studies aim to demonstrate whether there is a synergy between tumour targeting of a cytotoxic agent using DEBs and systemic treatment with agents designed to interfere with the angiogenesis and antiapoptosis/prosurvival pathways that are activated in tumour cells. There will, however, inevitably remain the issue of adverse side effects related to the systemic exposure of these complementary agents [17–19]. One potential solution to this problem could be locoregional codelivery of the drug combination using the DEB platform. Reported herein is the development and evaluation of a combination DEB for locoregional tumour therapy, whereby the drugs have

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been chosen and screened *in vitro* in an attempt to uncover any synergistic action of inhibition.

Rationale for drug selection

A series of known chemotherapeutic agents was selected for evaluation in combination within DEBs. Doxorubicin is an anthracycline antibiotic used in the treatment of a wide variety of cancers including many types of carcinoma and soft tissue sarcomas. Its mechanism of action is not particularly well understood [20], but is thought to have multiple targets, intercalating between the DNA double helix, inhibiting the progression of topoisomerase II and preventing DNA strand rejoining, all of which lead to interference in cell replication. It has been suggested that its optimum method of delivery is by a low level of sustained delivery [21]. Irinotecan and topotecan are members of the camptothecin drug family and have a more specific mode of action, binding to topoisomerase I, resulting in DNA strand damage [22,23]. Irinotecan is a prodrug that requires conversion into its active form, SN-38, by carboxylesterases present in some cells; topotecan does not require this conversion and so offers an interesting comparison in this study. The final drug selected was rapamycin (or sirolimus), which, together with its analogues such as everolimus, are finding increasing use in cancer therapy [24,25]. These drugs bind to FK-binding protein 12, which inhibits the mammalian target of the rapamycin (mTOR) pathway. This pathway has been implicated as central to a series of prosurvival pathways that provide cancer cells with an escape route when they become stressed, particularly in the case of cell hypoxia [26]. This drug is therefore of great interest when considering that hypoxia is intrinsically present in many tumour types, particularly pancreatic tumours [27–30] and, moreover, hypoxia may be induced following embolization of tumours [31–33].

Materials and methods

Chemotherapeutic agents and beads

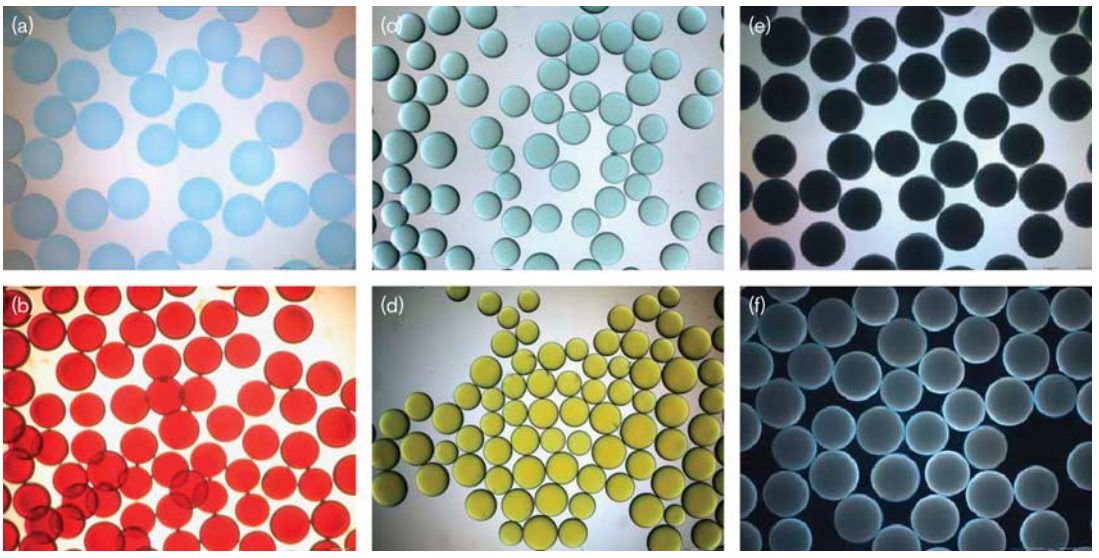
Doxorubicin hydrochloride was supplied as a red powder (> 98% purity; Zhejiang Hisun Pharmaceuticals, Zhejiang, China), irinotecan hydrochloride as a pale yellow powder (> 99% purity; Dabur Pharma Ltd, Ghaziabad, India), topotecan hydrochloride as a yellow powder (> 99% purity; Dabur Pharma Ltd) and rapamycin as a white powder (> 99% purity, LC Laboratories, Woburn, Massachusetts, USA). DC Beads (Biocompatibles UK Ltd, Farnham, UK) were provided in a 500–700 μm size range in sterile vials as 2 ml hydrated bead volume, suspended in a 1 mmol sodium phosphate solution (Biocompatibles UK Ltd). This larger bead size was selected for ease of individual bead isolation and handling in the cell viability assays.

Preparation of drug-eluting beads with different drugs

The loading of doxorubicin, irinotecan and topotecan into DC Bead was successfully achieved utilizing a simple ion-exchange process to produce the various single drug-loaded DEB following the well characterized procedures previously described in the literature [3,34–36] (Fig. 1a–d). Loadings of 32, 30 and 30 mg/ml were achieved for doxorubicin, irinotecan and topotecan, respectively.

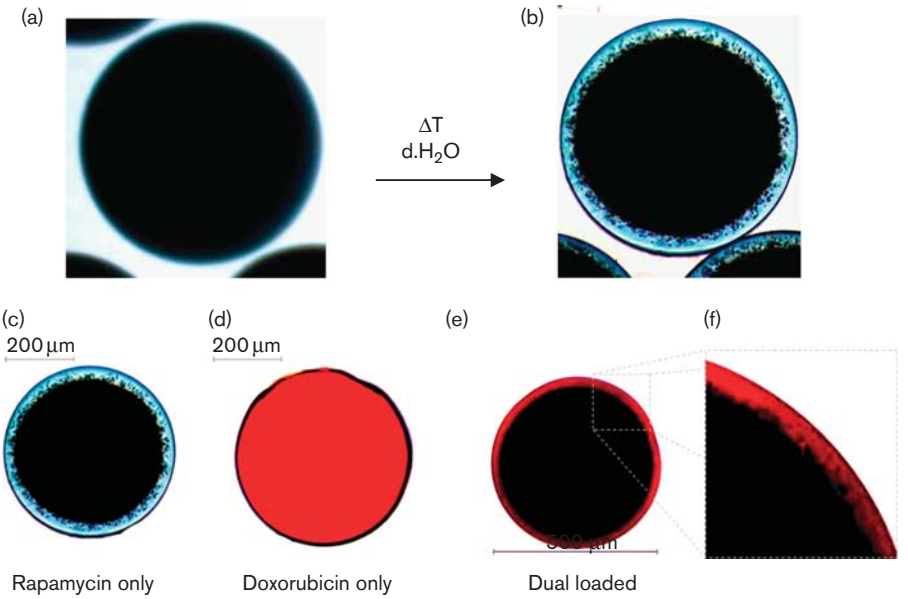
Rapamycin DEBs have not been described previously and given the drug's non-ionic nature and low water solubility,

Fig. 1



(a) Unloaded beads; (b) drug eluting beads (DEBs) loaded with doxorubicin; (c) DEBs loaded with irinotecan; (d) DEBs loaded with topotecan; (e) DEBs loaded with rapamycin (phase 0); (f) DEBs loaded with rapamycin (phase 3); viewed under an optical microscope (× 100 magnification).

Fig. 2



Optical micrograph of drug-eluting beads (DEBs) loaded with rapamycin before (a) and after (b) storage in distilled water. Optical micrographs of DEBs loaded with rapamycin (c), DEBs loaded with doxorubicin (d) and DEBs loaded with doxorubicin and rapamycin (e), with a magnification of the edge of these dual-loaded beads showing the outer surface devoid of rapamycin.

a new method of loading the beads had to be developed. An aliquot of DC Bead (1 ml) was washed five times with 1 ml dimethyl sulfoxide (DMSO) (Aldrich Chem. Co., Poole, UK). DMSO was selected as a solvent that could swell the bead network, dissolve the drug and crucially was miscible with water. Rapamycin (60 mg) in 1 ml DMSO was added and placed on a roller-mixer for 10 min to allow penetration into the structure. The beads were then transferred to water, which caused the drug to precipitate within the bead hydrogel network; this could be visualized under light microscopy as the drug particles prevent the transmittance of light through the bead, unlike the DEB with ionically bound drugs (Fig. 1b–e). The residual solution was removed and the remaining bead slurry was washed five times with 10 ml deionized water; the drug remained inside the bead after the excess rapamycin (as a precipitate in the washings) was removed and the DEB was washed several times to remove any surface-bound drug.

The extent of rapamycin loading was determined using ultraviolet (UV)-visible spectrophotometry at an absorbance of 279 nm (Lambda 25 UV; Perkin Elmer, Seer Green, Buckinghamshire, UK). The drug was extracted from the beads into DMSO (4×1 ml) and the solution was scanned to assess the total loading by comparing the absorbance with a standard curve. A loading of 21 mg/ml was achieved by this method. After loading, the rapamycin DEB turned from a transparent blue to an opaque light blue colour due to the white appearance of rapamycin and the blue tint of DC Bead (Fig. 1f).

After storage in water for a few weeks, it was noted that a small amount of rapamycin dissolved into solution, leaving a central rapamycin core, with the outer edge of the bead devoid of drug (Fig. 2). The process of loading rapamycin did not appreciably alter the bead size, unlike the ion-exchange process with the other drugs, which result in a decrease in the average bead diameter by ~20–30% [34,36]. To avoid unwanted elution, the rapamycin-loaded beads were stored dry or in minimal volume of water. The ability of the rapamycin DEB to elute drug *in vitro* was confirmed and is reported in the results.

Preparation of drug-eluting beads coloaded with doxorubicin and rapamycin

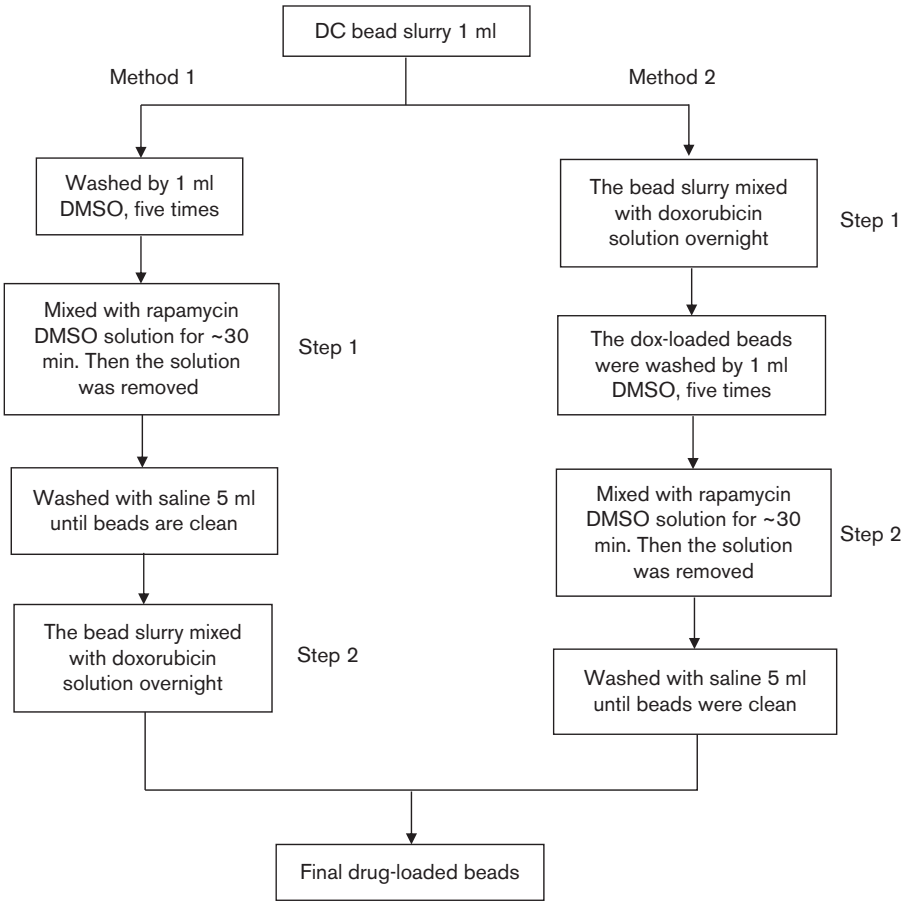
Two loading processes were performed for the coloading of doxorubicin and rapamycin (as outlined in Fig. 3).

Method 1

One millilitre of a DC Bead slurry was washed with 1 ml DMSO five times and mixed with 1 ml of 60 mg/ml rapamycin DMSO solution for 30 min. The loaded beads were then washed with 5 ml saline until no further white drug particles were observed. Rapamycin-loaded bead slurry of 0.5 ml was roller-mixed with 2.5 ml of 10.07 mg/ml doxorubicin solution overnight; the target doxorubicin loading was 25 mg/ml.

The doxorubicin loading was measured by the depletion method in either water or DMSO solution. The loading solution was taken and diluted 10 times and measured using UV-visible spectrophotometry at 483 nm to determine the concentration of doxorubicin residue in solution.

Fig. 3



Two methods for rapamycin and doxorubicin loading into a DC Bead. DMSO, dimethyl sulfoxide.

For the beads with rapamycin alone, 0.5 ml rapamycin-DEB (from first step, Method 1) was extracted by 1 ml DMSO five times, and the solution was combined in a 10 ml volumetric flask. The concentration of rapamycin was determined using UV-visible spectrophotometry at 279 nm.

For DEB coloaded with doxorubicin and rapamycin, 0.26 ml of DEB (from second step, Method 2) was extracted with 1 ml DMSO four times, and the extraction was collected in a 5 ml volumetric flask and DMSO was added to top the solution to the mark. The concentration of rapamycin was determined by subtraction of the absorbance of doxorubicin at 279 nm from the total absorbance at 279 nm, in which the doxorubicin absorbance at 279 nm was calculated from that at 483 nm according to the scan of solutions of known concentration. The doxorubicin loss during DMSO washing of loaded DEB before further loading of rapamycin (Method 2) was determined by measuring the combined DMSO washing solution in a volumetric flask at 483 nm. Drug loadings obtained were 25 mg/ml doxorubicin and 20 mg/ml rapamycin.

Method 2

One millilitre of a DC Bead slurry was loaded with doxorubicin solution overnight (2.5 ml of 10.07 mg/ml, target loading 25 mg/ml DC Bead). The doxorubicin-loaded beads were washed with 1 ml DMSO five times to remove residual water and 1 ml of 60 mg/ml rapamycin DMSO solution was mixed with the doxorubicin beads for about 30 min. Subsequently, following removal of the residual rapamycin DMSO solution, the beads were washed with 5 ml saline until no white drug particles were observed.

Elution characteristics of doxorubicin, rapamycin and dual-loaded doxorubicin/rapamycin drug-eluting beads

Doxorubicin and rapamycin were eluted from the coloaded DEB and compared with the elution of the single individual DEB [37]. Dual-loaded or singly loaded DEB (1 ml) was eluted into 400 ml PBS (Inverclyde, Bellshill, UK) and agitated. At certain time points, samples were taken (and replaced with fresh PBS to maintain sink conditions) and the amount of drug eluted was determined using UV-visible absorbance at 483 nm

(doxorubicin) and 279 nm (rapamycin). The amount of rapamycin eluted was calculated when both drugs were present by determining the amount of doxorubicin (from the absorbance at 483 nm) and subtracting the predetermined corresponding absorbance at 279 nm. The remaining value corresponded to the 279 nm absorbance due to rapamycin.

Drug-eluting bead imaging

Images of the DEBs were taken using an Olympus BX50F4 microscope equipped with a Colorview III camera (Olympus, Southend-on-Sea, UK). Approximately 200 beads were counted for size distribution studies using software (AnalySIS 5.0; Soft Image System GmbH, Munster, Germany), and optical photomicrographs were taken using different light/contrast backgrounds for morphological studies.

Studies of the effect of drug-eluting bead combinations on in-vitro tumour cell viability

Human hepatocellular liver carcinoma cell line, HepG2 (product Description HB-8065; purchased frozen from ATCC; LGC Standards, Teddington, Middlesex, UK), and human pancreatic adenocarcinoma cell line, PSN1 (94060601; purchased frozen from ECACC, Sigma Aldrich, Poole, UK), were used to screen the effects of DEB combinations on cell viability over time. HepG2 cells were grown in MEM (minimum essential medium Eagle) (E15-825; PAA Laboratories GmbH, Pasching, Austria), 10% foetal bovine serum (A15-144; PAA) and 1% nonessential amino acids (NeAA; Biowhittaker, Wokingham, UK). PSN1 cells were grown in RPMI (Roswell Park Memorial Institute) (E15-840; PAA) and 10% foetal bovine serum (A15-144; PAA). Cells (20 000) were seeded to each well of a sterile flat-bottomed 96-well plate (655180, Cellstar; Greiner Bio-One Ltd, Stonehouse, Gloucestershire, UK) and incubated for 20 h (37°C, 5% CO_2). The cultured cells were then subsequently used in a series of viability experiments.

Establishing a method for evaluating drug-eluting bead cytotoxicity using the MTS assay

A first set of experiments were performed using HepG2 cells prepared as in the previous section, to determine whether there was any intrinsic effect of the beads themselves on the viability of the cells. After the initial cell incubation period, the medium was replaced with 200 µl of fresh medium, to which either 1, 2, 3 or 10 unloaded DC Beads (nondrug loaded control) were added using a pipette (three replicates of $n = 6$). The plates were placed in an incubator (37°C, 5% CO_2) for 24, 48 and 72 h, after which the cell viability was tested using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) assay (Promega Ltd, Southampton, UK). Differences in cell viabilities between the different bead numbers and time points were analysed using analysis of variance, with a *P*-value of less than 0.05 being defined as significantly different.

Once the effect of the bead alone was known, a second set of experiments were performed in order to establish the appropriate number of DEBs that would result in an effective amount and rate of cell kill to allow a useful comparison between different drugs. Doxorubicin DEBs loaded with 25 mg/ml was selected for this evaluation due to the known efficacy of this drug towards liver cancer cells. Either 1, 3 or 10 doxorubicin DEBs were added to the wells using a pipette (three replicates of $n = 6$) and incubated (37°C, 5% CO_2) for 24, 48 and 72 h. Cell viability was again determined using the MTS assay and the results were analysed using analysis of variance between bead number and time points, with a *P*-value less than 0.05 being defined as significantly different.

Screening for the synergistic effects of different single drug-loaded drug-eluting bead combinations on cell cytotoxicity

A series of experiments were performed using both HepG2 and PSN1 cells, to compare the cell viability following exposure to single drug-loaded DEBs compared with a combination of two single drug-loaded DEBs containing different drugs and to look for synergistic activity (see Table 1 for an outline of the experimental design). After incubation of the cells, the cell medium was replaced with 200 µl fresh medium, to which either one single drug-loaded DEB was added or two DEBs containing different drugs in each (three replicates of $n = 6$), as per the experimental design. The plates were placed in an incubator (37°C, 5% CO_2) and the drug(s) were allowed to elute from the beads into the cell culture medium for 24, 48 and 72 h. The cell viability of drug-loaded beads was tested using the MTS assay described previously. The results for the best-performing single agent were compared against the drug combination using Student's *t*-test ($P < 0.05$ being significantly different) in order to determine whether the combination had any additional benefit.

Table 1 Experimental design for cell viability studies for combination drug-eluting beads

Cell viability experiment 1 (HepG2 and PSN1)		
DEBDOX	DEBIRI	DEBDOX + DEBIRI
DEBDOX	DEBTOP	DEBDOX + DEBTOP
DEBDOX	DEBRAP	DEBDOX + DEBRAP
DEBIRI	DEBTOP	DEBIRI + DEBTOP
DEBIRI	DEBRAP	DEBIRI + DEBRAP
DEBTOP	DEBRAP	DEBTOP + DEBRAP
Cell viability experiment 2 (HepG2 only)		
1 × DEBDOX	1 × DEBRAP	1 × DEBDOXRAP
2 × DEBDOX	2 × DEBRAP	2 × DEBDOXRAP
3 × DEBDOX	3 × DEBRAP	3 × DEBDOXRAP
4 × DEBDOX	4 × DEBRAP	4 × DEBDOXRAP

DEBDOX, drug-eluting beads loaded with doxorubicin; DEBIRI, drug-eluting beads loaded with irinotecan; DEBDOXRAP, drug-eluting beads loaded with doxorubicin and rapamycin; DEBRAP, drug-eluting beads loaded with rapamycin; DEBTOP, drug-eluting beads loaded with topotecan.

Comparing the effectiveness of single-drug loaded drug-eluting bead combination with a dual-loaded drug-eluting bead combination on cell cytotoxicity

On the basis of the results of the screening experiments, a dual drug-loaded DEB was prepared as described containing doxorubicin and rapamycin. A further set of experiments was performed according to the experimental design in Table 1, using HepG2 cells only, to compare the effectiveness of a combination of single-loaded doxorubicin DEB and rapamycin DEB with dual-loaded rapamycin/doxorubicin (rapa-dox) DEB. After incubation of the cells, the cell medium was replaced with 200 µl fresh medium, to which either 1, 2, 3 or 4 doxorubicin DEB and an equivalent number of rapamycin DEB, or 1, 2, 3 or 4 dual-loaded rapa-dox DEB were added (three replicates of $n = 6$), as per the experimental design in Table 1. The plates were placed in an incubator (37°C, 5% CO_2) and the drug(s) were allowed to elute from the beads into the cell culture medium for 24, 48 and 72 h. The cell viability was tested using the MTS assay described and the results for the combination of the single drug-loaded beads were compared with those of the dual-loaded beads using Student's *t*-test, with a *P*-value less than 0.05 being defined as significantly different.

In-vivo evaluation of drug-eluting beads using a nude mouse xenograft

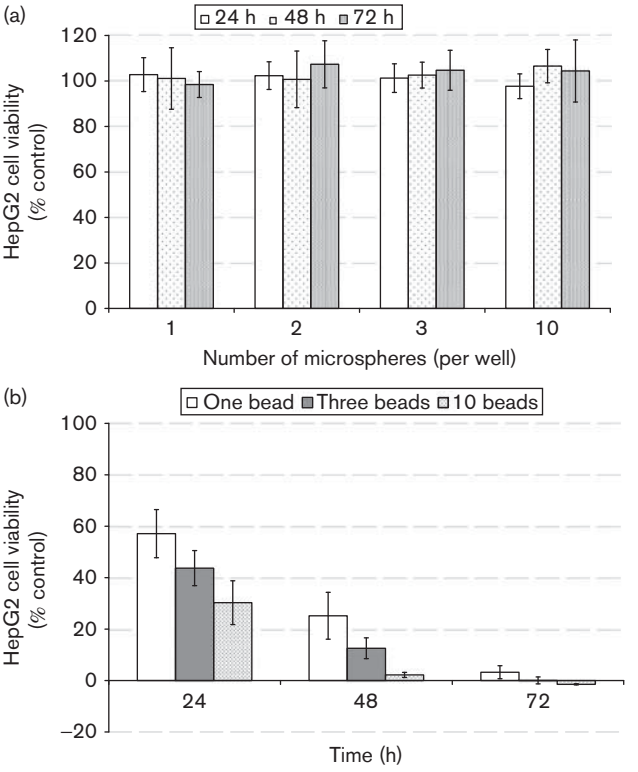
Mouse studies were performed by Exp. Pharmacol. & Oncol. GmbH, (Berlin-Buch, Germany) in accordance with local regulations (granted by the LAGeSo, State Office of Health and Social Affairs, Berlin). A HepG2 mouse model was not available at the time and therefore the studies were performed using the previously described PSN1 model [3]. In brief, PSN1 tumour cells were prepared from an in-vitro passage and at day 0, 1×10^7 cells were injected subcutaneously in female nude mice (Taconic M&B, Ry, Denmark). Mice were randomized to the scheduled treatment groups ($n = 3$ per group), which included an unloaded DC Bead control, doxorubicin DEB, rapamycin DEB and rapa-dox DEB. At day 5, when the tumours become palpable (0.2 cm^3), a DEB/alginate mixture (1 ml DEB + 3 ml 0.6% w/v alginate; CellMed AG (Alzenau, Germany), used to prevent bead backflow of DEB from the injection site as previously reported) was injected close to the subcutaneous growing tumour, using a 1 ml syringe (B.Braun, Melsungen, Germany) fitted with a G16 needle (0.6 × 25 mm; Terumo Europe, Heverlee, Belgium). The delivery volume was 100 µl per mouse. Tumour volumes and body weights were recorded twice weekly. Mice were sacrificed when the tumours reached an average size of greater than 1 cm^3.

Results

Development of a cytotoxicity assay for the evaluation of drug-eluting beads

An MTS assay for measuring cell viability was adapted to evaluate the cytotoxicity of the DEBs. Unloaded DC Beads were tested first and showed no effect on the viability of HepG2 cells incubated in the presence of either 1, 2, 3 or 10 microspheres per well. This demonstrated that there were no leachables from the carrier beads that exerted any effect on the growth of the cells (Fig. 4a). Doxorubicin DEBs were then tested using the same approach, as it is known that doxorubicin is cytotoxic towards this liver cancer cell line [38,39]. There was a dose–response effect on cell viability using 1, 3 or 10 beads per well, although this response was not linear (Fig. 4b). This may have been due to an inherent limitation of drug solubility in the small volume of cell incubate, which would restrict elution of larger amounts of drug in the wells containing larger bead numbers. It was therefore concluded that the use of one DEB per well was sufficient to detect a change in cell viability and allow comparison of cytotoxic effects between different DEB and their combinations. Other groups have considered methods for testing drug-eluting microspheres using cell-based in-vitro assays [40] but have rather opted to expose the cells to aliquots of drug that has been eluted from the microspheres in a separate vial. Our approach provides a simple method to compare between DEB but with the potential complication of elution into small

Fig. 4



(a) HEPG2 cell viability in contact with various numbers of unloaded DC Beads using the MTS assay. (b) HEPG2 cell viability after the addition of 1, 3 or 10 drug-eluting beads loaded with doxorubicin (loaded at 25 mg/ml). MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt.

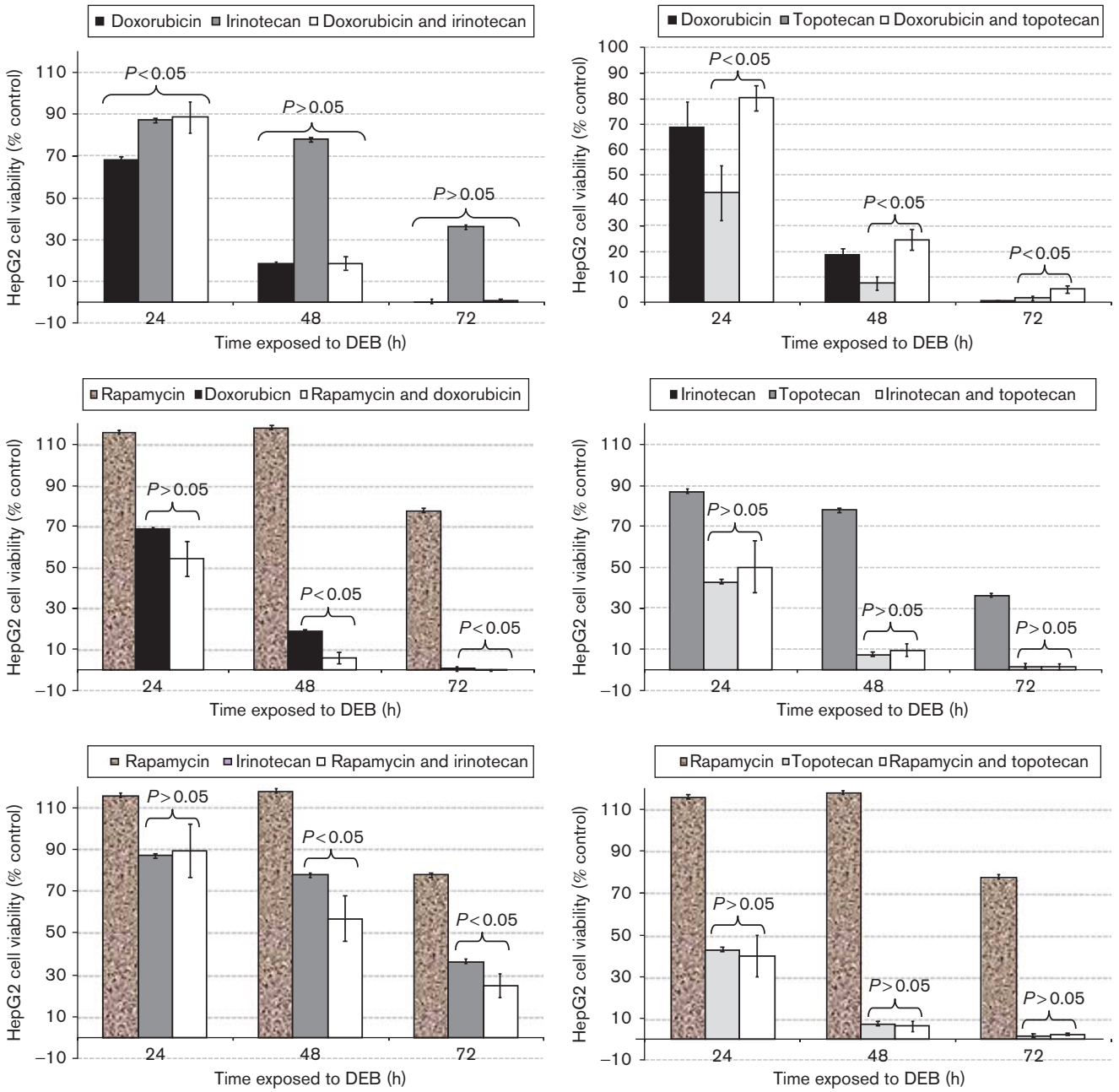
volumes of media containing a mixture of protein and other components.

Cytotoxicity of single drug-loaded drug-eluting beads and their combinations

Using the method previously described, the cytotoxicity of the different single drug-loaded DEBs towards HepG2 cells was compared with their combinations as outlined in the experimental design shown in Table 1. The results of these assays are shown in Fig. 5. The same experimental design was repeated using PSN1 cells, the results for which are shown in Fig. 6.

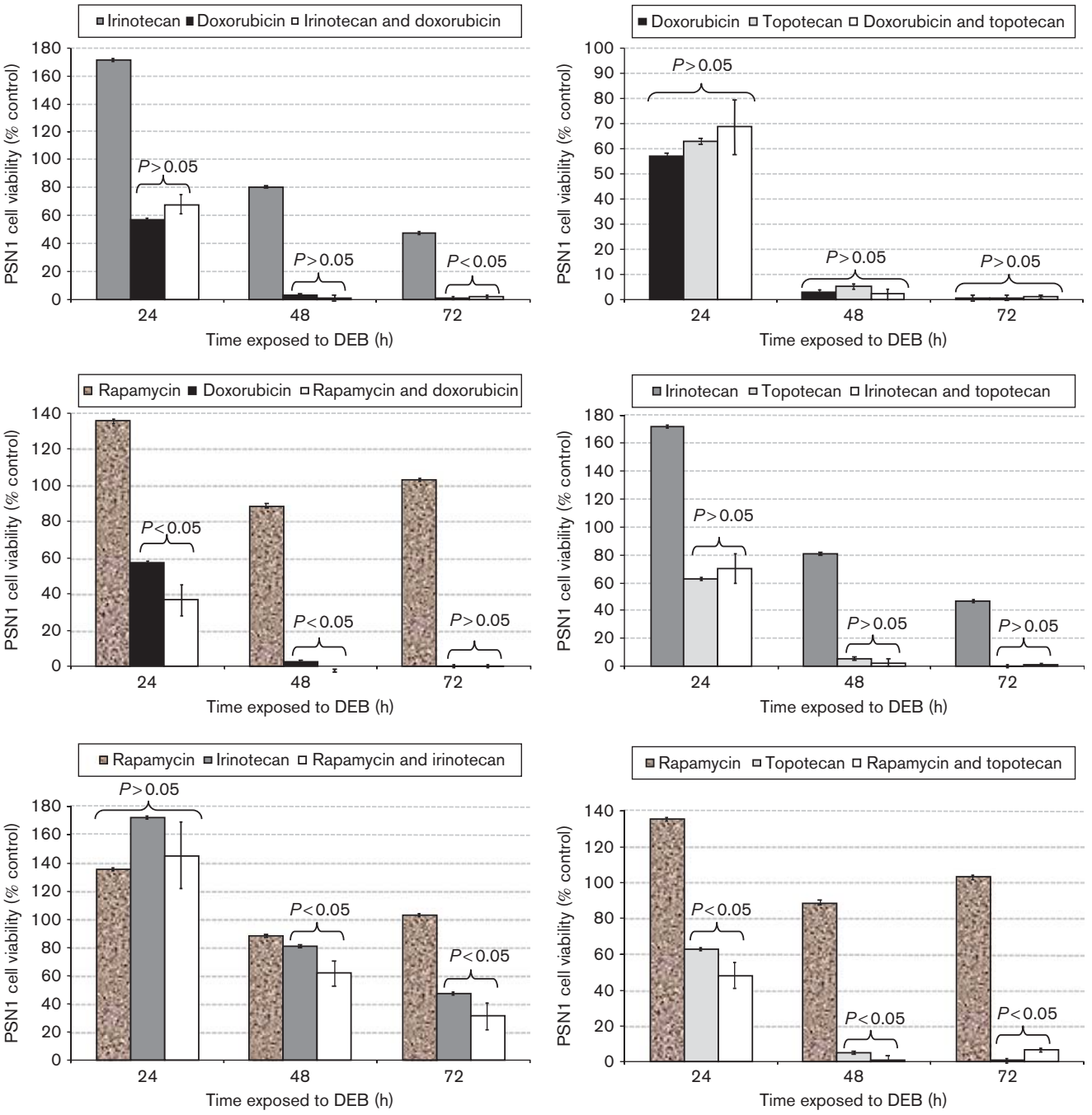
A statistical comparison was made between the single-loaded DEB with the greatest effect on cell kill and its combination with another DEB, in order to determine whether there was an increased cytotoxic effect with the

Fig. 5



Effect of various single drug-eluting bead (DEB) combinations on HEPG2 cell viability using the MTS assay. MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt.

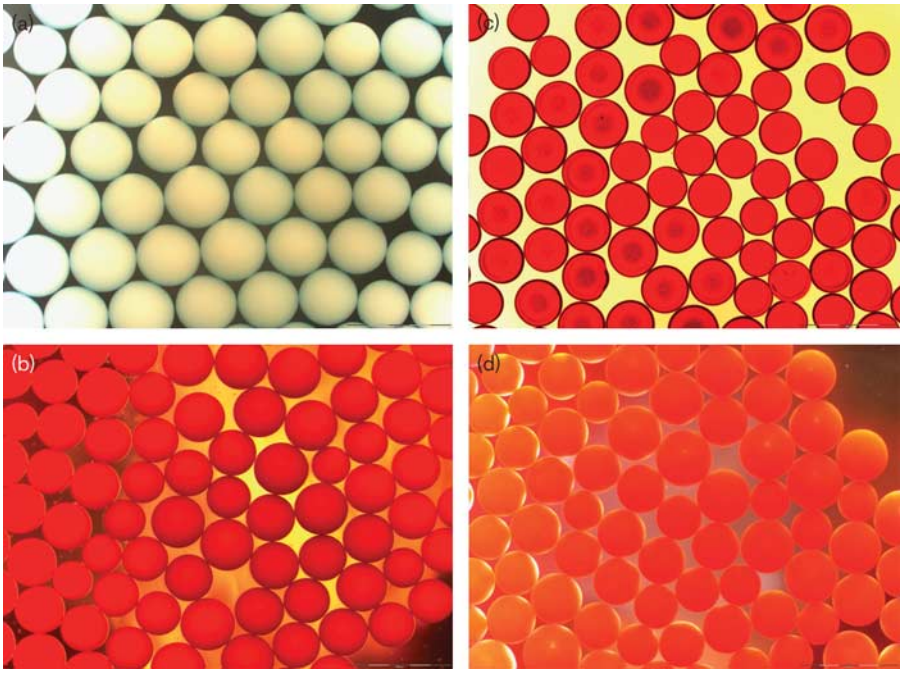
Fig. 6



Effect of various single drug-eluting bead (DEB) combinations on PSN1 cell viability using the MTS assay. MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt.

combination. In all cases of the various combinations of doxorubicin, irinotecan or topotecan DEBs, there was either no synergistic effect or perhaps a slight antagonistic effect of the combination on cell kill for both HepG2 and PSN1 cells. Rapamycin DEBs alone had only a minor effect on the cell viability of either cell line. However, for both the doxorubicin + rapamycin DEB combination and the irinotecan + rapamycin DEB combination, there was a synergistic effect resulting in significantly greater HepG2 cell kill at both 48 and 72 h. There was no such synergy with the topotecan + rapamycin DEB combination at any time point. Similarly, the doxorubicin + rapamycin DEB combination demonstrated synergistic effects of greater PSN1 cell kill at all time points, whereas irinotecan +

Fig. 7



Optical micrographs of product using Method 1: (a) drug-eluting beads (DEBs) loaded with rapamycin from step 1; (b) DEBs loaded with doxorubicin and rapamycin after step 2. Method 2: (c) DEBs loaded with doxorubicin from step 1; (d) DEBs loaded with doxorubicin and rapamycin after step 2.

rapamycin showed an increased effect at 48 and 72 h. Interestingly, for this cell line, topotecan + rapamycin also demonstrated a synergistic effect but only at 24 and 48 h, as at 72 h, the topotecan DEB alone was more effective.

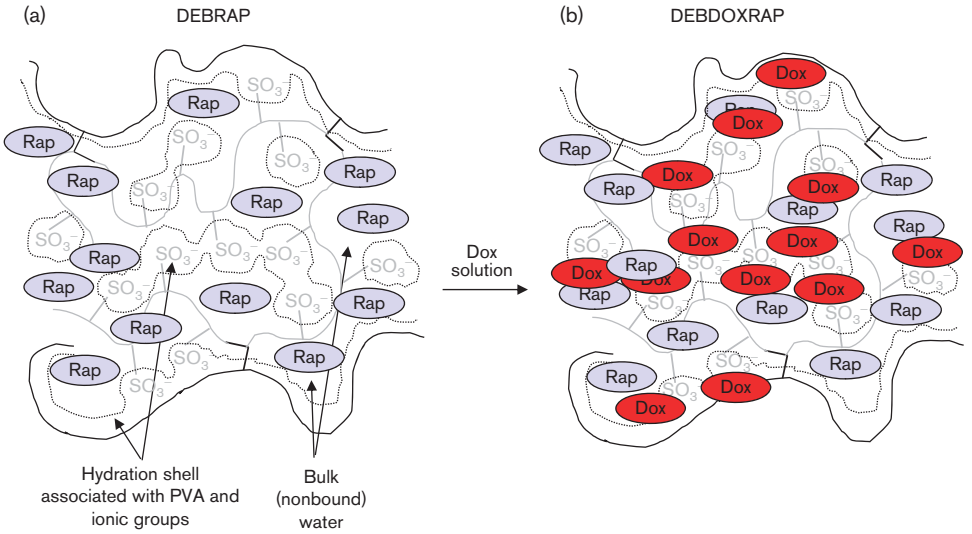
Preparation and characterization of a dual-loaded doxorubicin/rapamycin drug-eluting bead

In considering the combination of doxorubicin and rapamycin in one DEB, the question arose as to whether the presence of one drug would interfere with the ability to load the other. Two approaches were adopted: Method 1 sought to load the rapamycin first, followed by the doxorubicin, by a combination of the processes used to load the individual drugs (Fig. 3). Method 2 was the reverse, with doxorubicin loaded first, followed by the rapamycin loading step. Figure 7 shows the optical micrographs of the products obtained after each loading step from the two different methods. Method 1 was shown to be the preferred route to preparation of the dual drug-loaded DEB, as Method 2 required a DMSO wash of the doxorubicin DEB before rapamycin loading, which resulted in a loss of several milligrams of doxorubicin from the bead and produced a final waste solvent that contained significant amounts of both drugs.

It was found that doxorubicin could be loaded into the dual-loaded DEB up to the maximum loading of greater than 40 mg/ml [34], while still allowing for rapamycin loadings of up to ~30 mg/ml. This implies that the rapamycin does not interfere with the anionic binding sites required for cationic drug binding. This is supported by the optical micrographs of the dual drug-loaded DEB (Fig. 2) that have the characteristic red coloration from doxorubicin interaction with the sulfonate groups on the polymer backbone, but are also opaque to light transmittance, demonstrating that the rapamycin is precipitated into the water-filled interstitial spaces between the polymer chains (Fig. 8). Furthermore, when rapamycin alone is loaded, the final bead size range is the same as for unloaded DC Bead, the beads having been swollen by the DMSO but subsequently being washed out of the structure, allowing it to shrink to its original size. When doxorubicin alone is loaded into the beads, they shrink significantly due to the drug–polymer ionic interactions and drug–drug π-stacking interactions that displace water from the structure, causing it to contract [34]. For dual-loaded DEBs, prepared by either Method 1 or 2, the average sizes of the beads are the same, lying somewhat in between that of doxorubicin DEB and rapamycin DEB. It seemed that doxorubicin still causes a substantial shrinking of DEB diameter but the presence of the rapamycin precipitated within the water-filled spaces has the effect of restricting the overall contraction process (Fig. 9).

The presence of either drug does not appear to demonstrate a significant change in the elution profile

Fig. 8



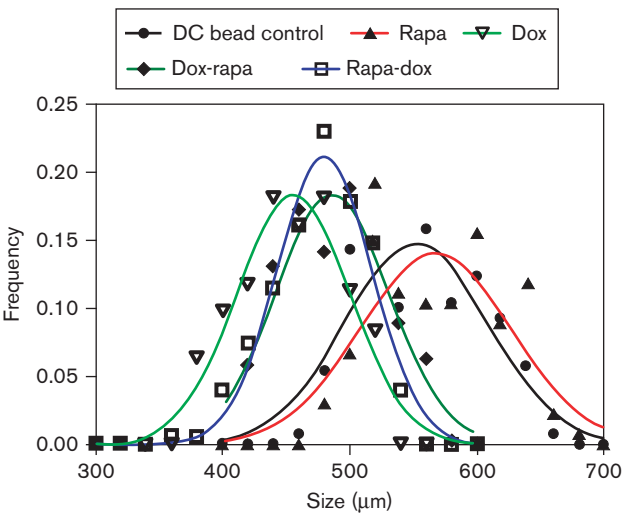
Proposed mechanism for dual-loaded drug-eluting beads (DEBs) loaded with doxorubicin and rapamycin. (a) DEBs loaded with rapamycin is formed by swelling the beads in dimethyl sulfoxide and then washing in water to precipitate the rapamycin into the bulk water-filled interstices in the hydrogel matrix; (b) DEBs loaded with doxorubicin and rapamycin can be subsequently formed by placing DEBs loaded with rapamycin in a solution of doxorubicin, whereby the doxorubicin is sequestered by ion exchange and interacts with the sulphonate groups and sits within the hydration shell of the polymer chains. PVA, polyvinyl alcohol.

of the other when loaded into the same bead as compared with their elution from the corresponding single drug-loaded DEB (Fig. 10). Doxorubicin has been shown to be released by an ionic exchange mechanism [37], whereas rapamycin appears to be solubility driven. The release rate of rapamycin could potentially be adjusted by using a more soluble rapamycin analogue such as everolimus.

Comparing the effectiveness of a single-drug drug-eluting bead combination with a dual-loaded drug-eluting bead combination on cell cytotoxicity

The viability of HepG2 cells was evaluated when exposed to a combination of single doxorubicin-loaded and rapamycin-loaded DEB compared with dual doxorubicin/rapamycin-loaded DEB according to the experimental design in Table 1. The resulting cell viability studies showed comparable values at all time points whether the drugs were in separate DEB or a single combination device (Bonferroni corrected Student's *t*-test $P > 0.1$ for all time points, Fig. 11a–c). This again supports the supposition that the elution is similar and amounts that are released from the DEBs into the wells are the same whether the DEB are dual loaded or not.

Fig. 9

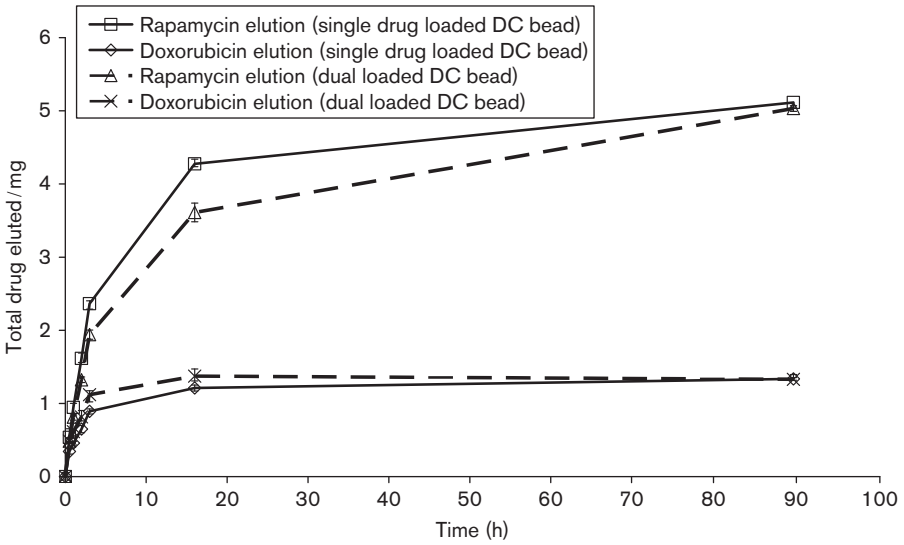


Size ranges for various doxorubicin, rapamycin and combination drug-eluting beads (DEBs) (Dox-rapa = dual DEB prepared by Method 1, Rapa-dox = dual DEB prepared by Method 2).

Efficacy of doxorubicin, rapamycin and dual drug-loaded drug-eluting beads in a mouse xenograft study

The results from the mouse xenograft study are presented in Fig. 12a and b. The control mice showed an increase in tumour size over 9 days (day 5–14), after which the mice were euthanized due to the tumour mass being greater than 10% of their body mass. Weight loss was observed at day 14 and was interpreted as a sign of sickness due to the tumour burden. The results show that doxorubicin (25 mg/ml) DEB reduced the tumour size to a minimum of 0.1 cm^3 over 13 days (day 18) after injection. After this time point, the tumour increased in size again. No signs of toxicity due to doxorubicin were observed as determined by mouse weight. Rapamycin DEB (20 mg/ml) showed no

Fig. 10



Comparison of drug elution from drug-eluting beads (DEBs) loaded with rapamycin and DEBs loaded with doxorubicin with DEBs loaded with doxorubicin and rapamycin (0.5 ml) into 400 ml PBS (mean ± SD; $n = 3$).

change in tumour size over the first 9 days (day 14). A marked increase in tumour size was observed after 13 days (day 18). The mice were euthanized following this time point due to the tumour mass being greater than 10% of their body mass. The mice demonstrated a decrease in weight at day 14 even though the tumour size remained constant. This suggests that the weight decrease may be due to the toxicity of rapamycin, not tumour burden. The weight continued to decline at later time points, as the tumour size increased. Rapamycin is described as a cytostatic drug and this effect can be seen before day 18, where the tumour neither increases nor decreases in size. This indicates that the drug is reaching enough cells within the tumour to prevent measurable growth. The increase in tumour size at day 18 may be due to the drug having fully eluted from the DEB and therefore failing to exhibit a cytostatic effect.

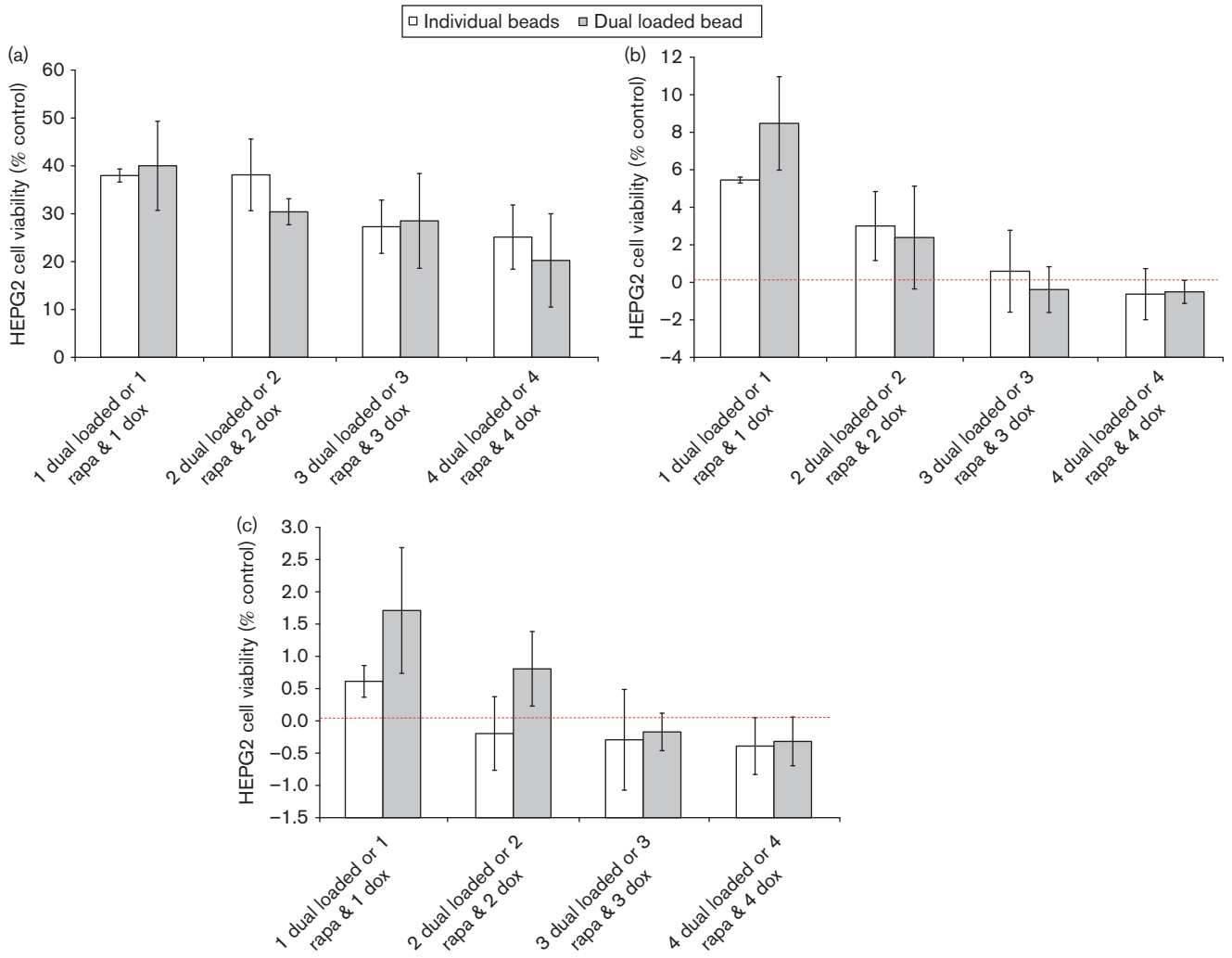
Discussion

Effectiveness of single-drug loaded drug-eluting bead combinations

From the cytotoxicity studies, there appears to be no apparent benefit in the combination of DEBs containing cytotoxic agents that have similar modes of action, that is, interference with DNA replication, albeit with different molecular targets within this process. Contrary to these results, some reports in the literature have shown that topoisomerase I and II inhibitors used in combination can have a complementary effect. Whitacre *et al.* [41] showed that inhibition of topoisomerase I by topotecan induces a compensatory increase in topoisomerase IIα (one of two topoisomerase isomers) and sensitivity to etoposide (a topoisomerase II inhibitor). The increase in topoisomerase II levels can increase cell kill induced by topoisomerase II poisons [42]. However, although it has been shown that topotecan can increase the cytotoxicity of etoposide in a sequential combination, others report that the simultaneous exposure to anthracyclines and camptothecins has shown antagonism in cell culture [43,44]. For these reasons, several studies investigating the dosing regimens of topoisomerase I and II inhibitors, such as topotecan and doxorubicin sequential administration, have been conducted [45]. The effects of sequential and simultaneous dosing have been confirmed *in vivo* using a mouse xenograft model using epotoside and irinotecan/topotecan [46]. Similar results were obtained by Kim *et al.* [47] on xenografts generated from a variety of cell lines working with irinotecan and doxorubicin.

Rapamycin DEB alone was relatively ineffective in terms of cytotoxicity but this was not unexpected, given its proposed mode of action in the inhibition of presurvival pathways. Its combination with the other DEBs, however, did result in some synergistic effects on cell kill of both HepG2 and PSN1 cell lines. This complementary action of rapamycin and doxorubicin has been noted in the literature, but no definitive mechanism has been established. One mechanism of synergy suggested is the activation and inactivation of nuclear factor κβ (NF-κβ). NF-κβ is a transcription factor that plays an important role in cell survival, cell proliferation and immune responses by expression of its target genes [48]. NF-κβ is generally regarded as an antiapoptotic mediator, which is generally what was observed in cancer cells, in which

Fig. 11



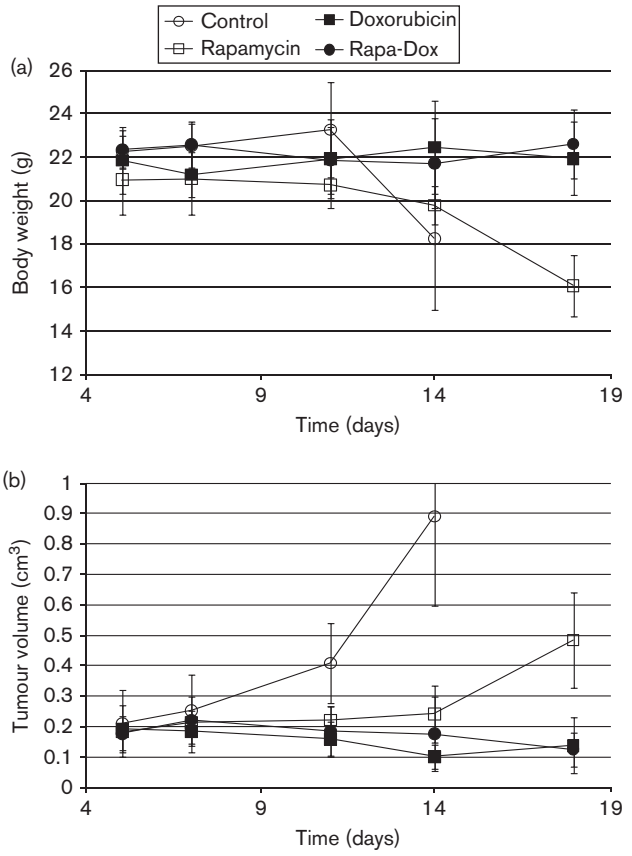
Comparison of the effect of combination of single drug-eluting beads (DEBs) loaded with doxorubicin and DEBs loaded with rapamycin vs. dual-loaded DEBs loaded with doxorubicin and rapamycin on HEPG2 cell viability using the MTS assay. (a) 24 h, (b) 48 h and (c) 72 h. MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt.

NF-κβ activation usually inhibits apoptosis induced by doxorubicin [49]. NF-κβ activity can be reduced using rapamycin. Rapamycin very specifically binds to FKBP51 and inhibits its isomerase activity, which is required for the function of the Iκβ kinase (IKK) complex [50]. Therefore, it is reasonable to assume that it counteracts NF-κβ activation by affecting the IKK cofactor [50]. The finding that rapamycin reduces the phosphorylating activity of IKK on its Iκβ [51] substrate supports this hypothesis and, likewise, rapamycin used in combination with doxorubicin has also been shown to reverse doxorubicin resistance in prostate cancer cells [52]. This supports the effects observed here with the combination of doxorubicin and rapamycin DEB, which was therefore the most promising option to take forward into further studies to generate a dual-drug combination DEB.

Effectiveness of doxorubicin : rapamycin dual-drug drug-eluting bead combinations

Single-drug and dual-drug DEB combinations of doxorubicin with rapamycin produced similar results in the cytotoxicity assays, demonstrating that drug release rates were similar whether combined in the same bead or in different beads. This supports the fact that the drugs do not interact with one another when loaded into the polymer structure. In the mouse xenograft model, rapamycin (20 mg/ml) and doxorubicin (25 mg/ml) dual-drug combination DEB reduced the tumour size in a manner comparable to the doxorubicin DEB until day 18. In contrast to rapamycin DEB alone, the drug combination DEBs did not show any signs of toxicity. Dual-loaded DEB did not show any significant improvement compared with doxorubicin DEB alone over the time period

Fig. 12



Effect of drug-eluting beads (DEBs) loaded with doxorubicin, DEBs loaded with rapamycin and DEBs loaded with doxorubicin and rapamycin vs. control (unloaded DEB) on (a) the body weight and (b) tumour size in a PSN1 mouse xenograft model.

observed. Had the study been longer, perhaps continued reduction would have resulted. However, this assumption needs to be tested in future experiments. The study did show a reduction in mouse toxicity for the dual-combination DEB compared with rapamycin-loaded DEB. Rapamycin toxicity has also been noted, as determined by weight in rat studies [53]. The mechanism for rapamycin toxicity reduction using the combination was not determined, but a group has observed that in yeast, elevated oxidative stress, a mechanism of doxorubicin activity, modifies mTOR complexes and prevents rapamycin binding [54]. This mechanism may be protecting the healthy mouse cells from the toxic effects of rapamycin.

Limitations to the study

Although this study has served to identify a potentially beneficial DEB combination for use in locoregional therapy, the methods used have their limitations. It has been widely reported that when drugs are used in combination, the ratio of the drugs actually delivered within the tumour cells may be a critical factor. Indeed, liposomal technologies have been developed in an attempt to ensure that specific drug combination ratios are maintained, as a change in the ratio can result in a shift from a synergistic to an antagonistic effect on efficacy [55,56]. In our in-vitro studies, we have made no attempt to control the relative drug release from the various DEB in order to achieve a specific ratio of the drug combination; the relative amounts of drug eluted into the wells will be controlled by the physicochemical properties of the drug such as its ion-exchange capability and solubility in cell culture media.

In terms of in-vivo methods of DEB evaluation, the nude mouse xenograft model may provide an initial screen for the efficacy and toxicity of the DEB, but could be improved with the use of longer study periods, multiple injections and more mice per test group in order to enhance the statistics. It cannot, however, be used to evaluate arterial embolization as the mouse vessels are too small, and hence lacks the effects of hypoxia induced by the embolization. Others have used a surgical approach in rats to achieve intrahepatic arterial infusion [57], but this too has its problems, and the rabbit VX2 tumour model remains the most reliable method for the evaluation of intra-arterial delivery [58], where the effects of hypoxia induced by arterial occlusion can be measured [33]. The inhibition of mTOR, the target of rapamycin, has also be shown to inhibit the activation of hypoxia-inducible factor-1α [59], a transcription factor that enhances cell survival under low oxygen conditions and has been shown to be upregulated in the VX2 tumour model postembolization [33]. This could confer a further advantage to a doxorubicin/rapamycin combination DEB if one of the drugs is a potential inhibitor of prosurvival pathways that could be activated as a consequence of intra-arterial occlusion, or even simply as a consequence of intrinsic hypoxia existing in rapidly growing malignancies such as pancreatic cancer. We intend, therefore, to report in the near future on our refinement to the in-vitro screening methods to allow a comparison of the effects of normoxic versus hypoxic conditions on the efficacy of the doxorubicin/rapamycin combination DEB.

Conclusion

Combinations of doxorubicin, irinotecan and topotecan DEB did not show signs of complementary activity at most time points and for both HepG2 and PSN1 cell types, with some antagonism evident with topotecan and doxorubicin DEB on HepG2 cells. Some differences were seen between the cell type and response to single-drug DEB combinations. A process was developed for the loading of rapamycin into DEB and the elution was shown to be solubility controlled. It was also shown that doxorubicin and rapamycin could be loaded into the same DEB without affecting the loading or the elution profile of the other. The combination of single drug-loaded doxorubicin and rapamycin DEBs was shown to

enhance cytotoxicity when compared with either drug alone, at all time points measured. This complementary killing efficacy was also demonstrated when the drugs were eluted from the dual-loaded doxorubicin/rapamycin DEB. The in-vivo nude mouse model seemed to demonstrate a similar effect for this dual-loaded DEB to doxorubicin DEB alone over the 21-day experimental period. The apparent toxicity of rapamycin DEB alone was reduced when compared with the dual-loaded DEB, which may be an indication of some effect regarding efficacy and perhaps signs of synergy.

Our studies have demonstrated that when selecting a particular drug combination, important consideration should be given to their respective mechanisms of action to ensure that they have a logical complementarity. Regardless of any limitations in our current screening approach, the combination of doxorubicin and rapamycin has demonstrated a synergistic action and the inclusion of both of these drugs in a single-combination DEB could provide the clinician with a simple therapy that could provide the benefits of a combination therapy without the adverse side effects experienced when these drugs are delivered systemically.

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Conflicts of interest

At the time that this work was carried out, R.E.J.F., Y.T. and A.L.L. were paid employees of Biocompatibles UK Ltd. R.E.J.F. and C.B. were PhD CASE students funded by Biocompatibles UK Ltd. A.W.L. is a consultant for Biocompatibles UK Ltd.

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