

# Expression and therapeutic relevance of heat-shock protein 90 in pancreatic endocrine tumors

Philipp Mayer\*, Andreas Harjung\*, Marco Breinig, Lars Fischer<sup>1</sup>, Volker Ehemann, Mona Malz, Hans Scherübel<sup>2</sup>, Sarah Britsch, Jens Werner<sup>1</sup>, Michael A Kern, Hendrik Bläker, Peter Schirmacher and Frank Bergmann

Institute of Pathology, University of Heidelberg, Im Neuenheimer Feld 220, D-69120 Heidelberg, Germany

<sup>1</sup>Department of General Surgery, University of Heidelberg, Im Neuenheimer Feld 110, D-69120 Heidelberg, Germany

<sup>2</sup>Medical Clinic - Gastroenterology and Gastrointestinal Oncology, Vivantes Klinikum Am Urban, Dieffenbachstrasse 1, D-10967 Berlin, Germany

(Correspondence should be addressed to F Bergmann; Email: frank.bergmann@med.uni-heidelberg.de)

\*(P Mayer and A Harjung contributed equally to this work)

## Abstract

Pancreatic endocrine tumors (PET) represent a heterogenous group of neoplasms. Although surgical resection is considered a safe and effective treatment for many PET, therapeutic options for inoperable and progressive PET are limited. The expression of heat-shock protein (HSP) 90 was investigated in 120 clinically and pathomorphologically well-characterized PET from 84 patients using immunohistochemistry. In addition, in 19 snap-frozen PET and in three healthy pancreatic tissues, we performed immunoblot analyses, and in 15 snap-frozen PET and in three healthy pancreatic tissues, we investigated the expression of HSP90 isoforms by means of semiquantitative RT-PCR. Functional tests were conducted using the human pancreas carcinoid cell line BON and the mouse insulinoma cell line  $\beta$ -TC-3. HSP90 was expressed in 95% of the PET patients. The transcript levels of the HSP90 isoforms HSP90 $\alpha$ , HSP90 $\beta$ , glucose-related protein 94, and TNF receptor-associated protein 1 were significantly increased in PET compared with non-neoplastic pancreatic tissues. The treatment of the cell lines BON and  $\beta$ -TC-3 with the HSP90 inhibitors 17-allylamino-17-demethoxygeldanamycin and 17-dimethylaminoethylamino-17-demethoxy-geldanamycin resulted in significant, dose-dependent reduction of cell viability, cell cycle arrest, and increased apoptosis. Furthermore, HSP90 inhibition induced the degradation and inactivation of several oncogenic HSP90 client proteins in a time- and dose-dependent manner. HSP90 inhibitors increased the therapeutic effects of doxorubicin and 5-fluorouracil in BON and  $\beta$ -TC-3 cells. HSP90 is expressed in the vast majority of PET and its inhibition reveals significant treatment effects *in vitro*. Thus, HSP90 qualifies as a promising new target.

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## Introduction

Pancreatic endocrine tumors (PET), which represent 1–2% of all pancreatic neoplasms, are biologically and clinically heterogeneous (Heitz *et al.* 2004). Based on a panel of clinical and morphological characteristics, PET are classified as benign well-differentiated PET, well-differentiated PET of uncertain behavior, well-differentiated endocrine carcinomas, and poorly differentiated endocrine carcinomas (Heitz *et al.* 2004). Furthermore, depending on clinical syndromes related

to hormone production by the tumors, PET are divided into functioning and nonfunctioning tumors (Heitz *et al.* 2004).

For the PET, surgical resection of the tumors is considered the therapy of first choice (Plockinger *et al.* 2004). This may also be applied to PET with hepatic metastasis and has been reported to result in 5-year survival rates for patients with operable malignant PET of up to 77% (Kazanjian *et al.* 2006). Nevertheless, treatment options are limited for inoperable and

progressive malignant PET, which present distant metastases at the time of diagnosis in up to 87% of cases (Kirshbom *et al.* 1999). Thus, current chemotherapeutic concepts for inoperable or progressive malignant PET with streptozotocin in combination with 5-fluoruracil (5-FU) or doxorubicin as first-line treatment lead to a significant tumor response in only 20–35% of the patients (Eriksson *et al.* 2009).

This urges the need for innovative nonsurgical treatment options for PET. In a variety of other neoplasms, including pancreatic ductal adenocarcinoma (Song *et al.* 2008), hepatocellular carcinoma (Breinig *et al.* 2009), breast cancer (Baselga 2010), non-small-cell lung cancer (Shimamura & Shapiro 2008), esophageal and gastric cancers (Lee *et al.* 2009), multiple myeloma (Richardson *et al.* 2010), acute myeloid leukemia (Lancet *et al.* 2010), and prostate cancer (Altieri 2010, Suzuki *et al.* 2010), heat-shock protein (HSP) 90 has been considered a promising new therapeutic target. HSP90 is a molecular chaperone that comprises 1–2% of total cellular protein content and regulates the correct folding, activity, function, and stability of over 200 client proteins (Trepel *et al.* 2010). The most important HSP90 isoforms are the highly inducible HSP90 $\alpha$  (HSP90AA) and the constitutively expressed HSP90 $\beta$  (HSP90AB), both of which are mostly found in the cytoplasm and constitute the HSP90A family (Csermely *et al.* 1998, Sreedhar *et al.* 2004, Chen *et al.* 2005). Higher eukaryotes possess their own endoplasmic reticulum (ER) isoform, glucose-related protein 94 (GRP94; Ni & Lee 2007), which constitutes the HSP90B family and is important for maintaining the ER-homeostasis (Pan *et al.* 2009), as well as their own mitochondrial isoform TNF receptor-associated protein 1 (TRAP1; Csermely *et al.* 1998), which is part of a mitochondrial pathway maintaining mitochondrial integrity (Kang *et al.* 2007).

Many client proteins of HSP90 are involved in signal transduction pathways whose dysregulation may drive cancer (Workman *et al.* 2007, Pearl *et al.* 2008, Trepel *et al.* 2010). In this context, the effects of HSP90 on several oncoproteins, e.g. B-RAF, may be enhanced by the presence of mutations, as in contrast to the wild-type proteins, the mutated forms of these oncoproteins are preferentially folded and protected by HSP90 (Grbovic *et al.* 2006, Pick *et al.* 2007). Furthermore, cancer cells depend on chaperones in general to manage cellular stress like hypoxia, acidosis, and nutrient deprivation, induced by the tumor environment, and stress associated with the activation of oncogenes (Workman 2004, Pearl *et al.* 2008).

Recently, HSP90 inhibitors have been considerably improved (Workman *et al.* 2007). 17-Allylamino-17-demethoxygeldanamycin (17-AAG) and 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), two of 14 HSP90 inhibitors currently undergoing clinical trials, are based on the natural product geldanamycin (Porter *et al.* 2010) and have shown relevant antitumor activity in preclinical studies (Hollingshead *et al.* 2005, Usmani *et al.* 2009, Trepel *et al.* 2010). Clinical trials have been initiated for both (Trepel *et al.* 2010), and phase III clinical trials involving 17-AAG are currently under way (Usmani *et al.* 2009).

The aim of this study was to investigate the expression and putative role of HSP90 as a therapeutic target in PET.

## Materials and methods

### Patients

One hundred and twenty-two PET from 86 patients, who had undergone resections for PET between 1991 and 2009, were included in the study (Supplementary Table 1, see section on supplementary data given at the end of this article). In 120 cases, formalin-fixed, paraffin-embedded tumor tissues were obtained. For histological evaluation, sections were stained with hematoxylin and eosin. The diagnosis was established according to the criteria recommended by the World Health Organization (2004). Freshly frozen tissue samples were available from 21 patients. Clinical data were obtained from the prospective database of the Department of General Surgery at the University of Heidelberg. The study was approved by the ethics committee of the University of Heidelberg.

The 86 patients (41 women and 45 men) included in our study had an average age of 53.7 years (range from 13 to 85 years) at the time of diagnosis. Clinically, a functional syndrome was reported in 30 cases (22 insulinomas, seven gastrinomas, and one glucagonoma). Seven patients were known to be affected by the multiple endocrine neoplasia type 1 syndrome.

The 122 tumors comprised 77 primary PET, four intrapancreatic recurrences (three pancreatic head and one tail), 26 lymph node metastases (corresponding primary obtained in 23 of these cases), 12 liver metastases (corresponding primary in ten cases), and three distant metastases other than liver (corresponding primary in two cases). Of the 77 primary PET, 33 were localized in the pancreatic head, 27 in the tail, 13 in the body, and four affected the body and tail. The average size of the primaries was 3.7 cm (range from 0.7 to

10 cm) in largest diameter. The number of mitotic figures in 10 high-power fields ranged from 0 to 100 (average 6.5). Correspondingly, the Ki-67 proliferation index ranged from 0 to 95% (average 8%). By means of immunohistochemistry, the tumors of 24 patients revealed positivity for insulin (28%), whereas an expression of gastrin was found in the tumors of 23 patients (27%). An expression of serotonin and glucagon was seen in tumors of nine patients each (10%). The tumors of four patients (5%) expressed somatostatin. Overlapping positivity for more than one hormone was observed in 17 cases. On the basis of the tumor size, the presence or absence of metastases, gross invasion, perineural invasion, and angioinvasion, as well as the mitotic activity of the tumors (mitotic count, Ki-67 proliferative activity), the diagnosis of a benign well-differentiated PET was made in 13 patients, well-differentiated PET with uncertain behavior in 16 patients, well-differentiated endocrine carcinoma in 51 patients, and poorly differentiated endocrine carcinoma in six patients.

Follow-up information could be obtained for 67 patients. After follow-up periods between 2 and 177 months (average 48.1 months), 40 patients (60%) showed no evidence of disease. Eight patients (12%) were alive with recurrent disease (local recurrence and/or distant metastases) between 26 and 164 months after surgery (average 62.4 months). Eighteen patients (27%) died from disease after follow-up periods between 1 and 196 months (average 29.9 months). One patient died 1 month after tumor enucleation of an unrelated cause. Local or systemic recurrences were not observed in patients with benign well-differentiated PET (average follow-up period 39.3 months).

### Immunohistochemical analysis

To evaluate the *HSP90* expression in PET and non-neoplastic pancreatic tissues, the immunohistochemical analyses were performed based on tissue microarrays using the avidin–biotin complex method with anti-HSP90 (1:70; Millipore, Billerica, MA, USA) as primary antibody, as described previously (Bergmann *et al.* 2009). Antigen retrieval was performed by heat pretreatment in a citrate buffer (pH 6.1) for 30 min. To confirm the tissue microarray-based results, in 15 selected cases, the stainings were repeated using regular whole tissue specimen. The latter displayed homogeneous staining patterns of HSP90 in all cases. For quantitative analyses of expression of *HSP90*, the respective immunohistochemical staining intensities were scored as negative (score: 0), weakly positive (score: 1), moderately

positive (score: 2), or strongly positive (score: 3) by three observers blinded to the diagnosis (F B, P M, and A H), as previously reported (Bergmann *et al.* 2009). The hormone expression was determined as described previously (Bergmann *et al.* 2009).

### Cell culture

For functional analyses, the murine insulinoma cell line  $\beta$ -TC-3 (obtained from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and the human carcinoid cell line BON were used and cultured, as described previously (Bergmann *et al.* 2009).

### Reagents

The HSP90 inhibitors 17-AAG and 17-DMAG (both from LC Laboratories, Woburn, MA, USA) were solubilized in dimethyl sulfoxide before use. For chemotherapeutics, 5-FU (Fluorouracil GRY; Teva, Aesch, Switzerland) and doxorubicin (Doxo Cell, Cell Pharm, Hannover, Germany) were obtained.

### Cell viability measurement

A total of 4000 cells per well ( $\beta$ -TC-3) or 5000 cells per well (BON) were seeded onto 96-well plates and allowed to attach overnight. After 24 h, the medium was replaced by 17-AAG, 17-DMAG, doxorubicin, and/or 5-FU in the concentrations indicated below. After the incubation time of 24 or 48 h, 25  $\mu$ l 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) stock solution (prepared as described by Rexen & Emborg (1992)) was added to each well and the plates were incubated for 2 h. Then, the formazan produced by the cells was dissolved by adding 150  $\mu$ l lysis buffer (prepared by dissolving SDS (Serva, Heidelberg, Germany) in a 1:1 mixture of water and *N,N*-dimethylformamide (VWR International, Fontenay-sous-Bois, France) to a concentration of 20% (w/v); pH in the lysing buffer was adjusted to  $\sim$ 2 by adding 32% HCl). The solubilized formazan product was spectrophotometrically quantified using a microplate spectrophotometer (Multiskan MS 6.0; Labsystems, Helsinki, Finland) at a wavelength of 490 nm with a reference at a wavelength of 690 nm. Assays were performed six times, and data were expressed as the mean  $\pm$  s.d. ( $n=6$ ).

### Cell cycle analysis and detection of apoptosis

Analyses for cell cycle were performed using a PAS II flow cytometer (Partec, Münster, Germany) equipped with a mercury vapor lamp (100 W) and a filter

**Table 1** Primers used to analyze the expression levels of the HSP90 mRNAs coding for the respective HSP90 isoforms

Gene	Direction of primer	Sequence (5' → 3')
HSP90AA1.1	Forward	GGGGTACCCTACGGGGAGCG
	Reverse	AGAGCGGAGGAAGAGCGCCA
HSP90AA1.2	Forward	TATAAGGCAGGCGCGGGGGT
	Reverse	ACCTCCTCCTCCTCCATCGGT
HSP90AB1	Forward	CGAGGGCGATGAGGATGCGT
	Reverse	GGGGAGCCAGGTGGGACAGG
HSP90B1	Forward	GCGGCTGGAGGTGTGAGGAT
	Reverse	CCCGAAGGTCAGCAGGACGC
TRAP1	Forward	TGGGCTCCAGCGTTGCACTG
	Reverse	AGCAGCTCCCGCTGAGGTT
18S	Forward	AAACGGCTACCACATCCAAG
	Reverse	CCTCCAATGGATCCTCGTTA

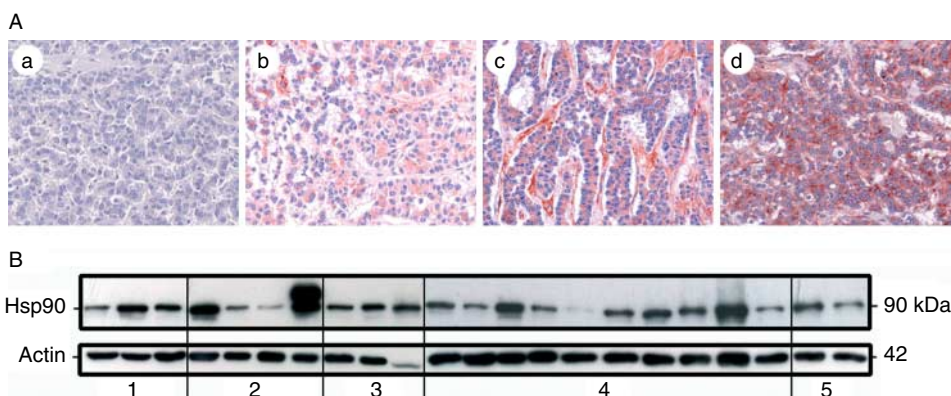
combination for 2,4-diamidino-2-phenylindole (DAPI)-stained single cells. Cells were harvested with trypsin/EDTA, treated with 2.1% citric acid/0.5% Tween 20, and stained with a phosphate buffer (7.2 g Na<sub>2</sub>HPO<sub>4</sub> × 2H<sub>2</sub>O in 100 ml distilled H<sub>2</sub>O) pH 8.0 containing DAPI, as described previously (Ehemann et al. 1999). Analyses for apoptosis and DNA fragmentation were performed using a FACS-calibur flow cytometer (Becton Dickinson, Franklin Lakes, NT, USA) equipped with a 488 nm air-cooled argon laser with filter combinations for propidium iodide. To detect apoptotic cells, cells were prepared according to Nicoletti stain with modifications (Ehemann et al. 1999, 2003) and measurements were acquired in FI-2 in logarithmic mode and calculated by setting gate over the first three decades. DNA fragmentation was detected with a terminal

deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) kit (Roche), as described previously (Ehemann et al. 2003). Multiparameter analyses (cell cycle vs TUNEL) were performed on a Galaxy Pro flow cytometer (Partec).

Cells (5 × 10<sup>5</sup>) were seeded onto 10 cm plates and allowed to attach for 24 h. Then, the medium was changed, 1 μM 17-AAG or 17-DMAG was added to β-TC-3 and 0.05 μM 17-AAG or 17-DMAG was added to BON. After an additional incubation time (24, 48, and 72 h), cell cycle analyses and Nicoletti staining were performed. TUNEL staining was performed after an incubation time of 48 h. Assays were performed in triplicate. Data are the mean (n = 3).

### Western immunoblot analysis

Western immunoblot analyses were performed with protein lysates obtained from the cell lines BON and β-TC-3 as well as from snap-frozen tissue samples, comprising four benign well-differentiated endocrine tumors, three well-differentiated endocrine tumors of uncertain behavior, ten well-differentiated endocrine carcinomas, two poorly differentiated endocrine carcinomas, and three non-neoplastic pancreatic tissues. The three snap-frozen non-neoplastic pancreatic tissue samples consisted lobules of predominantly exocrine cells, containing scattered islets. For protein isolation from cell lines, the cell monolayers were washed with PBS (PAA Laboratories, Cölbe, Germany) and lysed (10% Chaps (10×), 0.2% DTT (200×), and 2% protease inhibitor cocktail (Sigma)). The protein concentrations were quantified by Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies,



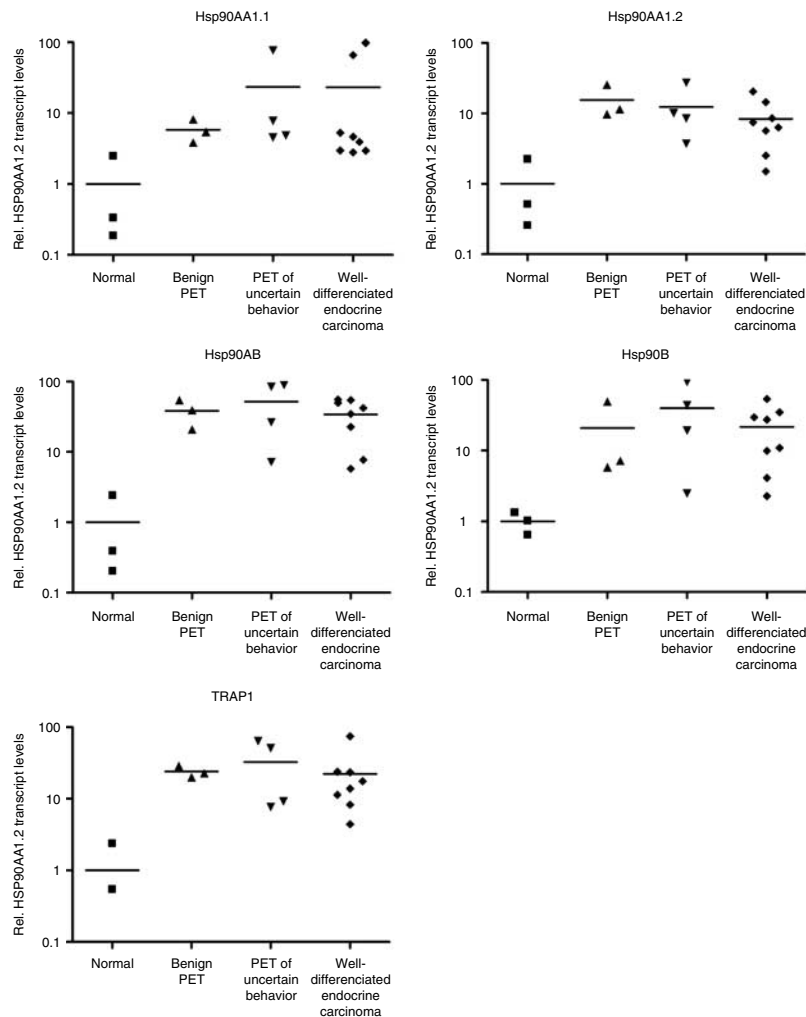
**Figure 1** HSP90 is expressed in PET. (A) Immunohistochemical reactivity for HSP90 in various PET. (a) No tumor staining, score: 0; (b) weak tumor staining, score: 1; (c) moderate tumor staining, score: 2; (d) strong tumor staining, score: 3. Original magnification: ×64. (B) HSP90 western immunoblot analysis revealed that HSP90 is expressed in all freshly frozen tissue samples. 1, non-neoplastic pancreatic tissues; 2, benign well-differentiated PET; 3, well-differentiated PET of uncertain behavior; 4, well-differentiated endocrine carcinoma; 5, poorly differentiated endocrine carcinoma. Actin served as a loading control.

Wilmington, DE, USA). Thirty micrograms of the respective protein were separated by SDS–PAGE (using 8–10% gels), transferred onto nitrocellulose membrane (Whatman, Dassel, Germany), probed with different antibodies (listed below), and detected by the Amersham ECL-detection system (GE Healthcare, Buckinghamshire, UK) and FUJI SuperRX medical X-ray films (Fujifilm, Düsseldorf, Germany). The following antibodies were used: HSP70 (1:6.000; Santa Cruz Biotechnologies, Santa Cruz, CA, USA), HSP90 (1:1.000; Assay Designs, Ann Arbor, MI, USA), MET (1:200; Santa Cruz Biotechnologies), phospho-p44/42 MAPK (pErk1/2, 1:500; Cell Signaling, Beverly, MA, USA), p44/42 MAPK (extracellular signal-regulated kinase 1/2 (Erk1/2), 1:1.000; Cell

Signaling), Phospho-Akt (Ser473, 1:500; Cell Signaling), Akt (1:1.000; Cell Signaling), poly(ADP-ribose) polymerase (PARP; 1:500; Cell Signaling), Raf1 (1:200; Santa Cruz Biotechnologies), IGF-IR $\beta$  (C-20) (1:200; Santa Cruz Biotechnologies), CDK4 (1:500; Invitrogen), and actin (1:10.000; MP Biomedicals, Munich, Germany). All western immunoblot analyses were performed three times.

## Real-time PCR

RT-PCR analyses were performed with total RNA isolated from three span-frozen benign well-differentiated endocrine tumors, four well-differentiated endocrine tumors of uncertain behavior, eight well-



**Figure 2** Transcript levels of HSP90 isoforms are elevated in PET compared with non-neoplastic pancreatic tissue. By means of semiquantitative RT-PCR, all *HSP90* mRNAs were significantly overexpressed in the freshly frozen PET samples compared with non-neoplastic pancreatic tissues ( $P < 0.005$ ). HSP90AA1.1 and HSP90AA1.2 code for HSP90 $\alpha$ , HSP90AB codes for HSP90 $\beta$ , HSP90B codes for Grp94, and TRAP1 codes for the identically named protein.

differentiated endocrine carcinomas, and three non-neoplastic pancreatic tissues. The three snap-frozen non-neoplastic pancreatic tissue samples consisted of lobules of predominantly exocrine cells, containing scattered islets. For isolation and purification of total RNA, the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) was used according to the manufacturer's protocol. For the semiquantitative real-time PCR, the TaqMan-PCR Technology (Prism 7300; Applied Biosystems, Darmstadt, Germany) was used. The following cycling program was applied using the SYBR Green (Absolute SYBR Green ROX Mix, Epsom, UK): 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 60 s (Prism 7300; Applied Biosystems). The primers are listed in Table 1. The amount of each mRNA coding for the respective HSP90 isoforms was detected by RT-PCR. For each experimental sample, the relative value obtained was normalized using the value derived from the house-keeping gene *18S* in the corresponding PCR. For specific normalization, the standard curve method was used. Assays were performed in triplicate.

### Statistical analyses

Statistical analyses were performed using the Student's *t*-test, the Wilcoxon rank-sum, or the log-rank test. All statistical analyses were two sided. For the MTT assays and the RT-PCR results, the Wilcoxon rank-sum test was used. Differences between treated and untreated cells in the percentages of cells in the distinct phases of cell cycle and in the percentages of cells in sub G1 phase were analyzed using the Student's *t*-test. The Kaplan–Meier survival curves were compared using the log-rank test. All statistical analyses took the number of samples into account. The asterisks in each graph indicate statistically significant changes given as *P* values: \**P* < 0.05, \*\**P* < 0.01. *P* values < 0.05 were considered statistically significant.

## Results

### HSP90 is expressed in almost all PET

In the immunohistochemical analyses, HSP90 was expressed in PET from 80 patients (Fig. 1A). The *HSP90* expression displayed a diffuse pattern. The expression of *HSP90* did not significantly correlate with malignant potential, functional activity, tumor size, mitotic index, Ki-67 positivity, or the immunohistochemical expression of insulin, gastrin, glucagon, or serotonin. Among the primary tumors, patients' overall survival was not dependent on the immunohistochemical expression of *HSP90* (*P* > 0.7408). In ten

non-neoplastic pancreatic tissues, immunohistochemistry revealed weak to moderate *HSP90* expression in islets and ducts and faint to weak expression in acinar cells.

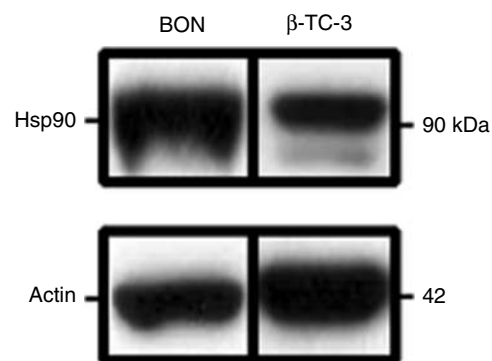
The results of the immunohistochemical analyses were confirmed by western immunoblot analyses, with all 19 examined tumors expressing HSP90 (Fig. 1B).

### Transcript levels of HSP90 isoforms are higher in PET than in non-neoplastic pancreatic tissue

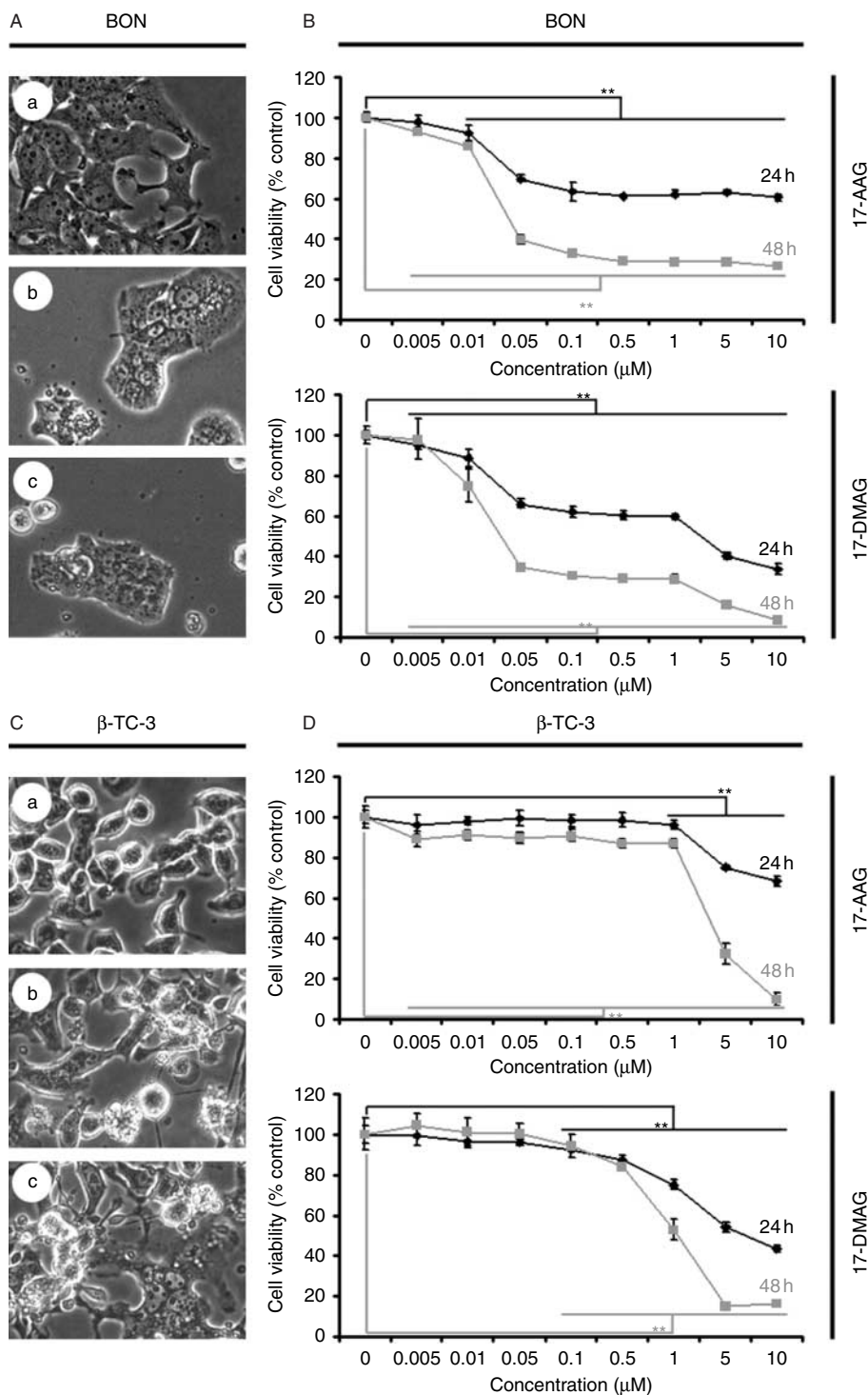
The comparison of the *HSP90* mRNA expression in non-neoplastic pancreatic tissues with the expression in PET (irrespective of the diagnosis) revealed a significant overexpression of all *HSP90* mRNAs in PET (*P* < 0.005, Fig. 2). For further statistical analysis, the samples were pooled according to their diagnosis. All *HSP90* mRNAs were significantly overexpressed in well-differentiated endocrine carcinomas compared with non-neoplastic pancreatic tissues (*P* < 0.025). The overexpression of the *HSP90* mRNAs in benign well-differentiated PET and in well-differentiated PET of uncertain behavior compared with non-neoplastic pancreatic tissues was not significant (*P* ≤ 0.100 and *P* < 0.058).

### 17-AAG and 17-DMAG reduce cell viability in PET cell lines

As shown in Fig. 3, both the human PET cell line BON and the murine PET cell line β-TC-3 expressed HSP90. As shown in Fig. 4, 17-AAG as well as 17-DMAG significantly reduced cell viability in both the cell lines in a time- and dose-dependent manner (*P* < 0.010). In BON, the half maximal growth inhibitory concentration after 48 h (IC50) was between 10 and 50 nM, in β-TC-3, ~ 1 μM.



**Figure 3** HSP90 is expressed in the PET cell lines BON and β-TC-3. The expression of HSP90 was detected in BON and β-TC-3 by western immunoblot analysis. Both cell lines expressed HSP90. Actin served as a loading control.



**Figure 4** HSP90 inhibition reduces cell viability in PET cells. (A) Human PET cell line BON. (a) Untreated, spindle-shaped to polygonal adherent cells. (b and c) After treatment with 1 μM 17-AAG or 17-DMAG for 24 h, the number of cells is reduced, with some cells showing apoptotic bodies (original magnification:  $\times 200$ ). (C) Murine PET cell line  $\beta$ -TC-3. (a) Untreated, spindle-shaped to polygonal adherent cells growing in multilayers. (b and c) After treatment with 1 μM 17-AAG or 17-DMAG for 24 h, some cells show apoptotic bodies (original magnification:  $\times 200$ ). (B and D) Cell viability was analyzed after 24 and 48 h with MTT assays. HSP90 inhibition significantly decreased cell viability of BON and  $\beta$ -TC-3 in a dose- and time-dependent manner ( $P < 0.010$ ).  $**P < 0.01$ .

### HSP90 inhibition induces apoptosis and cell cycle arrest in PET cell lines

In both BON and  $\beta$ -TC-3, a significant decrease in the percentage of cells in the S-phase was observed after inhibition with 17-AAG or 17-DMAG on the basis of G1 and G2/M arrest ( $P < 0.003$ , Fig. 5). Moreover, apoptosis significantly increased after inhibition of HSP90, for both inhibitors in both the cell lines (Fig. 6). In BON, inhibition of HSP90 leads to a significantly increased percentage of Nicoletti-positive cells after 48 and 72 h ( $P < 0.010$ ) and a significantly increased percentage of TUNEL-positive cells after 48 h ( $P < 0.016$ ) for both 17-AAG and 17-DMAG. In  $\beta$ -TC-3, a significant increase in the percentage of Nicoletti-positive cells was observed after inhibition with 17-AAG or 17-DMAG after 24, 48, and 72 h ( $P < 0.022$ ) whereas inhibition with 17-AAG or 17-DMAG resulted in a significant increase in TUNEL-positive cells after 48 h ( $P < 0.035$ ).

Confirming these results, in both the cell lines, nuclear PARP cleavage was increased after the administration of 17-AAG and 17-DMAG in a time-dependent manner (Fig. 7).

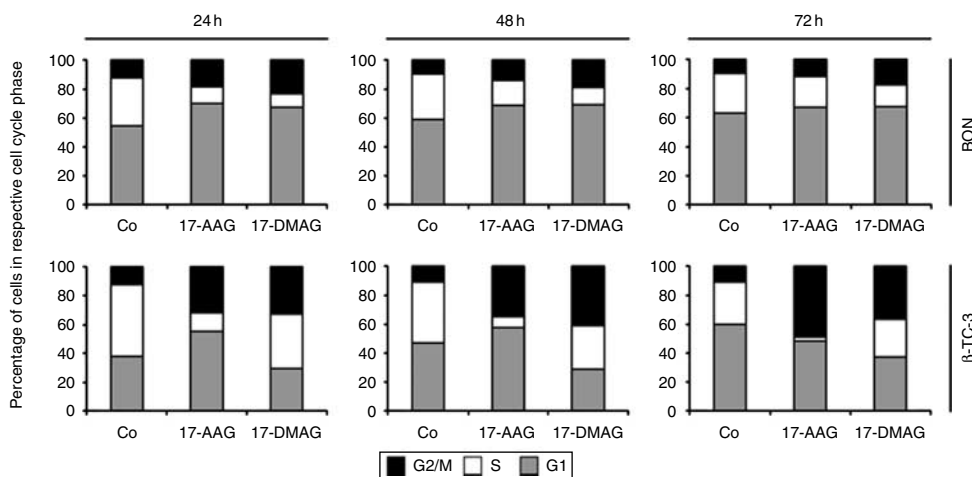
### HSP90 inhibition induces the depletion and inactivation of several oncogenic client proteins and increases the expression of HSP70 and HSP90 itself

In both the cell lines, HSP90 inhibition reduced the expression of several HSP90 client proteins in a time- and dose-dependent manner (Fig. 8). This could be

demonstrated for v-raf1 murine leukemia viral oncogene homolog 1 (RAF1), CDK4, and protein kinase B (AKT). In BON, HSP90 inhibition also reduced the expression of Met receptor (MET) in a time- and dose-dependent manner and of insulin-like growth factor receptor 1 (IGF1R) in a dose-dependent manner. Furthermore, in both the cell lines, the administration of 17-AAG or 17-DMAG inhibited the activation of AKT in a time- and dose-dependent manner and of Erk1/2 in a time-dependent manner, except for Erk1/2 in BON 48 h after treatment with 5  $\mu$ M 17-DMAG. However, HSP90 inhibition did not alter the expression of ERK1/2, which is not a client of HSP90. HSP90 inhibition also increased the expression of HSP70 and HSP90 itself.

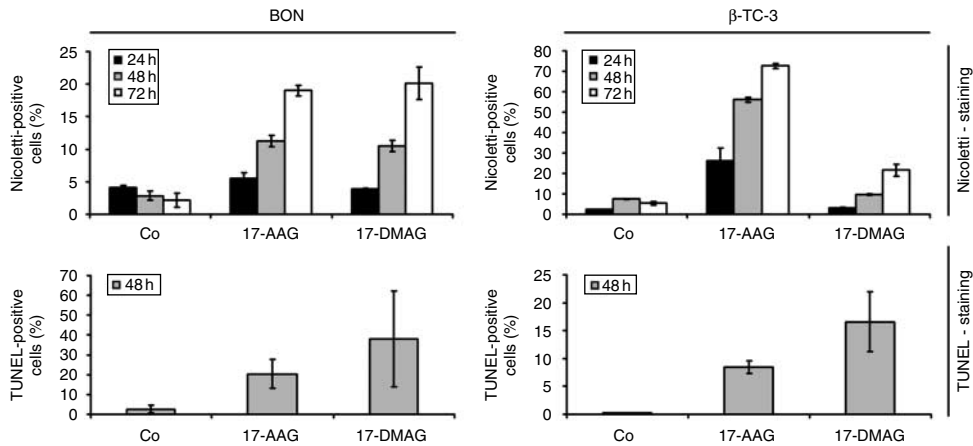
### HSP90 inhibition maximizes the effects of the established chemotherapeutic agents doxorubicin and 5-FU *in vitro*

As shown by MTT assays (Fig. 9), the combined application of one HSP90 inhibitor, 17-AAG or 17-DMAG, together with an established chemotherapeutic agent, doxorubicin or 5-FU, resulted in a further decreased cell viability of BON and  $\beta$ -TC-3. In both the cell lines, reduction of cell viability after combined applications of doxorubicin with either 17-AAG or 17-DMAG was significantly stronger than the application of the latter agents alone ( $P < 0.050$ ), except for the combination of 0.01  $\mu$ M doxorubicin plus 0.05  $\mu$ M 17-DMAG in BON after 24 h compared with 0.05  $\mu$ M 17-DMAG alone. The following combinations of 5-FU plus either 17-AAG or 17-DMAG reduced cell



**Figure 5** HSP90 inhibition induces cell cycle arrest in PET cells. BON cells were treated with 0.05  $\mu$ M HSP90 inhibitors and  $\beta$ -TC-3 cells with 1  $\mu$ M HSP90 inhibitors and the cell cycle distribution was analyzed after 24, 48, and 72 h with FACS measurement. HSP90 inhibition manifested a significant decrease in the percentage of BON and  $\beta$ -TC-3 cells in the S-phase on the basis of G1 and G2/M arrest ( $P < 0.003$ ).





**Figure 6** HSP90 inhibition induces apoptosis in PET cells. BON cells were treated with 0.05  $\mu$ M HSP90 inhibitors,  $\beta$ -TC-3 cells with 1  $\mu$ M HSP90 inhibitors, and the percentage of Nicoletti-positive and TUNEL-positive cells was analyzed after 24, 48, and 72 h with FACS measurement. In both BON and  $\beta$ -TC3, HSP90 inhibition significantly increased the percentage of Nicoletti-positive cells after 48 and 72 h ( $P < 0.010$ ) and TUNEL-positive cells after 48 h ( $P < 0.035$ ).

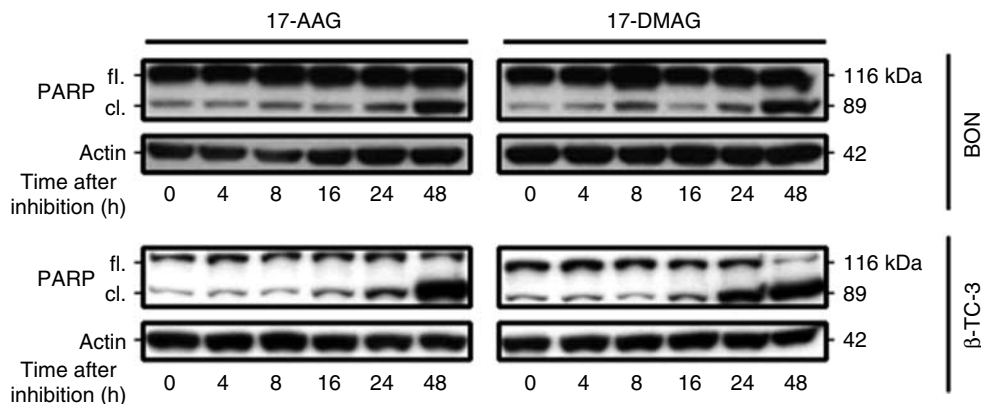
viability significantly stronger than the latter agents alone ( $P < 0.010$ ): in BON, 5-FU plus 17-AAG after 24 h, and in  $\beta$ -TC-3, 5-FU plus 17-DMAG after 24 and 48 h in all concentrations tested.

## Discussion

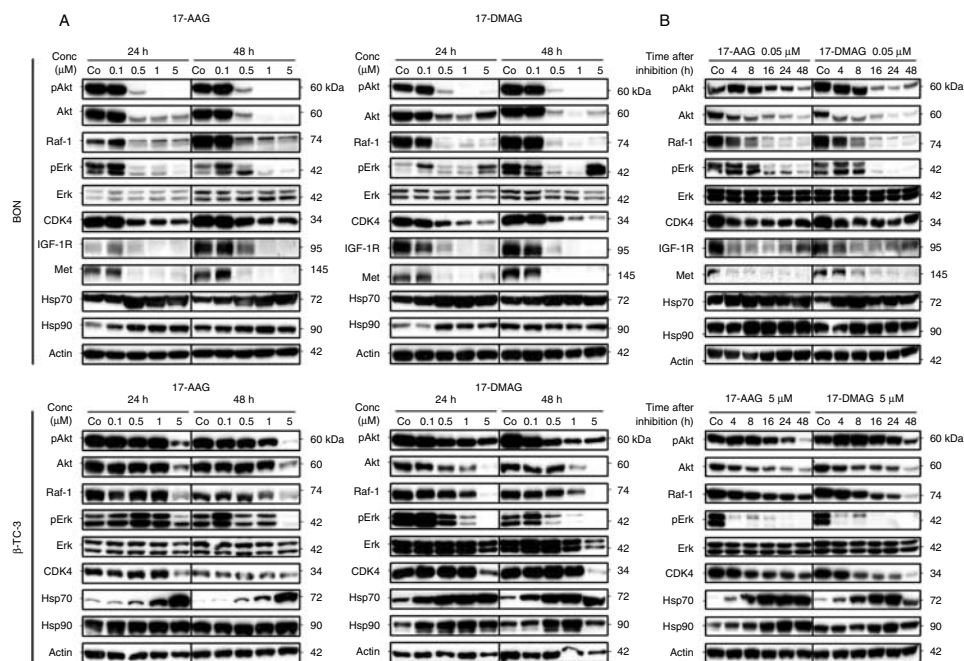
In this study, we show that HSP90 is expressed in the vast majority of PET, by means of immunohistochemistry and western immunoblot analyses. This was irrespective of clinical and pathological features including the patients' survival, malignant potential, functional activity, tumor size, mitotic index, Ki-67 positivity, or the immunohistochemical expression of insulin, glucagon, gastrin, and serotonin. Furthermore, our RT-PCR analyses indicate that the average transcript levels of all HSP90 isoforms investigated

are higher in PET than in non-neoplastic pancreatic tissues. Our findings on PET are in line with the previously reported overexpression of *HSP90* in different tumor entities, including colorectal cancer (Zhang *et al.* 2009), pancreatic ductal adenocarcinoma (Ogata *et al.* 2000), prostate carcinoma (Elmore *et al.* 2008), gastrointestinal stromal tumors (Kang *et al.* 2010), and other mesenchymal tumors (Kang *et al.* 2010). In colorectal cancer cells, a high expression of HSP90 $\alpha$  has been associated with invasiveness and metastasis (Milicevic *et al.* 2008). The HSP90 isoform GRP94 was reported to partly account for apoptosis resistance in pancreatic cancer cells (Pan *et al.* 2009).

The high expression of *HSP90* in PET raises the question regarding the biological role of HSP90 in these tumors. Our findings show that in PET, HSP90 safeguards various tumorigenic proteins *in vitro*,



**Figure 7** HSP90 inhibition induces PARP cleavage in PET cells. BON cells were treated with 0.05  $\mu$ M HSP90 inhibitors,  $\beta$ -TC-3 cells with 1  $\mu$ M HSP90 inhibitors, and PARP cleavage was determined semiquantitatively by western immunoblot analysis. In both cell lines, HSP90 inhibition increased PARP cleavage in a time-dependent manner. cl., cleaved; fl., full length.

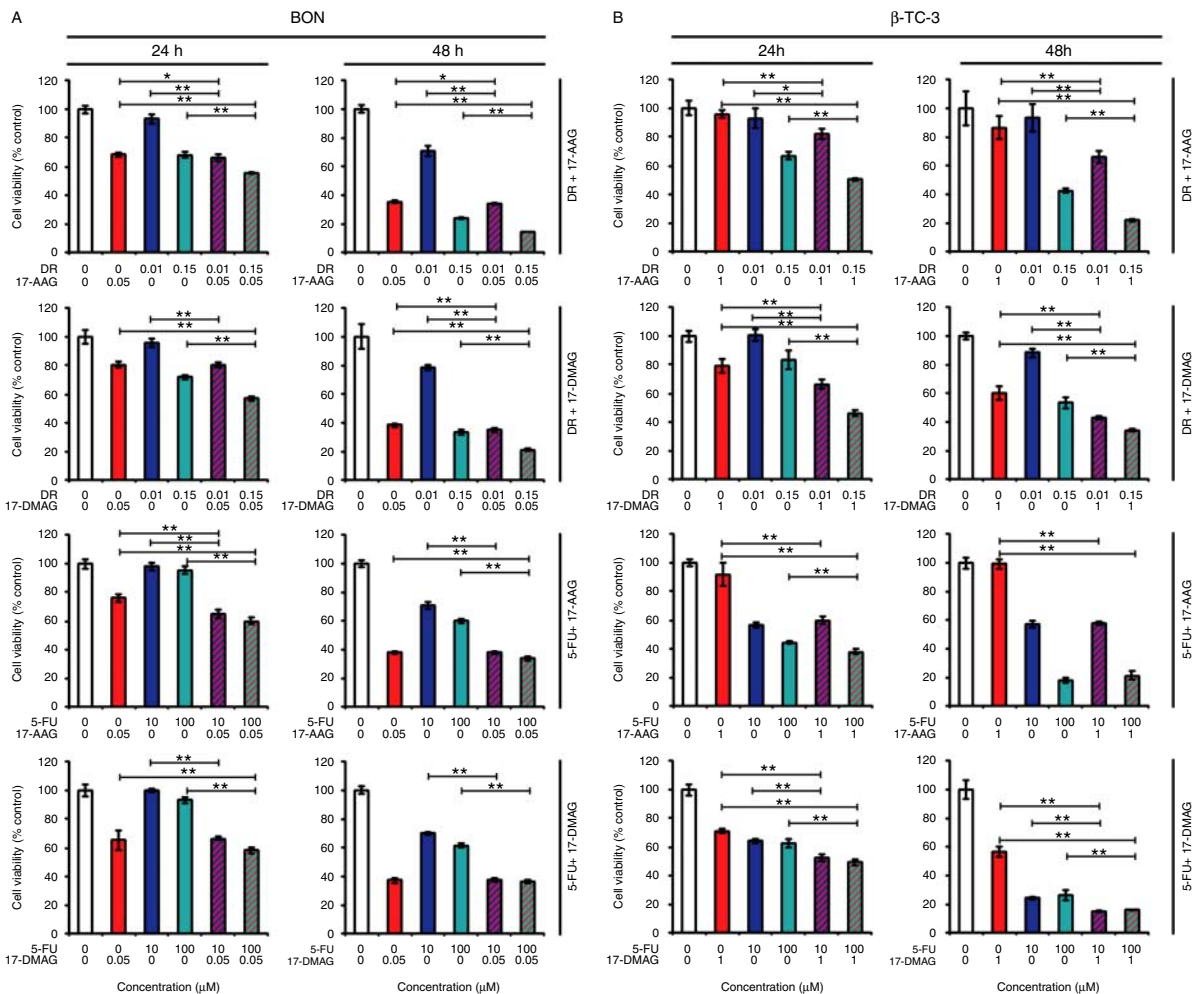


**Figure 8** HSP90 inhibition induces the degradation and inactivation of several oncogenic client proteins in a time- and dose-dependent manner. The concentration of the proteins was detected semiquantitatively by western immunoblot analyses. (A) BON and  $\beta$ -TC-3 cells were treated with increasing concentrations of HSP90 inhibitors, and the expression of the indicated proteins was investigated after 24 and 48 h with western immunoblotting. HSP90 inhibition induced a concentration-dependent depletion and/or inactivation of the displayed oncogenic client proteins. (B) BON cells were treated with 0.05  $\mu$ M HSP90 inhibitors,  $\beta$ -TC-3 with 5  $\mu$ M HSP90 inhibitors, and the expression of the indicated proteins was investigated after different time points with western immunoblotting. HSP90 inhibition induced a time-dependent depletion and/or inactivation of the displayed oncogenic client proteins. Actin served as a loading control. AKT, protein kinase B; CDK4, cyclin-dependent kinase 4; Erk, extracellular signal-regulated kinase; HSP70, heat-shock protein 70; HSP90, heat-shock protein 90; IGF1R, insulin-like growth factor receptor 1; MET, hepatocyte growth factor (HGF) receptor; pAKT, phosphorylated protein kinase B; pErk, phosphorylated extracellular signal-regulated kinase; RAF1, v-raf1 murine leukemia viral oncogene homolog 1.

namely RAF1, AKT, ERK1/2, CDK4, IGF1R, and MET. In the human PET cell line BON and the murine PET cell line  $\beta$ -TC-3, the HSP90 inhibitors 17-AAG and 17-DMAG lead to a degradation and/or inactivation of these proteins. The latter proteins are part of or associated with important cell signaling pathways, in particular the PI3K/AKT pathway that contributes to cell growth and survival (Hennessy *et al.* 2005) and the Ras–Raf–MEK–Erk pathway that controls cell proliferation (Mebratu & Tesfaygi 2009), and are shown to also affect PET (Guo *et al.* 2003, Sippel *et al.* 2003, Tannapfel *et al.* 2005, Missiaglia *et al.* 2010). Both pathways can be activated by IGF1R, which is thought to be part of an autocrine feedback loop-stimulating proliferation of PET cells (von Wichert *et al.* 2000), and MET, which seems to support metastasization in PET (Hansel *et al.* 2004). AKT, as part of the PI3K/AKT pathway, is activated in ~70% of PET (Guo *et al.* 2003), and its activation has been associated with a resistance to apoptosis and increased cell proliferation in part by increasing the activity of CDK4 (Hay 2005, Fatrai *et al.* 2006). ERK1/2, which

is part of the Ras–Raf–MEK–Erk pathway, was reported to be activated in PET to a much greater extent than in non-neoplastic  $\beta$  cells (Tannapfel *et al.* 2005). We show that upon HSP90 inhibition in PET cells, these key proteins are degraded or inactivated. Similar findings have been obtained in other tumor cells, e.g. derived from hepatocellular carcinoma (Breinig *et al.* 2009), breast cancer (Caldas-Lopes *et al.* 2009), monocytic leukemia (Peng *et al.* 2010), and esophageal cancer (Wu *et al.* 2009). Thus, HSP90 is likely to protect the function of these oncogenic factors in PET and thereby acts in a protumorigenic manner.

The aim of our study was to investigate the putative role of HSP90 as an innovative target for the therapy of PET. For inoperable and progressive malignant PET, treatment options are limited. Streptozotocin plus 5-FU or doxorubicin as first-line regime for systemic chemotherapy leads to a significant tumor response in only 20–35% of cases (Plockinger *et al.* 2004). Further medical treatment options include somatostatin analogs and interferon- $\alpha$  that were shown to result in the



**Figure 9** HSP90 inhibitors intensify the effect of established chemotherapeutic agents. (A) BON and (B)  $\beta$ -TC-3 cells were treated with HSP90 inhibitors, doxorubicin (DR), and/or 5-FU in the indicated concentrations, and cell viability was analyzed after 24 and 48 h with MTT assays. HSP90 inhibition intensified the effect of doxorubicin and 5-FU. \* $P < 0.05$ ; \*\* $P < 0.01$ .

reduction of tumor size in only 10–15% of cases (Plockinger *et al.* 2004).

Underlining the applicability of HSP90 as a potential new therapeutic target in PET, we show that the HSP90 inhibitors 17-AAG and 17-DMAG reduce cell viability in a concentration- and time-dependent manner both in BON and  $\beta$ -TC-3, inducing a cell cycle arrest, mostly on the basis of a G1 and G2/M arrest. Moreover, inhibition of HSP90 markedly increases apoptosis in both the cell lines. Comparable *in vitro* results have been obtained in various other neoplasms, including urinary bladder cancer (Karkoulis *et al.* 2010), leukemia (Yu *et al.* 2005), endometrial cancer (Gossett *et al.* 2005), ovarian cancer (Gossett *et al.* 2005), and breast cancer (Gossett *et al.* 2005).

The fact that BON cells are more susceptible to inhibition of HSP90 than  $\beta$ -TC-3 cells could be due to

a stronger dependence of BON cells on cell signaling pathways affected by inhibition of HSP90. Growth of BON cells was reported to be dependent on an autocrine feedback loop involving IGF1R leading to a constitutively activated PI3K/AKT signaling in BON cells (von Wichert *et al.* 2000). Underlining the importance of PI3K/AKT signaling in BON cells, inhibition of mammalian target of rapamycin (mTOR), which is a major downstream effector of AKT, potently induces growth inhibition in BON cells (Zitzmann *et al.* 2007, Missiaglia *et al.* 2010). In contrast, for  $\beta$ -TC-3, a specific dependence on signaling pathways affected by HSP90 inhibition has not been reported. Furthermore, 17-DMAG had a stronger effect on cell viability than 17-AAG in both BON and  $\beta$ -TC-3. In line with this, 17-DMAG was reported as a slightly more potent inhibitor of human HSP90 than 17-AAG

with half maximal inhibitory concentrations (IC50) for 17-AAG and 17-DMAG being  $119 \pm 23$  and  $62 \pm 29$  nM respectively (Ge et al. 2006).

The relevance of HSP90 as a therapeutic target structure in PET could be superior to single target proteins of the PI3K/AKT and the Ras–Raf–MEK–Erk pathways because HSP90 interferes with both the pathways. In this way, HSP90 inhibitors suppress the activation of feedback loops that are responsible for the compensatory activation of two pathways after the inhibition of a single component of one pathway (Carracedo et al. 2008, Grant 2008). Furthermore, in molecular aspects, PET comprise a group of very heterogenous neoplasms with a variety of oncogenetic proteins contributing to their tumorigenesis (Zikusoka et al. 2005) so that interference with multiple oncogenetic proteins by HSP90 inhibitors could show favorable therapeutic effects. It was stated that this multitargeted approach with HSP90 inhibitors could also prevent the development of chemoresistance (Pearl et al. 2008). Activated oncoproteins seem to depend more on the function of HSP90 than on their nontransformed counterparts (Karapanagiotou et al. 2009). In cancer cells, HSP90 exists in an activated superchaperone complex, which is hypersensitive to HSP90 inhibition, while under normal conditions HSP90 is mainly uncomplexed (Kamal et al. 2003).

We examined the effects of a combined therapy with HSP90 inhibitors and established chemotherapeutic agents, doxorubicin or 5-FU (Plockinger et al. 2004). In other tumor entities, HSP90 inhibitors enhanced the effect of doxorubicin or 5-FU *in vitro* (Burkitt et al. 2007, Abramson et al. 2009, Kaiser et al. 2010). For PET, we could demonstrate similar effects. 17-AAG and 17-DMAG significantly enhanced the effect of doxorubicin and 5-FU on cell viability *in vitro*. Overexpression of the HSP90 isoforms HSP90 $\beta$  and TRAP1 has been reported to be associated with resistance to doxorubicin or 5-FU in colorectal cancer cells (Bertram et al. 1996, Costantino et al. 2009). The transcript levels of the latter were elevated in our freshly frozen PET samples. Thus, HSP90 inhibitors could improve the so far poor response characteristics of PET to systemic chemotherapy (Eriksson et al. 2009).

HSP90 inhibitors have been applied successfully in clinical studies. 17-DMAG, currently being in phase I clinical studies, showed antileukemia activity in three out of 17 patients with acute myeloid leukemia (Lancet et al. 2010). In another clinical study on patients with advanced malignancies including adenocarcinomas of the pancreas, the administration of 17-DMAG as single agent resulted in stable disease in nine out of 31

patients for a median of 4 months (Kummar et al. 2010). 17-AAG resulted in prolonged stable disease in two patients with melanoma (Workman 2004). In Her2-positive breast tumors, the combination of 17-AAG and trastuzumab induced regressions in 23% of the cases, including patients that had primarily not been responsive to trastuzumab therapy (Modi et al. 2007). Moreover, multiple myeloma patients responded to the combination of 17-AAG and bortezomib, even if they had been refractory to bortezomib as a single agent (Biamonte et al. 2010). However, in a phase II trial, the administration of 17-AAG in patients with metastatic melanoma reported no objective clinical responses, probably as a result of very low 17-AAG concentrations in the tumors (Solit et al. 2008). Another study with 17-AAG in patients with metastatic melanoma was stopped because endpoints were not reached, although some evidence of 17-AAG activity had been observed (Pacey et al. 2010).

Currently ongoing clinical trials of HSP90 inhibitors include, among others, three clinical trials of IPI-504, a water-soluble derivative of 17-AAG (Hanson & Vesole 2009), in patients with advanced non-small cell lung cancer (NSCLC; <http://clinicaltrials.gov>). One trial examines the efficacy of IPI-504 in NSCLC patients with anaplastic lymphoma kinase (ALK) translocations after it has been shown *in vitro* that IPI-504 potently induces growth arrest and apoptosis in cells carrying ALK translocations (Normant et al. 2011). In another study, IPI-504 is administered together with the mTOR inhibitor everolimus in patients with KRAS mutant NSCLC (<http://clinicaltrials.gov>). Nevertheless, more recently, it has been suggested that mTOR inhibitors will be ineffective against cancer harboring KRAS mutations despite having PI3K/AKT/mTOR pathway activation (Mohseni & Park 2010). Moreover, a phase II double-blind study compares the impact of IPI-504 in combination with docetaxel to placebo in combination with docetaxel on life expectancy in NSCLC patients. Before this, the combination of IPI-504 and docetaxel had demonstrated additive efficacy in murine xenograft models and a phase Ib trial was undertaken to identify the maximum tolerated dose of IPI-504 in combination with docetaxel, reporting no irreversible toxicities in 16 patients with advanced solid tumors, among these six with NSCLC (Riely et al. 2009). According to these findings and the promising *in vitro* results of our study, the effects of a HSP90 inhibition in PET should be further evaluated in animal models before clinical tests may be designed for PET.

In summary, in this study, we show that HSP90 is expressed in the vast majority of PET and safeguards

many tumorigenic proteins. Inhibition of HSP90 results in a significant reduction of cell viability, cell cycle arrest, and increased apoptosis. The HSP90 inhibitors 17-AAG and 17-DMAG significantly increased the therapeutic effects of the doxorubicin and 5-FU in PET *in vitro*. Therefore, our findings suggest that HSP90 inhibition may be a valuable additive therapeutic strategy for advanced and progressed PET.

### Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-11-0227>.

### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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