Sustained in Vivo Regression of Dunning H Rat Prostate Cancers Treated with Combinations of Androgen Ablation and Trk Tyrosine Kinase Inhibitors, CEP-751 (KT-6587) or CEP-701 (KT-5555)\(^1\)

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ABSTRACT

The indolocarbazole analogue CEP-751 is a potent and selective tyrosine kinase inhibitor of the neurotrophin-specific trk receptors that has demonstrated antitumor activity in nine different models of prostate cancer growth in vivo. In the slow-growing, androgen-sensitive Dunning H prostate cancers, which express trk receptors, CEP-751 induced transient regressions independent of effects on cell cycle. Because androgen ablation is the most commonly used treatment for prostate cancer, we examined whether the combination treatment of CEP-751 with castration would lead to better antitumor efficacy than either treatment alone. For a 60-day period, H tumor-bearing rats received treatment with either castration, CEP-751 (10 mg/kg once a day s.c. for 5 days every 2 weeks), or a combination of both, or vehicle. Castration caused tumor regression, followed by tumor regrowth in 4–6 weeks, whereas intermittent CEP-751 treatments resulted in tumor regressions during each treatment, which were followed by a period of regrowth between intermittent drug treatment cycles. Overall, both monotherapies significantly inhibited tumor growth compared with the vehicle-treated control group. However, the combination of castration and concomitant CEP-751 produced the most dramatic results: significantly greater tumor regression than either therapy alone, with no signs of regrowth. A related experiment using an orally administered CEP-751 analogue (CEP-701), as the trk inhibitor, and a gonadotrophin-releasing hormone agonist, Leuprolide, to induce androgen ablation demonstrated similar results, indicating that these effects could be generalized to other forms of androgen ablation and other trk inhibitors within this class. In addition, when CEP-701 was given sequentially to rats bearing H tumors, which were progressing in the presence of continuous androgen ablation induced by Leuprolide, regression of the androgen-independent tumors occurred. In summary, these data demonstrate that CEP-751 or CEP-701, when combined with surgically or chemically induced androgen ablation, offer better antitumor efficacy than either monotherapy and suggest that each therapy produces prostate cancer cell death through complementary mechanisms.

INTRODUCTION

For nearly 60 years, androgen ablation therapy has been the mainstay of treatment for metastatic prostate cancer, resulting in significant palliation for the majority of patients. However, although androgen ablation results in measurable tumor regression for the majority of prostate cancer patients, it inevitably selects for androgen-independent prostate cancer progression, which becomes clinically apparent within an average of 18 months, and leads to a modest, if any, survival advantage (1). Mortality in this androgen-refractory stage has been uniform and has resulted in nearly 40,000 deaths per year in the United States alone (2). For these reasons, additional targets and therapies are needed in conjunction with androgen ablation.

Over the past two decades, a myriad of growth factors have been identified that stimulate prostate cell proliferation and could thus represent targets for therapy. Recent evidence suggests that the NGF\(^3\) pathway, which appears to function in an autocrine/paracrine manner to stimulate prostate cancer growth (3, 4) and survival (5), may represent one such appropriate target: (a) normal prostate epithelial cells express trkA, the high affinity tyrosine kinase receptor for NGF (5–9). In addition, both primary and metastatic prostatic adenocarcinomas as well as established prostate cancer cell lines express trkA, detected using immunocytochemical and reverse transcription-PCR analysis (5), although no mutations have been detected in the trkA gene of these cancers (10); (b) Djakiew et al. (11) have demonstrated that an NGF-like protein, secreted from a human stromal cell line, stimulates the growth of a human prostate cancer cell line (TSU-pr1), an effect which an anti-NGF antibody completely blocked (3); and (c) NGF and NGF-like proteins appear to increase the motility and invasiveness of prostate cancer cell lines (11, 12). Because these cell lines do not express detectable levels of the low-affinity NGF receptor, p75NGFR (13), these effects are likely propagated solely through the trkA receptor, which could therefore represent a specific target for therapy.

The indolocarbazole K252a is a well-known inhibitor of trk tyrosine kinase activity in vitro (14–17), whereas the more trk-selective, K-252a analogue CEP-751 (Table 1) was shown to have potent antitumor activity in vivo (5, 18). Furthermore, CEP-751 has been demonstrated to selectively inhibit prostatic cancer growth in various animal models irrespective of tumor growth rate, androgen sensitivity, or metastatic ability (5). In these initial studies, the antitumor effects of CEP-751 were independent of effects on androgen because exogenous androgen treatment maintained the serum testosterone levels of the host animals within the normal physiological range. Therefore, the present studies of intermittent CEP-751 as monotherapy or combined with androgen ablation were designed to determine whether the combination resulted in greater tumor regression than either monotherapy and to elucidate whether CEP-751 and androgen ablation caused tumor regression through independent pathways. Additionally, an orally absorbed derivative of CEP-751, known as CEP-701 (Table 1), was tested alone and in combination with chemical-induced androgen ablation (with the GnRH agonist Leuprolide) to determine whether the mode of androgen ablation was critical to the results, and whether other trk-inhibitory indolocarbazoles might also cause tumor regression independent of, or in addition to, androgen ablation.

As a test system, the Dunning R-3327 H rat prostate cancer was chosen because it mimics many of the characteristics of the human disease (19). Having spontaneously arisen in an aged Copenhagen rat, this prostate cancer is androgen sensitive and slow growing (>20-day doubling time; Ref. 19). Surgical castration of rats bearing Dunning H cancers causes an almost immediate cessation of tumor growth. How-
ever, just as in the human disease, the cancers invariably regrow in an androgen-independent state, usually detectable 5–6 weeks after castration (20).

MATERIALS AND METHODS

Materials. Nunc 96-well microtiter plates (Maxisorb) were purchased through Fisher Scientific (Pittsburgh, PA), and 96-well Multiscreen-DP plates were obtained from Millipore (Bedford, MA). Anti-phosphotyrosine 4G10 antibody was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Alkaline phosphatase goat anti-mouse IgG was supplied by Bio-Rad Laboratories (Hercules, CA). ELISA amplification system was purchased from Life Technologies, Inc. (Gaithersburg, MD). DMSO and ATP were supplied by Sigma Chemical Co. (St. Louis, MO). Protease-free and Histone H1 (electrophoretically homogeneous preparation) were purchased from Boehringer Mannheim (Indianapolis, IN). Phospholipase C-γ1 was generated as a fusion protein with glutathione S-transferase, following the procedure of Rotin et al. (21). [γ-32P]ATP (6000 Ci/mmol) was supplied by Amersham (Arlington Heights, IL). CEP-701 and CEP-751 were provided by Kyowa-Hakko Kagyo Co., Ltd. (Tokyo, Japan). All other reagents were of the highest purity commercially available.

CEP-751 (also known as KT-6587) was formulated (10 mg/ml) in a vehicle containing 40% polyethylene glycol (PEG 1000; Spectrum, Los Angeles, CA), 10% polyvinylpyrrolidone (C30; ISP, Boundbrook, NJ), and 2% benzyl alcohol ( Spectrum) in water (48%). CEP-701 (also known as KT-5555) was formulated at a 10 mg/ml concentration in a vehicle of Gelucire:propylene glycol (3:1), using Gelucire 44/14 (Gattefosse Corp., Westwood, MA) and propylene glycol, USP/NF (Spectrum Chemical, New Brunswick, NJ).

Enzymes. Recombinant human EGFR, PDGFRβ, trkA, and VEGFR2 cytoplasmic domains were expressed in a baculovirus insect cell system. Human β1RK domain was obtained from Stratagene (La Jolla, CA) as a purified preparation from baculovirus clones expressed in S99 insect cells. Purified protein kinase C from rat brain was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY) as a mixture of Ca2+-dependent isozymes (α, β, and γ).

Receptor-linked Tyrosine Kinase ELISA. Assays were performed as described for trkA kinase (22). Briefly, the 96-well microtiter plate was coated with substrate solution (human recombinant phospholipase C-γ-glutathione S-transferase fusion protein). Inhibition studies were performed in 100-μl assay mixtures containing 50 μM HEPES (pH 7.4) K+, level of ATP (1, 5, 20, 40, 50 μM ATP for EGFR, β1RK, PDGFRβ, trkA, and VEGFR2, respectively), 10 mM MnCl2, 0.1% BSA, 2% DMSO, and various concentrations of CEP-751 or CEP-701. The reaction was initiated by addition of prephosphorylated recombinant human kinase domain (50 μg/ml EGFR, 20 milliunits/ml β1RK, 10 ng/ml PDGFRβ, 1 μg/ml trkA, or 75 ng/ml VEGFR2) and allowed to proceed for 15 min at 37°C. An antibody to phosphotyrosine (4G10) was then added, followed by a secondary enzyme-conjugated antibody, alkaline phosphatase-labeled goat anti-mouse IgG. The activity of the bound enzyme was measured via an amplified detection system (Life Technologies, Inc.). Inhibition data were analyzed using the sigmoidal dose-response (variable slope) equation in GraphPad Prism.

Protein Kinase C Multiscreen “In-Plate” Assay. Protein kinase C activity was assessed using the Millipore Multiscreen TCA “in-plate” assay mixture containing 50 μM HEPES (pH 7.4), 10 mM MgCl2, 2.5 mM EGTA, 2.5 mM CaCl2, 0.1 mM MnCl2, 0.8 μM/ml dienol, 200 μM/ml histone H-1, 5 μM [γ-32P]ATP, 1.5 ng of protein kinase C, 0.1% BSA, 2% DMSO, and various concentrations of CEP-751 or CEP-701. The reaction was allowed to proceed for 10 min at 37°C, then quenched by adding trichloroacetic acid. The plates were washed several times with ice-cold 25% trichloroacetic acid using a filtration apparatus. Scintillation cocktail was added to the samples, followed by equilibration for 1 h. The radioactivity was determined in the Wallac MicroBeta 1450 PLUS scintillation counter.

Animals and Tumor Implantation. Adult male inbred Copenhagen rats (200–240 g) were obtained from Harlan Sprague Dawley (Indianapolis, IN) and maintained in cages (three rats/cage) with a commercial diet (Purina Formula M5001) and water ad libitum. The animals were housed under humidity- and temperature-controlled conditions with the light/dark cycle set at 12-h intervals. All the rats were quarantined for 1 week before experimental manipulation. As a donor, a 1–2 cm3 Dunning R-3327 H cancer, growing in the flank of a Copenhagen rat, was resected and minced into small pieces. One g of such mince was combined with 10 ml of Matrigel (Collaborative Research, Bedford, MA), and 100 μl of this mix (~10 mg of tumor tissue) were injected s.c. into the flank of recipient Copenhagen rats.

Immunocytochemical Analysis of Trk Expression. Tumor tissue was dissected and fixed in 10% buffered formalin and paraffin embedded. Immunocytochemical staining of 5-μm paraffin-embedded histological sections was then performed as described previously (5), except that mouse monoclonal antibodies were used as primary antibodies and the universal DAKO LSAB peroxidase kit from DAKO Corp. (Carpenteria, CA) was used for the immunoperoxidase detection with diaminobenzidine as a substrate. Anti-trk mouse monoclonal IgG, antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), using the Dako LSAB kit (32P/ml final concentration), was used to demonstrate the specificity of this antibody, control samples were incubated 10-fold excess of the defined peptide mapped to the COOH terminus of the precursor sequence of rat trkA used to generate the antibody.

Antitumor Efficacy of CEP-751 as an Adjuvant to Surgical Castration. Thirty-six rats bearing Dunning H cancers were divided into four groups of nine animals each, with the average tumor size for each group of 8 cm3. One group of rats (group 1) served as a vehicle control, whereas a second group of rats (group 2) underwent castration on day 0, as described previously (19). A third group (group 3) of rats received s.c. injections (10 mg/kg) of CEP-751 once daily for 5 consecutive days, followed by 9 days of no treatment. This intermittent dosing was repeated every 2 weeks so that the drug was administered on days 0–4, 14–18, 29–33, and 42–46 in the initial experiment. The fourth group (group 4) was castrated on day 0 and also received intermittent dosing of CEP-751. On day 0, 2-cm long, sealed silastic capsules, which were filled with testosterone, as described previously (24), were implanted s.c. into the flanks of rats in groups 1 and 3. This size silastic implant maintains serum testosterone in the physiological range of 1–3 ng/ml chronically for a period of 6 months (24). At the completion of the experiment, all tumor tissues were harvested, fixed in 10% buffered formalin, paraffin-embedded, and sectioned. These sections were then stained with H&E for histological examination.

Dose Response of Leuprolide Depot Injection versus Castration. To determine the appropriate dose for inducing androgen ablation in rats with the GnRH agonist Leuprolide (Lupron; TAP Pharmaceuticals, Deerfield, IL), a five-arm trial with eight rats in each group was undertaken. In the first group, control animals were treated with vehicle injections alone, whereas group 2 animals underwent surgical castration on day 0. For the remaining groups, Leuprolide was injected s.c. in the flank in a depot formulation, which releases the drug into the plasma circulation continuously over a 30-day period. Group 3 received a dose of 1.04 mg/kg, whereas groups 4 and 5 animals received doses of 5.2 and 12.5 mg/kg, respectively, based on a report published previously (25). Blood was drawn via the retroorbital vein from rats on days 1–4, 6, and 10, and serum testosterone was determined by RIA as described previously (24). On day 21, the animals were sacrificed; their prostates were removed, dissected into dorsolateral and ventral lobes, and weighed, and ventral prostates were processed for DNA content as described previously (24). This initial experiment revealed maximal androgen suppression with doses of 5.2 mg/kg or higher given every 21 days; thus, a 5.2 mg/kg dose was given every 21 days in all experiments.

Antitumor Effects of CEP-701 Alone and Combined with Leuprolide Injections. Forty rats bearing Dunning H cancers of a mean size of 8 cm3 were divided into four groups of 10 rats each. The first group (group 1) served as the control and received drug vehicle alone. A second group of rats (group 2) underwent androgen ablation by administration of 5.2 mg/kg Leuprolide in depot formulation every 21 days. A third group (group 3) received orally administered intermittent CEP-701 treatment at 10 mg/kg doses twice a day in volumes <0.5 ml via a stomach tube. The rats tolerated the therapy well, which they received intermittently for two 3-week periods from days 0–20 and days 32–53. The drug vehicle was administered to all rats not receiving CEP-701 treatment on an identical schedule in similar volumes. The fourth group (group 4) received treatment with both intermittent CEP-701 and Leuprolide depot injections. Similar to the previous experiment, 2-cm-long silastic tubes filled with testosterone were implanted in rats from groups 1 and 3 on day 0 to maintain physiological serum levels. After 63 days of Leuprolide treatments, the 10 remaining rats from group 2 received another injection of Leuprolide depot and then were separated into
two sets of five rats each. One set was treated with CEP-701 for 15 days, whereas the other set received vehicle. Upon completion of the experiment, the animals were sacrificed; their tumors were then formalin-fixed, paraffin-embedded, and step-sectioned. These sections were then stained with H&E and visualized using ×800. Pictures of representative fields measuring $373 \times 324 \times 5 \mu m (4.6 \times 10^{-7} \text{cm}^3)$ from each tumor section were acquired using a Kodak MDS-40 digital camera. Cancer cells were then counted from a minimum of 12 fields and averaged. The SE of the 12 fields was then calculated, and if it was >10% of the average, an additional 4 fields were counted. For each tumor counted (three to four per group), the average cancer cell number per cm$^3$ was then calculated (average cell count divided by the volume, $4.6 \times 10^{-7} \text{cm}^3$) for each treatment group, and the SE for each group was calculated.

Tumor Measurements. Tumors were measured at indicated intervals using a vernier caliper on animals anesthetized with methoxyflurane. Tumor volumes were then calculated using the formula:

$$V (\text{cm}^3) = 0.5236 \times [\text{length (cm)} \times \text{width (cm)} + \text{width (cm)}]/2$$

The tumors in each animal were individually normalized to their size at the start of the experiment, and the data were calculated as the change in tumor volume relative to the day 0 volume.

**RESULTS**

Neurotrophin Receptor Expression by Dunning H Rat Prostate Cancer Cells. Using reverse transcription-PCR, it was demonstrated that all three trk receptor mRNAs were expressed in Dunning H rat prostate cancers (S). To identify the specific cell types expressing the trk receptor proteins, histological sections of H tumors were immunocytochemically stained using a mouse monoclonal pan-trk antibody. These studies demonstrate that in the well-differentiated H rat prostate cancers, the malignant acinar cells express trk receptors whereas the normal host stromal cells do not (Fig. 1). Thus, the H prostate cancers express the appropriate cellular targets for evaluating the antitumor effects of inhibition of the trk tyrosine kinase. Previously, a series of indolocarbazole analogue was screened for their selection abilities to inhibit a variety of receptor and nonreceptor tyrosine kinases (18). Two of these, CEP-751 and CEP-701, were chosen for additional study on the basis of their potent ability to

Statistical Analysis. The paired t-Test and Mann-Whitney Rank Sum Test were applied for statistical analysis using the SigmaStat program.
10 mg of Dunning H cancer tissue was inoculated s.c. in Matrigel, and in Simultaneous Combination on Dunning H Tumors. When activity toward other kinases (Table 1).

rounded by stromal elements (Figure 3 well-differentiated adenocarcinomas composed of tumor acini sur-

the control group, which was treated with s.c. injections of vehicle four groups over the 2-month time period. As shown by this figure, administration as described above.

animals were then divided equally into four cohorts of nine tumor-

hagen rats. These tumor-bearing rats were allowed to go untreated for

CEP-751 monotherapy also resulted in tumor regression, which continued until the cessation of each daily drug treatment. Although tumor regrowth was apparent after drug withdrawal, significant tumor regression was observed during each subsequent CEP-751 dosing cycle (Fig. 2). Using immunocytochemical analysis, Dunning H tumor regression induced by CEP-751 has been demonstrated previously to result from both an inhibition of proliferation and an increase in apoptotic death of the H cancer cells (5). The net effect of the four 5-day intermittent treatments of CEP-751 over the course of 60 days was a mean 44% inhibition of tumor growth by volume. In addition to this decrease in volume after four intermittent treatments with CEP-751, the histology of the H tumors is different (i.e., the malignant acini are generally smaller; Fig. 3C).

The simultaneous combination of surgical castration and intermittent CEP-751 therapy produced the most durable and significant responses. In this group of rats, tumor regression within 5 days was significantly (P ≤ 0.05) greater than with either monotherapy and demonstrated no evidence of regrowth throughout the 60-day time course (Fig. 2). The differences in tumor volumes became even more statistically pronounced (P ≤ 0.01) once the tumors in the other treatment groups began to regrow. Furthermore, the histology of tumors from these animals that had been treated with the simultaneous combination of castration and CEP-751 is dramatically different compared with tumors from either the vehicle-treated animals or the single treatment groups. These combined-treatment tumors have the lowest number of malignant acini per volume, and the acini size is generally smaller (Fig. 3D), compared with all of the other treatment groups.

Because of the design of the experiment and the frequency of measurements and dosing, which necessitated multiple occasions of anesthesia, several rats died during the course of the experiment. Specifically, three rats died in the control group (group 1), one rat died in the castration group (group 2), and two rats each died in the CEP-751-treated group (group 3) and the combination treatment group (group 4). All of these deaths were temporally related to the administration of the anesthesia and were not felt to represent lethal toxicity from the therapies.

<table>
<thead>
<tr>
<th>Kinase</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;, nM CEP-751 (R = OCH&lt;sub&gt;3&lt;/sub&gt;)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;, nM CEP-701 (R = OH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>trkA</td>
<td>3.4 ± 0.2</td>
<td>3.7 ± 0.5</td>
</tr>
<tr>
<td>EGFR</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>9 ± 2</td>
<td>65 ± 7</td>
</tr>
<tr>
<td>Insulin receptor</td>
<td>678 ± 31</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>PDGFRβ</td>
<td>160 ± 7</td>
<td>773 ± 53</td>
</tr>
<tr>
<td>PKC</td>
<td>114 ± 7</td>
<td>218 ± 6</td>
</tr>
</tbody>
</table>

The inhibitory effects of CEP-701 or CEP-751 on the kinase activities of trkA, PDGFRβ, EGFR, VEGFR2, β-insulin receptor, and protein kinase C were evaluated using assays described in “Materials and Methods.” IC<sub>50</sub>s are reported as the mean of at least three determinations ± SE.
Effects of Intermittent Oral CEP-701 and Chemical Castration Treatment Alone and in Simultaneous Combination on Dunning H Tumors. To test whether the enhanced efficacy of combined CEP-751 treatment and surgical castration is unique or generalized to other trk inhibitors and other forms of androgen ablation, the experimental design was repeated using the GnRH agonist Leuprolide for androgen ablation and an orally active selective trk inhibitor, CEP-701. Before initiating this experiment, a dose of Leuprolide, which effectively lowers the serum testosterone to castration levels, had to be established. In a previous study in which Copenhagen rats were treated with 1 mg/kg every 3 weeks for 9 weeks, significant decreases in prostate weight and serum testosterone levels were seen (25). To compare the time courses of castration versus Leuprolide-induced androgen ablation, Copenhagen rats were either surgically castrated or injected s.c. in the flank with vehicle or Leuprolide at a dose of 1.04, 5.2, or 12.5 mg/kg. At 1, 2, 4, 6, and 10 days, blood was drawn for serum testosterone determination. After 21 days, the animals were sacrificed, their ventral prostates were removed and weighed, and the DNA content of these prostates was measured. Within 6 days, serum testosterone levels decreased from 2.11 ± 0.18 ng/ml in the intact vehicle-treated control group to <0.1 ng/ml in the surgically castrated rats, and rats were given either 5.2 or 12.5 mg/kg of Leuprolide (Table 2). At a dose of 12.5 mg/kg of Leuprolide, there was an initial rise in serum testosterone suggestive of the flare reaction seen clinically (26). Regardless, by 21 days of treatment with either 5.2 or 12.5 mg/kg of Leuprolide, the wet weights and DNA contents of the rat ventral prostates decreased to the same extent as those of the surgically castrated rats (Table 3). On the basis of these data, 5.2 mg/kg of Leuprolide was the highest dose that did not produce a flare reaction and thus was chosen as the standard dose. For all future studies, this dose was injected s.c. into the flank of rats every 3 weeks.

With an effective dose of Leuprolide in Copenhagen rats estab-

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 4</th>
<th>Day 6</th>
<th>Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>2.11 ± 0.15a</td>
<td>1.85 ± 0.21</td>
<td>2.01 ± 0.17</td>
<td>1.92 ± 0.21</td>
<td>1.86 ± 0.13</td>
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<tr>
<td>Surgery</td>
<td>&lt;0.1b</td>
<td>&lt;0.1b</td>
<td>&lt;0.1b</td>
<td>&lt;0.1b</td>
<td>&lt;0.1b</td>
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<tr>
<td>Leuprolide</td>
<td>1.04 mg/kg</td>
<td>1.99 ± 0.12</td>
<td>1.31 ± 0.29</td>
<td>0.29 ± 0.02b</td>
<td>NDc</td>
</tr>
<tr>
<td></td>
<td>5.2 mg/kg</td>
<td>2.19 ± 0.1</td>
<td>1.12 ± 0.14d</td>
<td>0.21 ± 0.01b</td>
<td>&lt;0.1b</td>
</tr>
<tr>
<td></td>
<td>12.5 mg/kg</td>
<td>3.16 ± 0.52</td>
<td>1.93 ± 0.5</td>
<td>0.48 ± 0.14</td>
<td>0.14 ± 0.02</td>
</tr>
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a Values are mean ± SE.
b P < 0.05 versus vehicle control.
c ND, not determined.

Fig. 3. Histology of Dunning H prostate cancer in rats treated with either vehicle, CEP-751, castration alone, or in combination. H&E-stained sections from vehicle-treated (A), castration-alone treated (B), intermittent CEP-751-alone treated (C), and combined castration and intermittent CEP-751-treated (D) tumors harvested on day 63 are shown. All photographs are at ×200.
established, the previous experimental design was repeated substituting Leuprolide for castration, and the O-desmethyl derivative of CEP-751 and CEP-701. Table 1 illustrates the chemical structures of CEP-701 compounds as well as its inhibitory properties. In this experiment, all of the rats designated for androgen ablation (groups 2 and 4) received a 5.2 mg/kg Leuprolide depot injection every 3 weeks, whereas CEP-701 was administered by gavage twice daily for two 21-day cycles (separated by 10 days of rest) to all of the rats in groups 3 and 4. As was observed in the previous CEP-751/castration experiment, each monotherapy inhibited growth of the tumors compared with the control group, whereas the combination treatment resulted in statistically significant tumor regression ($P \leq 0.01$) with no evidence of regrowth (Fig. 4).

The previous observation that the malignant cell density of tumors in animals treated with either castration, CEP-751, or the combination was qualitatively decreased when compared with vehicle-treated controls (Fig. 3) suggests that the effects of those treatments were underestimated by bidimensional tumor measurements. Therefore, to document this regression further, as well as to determine whether malignant cell density might estimate more accurately the degree of regression induced, H&E-stained tumor sections from each animal were counted for the number of H cancer cells/cm$^3$ of tumor. Table 4 demonstrates that there were statistically less cancer cells per tumor volume ($P < 0.05$) in the three treatment groups when compared with the vehicle-only group (i.e., 49, 57, and 19% tumor cell density relative to the vehicle control group for the Leuprolide alone, CEP-701 alone, and the combination groups, respectively). By multiplying these tumor cell density values by the total volume of each tumor at the end of the 63-day treatment period, the total number of tumor cells was calculated. Each of these values was then divided by the total number of starting cells at day 0 (calculated by multiplying the volume of each tumor at day 0 by the tumor cell density of the vehicle control group) to calculate the mean ± SE fold change in tumor cells over the 63-day observation period. These data (Table 4) demonstrate that androgen ablation monotherapy induced an approximate one-third decrease in the total number of cells, whereas intermittent CEP-701 monotherapy completely blocked any significant increase in total tumor cells over the 63-day period. In contrast, the combination treatment produced a $>80\%$ ($P < 0.05$) regression in the total tumor cell number (Table 4).

### Table 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dorsolateral prostate wet weight (mg/gland)</th>
<th>Ventral prostate wet weight (mg/gland)</th>
<th>Ventral prostate DNA content (μg/μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle control</td>
<td>273 ± 16</td>
<td>348 ± 13</td>
<td>863 ± 60</td>
</tr>
<tr>
<td>Surgical castration</td>
<td>90 ± 5</td>
<td>42 ± 9</td>
<td>216 ± 11</td>
</tr>
<tr>
<td>Leuprolide</td>
<td>104 mg/kg</td>
<td>137 ± 17</td>
<td>218 ± 20</td>
</tr>
<tr>
<td></td>
<td>5.2 mg/kg</td>
<td>116 ± 17</td>
<td>264 ± 43</td>
</tr>
<tr>
<td></td>
<td>12.5 mg/kg</td>
<td>101 ± 9</td>
<td>221 ± 23</td>
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$^a$ $P < 0.05$ versus vehicle control.

### Table 4

<table>
<thead>
<tr>
<th>Treatment</th>
<th>H-Tumor cell density at day 63 ($\times 10^9$ cells/cm$^3$)</th>
<th>Total no. of H-prostate cancer cells ($\times 10^7$ cells)</th>
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<tbody>
<tr>
<td>None</td>
<td>8.9 ± 0.35</td>
<td>6.96 ± 0.54</td>
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<tr>
<td>Vehicle</td>
<td>8.7 ± 0.22</td>
<td>16.00 ± 2.69</td>
</tr>
<tr>
<td>Leuprolide</td>
<td>4.3 ± 0.19</td>
<td>4.73 ± 0.07</td>
</tr>
<tr>
<td>CEP-701</td>
<td>5.0 ± 0.84</td>
<td>8.21 ± 0.07</td>
</tr>
<tr>
<td>Leuprolide and CEP-701</td>
<td>1.8 ± 0.10</td>
<td>1.32 ± 0.07</td>
</tr>
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</table>

$^a$ Values are expressed as mean ± SE.

$^b$ Total number cells = tumor volume $\times$ tumor cell density.

$^c$ Day 63.

$^d$ $P < 0.05$ compared with day 0.

Fig. 4. Effects of oral CEP-701, or chemical castration with Leuprolide alone or in simultaneous combination, on the growth of Dunning H prostate cancer in rats. CEP-701 treatment was given intermittently for two 21-day cycles separated by 10 days of no drug treatment, as indicated by the boxes on the x-axis. Results are presented as the means of tumor volumes relative to the tumor volumes at day 0 of treatment; bars, SE. All groups are statistically ($P < 0.05$) different from the vehicle group at all points after day 0. *, $P < 0.05$; **, $P < 0.01$ compared with either Leuprolide or CEP-701 monotherapy treatment groups.

Fig. 5. Effects of sequential CEP-701 treatment on the growth of Dunning H prostate cancer in Leuprolide-treated rats. Male rats bearing Dunning H cancer were treated with Leuprolide for 63 days before being divided into two groups. One group was subsequently treated with oral CEP-701 (10 mg/kg twice a day) and additional Leuprolide dosing, whereas the second group received additional Leuprolide dosing only. Results are presented as means of tumor volume relative to day 0 (i.e., day 63 of Leuprolide); bars, SE. *, $P < 0.05$; **, $P < 0.001$ relative to the Leuprolide-alone group.
ment (group 2) were subdivided, and half of them were treated with oral CEP-701 for 15 days. Fig. 5 demonstrates a significant 24% (P < 0.01), tumor regression caused by the addition of CEP-701 after just 8 days, although the tumors in animals given Leuprolide alone continued to grow in the presence of androgen ablation. When the tumor measurements from these groups were corrected for malignant cell density, the sequential treatment produces an overall effect similar to that of the concomitantly treated group. Specifically, the group treated for 15 days with the combination after 2 months of Leuprolide monotherapy produced tumors with an average cell number of 2.9 ± 0.64 × 10^5 cells, which represents an 80% decrease (P < 0.05) from the average day 0 cell number (14.7 ± 5.11 × 10^5 cells), similar to the 81% decrease induced by two 21-day cycles of CEP-701 and simultaneous Leuprolide treatment. These data suggest that initial treatment with androgen ablation therapy can be followed sequentially with CEP-701 treatment without loss of the additional antituumor effect of CEP-701.

**DISCUSSION**

Using the androgen-sensitive Dunning H rat prostate cancer model, the present experiments have demonstrated significant antitumor effects of the indolocarbazole analogues CEP-751 and CEP-701. Both drugs selectively inhibit the trk tyrosine kinase receptors with an IC₅₀ in vitro between 2 and 4 nM (18), and although these drugs differ slightly in their structure, bioavailability, and toxicity, there is no evidence that they inhibit tumor growth through separate mechanisms of action. Additionally, studies suggest CEP-751 and CEP-701 have selective antitumor activities in vivo (5), presumably through competitive inhibition of ATP-binding domains associated with enzymes including tyrosine kinases such as the trk receptors (18). These studies also demonstrate that treatment of H prostate cancer with CEP-751 produces tumor regression in the presence of physiological testosterone levels (5). Therefore, to investigate whether CEP-751 antitumor efficacy could be enhanced by androgen ablation, the treatments were combined.

The results of these experiments support several conclusions: (a) CEP-751, as shown previously, does induce tumor regression (5). Interestingly, six sequential cycles did not select for a drug-resistant population. Likewise, retreatment of Dunning H tumors with CEP-751 after 2 months of 5 mg/kg/day dosing and a period of withdrawal still resulted in significant regression (data not shown); (b) castration induces regression of Dunning H tumors as reported previously (19). These results validate our approach and technique; (c) the combination of both androgen ablation and intermittent CEP-751 (or CEP-701) initiated simultaneously exhibit greater antitumor effects in terms of both extent of tumor regression and inhibition of regrowth than either monotherapy. Furthermore, the enhanced effects of this combination are seen whether the drugs are given simultaneously with androgen ablation or sequentially at the time of tumor regrowth after androgen ablation. This finding has important implications for the development and use of these compounds in clinical trials; and (d) previous work on CEP-751 (5) and androgen ablation (19, 20) in this tumor model suggests that both induce apoptosis and inhibit survival pathways. These data suggest they do so through complementary pathways because progression to androgen-independent growth, after androgen ablation, does not select for cross-resistance to CEP-751 or CEP-701.

The limitations of this combination therapy warrant further investigation. For instance, how much CEP-751 is necessary to inhibit regrowth when combined with androgen ablation? Do both therapies require indefinite use, or can one or the other be stopped without further regrowth? Will these tumors eventually regrow, even with both therapies continuing? And finally, by treating low volume palpable cancers, can recurrence be prevented? These issues are presently being addressed experimentally, while clinically, Phase I trials with this class of trk inhibitors are ongoing.

**REFERENCES**

Sustained in Vivo Regression of Dunning H Rat Prostate Cancers Treated with Combinations of Androgen Ablation and Trk Tyrosine Kinase Inhibitors, CEP-751 (KT-6587) or CEP-701 (KT-5555)

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