Cardiac safety and antitumoral activity of a new nitric oxide derivative of pegylated epirubicin in mice

Luca Santucci[a], Andrea Mencarelli[b], Barbara Renga[b], Daniela Ceccobelli[b], Gianfranco Pasut[c], Francesco M. Veronese[c], Eleonora Distrutti[a] and Stefano Fiorucci[b]

The use of epirubicin is limited by the risk of a dilatory congestive heart failure that develops as a consequence of induction of a mitochondrial-dependent cardiomyocyte apoptosis. In a previous in-vitro study, we have provided evidence that a new formulation of pegylated epirubicin-bearing moieties that release nitric oxide, named BP-747, exerted a potent antitumoral activity against a colon cancer cell line, which was completely devoid of cytotoxic activity against cardiomyocytes. The aim of this study was to investigate the antitumoral and cardiotoxic profile of BP-747 in Caco-2 and SKOV-2 tumor-bearing mice. Epirubicin-induced cardiomyopathy was detected by clinical (survival, weight loss), anatomical (heart weight loss) and biochemical evaluations (measurement of serum troponin and creatine phosphokinase levels). The antitumoral activity was investigated by the measurement of tumor diameters and weight. In comparison with free epirubicin and pegylated epirubicin, BP-747 showed more potent antineoplastic effects, as demonstrated by the 95% reduction of tumor volume. Moreover, while administration of epirubicin and pegylated epirubicin resulted in the development of a severe anthracycline cardiomyopathy, BP-747-treated mice were virtually devoid of clinical and biochemical signs of cardiotoxicity. The present data provide evidence that addition of a nitric oxide-releasing moiety to pegylated epirubicin confers a new and unique cytotoxic profile to the drug. *Anti-Cancer Drugs* 18:1081–1091 © 2007 Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2007, **18:**1081–1091

Keywords: anthracycline cardiomyopathy, apoptosis, Caco-2, SKOV-2, xenograft

[a]Struttura Complessa di Gastroenterologia, Azienda Ospedaliera di Perugia and [b]Department of Clinical and Experimental Medicine, University of Perugia, Perugia and [c]Department of Pharmaceutical Sciences, University of Padova, Padova, Italy

Correspondence to Dr Luca Santucci, Struttura Complessa di Gastroenterologia, Azienda Ospedaliera di Perugia, Via Brunamonti 51, 06122, Perugia, Italy
Tel: +39 075 5783268; e-mail: lsant@unipg.it

Received 14 March 2007 **Revised form accepted** 9 April 2007

Introduction

Anthracyclines, such as doxorubicin and epirubicin (EPI), are clinically effective and widely employed antineoplastic drugs used in the treatment of hematologic malignancies, including leukemia and lymphoma, and solid tumors, such as breast cancer [1,2]. Their use, however, is limited by the risk of a delayed, life-threatening and irreversible form of dilatory congestive heart failure, with a mortality that exceeds 50% within 2 years [3–5]. Mechanistically, anthracycline-induced cardiomyopathy involves the generation of free radicals [6–10] and selective inhibition of cardiac-specific genes involved in cardiac development and function, such as CARP and SERCA2 [11–13], leading to extensive cardiomyocyte apoptosis.

A body of evidence suggests that modifications of existing anthracyclines might be an effective strategy for generating effective drugs with reduced cardiotoxicity [14]. Liposome encapsulation of pegylated (PEG) doxorubicin is an effective method, and several preclinical and clinical comparative studies have demonstrated that this derivative is less cardiotoxic than the free drug and also provides comparable or better objective clinical responses [15,16]. The different safety profile of liposome PEG doxorubicin is probably related to its modified pharmacokinetic, resulting in a reduced cardiac uptake and in a longer plasma half-life of doxorubicin [17,18].

Nitric oxide (NO) protects endothelial cells and cardiomyocytes from apoptosis induced by oxidative stress and doxorubicin [19–24] and increases the antitumoral activity of several agents, including doxorubicin [25], cisplatin [26], melphalan [27], fludarabine [28] and nonsteroidal antiinflammatory drugs [29,30]. In the search of a new approach to limit anthracycline cardiomyopathy, we hypothesized that a PEG conjugate of EPI-bearing moieties that release NO would result in a new chemical entity (NO-PEG-EPI, named BP-747) that possesses either the advantage of drug pegylation and the cardio-protective and antitumoral properties of NO. We have previously shown that, in comparison with free EPI and pegylated EPI (p-EPI), BP-747 exerts a more potent antitumoral activity against the colon cancer cell line Caco-2, while sparing both endothelial cells and cardiomyocytes from EPI-induced cell apoptosis [31]. In this study we have investigated whether BP-747 maintains these properties also *in vivo* using two experimental models of solid cancer in mice.

0959-4973

Materials and methods

Drug and animals

EPI was purchased from Tide Corporation (Hangzhou, PRC). BP-747 was synthesized as detailed elsewhere [31]. BALB/c and nude Swiss mice were from Charles River Laboratories (Monza, Italy), and were maintained under specific pathogen-free conditions in the Animal Care Facility of the University of Perugia. Protocols were approved by the Animal Study Committees of the University of Perugia according to governmental guidelines for animal care.

Toxicity studies

Tumor-free BALB/c mice (6–8 mice per group) were used to compare the toxicity of single high doses of EPI, p-EPI and BP-747. Sterile solutions of the drugs in 0.9% (w/v) NaCl were administered intraperitoneally as a single bolus injection of 10 or 20 mg/kg equivalent amount of EPI. Body weight and mortality were recorded daily for 28 days. At the end of the study period, surviving mice were killed, and blood and hearts were harvested.

Histological analysis

Mice were killed, and their hearts were immediately removed and fixed in 10% buffered formalin, dehydrated in graded ethyl alcohol, and embedded in paraffin. Sections of 4 μm thickness were stained with hematoxylin/eosin (H/E) or Sirius red (0.1% solution in saturated aqueous picric acid). The H/E-stained sections were analyzed under light microscopy for histopathological assessment. The degree of lesions on microscopic cross-sections of the heart was graded semi-quantitatively according to Working *et al.* [17] from 1 to 4: grade 1: very slight degree of change (scattered, single myocardial fibers affected); grade 2: slight degree of change (scattered small groups of altered myocardial fibers throughout the myocardium); grade 3: moderate degree of change (disseminated myocardial fiber change with occasional focal unaffected areas); grade 4: marked degree of change (confluent groups of affected fibers, most myocardial fibers affected). For the Picrosirius red staining, the sections were stained with 0.1% Sirius red F3BA and 0.25% Fast green FCF.

Serum troponin and creatine phosphokinase measurements

Myocardial injury was detected by measuring cardiac troponin and creatine phosphokinase (CPK) levels in serum from venous blood samples. The blood samples were centrifuged, and the serum was stored at −80°C until assayed. Serum cardiac troponin and CPK levels were measured using a Hitachi 717 automatic analyzer (Hitachi; Tokyo, Japan).

Measurements of heart caspases 3, 8 and 9

Mice were killed and hearts were removed; they were then homogenized in cell lysis buffer with a tissue homogenizer. The homogenated tissue was washed with lysis buffer, and 250 μg of these lysates was evaluated for caspases 3, 8 and 9 activities using specific assay kits (for caspases 3 and 8: Caspase Apoptosis Detection Kit, Santa Cruz, California, USA; for caspase 9: ApoAlert Caspase Assay Kit, Clontech, Palo Alto, California, USA).

TUNEL assay

Terminal transferase dUTP nick end-labeling (TUNEL) assay was performed to detect apoptosis in cardiac sections. Briefly, after pretreatment with proteinase K (20 μg/ml) and 3.0% hydrogen peroxide, paraffin-fixed slides were treated with a digoxigenin–dNTP complex for 1 h at 37°C (ApopTag Plus Peroxidase in Situ Apoptosis Detection Kit; Chemicon International, Temecula, California, USA). This complex binds to free 3-OH termini of nucleosome-sized DNA fragments that are formed during apoptosis and it is detected by anti-digoxigenin conjugate antibody. The bound complex was stained with a 3,3′-diaminobenzidine-based substrate. The slides were counterstained with 0.5% methyl green in 0.1 mol/l sodium acetate, dehydrated in xylene, mounted and examined by light microscopy. Mouse mammary tissue served as a positive control.

Murine solid tumor models

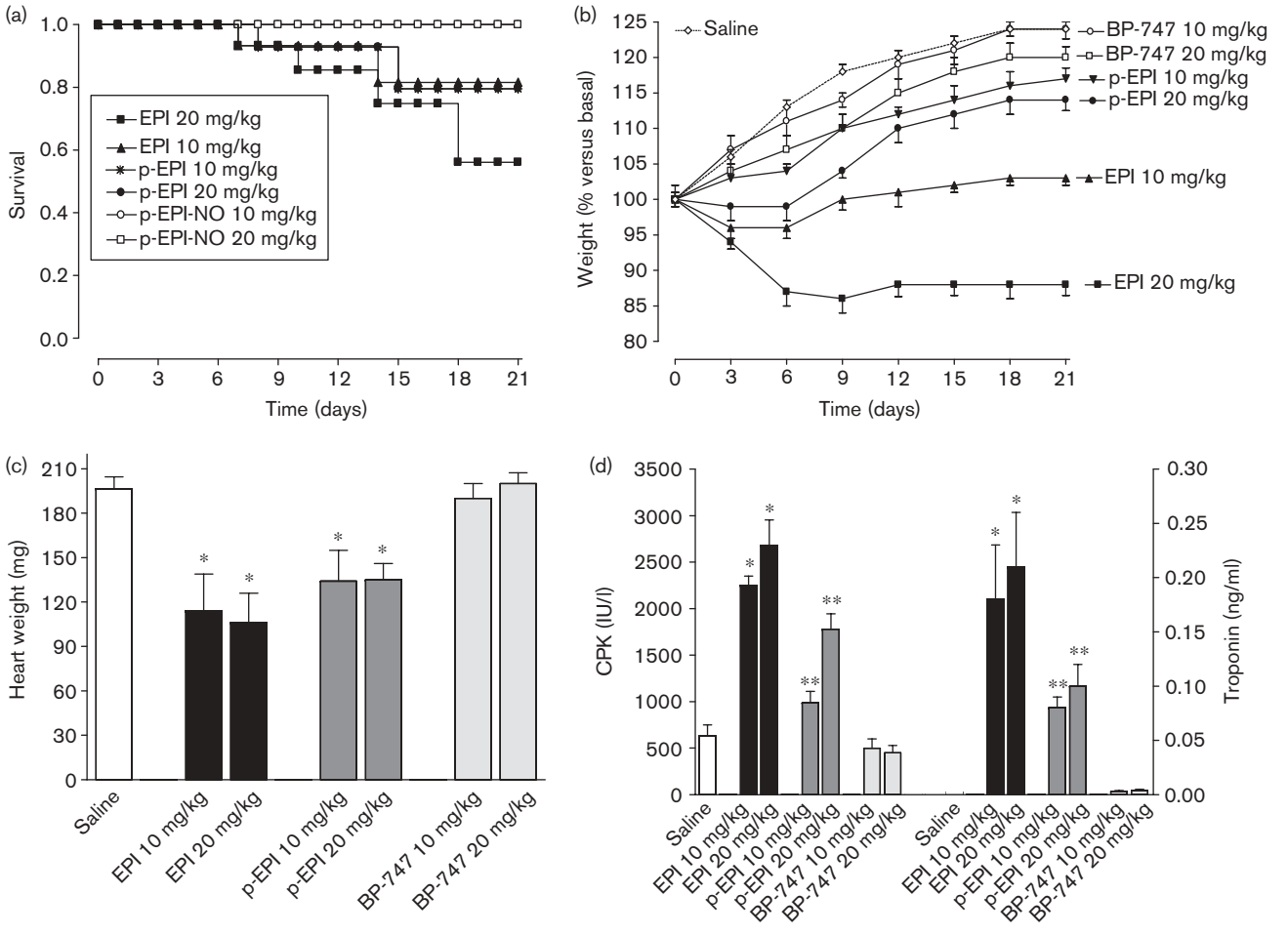
The human colon cancer cell line Caco-2 and the human ovarian cancer cell line SKOV-2 were purchased from American Type Culture Collection (Rockville, Maryland, USA) and were cultured in RPMI 1640 supplemented with 10% fetal calf serum at 37°C in a 5% CO_2 atmosphere. Caco-2 or SKOV-2 cells were injected subcutaneously in the left dorsal flank (1×10^6 cells per mouse) of 6- to 8-week-old Swiss nude male and female mice, respectively. Mice were monitored daily and tumor volume was measured with a caliper using the following formula: volume (mm^3) = length × width^2/2. When the tumors had grown to about 5 × 5 mm size, mice were randomly allocated in the treatment groups (6–8 animals per treatment group) such that the average volume of tumor in all the groups was about 100 mm^3. Mice were then treated intraperitoneally for 4 consecutive weeks with saline, EPI (5 mg/kg/week), p-EPI (5 mg/kg/week equivalent amount of EPI) or BP-747 (2.5 and 5 mg/kg/week equivalent amount of EPI). Body weight, mortality and tumor size were recorded until day 28, at which time surviving mice were killed and blood, heart and tumor were harvested.

Real-time reverse transcription-polymerase chain reaction

After killing the mice, the hearts and tumors were removed, weighed, immediately snap-frozen in liquid nitrogen and stored at −80°C until assayed. Total RNA was processed directly to cDNA by reverse transcription with Superscript III (Invitrogen, Milan, Italy). Quantification of the expression of mouse genes in heart tissue

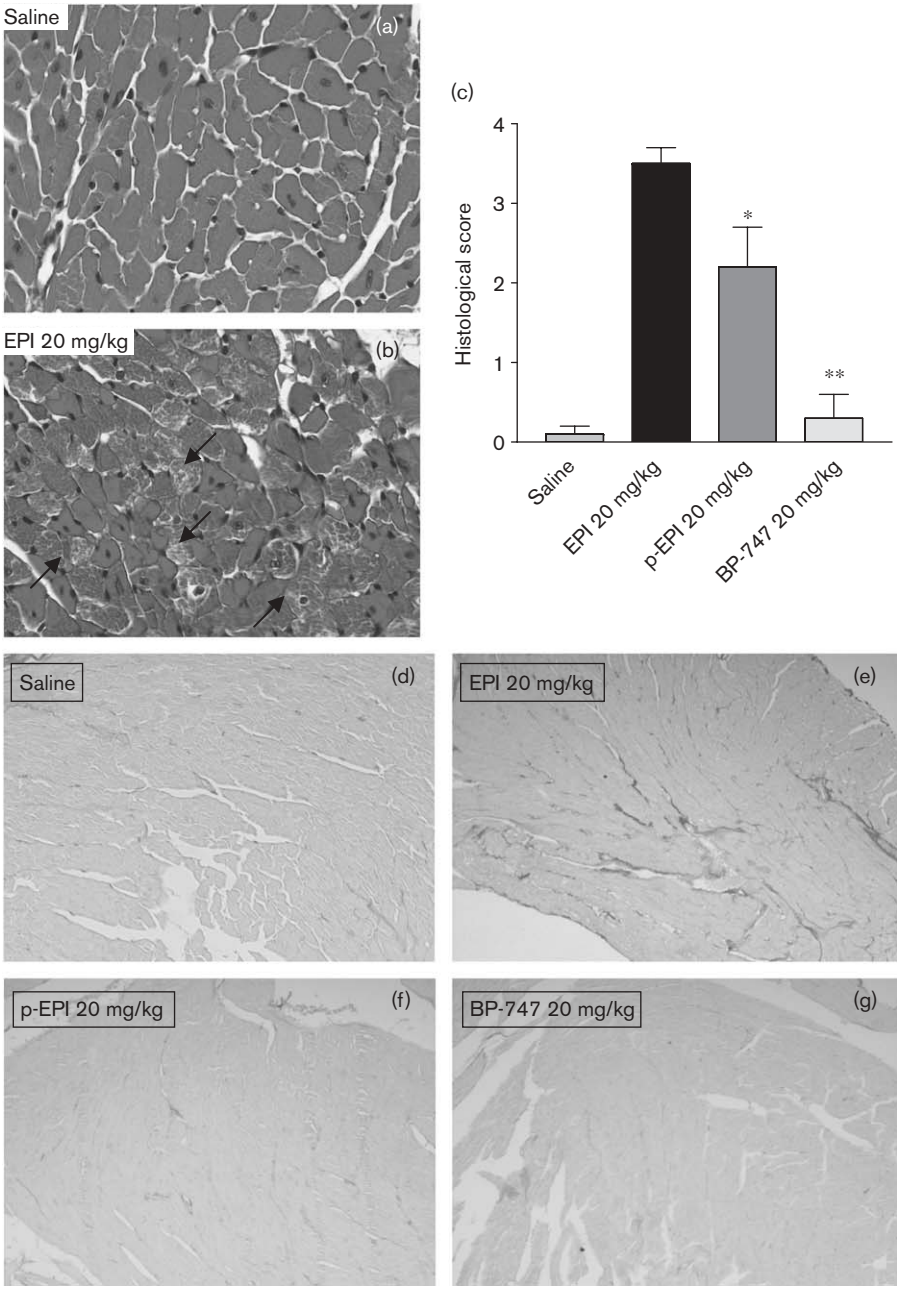
was performed by quantitative reverse transcription polymerase chain reaction (RT-PCR), using the following sense and antisense primers: CARP, 5′-gccaaggacagagaaggagac-3′ and 5′-tgcgagagttcttgtaggcat-3′ and SERCA-2, 5′-gtggaacctttgccgctcat-3′ and 5′-acgcacccgaacacccttat-3′. Quantification of the expression of human genes in colon carcinoma tissue was performed by quantitative reverse transcription-polymerase chain reaction (RT-PCR), using the following sense and antisense primers: survivin: 5′-acatctgtcacgttctccacac-3′ and 5′-atccatcatcttacgccagact-3′; Bcl-2: 5′-atgtgtgtggagagcgtcaa-3′ and 5′-atcaccaagtgcacctaccc-3′; Bax: 5′-ggggacgaactggacagtaa-3′ and 5′-cagttgaagttgccgtcaga-3′; galectin-1: 5′-gacgctaagagcttcgtgct-3′ and 5′-gcacacctctgcaacacttc-3′ and p53: 5′-gttccgagagctgaatgagg-3′ and 5′-tctgagtcaggcccttctgt-3′. All PCR primers were designed using PRIMER3-OUTPUT software using published sequence data from the National Center for Biotechnology Information database. Total RNA was isolated from specimens by TRIzol reagent (Invitrogen). One microgram of purified RNA was treated with DNase I for 15 min at room temperature, followed by incubation at 95°C for 5 min in the presence of 2.5 mmol/l ethylenediaminetetraacetic acid. The RNA was reverse transcribed with Superscript II (Invitrogen) in 20 µl reaction volume using random primers. For quantitative RT-PCR, 100 ng of template was dissolved in a 25 µl containing 0.3 µmol/l of each primer and 12.5 µl of 2XZ SYBR Green PCR Master mix (Bio-Rad, Hercules, California, USA). All reactions were performed in triplicate and the thermal cycling conditions were as follows: 2 min at 95°C, followed by 50 cycles of 95°C for 10 s and 60°C for 30 s in an iCycler iQ instrument (Bio-Rad). The mean value of the replicates for each sample was calculated and expressed as the cycle

Fig. 1



Effect of BP-747 on anthracycline cardiomyopathy. BALB/c mice (10 per group) were given saline, free EPI (10 or 20 mg/kg), p-EPI (10 or 20 mg/kg equivalent amount of EPI) or BP-747 (10 or 20 mg/kg equivalent amount of EPI) as a single intraperitoneal bolus injection and followed for 21 days to evaluate mortality (a) and weight (b). At the end of the study period, surviving mice were killed, and heart weight (c) and serum cardiac enzymes (d) were determined. *$P<0.05$ versus saline and BP-747-treated animals. **$P<0.05$ versus all groups. EPI, epirubicin; p-EPI, pegylated epirubicin; CPK, creatine phosphokinase.

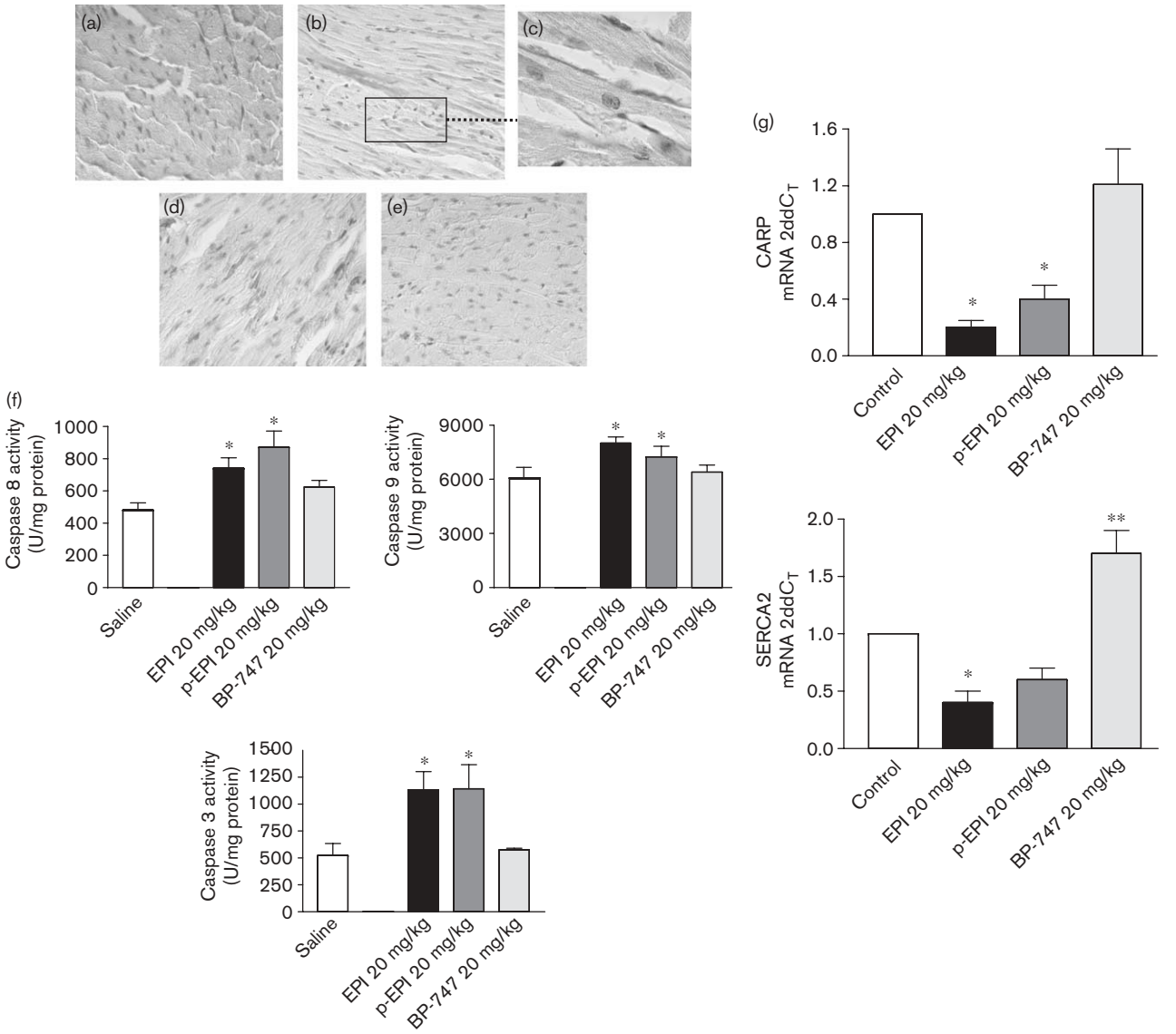
Fig. 2



Effect of BP-747 on anthracycline-induced heart pathological changes. Hematoxylin/eosin stained histological sections of the left ventricular myocardium from a saline-treated mouse (a; original magnification, ×200) and from a mouse killed 21 days after the administration of 20 mg/kg free EPI. Note the cytoplasmic vacuolization (arrows) (b; original magnification, ×200). (c) Histological score of heart harvested from mice killed 21 days after drug administration (six to eight mice per group). *$P<0.05$ versus all groups; **$P<0.05$ versus EPI and p-EPI-treated mice. (d–g) Sirius red staining of heart collagen content. Original magnification, ×200. BALB/c mice were given saline, free EPI (20 mg/kg), p-EPI (20 mg/kg equivalent amount of EPI) or BP-747 (20 mg/kg equivalent amount of EPI) as a single intraperitoneal bolus injection and killed 21 days after drug administration. EPI, epirubicin; p-EPI, pegylated epirubicin.

threshold (C_T); cycle number at which each PCR reaches a predetermined fluorescent threshold, set within the linear range of all reactions. The amount of gene expression was then calculated as the difference (ΔC_T) between the C_T value of the sample for the target gene and the mean C_T value of that sample for the endogenous control (glyceraldehyde-3-phosphate dehydrogenase). Relative expression was calculated as the difference

Fig. 3



Effect of BP-747 on anthracycline-induced cardiomyocyte apoptosis. TUNEL staining in ventricles from BALB/c mice treated with saline (a, magnification $\times 200$), 20 mg/kg free EPI (b and c, magnification $\times 200$ and $\times 400$, respectively), 20 mg/kg p-EPI (d, magnification $\times 200$) and 20 mg/kg BP-747 (e, magnification $\times 200$). (f) Effect of BP-747 on anthracycline-induced heart caspases activation. *$P<0.05$ versus saline and BP-747-treated mice. (g) Effect of BP-747 on heart CARP and SERCA2 mRNA expression. Mice were injected with a single dose of saline, 20 mg/kg free EPI, p-EPI and BP-747, and killed after 21 days. Data are the mean ± SE of four to six mice per group. *$P<0.05$ versus saline and BP-747-treated mice. **$P<0.05$ versus all groups. EPI, epirubicin; p-EPI, pegylated epirubicin.

($\Delta\Delta C_T$) between ΔC_T values of the test control sample for each target gene. The relative expression level was expressed as $2\Delta\Delta C_T$.

Statistical analysis

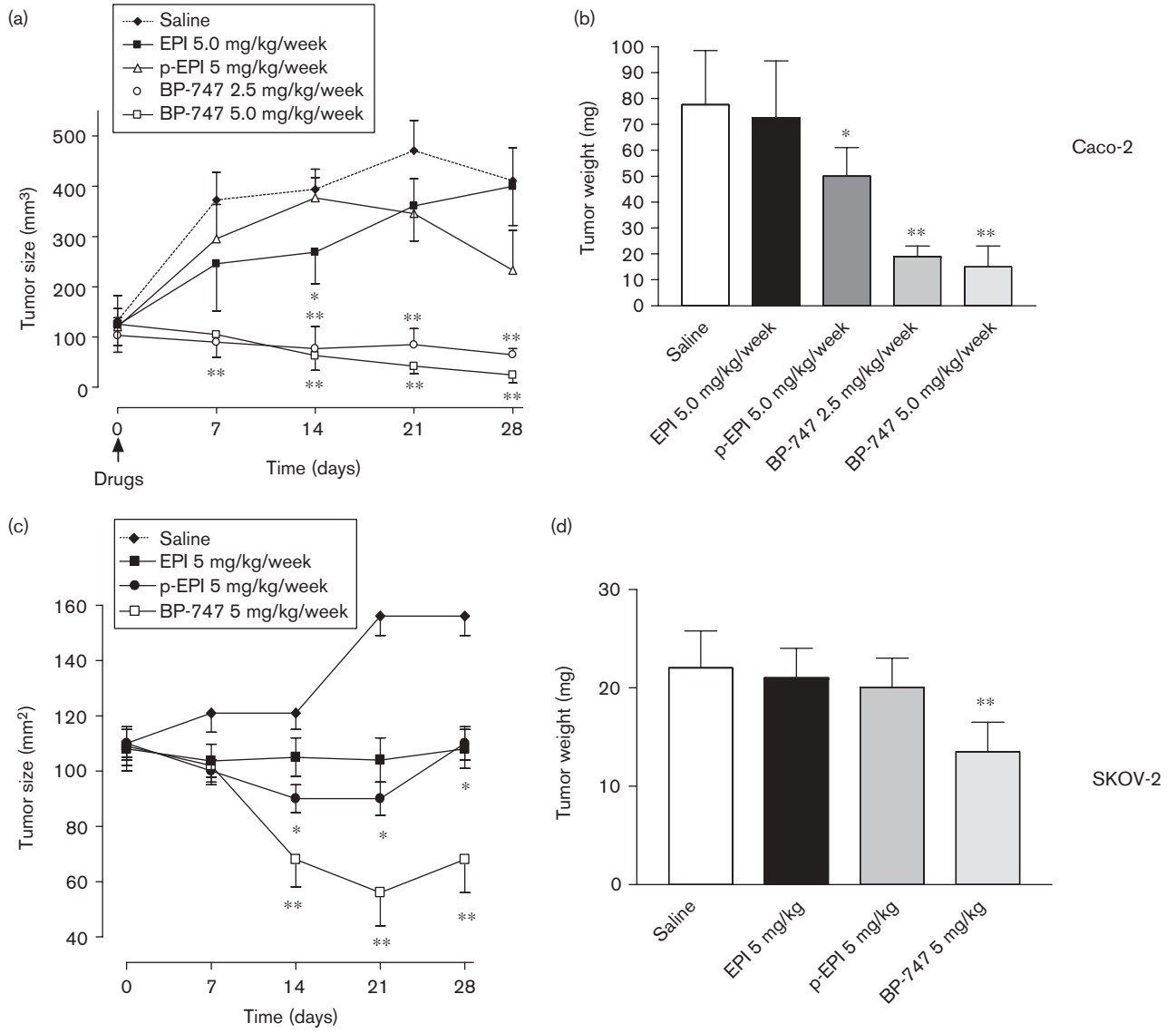
All values in the figures and text are expressed as mean ± SE. The variation between data sets was tested with analysis of variance and the significance was tested with unpaired *t*-tests, with a Bonferroni modification for multicomparison of data. Differences were considered significant when $P < 0.05$

Results

Effect of BP-747 on anthracycline cardiomyopathy

We have first investigated the heart toxicity of a single injection of high doses of EPI, p-EPI and BP-747 in normal, tumor-free, BALB/c mice. Mice treated with free EPI (10 and 20 mg/kg) developed severe signs of anthracycline cardiomyopathy, characterized by significant mortality (Fig. 1a), loss of body weight (Fig. 1b), general lethargy, ascites and hepatic enlargement. Moreover, EPI administration caused a 40–50% reduction of heart weight (from 196 ± 8 mg in saline-treated mice to

Fig. 4



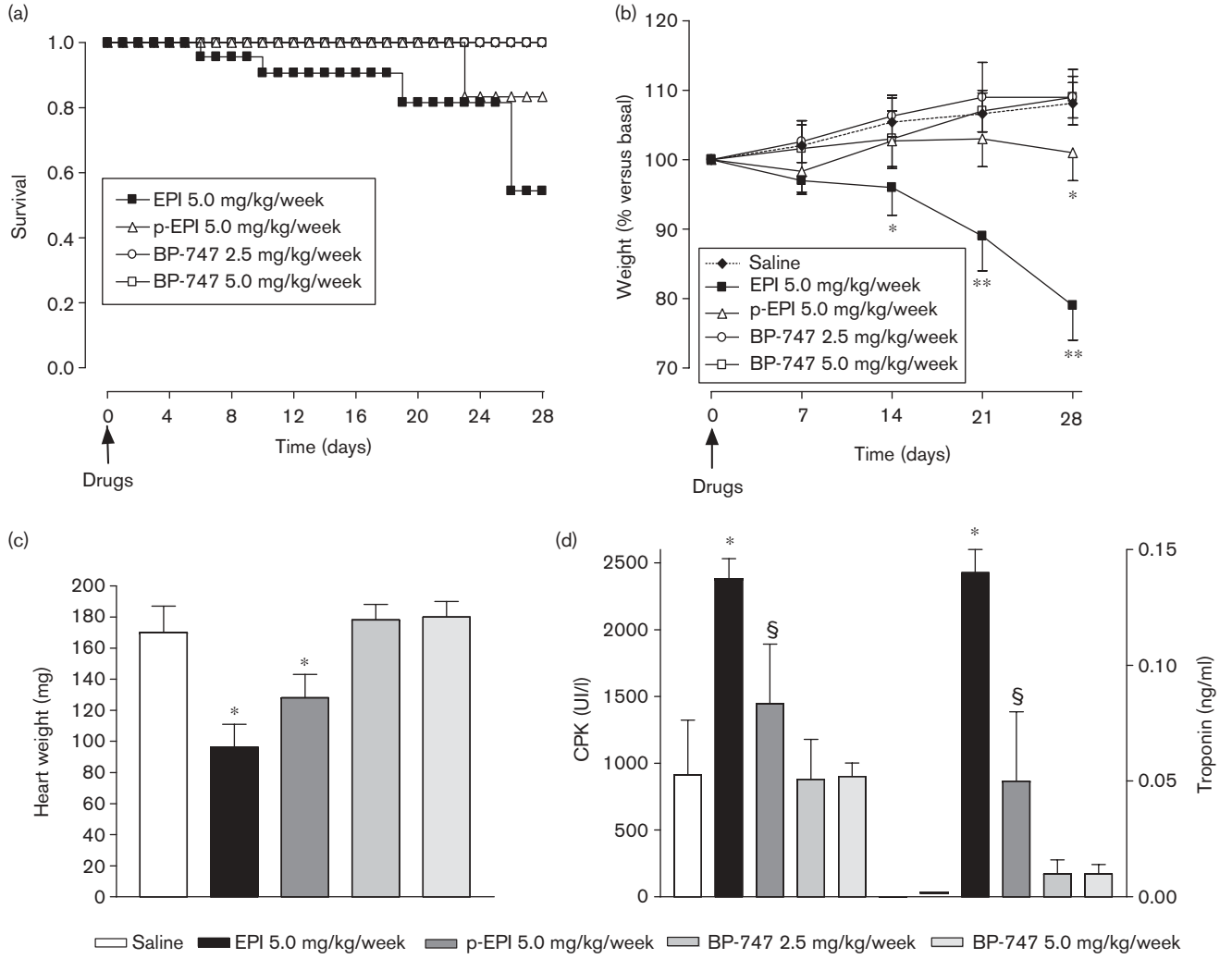
In-vivo antitumor effects of BP-747. Nude Swiss mice bearing Caco-2 (a and b) or SKOV-2 (c and d) xenografts were randomly allocated in the treatment groups (eight animals per treatment group) such that the average volume of tumor in all the groups was about 100 mm^2. Mice were then treated intraperitoneally for 4 weeks with saline, free EPI (5 mg/kg/week), p-EPI (5 mg/kg/week equivalent amount of EPI) or BP-747 (2.5 and 5 mg/kg/week equivalent amount of EPI). One week after the administration of the last dose, mice were killed, and tumor excised and weighed. Data are the mean ± SE of six to eight mice per group. *$P<0.05$ versus saline-treated mice; **$P<0.05$ versus all groups. EPI, epirubicin; p-EPI, pegylated epirubicin.

114 ± 25 mg and 105 ± 20 mg in mice treated with 10 and 20 mg/kg EPI, respectively, $P < 0.01$) (Fig. 1c), and 30% reduction of heart/body weight ratio (data not shown) in comparison to saline-treated mice. Serum troponin and CPK levels were markedly increased by EPI treatment (Fig. 1d). In comparison with free EPI, p-EPI showed a safer cardiotoxic profile; however, a significant cardiomyopathy was still evident as demonstrated by the marked reduction in heart weight (Fig. 1c), and increased serum levels of troponin and CPK (Fig. 1d). Conversely, BP-747-treated mice showed no clinical and anatomical signs of anthracycline cardiomyopathy (Fig. 1a–c) and normal serum cardiac enzyme levels (Fig. 1d).

Histological injury

Histology of the hearts from EPI-treated animals revealed the typical findings associated with anthracycline cardiomyopathy in humans: myocyte loss, myofibrillar

Fig. 5



Effect of chronic administration of BP-747 on anthracycline cardiomyopathy. Swiss nude mice bearing Caco-2 xenografts were treated for 4 weeks with saline, free EPI (5 mg/kg/week), p-EPI (5 mg/kg/week equivalent amount of EPI) or BP-747 (2.5 and 5 mg/kg/week equivalent amount of EPI). Mice were followed for 28 days to evaluate mortality (a) and weight (b). At the end of the study period, surviving mice were sacrificed, and heart weight (c) and serum cardiac enzymes (d) determined. *$P<0.05$ versus saline and BP-747-treated animals. **$P<0.05$ versus all groups. §$P<0.05$ versus EPI and BP-747-treated mice. EPI, epirubicin; p-EPI, pegylated epirubicin.

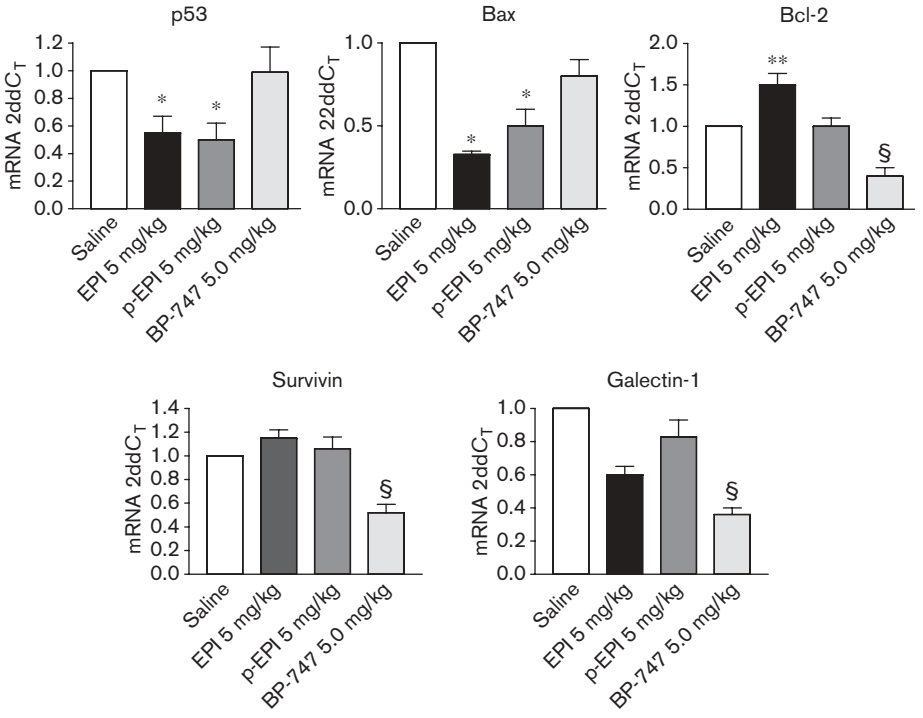
degeneration and extensive cytoplasmic vacuolization (Fig. 2b). Lesions were observed in almost all examined areas (Fig. 2c). These changes were partially attenuated by EPI pegylation, while BP-747-treated mice were devoid of significant histological damage (Fig. 2c). In addition, Sirius red staining demonstrated a significant attenuation of heart fibrosis in both p-EPI- and BP-747-treated mice when compared with mice treated with 20 mg/kg free EPI (Fig. 2d–g).

Effect of BP-747 on cardiomyocyte apoptosis and heart CARP and SERCA2 expression

Confirming previous studies EPI administration resulted in a marked increase of apoptotic, TUNEL-positive, ventricle cells (Fig. 3b and c). A similar pattern was observed in animals exposed to p-EPI (Fig. 3d). In contrast, the number of TUNEL-positive cells in BP-747-treated mice was similar to that of control mice (Fig. 3e). Consistent with the results of the TUNEL assay, EPI and p-EPI caused a robust induction of caspases 3, 8 and 9 activities (Fig. 3f), an effect that was not observed in animals treated with BP-747 (Fig. 3f).

Confirming previous studies, we found that EPI administration downregulates both CARP and SERCA2 mRNA expression, two of the genes that are specifically modulated in anthracycline-induced cardiomyopathy. CARP and SERCA2 downregulation was only partially

Fig. 6



Effect of BP-747 on p53, Bax, Bcl-2, survivin and galectin-1 mRNA colon tumor expression. Swiss nude mice bearing Caco-2 xenografts were treated for 4 weeks with saline, free EPI (5 mg/kg/week), p-EPI (5 mg/kg/week equivalent amount of EPI), or BP-747 (5 mg/kg/week equivalent amount of EPI). One week after the last injection of the drug, mice were killed, and tumor excised, immediately snap-frozen in liquid nitrogen and processed for RNA extraction as described in Materials and methods. Data are the mean ± SE of four to six mice per group. *$P<0.05$ versus saline and BP-747-treated mice. **$P<0.05$ versus EPI and p-EPI-treated mice; §$P<0.05$ versus all groups. EPI, epirubicin; p-EPI, pegylated epirubicin.

prevented by EPI pegylation (Fig. 3g). In contrast, BP-747 completely protected against EPI-induced downregulation of heart CARP and SERCA2 mRNA expression (Fig. 3g).

In-vivo antitumor activity of BP-747

The antitumor activity of BP-747 was investigated in nude Swiss mice bearing Caco-2 or SKOV2 tumor xenografts. After establishment of palpable tumors (mean tumor volume 100 mm^2) animals were randomly treated with one of the investigational drugs at the indicated doses for 4 weeks. At the dose of 2.5 and 5.0 mg/kg/week, BP-747 reduced tumor volume and weight by 85 and 95%, respectively (Fig. 4a and b; $P < 0.01$ versus free EPI and p-EPI). Interestingly, 40% of mice treated with 5 mg/kg BP-747 showed a complete regression of the palpable tumor. Similar results were obtained in the SKOV-2 model of xenograft, where BP-747 displayed a significant more potent antitumor activity in comparison with free EPI and p-EPI (Fig. 4c and d).

As observed in the acute toxicity protocol, chronic administration of free EPI in tumor-bearing nude mice resulted in the development of clinical, anatomical and biochemical signs of cardiomyopathy, that were only partially reduced by drug pegylation (Fig. 5a–d). Again, in mice treated with BP-747 the clinical, biochemical and anatomical signs of anthracycline-induced cardiomyopathy were virtually absent (Fig. 5a–d).

Finally we investigated the molecular mechanisms of antitumoral activity of BP-747 in colon cancer xenografts. In comparison with EPI and p-EPI, BP-747 administration increased mRNA expression of the proapoptotic proteins Bax and p53, whereas it reduced the mRNA expression of the antiapoptotic protein Bcl-2 (Fig. 6). Confirming and expanding previous in-vitro results, we found that EPI administration resulted in a robust induction of survivin mRNA expression, an antiapoptotic protein involved in cancer resistance against chemotherapy agents. In contrast, administration of BP-747 resulted in a reduction of survivin mRNA expression to levels significantly lower than control ($P < 0.05$) (Fig. 6). Finally, we found that BP-747 downregulated galectin-1 mRNA expression in colon cancer tissue, a protein that has been correlated with colon cancer growth and metastasis ($P < 0.05$ versus EPI and p-EPI).

Discussion

Anthracyclines are extensively used in the treatment of solid cancers, including breast and ovarian cancer, and hematological malignancies such as lymphoma [1,2]. The clinical use of these agents, however, results in a condition called 'anthracycline cardiomyopathy' in approximately 50% of patients [3]. Several formulations of anthracyclines have been developed to improve their delivery to tumors and to decrease cardiotoxicity [14]. One of the most investigated is PEG liposomal doxorubicin and recently a commercial formulation has been approved by the US Food and Drug Administration for the treatment of ovarian cancer and Kaposi's sarcoma [32,33]. Here we report on the antitumoral and cardiotoxic effects of a new anthracycline formulation in which the doxorubicin derivative EPI is complexed to a PEG molecule bearing NO-releasing groups [31]. As for the PEG liposomal doxorubicin, the rationale for the use of PEG in the BP-747 molecule was to prolong the plasma half-life of EPI, and to increase the penetration and accumulation of the chemotherapy agent into the tumor [33]. In addition, the new formulation takes advantage from the well-known cardio-protective and antitumoral properties of NO [19–30]. In a preliminary study we have found that BP-747 exerts a more potent antitumoral activity in the colon cancer cell line Caco-2 in comparison with free EPI and p-EPI, while it is virtually devoid of cytotoxicity against endothelial cells and cardiomyocytes [31]. Strikingly, here we demonstrate that BP-747 shows a very safe cardiotoxic profile also *in vivo* while providing a more potent antitumoral activity in two experimental models of solid tumors in comparison with free EPI or p-EPI. As mice treated with p-EPI still developed a clinically relevant cardiomyopathy, it can be concluded that the addition of the NO-releasing molecule to the PEG backbone is necessary to obtain a safe cardiac profile and confirms previous studies demonstrating that doxorubicin-induced cardiomyopathy is exacerbated in inducible NO synthase-deficient mice [24].

A number of in-vitro and in-vivo data support the notion that EPI-induced cardiomyopathy is the result of cardiomyocytes apoptosis [33]. Consistent with these findings, we found that administration of free EPI resulted in massive cardiomyocyte apoptosis; in contrast, in BP-747-treated mice the number of TUNEL positive cells were similar to control mice. This antiapoptotic effect was correlated to a remarkable inhibition of heart caspases activation by BP-747, in particular caspase 3 activation that was completely inhibited. As NO inhibits caspases activation and apoptosis in several cellular systems through the *S*-nitrosylation of critical cysteine residues on the protein [34–39], we speculate that the inhibition of heart caspases activation by BP-747 is mainly related to the NO-releasing moiety of the molecule. Therefore, we conclude that inhibition of apoptosis through the NO-dependent prevention of heart caspases activation is the main mechanism underlying the protective activity of BP-747 against anthracycline-induced cardiomyopathy.

Several cardiac-specific genes that are involved in the structural integrity and function of cardiomyocytes appear particularly susceptible to anthracyclines [11–13]. Confirming previous results, here we found that in-vivo administration of free EPI resulted in a marked downregulation of CARP and SERCA2 mRNA, which was only partially prevented by drug pegylation. On the other hand, BP-747 administration almost completely restores the mRNA expression of these sarcomeric proteins, suggesting a further cardioprotective mechanism against anthracycline-induced cardiomyopathy. The mechanism through which BP-747 prevents CARP and SERCA2 downregulation is at the moment unknown; however, the NO-releasing molecules seem to play a role also in this effect, as suggested by the fact that EPI pegylation alone failed to prevent mRNA downregulation and that the NO donor dinitrosyl iron complex increases SERCA2 gene expression in myocardial and skeletal muscles [40].

Whereas significant cardiotoxicity was not observed, BP-747 showed a potent antitumoral activity in both models of xenografts, confirming and strengthening the in-vitro results [31]. It can be speculated that the different cytotoxic profile of BP-747 *in vivo* is related to the ability of NO to activate the mitochondrial pathway of apoptosis selectively in highly proliferative cells, such as neoplastic cells [41,42]. This is confirmed by the observation that in cancer tissue harvested from BP-747-treated animals, the levels of Bax and p53 mRNA was markedly upregulated, while the levels of Bcl-2 mRNA were downregulated in comparison with free EPI.

Survivin, a member of the inhibitors of apoptosis proteins family that is expressed in most human tumor cells, is an antiapoptotic protein that has been implicated in cancer resistance against chemotherapy agents [43]. Interestingly, survivin overexpression confers protection to cancer cells from anthracycline-induced apoptosis and treatment with antisense survivin renders neoplastic cells susceptible to apoptosis induced by these drugs [44]. Consistent with the findings obtained with NO donors in human lung cancer cell lines [45] and with BP-747 in the Caco-2 cell line [31], here we found that BP-747 downregulates mRNA survivin in colon cancer tissue, suggesting that this effect may represent an additional molecular mechanism underlying the potent antineoplastic activity of this new anthracycline derivative. Finally, we found that BP-747 exerted an interesting inhibitory effect also on colon cancer galectin-1 mRNA expression. As galectin-1 expression has been correlated to colon cancer growth and metastasis [46,47], this effect may be

part of the anticancer activity of BP-747 in colon cancer xenograft growth.

In conclusion, in this study we have provided evidence that the addition of an NO-releasing moiety to pegylated EPI confers to the drug a new cytotoxic profile *in vivo*. Indeed, while NO increases the antitumoral activity of the chemotherapy agent, it confers protection against the development of anthracycline cardiomyopathy, suggesting a potential utility of this agent in human cancers.

References

1 Poole CJ, Earl HM, Hiller L, Dunn JA, Bathers S, Grieve RJ, *et al*. Epirubicin and cyclophosphamide, methotrexate, and fluorouracil as adjuvant therapy for early breast cancer. *N Engl J Med* 2006; **355**:1851–1862.

2 Pui CH, Evans WE. Treatment of acute lymphoblastic leukemia. *N Engl J Med* 2006; **354**:166–178.

3 Singal PK, Iliskovic N. Doxorubicin-induced cardiomyopathy. *N Engl J Med* 1998; **339**:900–905.

4 Jensen BV, Skovsgaard T, Nielsen SL. Functional monitoring of anthracycline cardiotoxicity: a prospective, blinded, long-term observational study of outcome in 120 patients. *Ann Oncol* 2002; **13**:699–709.

5 Swain SM, Whaley FS, Ewer MS. Congestive heart failure in patients treated with doxorubicin: a retrospective analysis of three trials. *Cancer* 2003; **97**:2869–2879.

6 Arola OJ, Saraste A, Pulkki K, Kallajoki M, Parvinen M, Voipio-Pulkki LM. Acute doxorubicin cardiotoxicity involves cardiomyocyte apoptosis. *Cancer Res* 2000; **60**:1789–1792.

7 Kelso EJ, Geraghty RF, McDermott BJ, Cameron CH, Nicholls DP, Silke B. Characterization of a cellular model of cardiomyopathy, in the rabbit, produced by chronic administration of the anthracycline epirubicin. *J Mol Cell Cardiol* 1997; **29**:3385–3397.

8 Sacco G, Giampietro R, Salvatorelli E, Menna P, Bertani N, Graiani G, *et al*. Chronic cardiotoxicity of anticancer anthracyclines in the rat: role of secondary metabolites and reduced toxicity by a novel anthracycline with impaired metabolite formation and reactivity. *Br J Pharmacol* 2003; **139**:641–651.

9 Childs AC, Phaneuf SL, Dirks AJ, Phillips T, Leeuwenburgh C. Doxorubicin treatment *in vivo* causes cytochrome *c* release and cardiomyocyte apoptosis, as well as increased mitochondrial efficiency, superoxide dismutase activity, and Bcl-2:Bax ratio. *Cancer Res* 2002; **62**: 4592–4598.

10 Wallace KB. Doxorubicin-induced cardiac mitochondrionopathy. *Pharmacol Toxicol* 2003; **93**:105–115.

11 Jeyaseelan R, Poizat C, Baker RK, Abdishoo S, Isterabadi LB, Lyons GE, *et al*. A novel cardiac-restricted target for doxorubicin. CARP, a nuclear modulator of gene expression in cardiac progenitor cells and cardiomyocytes. *J Biol Chem* 1997; **272**:22800–22808.

12 Aihara Y, Kurabayashi M, Arai M, Kedes L, Nagai R. Molecular cloning of rabbit CARP cDNA and its regulated expression in adriamycin-cardiomyopathy. *Biochim Biophys Acta* 1999; **1447**:318–324.

13 Olson RD, Gambliel HA, Vestal RE, Shadle SE, Charlier HA Jr, Cusack BJ. Doxorubicin cardiac dysfunction: effects on calcium regulatory proteins, sarcoplasmic reticulum, and triiodothyronine. *Cardiovasc Toxicol* 2005; **5**:269–283.

14 van Dalen EC, Michiels EMC, Caron HN, Kremer LCM. Different anthracycline derivatives for reducing cardiotoxicity in cancer patients. *Cochrane Database Syst Rev* 2006; **18**:CD005006.

15 Markman M, Gordon AN, McGuire WP, Muggia FM. Liposomal anthracycline treatment for ovarian cancer. *Semin Oncol* 2004; **31**:91–105.

16 Hofheinz RD, Gnad-Vogt SU, Beyer U, Hochhaus A. Liposomal encapsulated anti-cancer drugs. *Anticancer Drugs* 2005; **16**:691–707.

17 Working PK, Newman MS, Sullivan T, Yarrington J. Reduction of the cardiotoxicity of doxorubicin in rabbits and dogs by encapsulation in long-circulating, pegylated liposomes. *J Pharmacol Exp Ther* 1999; **289**: 1128–1133.

18 Gabizon A, Catane R, Uziely B, Kaufman B, Safra T, Cohen R, *et al*. Prolonged circulation time and enhanced accumulation in malignant exudates of doxorubicin encapsulated in polyethylene-glycol coated liposomes. *Cancer Res* 1994; **54**:987–993.

19 Chang J, Rao NV, Markewitz BA, Hoidal JR, Michael JR. Nitric oxide donor prevents hydrogen peroxide-mediated endothelial cell injury. *Am J Physiol* 1996; **270**:L931–L940.

20 Monastyrskaya E, Folarin N, Malyshev I, Green C, Andreeva L. Application of the nitric oxide donor SNAP to cardiomyocytes in culture provides protection against oxidative stress. *Nitric Oxide* 2002; **7**:127–131.

21 Hida A, Kawakami A, Miyashita T, Yamasaki S, Nakashima K, Tanaka F, *et al*. Nitric oxide acts on the mitochondria and protects human endothelial cells from apoptosis. *J Lab Clin Med* 2004; **144**:148–155.

22 Maejima Y, Adachi S, Morikawa K, Ito H, Isobe M. Nitric oxide inhibits myocardial apoptosis by preventing caspase-3 activity via *S*-nitrosylation. *J Mol Cell Cardiol* 2005; **38**:163–174.

23 Cole MP, Chaiswing L, Oberley TD, Edelmann SE, Piascik MT, Lin SM, *et al*. The protective roles of nitric oxide and superoxide dismutase in adriamycin-induced cardiotoxicity. *Cardiovasc Res* 2006; **69**:186–197.

24 Chaiswing L, Cole MP, Ittarat W, Szweda LI, St Clair DK, Oberley TD. Manganese superoxide dismutase and inducible nitric oxide synthase modify early oxidative events in acute adriamycin-induced mitochondrial toxicity. *Mol Cancer Ther* 2005; **4**:1056–1064.

25 Evig CB, Kelley EE, Weydert CJ, Chu Y, Buettner GR, Burns CP. Endogenous production and exogenous exposure to nitric oxide augment doxorubicin cytotoxicity for breast cancer cells but not cardiac myoblasts. *Nitric Oxide* 2004; **10**:119–129.

26 Wink DA, Cook JA, Christodoulou D, Krishna MC, Pacelli R, Kim S, *et al*. Nitric oxide and some nitric oxide donor compounds enhance the cytotoxicity of cisplatin. *Nitric Oxide* 1997; **1**:88–94.

27 Cook JA, Krishna MC, Pacelli R, DeGraff W, Liebmann J, Mitchell JB, *et al*. Nitric oxide enhancement of melphalan-induced cytotoxicity. *Br J Cancer* 1997; **76**:325–334.

28 Adams DJ, Levesque MC, Weinberg JB, Smith KL, Flowers JL, Moore J, *et al*. Nitric oxide enhancement of fludarabine cytotoxicity for B-CLL lymphocytes. *Leukemia* 2001; **15**:1852–1859.

29 Lavagna C, Del Soldato P, Burgaud JL, Rampal P. Nitric oxide-releasing non steroidal anti-inflammatory drugs: a new generation of anti-tumoral molecules. *Curr Cancer Drug Targets* 2003; **3**:407–426.

30 Yeh RK, Chen J, Williams JL, Baluch M, Hundley TR, Rosenbaum RE, *et al*. NO-donating nonsteroidal antiinflammatory drugs (NSAIDs) inhibit colon cancer cell growth more potently than traditional NSAIDs: a general pharmacological property? *Biochem Pharmacol* 2004; **67**: 2197–2205.

31 Santucci L, Mencarelli A, Renga B, Pasut G, Veronese F, Zacheo A, Germani A, *et al*. Nitric oxide modulates proapoptotic and antiapoptotic properties of chemotherapy agents: the case of NO-pegylated epirubicin. *FASEB J* 2006; **20**:765–767.

32 Gabizon A, Shmeeda H, Barenholz Y. Pharmacokinetics of pegylated liposomal doxorubicin: review of animal and human studies. *Clin Pharmacokinet* 2003; **42**:419–436.

33 Minotti G, Menna P, Salvatorelli E, Cairo G, Gianni L. Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. *Pharmacol Rev* 2004; **56**:185–229.

34 Kim YM, Talanian RV, Billiar TR. Nitric oxide inhibits apoptosis by preventing increases in caspase-3-like activity via two distinct mechanisms. *J Biol Chem* 1997; **272**:31138–31148.

35 Li J, Billiar TR, Talanian RV, Kim YM. Nitric oxide reversibly inhibits seven members of the caspase family via *S*-nitrosylation. *Biochem Biophys Res Commun* 1997; **240**:419–424.

36 Kim YM, Kim TH, Chung HT, Talanian RV, Yin XM, Billiar TR. Nitric oxide prevents tumor necrosis factor alpha-induced rat hepatocyte apoptosis by the interruption of mitochondrial apoptotic signaling through *S*-nitrosylation of caspase-8. *Hepatology* 2000; **32**:770–778.

37 Mital S, Barbone A, Addonizio LJ, Quaegebeur JM, Mosca RJ, Oz MC, *et al*. Endogenous endothelium-derived nitric oxide inhibits myocardial caspase activity: implications for treatment of end-stage heart failure. *J Heart Lung Transplant* 2002; **21**:576–585.

38 Fiorucci S, Mencarelli A, Palazzetti B, Del Soldato P, Morelli A, Ignarro LJ. An NO derivative of ursodeoxycholic acid protects against Fas-mediated liver injury by inhibiting caspase activity. *Proc Natl Acad Sci U S A* 2001; **98**:2652–2657.

39 Fiorucci S, Santucci L, Antonelli E, Distrutti E, Del Sero G, Morelli O, *et al*. NO-aspirin protects from T cell-mediated liver injury by inhibiting caspase-dependent processing of T_h1-like cytokines. *Gastroenterology* 2000; **118**:404–421.

40 Malyshev IY, Aymasheva NP, Malenyuk EB, Manukhina EB, Khaspekov GL, Mikoyan VD, *et al*. Nitric oxide increases gene expression of Ca^{2+}-ATPase in myocardial and skeletal muscle sarcoplasmic reticulum: physiological implications. *Med Sci Monit* 2000; **6**:480–485.

41 Beltran B, Mathur A, Duchen MR, Erusalimsky JD, Moncada S. The effect of nitric oxide on cell respiration: a key to understanding its role in cell survival or death. *Proc Natl Acad Sci U S A* 2000; **97**:14602–14607.
42 Brune B. Nitric oxide: NO apoptosis or turning it ON? *Cell Death Differ* 2003; **10**:864–869.
43 Ambrosiani G, Adida C, Altieri DC. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nat Med* 1997; **3**:917–921.
44 Wang Z, Fukuda S, Pelus LM. Survivin regulates the p53 tumor suppressor gene family. *Oncogene* 2004; **23**:8146–8153.
45 Chao JI, Kuo PC, Hsu TS. Down-regulation of survivin in nitric oxide-induced cell growth inhibition and apoptosis of the human lung carcinoma cells. *J Biol Chem* 2004; **279**:20267–20276.
46 Hittelet A, Legendre H, Nagy N, Bronckart Y, Pector JC, Salmon I, *et al*. Upregulation of galectins-1 and -3 in human colon cancer and their role in regulating cell migration. *Int J Cancer* 2003; **103**: 370–379.
47 Rabinovich GA. Galectin-1 as a potential cancer target. *Br J Cancer* 2005; **92**:1188–1192.