Combination Antiangiogenic and Androgen Deprivation Therapy for Prostate Cancer: A Promising Therapeutic Approach

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ABSTRACT

Purpose: Androgen ablation therapy leads to mild regression or stabilization of prostate cancer, followed by progression to the fatal androgen-independent state. Whereas androgen ablation diminishes tumor angiogenesis by suppressing vascular endothelial growth factor (VEGF) production, androgen-independent disease is marked by androgen-independent VEGF expression. We examined combined androgen ablation and inhibition of VEGF signaling in an androgen-sensitive human prostate cancer xenograft model (LNCaP) that is known to develop androgen-independent growth after androgen ablation.

Experimental Design: N-(4-Bromo-2-fluorophenyl)-6-methoxy-7-(1-methylpiperidin-4-yl)methoxy]quinazolin-4-amine (ZD6474) is an orally active inhibitor of VEGF receptor tyrosine kinase activity, with additional activity against epidermal growth factor receptor tyrosine kinase. ZD6474 (50 mg/kg/d, per os) was administered to groups of castrated and noncastrated athymic mice bearing established (4–616 mm³) LNCaP xenografts. To evaluate the extent of tumor regrowth after ZD6474, treatment was stopped after 40 days of continuous dosing, and subsequent tumor growth was monitored. Prostate-specific antigen expression was assessed to determine the effect of ZD6474 on androgen-regulated genes.

Results: In comparison with orchiectomy, ZD6474 treatment produced greater tumor growth inhibition (P < 0.001), inducing complete cytostasis for the duration of dosing. An analysis of serum prostate-specific antigen concentration and tumor weight indicated that ZD6474 did not have a direct effect on androgen-related gene expression. Combination therapy (castration plus ZD6474) produced a comparable therapeutic effect to treatment with ZD6474 alone (in noncastrated mice), for the duration of ZD6474 administration. However, when ZD6474 treatment was discontinued, the rate of tumor regrowth was significantly less in the combination group. Tumors from mice receiving combined treatment were also found to be more necrotic than tumors from mice receiving either androgen ablation or ZD6474 alone.

Conclusions: These data indicate that inhibition of VEGF signaling produces a highly significant inhibition of tumor growth in a human androgen-dependent prostate tumor model, which far exceeds that produced by androgen ablation alone. However, when ZD6474 treatment is removed, concurrent androgen ablation produces a greater inhibition of tumor regrowth than is observed in mice without androgen ablation. Increased necrosis observed in tumors from orchiectomized mice receiving ZD6474 also suggests benefit from combining anti-androgen and anti-VEGF signaling approaches.

INTRODUCTION

For patients presenting with metastatic prostate cancer, androgen ablation therapy leads to regression or disease stabilization in most (1). However, over the ensuing 2 to 3 years, prostate cancer progresses from this androgen-dependent disease to an androgen-independent state, which is invariably fatal (1). Progression toward androgen-independent correlates with up-regulation of autocrine and paracrine growth factor loops, including overexpression of vascular endothelial growth factor (VEGF; refs. 2 and 3). In prostate cancer, VEGF expression has been related to a more aggressive (4, 5) and metastatic (4, 6) phenotype, both in animal models and in human disease (7).

Studies have also demonstrated a link between androgen action and angiogenesis in androgen-responsive LNCaP (8), PC-82 and A-2 (9) human prostate cancer xenografts. These have shown both direct activation of programmed (apoptotic) cell death and indirect activation of apoptosis via a decrease in tumor angiogenesis, secondary to a reduction in tumor VEGF levels and the associated collapse of tumor vasculature. Studies with the Shionogi murine tumor, an androgen-dependent male mammary carcinoma, indicate that depletion of androgen by castration leads to initial regression of these tumors (10). VEGF is expressed at a high level during the initial tumor growth and decreases to an almost undetectable level 1 week after castration. Strikingly, during tumor relapse the dominant angiogenic factor is VEGF, despite the lack of androgen.
Taken together, these observations suggest that treatment of androgen-sensitive tumors with a combination of androgen ablation and agents that inhibit VEGF signaling may delay emergence of androgen-independent disease. We sought to examine the well-characterized androgen-sensitive LNCaP human prostate cancer xenograft model (11) and to evaluate the in vivo tumor responses to orchietomy alone or when combined with N-(4-bromo-2-fluorophenyl)-6-methoxy-7-[[1-methylpiperidin-4-yl]methoxy]quinazolin-4-amine (ZD6474), a potent, orally active, low M<sub>i</sub> inhibitor of VEGF receptor-2 (VEGFR-2; KDR) tyrosine kinase activity (12, 13).

MATERIALS AND METHODS

Cell Culture. The LNCaP human prostate cancer cells (14) were purchased from American Type Culture Collection (Manassas, VA, USA). LNCaP cells are tumorigenic when coinjected with extracellular matrix components (Matrigel) in the subcutaneous site of immunocompromised murine hosts, and the resultant tumors express VEGF mRNA and protein (8, 15). Tumor cells were grown in T-medium (Life Technologies, Carlsbad, CA) containing 5% heat-inactivated fetal bovine serum. Proliferating cells were detached from tissue culture plates by treatment with 0.25% trypsin and 1 mmol/L EDTA and resuspended in growth medium before subsequent manipulations or passaging.

Tumor Cell Cytotoxicity Assay. Freshly trypsinized cells were seeded at a density of 5,000 cells per well (in 200 μL of medium) in a 96-well microtiter plate. After 24 hours of incubation (37°C, 5% CO<sub>2</sub>), ZD6474 was added in a solution of dimethyl sulfoxide in medium (0.1% final dimethyl sulfoxide concentration), and plates were reincubated. After an additional 96-hour incubation, cultures were pulsed with 1 μCi per well of [³H]thymidine (Amersham Biosciences, Uppsala, Sweden) and reincubated for 4 hours. Finally, cells were harvested and assayed for incorporated tritium using a β counter.

In vivo Tumor Growth, Treatments, and Histologic Analysis. Six- to 8-week-old Swiss nude mice were obtained from Taconic (Germantown, NY) and were handled according to a protocol approved by the University of Virginia, Institutional Animal Care and Use Committee. Mice were given one injection in the anterior flank with 5 × 10<sup>6</sup> tumor cells suspended in 0.1 mL of serum-free medium with the addition of 100 μL of Matrigel (BD Biosciences, San Jose, CA). Once tumors were established, mice were randomized into control and treatment groups. Compounds were suspended in a 1% (v/v) solution of polyoxyethylene (12) sorbitan mono-oleate in deionized water and administered by daily oral gavage at 0.1 mL/10 g body weight.

In an initial experiment, the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor gefitinib (Iressa, ZD1839) was examined at a dose of 50 mg/kg/d versus a control (vehicle-treated) group. In subsequent experiments, the impact of ZD6474 on tumor growth was examined at a dose of 50 mg/kg/d in both tumor-bearing normal and castrated mice versus appropriate control groups. For castration, animals were anesthetized via an intraperitoneal injection of ketamine, xylazine, and acepromazine. In a sterile laminar flow hood, the abdominal and scrotal areas were sterilized, and abdominal pressure was applied to move the testes into the scrotum before excising both testes. The animal was recovered from anesthesia in a housing container.

Mice were examined twice a week, and tumors were measured with calipers across the greatest two diameters. At the time of euthanasia, tumors were removed by dissection away from adjacent organs and structures and weighed on an analytical balance. For histologic analysis, tissue samples were fixed in 10% neutral buffered formalin and processed through graded ethanol and xylenes for paraffin embedding and staining using standard methods (16).

For calculation of percent necrosis, five random fields per tumor were examined at low power (∼10) from tumors harvested at 40 days. The necrotic area(s) in each field was/were circumscribed electronically using the tools available in the ImagePro computerized image analysis package (Media Cybernetics) to create an area. A human operator carried out this identification and circling of the necrotic area for all analyzed issues. Using the total area of the section as the denominator, the percentage of necrosis was evaluated for each field. To obtain an average for a whole experimental tumor group, the percent necrosis for each field was averaged across the total number of tumors in the group.

Serum Prostate-Specific Antigen. At the time of euthanasia, blood (100 μL) was tapped from the left ventricle for a determination of serum prostate-specific antigen levels. After separation of the cellular material by centrifugation, serum prostate-specific antigen determinations were performed by the University of Virginia Medical Laboratories using the Bayer Centaur System. The procedure is a two-site sandwich immunoassay using direct chemiluminometric detection. Dilutions, as necessary, were performed using the manufacturer’s provided diluent. Prostate-specific antigen density was measured as the serum prostate-specific antigen concentration (ng/mL) divided by the mass of the tumor in grams immediately after excision from the animal. This mass was corrected for the degree of necrosis found on histology, so only viable tumor was used in the density calculation.

End Points and Statistical Analysis. Repeated measure models were used for the analysis of the in vivo tumor volumes calculated as described previously (12, 13). Several covariance structures, including AR-1 and random coefficient models, were used; and all yielded similar conclusions. The analyses reported in this paper are based on a random coefficient model with robust estimates of the covariance matrix (17). Specific comparisons between treatment groups were made using F-test based on contrasts. Statistical analyses were carried out in SAS PROC MIXED; the plots were prepared in GAUSS 5.0. Error bars represent 1 SE. Time from treatment initiation to the time that tumor volumes exceeded a cutoff value (a tumor volume of >300 mm<sup>3</sup>) were estimated with Kaplan-Meier (18) curves and compared across treatment groups with the log-rank test. Tumor volume cutoffs were used to avoid using a survival end point, which would have involved significant animal distress and to employ a threshold that was appropriate for comparing the tumor growth data across all groups.
RESULTS AND DISCUSSION

Effect of ZD6474 on LNCaP Growth In vitro. There are divergent reports as to whether inhibition of EGFR phosphorylation can reduce LNCaP cell growth in vitro (19, 20). Because ZD6474 has some EGFR tyrosine kinase inhibitory activity at higher doses, the ability of ZD6474 to inhibit tumor cell growth directly was examined in vitro. ZD6474 inhibited LNCaP proliferation in vitro with an IC_{50} value of 2.6 ± 0.3 μmol/L. This concentration is 43-fold greater than that required to inhibit VEGF-stimulated human umbilical vascular endothelial cell proliferation and consistent with IC_{50} values obtained in other tumor cell lines in vitro (12, 13). Free plasma drug levels at the dose examined (50 mg/kg) are unlikely to contribute to a direct antitumor effect in vivo (12, 13).

To further examine the effect of EGFR inhibition on the in vivo growth of LNCaP, we carried out an experiment to evaluate treatment with the selective EGFR tyrosine kinase inhibitor ZD1839 (gefitinib; Iressa). No effect was observed on tumor xenograft growth in this setting at a dose of 50 mg/kg/d per os (Fig. 1). Although this does not completely rule out the possibility that ZD1839 may have demonstrated an antitumor effect at a greater dose, ZD1839 has been shown to impart significant therapeutic benefit at 50 mg/kg/d in tumor models that are dependent on EGFR signaling, including DU145 prostate xenografts (21, 22). In contrast, ZD6474 is known to inhibit tumor growth in xenograft models that demonstrate intrinsic or acquired resistance to ZD1839, in addition to models that are known to be sensitive to EGFR inhibition (12, 23). Collectively, the data suggest that the effects on LNCaP xenograft growth in vivo associated with 50 mg/kg/d ZD6474, are most likely to be related to an effect on VEGF receptor signaling.

ZD6474 Is Superior to Orchiectomy in Maintaining Tumor Stasis of Established Tumors. Xenografts reached an average volume of 134 mm^3 (range 4–616 mm^3) before treatment. There were no significant differences in tumor volume among the groups at the start of treatment. After 38 days of ZD6474 treatment, significant differences in tumor volume were observed between the ZD6474 group and the orchiectomy (P < 0.001) and control groups (P < 0.001; Fig. 2A). Similarly, significant differences were observed between the combination therapy group and the orchiectomy (P < 0.001) and control groups (P < 0.001).

ZD6474 treatment was well tolerated, with no appreciable difference in body weight as a function of treatment time in either of the experimental groups. When body weights of mice from all four xenograft groups were analyzed, treatment with ZD6474 50 mg/kg/d for 40 days in control and orchiectomized mice induced a mean body weight loss of 5.1% compared with pretreatment values, whereas a 3.4% mean body weight loss was observed across the vehicle-treated controls and mice receiving orchiectomy alone, during this period.

Chronic once-daily oral administration of ZD6474 produced stasis of established tumor growth. This activity is consistent with previous studies, including one that showed that ZD6474 could inhibit growth of androgen-independent PC-3 prostate adenocarcinoma xenografts in female nude mice (12). Alternative experimental approaches to inhibit VEGF signaling have also demonstrated therapeutic benefit in xenografts of human cancer (22–25). Statistically significant differences (P < 0.05) were found between the ZD6474 treatment group and the orchiectomy group for days 13 to 38 posttreatment. This result may suggest that ZD6474 maintains tumor stasis, whereas tumors in the mice that have undergone androgen ablation become androgen refractory.

ZD6474 Does Not Affect Androgen-Responsive Gene Expression in Established Tumors. Serum prostate-specific antigen levels are key indicators of response to therapy in prostate cancer (26), and this is true for both localized and metastatic disease. In addition, in patients treated with androgen deprivation, prostate-specific antigen levels may initially drop more abruptly than the decrease in tumor burden. This is because prostate-specific antigen is an androgen-regulated gene (26). However, prostate-specific antigen expression is also influenced by non-androgen-dependent mechanisms (27). Therefore, because it is conceivable that ZD6474 may affect these latter mechanisms, we sought to compare the serum prostate-specific antigen density to control mice, indicating that ZD6474 does not affect tumor growth by affecting androgen-related gene expression. This finding also supports the belief that prostate-specific antigen monitoring would be an effective method of disease follow-up in clinical studies using ZD6474 alone and would not be vulnerable to the same caveats as when it is used in patients undergoing androgen ablation (26).

Fig. 1 Effect of vehicle (control) and ZD1839 (50 mg/kg/d) on the growth of LNCaP tumor xenografts. Xenografts were established subcutaneously in nude mice and reached an average (all treatment groups combined) volume of 335 mm^3 (range 4–1267 mm^3) before treatment. Once-daily oral administration of ZD1839 or vehicle was then started and continued for the duration of the experiment. Data points represent a mean from 22 mice in the vehicle-treated control group and 20 mice in the ZD1839 group, with SEs shown in one direction. Data represent tumor size (in cubic millimeters).
Chronic ZD6474 Therapy Does Not Result in More Aggressive Tumors on Treatment Discontinuation. Despite being well tolerated, antiangiogenic agents may not be used continuously in all cases. For example, patients undergoing deep-sited surgery will need physiologic angiogenesis to undergo proper healing and repair (28). Intermittent therapy involving periods of compound withdrawal may therefore be required. In addition, although ZD6474 has been continuously dosed to patients for greater than a year, episodic dosing may be necessitated with other VEGF receptor tyrosine kinase inhibitors, such as SU11248 (29).

Because chronic treatment with ZD6474 was highly effective in this model, we were able to examine the characteristics of tumor regrowth upon compound withdrawal and to determine whether long-term ZD6474 pretreatment induced more aggressive growth characteristics in resultant tumors. This is possible because tumors denied angiogenesis, although not able to expand in size, could still produce more aggressive variants due to continued genetic instability (30) in the face of continued cell division with concomitant cell turnover.

In animals treated with ZD6474 alone for 40 days and then monitored after compound withdrawal, tumor growth resumed after a delay of a few days (~15 days; Fig. 4A). The delay is also evident from the Kaplan-Meier analysis (Fig. 4B). This

Fig. 3 Prostate-specific antigen (PSA) density in LNCaP tumor xenografts. At the time of euthanasia, blood sampling from LNCaP tumor-bearing mice was carried out and sent for prostate-specific antigen evaluation. Tumors were measured and then excised and weighed. Calculation of prostate-specific antigen density was carried out as described in Materials and Methods. Box plots of tumor volume before excision and prostate-specific antigen density showing mean and SE are shown. Data represent a mean from 9 mice in the control group, 12 mice in the orchiectomy group, and 15 mice in the ZD6474 group. Although tumor volume and prostate-specific antigen density were statistically different (P < 0.01, ANOVA) between the orchiectomy and control group, ZD6474 inhibited tumor growth without a significant effect on prostate-specific antigen.

Fig. 2 Effect of vehicle (control), ZD6474 (50 mg/kg/d), orchiectomy, or ZD6474 (50 mg/kg/d) and orchiectomy on the growth of LNCaP tumor xenografts. Xenografts were established subcutaneously in nude mice and reached an average (all treatment groups combined) volume of 134 mm$^3$ (range 4–616 mm$^3$) before treatment. Once-daily oral administration of ZD6474 or vehicle was then started and continued for the duration of the experiment. Data points represent a mean from 9 mice in the control group, 12 mice in the orchiectomy group, 15 mice in the ZD6474 group, and 12 mice in the combination therapy group, with SEs shown in one direction. Castration was carried out at the same time as initiation of ZD6474. Data represent tumor size (in cubic millimeters). A, estimated tumor growth among groups at median initial tumor volume (134 mm$^3$). B, estimated tumor growth among groups at 90th percentile of tumor volume (339 mm$^3$). C, estimated tumor growth among groups at 25th percentile of tumor volume (30.4 mm$^3$).

Chronic ZD6474 Therapy Does Not Result in More Aggressive Tumors on Treatment Discontinuation. Despite being well tolerated, antiangiogenic agents may not be used continuously in all cases. For example, patients undergoing deep-sited surgery will need physiologic angiogenesis to undergo proper healing and repair (28). Intermittent therapy involving periods of compound withdrawal may therefore be required. In addition, although ZD6474 has been continuously dosed to patients for greater than a year, episodic dosing may be necessitated with other VEGF receptor tyrosine kinase inhibitors that possess broad-spectrum kinase activity, such as SU11248 (29).

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In animals treated with ZD6474 alone for 40 days and then monitored after compound withdrawal, tumor growth resumed after a delay of a few days (~15 days; Fig. 4A). The delay is also evident from the Kaplan-Meier analysis (Fig. 4B). This
observation is consistent with a previous report examining the VEGF receptor tyrosine kinase inhibitor, ZD4190, in which PC-3 prostate tumor regrowth was observed after a delay of ~10 days, after long-term treatment and compound withdrawal (31). The delay in tumor regrowth may in part be due to the time needed to remove compound from tissues and/or an overestimate of viable tumor tissue from caliper measurements, because chronic administration of ZD6474 can induce significant tumor necrosis (12). Careful examination of the rate of tumor growth of untreated and previously ZD6474-treated tumors suggested there was no significant difference between the two when the delay of approximately 15 days is taken into account (Fig. 4C).

When ZD6474 removal was examined in orchiectomized mice, caliper measurements of tumor volume indicated that the combination of androgen ablation with a fixed period of ZD6474 treatment was found to produce the greatest net therapeutic effect (Fig. 4A). Evidence of tumor growth, relative to the tumor volumes measured on the day of ZD6474 withdrawal, was only apparent 40 to 58 days after compound removal. The data suggest that the cytostatic effect of androgen ablation is maintained even after the discontinuation of ZD6474 in animals that were treated with both modalities. Importantly, if LNCaP tumors are not able to acquire androgen independence while on prolonged ZD6474 therapy, this would argue in favor of employing the combination, to ensure that effective androgen ablation was maintained should transient discontinuation of a VEGF signaling inhibitor be required.

Tumors from either the orchiectomy alone or orchiectomy with ZD6474 groups did not attain rapid exponential growth during the course of the experiment, preventing a complete assessment of whether the combination provided an additional advantage over what would be expected from simply adding the antitumor effects of the two approaches together. However, the rate of tumor regrowth in the combination group did not obviously exceed that observed in the orchiectomy alone group. These data suggest that long-term ZD6474 treatment and then withdrawal does not induce more aggressive growth characteristics in resultant tumors, either in hormone naïve or androgen-ablated animals.

Assessments of therapeutic efficacy based solely on caliper measurements of tumor volume can be subject to potential confounds, such as the inability to accurately assess the induction of necrosis within tumors (29, 32). To examine the consequences of treatment by immunohistology, we evaluated five tumors from each experimental group at day 40 after initiation of treatment. Tumor xenografts from mice treated with 50 mg/kg/d ZD6474 (once daily, per os) or from those undergoing orchiectomy were found to have a higher percentage of necrosis compared with vehicle-treated controls (48 ± 5% and 51.1 ± 5% versus 31 ± 7%, respectively, P = 0.047; Fig. 5). Interestingly, animals that were treated with the combination of orchiectomy and ZD6474 had tumors with more necrosis (73 ± 6% versus 51 ± 5%, P = 0.01) than was observed in either monotherapy group. Although not clearly demonstrated in the tumor regrowth experiments, more profound tumor necrosis in the combination group may afford some added therapeutic benefit.

In summary, treatment with ZD6474 alone (50 mg/kg/d) inhibited growth of androgen-dependent LNCaP prostate tumors...
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Fig. 5  Histologic analysis of five LNCaP tumor xenografts treated for 40 days. A, hematoxylin and eosin histologic sections (×10 objective) picked at random from control (i; vehicle-treated); orchiectomy (ii); ZD6474 (iii; 50 mg/kg/d) treated; and (iv) orchiectomy + ZD6474 (50 mg/kg/d) treated. All tumors were harvested at 40 days. A significant increase in the percentage of total tumor necrosis was noted in the ZD6474 (50 mg/kg/d, once daily, per os) plus orchiectomy group, compared with tumors from mice receiving either treatment alone. B, percentage of tumor necrosis as determined by morphometric image analysis of tumors described in A.

significantly and had a more profound effect than androgen ablation alone. When ZD6474 was administered to mice with or without concomitant androgen ablation and then withdrawn, the greatest inhibition of tumor regrowth was observed in androgen-ablated animals. Increased tumor necrosis was also observed in mice treated with the combination of ZD6474 and androgen ablation. Collectively, these data suggest that clinical testing of VEGF signaling inhibition in combination with anti-androgen agents is warranted.

ACKNOWLEDGMENTS
We gratefully acknowledge the technical assistance of Sandra Brave for producing in vitro cytotoxicity data.

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