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Differential efficacy of combined therapy with radiation and AEE788 in a high and low EGFR expressing androgen independent prostate tumor models

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Abstract

Purpose—To determine the efficacy of combining radiation (XRT) with a dual EGFR/VEGFR inhibitor, AEE788, in prostate cancer models with different levels of EGFR expression.

Methods and Materials—Immunoblotting was performed for EGFR, phosphorylated-EGFR (p-EGFR), and p-AKT in prostate cancer cells. Clonogenic assays were performed on DU145, PC-3 and HUVEC cells treated with XRT+/-AEE788. Tumor xenografts were established for DU145 and PC-3 on hindlimbs of athymic nude mice assigned to four treatment groups: 1) Control, 2) AEE788, 3) XRT, 4) AEE788+XRT. Tumor blood flow and growth measurements were performed using immunohistochemistry and imaging.

Results—AEE788 effectively reduced p-EGFR and p-AKT levels in DU145 and PC-3 cells. Clonogenic assays showed no radiosensitization for DU145 and PC-3 colonies treated with AEE788 +XRT. However, AEE788 caused decreased proliferation in DU145 cells. AEE788 showed radiosensitization effect in HUVEC and increased apoptosis susceptibility. Concurrent AEE788 +XRT compared to either alone led to significant tumor growth delay in DU145 tumors. In contrast, PC-3 tumors derived no added benefit to combined modality therapy. In the DU145 tumors, significant reduction in tumor blood flow with combination therapy was demonstrated by power Doppler sonography and tumor blood vessel destruction on immunohistochemistry. MS imaging demonstrated that AEE788 is bioavailable and heterogeneously distributed in DU145 tumors undergoing therapy.

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Conclusion—AEE788+XRT showed efficacy *in vitro/in vivo* with DU145-based cell models while PC-3-based were adequately treated with radiation alone without added benefit from combination therapy. These findings correlated with differences in EGFR expression and demonstrated effects on both tumor cell proliferation and vascular destruction.

Keywords

EGFR; Radiation therapy; Prostate Cancer; AEE788; VEGFR

INTRODUCTION

Prostate cancer is the second leading cause of cancer death in men with an estimated 230,090 men diagnosed with prostate cancer in 2005 (1). The first line of therapy includes surgery or radiation therapy (XRT) (2–4), with intermediate to high risk patients often receiving androgen suppression therapy (5). Once the disease progresses to an androgen independent disease status (AID), therapeutic options for prostate cancer patients diminish, with an overall survival of 6–12 months (6). Chemotherapy confers only a small survival benefit in patients with metastatic prostate cancer (7,8), suggesting a need for investigation of non-hormonal systemic therapy. In AID patients, combining radiation with systemic agents result in 20–30% therapy failure (9), therefore requiring better treatment approaches.

Studies have demonstrated that human prostate cancer cells (DU145 and PC-3) derived from androgen independent prostate tumors express epidermal growth factor receptor (EGFR) (10–13). Furthermore, in pre-clinical studies involving various cancer models, including prostate tumors, EGFR overexpression has been linked to proliferation, angiogenesis, and migration (14–16), and has an inverse relationship to tumor radiocurability (17,18). In prostate cancer, EGFR expression correlates with higher Gleason score, higher PSA values, and is an independent prognostic factor negatively impacting disease free survival (19). New small molecule drugs targeting the EGFR receptor have been developed and show promise in the clinical setting (20).

In addition to targeting EGFR, the use of vascular endothelial growth factor receptor (VEGFR) inhibitors is actively being investigated for cancer therapy (21). In fact, a number of preclinical studies have demonstrated that molecular compounds targeting VEGFR-2 (such as SU11248) when combined with radiation leads to improved tumor growth delay, partially due to significant tumor vascular destruction (22). Since there is a direct correlation between angiogenesis as well multiple angiogenesis related proteins with tumor aggressiveness and survival (23,24), analysis of angiogenesis-related proteins has been examined. In particular, VEGFR-2 expression has been reported suggesting that prostate tumors are highly vascular and may also benefit from therapy targeting VEGFR-2 (25). Furthermore, a recent study has suggested a significant advantage to combining both EGFR and VEGFR inhibitors *in vitro* to reduce the activation of Akt in endothelial cells (26).

AEE788, a dual tyrosine kinase inhibitor of both EGFR and VEGFR, now provides an avenue to investigate the effect of simultaneous blockade of EGFR and VEGFR (27–35) in cancer cells. We hypothesized that dual inhibition of both targets using AEE788 in prostate cancer will lead to improved tumor control when combined with radiation.

METHODS AND MATERIALS

Cell Culture, Animals, and Compounds

DU145 and PC-3 (ATCC, Rockville, MD) human prostate cancer cells and Human umbilical vein endothelial cells (HUVEC) were obtained from Cambrex (East Rutherford, NJ), and

cultivated *in vitro* according to the recommendations of the supplier. Five to six week old male athymic nude mice (nu/nu) were purchased from Harlan Laboratories and maintained in accordance to guidelines approved by the Vanderbilt Institutional Animal Care and Use Committee (IACUC). AEE788 was provided by Novartis Pharma (Basel, Switzerland). For cellular assays, AEE788 was dissolved in DMSO, and for *in vivo* experiments, AEE788 was dissolved in a suspension on N-methylpyrrolidine and PEG300 1:9 (v/v).

Western Blots

DU145 and PC-3 cells were grown in 100 mm dishes to 90% confluency. Cells were serum starved overnight and treated with DMSO (control) and AEE788 (500 nM or 1 μ M) for 2 hours and then stimulated with EGF (100 ng/ml) for 15 minutes at 37°C/5% CO₂. Cells were washed twice in PBS and lysed with M-PER (Pierce) supplemented with phosphatase and protease inhibitor cocktail mix (Sigma) according to the manufacturer recommendations at 4°C for 5 min prior to harvest. Remainder of the procedure has been described previously (22). Primary antibodies used were rabbit polyclonal antibodies for phosphorylated-EGFR (Tyr 1068, 1:500), EGFR (1:1000), phosphorylated-AKT (Ser473, 1:1000), and AKT (1:1000) from Cell Signaling Technology (Beverly, MA) and monoclonal anti-Actin (1:5000) from Santa Cruz Biotechnologies.

Clonogenic Assay

DU145, PC-3 and HUVEC cells were seeded in triplicate and distributed in different treatment groups: Control (DMSO) and AEE788 (100 nM, 500 nM, and 1 μ M) +/- radiation (0, 2, 4, and 6 Gy). Drug treatment was applied 2 hours prior to radiation treatment. Colonies were allowed to grow for 2 weeks prior to harvesting and assay performed as previously described (22).

In vitro cell proliferation assay

DU145 and PC-3 cells were plated in duplicate at 1×10^4 . The experimental groups were treated with 100 nM, 500 nM and 1 μ M AEE788 dissolved in DMSO as well as a control group (DMSO). Cells were counted using a Coulter counter at days 0, 2, 4, and 6.

Apoptosis assays

Apoptosis was determined by the translocation of phosphatidylserine revealed with Annexin-V staining. HUVEC cells undergoing apoptosis were distinguished from live and necrotic cells by the use of Annexin-V and propidium iodide (PI) staining using Apoptosis Detection Kit (BD PharMingen, San Diego, CA). Briefly HUVEC cells were either treated with AEE788 and were irradiated with 6 Gy and harvested 24 hours post irradiation. Camptothecin treated positive control cells were harvested at 2, 12 or 24 hours. Aliquots of 10^5 cells were incubated with Annexin and PI for 15 minutes at room temperature. The cells were then analyzed by flow cytometry, using a two-color FACS analysis (BD LSR II); live cells were considered as being Annexin-V⁻ and PI⁻. Apoptotic cells were considered the sum of early and late apoptotic cells; early apoptotic cells are Annexin-V⁺ and PI⁻; late apoptotic cells as both Annexin⁺ and PI⁺; and necrotic cells are only PI⁺. For each treatment, the average fold increase of apoptotic cells over control (+/- SEM) was calculated.

To confirm the results, apoptosis was also determined by 4', 6-diamidino-2-phenylindole (DAPI) staining. The treated cells were washed with PBS, fixed in 4% paraformaldehyde at room temperature for 10 minutes, and stained with 5 μ g/mL of DAPI at room temperature for 10 minutes. The nuclear morphology was observed under a fluorescent microscope (Zeiss HBO 100). Apoptosis was quantified by scoring the percentage of cells with apoptotic nuclear morphology at the single cell level. Condensed or fragmented nuclei were scored as apoptotic;

and five to seven randomly selected fields were captured using Axio vision software. The average percentage of apoptotic cells ($\pm$ SEM) was calculated.

Xenograft Models and Therapy

3.5×10^6 cells (DU145) or 5×10^6 cells (PC-3) were injected subcutaneously in the right hind-limbs of 30 athymic nu/nu mice. Three weeks post-injection, all mice were randomized in four treatment groups (n=5): (1) control, (2) AEE788 (25 mg/Kg), (3) XRT (2–3 Gy), and (4) AEE788+XRT. Animals received vehicle (N-methylpyrrolidine and PEG300 1:9 v/v) or 25 mg/kg of AEE788 by oral gavage for 7 days, two hours prior to radiation (3 Gy x 7 days). The (a) length, (b) width and (c) depth, of tumors were measured every 2 days, and tumor volumes calculated as $(a \times b \times c)/2$ derived from the ellipsoid formula.

Immunohistochemistry and TUNEL

Paraffin-embedded prostate cancer xenograft tissues collected from mice receiving 5 consecutive days of treatment (AEE788+/-XRT) were sectioned (5 μ m) and stained for Ki-67, von Willebrand Factor (VWF) and TUNEL as described previously (39). Sections were incubated for 30 min with rabbit anti-human VWF (1:900, Dakocytomation, Carpinteria, CA) and rabbit anti-human Ki-67 (1:1000, NovaCastra Laboratories Ltd., Newcastle, UK). Sections without primary antibody served as negative controls. The Dako Envision+HRP/DAB System (DakoCytomation) was used. TUNEL staining was performed following vendor specifications (DeadEnd Colorimetric TUNEL System, Promega, Madison, WI). For co-staining procedure, TUNEL was performed after staining to localize VWF.

Ultrasound Imaging

Prostate tumors (DU145 and PC-3) underwent power Doppler sonography prior to therapy and after five consecutive days of daily therapy. Tumors were imaged with a 10-5 MHz linear probe (Entos, Philips/ATL, Bothell, WA) attached to a US scanner (HDI 5000, Philips/ATL). A tumor cross-section consisting of at least 20 power Doppler US images was acquired in real time with a gain of 82%. Care was taken to minimize motion artifact during the scan. Data from power Doppler ultrasound imaging was analyzed as described previously (36).

MALDI

Frozen prostate tumor xenograft tissue samples corresponding to each treatment group: (a) control, (b) 25 mg/kg AEE788 (c) 25 mg/kg AEE788 + XRT (3 Gy), were harvested at 24 h and after 5 days of consecutive treatment, prepared, and MALDI-TOF mass spectra were acquired on a Voyager DE-STR mass spectrometer (Applied Biosystems, Foster City, CA, USA) following specifications previously described (37,38).

Statistical Analysis

All descriptive statistics including means and standard error of means (SEM) were performed. Unpaired student t-test were used to evaluate differences between control group and each treatment group in all *in vitro* and *in vivo* studies performed.

RESULTS

Differential Expression of EGFR in two prostate cancer cell lines

EGFR expression in prostate cancer cell lines, DU145 and PC-3, was assessed using immunoblot analysis. At baseline, there was a higher level of EGFR protein expression in the DU145 cells compared to the PC-3 cells (Fig. 1). When serum starved, only a faint level of phosphorylated EGFR activity was noted in both cell lines (Fig. 1, lanes 1 and 5). Treatment with recombinant human EGF (100 ng/mL) led to robust phosphorylation of EGFR in the

DU145 cells (Fig. 1, lane 6), and no induction of phosphorylation of EGFR in the PC-3 cells (lane 2). Pretreatment with AEE788, 2 hours prior to EGF treatment, led to abrogation of the EGFR phosphorylation in both prostate cancer cell lines (lanes 3 and 7). This inhibition, as expected, was much more apparent in the DU145 cells.

The highly EGFR expressing DU145 cells have reduced colony size when treated with AEE788 compared to the PC-3 cells

Clonogenic survival assay was performed on both DU145 (Panel A) and PC-3 (Panel B) cells by treating them with increasing doses of ionizing radiation (0, 2, 4, 6 Gy) and AEE788 (0, 500 nM, 1 μ M). Both prostate cancer cells demonstrated no impact on surviving fraction for up to 1 μ M AEE788 doses (Fig. 2A.1, 2B.1) when incubation times were short (2 h), though a reduction in individual surviving colony size was noted in DU145 cells, with no uniform reduction in colony size in PC-3 cells with the same dose of AEE788 treatment (data not shown). Interestingly, the lower EGFR expressing PC-3 cells were more sensitive to radiation treatment alone than the DU145 cells (Fig. 2A.1, 2B.1).

AEE788 leads to increased inhibition of cell proliferation in the DU145 cells

EGFR expression has often been linked with cell proliferation (14–16). Therefore, we studied the impact of EGFR inhibition on DU145 and PC-3 cell proliferative capacity. Cells were seeded in normal culture conditions, on day 0, and treated with 0, 100, and 500 nM AEE788 compound. Cells were harvested at days 2, 4, and 6 following treatment. As seen in Fig. 2A.2, there was a dose dependent reduction in cell numbers for the DU145 cells. Interestingly, even at the lower dose of 100 nM concentration, there was a reduction in cell proliferation for the DU145 cells ($p=0.062$) which express EGFR highly. The PC-3 cells only displayed a modest reduction (Fig. 2B.2, $p=0.174$) even at the higher 500 nM concentration of AEE788 treatment.

AEE788 can radiosensitize DU145 cells with longer incubation

Because of the differences seen during cell proliferation studies, we pretreated DU145 and PC-3 cells with AEE788 over 24 h to determine if longer exposures would modify the clonogenic survival assay. Interestingly, as shown in Fig. 2A.3 and 2B.3, 24 h incubation with AEE788 demonstrated a radiation enhancement at both drug concentrations (500 nM and 1 μ M) compared to vehicle control but only for DU145 cells (significant p values indicated on figure). The PC-3 showed no change in clonogenic survival.

AEE788 therapy leads to clonogenic radiosensitization and increased apoptosis in HUVEC

We next investigated the effect of AEE788+XRT in tumor vasculature endothelial cells. Combination therapy of AEE788 and radiation in human umbilical vein endothelial cells (HUVEC) resulted in significant reduction ($*p=0.001$) in the surviving fraction compared to radiation (XRT) alone (Fig. 3A). These results were normalized for plating efficiency (vehicle control=0.1616; 500 nM AEE788=0.13475; and 1 μ M AEE788=0.134). To further define the cytotoxic effect of AEE788 in HUVEC, we performed flow cytometry assessment of annexin V staining as a marker of apoptosis in HUVEC treated with AEE788 or vehicle +/- XRT. Treatment with AEE788 or XRT alone did not confer significant apoptosis. However, treatment with AEE788 in combination with XRT led to an increase in both early (Shift to Q4) and late apoptosis (Shift to Q2) which was more than additive (Fig. 3B, 3C, $*p<0.0001$ compared to XRT, AEE788 or DMSO alone). To confirm the flow cytometry data, we performed DAPI staining experiments using similar conditions. As shown in Fig. 3D and E, XRT alone provided a mild increase in pyknotic nuclei ($p=0.0007$), while combination AEE788 and XRT demonstrated a much more significant increase compared to either treatment alone ($p<0.0001$). AEE788 alone showed no change in pyknotic nuclei ($p=0.67$). Camptothecin treatment served

as a positive control. Endothelial cell apoptosis induction by combination therapy of AEE788 and XRT may be a primary mechanism for the radiosensitization effect noted on the clonogenic assay (Figure 3A).

Prostate xenograft tumor growth delay is increased in combination therapy group (AEE788 +XRT) for DU145 prostate tumor models

Optimal doses for AEE788 therapy in preclinical studies have been established (50 mg/Kg), when used as a single agent(29). For our studies, we investigated a lower dose (25 mg/kg) of AEE788 as doses needed for radiosensitizing effects are often lower than what is required for single agent activity, and often less toxic. Treatment groups included: 1) AEE788 (25 mg/kg), 2) XRT (2–3Gy), 3) XRT (2–3Gy) +AEE788 (25 mg/kg) 4) no treatment delivered consecutively for seven days. Tumor volumes were measured for up to 40 days after initiation of therapy. In the DU145 xenograft tumors, there was a marked increase in tumor growth delay in the animals that were treated with AEE788+XRT (Fig. 4A) compared to radiation alone (*p=0.044 compared to XRT). AEE788, even at the lower dose (25 mg/kg), was effective at inducing modest tumor growth delay in this model. In contrast, in the PC-3 xenografts (Fig. 4B), the lower dose of AEE788 did not confer a significant tumor growth delay. When these tumors were treated with 3 Gy x 7 doses, there was near complete abrogation of tumor growth (data not shown), correlating well with the higher radiosensitivity of these tumors seen in the clonogenic assays *in vitro*. Therefore, in order to determine whether there is any added benefit of the drug with radiation treatment, we reduced the radiation dose to 2 Gy per day x 7 fractions. Despite this reduction, in PC-3s, XRT alone and the AEE788+XRT treated group showed very similar tumor growth rate (Fig. 4B, ** p=0.727), suggesting no additional benefit conferred by the drug to the cytotoxic effects of radiation. Based on these results, we decided to focus our attention on DU145 xenograft tumors.

Combination therapy of AEE788 and XRT leads to significant reduction in tumor blood flow in the DU145 tumors

The same animals which were subjected to tumor volume measurement analysis in Fig. 4A were also measured longitudinally for tumor blood flow using non-invasive Doppler Ultrasound as described previously (36,39). As demonstrated in Fig. 5A, animals that were treated with XRT and AEE788 demonstrated statistically significant decrease in tumor blood flow compared to day 0 as assessed by percent change in power weighted pixel density (PWPDP) measurements (p=0.03). Animals treated with either vehicle, AEE788, or XRT alone did not demonstrate significant reduction in tumor blood flow (p=0.2, p=0.7, and p=0.5, respectively). Meanwhile, in the PC-3 tumor xenografts undergoing same treatment conditions, there was no significant reduction, but rather a slightly increased level in tumor blood flow at 5 days following AEE+XRT treatment (p=0.1) (figure 5 A.1 lower panel, p=0.1). In fact, in all of the PC-3 treatment groups, after 5 days of treatment, the tumor blood flow levels were increased compared to baseline levels, suggesting that AEE788 +/- radiotherapy failed to reduce tumor blood flow (figure 5.A.1 lower panel).

DU145 prostate tumor xenograft treated with combination therapy (AEE788+XRT) have reduced microvessel density, increased endothelial cell apoptosis, and reduced proliferative capacity

To confirm the Doppler ultrasound findings, we examined the vasculature of treated tumors immunohistochemically. Microvessel density (MVD) was assessed in DU145 tumors after 5 days of consecutive treatment with AEE788+/-XRT, measured by counting number of von Willibrand Factor (VWF) positive cells (shown in red) present in 10 random high power fields. Co-staining of VWF and TUNEL (shown in brown) was also performed in same tissue sections to assess for apoptotic endothelial cells *in vivo* with those positive for both indicated by black

arrows. Microvessel density of tumors within animals treated with AEE788+XRT was significantly reduced compared to control animals (8.86 ± 0.11 and 14.15 ± 0.93 respectively, $p=0.004$). In comparison, monotherapy with AEE788 or XRT on tumors did not display a significant reduction in MVD compared to untreated control animals (14.55 ± 2.49 and 12.32 ± 2.36 , $p=0.88$ and 0.51 , respectively) (Fig. 5B and Table 1). When level of apoptotic blood vessels was assessed in the treated tumors, all three treatment regimens, AEE788 (48.67 ± 4.05), XRT (49.67 ± 2.19), and AEE788+XRT (61.67 ± 3.84) led to a statistically significant elevation in blood vessel apoptosis compared to control (22.00 ± 2.08) ($p=.004$, $p=.0007$, and $p=.0008$, respectively) (Fig. 5B and Table 1). Moreover, there was a statistically significant elevation of apoptotic blood vessels in the AEE788+XRT group when compared to the XRT group ($p=.05$). When compared to the AEE788 treatment group, the tumors in the AEE788+XRT group displayed a trend for increased blood vessel apoptosis ($p=0.08$).

To assess for proliferative capacity of the tumors following 5 days of consecutive treatment with AEE788+XRT, Ki-67 staining was performed in DU145 prostate tumors. The tumors treated with both AEE788 and XRT (165.77 ± 25.12) had a statistically significant reduction in Ki-67 staining compared to the untreated tumors (285.36 ± 16.25) ($p=.016$) (Fig. 5C, and Table 1). However, tumors treated with AEE788 (158.57 ± 31.78) or XRT (195.62 ± 20.9) alone also showed significant reduction in Ki-67 staining compared to control tumors ($p=0.024$ and $p=0.028$, respectively) (Fig. 5C and Table 1).

AEE788 is detected using MALDI-imaging in prostate tumors

To determine whether AEE788 bioavailability in prostate tumors correlates with tumor blood flow reduction data (Fig 5A, 5A.1), we used MALDI-imaging, a technology that has been used to determine a drug's spatial biodistribution directly from frozen tissue sections(37). The bioavailability of AEE788 in DU145 prostate tumor xenograft sections was determined at various time points following oral administration of the compound. *In vitro*, AEE788 is ionized and detected in fragments at two specific sites with mass to charge (M/Z) ratios of 223 and 327 (Fig. 6A). DU145 prostate xenograft tumors sections were imaged for AEE788 (m/z 327, represented as blue pixels) at 24 h (Fig 6B lane 2), and after 5 days of consecutive therapy (Fig. 6B lanes 3–4). As seen in Fig. 6B lane 2, there was a sustained heterogeneous distribution of the AEE788 compound even 24 h post administration providing a favorable pharmacokinetics for use in combination with radiation therapy. Combined therapy with AEE788+XRT which led to tumor blood vessel destruction (Figure 5A), also demonstrated a reduction in the biodistribution of AEE788 in prostate tumors (Fig. 6B. lane 4). Tumor that was treated with vehicle demonstrates no AEE788 signal as expected (Fig. 6B lane 1).

DISCUSSION

Since EGFR and VEGFR expression has shown to be important for prostate cancer biology (12,19,25), there is significant rationale for treatment of these tumors with AEE788. We did investigate VEGFR-2 levels in HUVEC, PC-3, and DU145 and expression was very low in the prostate cancer lines compared to HUVEC which did not change with drug treatment (data not shown). Therefore, we postulated that the differences seen in our study were based on EGFR.

Previous studies have shown presence of higher EGFR expression in prostate cancers derived from androgen-independent prostate tumors (10–13). The two human prostate cancer cell lines chosen in our study, DU145 and PC-3, are both androgen-independent tumors. However, there is a differential expression of EGFR and phosphorylation level in these two cell lines being high for DU145 and low for PC-3 (Fig. 1). Interestingly, the two tumors demonstrated differential growth rates with a higher proliferation rate for DU145 cells and lower proliferation

for PC-3 cell (Fig 2A, 2B, 4). In the DU145 cells, blockade of EGFR with AEE788 led to growth inhibition which was not observed in the PC-3 cells. This suggests that EGFR levels in these androgen independent tumor cells are directly related to their proliferative capacity.

Treatment with AEE788 did abrogate the phosphorylation of EGFR in both cell lines. There was a strong down-regulation of the EGFR downstream target p-AKT in both DU145 and PC-3 cell lines. Interestingly, there was a robust activation of AKT even in PC-3 cells following serum starvation and EGF stimulation (100 ng) which is consistent with a previously published report (12). Although some have reported little to no difference in p-AKT for basal and serum starved PC-3 cells subject to EGF stimulation (40), the dose of EGF used in such studies was lower (50 ng) than the present study. Although the constitutive phosphorylation of downstream proteins, such as AKT in PC-3 is likely due to the PTEN negative status (40), our data indicates that EGF stimulation can enhance this AKT phosphorylation. The noted improved treatment efficacy with AEE788 in the DU145 cells that have high EGFR expression suggests that efficacy of EGFR targeted compounds may be dependent on cell's EGFR level and activity.

Previously, pre-clinical studies using ZD1839, an EGFR inhibitor, with standard chemotherapeutics demonstrated growth inhibition when used at higher doses in prostate xenografts (40). The lower doses of AEE788 chosen in our study were effective due to radiosensitization effect, primarily on the vasculature, but also likely due to anti-proliferative effect on the highly expressed EGFR levels in the DU145 tumor. Therefore, it appears that lower drug doses can be used when used as a radiosensitizer in appropriately selected tumors.

Based on our studies, the anti-vascular effect of radiation and AEE788 predominated. The endothelial cells displayed significant radiosensitization to increasing doses of AEE788 by our *in vitro* assays (Fig 3). Furthermore, in DU145 tumor xenografts, we found both histological and imaging (Doppler ultrasound) evidence of effective vasculature destruction following combined AEE788 and radiation treatments. A recent study has suggested the notion that HUVEC express phosphorylated-EGFR when subject to radiation and expression is abrogated by use of both EGFR and VEGFR inhibitors (26). It is unclear why AEE788 failed to sensitize the PC-3 vasculature to the cytotoxic effects of radiation, but it leads one to speculate that the tumor type has a direct impact on host (vasculature)'s response to targeted therapy.

Finally, the data suggests that not all prostate tumors will be effectively radio-sensitized by EGFR and VEGFR blockade. Identification of biomarkers which can predict for targeted therapy sensitivity may become clinically relevant. MALDI-TOF technology utilized in our studies holds promise to help in identification of such biomarkers.

CONCLUSION

The data presented supports the efficacy of AEE788 in DU145 prostate tumors and that low doses of AEE788 combined with ionizing radiation can lead to significant tumor growth delay in the highly EGFR expressing DU145 prostate tumors. Potential mechanism of action could include: (1) enhanced tumor vasculature destruction and (2) decreased proliferation of tumor cells surviving cytotoxic effects of radiation therapy.

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Fig. 1.

AEE788 attenuates prostate cancer response to EGF. Prostate cancer cells (DU145 and PC-3) were treated and subjected to Western blot analysis using antibodies for phospho-EGFR (p-EGFR), EGFR, phospho-AKT (p-AKT), AKT, and actin (loading control) for the indicated conditions. 1) PC-3 control cells (untreated), 2) PC-3 cells treated with 100 ng/mL EGF, 3) PC-3 cells treated with 500 nM AEE788 and 100 ng/ml of EGF, 4) PC-3 cells treated with 1000 nM AEE788 and 100 ng/ml of EGF, 5) DU145 controls (untreated), 6) DU145 cells treated with 100 ng/ml EGF, 7) DU145 cells treated with 500 nM AEE788 and 100 ng/ml EGF, 8) DU145 cells treated with 1000 nM AEE788 and 100 ng/ml EGF.

Fig. 2.

AEE788 does not affect surviving fraction, however it impacts proliferation. *A.1*. Clonogenic assay of DU145 prostate cancer cells treated with AEE788 (500 nM, 1 μ M) $\pm$ XRT (2 Gy, 4 Gy, 6 Gy) as indicated. *A.2*. Cell proliferation assay of DU145 cells treated with increasing doses of AEE788 (500 nM and 1 μ M). *B.1*. Clonogenic assay of PC-3 prostate cancer cells treated with AEE788 (500 nM) $\pm$ XRT (2, 4, 6 Gy) as indicated. *B.2*. Cell proliferation assay of PC-3 cells treated with increasing doses of AEE788 (500 nM and 1 μ M). * indicates p-values. SEM (Standard Error of Means).

Fig. 3.

AEE788 enhances cytotoxicity in endothelial cells. **A**. Clonogenic assay of HUVEC treated with AEE788+/-XRT as indicated. **B**, **C**. Apoptosis assay via flow cytometry for Annexin V and propidium iodide with or without XRT (0 or 6 Gy), treated with AEE788 (0 or 1 μ M) as indicated. Camptothecin time course as positive control is also shown. **D**, **E**. Apoptosis assay via DAPI staining for nuclear morphology with or without XRT (0 or 6 Gy). Camptothecin positive control is also shown. * indicates p-values. SEM (Standard Error of Means).

Fig. 4.

AEE788 treatment results in prostate tumor growth delay. DU145 (A) and PC-3 (B) xenografts tumors were treated as indicated were started when tumor reached $\sim 800 \text{ mm}^3$ (21 days after implantation for DU145 and 31 days for PC-3). AEE788 (25 mg/kg) was administered orally once per day for 7 days during the week of therapy. In AEE788+XRT and the XRT groups, 3 Gy for DU145 and 2 Gy for PC-3 was delivered to the mice hindlimbs containing the corresponding prostate tumor 2 hours after treatment with AEE788. Tumor volume was determined three times per week. $N=5$ for each treatment group. * indicates p-values. Red arrows indicate the end of the treatment protocol (day 7). SEM (Standard Error of Means).

Fig. 5. AEE788+XRT results in tumor blood flow reduction, tumor microvessel destruction, and decreased proliferation. *A & A.1.* Tumor blood flow assessment was performed using Doppler ultrasonography at day 5 following indicated treatments. Doses for AEE788 and XRT were as described for figure 4 and in Materials and Methods. Graph depicts percent changes in PWP at day 5 compared to day 0. *B.* Microvascular density and vasculature destruction (black arrows) in DU145 tumors following 5 days of indicated treatments were assessed using immunohistochemical analysis. Double staining for microvasculature (anti-VWF antibodies in red) and apoptotic cells (TUNEL in brown) was performed. *C.* Immunohistochemical analysis using anti-Ki67 antibodies on DU145 tumors 5 days following indicated treatments. SEM (Standard Error of Means).

Fig. 6. Imaging mass spectroscopy analysis of AEE788 biodistribution. **A.** Mass spectrometry analysis for AEE788. Ionized peaks detected at 327 and 223 m/z ratio represent AEE788. **B.** Spatial biodistribution of AEE788 in prostate cancer xenograft sections via MALDI-Q-TOF-MS. Bottom panels are MALDI-MS images of frozen DU145 prostate tumor tissue sections harvested at multiple time points after oral administration of AEE788 compound as indicated: lane 1) vehicle control treatment, lane 2) 24 hours post-treatment with 25 mg/kg AEE788, lane 3) AEE788 (25 mg/kg) for 5 consecutive days, lane 4) AEE788 (25 mg/kg)+XRT (3 Gy) for 5 consecutive days.

Table I

Quantification of Tumor Mean Vessel Density, Apoptosis and Proliferation

	Treatment Groups (Mean +/- SEM)			
	Control	AEE788	XRT	AEE788+XRT
VWF ^a	14.15+/-0.93	14.55+/-2.49	12.32+/-2.36	8.86+/-0.11
VWF+TUNEL ^a	22.00+/-2.08	48.67+/-4.05	49.67+/-2.19	61.67+/-3.84
Ki67 ^a	285.36+/-16.25	158.57+/-31.78	195.62+/-20.9	165.77+/-25.12

^a Assessment at 5 days of consecutive treatment. VWF, Von Willenbrand Factor, TUNEL, terminal deoxynucleotidyl-transferase-mediated-nick end labeling

The number of positive cells were counted in ten random fields at 400x magnification^a.

Three representative slides per group.