ORIGINAL ARTICLE

Enhanced efficacy of combined ^{213}Bi-DTPA-F3 and paclitaxel therapy of peritoneal carcinomatosis is mediated by enhanced induction of apoptosis and G2/M phase arrest

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Abstract

Purpose Targeted therapy with α-particle emitting radionuclides is a promising new option in cancer therapy. Stable conjugates of the vascular tumour-homing peptide F3 with the α-emitter ^{213}Bi specifically target tumour cells. The aim of our study was to determine efficacy of combined ^{213}Bi-diethylenetriaminepentaacetic acid (DTPA)-F3 and paclitaxel treatment compared to treatment with either ^{213}Bi-DTPA-F3 or paclitaxel both in vitro and in vivo.

Methods Cytotoxicity of treatment with ^{213}Bi-DTPA-F3 and paclitaxel, alone or in combination, was assayed towards OVCAR-3 cells using the alamarBlue assay, the clonogenic assay and flow cytometric analyses of the mode of cell death and cell cycle arrest. Therapeutic efficacy of the different treatment options was assayed after repeated treatment of mice bearing intraperitoneal OVCAR-3 xenograft tumours. Therapy monitoring was performed by bioluminescence imaging and histopathologic analysis.

Results Treatment of OVCAR-3 cells in vitro with combined ^{213}Bi-DTPA-F3 and paclitaxel resulted in enhanced cytotoxicity, induction of apoptosis and G2/M phase arrest compared to treatment with either ^{213}Bi-DTPA-F3 or paclitaxel. Accordingly, i.p. xenograft OVCAR-3 tumours showed the best response following repeated (six times) combined therapy with ^{213}Bi-DTPA-F3 (1.85 MBq) and paclitaxel (120 μg) as demonstrated by bioluminescence imaging and histopathologic investigation of tumour spread on the mesentery of the small and large intestine. Moreover, mean survival of xenograft mice that received combined therapy with ^{213}Bi-DTPA-F3 and paclitaxel was significantly superior to mice treated with either ^{213}Bi-DTPA-F3 or paclitaxel alone.

Conclusion Combined treatment with ^{213}Bi-DTPA-F3 and paclitaxel significantly increased mean survival of mice with peritoneal carcinomatosis of ovarian origin, thus favouring future therapeutic application.

Keywords Targeted radionuclide therapy · Tumour-homing peptide F3 · Peritoneal carcinomatosis · α-Emitter ^{213}Bi · Anticancer drug paclitaxel

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Introduction

Peritoneal carcinomatosis is the most common secondary cancer that affects the peritoneal cavity. Secondary peritoneal carcinomatosis is a frequent consequence of primary cancers of the gastrointestinal tract as well as of ovarian, breast, lung and uterine cancer [1]. Prognosis of peritoneal spread of a primary carcinoma is poor because the existing treatment strategies are less effective. These strategies include cytoreductive surgery as a single treatment or in combination with adjuvant intravenous or intraperitoneal chemotherapy [2]. In hyperthermic perfusion therapy chemotherapeutic compounds are heated to 41–43 °C to increase their cytotoxicity [3, 4]. Furthermore, intraperitoneal chemotherapy has proven superior to intravenous chemotherapy in patients with metastatic ovarian cancer [5].

To improve therapy of peritoneal disease, compounds should be applied that target surface proteins which are expressed by cancer cells. Those compounds can be used in targeted therapy as specific carrier molecules for cytotoxic radionuclides. Therapeutic efficacy of targeted therapy with β-particle emitting radionuclides has been investigated in numerous preclinical and clinical studies [6–12]. Nevertheless, therapeutic efficacy of intraperitoneal radioimmunotherapy using β-emitters is not satisfactory in most cases. Therefore, alternative treatment strategies using α-emitters are being increasingly pursued.

Alpha-particles eradicate single tumour cells or small tumour cell nodules very efficiently. Therefore, targeted therapy with α-emitters is a promising adjuvant strategy for treatment of disseminated tumour cells and micrometastatic disease characteristic for peritoneal carcinomatosis. Accordingly, radioimmunoconjugates composed of α-emitters and antibodies specifically targeting antigens expressed on tumour cells have been successfully administered intraperitoneally in the treatment of experimental peritoneal carcinomatosis of gastric [13, 14], colonic [15, 16] and ovarian origin [17, 18]. Moreover, in a first clinical phase I study nine women in complete remission after second-line chemotherapy for recurrent ovarian carcinoma were treated intraperitoneally with ^{211}At-MX35 F(ab′)$_2$. No adverse effects could be observed in all laboratory parameters analysed. Therefore, intraperitoneal α-emitter radioimmunotherapy of ovarian cancer patients is thought to be efficient without significant toxicity [19].

The 31 aa tumour-homing peptide F3 (KDEPQRRSAR LSAKPAPPKPEPKPKKAPAKK) corresponds to the nucleosomal binding domain of HMGN2. F3 shows specific binding to nucleolin present on the surface of proliferating tumour cells. In proliferating cells a cyclical transport of nucleolin occurs from the nucleus to the cell surface and back by a specific shuttle mechanism. In resting cells nucleolin is primarily expressed in the nucleus. Therefore, nucleolin is a promising target for tumour therapies that are aimed at selective eradication of proliferating tumour cells [20]. Internalization of F3 into the nucleus is extremely attractive in terms of therapeutic application of α-emitters. The selective transport of α-emitter-F3 conjugates to the nucleus of tumour cells enhances the probability for induction of lethal DNA double-strand breaks by α-particles [21]. Indeed, α-emitter-F3 radioconjugates have successfully been administered in tumour therapy. ^{213}Bi-diethylenetriaminepentaacetic acid (DTPA)-[F3]$_2$ has been shown to specifically accumulate in tumour tissue in vivo and to interfere with tumour growth of small tumours. Consequently, intraperitoneal injection of ^{213}Bi-DTPA-[F3]$_2$ significantly prolonged overall survival in a preclinical model of peritoneal carcinomatosis [22]. Conjugates of the α-emitter ^{225}Ac coupled to the tumour-homing peptide F3 are consistent with the concept of an atomic nanogenerator. Such a generator releases four α-particles within a tumour cell in the course of ^{225}Ac decay [23]. Therapeutic efficiency of stable conjugates of F3 and ^{225}Ac (^{225}Ac-DOTA-F3) as well as ^{213}Bi (^{213}Bi-DTPA-F3) has been compared in a mouse model of peritoneal carcinomatosis. Therapy with both conjugates increased survival of animals with negligible toxicity [24]. The aim of this study was to further improve therapeutic efficacy of ^{213}Bi-DTPA-F3 by additional administration of the cytostatic drug paclitaxel. For that purpose cytotoxic effects of combined ^{213}Bi-DTPA-F3 and paclitaxel treatment on OVCAR-3 cells were investigated in comparison to the effects of single treatments with ^{213}Bi-DTPA-F3 or paclitaxel. These analyses included alamarBlue and clonogenic assays as well as flow cytometric determination of the mode of cell death and cell cycle arrest. Moreover, therapeutic efficacy of single and combined treatments was analysed in a nude mouse model of peritoneal carcinomatosis of ovarian origin including monitoring of tumour development by bioluminescence imaging, histopathologic analysis of tumour spread and determination of overall survival.

Materials and methods

Cell culture

The ovarian carcinoma cell line OVCAR-3 (ATCC, Wesel, Germany) and the human embryonic kidney cell line HEK293T (gift of D. Saur, Technische Universität München) were cultured in RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with 10 % fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Biochrom). Cells were maintained at 37 °C and 5 % CO_2.

^{213}Bi labelling of the F3 peptide

^{213}Bi was eluted from a ^{225}Ac/^{213}Bi generator system provided by the Institute of Transuranium Elements (Karlsruhe, Germany) [25, 26]. To the ^{213}Bi eluate 100 μl ascorbic acid (40 mg/ml) and 100 μl ammonium acetate (3 M) were added. For ^{213}Bi labelling DTPA-conjugated F3 peptide (Bachem, Bubendorf, Switzerland) was supplemented with 37 MBq of ^{213}Bi per 5 μg of peptide. After 7 min at room temperature (RT) labelling efficacy was determined by instant thin-layer chromatography. DTPA (Sigma-Aldrich, Seelze, Germany) was labelled in the same way except that 500 μg DTPA were incubated with 37 MBq of ^{213}Bi. Labelling efficacy was >90 % in all experiments. Stability of the ^{213}Bi-DTPA-F3 peptide conjugate is appropriate for intraperitoneal therapeutic application (data not shown). Toxicities of the β-particle emitting daughter nuclides of ^{213}Bi that are released from the F3 peptide conjugates following decay of the α-emitter ^{213}Bi are negligible.

Cell binding and internalization of ^{213}Bi-DTPA-F3

A total of 1×10^7 OVCAR-3 cells were incubated in 1 ml culture medium with 37 kBq/ml ^{213}Bi-DTPA-F3 or ^{213}Bi-DTPA with or without 10 μM phenylarsine oxide (PAO, Sigma-Aldrich, Seelze, Germany) (0.1 % dimethyl sulphoxide final concentration) for 1 h at 37 °C. Membrane-permeable PAO inhibits the internalization of cell surface receptors [27, 28]. Cells were centrifuged at 400 g for 5 min at 4 °C. Supernatants were transferred to new tubes and cells were resuspended in ice-cold phosphate-buffered saline (PBS). Cells were pelleted again, supernatants were combined with previous supernatants and washing was repeated twice. Finally ^{213}Bi activities in the cell pellets and supernatants were measured using a gamma counter. Binding/internalization of ^{213}Bi-DTPA-F3 was calculated as percentage of ^{213}Bi activity in the cell pellet relating to total ^{213}Bi activity (cell pellet + supernatant).

Assessment of cell viability using the alamarBlue assay

OVCAR-3 cells (4×10^3 per well) were incubated with or without paclitaxel (60 ng/ml; 90 min) 24 h after seeding. Cells were washed once with culture medium and then incubated with or without ^{213}Bi-DTPA-F3 (0.37 MBq/ml). At indicated time points after initiation of treatment cell supernatants were supplemented with 10 % alamarBlue (AbD Serotec, Düsseldorf, Germany) and cells were incubated for 4 h at 37 °C. In the alamarBlue assay resazurin is reduced to red fluorescent resorufin by metabolically active cells. After incubation supernatants were transferred to new plates and stored at −20 °C. Cells received fresh medium and were incubated until the next alamarBlue assay.

Resorufin fluorescence was quantified according to the manufacturer's instructions.

Soft agar clonogenic assay

OVCAR-3 cells (2×10^5 per well) were incubated with or without paclitaxel (60 ng/ml; 90 min) 24 h after seeding. Cells were then treated with ^{213}Bi-DTPA-F3 for 16 h. Six-well plates were layered with 1.5 ml base agarose (0.75 ml 1 % agarose +0.75 ml 2× culture medium both pre-warmed to 40 °C) per well. Cells were detached with trypsin/ethylenediaminetetraacetic acid (EDTA) (Biochrom, Berlin, Germany) and resuspended in 2 ml culture medium. To a pre-warmed (40 °C) mixture of 0.7 % low melt agarose (3 ml) (Sigma-Aldrich) and 2× culture medium (3 ml) 0.2 ml of the cell suspension was added. After gentle mixing 1.5 ml of the agarose cell suspension (top agarose) was seeded per well in duplicates in six-well plates containing base agarose. Cells were incubated at 37 °C for 1 week and then stained with 0.5 ml of 0.005 % crystal violet for 1 h. Clones were counted microscopically.

Generation of luciferase-GFP-expressing OVCAR-3 cells

OVCAR-3 cells stably expressing luciferase and green fluorescent protein (GFP) (OVCAR-3-Luc-GFP) were generated by lentiviral transduction. At first HEK293T cells were transfected in a culture dish with a mixture of pLenti6/UbC/V5-DEST-Luc-GFP, psPAX2 and pMD2.G at a weight ratio of 1:1:1 using TurboFect (Fermentas, St.Leon-Rot, Germany). Culture supernatants containing replication incompetent lentiviral particles were harvested 24, 32 and 48 h after transfection, passed through 0.45 μm pore size filters, supplemented with 8 μg/ml polybrene (Sigma-Aldrich) and added immediately to OVCAR-3 cells grown to ~70 % confluence. Transduced cells were incubated for 72 h and single clones were obtained by limiting dilution cloning. GFP-positive clones were detected by fluorescence microscopy. Positive clones were expanded in culture medium containing 10 μg/ml blasticidin S (Life Technologies, Carlsbad, CA, USA). Clones were seeded in an opaque 96-well plate and supplemented with 2.5 mM D-luciferin. Luciferase activity was visualized by bioluminescence imaging.

Treatment of i.p. xenograft mice and monitoring of therapeutic efficacy

OVCAR-3-Luc-GFP cells (1×10^7 in 100 μl PBS) were injected intraperitoneally into 6- to 8-week-old severe combined immunodeficiency (SCID) mice (Charles River, Sulzfeld, Germany). Treatment was initiated 10 days after cell inoculation comprising four different

groups: PBS ($n=10$), paclitaxel ($n=10$), ^{213}Bi-DTPA-F3 ($n=11$) and combined paclitaxel + ^{213}Bi-DTPA-F3 ($n=9$). At day 10 after tumour cell inoculation animals of the paclitaxel group and the combined group received i.p. injections of 120 μg paclitaxel (in 100 μl PBS), respectively. Animals of the control group and the ^{213}Bi-DTPA-F3 group were i.p. injected with 100 μl PBS, respectively. At day 11 after tumour cell inoculation the ^{213}Bi-DTPA-F3 group and the combined group received i.p. injections of 1.85 MBq of ^{213}Bi-DTPA-F3 (in 100 μl PBS), while the control group and the paclitaxel group were treated i.p. with 100 μl PBS, respectively. According to previous studies [22, 24] treatments were repeated five times at the time points indicated in Fig. 5a. Tumour development was monitored noninvasively in two mice each of every treatment group using bioluminescence imaging 7, 12, 19, 26, 33 and 40 days after tumour cell inoculation. For that purpose anaesthetized animals were injected with 300 μl D-luciferin (50 mM in 0.9 % NaCl) i.p. Ten minutes after injection bioluminescence imaging was performed using a cooled charge-coupled device (CCD) camera (Hamamatsu, Herrsching am Ammersee, Germany).

Histopathologic evaluation of therapeutic efficacy

Thirteen days after the last tumour treatment three mice of every group (including the animals that were used for bioluminescence imaging) were sacrificed and small and large intestine including mesentery and tumours were dissected and fixed in 4 % formalin. Tumour nodules in the tissue sections stained with haematoxylin and eosin (H&E) were counted microscopically. Quantification of tumour areas was performed using the image analysis platform Definiens Enterprise Image Intelligence Suite (Definiens, Munich, Germany).

Flow cytometric analysis of cell death and cell cycle arrest

OVCAR-3 cells (1×10^6 per well) were incubated with or without paclitaxel (60 ng/ml; 90 min) 24 h after seeding. Subsequently cells were incubated with or without ^{213}Bi-DTPA-F3 (0.37 MBq/ml). At 24 and 48 h after treatment cells were detached using Accutase and washed twice with PBS. For cell death analysis the detached cells (+ cells from culture supernatants) were incubated with 5 μl/ml annexin V-fluorescein isothiocyanate (FITC) solution (BD Biosciences, Heidelberg, Germany) and 0.5 μg/ml propidium iodide (PI) for 15 min at 4 °C. PI and FITC fluorescence of cells were analysed by dual-parameter flow cytometry (Beckman Coulter). Percentages of necrotic and apoptotic cells were determined using the 'FlowJo' software. For cell cycle analysis detached cells were fixed in 80 % ethanol (2 h, 4 °C), incubated with RNase (0.1 mg/ml; 5 min RT), treated with pepsin (5 mg/ml in 50 mM HCl; 10 min 37 °C), stained with

PI (50 μg/ml) and subjected to flow cytometric analysis (FACScalibur, Becton Dickinson). Histograms were evaluated as described [29].

Detection of active caspase-3 by Western blotting

OVCAR-3 cells (5×10^5 per well) were incubated with or without paclitaxel (60 ng/ml; 90 min) 24 h after seeding. Subsequently cells were incubated with or without ^{213}Bi-DTPA-F3 (0.37 MBq/ml). Then, 24 and 48 h later detached cells (+ cells from culture supernatants) were lysed [2 % sodium dodecyl sulphate (SDS), 62.5 mM Tris–HCl, pH 6.8; 5 min 95 °C]. Lysates were incubated for 5 min at 95 °C. Lysates (30 μg each) were subjected to SDS polyacrylamide gel electrophoresis (PAGE) (14 %). Western blotting using polyclonal anti-active-caspase-3 antibody (Millipore, Schwalbach/Ts, Germany), peroxidase-conjugated monoclonal anti-β-actin antibody (clone 8226, Abcam, Cambridge, UK) and peroxidase-conjugated anti-rabbit IgG antibody (GE Healthcare, Hatfield, UK) was performed as described previously [30].

Statistical analysis

Software package SPSS Statistics version 17.0.0 for Microsoft Windows XP (SP3) was used for statistical analysis. Significance of survival as expressed in Kaplan-Meier curves was determined using the log-rank test. The level of statistical significance was set at $p < 0.05$.

Results

^{213}Bi-DTPA-F3 is internalized by OVCAR-3 cells

Alpha-emitters show maximum therapeutic efficacy when delivered into the nucleus of tumour cells. We have demonstrated that F3 successfully transports the α-emitter ^{213}Bi into the nucleus of MDA-MB-435 cells and efficiently targets xenograft tumours that express nucleolin [22]. To determine whether the monomeric F3 peptide is applicable for delivery of ^{213}Bi into OVCAR-3 cells, we compared binding/uptake of ^{213}Bi-DTPA-F3 and ^{213}Bi-DTPA. As shown in Fig. 1, approx. 10 % of ^{213}Bi-DTPA-F3 added was bound and/or internalized by OVCAR-3 cells after 1 h at 37 °C, whereas less than 1 % of ^{213}Bi-DTPA was bound and/or internalized. In proliferating tumour cells nucleolin, the cell surface receptor of the peptide ligand F3, constantly shuttles from the cell surface to the cell nucleus, thus transporting ^{213}Bi-DTPA-F3 as a payload [20]. To discriminate cell surface-bound from internalized F3, the cells were co-incubated with PAO, an inhibitor of endocytosis [27]. PAO reduced internalization of ^{213}Bi-DTPA-F3 but not
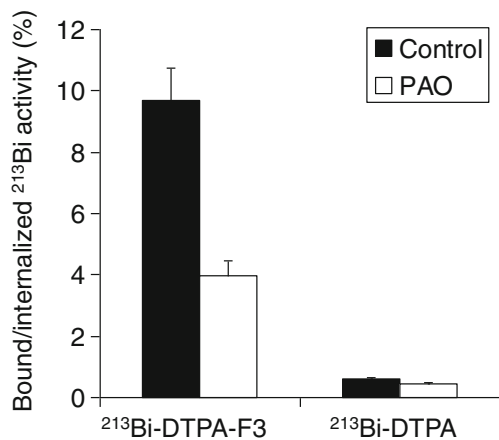
Fig. 1 Binding and internalization of ^{213}Bi-DTPA-F3 by OVCAR-3 cells. OVCAR-3 cells were incubated with 37 kBq/ml ^{213}Bi-DTPA-F3 or ^{213}Bi-DTPA for 1 h at 37 °C with or without the endocytosis inhibitor PAO (10 μM). After centrifugation of cells ^{213}Bi activity was measured in pelleted cells and supernatants. Bound/internalized ^{213}Bi activities were calculated (%) ($n=3 \pm$ SD)

of ^{213}Bi-DTPA indicating that approx. 4 % of ^{213}Bi-DTPA-F3 is bound to the cell surface and that approx. 6 % has been internalized (Fig. 1).

Combined treatment of OVCAR-3 cells with ^{213}Bi-DTPA-F3 + paclitaxel is more cytotoxic than treatment with either ^{213}Bi-DTPA-F3 or paclitaxel

The therapeutic effect of ionizing radiation can be enhanced by the chemotherapeutic drug paclitaxel [15, 31, 32]. In this study the effects of combined treatment with ^{213}Bi-DTPA-F3 and paclitaxel were investigated in terms of cell viability/growth (alamarBlue assay) and proliferation (clonogenic assay) in OVCAR-3 cells compared to treatments with either ^{213}Bi-DTPA-F3 or paclitaxel. The IC$_{50}$ values of ^{213}Bi-DTPA-F3 and paclitaxel have been determined to 370 kBq/ml and 60 ng/ml, respectively (data not shown). Therefore, these IC$_{50}$ concentrations of ^{213}Bi-DTPA-F3 and paclitaxel were applied in all subsequent in vitro experiments, unless indicated otherwise. As shown in Fig. 2a treatments with ^{213}Bi-DTPA-F3, paclitaxel and combined ^{213}Bi-DTPA-F3 + paclitaxel did not affect cell viability/growth within the first 24 h. At 48 and 72 h, however, treatments markedly reduced cell viability/growth compared to the untreated control, with combined ^{213}Bi-DTPA-F3 + paclitaxel showing the most pronounced effect. In clonogenic assays OVCAR-3 cells were treated with or without paclitaxel (60 ng/ml) and ^{213}Bi-DTPA-F3 up to 740 kBq/ml (Fig. 2b). Clonogenic cell survival was reduced by ^{213}Bi-DTPA-F3 in an activity concentration-dependent manner. This effect was enhanced by paclitaxel except for the highest activity concentration of 740 kBq/ml.
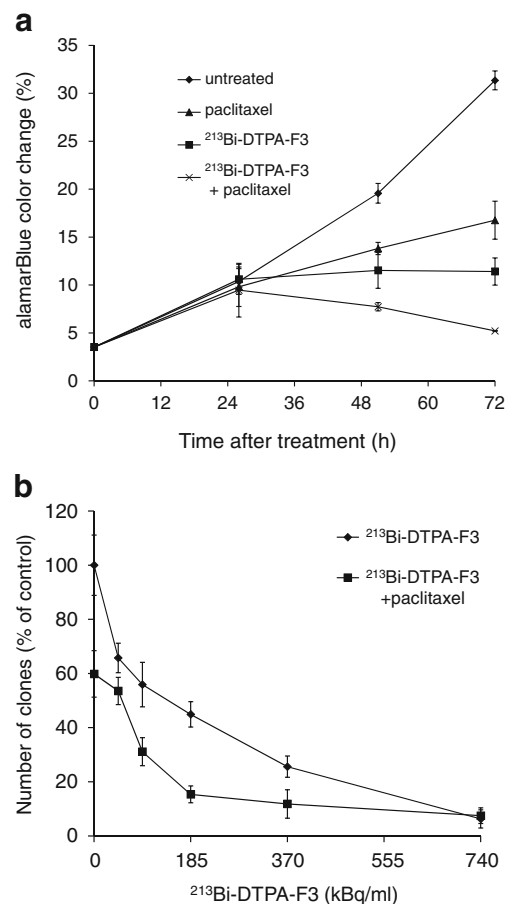

Fig. 2 Cell viability/survival after treatment of OVCAR-3 cells with paclitaxel, ^{213}Bi-DTPA-F3 or combined paclitaxel + ^{213}Bi-DTPA-F3. **a** Viability of OVCAR-3 cells was measured at different time points (26, 51 and 72 h) after treatment of cells with paclitaxel (60 ng/ml), ^{213}Bi-DTPA-F3 (0.37 MBq/ml) or combined paclitaxel + ^{213}Bi-DTPA-F3 using the alamarBlue® assay ($n=3 \pm$ SD). **b** Survival of OVCAR-3 cells was quantified in a clonogenic assay 8 days after treatment with different activity concentrations of ^{213}Bi-DTPA-F3 (46.3, 92.5, 185, 370, 740 MBq/ml) or a regimen including combined ^{213}Bi-DTPA-F3 and paclitaxel ($n=2 \pm$ SD)

Combined treatment of OVCAR-3 cells with ^{213}Bi-DTPA-F3 + paclitaxel results in enhanced induction of apoptosis and G2/M phase arrest

Quantification of cell death and cell cycle arrest in ^{213}Bi-DTPA-F3- and/or paclitaxel-treated OVCAR-3 cells was performed by flow cytometry. Combined ^{213}Bi-DTPA-F3 + paclitaxel treatment resulted in enhanced induction of apoptosis and necrosis as compared to single treatments with either ^{213}Bi-DTPA-F3 or paclitaxel after 48 h (Fig. 3a, b). Induction of apoptosis and necrosis by any of the treatments applied could be inhibited by the membrane-permeable caspase-3 inhibitor Z-VAD-FMK indicating that occurrence of necrosis is secondary to apoptosis. Caspase-3 activation, indicative of induction of apoptosis, as determined by Western
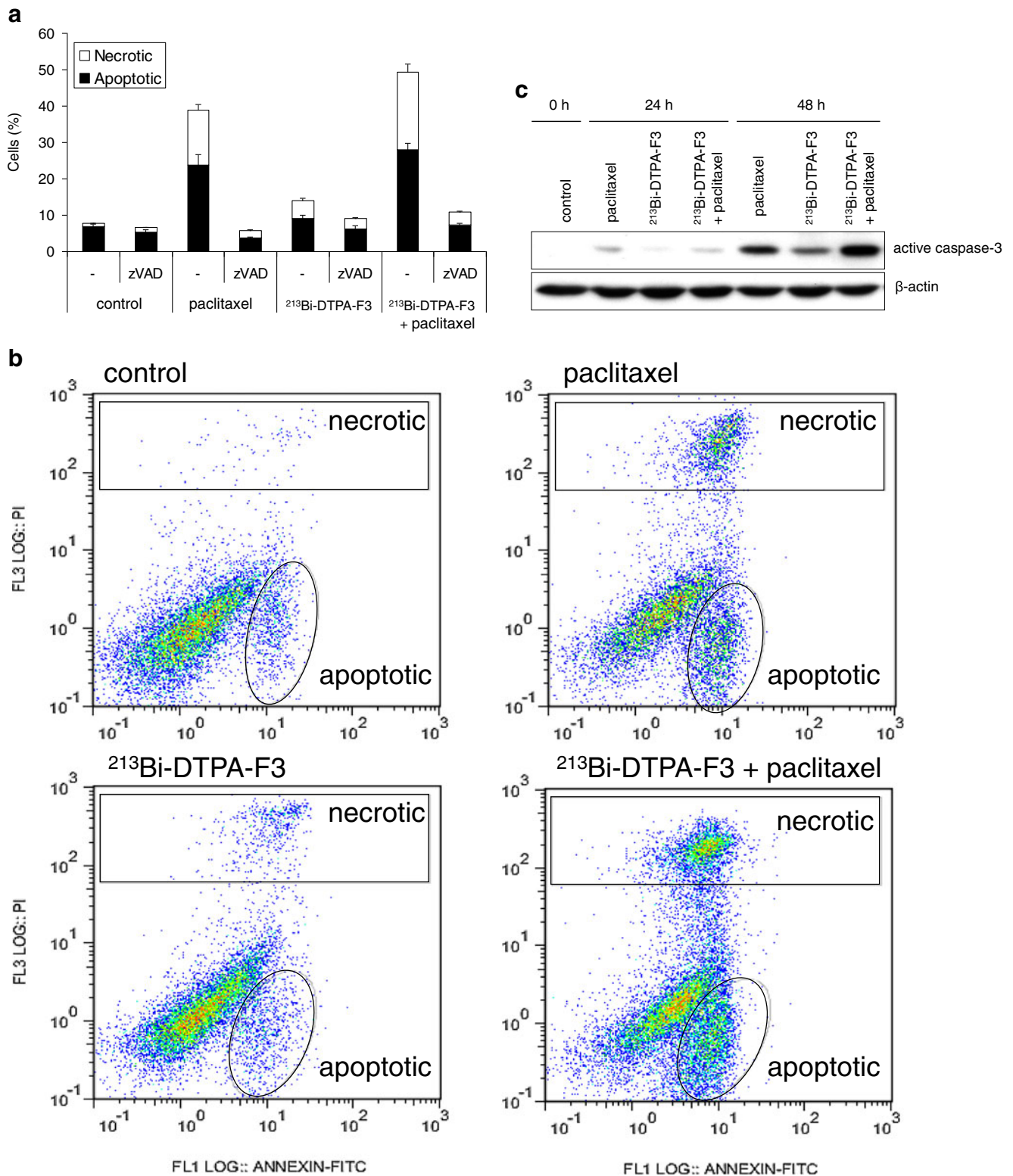
Fig. 3 Characterization of cell death in OVCAR-3 cells induced by treatment with paclitaxel and [213]Bi-DTPA-F3. OVCAR-3 cells were treated with paclitaxel (60 ng/ml), [213]Bi-DTPA-F3 (0.37 MBq/ml) or combined paclitaxel + [213]Bi-DTPA-F3 with or without the caspase inhibitor Z-VAD-FMK (50 μM); 48 h after treatment cells were incubated with annexin V-FITC and PI and subjected to flow cytometry. **a** Percentages of apoptotic and necrotic cells ($n=3 \pm$ SD). **b** Flow cytometric distribution of cells. Representative dot plots of triplicates are shown. Areas of necrotic (*rectangle*) and apoptotic (*ellipse*) cells are marked. **c** Western blot detection of active caspase-3 in cell lysates. Blots were re-probed with an anti-β-actin antibody

blotting correlated well with the flow cytometry results (Fig. 3c). Combined ^{213}Bi-DTPA-F3 + paclitaxel treatment resulted in the strongest caspase-3 activation of all treatments after 48 h. Moreover, ^{213}Bi-DTPA-F3 and/or paclitaxel treatments induced G2/M phase arrest (Fig. 4a, b). While ^{213}Bi-DTPA-F3 triggered a transient increase in G2/M phase cells peaking at 24 h that returned to the control level at 72 h, paclitaxel treatment induced less but sustained increase in G2/M phase cells. Combined ^{213}Bi-DTPA-F3 +

paclitaxel treatment induced the most pronounced increase in G2/M phase cells also peaking at 24 h, however dropping to the paclitaxel level at 72 h.

Combined ^{213}Bi-DTPA-F3 + paclitaxel treatment results in an enhanced inhibition of tumour growth

To investigate whether the enhanced effect of combined treatment with ^{213}Bi-DTPA-F3 + paclitaxel as observed in

Fig. 4 Cell cycle analysis of OVCAR-3 cells after treatment with paclitaxel, ^{213}Bi-DTPA-F3 or combined paclitaxel + ^{213}Bi-DTPA-F3. At 24, 48 and 72 h after treatment with paclitaxel (60 ng/ml), ^{213}Bi-DTPA-F3 (0.37 MBq/ml) or combined paclitaxel + ^{213}Bi-DTPA-F3, OVCAR-3 cells were fixed, stained with PI and subjected to single-parameter flow cytometry. a Percentages of OVCAR-3 cells in G2/M phase ($n=3 \pm$ SD). b Representative histograms showing the proportions of cells in G1, S and G2/M phase
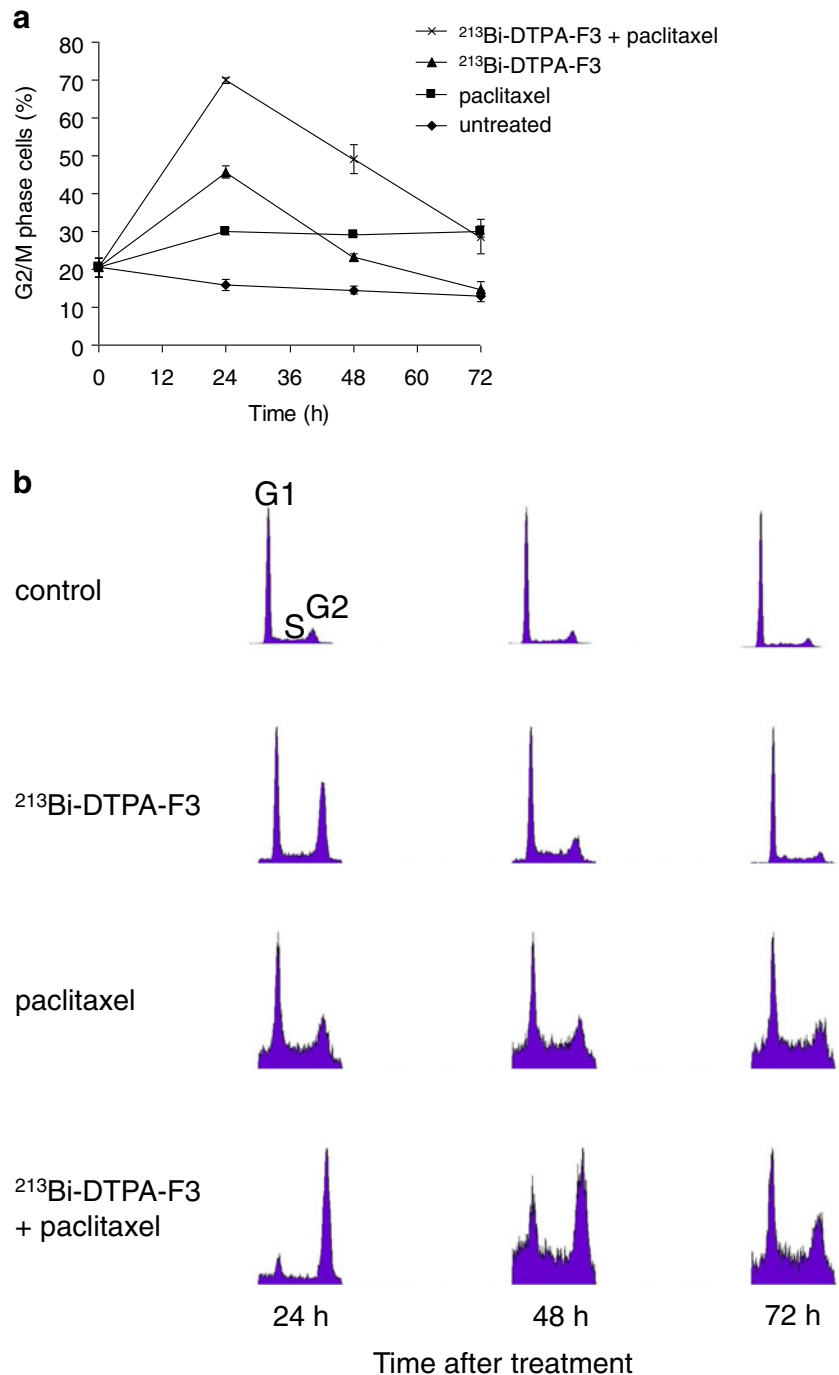
vitro can be translated to tumour treatment in a mouse model, we generated xenograft tumours by intraperitoneal injection of OVCAR-3-Luc-GFP cells into SCID mice. Ten days after tumour cell inoculation mice received 120 μg paclitaxel by intraperitoneal injection. On the following day 1.85 MBq of ^{213}Bi-DTPA-F3 was injected intraperitoneally. Treatments were repeated five times every third or fourth day (Fig. 5a). Control mice were injected with PBS instead of ^{213}Bi-DTPA-F3 and paclitaxel. Tumour development was monitored by bioluminescence imaging (Fig. 5b). On day 7 after tumour cell inoculation, i.e. 3 days before initiation of tumour treatment, most tumours had grown to a detectable size. The PBS-treated control mice displayed a strong increase in the bioluminescence signal over time indicative of uninhibited tumour growth. In the ^{213}Bi-DTPA-F3-treated mice the bioluminescence signal decreased until day 33 after cell inoculation and subsequently increased again as shown at day 40 (Fig. 5b). In the paclitaxel-treated mice the bioluminescence signal remained more or less constant until day 33 after tumour cell inoculation indicative of tumour stasis. However, at day 40 the signal had increased again. In contrast, in the combined treatment group (^{213}Bi-DTPA-F3 + paclitaxel) no signal was detectable from day 19 until day 40 after tumour cell inoculation indicating complete tumour clearance or tumour regression below detection limits.

Combined treatment with ^{213}Bi-DTPA-F3 + paclitaxel results in prolonged survival of xenograft tumour-bearing mice

Inhibition of tumour growth in xenograft mice after combined ^{213}Bi-DTPA-F3 + paclitaxel treatment also resulted in prolonged survival of animals. Xenograft mice that had been treated with combined ^{213}Bi-DTPA-F3 + paclitaxel significantly differed in overall survival from the other three treatment groups (PBS: $p=0.00051$; paclitaxel: $p=0.00055$; ^{213}Bi-DTPA-F3: $p=0.002$). As shown in Fig. 5c, d treatment with paclitaxel did not significantly prolong survival compared to treatment with PBS resulting in a mean survival of approximately 40 days each ($p=0.34$). Treatment with ^{213}Bi-DTPA-F3 significantly prolonged survival to 84 days both compared to PBS ($p=0.000074$) and paclitaxel ($p=0.000083$), while combined ^{213}Bi-DTPA-F3 + paclitaxel treatment increased mean survival to 121 days. Although tumour cells could no longer be detected by bioluminescence imaging up to 40 days after tumour cell inoculation following combined ^{213}Bi-DTPA-F3 + paclitaxel treatment (Fig. 5b), animals of the combined treatment group finally died from intraperitoneal tumour spread. This suggests that single tumour cells that escaped therapy caused fatal tumour growth in the end.

Fig. 5 Efficacy of repeated i.p. treatment of xenograft mice with PBS, paclitaxel, ^{213}Bi-DTPA-F3 or combined paclitaxel + ^{213}Bi-DTPA-F3. Xenografts were generated by i.p. injection of 1×10^7 OVCAR-3-Luc-GFP cells into SCID mice. **a** Treatment plan for i.p. xenograft mice: animals were injected six times either with paclitaxel (120 μg) or ^{213}Bi-DTPA-F3 (1.85 MBq) every third/fourth day starting at day 10 (paclitaxel) or 11 (^{213}Bi-DTPA-F3) after cell inoculation. Mice receiving combined treatment were injected i.p. six times with paclitaxel (120 μg) and ^{213}Bi-DTPA-F3 (1.85 MBq) on 2 consecutive days. Control mice were i.p. injected with PBS (100 μl). **b** Monitoring of tumour development by bioluminescence imaging. **c, d** Survival of i.p. xenograft mice. **c** Kaplan-Meier plot. **d** Mean survival ± SEM

Combined i.p. treatment with ^{213}Bi-DTPA-F3 + paclitaxel efficiently eradicates xenograft tumours

The efficacy of the different treatment protocols (Fig. 5a) was also evaluated via histopathologic analysis. For that purpose the mice subjected to bioluminescence imaging analysis were sacrificed at day 41 after tumour cell inoculation and small and large intestine including mesentery and tumours were dissected and processed for histopathologic analysis. H&E-stained slices of abdominal organs of control mice revealed that intraperitoneal tumours grew as independent nodules with a typical gland-like histology (Fig. 6a). Tumour treatment with paclitaxel or ^{213}Bi-DTPA-F3 resulted in drastic reduction of both the number and size of tumour nodules (Fig. 6b, c). No tumour nodules were detectable in sections from mice that had received combined ^{213}Bi-DTPA-F3 + paclitaxel treatment. Because these animals died approximately 120 days after tumour cell inoculation, single tumour cells that could not be detected histopathologically must have escaped treatment finally causing death of animals due to tumour regrowth.

Discussion

Peritoneal carcinomatosis is characterized by the dissemination of tumour cells throughout the peritoneal cavity. Most commonly intraperitoneal dissemination of tumour cells occurs from gastrointestinal and ovarian malignancies [1]. Prognosis of peritoneal spread of a primary carcinoma is poor because the treatment strategies applied such as cytoreductive surgery together with adjuvant intravenous or intraperitoneal chemotherapy are not efficient. Therefore, new therapeutic options based on tumour targeting are being increasingly pursued [2]. We have investigated efficacy of targeted therapy with ^{213}Bi-DTPA-F3 conjugates that specifically bind to nucleolin on the surface of ovarian tumour cells in an animal model of peritoneal spread. Nucleolin constantly shuttles from the cell surface to the nucleus and back in proliferating tumour cells. Upon binding of the ^{213}Bi-DTPA-F3 conjugates to nucleolin they are cotransported to the nucleus, thus increasing the probability of
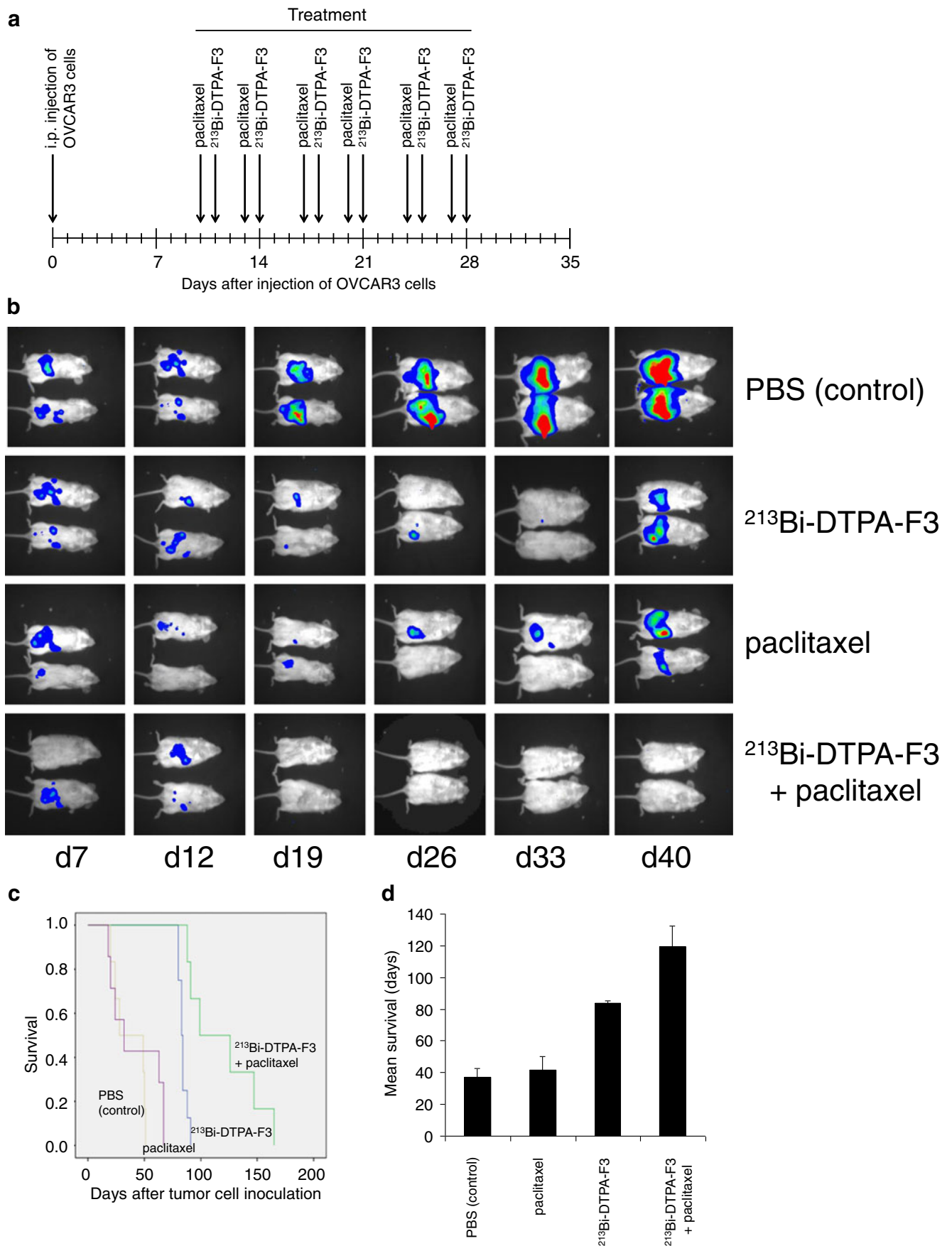
Fig. 6 Histopathologic analysis of intraperitoneal tumour spread after repeated i.p. treatment of xenograft mice with PBS, paclitaxel, ^{213}Bi-DTPA-F3 or combined paclitaxel + ^{213}Bi-DTPA-F3. Xenograft mice were treated as outlined in Fig. 5. **a** H&E-stained sections of mesentery of small and large intestine 41 days after inoculation of OVCAR-3 tumour cells; *arrows* point at tumour nodules; top overview, scale bars: 2 mm; bottom magnification of indicated areas, scale bars: 50 μM. **b** Number of tumour nodules per section and **c** tumour area (mm^2) per section (n=2 ± SD)
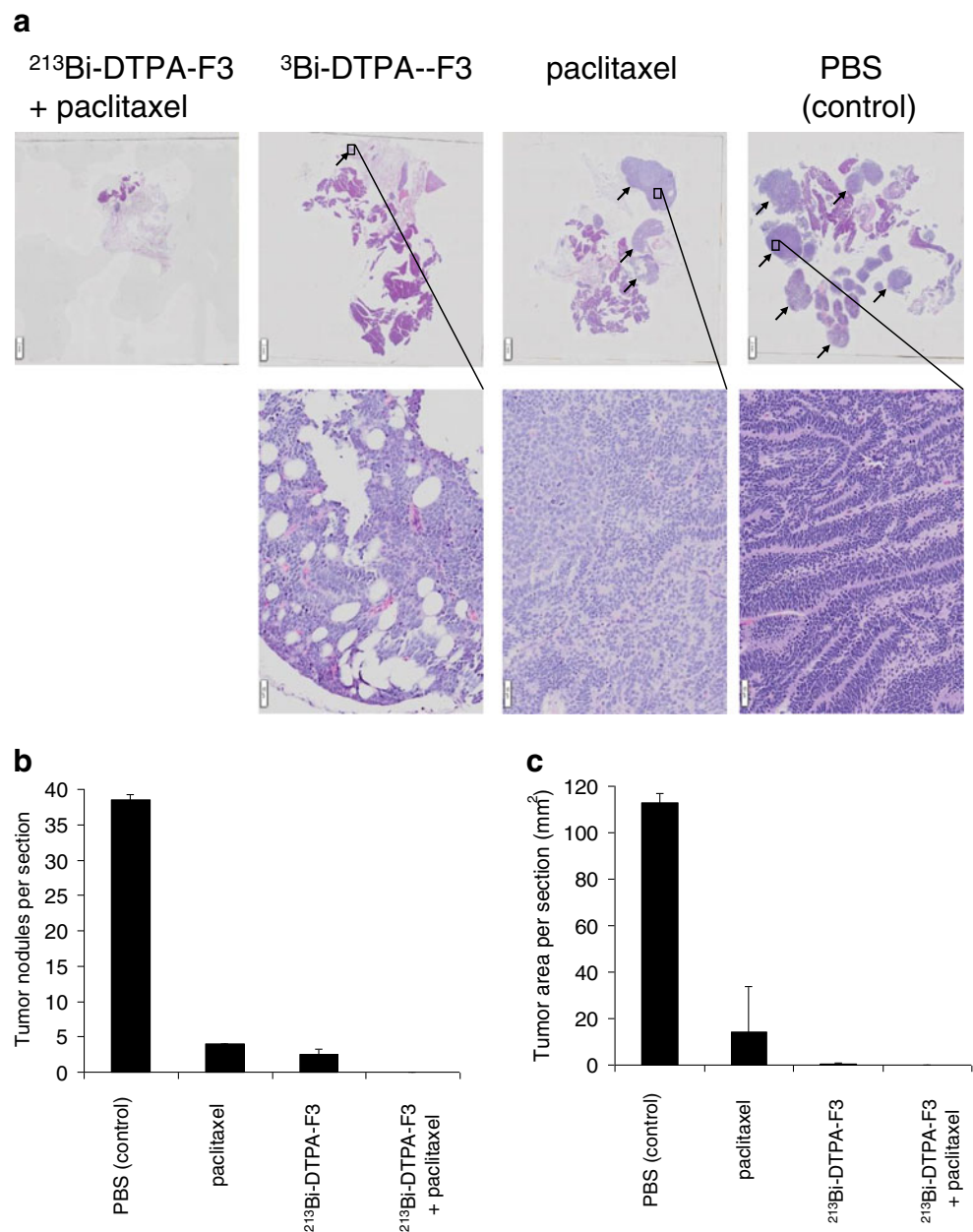
induction of lethal DNA double-strand breaks by the α-emitter ^{213}Bi. Efficacy of ^{213}Bi-DTPA-F3 treatment was compared with treatment with the cytostatic drug paclitaxel and with combined ^{213}Bi-DTPA-F3 + paclitaxel treatment. Repeated therapy with ^{213}Bi-DTPA-F3 significantly prolonged survival of mice compared to animals that were mock-treated (PBS) or received paclitaxel. Moreover, combined repeated intraperitoneal application of ^{213}Bi-DTPA-F3 and paclitaxel again improved therapeutic efficacy compared to ^{213}Bi-DTPA-F3 therapy (Fig. 5). This suggests that paclitaxel can potentiate cytotoxic effects on tumour cells induced by ^{213}Bi-DTPA-F3.

Nevertheless, even repeated combined treatment with ^{213}Bi-DTPA-F3 and paclitaxel did not result in complete remission of tumour nodules. As shown in Fig. 6 the histopathologic investigation of intraperitoneal tumour spread at day 41 after tumour cell inoculation did not reveal any signs of tumour cell infiltration on mesenteries of small and large intestine after combined ^{213}Bi-DTPA-F3 and paclitaxel treatment. However, these animals died from tumour regrowth approximately 120 days after tumour cell inoculation (Fig. 5d). This means that single tumour cells must have survived the therapeutic regimen though not detectable by histopathologic investigation. In future trials the number of treatment cycles could be increased because no signs of significant toxicity have been detected both after application of ^{213}Bi-F3 conjugates [24] and paclitaxel (data not shown).

Another discrepancy with respect to the results of the different therapeutic regimens obviously affects mock-treated (PBS) and paclitaxel-treated animals. As shown in Fig. 6, treatment with paclitaxel significantly reduced both the number of tumour nodules and the tumour area compared to mock treatment. In contrast, data on mean survival of these two treatment groups show almost identical results (Fig. 5d). Consequently, treatment with paclitaxel did not significantly prolong survival compared to mock treatment. Because paclitaxel efficiently eradicated tumour cells—in contrast to PBS (Fig. 6)—this could mean that treatment with paclitaxel possibly created aggressive tumour cells showing fast proliferation finally triggering death of animals at almost the same time point as it occurred in the control group. On the other hand, death of these animals might have been caused by small tumour nodules that induced internal haemorrhage of the aorta combined with the development of ascites. Such haemorrhages may also happen during tumour shrinkage or may be even caused by intra-abdominal mechanical changes due to reduction of tumour size.

Targeted therapy with α-emitters of disseminated intraperitoneal disease has been applied successfully in different animal models of gastric [13, 14], colonic [15, 16] and ovarian origin [17, 18]. Therapeutic efficacy of α-emitters predominantly is based on induction of DNA double-strand breaks that trigger persistent cell cycle arrest at G2/M phase finally resulting in cell death. G2/M phase arrest has been demonstrated for HSC45-M2 gastric cancer cells following treatment with ^{213}Bi-d9MAb immunoconjugates [33]. G2/M cell cycle arrest could be confirmed also in tumour cells of mice bearing LS-174T intraperitoneal xenografts after ^{212}Pb radioimmunotherapy [34]. Moreover, the anticancer drug paclitaxel is known to induce G2/M phase arrest. Therefore, combined treatment with α-emitter conjugates and paclitaxel should increase the number of cells arrested in G2/M phase compared to treatment with either α-emitter or paclitaxel alone. Indeed, our results clearly reveal the expected increase in G2/M phase cells as induced by the combined treatment (Fig. 4).

Accordingly, our results clearly demonstrate that the therapeutic effect of α-emitters can be potentiated by additional application of the cytostatic drug paclitaxel. Potentiation of the cytotoxic effects of particle radiation with cytostatic drugs has been described also in previous studies.

In a mouse model with s.c. xenografts of the Le^y-positive breast adenocarcinoma cell line MCF-7, i.v. treatment with β-emitter (^{90}Y) or α-emitter (^{213}Bi) hu3S193 immunoconjugates targeting the Le^y antigen in combination with paclitaxel significantly prolonged survival compared to treatment with the radioimmunoconjugates only [35, 36]. In a mouse model with s.c. Le^y-positive DU145 prostate xenografts combined treatment with ^{177}Lu-hu3S193 immunoconjugates and the

epidermal growth factor receptor (EGFR) inhibitor AG1478 or the cytostatic drug docetaxel significantly improved therapeutic efficacy [37].

Experimental treatment of disseminated peritoneal disease with α-emitter immunoconjugates in combination with cytostatic drugs has been investigated in a nude mouse model with i.p. LS-174T human colon cancer xenografts. LS-174T cells are efficiently targeted by trastuzumab due to overexpression of HER2/neu. By development of a treatment regimen combining multiple doses of gemcitabine (50 mg/kg) with ^{212}Pb radioimmunotherapy (370 kBq) median survival increased up to 196.5 days [38]. Therapeutic efficacy of ^{212}Pb-trastuzumab also increased in combination with the chemotherapeutic paclitaxel. In athymic mice bearing 3-day intraperitoneal LS-174T xenografts, 600 μg paclitaxel applied 24 h before radioimmunotherapy extended the median survival from 44 to 171 days [15]. Paclitaxel also provided benefit as to the therapeutic success of ^{213}Bi-trastuzumab treatment of experimental peritoneal carcinomatosis: Median survival increased to 198 days with paclitaxel (600 μg) applied 24 h before ^{213}Bi-trastuzumab (18.5 MBq) followed by another injection of paclitaxel [15]. These results also demonstrate that application of paclitaxel 24 h before ^{213}Bi-trastuzumab is superior to other orders of application.

An enhanced, additive therapeutic benefit was also achieved by dual targeting of two distinct tumour surface molecules with ^{213}Bi-labelled antibodies. Concurrent α-radioimmunotherapy with ^{213}Bi-trastuzumab and ^{213}Bi-HuCC49ΔCH2 [targeting the tumour associated glycoprotein 72 (TAG-72)] significantly prolonged median survival [16]. Additional application of cytostatic drugs could further improve therapeutic efficacy.

In conclusion, multimodality therapy combining targeted radionuclide therapy with administration of cytostatic drugs is a promising therapeutic concept both after i.v. and i.p. application. Therefore, this concept should soon find its way into clinical practice.

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