Blocking heat shock protein-90 inhibits the invasive properties and hepatic growth of human colon cancer cells and improves the efficacy of oxaliplatin in p53-deficient colon cancer tumors in vivo

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Abstract
We recently showed that inhibition of heat shock protein 90 (Hsp90) decreases tumor growth and angiogenesis in gastric cancer through interference with oncogenic signaling pathways. However, controversy still exists about the antimetastatic potential of Hsp90 inhibitors. Moreover, in vitro studies suggested that blocking Hsp90 could overcome p53-mediated resistance of cancer cells to oxaliplatin. We therefore hypothesized that blocking oncogenic signaling with a Hsp90 inhibitor would impair metastatic behavior of colon cancer cells and also improve the efficacy of oxaliplatin in vivo. Human colon cancer cells (HCT116, HT29, and SW620) and the Hsp90 inhibitor 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG) were used for experiments. In vitro, 17-DMAG substantially inhibited phosphorylation of epidermal growth factor receptor, c-Met, and focal adhesion kinase, overall resulting in a significant decrease in cancer cell invasiveness. Importantly, 17-DMAG led to an up-regulation of the transcription factor activating transcription factor-3, a tumor suppressor and antimetastatic factor, on mRNA and protein levels. In a cell death ELISA, 17-DMAG markedly induced apoptosis in both p53-wt and p53-deficient cells. In vivo, 17-DMAG significantly reduced tumor growth and vascularization. Furthermore, blocking Hsp90 reduced hepatic tumor burden and metastatic nodules in an experimental model of hepatic colon cancer growth. Importantly, combining oxaliplatin with 17-DMAG in vivo significantly improved growth inhibitory and proapoptotic effects on p53-deficient cells, compared with either substance alone. In conclusion, inhibition of Hsp90 abrogates the invasive properties of colon cancer cells and modulates the expression of the antimetastatic factor activating transcription factor-3. Hence, targeting Hsp90 could prove valuable for treatment of advanced colorectal cancer by effectively inhibiting colon cancer growth and hepatic metastasis and improving the efficacy of oxaliplatin.

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Introduction
Invasion into adjacent tissues and metastasis to distant sites are major features of malignant cancer cells, which are complex processes that require coordinated actions of a large assortment of growth factors and their receptors, as well as downstream signaling intermediates (1). With respect to colorectal cancer metastasis, the epidermal growth factor (EGF) receptor (EGFR) system and c-Met, the primary receptor for hepatocyte growth factor/scatter factor (HGF), have been shown to play an important role (2–5). Both receptor systems are overexpressed in colorectal cancer and colorectal cancer liver metastases. In addition, both kinase systems are involved in carcinogenic processes such as cell proliferation, motility, survival, apoptosis, and tumor angiogenesis (3, 6–8). Hence, EGFR and c-Met systems represent interesting molecular targets for reducing metastasis and angiogenesis of colorectal cancer (4, 9). However, development of c-Met inhibitors has been challenging with studies currently ongoing.

Interestingly, inhibition of heat shock protein 90 (Hsp90) may be a novel approach to achieve effective interference with the function of both receptor systems and respective downstream signaling intermediates. Hsp90 is a molecular chaperone that is ubiquitously and abundantly expressed. There are numerous proteins involved in the control of physiologic and pathophysiologic processes that require Hsp90 for their biogenesis, regulation, and function (10, 11). In cancer, the expression of Hsp90 is increased at 2- to 10-fold higher levels when compared with that of normal tissues (12). Indeed, compounds that inhibit Hsp90 action, such as geldanamycin or its derivates 17-allylamino-17-demethoxygeldanamycin (17-AAG) and 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG),
have been shown to effectively inhibit tumor growth in several preclinical xenograft models (13–16), and the efficacy of 17-AAG is currently being investigated in cancer patients in clinical phase I/II trials (17–20).

Importantly, a number of cancer-associated proteins have been identified as Hsp90 clients, including EGFR family members, c-MET, AKT, mutant p53, mitogen-activated protein kinase (MAPK)/extracellular signal–regulated kinase (Erk), and the transcription factor hypoxia-inducible factor-1α (8, 21–23). Because these are important participants in pathways driving tumor cell survival, proliferation, and progression, Hsp90 seems to be an excellent target in cancer therapy (22, 24, 25). In a previous study, we showed that blocking Hsp90 significantly interferes with EGFR signaling in gastric cancer, which, in part, is mediated through down-regulation of EGFR itself (26). Moreover, we also showed that targeting multiple pathways does not require therapy with Hsp90 inhibitors at maximum tolerated doses to achieve potent inhibition of tumor growth and angiogenesis. Nevertheless, controversies still exist about the antimetastatic potential of Hsp90 inhibitors. In one report, Price et al. (27) have shown an enhanced incidence of bone metastasis and osteolytic lesions with 17-AAG treatment in a human breast cancer mouse model. This crucial aspect has not been investigated before initiating clinical trials with Hsp90 inhibitors for patients in clinical phase I/II trials (17–20).

Importantly, an increase in the inhibition of MAPK/Erk kinase 1/2, phospho-Ser172/181 MAPK/Erk kinase 1/2, Akt, phospho-Thr308 Akt, p44/42 MAPK, phospho-Tyr202/204 p44/42 MAPK, signal transducer and activator of transcription-3, phospho-Tyr385 signal transducer and activator of transcription 3, focal adhesion kinase (FAK), phospho-Tyr569 FAK, phospho-Tyr319 c-Met, and EGFR were purchased from Cell Signaling Technologies. Anti-β-actin and anti–activating transcription-factor-3 (ATF3) antibodies were purchased from Santa Cruz Biotechnology. β-Actin serves as a loading control in Western blot analyses. Recombinant human EGF and HGF were obtained from R&D Systems.

Materials and Methods

Cells and Culture Conditions

The human colorectal cancer cell lines HCT116, SW620, and HT29 were obtained from American Type Culture Collection. Cells were cultured in RPMI 1640 or DMEM (Life Technologies, Inc.) supplemented with 20% FCS (HCT116 and SW620) or 10% FCS (HT29) and were maintained in 5% CO2 at 37°C. The human colorectal cancer cell lines HCT116, HT29, and SW620 were seeded into 96-well plates (1 × 103 per well) and exposed to various concentrations of 17-DMAG for 24 or 48 h at 37°C. We used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay to assess cell viability, as previously described (30).

Immunoblot Analysis of Signaling Intermediates

To determine the effects of Hsp90 inhibition on signaling intermediates, cancer cells were incubated for 16 h with FCS-RPMI containing 17-DMAG (250 nmol/L) before stimulation with either recombinant human EGF (100 ng/mL, 10 and 30 min) or HGF (50 ng/mL, 10 and 30 min) under serum-reduced conditions (1% FCS-RPMI). Pretreatment of cells for a minimum of 16 h is required to down-regulate/inhibit Hsp90 client proteins, as recently reported (8, 16, 26). Whole-cell lysates were prepared, as described elsewhere (30). Protein samples (75 μg) were subjected to Western blotting on a denaturing 10% SDS-PAGE.

ELISA for Vascular Endothelial Growth Factor-A Secretion

To determine changes in vascular endothelial growth factor (VEGF)-A secretion by cancer cells, we used an ELISA kit specific for human VEGF-A (BioSource Europe), as previously described (30). Colorectal cancer cells were plated at 40% to 50% density and incubated with or without 17-DMAG (250 nmol/L) for 24 or 48 h. Analyses of culture supernatants were done according to the manufacturers’ protocol. After harvesting the cells by trypsinization, they were counted. Detected VEGF-A levels were calculated as picograms per milliliter per 1,000 viable cells.

Real-time PCR analyses

For real-time PCR, total RNA was isolated using Trizol Reagent (Invitrogen) and subsequently purified by ethanol precipitation. For each RNA sample, a 1-μg aliquot was reverse transcribed into cDNA using the Superscript II Kit (Qiagen). Primer pairs were as follows: VEGF165, 5′-GCACCCATGGCAGAAGGAG and 3′-TGAGCCCGGACAATACAC. Primers were optimized for MgCl2 and annealing, and PCR products were confirmed by gel electrophoresis. Real-time PCR was done using the
LightCycler system and Roche Fast-Start Light Cycler-Master Hybridization Probes master mix (Roche Diagnostics). Changes in VEGF-A expression were determined after 24 and 48 h of exposure to 17-DMAG (250 nmol/L).

**Migration and Invasion Assays**

To determine the effect of 17-DMAG treatment on cancer cell motility in vitro, migration assays were done using modified Boyden chambers, as described elsewhere (2). Briefly, 1 × 10^5 cells were resuspended in 1% FCS-RPMI and seeded into inserts with 8-μm filter pores, which were either uncoated (migration assay) or Matrigel coated (invasion assay; Becton Dickinson Bioscience). 1% FCS-RPMI, with or without EGF (100 ng/mL) or HGF (50 ng/mL) added, served as a chemoattractant. Cells were fixed and stained (Diff-Quick reagent, Dade Behring) after 48 h, counted in four random fields, and average numbers were calculated. For experiments, two different doses of 17-DMAG (100 and 250 nmol/L) were investigated to minimize the bias through cytotoxic effects in this particular assay.

**Scratch Assay**

To validate experiments on cell migration, scratch assays were done. In brief, cancer cells were plated into six-well cell culture plates and grown to 90% density in 10% FCS-containing RPMI medium within 24 h. Plates were then rinsed with serum-free medium and cells subsequently serum starved overnight. One 2-mm-wide scratch was drawn across the cell layer using a pipette tip. After rinsing with serum-free medium, RPMI medium containing 250 nmol/L 17-DMAG was added to the cells. Plates were photographed after 6, 24, and 48 h and scratch width was measured.

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**Figure 1.** Effect of Hsp90 inhibition on signaling in colorectal cancer cells. Western blot analyses for activated signaling intermediates. A, HCT116 cells were treated with 17-DMAG (16 h) and subsequently stimulated with EGF for indicated time points. 17-DMAG down-regulated EGFR and inhibited downstream signaling. B, HCT116 and SW620 (bottom) cells were treated with 17-DMAG (16 h) and subsequently stimulated for the indicated time points with HGF. Blocking Hsp90 reduced HGF-mediated activation of c-Met. In addition, phospho-MAPK/Erk kinase (Mek), phospho-p44/42 MAPK, phospho-Akt, phospho-signal transducer and activator of transcription 3 (STAT3), and phospho-FAK were diminished. β-Actin served as loading control. All results were confirmed in a second colorectal cancer cell line (SW620). C, HCT116 were exposed to 17-DMAG for indicated time. By Western blotting, blocking Hsp90 led to an increase in ATF3 protein expression. D, real-time PCR showed that treatment with 17-DMAG also induced ATF3 mRNA expression in colon cancer cells (HCT116 and SW620). ATF3 mRNA expression is normalized to β-actin.
were either paraffin embedded or snap frozen.

In the growth of colorectal liver metastases, HCT116 cells (10 mg/kg; twice per week), respectively. Tumor volumes dose 17-DMAG (15 mg/kg/d; ref. 26) and/or oxaliplatin combination with chemotherapy on the growth of colorectal cancer cells. Treatment with 17-DMAG and/or oxaliplatin to evaluate the effects of Hsp90 inhibition in implanted into the subcutis (right flank) of BALB/c nude mice to determine the specific enrichment of mononucleosomes and oligonucleosomes released into the cytoplasm (enrichment factor) of the treated cells, the following formula was used:

\[
\frac{\text{milliunits of the sample (dying/dead cells)/milliunits of corresponding negative control}}{2}
\]

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counted in four fields per tumor section at 26. For immunohistochemical analyses, tumors were either paraffin embedded or snap frozen.

The effects of Hsp90 inhibition on the growth of human colorectal cancer cells were evaluated in a s.c. xenograft model, as described (26). The water-soluble Hsp90 inhibitor 17-DMAG was used for these experiments. In brief, 1 × 10⁶ human colorectal cancer cells (HCT116) were injected into the subcutis (right flank) of nude mice. After implantation, tumors were allowed to grow 7 days (volume ~ 50 mm³) before treatment was initiated. Mice were randomized into one of two groups (n = 9–10 per group) receiving either vehicle (saline) or 17-DMAG (15 mg/kg/d). Tumor diameters were measured every other day, and tumor volumes were calculated (width² × length × 0.5). For immunohistochemical analyses, tumors were either paraffin embedded or snap frozen.

Hepatic Tumor Growth. In a following orthotopic experiment to determine the effects of Hsp90 inhibition on the growth of colorectal liver metastases, HCT116 cells (1 × 10⁶) were injected into the liver of athymic BALB/c nude mice. After randomization into one of two groups, treatment with 17-DMAG 15 mg/kg/d or vehicle (saline) was started on day 7. On day 28, following necropsy, liver weights were measured and nodules counted.

Chemotherapy Model. SW620 cells (2 × 10⁶) were implanted into the subcutis (right flank) of BALB/c nude mice to evaluate the effects of Hsp90 inhibition in combination with chemotherapy on the growth of colorectal cancer cells. Treatment with 17-DMAG and/or oxaliplatin versus vehicle (saline) was started on day 9 after tumors were established. Mice were treated with low-dose 17-DMAG (15 mg/kg/d; ref. 26) and/or oxaliplatin (10 mg/kg; twice per week), respectively. Tumor volumes were determined every other day as described above.

Immunohistochemical Analyses of Tumor Vascularization, Cancer Cell Proliferation, and Apoptosis

Multiple cryosections and paraffin-embedded sections were obtained from tumors for all immunohistochemical analyses. CD31-positive vessel area was assessed with rat anti-mouse CD31/platelet-endothelial cell adhesion molecule 1 antibody (PharMingen) and peroxidase-conjugated goat anti-rat IgG (The Jackson Research Laboratory) on frozen sections, as previously described (30). Antibody binding was visualized with stable diaminobenzidine. 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 done by converting images to grayscale and setting a consistent threshold for all slides using ImageJ software (version 1.33, NIH). Vessel areas were expressed as pixels per high-power field (26). To determine the amount of proliferating tumor cells, mice received i.p. injections of bromodeoxyuridine (BrdUrd; Sigma-Aldrich; 1 mg/mouse) 2 h before termination of animal studies. A commercially available BrdUrd detection kit (Becton Dickinson) was used to visualize BrdUrd uptake of cells in sections of tumors. Briefly, sections were incubated with anti-BrdUrd antibody solution, followed by streptavidin-conjugated horseradish peroxidase–linked goat anti-mouse IgG2. Antibody binding was visualized by incubating slides in diaminobenzidine, with the aid of hematoxylin counterstaining. BrdUrd-positive tumor cells were counted in four fields per tumor section at ×20 magnification and averages were calculated (26). For analyses of tumor cell apoptosis, a commercially available terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling detection kit was used (Promega Corp.) according to the manufacturer’s instructions. Briefly, the 3’OH DNA ends were labeled with a biotinylated nucleotide, coupled with horseradish peroxidase–labeled streptavidin, and then detected hydrogen peroxide and diaminobenzidine. Apoptotic nuclei were stained dark brown. Four fields at ×40 magnification were selected in each tumor at the proliferation front, and the terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling–positive cells were counted. Data are expressed as number of apoptotic cells per high-power field. Using ImageJ software (NIH), necrotic areas of the tumor sections were measured. Results are expressed as percentage of necrosis in relation to total tumor area.

Statistical Analyses and Densitometry

Results of in vivo experiments were analyzed for significant outliers using the Grubb’s test for detecting outliers.4 Tumor-associated variables in in vivo experiments were tested for statistical significance using the Mann-Whitney U test for nonparametric data. The two-sided Student t test was applied for analysis of in vitro data. All results are expressed as the mean ± SE.

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Effects of Hsp90 Inhibition on EGFR and c-Met Signaling in Human Colon Cancer Cells

Because the EGF and c-Met receptor systems both represent important mediators of colon cancer growth and metastasis (2, 4, 9), we focused on investigating the effects of 17-DMAG on EGF- and HGF-induced signaling pathways in human colon cancer cells (HCT116 and SW620). Treatment with 17-DMAG substantially disrupted EGF signaling in terms of EGF R down-regulation, inhibition of MAPK/Erk kinase activation, and diminishing downstream phosphorylation of the substrates Erk1/2 and Akt (Fig. 1A). Moreover, constitutive signal transducer and activator of transcription-3 phosphorylation was diminished on Hsp90 blockade. Importantly, inhibition of Hsp90 with 17-DMAG (250 nmol/L) significantly abrogated the EGF- and HGF-mediated invasive properties of colorectal cancer cells (HCT116) compared with control (*, P < 0.01, for all). Experiments were done in triplicates.

As a functional consequence of multiple signaling pathway and receptor inhibition and up-regulation of ATF3, 17-DMAG significantly inhibited EGF- and HGF-induced cancer cell migration and invasiveness, as determined in modified Boyden chambers (Fig. 2A and B). In scratch assays, treatment of HCT116 cell with 17-DMAG markedly impaired cell migration, compared with controls, which had completely filled the gap within 24 h (data not shown). Moreover, as another consequence, blocking Hsp90 also significantly reduced constitutive VEGF secretion in colon cancer cells after 24 and 48 h of treatment (Supplementary Fig. S1). These ELISA results were

5 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
additionally confirmed by real-time PCR (data not shown). Together, these experiments provide evidence that blocking Hsp90 in colon cancer cells not only affects tumor angiogenesis but also harbors the potential to reduce colon cancer metastasis through direct interference with promigratory signaling components.

**Effect of Hsp90 Inhibition on s.c. and Hepatic Tumor Growth In vivo**

To estimate the effects of Hsp90 blockade on colon cancer growth and vascularization in vivo, we first used a s.c. xenograft model of colon cancer (HCT116). Treatment with 17-DMAG (15 mg/kg/d, i.p.) significantly inhibited tumor growth of p53-wt colon cancer cells (Fig. 3A). This was also reflected by measuring final tumor weights, which also were significantly reduced in 17-DMAG–treated mice (data not shown). Importantly, as determined by immunohistochemical staining, therapy with 17-DMAG significantly reduced tumor vascularization (CD31-positive vessels; Fig. 3B) and tumor cell proliferation (BrdUrd-positive cells; Fig. 3C).

Next, we addressed the question on whether Hsp90 inhibition would also be effective in reducing the growth of colon cancer cells in the liver because the microenvironment plays a critical role for the efficacy of targeted therapies. For this purpose, human colon cancer cells were directly implanted into the liver of mice. Indeed, treatment with 17-DMAG significantly reduced hepatic tumor burden, compared with controls, as reflected by liver weights at the end of the experiment (Fig. 4A). Normal non–tumor-bearing mice served as an additional control, showing that...
17-DMAG (15 mg/kg/d) itself did not significantly alter the liver weight (data not shown). In addition to measuring liver weights of tumor-bearing mice, hepatic tumor nodules were counted, revealing that blocking Hsp90 with 17-DMAG significantly reduced the number of hepatic metastases (Fig. 4B). Mouse body weights did not statistically differ among treatment groups. We conclude from these experiments that targeting Hsp90 inhibits both s.c. ("primary") and hepatic growth of colon cancer. Our data suggest that this inhibition is directly on the tumor cells and indirectly through inhibition of angiogenesis.

**Effect of Hsp90 Inhibition on Susceptibility of Colon Cancer Cells to Chemotherapy In vitro and In vivo**

The potential of Hsp90 inhibitors to improve susceptibility to chemotherapy has been suggested by studies in other cancer entities; however, the effects of 17-DMAG on the chemosensitivity of colon cancer cells to oxaliplatin have not been fully investigated to date. It has been reported that Hsp90 inhibitors promote oxaliplatin-dependent caspase activation and cytotoxicity by down-regulating antiapoptotic signaling through the transcription factor nuclear factor αB in colon cancer cell lines (28). Moreover, a recent study showed that Hsp90 inhibitors harbor the potential to inhibit G1-S transition in p53-deficient colon cancer cells, thereby enhancing cell cycle effects of oxaliplatin (29). Hence, we sought to investigate whether these solely

![Figure 4](https://example.com/figure4.jpg)

**Figure 4.** Effect of Hsp90 inhibition of hepatic growth of colon cancer cells. HCT116 cells were injected into the livers of BALB/c nude mice (1 × 10⁶ per mouse; n = 7–10 per group). Treatment with 17-DMAG 15 mg/kg/d (i.p. injection) was started on day 7. On day 28, necropsy liver weights were measured and nodules counted. **A**, treatment with 17-DMAG significantly inhibited hepatic tumor burden, as reflected by final liver weights (*, P < 0.05). **Columns**, mean; **bars**, SE. **B**, therapy with 17-DMAG significantly reduced hepatic tumor nodules compared with controls (bar, median; *, P < 0.05).
inhibitors can be used for inhibiting colon cancer growth and angiogenesis and for improving the antineoplastic efficacy of oxaliplatin on \( p53 \)-mutated tumors \textit{in vivo}.

**Discussion**

In this study, we show that inhibition of Hsp90 harbors the potential to substantially decrease the migratory and invasive properties of colon cancer cells, which, in part, is mediated through inhibition of EGFR, c-Met, and FAK. Moreover, we provide the first evidence that Hsp90 inhibitors are effective in reducing the hepatic growth of colon cancer and in improving the efficacy of oxaliplatin in \( p53 \)-deficient colon cancer tumors \textit{in vivo}.

The fact that Hsp90 is an interesting molecular target for cancer therapy has been reported and validated in various preclinical studies. Indeed, the antineoplastic efficacy of geldanamycin derivates and other synthetic inhibitors is still under active development. However, controversy continues about the antimetastatic potential of Hsp90 inhibitors. We and others have shown that certain signaling components, such as EGFR, c-Met, and FAK, are inhibited by 17-AAG/17-DMAG treatment, hence leading to reduced cancer cell migration \textit{in vitro}. However, our study is the first to show that blocking Hsp90 leads to a marked up-regulation of a transcription factor that has been considered to function as a tumor suppressor and antimetastatic factor ATF3. ATF3 may act both as an inducer and as a repressor.

**Figure 5.** Effect of Hsp90 inhibition on induction of apoptosis in colorectal cancer cells. Cell Death Detection ELISA kit was used to measure DNA fragmentation as a marker for apoptosis. After 24 h, cells were treated with 17-DMAG (250 nmol/L) and/or oxaliplatin (1 \( \mu \)mol/L) and allowed to grow for an additional 24 and 48 h. The specific enrichment of mononucleosomes and oligonucleosomes released into the cytoplasm of the treated cells was additionally calculated (enrichment factor). A, in HCT116 cells (\( p53 \)-wt), combination therapy led to improved apoptotic effects compared with either substance alone at 24 h. In \( p53 \)-deficient cells, such as HT29 colon cancer cells (B) and SW620 cells (C), 17-DMAG seemed to be superior to oxaliplatin. Columns, fold induction of DNA fragmentation.
of transcription for genes such as \textit{gadd153}/Chop10, which is associated with cell growth (34), and matrix metalloproteinase-2, which is associated with invasion (32, 35). Importantly, ATF3 is repressed in colon cancer, indicating that it may be involved in the tumorigenic process. Bottone et al. (31) recently showed that the anti-invasive activity of cyclooxygenase-2 inhibitors or nonsteroidal anti-inflammatory drugs involves up-regulation of ATF3. RNA interference of ATF3 led to a significant increase in the invasive properties of cancer cells, suggesting that ATF3 acts as a repressor of metastasis. Nevertheless, to date, \textit{in vivo} effects are not quite clear because Price et al. (27) have elegantly shown that Hsp90 inhibitors promote the formation of bone metastases through activation of osteoclasts in an experimental model of breast cancer. On the other hand, because formation of bone metastases is not a predominant phenomenon of colon cancer, we suggest that the effect of Hsp90 blockade on the metastatic potential of cancer cells is highly dependent on tumor entity and tumor cell line. The role of Hsp90 inhibitors for reducing the hepatic growth of colon cancers has not been investigated to date. Formation of colorectal liver metastases represents a relevant clinical problem, and only few patients qualify for surgery. With results from this study, we provide evidence that blocking Hsp90 could indeed decrease hepatic “metastatic” tumor growth of colon cancer, thus offering a novel approach for targeted therapy concepts for downsizing of colorectal cancer liver metastases.

Interestingly, the presence of \textit{p53}-deficiency has been associated with an increased incidence of liver metastasis and progression in patients with colorectal cancer (36). In addition, \textit{p53} status has been shown to be relevant for susceptibility of colon cancer cells to chemotherapy with oxaliplatin. Because oxaliplatin is now widely used for
multimodality regimens in treating advanced or metastatic (liver) colorectal cancer, we focused on investigating the effects of 17-DMAG in combination with oxaliplatin on $p53$-wt and $p53$-deficient colon cancer cells. This aspect has, in part, been addressed in a recent study by Rakitina et al. (28), showing that the geldanamycin derivate 17-AAG could prevent S-phase accumulation (G1-S transition), which is induced by oxaliplatin in $p53$-deficient colon cancer cells (HT29), thereby improving apoptotic effects by 17-AAG in vitro. In an earlier study, authors similarly used a cell death detection ELISA to detect effects of Hsp90 inhibition with or without addition of oxaliplatin on colon cancer cell apoptosis. In concordance with our results, Rakitina et al. (28) found that combination therapy may lead to additive/synergistic apoptotic effects in HCT116 cells ($p53$-wt). However, in our hands, $p53$-deficient cells solely responded to 17-DMAG whereas only a modest effect of oxaliplatin alone was detectable. A critical determinant for interpreting results seems to be the time point when evaluating apoptosis (24 versus 48 h). The best apoptotic effect achievable in $p53$-deficient cells was in our experiments observed with 17-DMAG after 48 h, but we did not detect clear synergistic effects by combination therapy in such $p53$-deficient cells in vitro. However, Rakitina et al. (28) also showed that interference with the nuclear factor-$\kappa$B pathway through Hsp90 inhibitors improves susceptibility to oxaliplatin, suggesting that these potential additive or synergistic effects might be more complex and difficult to detect by in vitro analyses. However, the hypothesis that in vivo the efficacy of oxaliplatin could be substantially improved by adding Hsp90 inhibitors to the regimen had not been tested to date. In our first model, we showed that 17-DMAG could decrease hepatic growth of colon cancer cells in vivo; hence, we subsequently addressed the question of whether combination therapy indeed would be more effective in vivo. For this purpose, we again used a s.c. tumor model because continuous evaluation of tumor growth in the liver is challenging. With this study, we were able to show for the first time that 17-DMAG, administered below the maximum tolerated dose (26), improves the antineoplastic efficacy of oxaliplatin in $p53$-deficient colon cancer cells in vivo. Moreover, the (indirect) antiangiogenic potential of Hsp90 inhibition was confirmed by CD31 staining in the 17-DMAG therapy groups, suggesting that blocking Hsp90 can improve antineoplastic effects of oxaliplatin and lead to inhibition of tumor angiogenesis in colon cancer. Importantly, by analyses for tumor cell apoptosis and by measuring necrotic areas in tumors, we confirmed that combining oxaliplatin with a Hsp90 inhibitor indeed leads to improved antineoplastic efficacy in vivo. In contrast to this finding, the antiangiogenic effect of Hsp90 inhibitors has been suggested by a few studies (24, 26, 37, 38). Notably, in one study, Sanderson et al. (39) showed that 17-AAG, administered at maximum tolerated dose, significantly reduces the expression of three VEGF receptors (VEGF-R1, VEGF-R2, and VEGF-R3) in mouse vasculature in vivo. However, we did not detect changes in vascular morphology or permeability, suggesting that Hsp90 inhibitors, administered below maximum tolerated dose, might not have a biologically significant direct effect on vasculature in vivo.

In conclusion, this present study shows that inhibition of Hsp90 abrogates the invasive properties of colon cancer cells in vitro and effectively inhibits growth, angiogenesis, and hepatic tumor growth in vivo. Because $p53$ status in colon cancer cells is a relevant determinant of susceptibility to oxaliplatin, and Hsp90 inhibition leads to the improved efficacy of oxaliplatin in $p53$-deficient cancer cells in vivo, our data suggest Hsp90 antagonists to be valuable for improving therapy of advanced colorectal cancer and for the (neo-adjuvant) therapy of colorectal liver metastases. Further studies will be required to define the risk of surgery when treating with novel Hsp90 inhibitors perioperatively.

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References


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