Hsp90 stabilizes Cdc25A and counteracts heat shock-mediated Cdc25A degradation and cell-cycle attenuation in pancreatic carcinoma cells

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Received May 11, 2012; Revised and Accepted July 19, 2012

Pancreatic cancer cells escape most treatment options. Heat shock protein (Hsp)90 is frequently over-expressed in pancreatic carcinomas and protects a number of cell-cycle regulators such as the proto-oncogene Cdc25A. We show that inhibition of Hsp90 with geldanamycin (GD) destabilizes Cdc25A independent of Chk1/2, whereas the standard drug for pancreas carcinoma treatment, gemcitabine (GEM), causes Cdc25A degradation through the activation of Chk2. Both agents applied together additively inhibit the expression of Cdc25A and the proliferation of pancreas carcinoma cells thereby demonstrating that both Cdc25A-destabilizing/degrading pathways are separated. The role of Hsp90 as stabilizer of Cdc25A in pancreatic carcinoma cells is further supported by two novel synthetic inhibitors 4-tosylcyclonovobiocic acid and 7-tosylcyclonovobiocic acid and specific Hsp90AB1 (Hsp90) shRNA. Our data show that targeting Hsp90 reduced the resistance of pancreas carcinoma cells to treatment with GEM.

INTRODUCTION

Pancreatic cancer is the tenth most common type of cancer in Western countries and ranks fourth in cancer mortality statistics and in spite of intensive research and significant improvement in the survival of pancreatic cancer patients (1–3). This cancer entity is still among the most malignant ones. Because of the lack of an effective screening test hampering early detection, the absence of symptoms, limited effective therapies and ultimately a high rate of relapse prognosis is very poor with a 5-year survival rate lower than 5% and a 1-year survival rate lower than 20% (4). Owing to metastasis, over 80% of these carcinomas are not resectable (5) and therefore systemic chemotherapy plays an important role in the treatment of this extremely aggressive cancer with the goal to provide symptomatic relief and prolong survival. Besides 5-fluorouracil, gemcitabine (GEM) was identified as the second main treatment option (6) but in particular metastatic pancreatic cancer is highly chemoresistant and response rates of single-agent therapies are <20% (4). Because of this lack of effective therapy, research for new capable treatment options represents an important challenge. Heat shock proteins (Hsps) represent a highly conserved set of proteins that have a pivotal role in cell-cycle progression and cell death (apoptosis) as well as in maintaining cellular homeostasis under stress (7). Various insults such as hypoxia, ischemia, exposure to UV light or

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chemicals, nutritional deficiencies or other stress rapidly induce their expression (8,9) and Hsp90A (further on termed Hsp90) over-expression was shown among others, e.g. for pancreatic, breast and lung cancer and for leukaemia (7). In a recent study, we could show that heat shock (HS) induces Cdc25A degradation and that Hsp90 stabilizes Cdc25A in HeLa and HEK293 cells (10). The cell-cycle promoting phosphatase Cdc25A is a proto-oncogene and indispensable for embryonic development (11) and can substitute for Cdc25B and Cdc25C. Therefore, Cdc25A is mandatory for cell-cycle progression and the fact that HS, in the presence of the Hsp90 inhibitor geldanamycine (GD, which is currently investigated in clinical trials), destabilizes Cdc25A in HEK293 and HeLa cells tempted us to test whether this is also the case in pancreas carcinoma cells. As there is still no cure for this cancer entity and GEM therapy has more of a palliative than life-extending effect, we investigated whether Hsp90 inhibition in combination with high-fever-range HS might affect pancreas carcinoma cells and contributes to cell-cycle arrest.

**RESULTS**

**HS and GD cause destabilization of Cdc25A and other cell-cycle regulators**

BxPC-3 cells were treated with HS (41.5°C, 90 min) or with 250 nm GD (Fig. 1), or both, and the protein expression of Cdc25A, B and C was investigated. Whereas HS had no effect and GD only little effect on the expression of Cdc25 proteins, the combination of HS plus GD dramatically suppressed the expression of Cdc25A, B and C in BxPC-3 cells (Fig. 2A).

The expression of Cdc25A reversed to the control level when cells were cultivated for further 6 h in the absence of GD (post-treatment) before cells were lysed for western blot analyses. In contrast, Cdc25B levels of BxPC-3 cells were still reduced post-treatment with HS plus GD. The protein level of cyclin D1, another cell-cycle regulating proto-oncogene, was also down-regulated upon combinatorial treatment and also within the post-treatment period.

Cdc25A is a proto-oncogene that was shown to be an Hsp90 client in HEK293 and HeLa cells (10). Since it is not known to which part of Hsp90 Cdc25A attaches we performed binding studies using immobilized His-tagged full-length and truncated constructs of Hsp90α and Hsp90β, which were exposed to BxPC-3 cell extracts (Fig. 2B).

Western blot analysis revealed that Cdc25A bound with stronger affinity to the C-terminus of Hsp90α than to the N-terminus and the binding affinity to the full-length protein was weaker than to the C-terminal construct. Interestingly, Cdc25A bound with a similar affinity also to the Hsp90β full-length protein as well as to the Hsp90β N-terminus.

To test whether this was a cell line effect, the expression of Cdc25s and cyclin D1 was analysed also in two other pancreas carcinoma cell lines, PANC-1 and ASPC-1 (Fig. 3A and B).

In PANC-1 HS alone had an already strong suppressing effect on Cdc25A and GD further reduced its expression below detection limit. The removal of GD for a 6 h post-treatment period reversed the levels of Cdc25A and Cdc25B back to that of control. Post-treatment of PANC-1 and ASPC-1 with HS plus GD still suppressed Cdc25C. This indicated that Cdc25A and Cdc25C were regulated by a mechanism that was common to all three pancreas carcinoma cell lines. Immediately after treatment, also Cdc25B expression responded similarly in the three cell lines. In the post-treatment period, Cdc25B levels even increased in BxPC-3 cells that experienced combinatorial treatment. Cyclin D1 decreased upon HS in PANC-1 and ASPC-1 cells and recovered in ASPC-1 to control levels upon post-treatment incubation, whereas in PANC-1 cells cyclin D1 even increased during HS post-treatment.

Such as Cdc25C (12), Wee1 is a client of Hsp90 in HCT116 colon cancer cells (13). Therefore, we tested whether HS and GD treatment would affect Wee1 stability also in pancreas carcinoma cell lines. Indeed, Wee1 levels decreased in BxPC-3 cells upon treatment with GD, and HS and GD and this caused also the reduction in phosphorylated (active) Wee1 kinase and consequently the reduction in the phosphorylation level of its target Cdc2 (Fig. 4A). Furthermore, in PANC-1 cells Wee1 became down-regulated after treatment with HS, and GD and HS which resulted in decreased phosphorylation levels of Cdc2 (Fig. 4B; we abstained to investigate the phosphorylation level of Wee1 in PANC-1, because the changes in Wee1 phosphorylation in BxPC-3 cells resulted from altered Wee1 protein expression levels rather than post-translational modification). This implicated that Cdc2 became activated and induced the cell-cycle and proliferation. In contrast, the down-regulation of cyclinD1, which reflects the status of cycling and therefore proliferating cells, indicated an attenuation of cell proliferation. The dis-regulated expression of cell-cycle protagonists may induce cell-cycle inhibitors to arrest cell-cycle progression in order to re-orchestrate the cell-cycle. Hence, we investigated the expression/activation of p53 and p21 in Bx-PC-3 cells after treatment with HS and GD, but neither p53 became activated nor p21 induced (Fig. 5A). P53 is a client of Hsp90 (14) and, therefore, p53 became degraded upon treatment with GD and its expression was completely suppressed by the combinatorial treatment with HS. HS causes Chk2 activation and induces Cdc25A degradation in HEK 293 and HeLa cells (10) and also exposure to UV causes Chk activation and Cdc25A degradation (15). In BxPC-3 cells, the check point kinases Chk1 and Chk2 remained inactive upon HS, GD or HS and GD (Fig. 5B).

Ser75 and Ser177 of Cdc25A are specifically phosphorylated by Chk1 and Chk2, respectively, tagging it for proteasome-mediated degradation. Since neither of the checkpoint kinases became activated, also the constitutive phosphorylation of Cdc25A did not increase at the specific amino acid residues.

To test whether the effects of HS and GD on cell-cycle proteins were specific for pancreas carcinoma cells the experiments were expanded to breast cancer cell lines. In the highly metastatic ERnegative breast cancer cell line, MDA-MB-231 Cdc25 family proteins and Wee1 were degraded and consequently Cdc2 was de-phosphorylated upon HS, whereas GD single treatment reduced the expression of Cdc25B, Cdc25C and Wee1 only weakly and Cdc25A and Cdc2 remained unchanged (Table 1; Fig. 6A). In the ERpositive MCF-7 breast cancer cell line the levels of Cdc25A and Wee1 were weakly reduced upon single
treatments and strongly reduced (also Cdc2 phosphorylation) upon combinatorial (HS and GD) treatment. Derivatives of MCF-7 that were made resistant to tamoxifen and fulvestrant maintained their sensitivity to HS and GD and the expression of Cdc25A and Wee1 and the phosphorylation of Cdc2 were down-regulated (Table 2; Fig. 6B). Hence, HS-induced degradation of Cdc25s, which was enforced by GD, was a general phenomenon and not limited to pancreas carcinoma cells, and Hsp90 protected the proto-oncogene Cdc25A from constitutive and high-fever-range-induced degradation. This implicates that HS and GD treatment caused Cdc25A destabilization and attenuated cell-cycle progression independent of DNA checkpoint activators.

Novel Hsp90 inhibitors and specific knock-down destabilize Cdc25A

To obtain additional proof that Cdc25A is a client of Hsp90 in pancreas carcinoma cells, BxPC-3 cells were treated with two novel synthetic Hsp90 inhibitors, 4- and 7-toslycyclonovobiocic acid (4-TCNA and 7-TCNA) (Fig. 1). Although GD binds the N-terminal ATP-binding pocket of Hsp90 and impairs its chaperone function, the coumarin antibiopicnovobiocin was demonstrated to bind an ATP-binding domain in the C-terminal region of Hsp90 (16) and the removal of the noviose moiety together with the introduction of a tosyl substituent at C-4 or C-7 position of the coumarin nucleus provided 4-TCNA and 7-TCNA as lead compounds (17). These two analogues were shown to down-regulate a subset of Hsp90 client proteins in breast, colon, ovarian and endometrial cancer cell lines with IC50 doses ~50 μM (18,19). The poor solubility under physiological conditions might be the reason for these high concentrations. However, 7-TCNA destabilized Cdc25C, which is a bona fide client of Hsp90 (12). In combination with HS both, 4-TCNA and 7-TCNA, caused the down-regulation of Cdc25C thereby demonstrating the specificity of these inhibitors (Fig. 7A). Even more pronounced was the effect of 4-TCNA and

Figure 1. Structure of geldanamycin (GD), 4-TCNA and 7-TCNA.
7-TCNA on the expression of Cdc25A further indicating that Cdc25A is a client of Hsp90 in BxPC-3 cells.

Wee1 phosphorylates Tyr38 of Hsp90 thereby preventing GD from association with the N-terminus of Hsp90 (20). Therefore, when Wee1 is active, cells are relatively insensitive to GD. Accordingly, when Wee1 becomes inhibited by Wee1 inhibitor II, this augmented sensitivity to GD (Fig. 7B). Since 4-TCNA does not bind to the N-terminus of Hsp90, inhibition of Wee1 did not augment the proliferation-inhibitory effect of 4-TCNA evidencing that the inhibitory mechanism of 4-TCNA is different from that of GD.

To demonstrate that Cdc25A binds to Hsp90 also in intact pancreas carcinoma cells, Hsp90 was detected by western blotting after immunoprecipitation of endogenous Cdc25A from the cell lysate (Fig. 7C). Treatment of BxPC-3 cells with 4-TCNA, but not with GD for 2 h reduced the amount of Cdc25A-bound Hsp90. Therefore, 4-TCNA interfered with the association of Cdc25A to Hsp90. This was consistent with the expression of Cdc25A further indicating that Cdc25A is a client of Hsp90 in BxPC-3 cells. 

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with the observation that Cdc25A bound also to the C-terminus of Hsp90a (and not only to the N-terminus of Hsp90b) and thus, in the same region as 4-TCNA. To provide firm evidence that Cdc25A is a client of Hsp90 the mRNA expression of the constitutively expressed gene (Hsp90AB1/Hsp90b) was knocked-down by specific shRNA (Fig. 8A) and also Hsp90 protein expression was reduced to a different extent in the 10 analysed clones (Fig. 8B). As anticipated, in the knock-down clone (no. 2 from Figs 8A and B) the expression of the client proteins Cdc25A, Cdc25C, Wee1 and p53 was down-regulated (Fig. 8C). However, HS did not further reduce the expression levels of Wee1, Cdc25C and Cdc25A in the knock-down clone and this could have been due to the activation of the residual level of Hsp90.

BrdU incorporation studies together with FACS analyses confirmed that directly after HS BxPC-3 cells were arrested in the G1 phase, whereas Hsp90 knock-down cells were arrested in G2 and the incorporation of BrdU in these cells was inhibited during the S phase (Fig. 8D). Therefore, Hsp90 significantly contributes to BxPC-3 pancreas carcinoma cell-cycle progression.

GD and gemcitabine additively inhibit Cdc25A and cell proliferation

A current chemotherapy against pancreas carcinoma is with GEM. Although pancreas carcinoma cells were reported to be a devoid of functional DNA checkpoints (21), GEM induced the phosphorylation of Chk2, but not Chk1 (Fig. 9A). This was accompanied by inhibition of Cdc25A expression. Therefore, we studied whether GD and HS in combination with GEM could support the anti-neoplastic effect in pancreas carcinoma cells. The short incubation times of the previous experiments (1 h preincubation with GD followed by 1.5 h HS) just served to study the basic cellular mechanisms of HS and in this part of the work, we also investigated the effects of GEM and HS after longer incubation times. For this BxPC-3 cells were incubated with GEM and GD for 8 h, whereas HS still lasted only for 90 min because in clinical applications this is the usually applied and tolerated time, which does not threat the patients (22–24). As shown before, HS or GD alone had no effect on Cdc25A expression after this incubation period but the combination of HS and GEM, or GD and GEM or HS and GD and GEM reduced the expression of Cdc25A below the levels of GEM treatment alone (Fig. 9B). On Cdc25C, GEM had only an effect in combination with HS. In long-term experiments (72 h), cell numbers were measured but for this, the concentrations of GEM and GD were reduced to inhibit BxPC-3 cell proliferation not more than 50%. In detail, 5 nM GEM reduced the cell number by ≏40% and 10 nM GD by nearly 20% (Fig. 9C). The effect of the combination of 5 nM GEM and 10 nM GD was roughly additive reducing the cell number by more than 55%. A single HS (90 min) at the beginning of the experiment had no additional effect in long-term experiments. In knock-down cells, GEM alone reduced the cell number by 53% which was similar to the cell number reduction achieved by GEM and GD in wild-type cells. Interestingly, in knock-down cells, HS and GEM further reduced the cell number by 70%. Thus, the exposure to HS and the targeting of Hsp90 strongly supported GEM standard treatment of pancreas carcinoma cells.

DISCUSSION

Pancreas cancer cells are highly resistant to various in vitro treatments and also clinical therapy regimens are largely ineffective (25). GEM is the main standard agent and the major beneficial effect seems to be a palliative one (26). Pancreas cancer cells tend to acquire resistance to GEM and this was
reported to be caused by the activation of the NF-kB pathway (27,28). Undoubtedly, the resistance to drug treatment involves mutations of p16INK4a or alternatively, mutations of p53 (29,30). Furthermore, disease progression correlates with an inactivation of the Chk2 DNA damage check point (21). Nevertheless, we demonstrate that isolated BxPC-3 pancreas carcinoma cells respond to GEM treatment with the phosphorylation of Chk2, Cdc25A degradation and concomitant cell-cycle attenuation. Recently, it was shown that HS-induced Chk2 in HEK293 cells (10) and hence, the observation that HS did not induce Chk2 phosphorylation in BxPC-3 pancreas carcinoma cells was unexpected. Thus, the activation of Chk2 by GEM or by HS was through distinct pathways, whereby the HS-induced pathway remained silent in BxPC-3 cells. Despite this fact, HS caused cell-cycle arrest in a background of inhibited Hsp90 (by GD) or reduced Hsp90 expression (by shRNA). However, the attenuation of cell-cycle progression was transient and only occurred immediately after HS-treatment and this was most likely due to disabled DNA checkpoint activation and the lack of p21 induction. The Hsp90 client p53 was also degraded, which, however, does not play a role in this scenario, because p53 is anyway mutated in BxPC-3 cells (31). Despite the transient nature of cell-cycle inhibition, we show that the cell-cycle was arrested independent of functional DNA check point kinases Chk1 and Chk2. Otherwise we would have detected the specific and destabilizing Cdc25A phosphorylations at Ser75 and Ser177. Instead, targeting Hsp90 was sufficient to attenuate cell-cycle progression and cell proliferation, which correlated with the destabilization of the Cdc25 family of phosphatases and other cell-cycle regulators such as Wee1, cyclin D1 and to some extent also Cdc2. Interestingly, Wee1 prevents inhibition of Hsp90 (20) by GD and therefore, the simultaneous inhibition of Wee1 by Wee1 inhibitor II and Hsp90 by GD synergized in attenuating cell-cycle progression, which can be conceptual for the development of a new therapeutic strategy.

We already provided evidence that HS caused the degradation of Cdc25A, B and C in HEK293 and HeLa cells through the activity of Chk2 and inhibition of Hsp90 just accelerated the destabilization of Cdc25A in these cell lines (10). Degradation of Cdc25A was due to phosphorylations at Ser75 and Ser177 (through p38 and Chk2) and a subsequent sequestration of Cdc25A by 14.3.3 proteins to the cytoplasm where Cdc25A became ubiquitinylated and subjected to proteasomal destruction (10,32–34). Here, we show for the first time that the degradation of Cdc25A depended on inhibition (reduction) of Hsp90 without the necessity to activate Chk2.

The combination of HS together with the Hsp90 inhibitor GD resulted in an impressive suppression of Cdc25A, B and C particularly in BxPC-3 and PANC-1 cells. This effect was
not restricted to pancreas carcinoma cells but was also observed in MDA-MB-231 and MCF-7 breast cancer cells and tamoxifen- and fulvestrant-resistant derivatives. Also the novel synthetic Hsp90 inhibitors 4-TCNA and 7-TCNA strongly down-regulated Cdc25A expression, which substantiated the hypothesis that inhibition of Hsp90 can be a strategy to combat pancreas cancer cell expansion. We observed that HS did not produce an additional effect on Cdc25A, Cdc25C or Wee1 degradation in BxPC-3 cells probably due to the activation of the residual level of Hsp90, which was incompletely knocked down by specific shRNA. Hsp90-dependent cell-cycle inhibition was only short but very strong. This effect was vastly prolonged when GD (+ GEM) was applied for the entire experimental period (which better reflects a clinical setting) despite a single and short (90 min) HS exposure.

The nucleoside analogue GEM stalls the replication fork setting) despite a single and short (90 min) HS exposure. Chemoluminescence was developed by the ECL plus detection kit (GE Healthcare, Buckinghamshire, UK) and analysed using a Lumi-Imager F1 Workstation (Roche, Basel, Switzerland).

**MATERIALS AND METHODS**

**Cell culture**

BxPC-3, AsPC-1 and PANC-1 pancreatic cancer cell lines and MCF-7 and MDA-MB-231 breast cancer cell lines were purchased from ATCC. BxPC-3 and AsPC-1 cells were cultured in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS), 1% l-glutamine, 1% sodium pyruvate and 1% penicillin/streptomycin. PANC-1 cells were grown in high-glucose DMEM medium supplemented with 10% heat inactivated FCS, 1% l-glutamine and 1% penicillin/streptomycin. MCF-7 and MDA-MB-231 breast cancer cells were cultivated in DMEM/F-12 1:1 medium supplemented with 10% heat inactivated FCS, 1% l-glutamine and 1% penicillin/streptomycin. TR and FR resistance were obtained by treating MCF-7 cells with increasing concentrations (up to 500 nM) of TR and FR, respectively, and the resistant cell lines [TR resistant (TR500-MCF-7); FR resistant (FR500-MCF-7)] were grown in DMEM/F-12 1:1 medium supplemented with 10% heat inactivated FCS, 1% l-glutamine, 1% penicillin/streptomycin and 500 nM the corresponding anti-oestrogen.

All cells were grown at 37°C in a humidified atmosphere containing 5% CO₂. If not mentioned otherwise, all media and supplements were obtained from Invitrogen Life Technologies (Karlsruhe, Germany).

**Heat shock and inhibitor treatment**

Cells were grown up to 80% confluence and after pre-incubation with 250 nM GD for 1 h, cells were exposed to 41.5°C for 1.5 h in a humidified atmosphere containing 5% CO₂. After HS, treatment cells were prepared for analysis as described thereafter.

**Western blotting**

After incubation with corresponding compounds and exposure to 41.5°C, HS cells were harvested, washed twice with cold PBS and lysed in a buffer containing 150 mM NaCl, 50 mM Tris, 1% Triton-X-100, 1 mM phenylmethylsulfonylfluoride and 2.5% PIC (Cat#P8849 Sigma, Munich, Germany). After centrifugation (12 000g) for 20 min at 4°C the supernatant was stored at −20°C until further analysis. Equal amounts of protein samples were separated by polyacrylamide gel electrophoresis and electrotransferred onto PVDF-membranes (Hybond-P, Amersham), 4°C overnight. Staining membranes with Ponceau S controlled equal sample loading. After washing with Tris-buffered saline (TBS) pH 7.6, membranes were blocked in 5% non-fat dry milk in TBS containing 0.1% Tween-20 for 1 h. Membranes were incubated with the first antibody (in blocking solution, dilution 1:500–1:1000) by gently rocking at 4°C overnight, washed with TBS containing 0.1% Tween-20 and further incubated with the second antibody (peroxidase-conjugated swine anti-rabbit IgG or rabbit anti-mouse IgG, dilution 1:2000–1:5000 in blocking solution) for 1 h. Chemiluminescence was developed by the ECL plus detection kit (GE Healthcare, Buckinghamshire, UK) and analysed using a Lumi-Imager F1 Workstation (Roche, Basel, Switzerland).

**Reagents and antibodies**

TR (Cat# T5648), FR (Cat# I4409), GD (Cat# G3381), hexadimethrine bromide (Cat# H9268) and puromycin (Cat# P9620) were purchased from Sigma. The Amersham ECLPlus Western Blotting Detection System was from GE Healthcare and Weel Inhibitor II from Calbiochem (Merck, Darmstadt, Germany).

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**Table 2.** Densitometry data of western blot results of different cell-cycle regulators in breast cancer cells

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After pre-incubation with 250 nM GD for 1 h, cells were exposed to 41.5°C for 1.5 h. Cells were lysed directly after HS. To compare relative expression levels with untreated control, values were standardized to β-actin.
The synthetic HSP90 inhibitors 4-TCNA and 7-TCNA were provided by Dr Mouâd Alami, Université Paris Sud.

Antibodies

Mouse monoclonal (ascites fluid) anti-β-actin clone AC-15 Cat# A5441 was from Sigma. Anti cyclin D1 (M-20) Cat# sc-718, p21 (C-19) Cat# sc-397, Cdc25A (F-6) Cat# sc-7389, Cdc25B (C-20) Cat# sc-326, Cdc25C (C-20) Cat# sc-327 were from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA, USA). Phospho-Wee1 (Ser642) (D47G5) Cat# 4910, Wee1 Cat# 4936, phospho-Chk2 (Thr 68) Cat# 2661, Chk2 Cat# 2662, phospho-Chk1 (Ser345) Cat# 2341, Chk1 Cat# 2345, phospho-p53 (Ser20), acetylated-p53 (Lys382) Cat# AP3046 and Hsp90 Cat# 4877 were from Cell Signaling (Danvers, MA, USA). p53 antibody Cat# 1767 was purchased from Immunotech (Marseille, France), phospho-Cdc25A (Ser75) Cat# ab47279 from Abcam (Cambridge, UK) and phospho-Cdc25A (Ser177) Cat# AP3046 was from Abgent (San Diego, CA, USA). Anti-mouse and anti-rabbit IgG were from Dako (Glostrup, Denmark).

Quantitative RT–PCR

BxPC-3 cells (0.25 × 10^5) were seeded in six wells and after 24 h cultivation they were harvested and homogenized using Qia-shredder (Cat# 79 654, Qiagen, Hilden, Germany). The cells were further processed according to the instructions of the RNaseasy Mini Kit (Cat# 74 104, Qiagen). The final RNA concentration was measured using a NanoDrop Fluorospectrometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). cDNA synthesis from 1 μg RNA was performed using Superscript-first-strand synthesis systems for RT–PCR (Cat# 11904–018, Invitrogen, Carlsbad, CA, USA). Hsp90AB1 transcript levels were investigated by real-time PCR using the Taqman detection system (Applied Biosystems, Carlsbad, CA, USA). The housekeeping-gene glyceralaldehyde 3-phosphate dehydrogenase (GAPDH) served as a reference gene. Assay ID numbers of the Taqman gene expression kits were: GAPDH: HS99999905_m1; HSP90AB1 (this is the constitutively expressed form of Hsp90A, which for reasons of simplicity is shortened throughout the manuscript to Hsp90): HS01546474_g1. Cycle program (95 °C for 10 min to activate polymerase followed by 40 cycles of 95°C for 15 s and 60°C for 1 min) was started on an Abi Prism 7000 Sequence Detection System (Applied Biosystems). Real-time PCR was performed in duplicates for each cDNA template and gene investigated. Negative controls, containing water instead of cDNA, confirmed the absence of RNA/DNA in all reagents applied in the assay.

Lentiviral shRNA transduction

BxPC-3 cells (1 × 10^4) were seeded in 24-well plates and were cultivated overnight. The next day hexadimethrine
bromide (8 ng/ml) and 1 × 10^5 transducing units of the lentiviral shRNA vectors were added. Transduction particles (HSP90AB1: TRCN0000008748; clone ID NM_007355.2-232s1c1; negative control: Cat# SHC002H) were obtained from Sigma. After 24 h of incubation, fresh media and further 24 h later puromycin (10 μg/ml) were added to identify resistant BxPC-3 Hsp90 knock-down colonies (BxPC-3 knock-down).

**Proliferation inhibition analysis**

BxPC-3 and BxPC-3 knock-down cells (1 × 10^5) were seeded in six-well plates, cultivated overnight and treated with 5 nM GEM and 10 nM GD, respectively. A single HS was performed 1 h after the beginning of the treatment. To avoid unspecified effects caused by the solvent, DMSO concentration was the same in all samples (0.05%). Cell counts were determined after 72 h using a Casy TTC cell counter (Roche).

**BrdU incorporation**

BxPC-3 and BxPC-3 knock-down cells were seeded in six-wells, pre-treated with GD for 1 h and incubated with 10 μM BrdU exposed to 41.5°C for 1.5 h and prepared following the instructions of the manufacturer (BrdU Flow Kit, BD Pharmingen). The BrdU incorporation was measured and analysed by a FACSCalibur flow cytometer. Experiments were performed in sextuple. Asterisks indicate significance compared with the corresponding control (P < 0.05) and error bars indicate ± SD.

**Binding of Cdc25A to immobilized Hsp90 constructs**

Binding studies of Cdc25A to immobilized Hsp90 constructs were carried out using Ni^{2+}-IDA affinity column. Column material was equilibrated with binding buffer (0.1 M NaCl, 50 mM Tris–HCl pH 7.2) and subsequently 0.25 pmol of following His tagged Hsp90 constructs were bound (1 h, RT, gently shaking).
Full-length Hsp90α (number of amino acids 752, MW: 87 kDa)

The protein sequence of the used Hsp90 construct was:

GSSHHHHHHSSGLVPRGSHAREETQDQPMEEEVEET FAFQAEIAQLMSLINTFYSNKEIFLRELISNSSDLKIR YESLTDPSKDLGSHELHINLIPKQDRTLTVTDTGI1MT
KADLINNLGTIAKSGTAFMEALQAGADISMQGGGQV
FYSAYLVAEKTVTHNHDDEYQAWESSAGGSFTVRT
DTGEMGRGTKVILHLEDQYTELEYEERKIEVKKHSQ
FIGYPITLDFVEKIDVESDDEAEEDKEEKEKKKKE
SEDKEPIEDVSDEEEKDKGDKKKKKKEKYEIDOEE
LNKTIPWTRNPDDITNEEYGEFKSNLDNEDWEDLAVK
HFSVEGQLEFRAFLFPRAPFDLFEKNNKKNNLYV
RRVFMHDNCEILEPYNFRIFVVDNPDLPNSREMLQ
QSKILKVRKNLVKKCELFTEAEDKENCYKFFYQFOS
KNIIKGHDSQNRKKSSELLRRYTSASGDEMSLKDYCT
TRMKENQKHIIYITGETKDVANSFAVERLRKHGELV

YMIEPIDEYCVQLKEEFGKTVSVEKTELEPEDEEEK
KKQEEKKKTENLCKIMKDELKVEVVSNRVLGTP
CTIVTSYGTWANMERMKQAQLRDNSTMGYMAAKK
HLEINPDHIIETLRKAEADKDNCVSDYLDILYETKLF
SSGFSLDPQTHANIRYRMKLGIDDDPTAADSAA
VTEEMPPLEDTDTSRMEEVD*.

N-terminal domain of Hsp90α (number of amino acids 281, MW: 31 kDa)

The protein sequence of the used Hsp90 construct was:

GSSHHHHHHSSGLVPRGSHAREETQDQPMEEEVEET ETDFAFQAEIAQLMSLINTFYSNKEIFLRELISNSSDLKIR
YESLTDPSKDLGSHELHINLIPKQDRTLTVTDTGI1MT
KADLINNLGTIAKSGTAFMEALQAGADISMQGGGQV
FYSAYLVAEKTVTHNHDDEYQAWESSAGGSFTVRT
DTGEMGRGTKVILHLEDQYTELEYEERKIEVKKHSQ
FIGYPITLDFVEKIDVESDDEAEEDKEEKKKKE
SEDKEPIEDVSDEEEKDKGDKKKKKKEKYEIDOEE
LNKTIPWTRNPDDITNEEYGEFKSNLDNEDWEDLAVK
HFSVEGQLEFRAFLFPRAPFDLFEKNNKKNNLYV
RRVFMHDNCEILEPYNFRIFVVDNPDLPNSREMLQ
QSKILKVRKNLVKKCELFTEAEDKENCYKFFYQFOS
KNIIKGHDSQNRKKSSELLRRYTSASGDEMSLKDYCT
TRMKENQKHIIYITGETKDVANSFAVERLRKHGELV

YMIEPIDEYCVQLKEEFGKTVSVEKTELEPEDEEEK
KKQEEKKKTENLCKIMKDELKVEVVSNRVLGTP
CTIVTSYGTWANMERMKQAQLRDNSTMGYMAAKK
HLEINPDHIIETLRKAEADKDNCVSDYLDILYETKLF
SSGFSLDPQTHANIRYRMKLGIDDDPTAADSAA
VTEEMPPLEDTDTSRMEEVD*.
The protein sequence of the used Hsp90 construct was:

MGSSHHHHPQPSGPQEEVQSLTPQPSGKSFVQPVPSVRQESKAFNLKGGVEQLY
DSKVEQLEFARALLFVPRRAPFDLFSRKGDKKIKKIEKMKVaGKQKIKKQ
KIHDSQNLQLKVEFVQGKGKSGVQQLKEFKGKLTVSVGKTELDPEEKKKQE
KKKKTKFENLCKMIDEKILEKVEVYVSRLTVSVCITSTYGG

Full-length Hsp90β (number of amino acids 749,MW: 85 kDa)

The protein sequence of the used Hsp90 construct was:

MGSSHHHHSSGLVPRGSHPMEEVHGEVEETVAFQAEIAQLMSLIINTFSNKEIFLRELISNASDLARIYE
SLTDPSSKLDGSKELIDIPQERTTLTVGTGMKTADLNLNGTIASKGKAMELQAGADISMQPGFQVFY
SAYLVKEVVKVTVKHDQEYWESASSGGSFTVRAH

N-terminal domain of Hsp90β (number of amino acids 259, MW: 29 kDa)

The protein sequence of the used Hsp90 construct was:

MGSSHHHHSSGLVPRGSHPMEEVHGEVEETVAFQAEIAQLMSLIINTFSNKEIFLRELISNASDLARIYE
SLTDPSSKLDGSKELIDIPQERTTLTVGTGMKTADLNLNGTIASKGKAMELQAGADISMQPGFQVFY
SAYLVKEVVKVTVKHDQEYWESASSGGSFTVRAH

Immunoprecipitation

For immunoprecipitation of endogenous Cdc25A/HSP90 complexes upon various treatments, BxPC-3 total lysates were prepared in NP40-containing lysis buffer (40 mM HEPES pH 7.5, 120 mM NaCl, 1 mM EDTA, 10 mM 2-glycerophosphate, 50 mM NaF and 0.5% NP40 supplemented with 2 μg/ml of aprotinin, 2 μg/ml of leupeptin, 0.3 μg/ml of benzamidin-chlorid, 10 μg/ml of trypsin inhibitor) in a 30–60 min incubation at 4°C. Anti-Cdc25A (F-6) (Santa Cruz, #sc-7389, Santa Cruz) was added to the cleared lysates and incubated with constant rotation for 2 h at 4°C. About 20 ml of a 50% slurry of protein A/G sepharose was then added and the incubation continued for another 60 min. The immunoprecipitates were washed four times with 40 μl lysis buffer and then separated by SDS-PAGE. The proteins were transferred to nitrocellulose and visualized by immunodetection using antibodies specific for the Cdc25A and Hsp90β proteins.
following proteins were used: HSP90 (BD Transduction Laboratories, #610 418, Franklin Lakes, NJ, USA) and Cdc25A (F-6) mouse monoclonal antibodies were detected using anti-mouse IgG, an HRP-linked heavy and light chain antibody from goat (Bethyl Laboratories, #A90-116P, Montgomery, TX, USA). Signals were visualized with the enhanced chemiluminescence method (Pierce, Thermo Fisher Scientific, #32 106).

Statistics
All experiments were performed in triplicate and analysed by r-test using Prism 5.0 program (GraphPad, San Diego, CA, USA).

ACKNOWLEDGEMENT
We wish to thank Toni Jaeger for preparing the figures.

Conflict of Interest statement. None declared.

FUNDING
This work was supported by the Fellinger Cancer Research Association (Fellinger Krebsforschung Gemeinnütziger Verein) with a grant to G.K. as a mission-oriented grant (Auftragsforschung), and by the Herzfelders Family foundation with a grant to T.S.

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