



## Evaluation of combination therapy schedules of doxorubicin and an acid-sensitive albumin-binding prodrug of doxorubicin in the MIA PaCa-2 pancreatic xenograft model

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### ABSTRACT

In this work, we evaluated combinations of doxorubicin with INNO-206, a (6-maleimidocaproyl)hydrazone derivative of doxorubicin (DOXO-EMCH) that is currently undergoing two phase II clinical trials, in a primarily chemoresistant tumor indication, *i.e.* pancreatic cancer. Thus, we compared the antitumor efficacy and tolerability of the following weekly intravenous treatments in the MIA PaCa-2 xenograft model:  $3 \times 6$  mg doxorubicin (MTD),  $3 \times 24$  mg/kg DOXO-EMCH (doxorubicin equivalents, MTD),  $3 \times 3$  mg/kg doxorubicin followed 6 h later by  $3 \times 12$  mg/kg DOXO-EMCH, and  $3 \times 12$  mg/kg DOXO-EMCH followed 6 h later by  $3 \times 3$  mg/kg doxorubicin. Whereas therapy with doxorubicin only produced a moderate tumor inhibition, all other therapy arms induced complete and partial remissions up to the end of the experiment on day 43. Although the total amount of doxorubicin equivalents is 72 mg/kg when DOXO-EMCH is administered alone, but only 45 mg/kg doxorubicin equivalents are administered in the combination regimens, the antitumor efficacy in all treated groups was essentially identical, a surprising finding of this study. However, there were significant differences in the tolerability as assessed by the body weight changes: whereas therapy at the MTD of DOXO-EMCH ( $3 \times 24$  mg/kg) produced a body weight loss of  $-16\%$  including one death, therapy with  $3 \times 12$  mg/kg DOXO-EMCH followed 6 h later by  $3 \times 3$  mg/kg doxorubicin produced  $-7\%$  body weight loss, and  $3 \times 3$  mg/kg doxorubicin followed 6 h later by  $3 \times 12$  mg/kg DOXO-EMCH produced a body weight gain of  $+2\%$  as a clear indication of minimal systemic toxicity. In addition, cell culture experiments revealed additive to synergistic effects when MIA PaCa-2 cells were exposed to doxorubicin followed 6 h later to exposure of the albumin-bound form of DOXO-EMCH spanning a ratio of 1:5 to 5:1 (analyzed for synergistic, additive or antagonistic effects using the software program CalcuSyn<sup>®</sup>). This animal study demonstrates that the time-dependent schedule of an albumin-binding prodrug and a free drug has a critical influence on the overall tolerability. A combination of doxorubicin and DOXO-EMCH is currently being investigated in a phase Ib study.

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### 1. Introduction

INNO-206 (DOXO-EMCH), the (6-maleimidocaproyl)hydrazone of doxorubicin is an albumin-binding prodrug of doxorubicin with acid-sensitive properties (see Fig. 1) that has demonstrated superior antitumor efficacy in eight tumor models compared to doxorubicin including three mamma carcinoma xenografts models (MDA-MB-435, MCF-7, M3366), an ovarian carcinoma model

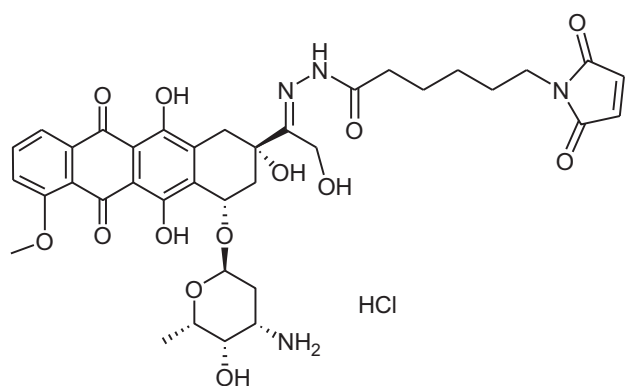
(A2780), an orthotopic renal cell cancer (RENCA) and an orthotopic pancreatic cancer model (AsPC-1) (Graeser et al., 2009; Kratz et al., 2002) as well as a multiple myeloma model (Sanchez et al., 2010).

After intravenous administration, DOXO-EMCH rapidly binds to the cysteine-34 position of circulating albumin and accumulates in solid tumors due to passive targeting. It has shown objective responses in a phase I study (Unger et al., 2007), and a phase Ib study against solid tumors as well as an overlapping phase II study in patients with soft tissue sarcoma are ongoing (see <http://www.cytrx.com>). Furthermore, a phase 2 clinical trial evaluating the preliminary efficacy and safety of DOXO-EMCH in patients with advanced pancreatic ductal adenocarcinomas within the US Pancreatic Cancer Dream Team in five centers was recently initiated (see <http://www.cytrx.com>).

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**Fig. 1.** Chemical structure of the (6-maleimidocaproyl)hydrazone derivative of doxorubicin (DOXO-EMCH, renamed INNO-206).

In a preliminary *in vivo* study, we reported on the anticancer effects and tolerability of a combination of DOXO-EMCH dosed at half its maximum tolerated dose (MTD), *i.e.*  $3 \times 12$  mg/kg, with a suboptimal dose of doxorubicin ( $3 \times 4$  mg/kg) in an aggressively growing ovarian carcinoma xenograft model (A2780) (Kratz et al., 2012). The combination achieved complete remissions as did therapy with DOXO-EMCH alone when dosed at its MTD ( $3 \times 24$  mg/kg), but was far better tolerated.

Due to these encouraging results, we extended our combination trials to a more chemoresistant tumor indication, *i.e.* pancreatic cancer. Pancreatic cancer is a common malignant disease worldwide that responds poorly to chemotherapy, the 5-year survival rate after diagnosis being less than 5% (Vincent et al., 2011). For locally advanced and for metastatic disease, which collectively represent over 80% of individuals, median survival is approximately 10 and 6 months, respectively, when treated with either 5-fluorouracil or gemcitabine (Burris et al., 1997; Zhu et al., 2011).

In this work, we evaluated the antitumor efficacy of DOXO-EMCH in the MIA PaCa-2 pancreatic cancer xenograft model alone and in comparison to doxorubicin at their respective maximum tolerated doses. In addition, we administered a combination of both drugs at half of their maximum tolerated doses and either dosed doxorubicin 6 h before administration of DOXO-EMCH or reversed the schedule and dosed DOXO-EMCH 6 h prior to doxorubicin. DOXO-EMCH as well as the combination therapy schedules were highly effective producing complete and partial remissions compared to only a moderate tumor inhibition induced by therapy with doxorubicin. Of note was that although the combination therapy showed strong antitumor effects comparable to those induced by DOXO-EMCH, there was a significant difference in the tolerability between the treated groups with a regimen where doxorubicin was administered 6 h before administration of DOXO-EMCH demonstrating no body weight loss at all. Furthermore, this drug combination and dose schedule demonstrated synergy against MIA PaCa-2 cells.

## 2. Materials and methods

DOXO-EMCH was dissolved in sterile 10 mM sodium phosphate, 5% D-(+)-glucose (pH 5.8) and the respective dose administered intravenously within 30 min after dissolution. All doses of DOXO-EMCH administered in the studies are stated in doxorubicin equivalents. Adrimedac<sup>®</sup> from medac, Germany, was used as the doxorubicin reference ( $c = 2$  mg/mL). The albumin-bound form of DOXO-EMCH (HSA-DOXO-EMCH) for cell culture experiments was prepared as reported previously and serially diluted with sterile 4 mM sodium phosphate buffer, 150 mM NaCl (pH 7.4). Doxorubicin HCl (from Yic-Vick, Hong Kong) was prepared as a stock solution

of 10 mM in sterile water for injection (Braun, Germany), and then serially diluted in the same vehicle.

### 2.1. *In vivo* experiments

For *in vivo* testing in the MIA PaCa-2 xenograft model, female NMRI: nu/nu mice (Taconic, Denmark) were used. The mice were held in individually ventilated cages (IVC) under sterile and standardized environmental conditions ( $25 \pm 2$  °C room temperature,  $50 \pm 10\%$  relative humidity, 12 h light–dark-rhythm). They received autoclaved food and bedding (ssniff, Soest, Germany) and acidified (pH 4.0) drinking water *ad libitum*. All animal experiments were performed under the auspices of the German Animal Protection Law. MIA PaCa-2 cells ( $5 \times 10^6$  cells/mouse) were transplanted subcutaneously (*s.c.*) into the left flank region of each mouse on day zero. Mice were randomly distributed to the experimental groups (7 mice per group). Treatment was initiated at day 10 when the tumors were grown to a size of  $\sim 60$  mm<sup>3</sup>. Treatment was initiated at day 10 when the tumors were grown to a size of  $\sim 60$  mm<sup>3</sup>. Mice were treated on a weekly schedule with 6 mg/kg doxorubicin (MTD), 24 mg/kg DOXO-EMCH (doxorubicin equivalents) (MTD), and a combination of 12 mg/kg DOXO-EMCH followed by 3 mg/kg doxorubicin in a 6 h interval on the day of injection, or a combination of 3 mg/kg doxorubicin followed by 12 mg/kg in a 6 h interval on the day of injection. All compounds were injected once a week for three weeks (days 10, 17, 24). The injection volume was 0.2 mL/20 g body weight. Tumor size was measured twice weekly with a caliper-like instrument in two dimensions. Individual tumor volumes (*V*) were calculated with the formula  $V = (\text{length} \times [\text{width}]^2) / 2$  and related to the values on the first day of treatment (relative tumor volume, RTV). The experiment was ended on day 43. Statistical analysis was performed with the *U*-test (Mann and Whitney) with  $p < 0.05$ . The body weight of mice was determined every 3–4 days.

### 2.2. Immunohistochemical staining of CD31

Control tumors for immunohistochemical staining of CD31 were isolated at the end of the experiment, shock frozen and stored at  $-80$  °C. Five  $\mu$ m thick cryosections (four slices/tumor) were prepared and pre-treated with 3% H<sub>2</sub>O<sub>2</sub> for 5 min, with avidin–biotin for 10 min each and 10% goat serum for 30 min in order to block endogenous enzyme activity. Slices were then incubated with a specific anti-mouse-CD31-antibody (rat anti-mouse CD31 (PECAM-1) monoclonal antibody MEC13.3, BD Pharmingen, cat. 550274) for 30 min followed by incubation with the secondary anti-rat antibody (goat anti-rat IgG, H+L chain specific, HRP-Conjugate, Southern Biotech, cat. 3050-05). Diaminobenzidine (Liquid DAB+, DakoCytomation, cat. K3468) was used as chromogen to visualize the antibody. Cryosections were finally counterstained with Mayer's Hematoxylin (DakoCytomation, cat. S3309) followed by dehydration and sealed with cover slips. Pictures were taken using an Axioskop 40 microscope (Zeiss, Germany) and analyzed with the software AxioVision 4.5 (Zeiss, Germany) applicable for measurement and counting of microscopic objects. Several pictures were taken per tumor ( $25\times$  and  $100\times$  magnification) and three representative pictures were chosen to visualize the vascularization pattern of MIA PaCa-2 xenografts.

### 2.3. Cytotoxicity assay and analysis of data with CalcuSyn<sup>®</sup>

Human pancreatic cancer MIA PaCa-2 cell lines were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 100 units/mL penicillin and 2 mg/mL streptomycin (Sigma Aldrich, Germany). Cells were exposed to the following compounds: (1) control group treated with vehicle

(sterile 4 mM sodium phosphate buffer, 150 mM NaCl–pH 7.4), (2) doxorubicin, (3) HSA-DOXO-EMCH, (4) doxorubicin followed 6 h later with HSA-DOXO-EMCH, and (5) HSA-DOXO-EMCH followed 6 h later with doxorubicin. The drugs were added in 1:3 serial dilution to give a final cellular concentration that ranged from 150  $\mu$ M to 2.5 nM. Constant ratios of 1:1; 1:5 and 5:1 ratios of HSA-DOXO-EMCH and doxorubicin were used for groups 4 and 5.

#### 2.4. MTT assay of cell viability

The number of viable cells remaining after treatment was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Aldrich, Germany) reduction assay as previously described (Sobottka and Berger, 1992). Cells were seeded in 96 well microtiter plates at a concentration of  $5 \times 10^4$ – $10^5$  cells/well. Following 72 h of treatment with various concentrations of the tested agents, MTT was added to each well at a final concentration of 0.5 mg/mL and incubated for 2 h at 37 °C. The formazan salt resulting from the reduction of MTT was solubilized in DMSO and the absorbance was read at 570 nm using an automatic plate reader. Mean fractional cell survival, defined as (treated – blank)/(untreated control – blank) was determined from 4 replicate wells per concentration. Individual dose response curves were obtained from two independent experiments, and the median effective dose,  $D_m$ , computed using CalcuSyn™ software (Biosoft, Cambridge, UK).

#### 2.5. Evaluation of drug interaction

Drug interaction was assessed by the combination index method. The combination index method is based on that described by Chou and Talalay and was determined with CalcuSyn™. The combination index (CI) is a quantitative measure of the degree of drug interaction in terms of additive effect (CI = 1), synergism (CI < 1), or antagonism (CI > 1) for a given fraction of cells affected (Chou and Talalay, 1984). The combination index (CI) values can be calculated at different “Effect levels”, i.e. at ED<sub>50</sub>, ED<sub>75</sub> and ED<sub>90</sub>. Antagonistic, additive, or synergistic effects according to the CalcuSyn™ software are defined as follows: 0.1–0.3 strong synergism; 0.3–0.7 synergism; 0.7–0.85 moderate synergism; 0.85–0.9 slight synergism; 0.9–1.1 additivity; 1.1–1.2 slight antagonism; 1.2–1.45 moderate antagonism; 1.45–3.3 antagonism; and finally 3.3–10 strong antagonism. Furthermore, the dose–reduction index (DRI) is a measure of how much the dose of each drug in a synergistic combination may be reduced at a given effect level compared with the doses for each drug alone, i.e. it defines the extent of dose reduction. The DRI is thus an important parameter when evaluating combination therapy *in vivo* where dose reduction leads to reduced toxicity toward the host while retaining the therapeutic efficacy (Chou, 2006).

#### 2.6. Statistical analysis

Data are presented as mean  $\pm$  SD which were analyzed using the software program GraphPad Instat (version 2).

### 3. Results and discussion

Pancreatic cancer responds poorly to clinically established low-molecular weight anticancer drugs, and progress obtained in combination chemotherapy regimens based on gemcitabine and 5-fluorouracil have been modest (Heinemann et al., 2006; Neoptolemos et al., 2010; Song et al., 2008). It is more likely that therapeutic advances will be achieved by new targeted approaches or drug delivery systems.

Our research group has been working intensively on the concept and on the chemical aspects of a prodrug concept for drug targeting which exploits endogenous albumin as a drug carrier. The salient feature of the prodrug technology is characterized by an *in situ* binding of thiol-binding prodrugs to the cysteine-34 position of circulating albumin, i.e., low-molecular weight prodrugs that bind specifically and selectively to albumin are injected directly into the blood stream and are then transported in their albumin-bound form to their site of action where they release the respective drug. The (6-maleimidocaproyl)hydrazine of doxorubicin (DOXO-EMCH, renamed INNO-206, now aldodoxorubicin) emerged as a lead compound of this technology. The prodrug binds rapidly and selectively to circulating serum albumin and after tumor uptake releases doxorubicin at the tumor site due to incorporation of an acid-sensitive hydrazone bond.

The accumulation of DOXO-EMCH in tumor tissue combined with a favorable biodistribution (Kratz, 2007) is due to the pathophysiology of tumor tissue, characterized by a high metabolic turnover, angiogenesis, hypervascularity, a defective vascular architecture and an impaired lymphatic drainage, the so-called EPR (enhanced permeation and retention) effect (Kratz and Beyer, 1998). Endogenous albumin acts as the drug carrier for DOXO-EMCH, and this protein is emerging as a versatile protein carrier for tumor drug targeting and for improving the pharmacokinetic profile of peptide- or protein-based drugs (Kratz, 2008).

Due to the encouraging results that we had observed when combining the albumin-binding prodrug DOXO-EMCH with doxorubicin in a fast growing A2780 ovarian cancer xenograft model (Kratz et al., 2012), we investigated the anticancer effect of a combination of DOXO-EMCH and doxorubicin in comparison to the effect of the single agents at their respective maximum tolerated doses (MTDs) in a slower growing and essentially chemoresistant xenograft model, i.e. the MIA PaCa-2 xenograft model.

Table 1 gives an overview of the design of the experiment and the parameters that were assessed.

We rated the MIA PaCa-2 xenograft model as a feasible animal model for assessing our combination protocols for two reasons: (1) it only responds poorly to anticancer agents such as 5-fluorouracil, doxorubicin, and cisplatin (<60% tumor inhibition) and modestly to gemcitabine (69% tumor inhibition) (Schultz et al., 1993), and (2) combination experiments with either two anticancer agents or an anticancer agent combined with NF-kappa inhibitors, MEK inhibitors, or opioid growth factors have been carried out in this model, such as a time-dependent schedule of an orally administered fluorouracil derivative (S1) and gemcitabine (Nakahira et al., 2008; Yoshizawa et al., 2009), a combination of glufosamide and gemcitabine (Ammons et al., 2007), combinations of uracil-tegafur and gemcitabine (Tsuje et al., 2006), a combination of a NF-kappa inhibitor apigenin and gemcitabine (Lee et al., 2008), a combination of a mitogen-activated protein kinase (MEK) inhibitor and rapamycin (Chang et al., 2009), and combination chemotherapy with gemcitabine and an opioid growth factor (Zagon et al., 2005), but progressive diseases, minor responses or stable diseases being the best results that have been achieved with any of these combinations.

We have previously shown that the therapeutic efficacy of DOXO-EMCH vs. either doxorubicin or gemcitabine on the pancreatic tumor was clearly superior ( $p=0.0041$  vs. control) in an orthotopic pancreatic cancer model (AsPC-1), with both free drugs being essentially inactive (Graesser et al., 2009). We therefore did not include gemcitabine in the present study also because to the best of our knowledge we are not aware of any published *in vivo* data which demonstrates remission in pancreatic carcinoma models.

When we performed our first orientating combination trial in the A2780 ovarian carcinoma xenograft model at the beginning of

**Table 1**  
Mortality, body weight change, and tumor response of MIA PaCa-2 tumor-bearing nude mice treated with specified dose schedules of doxorubicin, DOXO-EMCH and combinations of the drugs in a 6 h interval.

No. of mice	Substance	Days of administration	Dose <sup>a</sup> (mg/kg)	Mortality (at day)	BWC [%] (maximum on day)	RTV T/C (%) (optimum on day)	No. of PD, SD, PR, and CR
7	Glucose	10,17,24					
7	Doxorubicin	10,17,24	6		–6 (d 18)	6.5 (d 29) <sup>b</sup>	3 PD, 4 SD
7	DOXO-EMCH	10,17,24	24	1 (d 39)	–20 (d 36)	1.4 (d 36) <sup>b,c</sup>	1 PD, 1 SD, 5 CR
7	DOXO-EMCH	10,17,24	12		–9 (d 14,21)	1.9 (d 29) <sup>b,c</sup>	1 PD, 2 SD, 4 CR
7	Doxorubicin (6 h later)	10,17,24	3				
7	Doxorubicin	10,17,24	3		–10 (d 18)	1.7 (d 39) <sup>b,c</sup>	1 SD, 3 PR, 3 CR
7	DOXO-EMCH (6 h later)	10,17,24	12				

<sup>a</sup> Doxorubicin equivalents.

<sup>b</sup> Statistically significant to control.

<sup>c</sup> Statistically significant to doxorubicin.

Mann–Whitney test,  $p < 0.05$ .

PD, progressive disease ( $\pm 25\%$  TV); PR, partial remission ( $< 30\%$  TV); CR, complete remission (disappearance of tumor).

2011, the rationale was in essence empirical based on a hypothesis that a combination of the freely diffusing doxorubicin with the accumulation of the albumin-bound drug DOXO-EMCH mediated by the EPR effect would have advantages over therapy with DOXO-EMCH alone (Kratz et al., 2012). Of note is that DOXO-EMCH was administered 6 h before doxorubicin intravenous application.

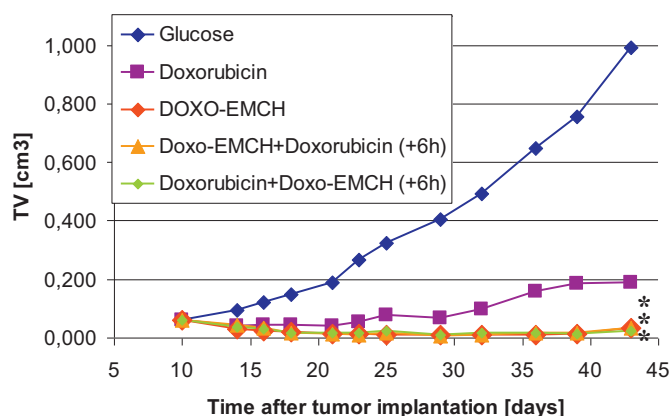
In the MIA PaCa-2 pancreatic carcinoma xenograft model we additionally investigated whether the time-dependent schedule of administering DOXO-EMCH and doxorubicin would produce a different picture of antitumor activity and tolerability. Hence, we compared the following weekly schedules (i.v. administration):  $3 \times 6$  mg doxorubicin (MTD),  $3 \times 24$  mg/kg DOXO-EMCH (doxorubicin equivalents, MTD),  $3 \times 3$  mg/kg doxorubicin followed 6 h later by  $3 \times 12$  mg/kg DOXO-EMCH, and  $3 \times 12$  mg/kg DOXO-EMCH followed 6 h later by  $3 \times 3$  mg/kg doxorubicin. The results of this experiment are shown in Fig. 2.

Whereas therapy with doxorubicin only produced a moderate tumor inhibition, all other therapy arms induced long-term remissions until the end of the experiment on day 43. However, there were significant differences in the tolerability as assessed by the body weight changes: therapy at the MTD of DOXO-EMCH ( $3 \times 24$  mg/kg) produced a body weight loss of  $-16\%$  at the end of the experiment including one death on day 39 and three animals

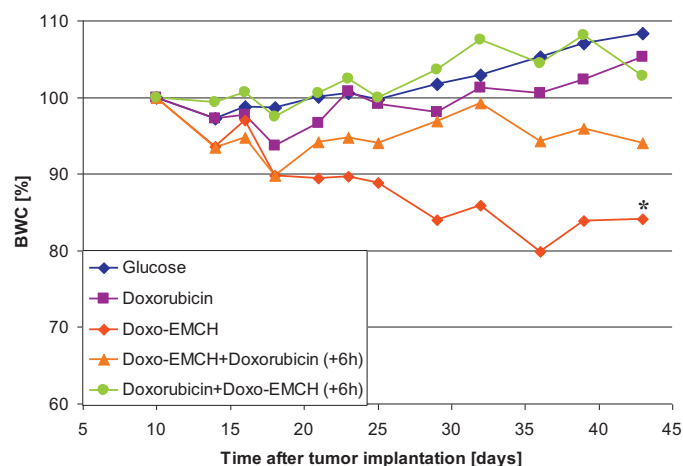
had to be sacrificed due to hind leg paralysis on day 43 (Table 1 and Fig. 3).

In contrast, therapy with  $3 \times 12$  mg/kg DOXO-EMCH followed 6 h later by  $3 \times 3$  mg/kg doxorubicin produced  $-7\%$  body weight loss, but  $3 \times 3$  mg/kg doxorubicin followed 6 h later by  $3 \times 12$  mg/kg DOXO-EMCH produced a body weight gain of  $+2\%$  as a clear indication of minimal systemic toxicity (see Fig. 3). The decrease in body weight in the animals treated with DOXO-EMCH alone was statistically significant to control animals, and to the groups treated with doxorubicin or both the time-dependent combinations of DOXO-EMCH and doxorubicin ( $p < 0.05$  – see Fig. 3). This animal study is to the best of our knowledge the first investigation demonstrating that a combination of an albumin-binding prodrug with its free parent drug not only has clear therapeutic advantages over free doxorubicin dosed at its MTD but that the time-dependent schedule has a critical influence on the overall tolerability and results with respect to tumor remissions without causing any body weight loss.

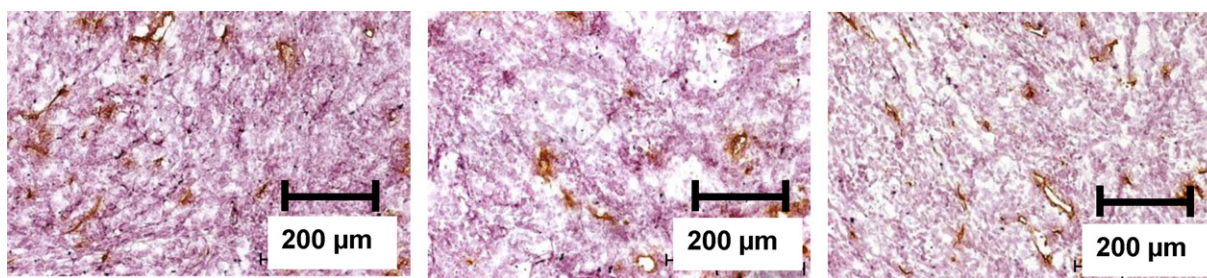
It is instructive to peruse the responses of the seven individual mice in each group – see Table 1 and Fig. 2S A–C (attached as supporting information): 1 PD, 1 SD, 5 CR for the DOXO-EMCH treated groups, 1 PD, 2 SD, 4 CR for therapy with DOXO-EMCH followed



**Fig. 2.** Therapeutic efficacy of a three weekly schedule of doxorubicin (6 mg/kg), DOXO-EMCH (24 mg/kg) and a combination of doxorubicin and DOXO-EMCH (3 mg/kg with 12 mg/kg DOXO-EMCH being administered 6 h later on the day of administrations, days 10, 17, and 24) and a combination of DOXO-EMCH and doxorubicin (12 mg/kg with 3 mg/kg doxorubicin being administered 6 h later on the day of administrations, days 10, 17, and 24) in nude mice bearing subcutaneously growing MIA PaCa-2 pancreatic carcinoma xenografts (7 animals per group).



**Fig. 3.** Body weight changes under therapy with doxorubicin ( $3 \times 6$  mg/kg), DOXO-EMCH ( $3 \times 24$  mg/kg) and a combination of doxorubicin and DOXO-EMCH ( $3 \times 3$  mg/kg with  $3 \times 12$  mg/kg DOXO-EMCH being administered 6 h later on the day of administrations, days 10, 17, and 24) and a combination of DOXO-EMCH and doxorubicin ( $3 \times 12$  mg/kg with  $3 \times 3$  mg/kg doxorubicin being administered 6 h later on the day of administrations, days 10, 17, and 24) against the pancreatic carcinoma xenograft MIA PaCa-2 (7 animals per group). \*Statistically significant vs. control, and the groups treated with doxorubicin and both time-dependent combinations of DOXO-EMCH and doxorubicin, group,  $p < 0.05$ .



**Fig. 4.** Immunohistochemical staining of the CD31 antigen that makes up a large portion of endothelial cell intercellular junctions and serves as a marker for the extent of microvessel density, shown for three MIA PaCa-2 control tumors.

6 h later by doxorubicin, and 1 SD, 3 PR, 3 CR for therapy with doxorubicin followed 6 h later by DOXO-EMCH (PD=progressive disease, SD=stable disease, PR=partial remission, CR=complete remission). Although these individual responses should not be over-interpreted considering that there was no statistically significant difference between the overall response of these three groups, it is of note that complete remissions are achieved which is rarely observed in pancreatic xenograft models, and the antitumor response within the group varies underscoring that xenograft models are also characterized by a certain degree of heterogeneity.

What are the most likely reasons to explain the surprising *in vivo* effects of the combination experiments carried out with the albumin-binding prodrug of doxorubicin, DOXO-EMCH, in the A2780 ovarian cancer and MIA PaCa-2 xenograft model? The first unexpected result is that a combination of DOXO-EMCH with doxorubicin – irrespective which drug is dosed first within a 6 h interval – is as effective as therapy with DOXO-EMCH alone although the total amount of doxorubicin administered is considerably lower in the combination therapy (48 mg/kg in the A2780 model and 45 mg/kg in the MIA PaCa-2 model) compared to 72 mg/kg for DOXO-EMCH at its MTD. This difference serves as an explanation for the better tolerability in terms of body weight loss (–16% BWC for 3 × DOXO-EMCH, –7% BWC for DOXO-EMCH → doxorubicin and +2% for doxorubicin → DOXO-EMCH) (see Fig. 3) but considering that neither 3 × 12 mg/kg DOXO-EMCH nor 3 × 3 mg/kg doxorubicin would achieve tumor remissions, it is indeed intriguing that the combination of the two drugs is so effective. Of interest is also that dosing doxorubicin before DOXO-EMCH results in a better tolerability compared to the reversed schedule. The most likely explanation is that in the first case the total exposure of doxorubicin to mice is shorter due to the rapid half-life of doxorubicin which is biphasic with a half-life of only ~1 min for the first elimination phase (Baurain et al., 1979).

The promising *in vivo* results when combining doxorubicin with an acid-sensitive prodrug that binds covalently to endogenous albumin are indeed surprising, because single doses of either agent in this experiment would not lead to tumor regressions as we have shown previously in three xenograft animal models (Kratz et al., 2000, 2002). In the three weekly schedule, doxorubicin at 3 or 4 mg/kg (total cumulative dose 9–12 mg/kg) is not the optimal schedule for doxorubicin in nude mice xenograft models (which is 2 × 8 mg/kg – cumulative dose 16 mg/kg) as has been shown convincingly in the early 1990s by the drug antibody group at Bristol–Myers (Trail et al., 1992) as well as by us in work on acid-sensitive transferrin and albumin conjugates of doxorubicin (Kratz et al., 2000). Splitting the doxorubicin doses into smaller doses results in a loss of antitumor activity due to the pharmacokinetics of doxorubicin and the concomitant lower plasma peak levels which do not translate into relevant antitumor efficacy (Trail et al., 1992).

The observation that the antitumor efficacy of the combination schedules of DOXO-EMCH and doxorubicin dosed at considerably

lower doses than for DOXO-EMCH are highly effective in the MIA PaCa-2 pancreatic tumor model achieving long-term remissions identical to therapy with DOXO-EMCH alone at its MTD (3 × 24 mg/kg) suggests that a different mode of action than mere additive cytotoxic effects is accountable for this unexpected result. Considering that doxorubicin at low doses has a pronounced apoptotic effect on endothelial cells (Albertsson et al., 2003; Lennernas et al., 2003), doxorubicin at the low dose of 3 × 3 mg/kg could act as an antiangiogenic rather than a tumor cell killing agent and expand the interstitial space, vessel diameter and blood-perfused area subsequently enhancing the delivery of albumin-bound DOXO-EMCH to the tumor interstitium. Although this working hypothesis for the combination therapy with DOXO-EMCH and doxorubicin needs to be corroborated by other methods such as intravital and fluorescence microscopy as well as microvessel density combined with intratumoral doxorubicin distribution studies, investigations by Tong et al. (2004) have already shown that antiangiogenic agents lead to a more uniform and deeper tumor penetration of albumin. When pre-treating tumor-bearing mice with the anti-VEGF antibody DC101, the penetration length for albumin from microvessels in the tumor increases from  $7.26 \pm 1.11 \mu\text{m}$  (untreated) to  $11.23 \pm 1.41 \mu\text{m}$  (Tong et al., 2004).

Preliminary support of this interpretation are the immunohistochemical staining results with CD31 in MIA PaCa-2 control tumors (Fig. 4) which can be used as a measure of vascularization and reveal that the microvessel density was moderate to good in control tumors.

In order to discover whether this obviously more than additive effect of the drug combinations was also due to a synergistic interplay on a cellular level, we carried out a series of *in vitro* combination experiments with the albumin-bound form of DOXO-EMCH, HSA-DOXO-EMCH, and doxorubicin in the MIA PaCa-2 pancreatic carcinoma cell line.  $\text{IC}_{50}$  values for HSA-DOXO-EMCH and doxorubicin were determined and constant ratios of drug combinations (5:1, 1:1 and 1:5) were serially diluted in semi-logarithmic steps with either doxorubicin or HSA-DOXO-EMCH being added 6 h before the other drug. The MIA PaCa-2 cells were exposed to the drugs or drug combinations for 72 h, and cell viability was quantified using an MTT assay. In order to determine whether the combination of doxorubicin and HSA-DOXO-EMCH showed antagonistic, additive, or synergistic effects, combination indexes were calculated and interpreted with the software programme CalcuSyn™ based on the method described by Chou (Chou, 2006; Chou and Talalay, 1984). The results are shown in Table 2A and B and Fig. 5A and B.

The MTT cell viability assay showed that doxorubicin and HSA-DOXO-EMCH inhibited the growth of MIA PaCa-2 pancreatic cancer cells in a concentration dependent manner with a  $D_m$  of  $0.32 \pm 0.08$  and  $6.36 \pm 0.33 \mu\text{M}$  which corresponds to the  $\text{IC}_{50}$  value (Table 2). Median effect analysis of drug combinations (Table 2A and B, Fig. 5A and B) revealed different levels of interactions at the different effect levels and ratios tested. The addition of HSA-DOXO-EMCH to

**Table 2**  
Dose–effect relationships of the single drugs (doxorubicin and HSA-DOXO-EMCH) and drug combinations. (A) Doxorubicin followed by addition of HSA-DOXO-EMCH after 6 h and (B) HSA-DOXO-EMCH followed by addition of doxorubicin after 6 h in MIA PaCa-2 pancreatic cancer cell lines. Data are presented as mean  $\pm$  S.D.

Drug or drug combination	Parameters			CI values at			DRI values at		
	$D_m$	$m$	$r$	ED <sub>50</sub>	ED <sub>75</sub>	ED <sub>90</sub>	ED <sub>50</sub>	ED <sub>75</sub>	ED <sub>90</sub>
Doxorubicin	0.32 $\pm$ 0.08	0.71	0.95	N/A	N/A	N/A	N/A	N/A	N/A
HSA-DOXO-EMCH	6.36 $\pm$ 0.33	0.92	0.96	N/A	N/A	N/A	N/A	N/A	N/A
Doxorubicin + HSA-DOXO-EMCH (1:1)	0.06 $\pm$ 0.02	0.36	0.93	0.51 $\pm$ 0.15	0.68 $\pm$ 0.14	0.93 $\pm$ 0.09	2.5 $\pm$ 0.77	1.7 $\pm$ 0.36	1.2 $\pm$ 0.12
Doxorubicin + HSA-DOXO-EMCH (1:5)	0.21 $\pm$ 0.03	0.39	0.95	0.72 $\pm$ 0.18	1.1 $\pm$ 0.15	1.8 $\pm$ 0.13	13.0 $\pm$ 4.0	12.0 $\pm$ 2.5	11.0 $\pm$ 1.2
Doxorubicin + HSA-DOXO-EMCH-EMCH (5:1)	0.09 $\pm$ 0.01	0.44	0.95	0.10 $\pm$ 0.01	0.18 $\pm$ 0.03	0.35 $\pm$ 0.12	2.0 $\pm$ 0.47	1.80 $\pm$ 0.49	1.5 $\pm$ 0.35
							11.0 $\pm$ 0.16	0.94 $\pm$ 0.29	3.2 $\pm$ 1.2
							121 $\pm$ 4	123 $\pm$ 15	127.0 $\pm$ 34.0

Drug or drug combination	Parameters			CI values at			DRI values at		
	$D_m$	$m$	$r$	ED <sub>50</sub>	ED <sub>75</sub>	ED <sub>90</sub>	ED <sub>50</sub>	ED <sub>75</sub>	ED <sub>90</sub>
HSA-DOXO-EMCH	6.36 $\pm$ 0.33	0.92	0.96	N/A	N/A	N/A	N/A	N/A	N/A
Doxorubicin	0.32 $\pm$ 0.08	0.71	0.95	N/A	N/A	N/A	N/A	N/A	N/A
HSA-DOXO-EMCH + doxorubicin (1:1)	0.14 $\pm$ 0.02	0.34	0.99	1.30 $\pm$ 0.17	2.20 $\pm$ 0.86	2.2 $\pm$ 0.68	0.95 $\pm$ 0.13	0.69 $\pm$ 0.06	0.52 $\pm$ 0.16
HSA-DOXO-EMCH + doxorubicin (1:5)	0.54 $\pm$ 0.09	0.53	0.95	1.90 $\pm$ 0.48	1.30 $\pm$ 0.2	1.1 $\pm$ 0.07	4.90 $\pm$ 0.64	4.90 $\pm$ 0.42	4.9 $\pm$ 1.5
HSA-DOXO-EMCH + doxorubicin (5:1)	0.34 $\pm$ 0.05	0.48	0.95	0.39 $\pm$ 0.09	0.59 $\pm$ 0.05	0.93 $\pm$ 0.09	1.90 $\pm$ 0.54	1.70 $\pm$ 0.02	1.6 $\pm$ 0.49
							0.79 $\pm$ 0.19	1.40 $\pm$ 0.41	2.6 $\pm$ 0.86
							2.90 $\pm$ 0.74	1.80 $\pm$ 0.12	1.1 $\pm$ 0.13
							31.0 $\pm$ 6.4	39.0 $\pm$ 8.0	49.0 $\pm$ 15

$D_m$ : the median-effect dose, equivalent to the IC<sub>50</sub> value;  $m$ : an exponent signifying the sigmoidal shape of the dose effect curve:  $m = 1$ ,  $>1$ , and  $<1$  indicates hyperbolic, sigmoidal, and negative sigmoidal shape, respectively;  $r$  value: the linear correlation coefficient of the median-effect plot. DRI: dose reduction index was measured by comparing the doses required to reach a given degree of inhibition when using the drug as a single agent and in combination.

doxorubicin after a 6 h interval at a 1:1 ratio produced synergism at ED<sub>50</sub> and ED<sub>75</sub> (CI = 0.51 and 0.68, respectively), whereas this combination ratio became additive at higher fraction of cell kill with an ED<sub>90</sub> of 0.93 (Table 2A and Fig. 5A). Likewise, the combination of doxorubicin and HSA-DOXO-EMCH at a 1:5 ratio produced slight synergism at ED<sub>50</sub> (CI = 0.72), whereas this combination was additive to antagonistic at ED<sub>75</sub> and ED<sub>90</sub> (1.1 and 1.8, respectively). Of note is that the highest and strong synergistic effect in this dose schedule of doxorubicin and HSA-DOXO-EMCH was when at a 5:1 ratio with CI values reaching 0.1, 0.18 and 0.35 for ED<sub>50</sub>, ED<sub>75</sub> and ED<sub>90</sub>.

In contrast, reversing the dose schedule, *i.e.* adding HSA-DOXO-EMCH 6 h before doxorubicin showed an antagonistic type of interaction for the ratios of 1:1 and 1:5 (doxorubicin:HSA-DOXO-EMCH) (Table 2B and Fig. 5B), and a synergistic to additive effect only at a ratio doxorubicin:HSA-DOXO-EMCH of 5:1 (CI = 0.39, 0.59 and 0.93 for ED<sub>50</sub>, ED<sub>75</sub> and ED<sub>90</sub>).

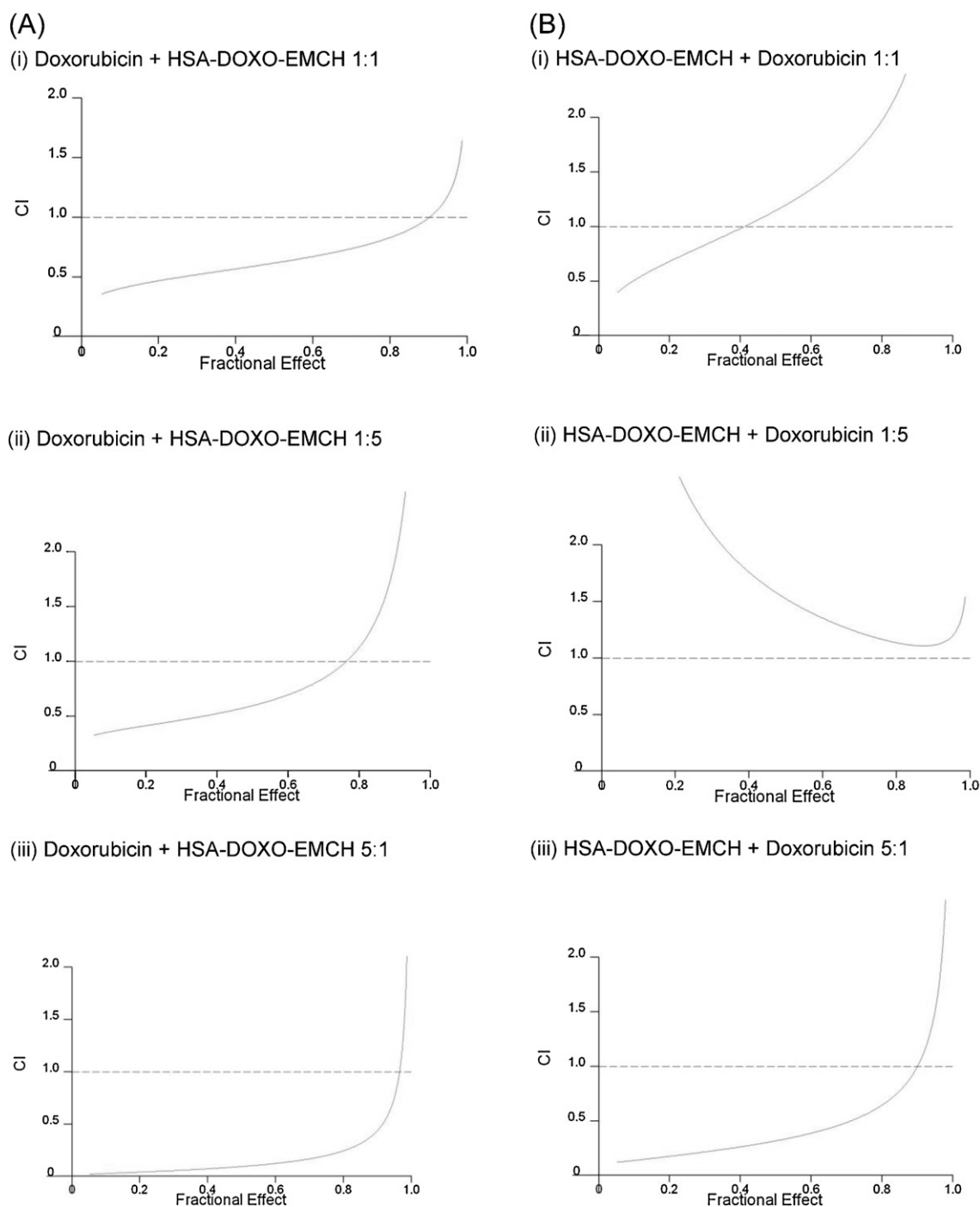
Taken together, our cell culture results revealed a primarily synergistic effect when doxorubicin was added 6 h before HSA-DOXO-EMCH and a 5:1 ratio of HSA-DOXO-EMCH:doxorubicin showed the highest synergistic profile. Although, the cleavage rate of HSA-DOXO-EMCH to doxorubicin at pH 5.0 is fast (half-life at 37 °C, ~25 min) (Kratz, 2007), it is difficult to predict precisely the exact ratio of HSA-DOXO-EMCH to doxorubicin in the MIA PaCa-2 cells after different incubation periods. The results, however, clearly suggest that the sequence of administration as well as the ratio has an effect on the synergistic mode of action that both drugs can exert. Of note in this context is that the intracellular accumulation of doxorubicin and acid-sensitive albumin conjugates of doxorubicin differs considerably as shown by us in confocal microscopy studies using fluorescent markers for mitochondria, golgi apparatus and lysosomes (Beyer et al., 2001). These results show accumulation of free doxorubicin primarily in the nucleus of tumor cells, whereas acid-sensitive albumin-doxorubicin conjugates accumulate in mitochondria and the golgi apparatus.

In summary, the encouraging *in vivo* results presented in this work in a pancreatic cancer xenograft model that in best case responses with stable disease when treated with clinically established anticancer agents, is an impetus for investigating further

combinations of albumin-binding prodrugs with other anticancer agents, albumin-based drug delivery systems as well as antiangiogenic inhibitors and clarifying the mode of action for the observed complementary effects. Several complementary and/or synergistic effects on the cellular as well as physiological level appear to be responsible for a favorable and homogenous distribution of the drugs in the tumor mass and within tumor cells and are worthy of further detailed investigation. After injection directly into the blood stream, DOXO-EMCH binds *in situ* to the cysteine-34 position of circulating albumin and is then transported in the albumin-bound form to the tumor site. It contains an acid-sensitive hydrazone linker that allows doxorubicin to be released either extracellularly in the slightly acidic environment often present in tumor tissue (pH range 6.0–6.8) (Tannock and Rotin, 1989) or intracellularly in acidic endosomal (pH 5.0–6.5) or lysosomal (pH 4.0–5.0) compartments after cellular uptake of the albumin conjugate by the tumor cell.

A number of factors will influence the EPR effect in preclinical animal models: the size and type of the tumor as well as the tumor model (subcutaneous, intramuscular, or orthotopic implantation sites, or spontaneous or chemically induced growing tumors) which all affect vascularization and the extent of hypoxic and necrotic areas (Matsumura and Maeda, 1986). Techniques such as intravital imaging have provided a detailed insight into the tumor microcirculation and microenvironment confirming hyper-permeability, a heterogeneous and compromised blood flow, and an absence of functional lymphatic vessels. However, the parameters which cause the EPR effect also result in an elevated interstitial fluid pressure that hinders the delivery of therapeutic agents to tumors (Fukumura et al., 2010). Although the EPR effect is presumably universal to all solid tumors, the extent of the EPR effect can be predicted to vary considerably within the tumor, and a passive tumor targeting approach may not reach all parts of the tumor (Kratz, 2007). Furthermore, drug penetration into the tumor can be influenced by metronomic and/or antiangiogenic therapy (Kratz and Warnecke, 2012).

Finally, tumor distribution of albumin is also mediated by transcytosis initiated by binding of albumin to a cell surface 60-kDa glycoprotein (gp60) receptor (albondin) as demonstrated for the albumin nanoparticle nab-paclitaxel (Abraxane®) and proposed



**Fig. 5.** Median effect plots of drug combinations in the MIA PaCa-2 pancreatic cancer cell line: combination index (CI) vs. effect level plots; (A) doxorubicin followed by addition of HSA-DOXO-EMCH after 6 h and incubated for further 66 h at a ratio of (i) 1:1, (ii) 1:5, (iii) 1:5. (B) HSA-DOXO-EMCH followed by addition of doxorubicin after 6 h and incubated for further 66 h at a ratio of (i) 1:1, (ii) 1:5, (iii) 5:1.

for albumin-binding single chain antibodies (Kratz and Elsadek, 2012).

A phase Ib study investigating the combination of doxorubicin and escalating doses of DOXO-EMCH (INNO-206) is underway (see [www.cytrx.com](http://www.cytrx.com)). Furthermore, we are extending our research to explore the role of gp60 receptor-mediated transcytosis as well as subsequent binding to SPARC (Secreted Protein, Acidic and Rich in Cysteine) for the tumor uptake and distribution of DOXO-EMCH.

#### Conflict of interest

The authors declare that they have no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ijpharm.2012.11.003>.

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