Tumor cells can't stand the heat

*Boosting the effectiveness of hyperthermia in cervical carcinoma*

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Chapter 9

HSP90-inhibitor Ganetesbip potentiates radiosensitizing and chemosensitizing effects of hyperthermia

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Abstract

Hyperthermia (HT) - application of supra-physiological temperatures to cells, tissues or organs - is a pleiotropic treatment that affects most aspects of cellular metabolism, but its effects on DNA are of special interest in the context of cancer research and treatment. HT inhibits repair of various DNA lesions, including double strand breaks (DSBs), making it a powerful radiosensitizer and chemosensitizer, with proven clinical efficacy in therapy for various types of cancer, including tumors of head, neck, bladder and cervix. Despite the positive results of multiple clinical trials, some patients subjected to treatment protocols that include HT do not respond to therapy or experience relapse of the disease. Therefore, approaches that boost HT efficacy may improve treatment outcome. Here, we show that treatment with a relatively low dose of HSP90-inhibitor Ganetespib during a single, short period of time, potentiates cytotoxic as well as radiosensitizing and chemosensitizing effects of HT in cervix cancer cell lines. Treatment with Ganetespib alone had virtually no effect on survival of non-heated cells. Our results thus suggest that HSP90 inhibition is a straightforward and efficient approach to improve HT treatment efficacy with no or limited additional systemic toxicity, paving the way for studies in vivo.
Introduction

DNA damaging agents, such as ionizing radiation, topoisomerase inhibitors, DNA intercalators or cross-linkers, are among the most effective anti-cancer agents exploited in diverse clinical relevant therapies. However, to maintain the integrity of genetic information, cells evolved intricate DNA repair mechanisms. These mechanisms are essential for survival and reproduction in healthy cells, but they also protect DNA of cancer cells against genotoxic agents of endogenous origin, as well as from those used in the clinic, effectively increasing their resistance to therapy (Helleday et al. 2008).

DNA double strand breaks (DSBs) are arguably the most dangerous DNA lesions induced by anti-cancer treatments. In mammalian cells, DSB repair is executed by two major pathways, non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ is a robust and conceptually simple mechanism, active throughout the cell cycle, that relies on a direct rejoining of the broken DNA ends, often at the cost of inducing nucleotide deletions or insertions (Davis & Chen 2013). Major players involved in the classical NHEJ pathway (c-NHEJ) include the KU70/KU80 heterodimer, DNA-PKcs kinase, and XRCC4/LIG4 complex. The backup NHEJ pathway (b-NHEJ) relies on poly-(ADP-ribose)-polymerase 1 (PARP1), MRE11, CtIP, Polθ and LIG3 and sustains NHEJ activity in the absence of c-NHEJ.

In contrast to NHEJ, HR is a precise mechanism that can utilize an intact DNA fragment as a repair template (Jasin & Rothstein 2013). HR is initiated by the resection of DNA ends, resulting in 3’ single stranded overhangs, which are instantly coated by RPA. RAD51, aided by BRCA2 and other accessory factors like PALB2, subsequently evicts and replaces RPA. The RAD51 nucleoprotein filament then searches for and invades the homologous DNA that serves as a template for DNA synthesis. Resolution of the heteroduplex structures is followed by filling of the remaining gaps and ligating strand ends. The activity of HR is tightly coupled to cell cycle progression and is limited to the S and G2 phases of the cell cycle.

Given the involvement of DSB repair in the resistance to DNA-damaging agents, inactivation of NHEJ and HR in cancer cells could increase their sensitivity to therapy. Small molecules designed to interfere with various DNA repair pathways have been developed and are being tested in multiple preclinical and clinical studies (Helleday et al. 2008), with notable example of PARP1-inhibitors (Lord et al. 2015). Despite considerable efforts, however, safe, potent, selective and bioavailable inhibitors of DSB repair have yet to emerge (Gavande et al. 2016).

Hyperthermia (HT) - elevation of the tumor temperature above physiological levels, usually to 40-42.5°C - is a clinically applied anti-cancer therapy
that affects multiple aspects of cellular metabolism, including DNA repair (Oei et al. 2015). HT is an excellent radiosensitizer and chemosensitizer, as demonstrated by in vitro and in vivo studies, as well as by randomized clinical trials (Issels 2008; Issels et al. 2010; Oei et al. 2015). One important feature of HT is that its application can be limited to the tumor volume, while sparing most of the non-transformed surrounding tissues. Notably, HT efficiently inhibits HR, likely by inducing degradation of its essential protein BRCA2 (Krawczyk et al. 2011; Genet et al. 2013), as well as NHEJ, possibly in part by affecting DNA-PK (Ihara et al. 2014). This may explain how HT sensitizes cells to agents such as ionizing radiation or cisplatin, because DNA lesions induced by these agents require HR and NHEJ for repair.

The radiosensitizing and chemosensitizing effects of HT are desirable in anti-cancer therapies, but they are counteracted by chaperone proteins that protect cells from the effects of various forms of stress, including heat. Heat shock proteins (or HSPs) are a subgroup of chaperone proteins that strongly respond to increased temperatures to regulate various genes and metabolic pathways as well as to physically protect their client proteins from heat-induced unfolding, inactivation and degradation (Calderwood & Ciocca 2008). One member of this group, HSP90, is of special interest in the context of cancer treatment and HT. HSP90 is an evolutionarily conserved chaperone, crucial in mammalian proteostasis, with affinity for a vast number of client proteins (Taipale et al. 2014). In addition to its important functions in normal cells, the chaperone can promote stability of misfolded oncogenic proteins in cancer cells, allowing the development of oncogene addiction and therapy resistance (Whitesell et al. 2014). Inhibition of this chaperone affects the stability of some essential DNA repair factors, including BRCA1, BRCA2, RAD51, CHK1 and DNA-PKcs (Pennisi et al. 2015). Recently, we reported that inhibition of HSP90 can enhance the effects of HT on DSB repair, likely, at least in part, by stimulating HT-induced degradation of BRCA2 (Krawczyk et al. 2011).

Here we show that Ganetespib, a new-generation HSP90-inhibitor, enhances the induction of DNA damage and cell killing by HT. Moreover, we demonstrate that Ganetespib potentiates HT-induced sensitization of cervix cancer cells to a number of DSB-inducing agents, suggesting that HSP90 inhibition is a simple and effective strategy to improve the outcomes of clinical treatments involving HT.

Results

HSP90-i Ganetespib potentiates the inhibitory effects of HT on HR
The semi-synthetic derivative of the antibiotic geldanamycin 17-DMAG is a HSP90-inhibitor that stimulates degradation of BRCA2 and inactivation of HR by
Figure 1. HSP90-inhibitor Ganetespib potentiates the inhibitory effects of HT on HR. (A) SiHa cells were incubated with the indicated concentrations of Ganetespib (HSP90-i) for 30 min at 37 °C, then for an additional hour at 37 or 42 °C. Next, cells were lysed and lysates were analyzed by western blotting, using antibodies against BRCA2 and Cyclin A (loading control). (B) Cells were treated as in (A), except Ganetespib was used at a concentration of 30 nM. At the indicated time after treatment, cells were irradiated with α-particles, fixed 30 min later and stained using antibodies against γ-H2AX (red) and RAD51 (green). The pictures are representative for cells irradiated 6 h after the treatment. The graphs show average percentage of cells containing tracks of γ-H2AX foci that were also positive for RAD51.
HT (Krawczyk et al. 2011). 17-DMAG also potentiates hyperthermic sensitization of cancer cells to ionizing radiation \textit{in vitro} and to PARP1 inhibition \textit{in vitro} and \textit{in vivo}. Importantly, the drug showed only limited cytotoxicity as a single agent, suggesting that HSP90 inhibition is an effective approach to potentiate effects of HT.

In the current study, we focused on Ganetespib, a new-generation, specific and well-tolerated HSP90-inhibitor that has been extensively studied \textit{in vitro} in animal models and in multiple clinical trials (Proia & Bates 2014). Since HT is routinely applied to a subset of cervical cancer patients (Datta et al. 2016), we used two cervical cancer cell lines, SiHa and HeLa. To confirm that Ganetespib promotes the inhibitory effects of HT on DSB repair, we first analyzed HT-induced changes in the levels of BRCA2 protein. As expected, we found that treatment for 60 min at 42°C reduced the levels of BRCA2 (Figure 1A). Importantly, addition of Ganetespib 30 min prior and during to the HT treatment further enhanced BRCA2 degradation in a dose-dependent manner. We found that 1.5 h treatment with Ganetespib alone (up to 100 nM) had only modest effects on clonogenic cell survival, but this was enhanced after HT, at least at Ganetespib concentrations exceeding 3 nM (Figure S1A). We therefore decided to use 30 nM concentration of Ganetespib in the subsequent experiments. One of the hallmarks of HT-induced HR deficiency is a disturbed accumulation of RAD51 at sites of DSBs (Krawczyk et al. 2011; Genet et al. 2013). Indeed, we found that HT temporarily inhibited recruitment of RAD51 to α-particle-induced DSBs. This effect was enhanced by Ganetespib, as RAD51 accumulation was impaired for considerably longer periods of time, in both SiHa and HeLa cells (Figure 1B and S1B). Treatment with Ganetespib alone did not affect RAD51 accumulation. These results confirm that Ganetespib can potentiate the inhibitory effects of HT on HR in cervical cancer cells, \textit{in vitro}.

**Inhibition of HSP90 enhances induction of DNA damage by HT**

HT has been suggested to induce DSBs, in at least two ways (Velichko et al. 2012). First, the elevated temperature may induce damage directly, as heating leads to the focal accumulation of some repair factors, which is considered to mark sites of ongoing DSB repair (Rogakou et al. 1998; Rogakou et al. 1999). Second, HT has been shown to inhibit topoisomerase 1 (TOP1) cleavage complexes, which may lead to DSB formation when replication forks collide with TOP1-induced single stranded breaks (SSBs) in the S-phase following HT treatment (Velichko et al. 2015). To examine whether HSP90 inhibition can potentiate these effects as well, we first analyzed the induction of γ-H2AX foci at various time points (up to 48 h after HT treatment). We observed, in both cell lines, that HT led to increased frequencies of foci-containing cells immediately after treatment, and that these frequencies diminished at later time points (Figure 2A and S2A). After treatment with Ganetespib alone, the frequencies of foci-positive cells...
Figure 2. Inhibition of HSP90 enhances induction of DNA damage by HT. Cells were incubated for 30 min with 30 nM Ganetespib (HSP90-i) at 37°C, then for 60 min at 37 or 42°C (HT). Medium was refreshed and cells were incubated at 37°C for the indicated time period, fixed and stained for γ-H2AX (A) and DNA (B). (A) Average percentages of cells with more than 5 γ-H2AX foci after the indicated treatments. (B) The pictures (top panel) show micronuclei (MN)-containing HeLa cells at 48 h. The graphs (bottom panel) show the average frequencies of MN-containing cells.
were not affected immediately, but they did moderately increase at later time points (2-24 h), only to return to the baseline at 48 h after treatment. Combination of HT with Ganetespib led to a further increase of foci-positive cell frequencies, especially at later time points (at 16 and 24 h after treatment). γ-H2AX is an indirect marker of DSB formation and, especially after HT, it may mark sites of other lesions (Hunt *et al.* 2007).

Therefore, we subsequently measured the induction of micronuclei (MN), which are a direct consequence of unrepaired DSBs (Fenech *et al.* 2011)(Figure 2B). Our results show that HT alone increased MN+ cell frequency at times >8 h after treatment, strongly suggesting induction of DSBs. In contrast, treatment with Ganetespib alone did not significantly affect MN+ cell numbers, whereas combinational treatment (HT + Ganetespib) led to an increased MN induction at later time points (at 24 and 48 h), as compared to HT alone (Figure 2B and S2B). The frequency of MN+ cells after the combinational treatment at 48 h was 3.5-fold (HeLa cells) and 6-fold higher (SiHa cells) than in untreated control cells. Combined, these results strongly suggest that HT does induce DSBs, probably indirectly, and that this effect is potentiated by HSP90 inhibition.

**Inhibition of HSP90 enhances radiosensitizing and chemosensitizing effects of HT**

Since HSP90 inhibition enhances the effects of HT on DSB repair, we investigated whether Ganetespib can potentiate cytotoxicity of treatment combining various DSB-inducing agents (radiation and chemotherapy) with HT. We focused on ionizing radiation (RT), cisplatin (cDDP), gemcitabine (Gem.) and etoposide (Etop.) - chemotherapeutics that are known to induce DSBs and that are relevant in clinical cancer treatment (Galmarini *et al.* 2002; Larsen *et al.* 2003; Dasari & Tchounwou 2014). To measure the cytotoxic effects of these agents, alone or in combination with HT/Ganetespib, we performed clonogenic assays (Figure 3). RT directly induces DSBs that are then repaired by NHEJ or HR, depending on the cell cycle phase and HT has been shown to sensitize cancer cells and tumors to RT (Overgaard *et al.* 1995). When combined with RT alone, Ganetespib did not induce additional cytotoxicity (Figure 3B), suggesting that short
Figure 3. Inhibition of HSP90 enhances radiosensitizing and chemosensitizing effects of HT. (A) Schematic overview of the treatment schedule for the clonogenic assay, results of which are presented in B-E. (B) Normalized clonogenic survival fraction after increasing doses of ionizing radiation (RT) alone or combined with hyperthermia (HT) and/or Ganetespib (HSP90-i). Bar graphs (right panels) represent the enlargement of the 2 Gy dose (red dotted box). (C) Normalized clonogenic survival fraction after increasing concentration of cisplatin (Cispl.) alone or combined with HT and/or HSP90-i. At the highest concentration (33 μM) no clones were detectable (n.d.). Bar graphs (right panels) represent the enlargement of the 3.3 μM concentration (red dotted box). (D) Relative clonogenic survival fraction after increasing concentration of gemcitabine (Gem.) alone or combined with HT and/or HSP90-i. The red dotted line indicates the cell survival after treatment with gemcitabine alone. (E) Normalized clonogenic survival fraction after increasing concentration of etoposide (Etop.) alone or combined with HT and/or HSP90-i. Bar graphs (right-hand side panels) represent the survival at 3.3 μM concentration of Ganetespib. The graphs show the average of three independent experiments, except for the 33 μM cisplatin combined with HT and HSP90-i in Hela cells, where n = 1.
Inhibition of HSP90 is insufficient for detectable downregulation of DSB repair. However, we detected a small but statistically significant decrease in survival after addition of Ganetespib to RT when it was combined with HT, even at the relatively low dose of 2 Gy, in both cell lines (Figure 3B and S3A). Cisplatin is an effective DNA cross-linking agent and repair of cisplatin-induced lesions in replicating cells requires HR and nucleotide-excision repair (McHugh et al. 2001; Crul et al. 2003). HT has been reported to be a strong sensitizer to cisplatin (Bergs et al. 2007), which is confirmed by our results (Figure 3C). This in contrast to Ganetespib treatment alone, which did not enhance cytotoxicity of cisplatin, similar to that of RT. However, Ganetespib further enhanced cytotoxicity of cisplatin/HT combinational treatment, at least at cisplatin concentrations exceeding 0.9 μM (Figure 3C and S3B). Gemcitabine is a clinically-applied nucleoside analog that directly targets HR (Wachters et al. 2003; Kobashigawa et al. 2015), but its main mechanism of action involves inhibition of DNA synthesis (de Sousa Cavalcante & Monteiro 2014), which can lead to collapse of replication forks and induction of DSBs (Ewald et al. 2007). We found that the cytotoxicity of a 24 h incubation period with gemcitabine was generally potentiated by HT, but not by Ganetespib alone (Figure 3D and S3C).

A combination of HT, gemcitabine and Ganetespib, however, significantly decreased cell survival, as compared to the HT/gemcitabine combination treatment. This was observed at nearly all tested concentrations of gemcitabine, in both HeLa and SiHa cells (Figure 3D and S3C). Finally, we tested whether Ganetespib potentiates cytotoxicity of etoposide, an inhibitor of topoisomerase 2 (TOP2) which blocks the TOP2/DNA cleavage complexes, leading to DSB formation (Wu et al. 2011). HT did not sensitize SiHa cells to etoposide, and there was only a moderate sensitization in HeLa cells, at concentrations exceeding 1 μM (Figure 3E and S3D). HeLa cells were similarly sensitized by addition of Ganetespib alone or HT alone. The combination of Ganetespib and HT did not decrease cell survival in SiHa cells and only slightly (and not significantly, p=0.09) in HeLa cells. Combined, these results suggest that chemical inhibition of HSP90 can potentiate the cytotoxicity of combinational approaches including HT and some, but not all, chemotherapeutic agents that inflict DNA damage. At the same time, it is apparent that the treatment with Ganetespib alone does not induce significant toxicity in vitro.

Inhibition of HSP90 combined with HT and RT/cDDP affects cell cycle progression and cell fate
To further explore how HSP90 inhibition enhances the cytotoxic effects of HT in combination with radiation and chemotherapy, we recorded time-lapse movies of HeLa and SiHa cells after single-agent and different combinational treatments including HT, RT and cDDP. We focussed on the latter agents because these modalities are often combined with HT for treatment of cervical cancer (Lutgens
Figure 4. Treatments combining inhibition of HSP90 with HT and RT/cDDP affect the cell cycle progression and cell fate. (A and C-E) Cells were preincubated with vehicle or 30 nM Ganetespib (HSP90-i) for 30 min. at 37°C, then mock-treated or exposed to 2 Gy of RT and subsequently incubated at 37 (control) or 42°C (HT) for one hour. Medium was then refreshed and cells were imaged for 96 h at time intervals of 15 min. (F-I) Cells were preincubated with vehicle or 30 nM HSP90-i and/or 3.3 μM cisplatin (cDDP) for 30 min. at 37°C and then incubated at 37 (control) or 42°C (HT) for one hour. Medium was refreshed and cells were imaged for 96 h at time intervals of 15 min. (A) Representative pictures.
of SiHa and HeLa cells at 48 h after the indicated treatments. (B) Average cell cycle time of untreated SiHa and HeLa cells. (C and G) Average percentage of cells that successfully divided within a single (23h for SiHa and 24h for HeLa) or double (48 h) cell cycle time (+ two standard deviations). (D and H) Cell cycle times measured as the time between the first and second mitosis after the indicated treatments. (E and I) Fate of the daughter cells directly after the second mitosis. Graphs represent the average of two independent experiments, except for cDDP+HSP90-i where n = 1. The numbers of cells analyzed in each individual treatment/measurement group are shown in Figure S4.
et al. 2010; Heijkoop et al. 2014) and because our previously discussed results indicate that HSP90 inhibition generally enhances the cytotoxicity of these agents when combined with HT (Figure 3). All treatment protocols mirrored those used for measuring the clonogenic survival (Figure 3A), except after refreshment of the medium cells were transferred to a live-cell microscope and time-lapse images were recorded for up to 96 h. Sample images captured after selected treatments are shown in Figure 4A and 4F. We measured various parameters related to cell cycle progression, cell division and cell fate. First, we determined the average duration of the cell cycle under normal conditions, in both cell lines (Figure 4B). Next, we focussed on RT as DNA-damaging agent and quantified the percentage of treated and control cells that were able to enter mitosis within 23 h or 48 h (corresponding to a single or double cell cycle time plus two standard deviations, respectively, Figure 4B) after the treatment (Figure 4C). We observed that treatment without HT, including exposure to 2 Gy of RT, did not significantly affect cell cycle progression, since nearly all cells were able to enter mitosis within the first 23 h after treatment. In contrast, HT-based treatments reduced the percentage of cells that entered mitosis during the first 23 or 48 h. The largest reduction was observed after the HT/Ganetespib combination, with or without RT. Additionally, HT-based treatments increased the frequency of abnormal first mitoses (Figure S4A). These differences were not accompanied by an altered duration of the cell cycle in cells that successfully completed the first mitosis (Figure 4D), but the frequencies of these cells were strongly reduced after HT-based double and triple-combinational treatments (Figure S4B). Furthermore, these treatments generally caused considerably increased frequencies of abnormal cell division, senescence and apoptosis (Figure 4E). One notable exception was the triple-modality treatment of SiHa cells, which did induce substantial cell cycle delay (Figure 4C) but did not cause abnormalities in those cells that were able to successfully divide (Figure 4E).

Treatments with cDDP showed even stronger effects of double- and triple modalities involving HT and, interestingly, of the cisplatin/Ganetespib double treatment. This was apparent in quantification of successful cell divisions (Figure 4G), abnormal first mitosis (Figure S4C) and of cell fate after the second mitosis (Figure 4I). Also here, frequencies of these cells were strongly reduced after HT-base double and triple-combinational treatments (Figure S4D). Similarly to experiments involving RT, most combinations, except for triple modality in SiHa cells, did not considerably affect the length of the first cell cycle after treatment (in those cells that were able to successfully divide) (Figure 4H). Importantly, the triple-combination regimen was clearly superior in causing disturbance of the cell cycle and mitosis as well as apoptosis and senescence. In conclusion, these observations generally confirm that HSP90 inhibition potentiates cytotoxicity of combinational treatments including HT and cDDP/RT. They also provide further details on how this toxicity is manifested in living cells.
Discussion

Strategies for efficient and targeted inhibition of DNA repair can help to improve clinical cancer therapies that rely on induction of DNA damage to destroy malignant cells. One example of such strategy is inhibition of PARP1, a protein involved in repair of SSBs, DSBs and in the regulation of the chromatin environment (Wang et al. 2012). PARP1-inhibitors have been exploited to target HR-deficient tumors, while sparing HR-proficient healthy tissues, in a reframed synthetic-lethality approach (Farmer et al. 2005; Bryant et al. 2005). Our previous in vitro and in vivo results suggested that mild hyperthermia in clinically-obtainable temperature range (40-42.5°C) can be used for on-demand induction of HR deficiency in cells and tissues (Krawczyk et al. 2011) and implied that HSP90 inhibition can potentiate this effect. In the current study, we further explored this hypothesis and showed that Ganetespib - a new-generation HSP90-inhibitor - enhances HT-mediated degradation of BRCA2 and inhibition of HR (Figure 1). These data are in line with studies showing that BRCA2 is a client of HSP90 and that HSP90 inhibition affects BRCA2 stability (Noguchi et al. 2006). In addition to BRCA2, HSP90 manages multiple other proteins associated with DNA repair, including important HR factors BRCA1 and RAD51, and the stability of these factors is affected by inhibition of HSP90 (Noguchi et al. 2006; Stecklein et al. 2012). In fact, the combination of a HSP90-inhibitor with HT emerges as a self-reinforcing strategy to disable HR, creating on-demand conditions of stimulated synthetic lethality.

Except for the effects on HR, our results suggest induction of DSBs after longer periods of time (16-48 h) after HT and - to a larger degree - after HT/Ganetespib treatment (Figure 2). In particular, the highly increased MN formation is a strong indicator of cells entering mitosis with unrepaired DSBs (Fenech et al. 2011). Induction of MN formation by HT has been reported over three decades ago (Rofstad et al. 1984), but its stimulation by HSP90 inhibition is a novel observation. The late appearance of MN and γ-H2AX foci suggests that they are not directly induced by treatment, but rather arise with the progression of the cell cycle. This is in line with the hypothesis that the induction of DSBs after HT is caused by inhibition of TOP1, leading to formation of SSBs. Unrepaired SSBs can then derail replication forks and result in DSB formation in the next S-phase (Velichko et al. 2015). Such one-ended DSBs are likely similar to those hypothetically induced after PARP1 inhibition. Since collapsed replication forks require HR for repair (Arnaudeau et al. 2001), inhibition of HR by HT contributes to the resulting toxicity, but this contribution may be limited by the temporary and reversible character of HT-mediated HR suppression (Figure 1B).

The observation of HR inhibition by HT/Ganetespib invites the combination of stimulated synthetic lethality with induction of DSBs in the temporary therapeutic window of HR deficiency. This is clearly
supported by our results showing considerable potentiation of HT-induced radiosensitization and chemosensitization by Ganetespib (Figure 3 and 4). Similar to late DSB induction by HT/Ganetespib treatment, clonogenic cell death and late effects on the cell cycle and division capabilities were observed when treatment was combined with DSB-inducing agents, like RT, cisplatin and gemcitabine. In contrast to these agents, we did not detect significant thermal sensitization of cells to the TOP2 inhibitor etoposide, whether or not Ganetespib was present during HT treatment. This can be explained by the previously described inhibitory effects of HT on the formation of etoposide/TOP2/DNA cleavage complex, which may effectively reduce the efficiency of DSB induction and treatment cytotoxicity (Kampinga 1995).

Our results show at least two different aspects of treatments comprising HSP90 inhibition and HT that can be beneficial in cancer treatment. First, the treatments produce DSBs in dividing cells and likely also cause cytotoxicity by other mechanisms. In nearly all experiments, we observed that Ganetespib considerably potentiates cell killing by HT, in line with the recent study that reported enhancement of the effects of HT by 17DMAG (Miyagawa et al. 2014). It is worth noting that Ganetespib alone can also sensitize cancer cells to radiation and some chemotherapeutic drugs (Nagaraju et al. 2014; Chettiar et al. 2016; Wang et al. 2016; Kramer et al. 2016). Second, the increased inhibition of HR (and, potentially, other DNA repair mechanisms) by HT and Ganetespib sensitizes cells to multiple DSB-inducing agents, indirectly increasing their cytotoxicity. Importantly for clinical application, our results imply that a single, short pulse of Ganetespib, combined with HT, is sufficient for a temporary but considerable HR downregulation (Figure 1)(Krawczyk et al. 2011). This approach is conceptually in contrast with the long-term application of Ganetespib that has been tested in clinical trials (Proia & Bates 2014), including the phase III trial of Ganetespib in combination with docetaxel which failed in patients with advanced non-small cell lung cancer (https://clinicaltrials.gov/ct2/show/NCT01798485). Long-term exposure to Ganetespib has been found to be well tolerated (Proia & Bates 2014) and the short application that is required for boosting HT efficacy should be safe in clinical practice. Strategies allowing potentiation of the cytotoxic and sensitizing effects of HT can lead to improved therapy outcomes via multiple avenues, e.g. by inducing stronger cytotoxicity while sparing the non-heated healthy tissues, by allowing reduction of the required dose of DNA-damaging agents or by rendering the HT treatments effective at decreased temperatures. Our study suggests that inhibition of HSP90 is such strategy, with limited systemic side-effects, paving the way for rational design of improved HT treatment protocols and for in vivo studies involving animal models.
Competing interests
Authors declare that they have no competing interests.

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Materials and methods

Cell lines and cell culture. SiHa and HeLa cervical cancer cell lines were obtained from the American Type Culture Collection (ATCC) and cultured in EMEM medium (Lonza) enriched with 10% fetal bovine serum (FBS) and 1% of penicillin/streptomycin (Gibco, 10000 U/mL). Cells were maintained at 37°C, in an atmosphere containing 5% CO₂. During the experiments with hyperthermia (HT), cells were incubated for 65 min at 42°C in an atmosphere containing 5% CO₂ (the medium in the wells needed approx. 5 min to reach the target temperature of 42°C). In the remaining part of the manuscript, we refer to HT treatment as 60 min or 1 h. For irradiation with α-particles, cells were cultured on custom-made dishes with 4 μm-thick polypropylene bottom, as described earlier (Stap et al. 2008).

Chemical agents and ionizing radiation. Cells were treated with the indicated concentrations of Ganetespib (STA-9090, Synta Pharmaceuticals), cisplatin (cDDP; Platosin®, Pharmachemie), gemcitabine (Actavis) and etoposide (Sigma Aldrich). In experiments involving irradiation, cells were exposed to the indicated doses of radiation from a 137Cs source (0.7 Gy/min) or from a 241Am α-particle source. To produce linear tracks of DSBs, cells were irradiated through the polypropylene bottom of culture dishes for 1 min, with the alpha-particle source positioned under an angle of approximately 45° directly below the bottom of the dish (Stap et al. 2008).

Immunohistochemistry. Cells were plated 24 h prior to treatment in the presence or absence of 30 nM Ganetespib for 90 min in combination with or without HT during the last 60 min. After 30 min for experiments with α-particle irradiation or after the indicated periods of time in the other experiments, cells were fixed with 2% paraformaldehyde. The fixed samples were washed twice with PBS and incubated in TNBS (PBS supplemented with 1% FBS and 0.1% Triton-X) for 30 min. Samples were incubated with the primary mouse anti-γ-H2AX (1:100, Millipore) and rat anti-Rad51 (1:50) (Essers et al. 2002) antibodies diluted in TNBS for 2 h. After two times washing, samples were incubated with the secondary anti-mouse-Cy3 and anti-rat-FITC (both 1:100, Jackson ImmunoResearch Laboratories) diluted in TNBS for 1 h. Finally, mounting gel containing DAPI (Thermo Scientific) was added and samples were covered with glass coverslips. Slides were imaged and scored using the wide-field fluorescence microscope (DM-RA and DM-RXA, Leica).

Clonogenic assays. At 24 h before treatment, 2×10⁶ cells were plated into a 10 cm dish. On the day of the experiment, cells were trypsinized, counted and plated in triplicates of two densities per condition in a 6-well plate. After 4 to 6 h incubation required for cell attachment, cells were treated with 30 nM Ganetespib for 90 min. with or without cisplatin or etoposide. After the first 30 min of incubation at 37°C, plates were either transferred to a 42°C water bath or were incubated at 37°C for the remaining 60 min. In experiments involving RT, cells were irradiated after the 30 min. treatment with Ganetespib and immediately prior to the HT treatment. Directly after HT, cells were washed with PBS and incubated in fresh medium for 8 (HeLa) or 13 (SiHa) days. Next, colonies were fixed, stained and counted according to previously published protocol (Franken et al. 2006). In experiments involving gemcitabine, cells were treated for 24 h, starting directly after cell culture until the start of the experiment (0 h). A schematic
overview of all treatment schedules can be found in Figure 3A.

**Time-lapse microscopy.** At 24 h prior to treatment, cells were plated in 6- or 12-well plates at a density of 15,000 or 7,000 cells per well, respectively. After treatment, cells were washed with PBS and fresh medium was added. The medium was covered with a layer of mineral oil (Sigma-Aldrich) to prevent evaporation during imaging. Cells were imaged for 96 h, at intervals of 15 min, using a wide-field phase-contrast microscope (Leica). The cell cycle time was defined as the time between the first and second successful mitosis observed after treatment; senescence as absence of cell division for at least 48 h and abnormal mitosis as division that gave rise to abnormal progeny.

**Western blotting.** Cells were directly harvested after treatment and lysed in Laemmli sample buffer (4% SDS, 20% glycerol and 120 MM Tris pH 6.8). Protein levels were quantified with the Lowry protein assay. A total of 50 μg protein supplemented with bromophenol blue and β-mercaptoethanol was loaded and runned on a SDS/PAGE gel. The protein samples were transferred to a PVDF membrane, incubated for 1 h in blocking buffer at 4°C (PBS with 0.05% Tween (PBS-T) and 3% nonfat dry milk) and overnight incubated with the primary antibodies mouse anti-BRCA2 and rabbit anti-cyclin A (both 1:1000 in PBS-T; OP95 (Ab-1) Merck Millipore and C-19, Santa Cruz Biotechnology). The membrane was washed five times for 8 min with PBS-T and incubated with the secondary antibodies: horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG and HRP-conjugated donkey anti-rabbit IgG (both 1:2000 in PBS-T, Jackson ImmunoResearch Laboratories) for 3 h at 4°C. The proteins on the membrane were visualized with enhanced chemiluminescence (ECL) substrate and imaged with the Alliance western blot imaging machine.

**Statistics.** For the statistical analysis, the unpaired t-test was used to compare two different groups. Statistical analysis was not performed in Figure 4, because values represent the averages of only two independent experiments and one experiment for double treatment with cisplatin+HSP90-i. In addition, no statistics could be performed for the highest concentration of cisplatin in HeLa cells in Figure 3C, because n=1 for the triple treatment (also not shown in Figure S3C). Graphs summarizes the results of three independent experiments, unless stated otherwise, with error bars indicating standard deviation. Asterisks indicate statistical significance with the p-values as follows: * < 0.05, ** < 0.01, *** < 0.001.
Supplementary Figures

Supplementary Figure 1. HSP90-inhibitor Ganetespib potentiates the inhibitory effects of HT. (A) Normalized clonogenic survival fraction after increasing concentrations of Ganetespib (HSP90-i) for 1.5 h with incubation of the last hour at 37 (control) or 42°C (HT). 30 nM (marked by the red dotted box) was selected as working concentration for the remaining experiments. (B) Results of the statistical analysis (unpaired t-test, p-values) from Figure 1B.

Supplementary Figure 2. Inhibition of HSP90 enhances induction of DNA damage by HT. (A) Results of the statistical analysis (unpaired t-test, p-values) from Figure 2A. (B) Results of the statistical analysis (unpaired t-test, p-values) from Figure 2B. The dark-blue columns (right) contain the p-values corresponding to the data points marked by asterisks in the indicated figures.
Supplementary Figure 3. Inhibition of HSP90 enhances radiosensitizing and chemosensitizing effects of HT. (A-D) Results of the statistical analysis (unpaired t-test, p-values) from Figure 3A-D. The dark-blue columns (right) contain the p-values corresponding to the data points marked by asterisks in the indicated figures.

### A. Irradiation

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<td>0.8149</td>
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<td>4 Gy</td>
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<td>0.0204</td>
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### B. Cisplatin

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<td>0.3 μM</td>
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<td>0.6244</td>
<td>0.7628</td>
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<td>0.0024</td>
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<td>3.3 μM</td>
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<td>0.0001</td>
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<td>9.9 μM</td>
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<td>0.0177</td>
<td>0.0002</td>
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<td>33 μM</td>
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<td>-</td>
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### C. Gemcitabine

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<td>100 nM</td>
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### D. Etoposide

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<tr>
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<tr>
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<td>0.5509</td>
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<tr>
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<td>0.4083</td>
<td>0.1464</td>
<td>0.7966</td>
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<tr>
<td>10 μM</td>
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<td>0.8311</td>
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Supplementary Figure 4. Treatments combining inhibition of HSP90 with HT and RT/cDDP affect cell cycle progression and cell fate. Cells were treated and imaged as described in legend of Figure 4. (A and C) Average percentages of cells that experienced abnormalities during the first mitosis following the treatment. (B and D) Tables showing the numbers of cells analyzed in each individual treatment group (data from these analyses is presented in Figure 4). Notably, the numbers of cells that could be adequately analyzed are dramatically reduced in the double/triple treatment groups.
References


Chapter 9 | HSP90 inhibition enhances effects of hyperthermia


