Targeting HSP90 by the Novel Inhibitor NVP-AUY922 Reduces Growth and Angiogenesis of Pancreatic Cancer

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Abstract. *Aim: To evaluate the impact of heat-shock protein 90 (HSP90) blockade by the novel inhibitor NVP-AUY922, on tumor growth and angiogenesis in pancreatic cancer. Materials and Methods: Effects of NVP-AUY922 on signaling pathways were evaluated by western blotting. Cell motility of cancer cells, pericytes and endothelial cells was investigated in Boyden chambers. Impact of HSP90 blockade on pancreatic tumor growth and angiogenesis were studied in in vivo tumor models. Results: NVP-AUY922 effectively inhibited cancer cell growth. Moreover, HSP90 inhibition potently interfered with multiple signaling pathways in cancer cells, as well as endothelial cells and pericytes, leading to significant reduction of pro-migratory and invasive properties of these cell types. In vivo, treatment with NVP-AUY922 significantly inhibited growth and vascularization of pancreatic cancer at doses far below the maximum tolerated dose. Conclusion: HSP90 blockade by the novel synthetic inhibitor NVP-AUY922 effectively reduces pancreatic cancer progression through direct effects on cancer cells, as well as on endothelial cells and pericytes.*

Pancreatic cancer represents a highly aggressive tumor entity, with a 5-year survival for all stages of less than 5% (1). The only potential curative treatment option for patients with a pancreatic adenocarcinoma is a combination of complete surgical tumor removal and adjuvant therapy, but fewer than 15% of patients present with resectable disease (2). Due to the aggressive nature of pancreatic cancer, and continued lack of potent therapeutic options, development of novel approaches, including targeted therapy concepts, for improvement of patient outcome is required. However, the identification of valid molecular targets remains a challenge.

In this context the molecular chaperone heat-shock protein 90 (HSP90) has recently been recognized as a promising target in oncology, as it supports the correct conformation, stabilization, activation and intracellular disposition of the so-called client proteins, including a wide variety of oncogenic molecules and signaling intermediates [*e.g.* epidermal growth factor receptor (EGFR), human epidermal growth factor receptor-2 (HER-2), vascular endothelial growth factor (VEGF), AKT, extracellular signal-regulated kinase (ERK), and hypoxia-inducible factor (HIF)-1α] (3, 4). Consequently, inhibition of HSP90 function results in the simultaneous disruption of numerous signal transduction pathways that are involved in characteristic processes of the malignant phenotype, such as invasion, angiogenesis, and metastasis (5). Recent studies indicate that HSP90 is expressed at a 2- to 10-fold higher level in tumor cells than in normal cells (6), and that HSP90 in cancer cells has a significantly higher binding affinity to HSP90 inhibitors, compared to HSP90 from nonmalignant cells (7). Interestingly, HSP90 is abundantly expressed in pancreatic cancer, as well as other types of cancer (8). Against this background, we were able to demonstrate that pancreatic cancer cells harbor multiple oncogenic HSP90 client proteins, which can effectively be controlled by HSP90 inhibition using geldanamycin derivates, thus leading to significant growth reduction *in vivo* (9).

In the present study, we follow-up on this aspect, using NVP-AUY922, a novel synthetic inhibitor of HSP90, belonging to the family of resorcinylic isoxazoles. NVP-AUY922 is the most potent synthetic small-molecule inhibitor of HSP90 described so far, which has already been shown to elicit antineoplastic activity in some tumor models (10, 11).

It becomes increasingly clear that there are complex interactions between cancer cells and the tumor stroma ultimately leading to pancreatic cancer progression (12). Hence, we sought not only to investigate effects of HSP90

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blockade by NVP-AUY922 on tumor cells themselves, but also focused on the impact of this compound on important cell components of the tumor microenvironment known to be crucial for growth and metastasis of pancreatic cancer (13). The vascular endothelial growth factor receptor 2 (VEGF-R2) system, with its ligand VEGF-A, which is primarily provided by secretion from tumor cells is one of the major systems involved in pancreatic tumor angiogenesis (13, 14). VEGF-induced activation of VEGF-R2 not only promotes migration and proliferation of endothelial cells which are crucial steps for initiating angiogenesis (15), but VEGF also has been identified as an antiapoptotic survival factor for endothelial cells (16). Therefore, interference with the VEGF-R2 signaling pathway might have therapeutic efficacy by preventing angiogenesis, as well as by causing endothelial cells in the tumor microenvironment to regress. Another important step in tumor angiogenesis is characterized by the recruitment of pericytes [vascular smooth muscle cells (VSMCs)] into tumors to stabilize the tumor vasculature which in part involves the VEGF-R and the platelet-derived growth factor (PDGF)-B/PDGF receptor (PDGF-R) system (17, 18). This migratory process is at least in part mediated by activation of MEK/ERK signaling cascades, which in turn are affected by inhibition of HSP90 (19).

Thus, for the present study, we hypothesized that targeting both the tumor cells and important cell components of the surrounding tumor microenvironment through blockade of HSP90 by NVP-AUY922, could prove valuable as a multitargeted approach for effectively inhibiting pancreatic cancer progression *in vivo*.

Materials and Methods

Cells and reagents. The human pancreatic cancer cell lines HPAF-II, BxPC-3, and MiaPaCa-2 were obtained from the American Type Culture Collection (Manassas, VA, USA), and the metastatic L3.6pl cell line was kindly provided by Dr. I. J. Fidler (The University of Texas, MD Anderson Cancer Center, Houston, TX, USA). Human umbilical vein endothelial cells (HUVECs) and VSMCs were purchased from PromoCell (Heidelberg, Germany). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Darmstadt, Germany) supplemented with 10% or 15% fetal bovine serum (FBS; Life Technologies, Darmstadt, Germany) and maintained in 5% CO_2 at 37˚C, as described elsewhere (20). For *in vivo* experiments, trypsinized cells were resuspended in Hank's balanced salt solution (HBSS; Life Technologies). Recombinant human epidermal growth factor (EGF), VEGF-A, and PDGF-B were purchased from R&D Systems (Minneapolis, MN, USA). The synthetic HSP90 inhibitor NVP-AUY922 was kindly provided by Novartis (Novartis Oncology, Basel, Switzerland) and dissolved for *in vitro* experiments in dimethyl sulfoxide (DMSO; Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). For *in vivo* studies, the mesylate salt of NVP-AUY922 was formulated in 5% glucose in water and was administered by intraperitoneal injection. Fresh solutions were made at each administration.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide analyses. To evaluate the cytotoxic potential of NVP-AUY922, pancreatic cancer cells, as well as HUVECs and VSMCs, were seeded into 96-well plates (1×10^3 cells per well) and exposed to different concentrations of NVP-AUY922 for the indicated times at 37˚C. Respective concentrations of DMSO were added to controls accordingly. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess cell viability, as previously described (21).

Immunoblot analysis of signaling intermediates. To determine the effects of NVP-AUY922 on signaling intermediates, western blot analysis was performed. Experiments were carried out in triplicates. Unless otherwise indicated, cells were incubated with NVP-AUY922 (10 nmol/l) for 20 h before stimulation with EGF (40 ng/ml), VEGF-A (50 ng/ml), or PDGF-B (10 ng/ml). Whole cell lysates and nuclear extracts were prepared as described elsewhere (9). Protein samples (75 μg) were subjected to western blotting using a denaturing 10% sodium dodecyl sulfate polyacrylamide gel. Membranes were sequentially probed with antibodies specific for phospho-MEK, MEK, phospho-AKT^{Ser473}, AKT, phospho-ERK$^{Thr202/Tyr204}$, ERK, phospho-signal transducer and activator of transcription (STAT)3^{Tyr705}, STAT3, HSP70, HER-2, cMET, focal adhesion kinase (FAK) (all from Cell Signaling Technologies, Beverly, MA, USA); phospho-VEGF-R2, VEGF-R2, phospho-PDGF-Rβ, PDGF-Rβ, β-Actin (all from Santa Cruz Biotechnologies, Santa Cruz, CA, USA); and HIF-1α (Novus Biologicals, Merck, Darmstadt, Germany). Antibodies were detected by enhanced chemiluminescence (Amersham Bioscience, Piscataway, NJ, USA). Western blot analyses of tumor tissue samples were carried out likewise after tissue lysis using an extraction buffer, as described elsewhere (21).

Motility assays. To determine the effects of NVP-AUY922 (10 nmol/l) on cell motility *in vitro*, migration and invasion assays were performed using modified Boyden chambers, as described elsewhere (22). Briefly, 1×10^5 cells were resuspended in 1% FCS-DMEM and seeded into uncoated (migration) or coated (invasion) inserts with 8-mm filter pores, and 10% FCS-DMEM, with or without EGF (40 ng/ml), VEGF-A (50 ng/ml) or PDGF-B (10 ng/ml), serving as chemoattractants. After 24 h and 48 h, cells were fixed and migrated cells were stained (Diff-Quick reagent; Dade Behring, Newark, NJ, USA). Cells were counted in four random fields, and average numbers were calculated.

Animal models. Eight-week-old male athymic nude mice (BALB/c$^{nu/nu}$; Charles River, Sulzfeld, Germany) were used for experiments, as approved by the Institutional Animal Care and Use Committee of the University of Regensburg and the regional authorities. In addition, experiments were conducted according to *Guidelines for the Welfare of Animals in Experimental Neoplasia,* published by The United Kingdom Coordinating Committee on Cancer Research (23). The effects of HSP90 inhibition on the growth of human pancreatic cancer cells (L3.6pl) were first investigated in a subcutaneous tumor model. Cancer cells (1×10^6) were injected into the subcutis of the right flank of nude mice. Mice were randomized (n=10 per group) and assigned to treatment groups. Intraperitoneal injections of NVP-AUY922 (50 mg/kg/week or 3×25 mg/kg/week) were started on day 7 after tumor cell implantation, when tumors became palpable. Tumor diameters were measured every other day and tumor volumes were calculated (width^2×length× 0.5). On day 17 after tumor cell inoculation, mice were sacrificed and excised tumors were measured and weighed.

The effects of HSP90 inhibition by NVP-AUY922 were additionally evaluated in an orthotopic pancreatic cancer model. In brief, 5×10^5 human pancreatic cancer cells (L3.6pl) were injected into the pancreas of mice. Tumors were allowed to grow for seven days after implantation before treatment was initiated. Mice were randomized into groups (n=10 per group), receiving either vehicle (saline) or NVP-AUY922 (50 mg/kg/week) by *i.p.* injections. On day 26 after tumor cell inoculation, the experiment was terminated and excised tumors were measured and weighed. For immunohistochemical analyses, tumors were either paraffin embedded or placed in optimum cutting temperature (OCT) solution.

Immunohistochemical analysis of vessel area. Multiple cryosections were obtained from tumors for immunohistochemical analyses. For assessment of vessel area, rat anti-mouse CD31/platelet endothelial cell adhesion molecule 1-specific antibody (PharMingen, San Diego, CA, USA) and peroxidase-conjugated goat anti-rat IgG (Jackson Research Laboratories, West Grove, PA, USA) were used. Antibody binding was visualized using diaminobenzidine (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). Images were obtained in four different quadrants of each tumor section (2 mm inside the tumor-normal tissue interface) at ×40 magnification. Measurement of vessel area of CD31-stained vessels was made by converting images to grayscale and setting a consistent threshold for all slides using ImageJ software (version 1.33; NIH, Bethesda, MD, USA). Vessel areas are expressed as pixels per high-power field (21).

Statistical analyses. Statistical analyses were performed using SigmaStat (version 3.0; Systat Software GmbH, Erkrath, Germany). Results of *in vivo* experiments were analyzed for outliers using Grubb's test (http://www.graphpad.com). Tumor-associated variables in *in vivo* experiments were tested for statistical significance using the Mann-Whitney *U*-test. The two-sided Student's *t*-test was applied for analysis of *in vitro* data. All results are expressed as the mean±SEM.

Results

Effects of HSP90 inhibition by NVP-AUY922 on growth of pancreatic cancer cells in vitro. To determine potential cytotoxic and antiproliferative effects of NVP-AUY922, the human pancreatic cancer cell lines BxPC3, L3.6pl, HPAF-II, and MiaPaCA-2 were exposed to NVP-AUY922 at different concentrations (1-100 nM) for 48 to 72 h and cell viability was determined by MTT assays. Results show that treatment with NVP-AUY922 led to a significant dose-dependent inhibition of pancreatic cancer cell growth *in vitro* (Figure 1A, and B). As previously reported for other types of cancer cells (*e.g.* non-small cell lung cancer, breast cancer, and gastric cancer) (10, 24, 25), the half maximal inhibitory concentration (IC_{50}) values of NVP-AUY922 for pancreatic cancer cells also fell in the nanomolar range, at 10 nM (all cell lines). This dose was used for all subsequent *in vitro* experiments. We conclude from these experiments that NVP-AUY922 exhibits anti-neoplastic activity towards pancreatic cancer cells.

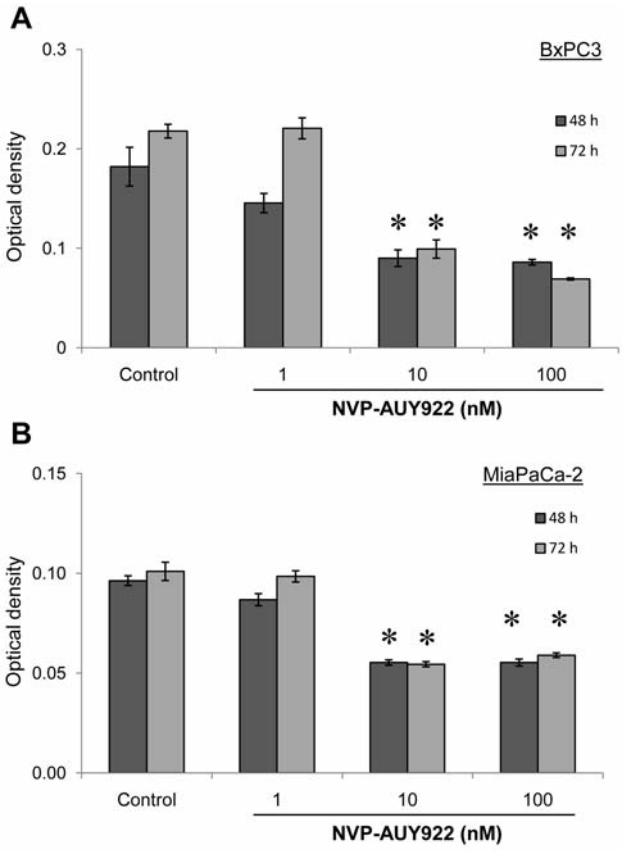

Figure 1. *Growth-inhibitory effects of heat-shock protein 90 inhibitor NVP-AUY922 on pancreatic cancer cells in vitro. Cytotoxic impact of NVP-AUY922 on various human pancreatic cancer cell lines (HPAF-II, BxPC3, L3.6pl, MiaPaCa-2) was determined using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) analyses. Treatment with NVP-AUY922 potently reduced growth of pancreatic cancer cells in a dose-dependent (1 nM, 10 nM, 100 nM) manner in vitro. Results are shown for BxPC3 (A) and MiaPaCa-2 (B) pancreatic cancer cells. *p<0.05 compared to control. Data are expressed as bars=SEM.*

Effects of NVP-AUY922 on cell signaling of pancreatic cancer cells in vitro. To identify intracellular signaling pathways that are affected by inhibition of HSP90, we next investigated whether treatment with NVP-AUY922 leads to alterations in constitutively activated pathways that have been associated with growth of pancreatic cancer. Western blot analyses showed that treatment with NVP-AUY922 effectively inhibited constitutive phosphorylation of MEK and $ERK^{Thr202/Tyr204}$, while expression of HSP70 was strongly induced (Figure 2A). Moreover, the activation of $STAT3^{Tyr705}$, an important transcription factor for regulating VEGF-A in cancer cells, was markedly blocked by NVP-AUY922. In addition, the expression of FAK, yet another essential mediator of cancer cell invasiveness, was reduced by NVP-AUY922 in these cells. Furthermore, NVP-AUY922

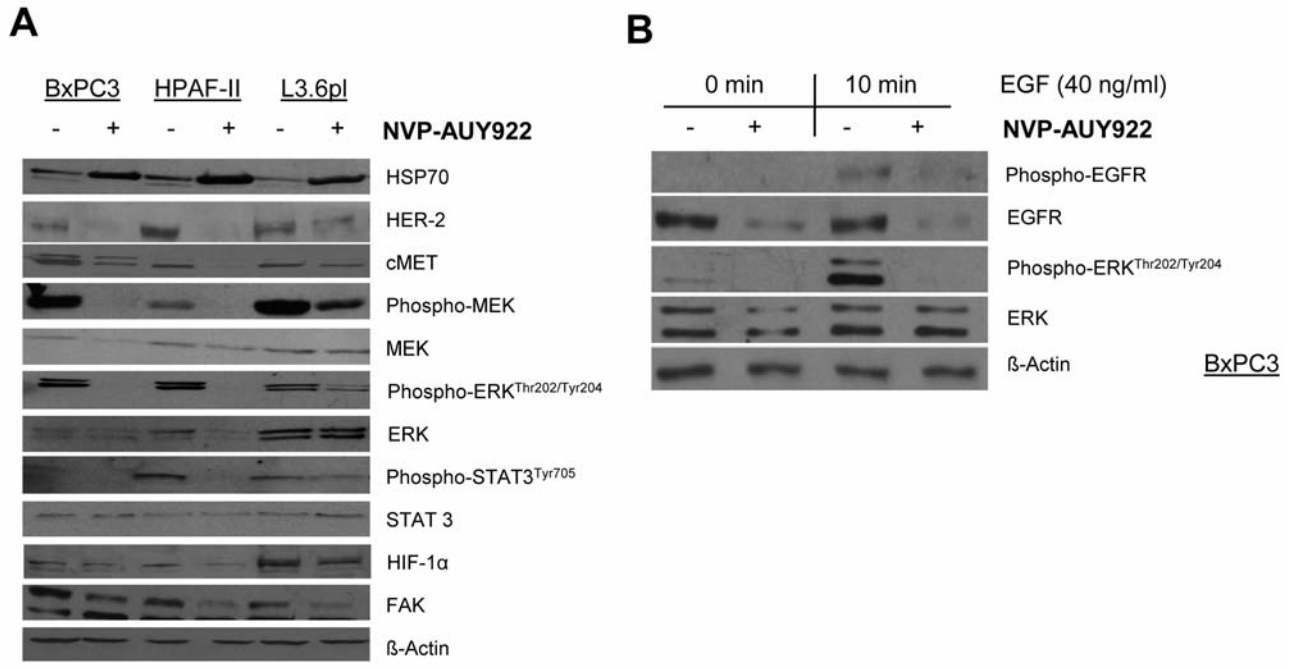

Figure 2. *Effects of heat-shock protein 90 (HSP90) inhibition by NVP-AUY922 on activation of signaling pathways in vitro. A: BxPC3-, HPAF-II-, and L3.6pl pancreatic cancer cells were incubated in the presence or absence of NVP-AUY922 (10 nM) for 20 h. Western blot analyses show that blockade of HSP90 by NVP-AUY922 led to a solid inhibition of the constitutive phosphorylation of MEK, extracellular signal-regulated kinase (ERK)$^{Thr202/Tyr204}$, and signal transducer and activator of transcription (STAT)3^{Tyr705}, while expression of HSP70 was strongly induced. Moreover, NVP-AUY922 also reduced the expression of focal adhesion kinase (FAK) and the transcription factor hypoxia-inducible factor (HIF)-1α, while that of human epidermal growth factor receptor-2 (HER-2) and cMET were down-regulated. B: Treatment with NVP-AUY922 (10 nM for 20h) reduced the expression and the epidermal growth factor (EGF)-mediated (40 ng/ml) activation of EGFR and substantially disrupted EGF signaling in terms of diminishing downstream phosphorylation of ERK$^{Thr202/Tyr204}$.*

led to a substantial down-regulation of the constitutive expression of HER-2 and cMET.

Since expression of EGFR and activation of EGFR-dependent pathways are known to play important roles in the biological aggressiveness of human pancreatic adenocarcinoma (26), we next addressed the question whether NVP-AUY922 could be used for interfering with activation of pathways involved in EGF signaling in pancreatic cancer cells. Indeed, treatment with NVP-AUY922 reduced the expression and activation of EGFR and substantially disrupted EGF signaling in terms of diminishing downstream phosphorylation of ERK$^{Thr202/Tyr204}$ (Figure 2B). Together, these experiments show that HSP90 inhibition by NVP-AUY922 significantly affects important growth factor-mediated and angiogenesis-related signaling cascades in pancreatic cancer cells.

Effects of NVP-AUY922 on motility of pancreatic cancer cells in vitro. Next, we addressed the effect of HSP90 blockade by NVP-AUY922 on functional aspects of tumor cell activity, including cancer cell migration and invasion. The basis for this examination comes from studies that identified FAK as being a client protein of HSP90 (4, 27), suggesting that cancer cell motility might be impaired by inhibiting HSP90. In western blot analysis, we observed down-regulation of FAK in BxPC3, HPAF-II, and L3.6pl pancreatic cancer cells (Figure 2A). To determine the effects of HSP90 inhibition on constitutive as well as on EGF-mediated cell motility, pancreatic cancer cells were subjected to migration and invasion assays. EGF significantly induced cancer cell migration and invasiveness (Figure 3A, and B). The presence of NVP-AUY922 (10 nM) significantly blocked cancer cell migration and invasion both in the absence and presence of EGF ($p<0.05$ for both). These results suggest that inhibitors of HSP90 have the potential to reduce invasiveness and metastatic spread of pancreatic cancer cells.

Effects of NVP-AUY922 on HUVECs in vitro. Complex interactions between cancer cells and the tumor stroma have been identified as being crucial for pancreatic cancer growth and metastasis (12, 13). In this context, the VEGF-A/VEGF-R2 system plays a predominant role as its activation has been shown to promote proliferation and motility of endothelial

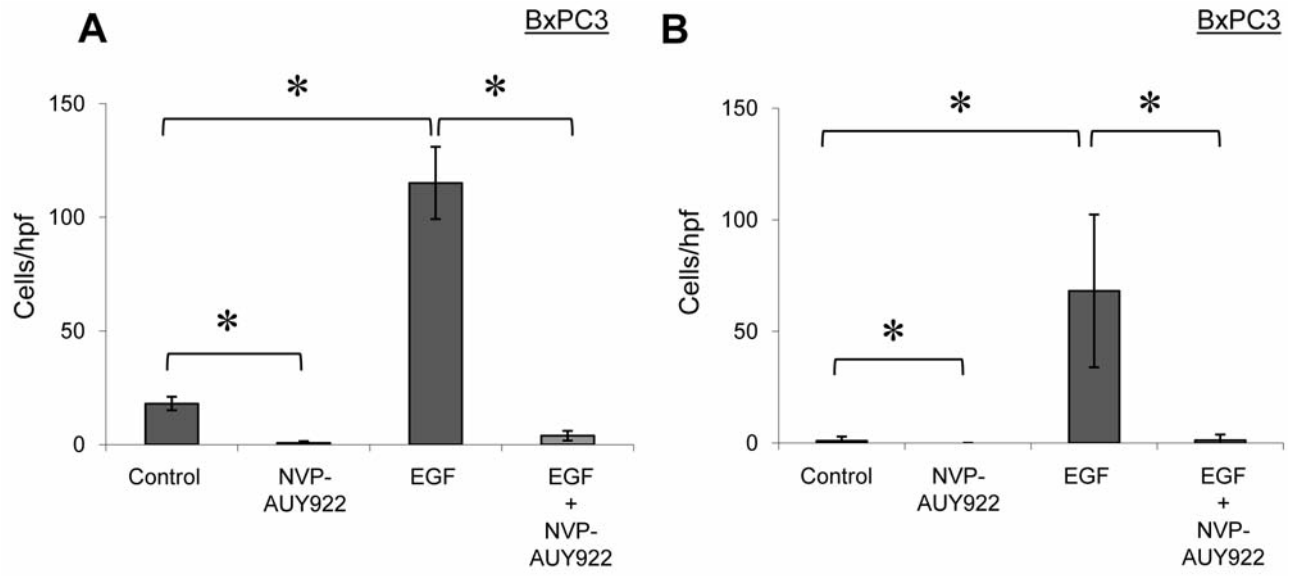

Figure 3. *Impact of heat-shock protein 90 (HSP90) blockade on the motility of pancreatic cancer cells in vitro. Changes in tumor cell migration and invasion were investigated in uncoated (migration) or coated (invasion) modified Boyden chambers. A: NVP-AUY922 (10 nM) significantly reduced both, constitutive and EGF-mediated (40 ng/ml) migratory properties of BxPC3 pancreatic cancer cells. *p<0.05. B: Similarly, HSP90 inhibition by NVP-AUY922 (10 nM) effectively blocked constitutive invasion of pancreatic cancer cells (BxPC3) and significantly inhibited response to stimulation with EGF (40 ng/ml). *p<0.05. Data are expressed as bars=SEM.*

cells (15), representing an important component of the tumor microenvironment. Therefore, we next investigated whether NVP-AUY922 could also elicit antiangiogenic effects through direct interaction with HUVECs by interference with the VEGF-R2 pathway. To test this hypothesis, we used MTT and western blot analyses, as well as migration assays, to determine the functional impact of NVP-AUY922 on HUVECs. In accordance with previous experiments by Eccles *et al*. (10), HUVECs were highly sensitive to NVP-AUY922, as revealed by MTT analyses (IC_{50}=10 nM) (data not shown). Moreover, treatment of HUVECs with NVP-AUY922 showed substantial inhibition of the activation of VEGF-R2, while phosphorylation of $ERK^{Thr202/Tyr204}$, AKT^{Ser473}, and $STAT3^{Tyr705}$ was potently blocked by HSP90 inhibition (Figure 4A). Additionally, NVP-AUY922 led to a significant reduction of constitutive, as well as VEGF-A-driven, migratory properties of HUVECs (Figure 4B).

Effects of NVP-AUY922 on VSMCs in vitro. The recruitment of pericytes into tumors in order to stabilize the tumor vasculature represents another important step in tumor angiogenesis. This migratory process involves at least in part the activation of the PDGF-B/PDGF-Rβ system (17, 18). Therefore, we next sought to investigate effects of HSP90 blockade by NVP-AUY922 on VSMCs. Interestingly, western blot analyses showed that treatment with NVP-AUY922 (10 nM for 20 h) substantially depleted PDGF-B-mediated activation of key angiogenic HSP90 client proteins, including phospho-$ERK^{Thr202/Tyr204}$ and phospho-AKT^{Ser473}, while expression of PDGF-Rβ was down-regulated (Figure 5A). Furthermore, as revealed by migration assays, treatment with NVP-AUY922 significantly inhibited both constitutive as well as PDGF-B-mediated migratory properties of VSMCs (Figure 5B). Together with the findings in HUVECs, the results of these experiments show that HSP90 blockade by NVP-AUY922 elicits relevant direct effects on the tumor microenvironment by impairing endothelial cell as well as pericyte migration.

Effects of NVP-AUY922 on growth and vascularization of pancreatic cancer cells in vivo. To estimate growth-inhibitory and antiangiogenic effects of NVP-AUY922 *in vivo*, we first used a subcutaneous tumor model (L3.6pl cells). Treatment with NVP-AUY922 (50 mg/kg/week or 3×25 mg/kg/week) significantly reduced the growth of pancreatic tumors, compared to that in controls (Figure 6A). This reduction in tumor growth was also reflected in the final weights of excised tumors on day 17, which were significantly lower in the NVP-AUY922-treated groups (Figure 6B).

To test whether HSP90 inhibition with NVP-AUY922 indeed reduces pancreatic cancer growth in the appropriate tumor microenvironment *in vivo*, we subsequently used an orthotopic model of pancreatic cancer (L3.6pl cells). Mice

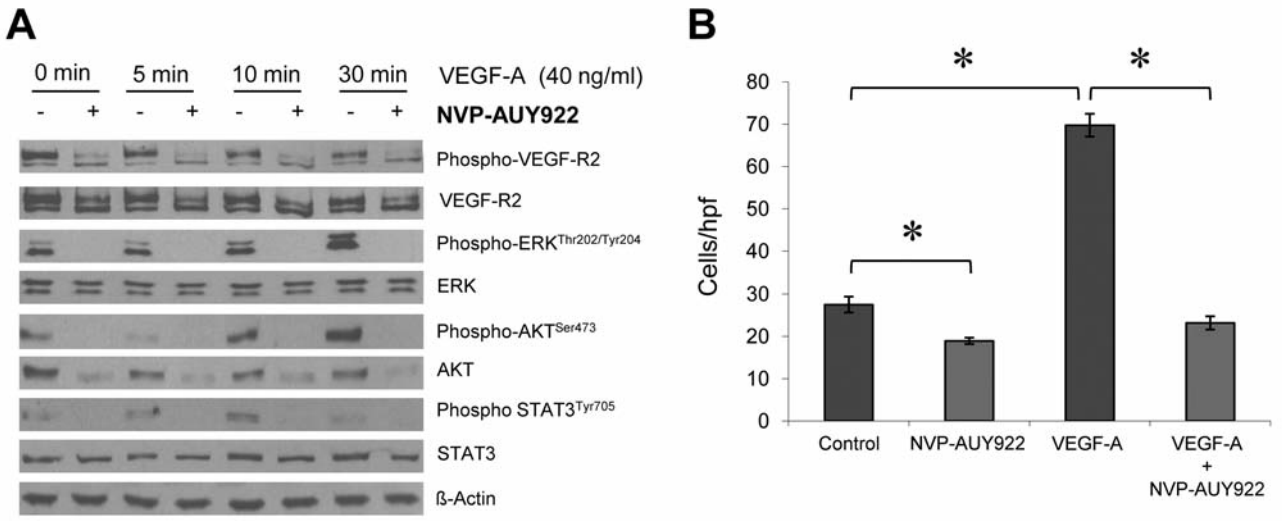

Figure 4. *Impact of NVP-AUY922 on human umbilical vein endothelial cells (HUVECs) in vitro. A: In HUVECs, NVP-AUY922 (10 nM for 20 h) markedly reduced vascular endothelia growth factor-receptor (VEGF-R)2 activation, as well as phosphorylation of extracellular signal-regulated kinase (ERK)$^{Thr202/Tyr204}$, AKT^{Ser473t}, and signal transducer and activator of transcription (STAT)3^{Tyr705} as revealed by western blot analyses. B: HSP90 inhibition by NVP-AUY922 significantly reduced constitutive migration of HUVECs in vitro. Stimulation of HUVECs with vascular endothelia growth factor (VEGF)-A (40 ng/ml) effectively enhanced migratory properties of the cells. This effect was also significantly blocked by NVP-AUY922. *p<0.05. Data are expressed as bars=SEM.*

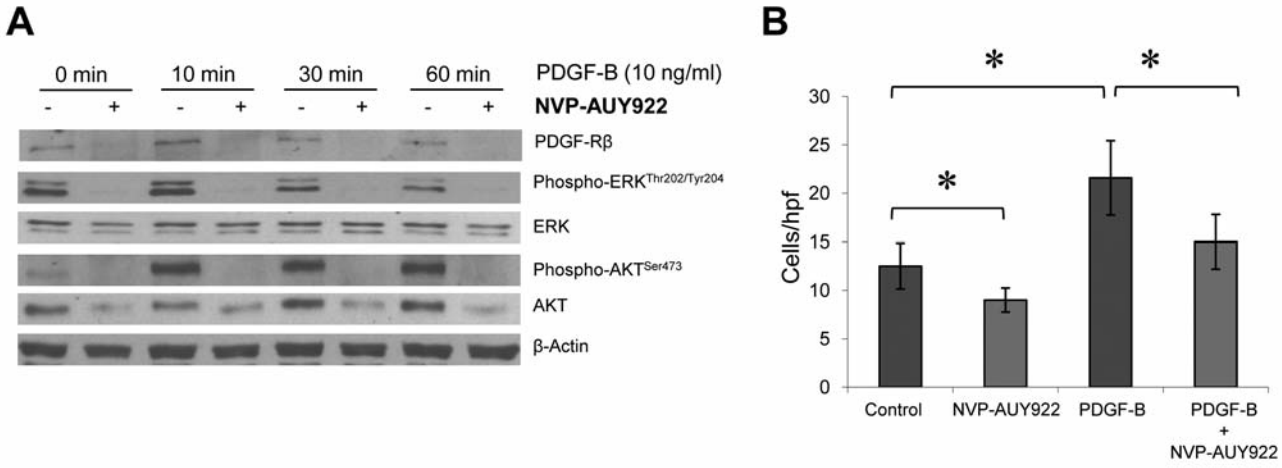

Figure 5. *Effects of NVP-AUY922 on vascular smooth muscle cells (VSMCs) in vitro. A: Western blot analyses show that activation of platelet-derived growth factor receptor (PDGF-R)β and phosphorylation of extracellular signal-regulated kinase (ERK)$^{Thr202/Tyr204}$ and AKT^{Ser473} were substantially inhibited by NVP-AUY922 (10 nM for 20h). B: As a functional consequence, both constitutive as well as PDGF-B-mediated (10 ng/ml) migration of VSMCs was significantly abrogated. *p<0.05. Data are expressed as bars=SEM.*

received either NVP-AUY922 (50 mg/kg) or vehicle, starting on day 7 post tumor cell implantation. On day 26, the experiment was terminated as mice in the control group became moribund because of tumor burden. Analysis of pancreatic tumor burden (tumor volume and tumor weight) shows that mice in the NVP-AUY922 therapy arm had developed significantly smaller tumors as compared to mice in the control group (Figure 6C and D). Importantly, mouse body weights did not statistically differ between these two groups. In addition, vascularization of L3.6pl tumors in terms of CD31-positive vessel area was significantly reduced in tumor sections of the NVP-AUY922-treated group. In conclusion, these results show that NVP-AUY922 substantially inhibits *in vivo* growth of pancreatic cancer through direct effects on tumor cells, and also through inhibition of angiogenesis.

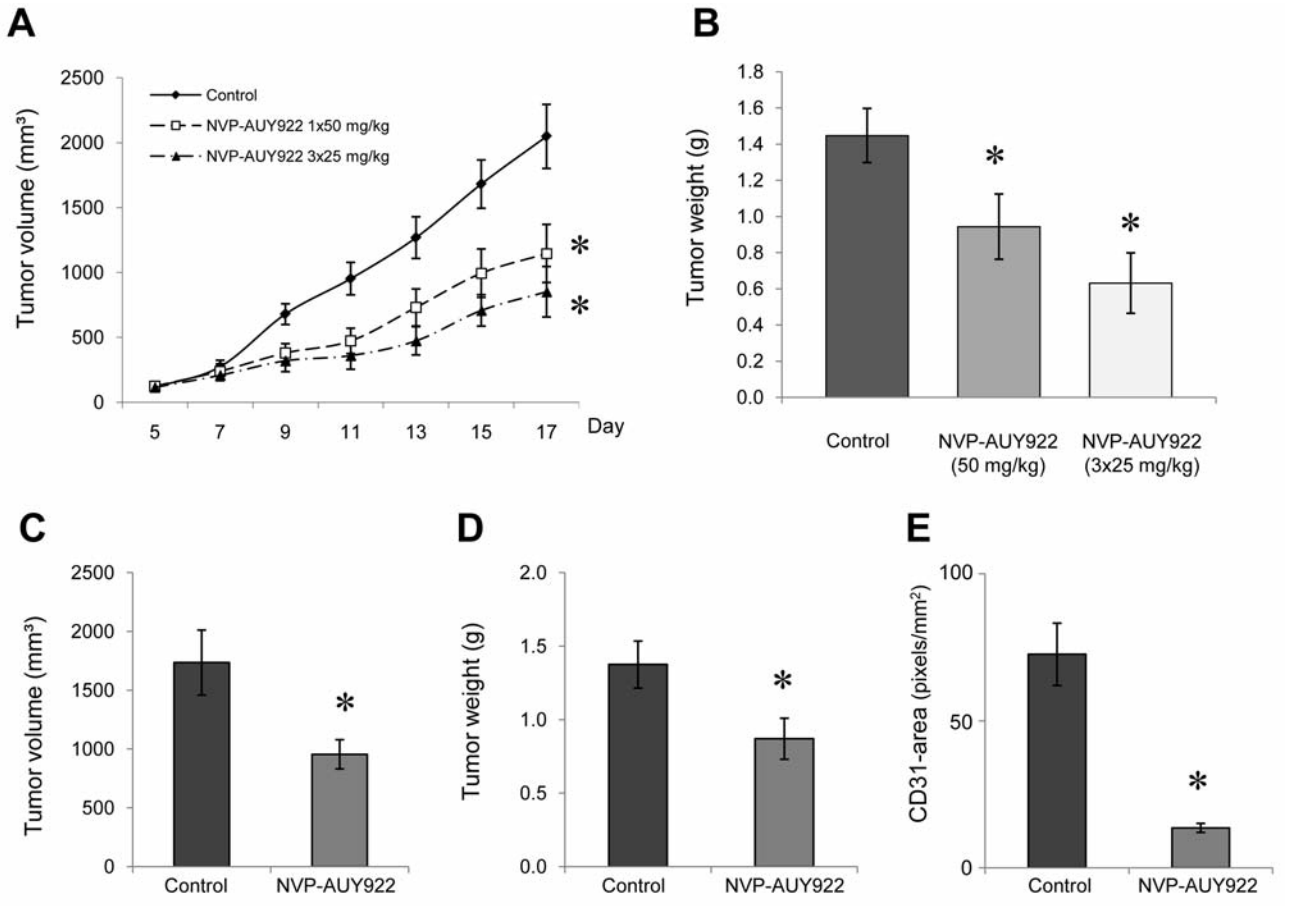

Figure 6. *Effects of heat-shock protein 90 (HSP90) blockade by NVP-AUY922 on pancreatic tumor growth and vascularization in vivo. Efficacy of NVP-AUY922 was first determined in a subcutaneous tumor model using L3.6pl pancreatic cancer cells, where mice received either NVP-AUY922 (50 mg/kg/week or 3×25 mg/kg/week), or vehicle, starting on day seven after tumor cell inoculation (n=10/group). A: Treatment with NVP-AUY922 significantly reduced tumor growth rates in vivo (*p<0.05 for both). B: Final weights of excised tumors were significantly lower in the NVP-AUY922 group (*p<0.05 for both). The effects of NVP-AUY922 on tumor growth were additionally investigated in an orthotopic L3.6pl tumor model in mice (n=10/group), where animals were treated either with NVP-AUY922 (50 mg/kg), or vehicle, seven days after tumor cell implantation. NVP-AUY922 significantly reduced orthotopic tumor growth, as reflected by final tumor volumes (C) and tumor weights (D) at the time of necropsy (*p<0.05). E: Blocking HSP90 with NVP-AUY922 led to a substantial reduction in vascularization (CD31-positive vessel area) of pancreatic tumors (*p<0.01). Data are expressed as bars=SEM.*

Discussion

In this study, we demonstrated the antineoplastic efficacy of NVP-AUY922, a new-generation synthetic HSP90 inhibitor, in pancreatic cancer. The small molecule NVP-AUY922 exhibits potent cytotoxic effects towards pancreatic cancer cells, effectively disrupts multiple oncogenic signaling cascades, and also harbors the potential to reduce cancer cell motility *in vitro*. Importantly, the additional antiangiogenic effects of NVP-AUY922 appear, at least in part, to be mediated through direct effects on endothelial cells, as well as pericytes, in terms of interfering with proangiogenic signaling pathways and inhibiting cell migration. As a functional consequence of these multifactorial effects on cancer cells, as well as on important cells of the tumor microenvironment, targeting HSP90 by NVP-AUY922 translates into potent inhibition of tumor growth and vascularization in pancreatic cancer models *in vivo*. Therefore, our results demonstrate that blocking HSP90 with the novel inhibitor NVP-AUY922 represents an efficacious approach for the herapy of pancreatic cancer.

Our data show that NVP-AUY922 exhibits strong cytotoxic effects on pancreatic cancer cells (Figure 1). None of the cell lines investigated in this study exhibited resistance to single treatment with NVP-AUY922, but growth of the pancreatic cell lines was inhibited in the nanomolar range of the compound. These findings are comparable with the results for NVP-AUY922 in gastric cancer cell lines

(2-40 nM range) (25), as well as breast cancer cells with IC_{50} values of 3.1-8.8 nM of NVP-AUY922 (11). To date, 17-allylamino-17-demethoxygeldanamycin (17-AAG) has been the most investigated HSP90 inhibitor in both preclinical and clinical studies (28). However, NVP-AUYP922 has been suggested to be the most potent inhibitor of HSP90 developed to date (10, 11). As for pancreatic cancer, a previous study reported that treatment with 17-AAG was effective, with IC_{50} values of 70-400 nM (29). Therefore, our data (IC_{50}=10 nM) support a more potent antiproliferative impact of NVP-AUY922 over 17-AAG in pancreatic cancer cell lines *in vitro*.

Novel therapeutic concepts aiming at simultaneously targeting multiple signaling pathways have also been implicated for the treatment of pancreatic cancer, since these approaches may prevent the development of molecular escape mechanisms towards a selective targeted therapy and help to overcome chemoresistance (5, 30). In this context, the use of HSP90 inhibitors has become a popular approach to interfere with a broad range of oncogenic signaling components in tumor cells (3). The molecular fingerprint of HSP90 inhibition is characterized through induction of HSP70 coupled with the depletion of client proteins (31). In our study, the results show that treatment of pancreatic cancer cells with NVP-AUY922 led to a robust induction of HSP70, while the expression or phosphorylation of oncogenic key receptor systems and signaling intermediates, as well as transcription factors associated with pancreatic cancer progression, was dramatically reduced (Figure 2A). As EGFR is known to be a prominent client protein of HSP90 and overexpression of EGF and EGFR by various types of tumor entities, including pancreatic cancer, has been demonstrated to correlate with metastasis, apoptosis and chemoresistance, as well as poor prognosis (26, 32, 33), interfering with EGFR signaling might be a promising therapeutic strategy. A recent Phase II clinical trial using cetuximab, a monoclonal antibody to EGFR, in combination with gemcitabine for locally advanced or pancreatic cancer expressing EGFR revealed a 12.2% partial response, and 63.4% of patients presented stable disease (34). These encouraging results have now led to a Phase III clinical trial of cetuximab plus gemcitabine and clearly promote the approach of inhibiting EGFR signaling in pancreatic cancer. In the present study, blockade of HSP90 function by NVP-AUY922 potently inhibited EGF-mediated activation of EGFR and additionally led to marked down-regulation of the receptor itself (Figure 3B). Moreover, treatment of pancreatic cancer cells with NVP-AUY922 abrogated EGF-mediated phosphorylation of $ERK^{Thr202/Tyr204}$. As a functional consequence, both EGF-mediated cancer cell migration, as well as invasion, *in vitro,* were significantly inhibited (Figure 3A, and B). Thus, our data indicate that HSP90 inhibition by NVP-AUY922 elicits strong antitumoral activity against pancreatic cancer cells, which is at least in part mediated through potent interference with the EGFR pathway. Taken together, these findings make inhibitors of HSP90 very attractive in therapy concepts for treating pancreatic cancer, as EGFR is frequently overexpressed and constitutively active in this type of cancer (26, 32).

The process of angiogenesis is essential for cancer growth and metastasis. Angiogenic activity not only involves tumor cells, but also various cell populations of the tumor microenvironment, such as endothelial cells and pericytes (35). The VEGF/VEGF-R system has been identified as the main angiogenic driving force (36). Other signaling elements playing crucial roles in angiogenesis by affecting endothelial cell proliferation and survival include the phosphatidylinositol 3-kinase (PI-3K)/AKT pathway as well as the RAF/MEK/mitogen-activated protein kinase (MAPK) pathway (37, 38). Additionally, various transcription factors and growth factors, such as STAT3 and PDGF-R have been identified to mediate angiogenic stimuli in endothelial cells and pericytes (39, 40). Because of the unique ability to simultaneously target multiple signaling elements, it has been suggested that these non-malignant angiogenic cells within the tumor microenvironment may also be affected by HSP90 inhibition. Our results show that treating HUVECs with NVP-AUY922 led to substantial inhibition of VEGF-mediated activation of VEGF-R2 (Figure 4A). Furthermore, blocking HSP90 also resulted in the inhibition of $ERK^{Thr202/Tyr204}$, AKT^{Ser473} and $STAT3^{Tyr705}$, which are all important components of angiogenic signaling. In addition, migration assays revealed a significant reduction of migratory properties of HUVECs, following treatment with NVP-AUY922 (Figure 4B). These findings are in line with the results of other groups investigating the antiangiogenic impact of HSP90 antagonists. Sanderson *et al*. demonstrated that 17-AAG significantly inhibited endothelial cell migration and invasion, while expression of VEGF-R2 and of other HSP90 client proteins was significantly reduced (41). Similar to these findings, Kaur and co-workers have also shown the water-soluble 17-AAG analog 17-(Dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG) to substantially block endothelial cell migration, as well as invasion and the ability to form capillary-like structures (42). Together with the results of these studies, our data clearly support the hypothesis that HSP90 inhibition may be useful to effectively interfere with angiogenesis. Importantly, the dramatic effect on endothelial cells suggests that the antiangiogenic impact of HSP90 inhibitors such as NVP-AUY922 are mediated through direct effects on endothelial cells.

So far, antiagiogenic treatment strategies have mainly focused on endothelial cells. However, recent studies implicate that pericytes also play crucial roles in the angiogenic process (43, 44). Pericyte recruitment and covering of endothelial cells for stabilization and maturation of vessel structure is dependent on PDGF-Rβ signaling (43). Hence, interference

with the PDGF pathway should elicit antiangiogenic activity. Indeed, we found that NVP-AUY922 strongly reduced the expression of PDGF-Rβ, while the PDGF-B-mediated activation of $ERK^{Thr202/Tyr204}$ and AKT^{Ser473} was potently diminished (Figure 5A). Importantly, NVP-AUY922 significantly inhibited PDGF-B-induced cell migration of VSMCs (Figure 5B). Again, these observations suggest, that targeting HSP90 by NVP-AUY922 could prove valuable for improving antiangiogenic activity of antineoplastic treatment strategies in therapy of pancreatic cancer through additional direct effects on pericytes.

The potential growth-inhibitory and antiangiogenic effects of HSP90-targeted therapy were investigated in subcutaneous, as well as orthotopic, *in vivo* tumor models with L3.6pl pancreatic cancer cells. To our knowledge, our study is the first to show that the novel synthetic HSP90 inhibitor NVP-AUY922 potently reduces tumor growth and vascularization of pancreatic tumors (Figure 6). Moreover, in contrast to reported studies that investigated the antineoplastic efficacy of NVP-AUY922 for malignancies other than pancreatic cancer, we used doses of only 50 mg/kg once a week or 25 mg/kg thrice a week, which is far below the maximum tolerated dose, instead of daily injections of 50 mg/kg (10). This aspect is important for minimizing potential side-effects of HSP90-targeted therapy. Here, we provide evidence that the principle of targeting multiple oncogenic pathways is efficient and does not require the use of high doses of HSP90 inhibitor.

Conclusion

HSP90 inhibitor NVP-AUY922, a novel resorcinylic isoxazole, is the most potent synthetic small-molecule inhibitor reported so far. In this article, we showed that the inhibition of HSP90 by NVP-AUY922 impairs oncogenic signaling in pancreatic cancer cells and elicits strong antiangiogenic activity through direct effects on endothelial cells, as well as pericytes. These effects are associated with a significant reduction in tumor growth and vascularization in pancreatic cancer models *in vivo*. Inhibition of HSP90 by NVP-AUY922 may therefore be a valuable addition to molecular-targeted therapy concepts for the treatment of pancreatic cancer.

Conflict of Interest

The Authors declare no conflict of interest.

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References

1 Shaib YH, Davila JA and El-Serag HB: The epidemiology of pancreatic cancer in the United States: changes below the surface. Aliment Pharmacol Ther *24(1)*: 87-94, 2006.

2 Muller MW, Friess H, Koninger J, Martin D, Wente MN, Hinz U, Ceyhan GO, Blaha P, Kleeff J and Buchler MW: Factors influencing survival after bypass procedures in patients with advanced pancreatic adenocarcinomas. Am J Surg *195(2)*: 221-228, 2008.

3 Whitesell L and Lindquist SL: HSP90 and the chaperoning of cancer. Nat Rev Cancer *5(10)*: 761-772, 2005.

4 Zhang H and Burrows F: Targeting multiple signal transduction pathways through inhibition of Hsp90. J Mol Med *82(8)*: 488-499, 2004.

5 Xu W and Neckers L: Targeting the molecular chaperone heat shock protein 90 provides a multifaceted effect on diverse cell signaling pathways of cancer cells. Clin Cancer Res *13(6)*: 1625-1629, 2007.

6 Gooljarsingh LT, Fernandes C, Yan K, Zhang H, Grooms M, Johanson K, Sinnamon RH, Kirkpatrick RB, Kerrigan J, Lewis T, Arnone M, King AJ, Lai Z, Copeland RA and Tummino PJ: A biochemical rationale for the anticancer effects of Hsp90 inhibitors: slow, tight binding inhibition by geldanamycin and its analogues. Proc Natl Acad Sci *103(20)*: 7625-7630, 2006.

7 Kamal A, Thao L, Sensintaffar J, Zhang L, Boehm MF, Fritz LC and Burrows FJ: A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. Nature *425(6956)*: 407-410, 2003.

8 Ogata M, Naito Z, Tanaka S, Moriyama Y and Asano G: Overexpression and localization of heat-shock protein mRNA in pancreatic carcinoma. J Nihon Med Sch *67(3)*: 177-185, 2000.

9 Lang SA, Moser C, Gaumann A, Klein D, Glockzin G, Popp FC, Dahlke MH, Piso P, Schlitt HJ, Geissler EK and Stoeltzing O: Targeting heat-shock protein 90 in pancreatic cancer impairs insulin-like growth factor-I receptor signaling, disrupts an interleukin-6/signal-transducer and activator of transcription 3/hypoxia-inducible factor-1alpha autocrine loop, and reduces orthotopic tumor growth. Clin Cancer Res *13(21)*: 6459-6468, 2007.

10 Eccles SA, Massey A, Raynaud FI, Sharp SY, Box G, Valenti M, Patterson L, de Haven Brandon A, Gowan S, Boxall F, Prodromou C, Pearl C, James K, Matthews PT, Cheung KM, Kalusa A, Jones K, McDonald E, Barril X, Brough PA, Cansfield J, Dymock B, Drysdale MJ, Finch H, Howes R, Hubbard RE, Surgenor A, Webb P, Wood M, Wright L and Workman P: NVP-AUY922: a novel heat-shock protein 90 inhibitor active against xenograft tumor growth, angiogenesis, and metastasis. Cancer Res *68(8)*: 2850-2860, 2008.

11 Jensen MR, Schoepfer J, Radimerski T, Massey A, Guy CT, Brueggen J, Quadt C, Buckler A, Cozens R, Drysdale MJ, Garcia-Echeverria C and Chène P: NVP-AUY922: a small molecule HSP90 inhibitor with potent antitumor activity in preclinical breast cancer models. Breast Cancer Res *10(2)*: R33, 2008.

12 Kim EJ and Simeone DM: Advances in pancreatic cancer. Curr Opin Gastroenterol *27(5)*: 460-466, 2011.

13 Solorzano CC, Baker CH, Bruns CJ, Killion JJ, Ellis LM, Wood J and Fidler IJ: Inhibition of growth and metastasis of human pancreatic cancer growing in nude mice by PTK 787/ZK222584, an inhibitor of the vascular endothelial growth factor receptor tyrosine kinases. Cancer Biother Radiopharm *16(5)*: 359-370, 2001.

14 Buchler P, Reber HA, Ullrich A, Shiroiki M, Roth M, Buchler MW, Lavey RS, Friess H and Hines OJ: Pancreatic cancer growth is inhibited by blockade of VEGF-RII. Surgery *134(5)*: 772-782, 2003.

15 Leung DW, Cachianes G, Kuang WJ, Goeddel DV and Ferrara N: Vascular endothelial growth factor is a secreted angiogenic mitogen. Science *246(4935)*: 1306-1309, 1989.

16 Gerber HP, Dixit V and Ferrara N: Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. J Biol Chem *273(21)*: 13313-13316, 1998.

17 Guo P, Hu B, Gu W, Xu L, Wang D, Huang HJ, Cavenee WK and Cheng SY: Platelet-derived growth factor-B enhances glioma angiogenesis by stimulating vascular endothelial growth factor expression in tumor endothelia and by promoting pericyte recruitment. Am J Pathol *162(4)*: 1083-1093, 2003.

18 Yamagishi S, Yonekura H, Yamamoto Y, Fujimori H, Sakurai S, Tanaka N and Yamamoto H: Vascular endothelial growth factor acts as a pericyte mitogen under hypoxic conditions. Lab Invest *79(4)*: 501-509, 1999.

19 Graf K, Xi XP, Yang D, Fleck E, Hsueh WA and Law RE: Mitogen-activated protein kinase activation is involved in platelet-derived growth factor-directed migration by vascular smooth muscle cells. Hypertension *29(1 Pt 2)*: 334-339, 1997.

20 Stoeltzing O, Liu W, Reinmuth N, Fan F, Parikh AA, Bucana CD, Evans DB, Semenza GL and Ellis LM: Regulation of hypoxia-inducible factor-1alpha, vascular endothelial growth factor, and angiogenesis by an insulin-like growth factor-I receptor autocrine loop in human pancreatic cancer. Am J Pathol *163(3)*: 1001-1011, 2003.

21 Lang SA, Klein D, Moser C, Gaumann A, Glockzin G, Dahlke MH, Dietmaier W, Bolder U, Schlitt HJ, Geissler EK and Stoeltzing O: Inhibition of heat-shock protein 90 impairs epidermal growth factor-mediated signaling in gastric cancer cells and reduces tumor growth and vascularization *in vivo*. Mol Cancer Ther *6(3)*: 1123-1132, 2007.

22 Bauer TW, Liu W, Fan F, Camp ER, Yang A, Somcio RJ, Bucana CD, Callahan J, Parry GC, Evans DB, Boyd DD, Mazar AP and Ellis LM: Targeting of urokinase plasminogen activator receptor in human pancreatic carcinoma cells inhibits c-Met- and insulin-like growth factor-I receptor-mediated migration and invasion and orthotopic tumor growth in mice. Cancer Res *65(17)*: 7775-7781, 2005.

23 Workman P, Balmain A, Hickman JA, McNally NJ, Rohas AM, Mitchison NA, Pierrepoint CG, Raymond R, Rowlatt C and Stephens TC, Wallace J and Straughan DW: UKCCCR guidelines for the welfare of animals in experimental neoplasia. Lab Anim *22(3)*: 195-201, 1988.

24 Stingl L, Stuhmer T, Chatterjee M, Jensen MR, Flentje M and Djuzenova CS: Novel HSP90 inhibitors, NVP-AUY922 and NVP-BEP800, radiosensitise tumour cells through cell-cycle impairment, increased DNA damage and repair protraction. Br J Cancer *102(11)*: 1578-1591, 2010.

25 Lee KH, Lee JH, Han SW, Im SA, Kim TY, Oh DY and Bang YJ: Antitumor activity of NVP-AUY922, a novel heat-shock protein 90 inhibitor, in human gastric cancer cells is mediated through proteasomal degradation of client proteins. Cancer Sci *102(7)*: 1388-1395, 2011.

26 Yamanaka Y, Friess H, Kobrin MS, Buchler M, Beger HG and Korc M: Coexpression of epidermal growth factor receptor and ligands in human pancreatic cancer is associated with enhanced tumor aggressiveness. Anticancer Res *13(3)*: 565-569, 1993.

27 Zagzag D, Nomura M, Friedlander DR, Blanco CY, Gagner JP, Nomura N and Newcomb EW: Geldanamycin inhibits migration of glioma cells *in vitro*: a potential role for hypoxia-inducible factor (HIF-1alpha) in glioma cell invasion. J Cell Physiol *196(2)*: 394-402, 2003.

28 Ramanathan RK, Egorin MJ, Eiseman JL, Ramalingam S, Friedland D, Agarwala SS, Ivy SP, Potter DM, Chatta G, Zuhowski EG, Stoller RG, Naret C, Guo J and Belani CP: Phase I and pharmacodynamic study of 17-(allylamino)-17-demethoxygeldanamycin in adult patients with refractory advanced cancers. Clin Cancer Res *13(6)*: 1769-1774, 2007.

29 Adachi S, Yasuda I, Nakashima M, Yamauchi T, Yamauchi J, Natsume H, Moriwaki H and Kozawa O: HSP90 inhibitors induce desensitization of EGF receptor *via* p38 MAPK-mediated phosphorylation at Ser1046/1047 in human pancreatic cancer cells. Oncol Rep *23(6)*: 1709-1714, 2010.

30 Xiong HQ, Rosenberg A, LoBuglio A, Schmidt W, Wolff RA, Deutsch J, Needle M and Abbruzzese JL: Cetuximab, a monoclonal antibody targeting the epidermal growth factor receptor, in combination with gemcitabine for advanced pancreatic cancer: a multicenter phase II trial. J Clin Oncol *22(13)*: 2610-2616, 2004.

31 Banerji U, O'Donnell A, Scurr M, Pacey S, Stapleton S, Asad Y, Simmons L, Maloney A, Raynaud F, Campbell M, Walton M, Lakhani S, Kaye S, Workman P and Judson I: Phase I pharmacokinetic and pharmacodynamic study of 17-allylamino, 17-demethoxygeldanamycin in patients with advanced malignancies. J Clin Oncol *23(18)*: 4152-4161, 2005.

32 Kuwahara K, Sasaki T, Kuwada Y, Murakami M, Yamasaki S and Chayama K: Expressions of angiogenic factors in pancreatic ductal carcinoma: a correlative study with clinicopathologic parameters and patient survival. Pancreas *26(4)*: 344-349, 2003.

33 Ghaneh P, Kawesha A, Evans JD and Neoptolemos JP: Molecular prognostic markers in pancreatic cancer. J Hepatobiliary Pancreat Surg *9(1)*: 1-11, 2002.

34 Burris HA, 3rd, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, Cripps MC, Portenoy RK, Storniolo AM, Tarassoff P, Nelson R., Dorr FA, Stephens CD and Von Hoff DD: Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. J Clin Oncol *15(6)*: 2403-2413, 1997.

35 Kerbel RS: Tumor angiogenesis. N Engl J Med 358(19): 2039-2049, 2008.

36 Ferrara N, Heinsohn H, Walder CE, Bunting S and Thomas GR: The regulation of blood vessel growth by vascular endothelial growth factor. Ann NY Acad Sci *752*: 246-256, 1995.

37 Phung TL, Ziv K, Dabydeen D, Eyiah-Mensah G, Riveros M, Perruzzi C, Sun J, Monahan-Earley RA, Shiojima I, Nagy JA, Lin MI, Walsh K, Dvorak AM, Briscoe DM, Neeman M, Sessa WC, Dvorak HF and Benjamin LE: Pathological angiogenesis is induced by sustained Akt signaling and inhibited by rapamycin. Cancer Cell *10(2)*: 159-170, 2006.

38 Srinivasan R, Zabuawala T, Huang H, Zhang J, Gulati P, Fernandez S, Karlo JC, Landreth GE, Leone G and Ostrowski MC: Erk1 and Erk2 regulate endothelial cell proliferation and migration during mouse embryonic angiogenesis. PLoS One *4(12)*: e8283, 2009.

39 Yang X, Qiao D, Meyer K and Friedl A: Signal transducers and activators of transcription mediate fibroblast growth factor-induced vascular endothelial morphogenesis. Cancer Res *69(4)*: 1668-1677, 2009.
40 Zhang J, Cao R, Zhang Y, Jia T, Cao Y and Wahlberg E: Differential roles of PDGFR-alpha and PDGFR-beta in angiogenesis and vessel stability. FASEB J *23(1)*: 153-163, 2009.
41 Sanderson S, Valenti M, Gowan S, Patterson L, Ahmad Z, Workman P and Eccles SA: Benzoquinone ansamycin heat-shock protein 90 inhibitors modulate multiple functions required for tumor angiogenesis. Mol Cancer Ther *5(3)*: 522-532, 2006.
42 Kaur G, Belotti D, Burger AM, Fisher-Nielson K, Borsotti P, Riccardi E, Thillainathan J, Hollingshead M, Sausville EA and Giavazzi R: Antiangiogenic properties of 17-(dimethylamino-ethylamino)-17-demethoxygeldanamycin: an orally bioavailable heat shock protein 90 modulator. Clin Cancer Res *10(14)*: 4813-21, 2004.
43 Ostman A: PDGF receptors-mediators of autocrine tumor growth and regulators of tumor vasculature and stroma. Cytokine Growth Factor Rev *15(4)*: 275-286, 2004.
44 Bergers G, Song S, Meyer-Morse N, Bergsland E and Hanahan D: Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. J Clin Invest *111(9)*: 1287-1295, 2003.

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