In Vitro Combined Modality Treatment of Prostate Carcinoma Cells with 17-(allylamino)-17-demethoxygeldanamycin and Ionizing Radiation

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Abstract. Background: The effect of 17-(allylamino)-17-demethoxygeldanamycin (17-AAG), a benzoquinone-ansamycin-type Hsp90-inhibitor, on the expression of focal adhesion kinase (FAK) and, when combined with ionizing radiation, on the clonogenicity of prostate cancer cells were determined. Materials and Methods: FAK was analyzed by Western immunoblot. Prostate carcinoma cells were exposed either to 17-AAG alone or combined with a single radiation fraction of 3 Gy. Results: FAK concentrations were reduced by 17-AAG in a time-dependent manner. Treatment with 100 nM 17-AAG for 24 h reduced clonogenicity by 90%. The plot of surviving fraction versus radiation energy dose yielded roughly parallel graphs for solvent- and 17-AAG-treated cells. Conclusion: 17-AAG induced rapid degradation of FAK. A single radiation fraction of 3 Gy did not enhance the dose-dependent drug-effect on survival. In this sequence, the combined effect of both modalities towards clonogenicity was largely additive.

Targeting molecular abnormalities specific for neoplastic cells may enable oncologists to treat patients with drugs with a favorable therapeutic index. Heat shock protein 90 (Hsp90) is a ubiquitous chaperone, which has physiological functions, not only in the cellular reaction to stressful conditions like heat shock, but also in the phenotypic buffering of mutations (1, 2) and in the maintenance of regulatory proteins in a latent functional state (3). Roughly a quarter of the malignant neoplasias analyzed for Hsp90-protein or -mRNA exhibited elevated concentrations (4-12). The characterization of specific inhibitors of Hsp90 opened up new avenues for the study and treatment of this diverse group of tumors (13). For these drugs, a correlation was found for the ability to bind Hsp90 and to induce cytotoxicity (14).

Prostate cancer, the most common malignant tumor in men, is highly resistant to conventional cytostatic agents. For metastatic disease, anti-androgenic therapy is usually the first systemic treatment administered and, in the event of subsequent failure, is followed by either contrasexual therapy or the application of cytotoxic agents. Only a very modest influence of conventional cytotoxic drugs on survival could be demonstrated (15-17). In locally advanced stages, anti-androgenic therapy is usually the first systemic treatment administered followed by radiotherapy.

Several therapeutic strategies compete for the treatment of locoregionally-confined, early disease. The strategies include surgical resection, percutaneous radiotherapy using conformal or intensity-modulated techniques (IMRT) or the implantation of radioactive seeds, the treatment results being roughly similar for patients with comparable stages and comorbidities. For patients with adverse prognostic factors, current cure rates are not satisfactory, thereby underlining the need to improve the therapeutic results.

For a variety of reasons, the escalation of total dose of percutaneous radiation using IMRT, although beneficial, does not completely solve the problem of localized prostate cancer, i.e., by improving on the 5-year survival probability by roughly 80%. Only patients with advanced stages seemed to benefit from escalating the total radiation dose (18). The optimal dose is still under investigation, but increasing the total dose is paralleled by an expected increase in local toxicity, especially rectal (19). Pretreatment with testosterone-lowering drugs improved the outcome of patients treated with percutaneous radiation (20) and, for patients with locally advanced disease, lifelong anti-androgenic therapy is recommended (21, 22). Nonetheless, despite these successes, there is still the need for effective systemic...
therapies that either augment the local radiation effect or show activity towards micrometastases. Simultaneous chemoradiation may be the route to further increasing local response and survival for patients with localized prostate cancer. Hsp90-inhibitors are among the candidate drugs for such a combined modality approach. Recently, it was demonstrated that Hsp90 in malignant cells is present in heteroprotein-complexes, with a higher affinity for benzoquinone-based Hsp90-inhibitors compared to Hsp90 in non-neoplastic cells (23). In vitro and in vivo treatments with Hsp90-inhibitors resulted in the depletion of pre-existing, regulatory proteins of oncological relevance, such as ErbB2, focal adhesion kinase (FAK) or Raf (24-27). These were the incentives to examine the combined activity of ionizing radiation and of one of the above compounds on survival parameters of prostate carcinoma cells. In the work presented here, 17-(allylamino)-17-demethoxygeldanamycin (17-AAG, Figure 1), a benzoquinone-ansamycin already in clinical phase I testing (28, 29), was investigated.

Materials and Methods

Cell lines, drugs and reagents. PC-3M cells are a subline of PC-3 cells, which were established from a lumbar metastasis of a patient with prostate cancer (30). PC-3M cells originate from a liver metastasis, which developed after injection of PC-3 prostate carcinoma cells into the spleen of a mouse (31). The cells were metastasis, which developed after injection of PC-3 prostate carcinoma cells into the spleen of a mouse (31). The cells were propagated in the same way as the PC-3M cell line. 17-AAG was a kind gift from Dr. Neckers, Tumor Cell Biology Section, Urologic Oncology Branch, National Cancer Institute (Bethesda, MD, USA; structural formula in Figure 1) and it was dissolved as a 1 mM stock solution in DMSO.

Western blot. Subconfluent cells were lysed on ice in TNESV (50 mM Tris-HCl pH 7.4, 1% Nonidet P-40, 2 mM EDTA, 100 mM NaCl, 1 mM orthovanadate) containing 20 μg/ml aprotinin, 20 μg/ml leupeptin and 1 mM phenylmethylsulfonylfluoride. The lysate was cleared by centrifugation for 10 min at 12000 xg. The BCA-protein assay (Pierce) was used for protein determinations. Protein aliquots were boiled for 5 min in Laemmli buffer and resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Wet transfer onto activated polyvinylidene fluoride-based (PVDF) Immobilon-P membrane (Millipore) was followed by blocking for 1 h at room temperature in 5% nonfat dry milk dissolved in PBST (0.05% (v/v) Tween 20 in PBS). Incubations with mouse-monoclonal antibody clone #77 (Transduction Laboratories) as the primary and sheep-anti-mouse horseradish-peroxidase-linked secondary antibody (Amersham) were carried out for 1 h at room temperature, followed by five washes in PBST. Enhanced chemiluminescence with Supersignal (Pierce) was applied according to the manufacturer’s instructions using X-Omat AR films (Kodak). Band intensities were analyzed densitometrically using Scion image software.

Results

Irradiation and determination of clonogenicity. The cells were trypsinized, counted, replated and allowed to adhere for at least 16 h prior to treatment. Cell numbers were determined by trypan blue staining and counting in a Neubauer chamber. Prespecified numbers of cells were inoculated into 6-cm dishes. They were irradiated with 250 kV-photons with 1 mm Cu-filtering and a surface-focus-distance of 40 cm (RT250, Philips, Hamburg, Germany). All doses refer to the surface-dose of a 6-cm tissue culture dish filled with 3 ml medium. Six to 8 days after irradiation, resultant colonies were washed twice in PBS, followed by 30-min incubation in 1% crystal violet dissolved in 20% (v/v) methanol. Thereafter, the colonies were washed twice with deionized water, dried at room temperature and counted. To qualify as a colony, 50 cells had to be present. All experiments were performed in triplicate and the median of these three values are depicted in the figures.

To characterize the cytotoxicity of 17-AAG, PC-3M cells were exposed to increasing doses for 24 h. The half-maximal growth inhibition was determined from Figure 2 as approximately 10 nM (17-AAG). This value is in agreement with previous reports, which described the growth inhibition of eukaryotic cells by Hsp90-inhibitors in the submicromolar drug concentration range (14).

The time-dependency of the clonogenicity of PC-3M cells treated with 17-AAG is depicted in Figure 3. A concentration of 100 nM 17-AAG resulted in reductions of colony numbers that were discernible after 24 h. With even longer treatment times, the clonogenicity was roughly 50% of solvent-treated controls. The estimate for the 48-h time-point is less certain, since only two experiments were carried out in this case. Among the time-points based on three estimates, only the data for the 6-h treatment differed with statistical significance (paired t-test, \( p=0.031 \)), indicating that the treatment with 100 nM 17-AAG for 2 days was only marginally toxic.
The effect of a single fraction of 3 Gy on cell survival in the presence of escalating doses of 17-AAG was subsequently examined. As shown in Figure 4, no influence on the clonogenicity of the cells from the addition of ionizing radiation was discernible. The magnitude of the drug-dose effect was much larger than the radiation impact.

To determine the radiation-dose dependence of the clonogenicity in the presence or absence of 17-AAG, the PC-3M cells were treated with 100 nM 17-AAG or an equivalent volume of solvent DMSO, with or without 0-5 Gy ionizing radiation. In the absence of radiation the clonogenicity was reduced to 20% of the control (Figure 5). Depending on the radiation dose, a steady reduction of resultant colony numbers was observed. All but the 5 Gy data are statistically different (paired t-test). The same trend was observed for the DU145 cells: upon increasing the radiation doses, a decrease in colony number (clonogenicity) was observed. Three out of six estimates reached statistical significance (paired t-test; 0, 1 and 3 Gy; Figure 6).

Most Hsp90 client proteins are severely depleted after 24 h of treatment with an Hsp90-inhibitor. One of these clients, FAK, is sensitive to Hsp90 inhibition by geldanamycin, a benzoquinone-ansamycin-like 17-AAG (25). Whether 17-AAG shares the ability to influence cellular FAK levels was also investigated. The results of a time-course experiment with DU145 cells treated with 100 nM 17-AAG are provided in Figure 7. A progressive decrease of the cellular FAK concentration was observed (if the 4-h estimate is set at 100%: 79% after 7 h; 43% after 24 h), confirming that 17-AAG exerted its activity towards the molecular targets within the time-frame of these radiobiological studies.

**Discussion**

The antitumor activity of geldanamycin (GA), the prototype of a benzoquinone-ansamycin-based Hsp90-inhibitor, was recognized early on and was mentioned in the first report on
the isolation of this compound (33). For some time, GA and its derivatives were classified as tyrosine kinase inhibitors, since a reduction of the activity of regulatory protein kinases was found as a consequence of the in vitro treatment of eukaryotic cells with these drugs (34). Later, Miller et al. discovered that the ErbB2-receptor, a tyrosine kinase which confers a dismal prognosis when overexpressed in breast cancer cells (35), was destabilized and degraded after exposure to herbimycin A or to other GA-analogs (24). Benzoquinone-ansaymcin-type Hsp90-inhibitors bind to a pocket at the aminoterminal end of Hsp90 and thus block a site with ATPase-activity located inside (36, 37). This induces the destabilization of Hsp90 client proteins due to proteasomal degradation (38). Obviously, the Hsp90 clients depend on constant interaction with Hsp90 as a molecular chaperone. Several molecules with prominent roles in the development or the maintenance of the malignant state are among these clients: ErbB2 (39), Raf (26), mutant p53 (40), FAK (25) and others (41). In a preclinical study, GA was tested in dogs to assess potential side-effects and was found to be too hepatotoxic for further development (42). As a result of a search for less toxic derivatives, 17-AAG was characterized (43) and has completed phase I testing (28, 29).

Since prostate cancer is the most common malignancy in men, we chose the established permanent prostate cancer cell lines PC-3M and DU145 as therapeutic models. Currently, chemotherapy has only a very limited role in the management of prostate cancer patients. It is primarily in use for patients with metastasized, hormone-refractory disease, but even newer combination treatments have only very limited activity (15-17).

Our data indicate that the antitumor activity of 17-AAG is time- and dose-dependent and that most of the interaction with ionizing radiation is additive. The clonogenicity of both cell lines was markedly reduced by Hsp90-inhibitors.

This is the first report, to our knowledge, of a depletion of FAK by 17-AAG. Bearing in mind the multitude of Hsp90 client protein interactions, which are the targets of Hsp90-inhibitors, a simple cause-effect relationship between 17-AAG-induced degradation of FAK and 17-AAG-dependent survival-effects is unlikely. These results complement earlier findings by Russel et al. on the interaction of ionizing radiation and 17-AAG (44). However, a major difference is that, in their study, the DU145 and PC-3 cells (not PC-3M) were treated with the drug for 24 h prior to, instead of following, radiation. Under these circumstances, a radiosensitization was observable for a comparable drug-dose range.

In another study, prostate cancer spheroids derived from the cell lines LNCaP and CWR22Rv1 were investigated (45). Similar to our findings, the application of conventional fractions of radiation in combination with 100 nM 17-AAG resulted in additive effects on spheroid growth. Only high radiation (6 Gy) and drug doses (1000 nM 17-AAG) were supra-additive in this respect. Since the sequencing of

![Figure 5. Radiation dose-response of PC-3M cells treated for 24 h with 100 nM 17-AAG in combination with increasing doses of single fraction orthovoltage irradiation during the first hour (mean±SEM). Inoculated cell numbers (three experiments): DMSO: 700; 17-AAG: 1400. SF: surviving fraction](image)

![Figure 6. Clonogenicity of DU145 cells treated for 24 h with 100 nM 17-AAG in combination with increasing doses of single fraction orthovoltage irradiation during the first hour (mean±SEM). Inoculated cell numbers: DMSO: 700; 17-AAG: 1400. SF: surviving fraction](image)
chemotherapy and Hsp90 inhibition has a major impact on cellular survival in vitro (46), the authors also investigated this issue. The sequences radiation → 17-AAG and 17-AAG → radiation decreased growth equally, when 100 nM 17-AAG and 2 Gy single-dose irradiation were administered. For a radiation-dose of 6 Gy and a drug-dose of 100 nM, the sequence 17-AAG → radiation reduced spheroid growth more than the reverse did. One conclusion from these experiments, including our own, is that the sequencing of Hsp90 inhibition and ionizing radiation determines the occurrence of radiosensitization.

The in vitro results presented here further support the translation of Hsp90-inhibitors into the clinical setting. In prostate cancer xenografts, the depletion of ErbB2, Akt or the androgen-receptor by 17-AAG is possible at non-toxic doses (47). In phase I studies, these dose-levels were also reached in humans (28). As with other novel targeted therapies, it has to be left to future clinical research to determine the therapeutic value of these compounds, the optimal dosing and sequencing of modalities. Appropriate patient selection, for example, for those with tumors overexpressing Hsp90-protein or -mRNA may be necessary to achieve maximum benefit. The combination of Hsp90-inhibitors with ionizing radiation will perhaps turn out to be beneficial in locally advanced disease stages. Such an approach is already under investigation with conventional therapeutics (48).

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References


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