

# Cell Cycle-independent Death of Prostate Adenocarcinoma Is Induced by the *trk* Tyrosine Kinase Inhibitor CEP-751 (KT6587)<sup>1</sup>

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## ABSTRACT

Advanced prostate cancer remains largely incurable, primarily because the very low growth fraction present in these tumors makes them generally resistant to treatment with standard chemotherapeutic agents that target cell division. Effective therapies should therefore induce death of prostate cancer cells, independent of their growth rate. *trkA*, the high-affinity tyrosine kinase-linked receptor for nerve growth factor, has been implicated in prostatic cancer growth and may represent a molecular target for therapeutic agents. At low mg/kg doses, the *trk* tyrosine kinase inhibitor CEP-751 (KT6587) inhibits prostatic cancer growth in nine different animal models independent of the tumor growth rate, androgen sensitivity, metastatic ability, or state of tumor differentiation. CEP-751 is selective for cancerous *versus* normal prostate cells and affects the growth of only a limited number of nonprostate tumors. Importantly, CEP-751 induces cell death of prostate cancer cells in a cell cycle-independent fashion and, therefore, represents a novel therapeutic approach to the management of both hormone-dependent and hormone-independent prostate cancer.

## INTRODUCTION

Prostate cancer is the most frequently diagnosed cancer in men and is responsible for approximately 41,000 deaths in the

United States annually (1). Early-stage, organ-confined prostate cancer is managed with some success via observation, surgery, or radiation, with the patient outliving the disease in many cases (1). Prostate cancer cells, like the normal prostatic glandular cells of their origin, are often responsive to androgen; therefore standard therapy for late-stage metastatic prostatic cancer is surgical or chemical androgen ablation. Although androgen ablation may have modest survival benefit, such therapy is mainly palliative, and androgen-insensitive tumors eventually develop that ultimately lead to the death of the patient (2). Due to the very low daily rate of proliferation of both androgen-dependent and androgen-independent prostate cancer cells (3), this disease has been refractory to treatment with standard chemotherapeutic agents, the cytotoxicity of which is dependent upon high rates of cellular division (4). Thus, an optimal drug for the treatment of prostate cancer will be an agent that induces death of the cancer cells in a cell proliferation-independent fashion (5).

The neurotrophins (*i.e.*, NGF,<sup>3</sup> brain-derived neurotrophic factor, NT-3, and NT-4) and their respective high-affinity tyrosine kinase-linked receptors (*i.e.*, *trkA* for NGF, *trkB* for brain-derived neurotrophic factor and NT-4, and *trkC* for NT-3; Ref. 6) have been implicated in prostate cancer growth. Both normal and malignant prostatic tissues locally produce measurable amounts of NGF and NT-4 (7-9), and human prostate stromal cells produce a mitogen for prostatic cancer cells, which can be immunodepleted with anti-NGF sera (10). Growth of prostate epithelial cells in soft agar is induced by NGF but not by a variety of other growth factors, including acidic fibroblast growth factor, transforming growth factor  $\beta$ , insulin-like growth factor-1, PDGF, epidermal growth factor, transforming growth factor  $\alpha$ , or keratinocyte growth factor (11). Using Scatchard binding analysis, Pflug *et al.* (12) demonstrated that normal human prostatic glandular cells express high affinity ( $K_d = \sim 1 \times 10^{-11}$  M) *trkA* receptors. *trkA* has also been shown by immunohistochemical analysis to be expressed in luminal epithelial cells of normal human prostate tissue (13). These data provide evidence that neurotrophins and *trks* are expressed in the prostate and may influence prostate cell growth.

To further evaluate the potential roles of the *trks* in prostate cancer, *trk* expression has been characterized in a series of human clinical specimens and in established human and rat prostate cancer cell lines. In addition, the anticancer efficacy of

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<sup>3</sup> The abbreviations used are: NGF, nerve growth factor; NT, neurotrophin; PDGF, platelet-derived growth factor; qd, once per day; BID, twice per day; BrdUrd, bromodeoxyuridine; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; RT-PCR, reverse transcription-PCR; T/C, treated *versus* control.

a potent trk inhibitor has been experimentally determined in animal models of both human and rat prostate cancer.

## MATERIALS AND METHODS

**Reagents and Animals.** CEP-751 (KT6587) was synthesized in the laboratories of Kyowa Hakko Kogyo (Tokyo, Japan) and was solubilized for *in vivo* experimentation as described previously (14).

Four to six week old NCr-nu or B6C3F1 mice were obtained under a contract with the National Cancer Institute. One hundred fifty-g inbred Copenhagen male rats were supplied by Harlan Sprague Dawley (Indianapolis, IN). Mice were maintained five per cage, and rats were maintained four per cage. All animals were given a commercial diet (Teklad Labchow) and water *ad libitum*. Animals were housed under humidity- and temperature-controlled conditions, and the light/dark cycle was set at 12-h intervals. All animal studies were performed according to animal protocols approved by the Cephalon, Johns Hopkins School of Medicine, or Southern Research Institute Institutional Animal Care and Use committees.

**Antitumor Experiments.** Tumors were measured in unanesthetized animals every 3–4 days using a vernier caliper. Tumor volume was calculated using the following formula: volume ( $\text{mm}^3$ ) =  $(L \times W \times [(L + W)/2]) \times 0.526$  as described previously (15). Body weights were also obtained at these times. Dosing volumes (3 ml/kg, s.c.) were adjusted as necessary twice a week.

LNCaP, DU145, PC-3, TSU-Pr1, and PC-82 human prostate cancer cell lines were tested as xenografts in adult male nude mice. G, MAT-Lu, AT-2, and H sublines of the Dunning R-3327 system of serially passageable rat prostate cancers were tested as growing tumors in Copenhagen rats. The initial development and characteristics of these lines has been reviewed recently (16, 17). The *in vivo* histology, androgen sensitivity, metastatic ability, and growth rate were determined as described previously (18). Adult female nude mice served as hosts for SK-OV-3, OVCAR-3, SK-Mel-5, MCF-7, and MX-1 human cancer cell lines; adult female B6C3F1 mice served as hosts for B16 and Lewis lung experiments.

The androgen-responsive PC-82 human and Dunning R-3327 H rat prostate cancers were established by s.c. implantation in the flank of ~20 mg of minced tumor tissue in 0.2 ml of Matrigel (Collaborative Research, Beltsville, MD) as described by Passiniti *et al.* (19). Four months post-tumor implantation, host animals were implanted with testosterone-filled capsules fabricated from sealed silastic tubing of sufficient size to maintain the serum testosterone within the physiological range for rats (*i.e.*, 1–2 ng/ml) or mice (*i.e.*, 2–4 ng/ml). The silastic capsules were formulated, and the serum testosterone was determined as described previously (20). After implanting the silastic capsules, tumor-bearing animals were randomized into vehicle *versus* CEP-751 treatment groups (*i.e.*, 14 mg/kg s.c. BID for 52 days for PC-82 tumor mice studies and 10 mg/kg s.c. qd for 10 days for H tumor rat studies).

For experiments using the androgen-independent human TSU-Pr1 cell line, nude mice were inoculated with  $3 \times 10^6$  viable cells. For the antitumor experiments using rat G, MAT-Lu, and AT-2 prostate cancers, adult male Copenhagen rats

were inoculated with  $1 \times 10^6$  viable cells of each of the sublines. In each case, cells were obtained from exponentially growing cultures of these serially passageable cell lines as described previously (18). When these tumors reached ~200–1000  $\text{mm}^3$ , animals were randomized into groups receiving daily s.c. injections of either vehicle or CEP-751. CEP-751 was administered at 10 mg/kg s.c. BID for 17 days for the TSU-Pr1, at 10 mg/kg s.c. BID for 14 days for the G experiment, or at 10 mg/kg s.c. qd for 10 days for the MAT-Lu and AT-2 experiments.

For all experiments except those described above, trocar pieces of the appropriate tumors (~2 × 3 mm) were implanted s.c. on the right flank of adult hosts. When tumors became palpable, the mice were randomized into two groups of 10 animals each. One group received CEP-751; the second group was vehicle treated. This protocol was followed for all experiments except that involving B16 tumors in which administration of CEP-751 or vehicle was begun 1 day after tumor implantation to ensure at least 10 days of dosing before sacrifice. DU145 and PC-3 tumor-bearing animals received 24 mg/kg s.c. BID CEP-751 for 28 days; Lewis lung animals received 9.3 mg/kg s.c. BID CEP-751 for 10 days; B16 animals received 10.5 mg/kg s.c. BID CEP-751 for 19 days. SK-OV-3, OVCAR-3, SK-Mel-5, MCF-7, and MX-1 animals received 21 mg/kg s.c. BID CEP-751 for 29, 29, 31, 25, and 18 days, respectively.

Animals were sacrificed 1 day after the last day of treatment in all experiments. The LNCaP, TSU-Pr1, PC-82, MAT-Lu, and H antitumor experiments were performed at the Johns Hopkins Oncology Center. The G and AT-2 antitumor experiments were performed at Cephalon, Inc. DU145, PC-3, SK-OV-3, OVCAR-3, B16, SK-Mel-5, MCF7, MX-1, and Lewis lung antitumor experiments were performed under contract to Cephalon at Southern Research Institute (Birmingham, AL).

**Immunocytochemical Analysis of trk Expression.** Rat and human tissues were fixed in 10% buffered formalin and processed for routine embedding in paraffin. All normal human prostate tissue and prostate cancer samples were obtained from the archival collection of the Department of Pathology, Johns Hopkins School of Medicine. Five- $\mu\text{m}$  sections were cut and mounted on Chem-Mate capillary gap plus microscope slides (BioTek Solutions). All slides were processed using the micro-capillary technique with Chem-Mate reagents (BioTek Solutions) according to the manufacturer's protocol. The sections were deparaffinized with xylene at room temperature, rehydrated, and then treated with steam heat for induction of antigen retrieval according to the BioTek protocol. These slides were then processed for immunocytochemical detection of expression of trks using purified rabbit polyclonal IgG antibodies specific for trk isoforms obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). As controls, each of these antibodies was preincubated overnight with a 10-fold excess by weight of the defined peptide sequence used to generate the antibodies. The defined peptides were obtained from Santa Cruz Biotechnology, Inc. For the trkA antibodies, the epitope corresponded to amino acids 763–777, located adjacent to the carboxyl terminus; for the trkB antibodies, the epitope corresponded to amino acids 794–808, located adjacent to the carboxyl terminus; for the trkC antibodies, the epitope corresponded to amino acids 798–812, located at the carboxyl terminus. The primary antibody solution

contained 0.25  $\mu\text{g}$  of IgG per ml. After incubation with the primary antibodies at 37°C for 2 h, the slides were washed with PBS and incubated with peroxidase-labeled polymer-conjugated antirabbit antibody (Envision Systems, DAKO Corp.) for 1 h. The final reaction product was visualized with 3-amino-9-ethylcarbazole solution (DAKO). The slides were counterstained with hematoxylin. Specific staining was determined by comparing the slides incubated with primary antibodies *versus* primary antibodies preincubated with 10-fold excess peptide to which the antibodies were made. Staining was evaluated as homogeneous when >80% of prostate cancer cells were positive, heterogeneous when >20 but <80% of the cells were positive, and negative when <20% of the cells were positive.

To evaluate the percentage of cells positively stained with each of the trk antibodies, histological fields, selected using systemic random sampling techniques (21) under 200 $\times$  magnification were quantitated using a point-counting morphometric technique (21). This technique involves the use of a 100-point intersecting grid reticule placed in the eye piece. Using this grid, the number of cancer cells per field that intersect a grid point (*i.e.*, denominator) was recorded, as well as the number of the cells that intersect the grid that were positively stained by the particular trk antibody (*i.e.*, numerator). Sufficient fields (*i.e.*, usually four or five per slide) were examined so that >200 total cells were evaluated per slide. The total number of cells that intersected the grid that were positive for trk staining was divided by the total number of cells that intersected the grid, and this fraction was multiplied by 100 to obtain the percentage of cells expressing the various trks.

**RT-PCR Detection of the trk Receptors in Prostate Cancer Cell Lines.** RNA was isolated from cell lines and tissue samples using the TRIzol Reagent (Life Technologies, Inc.). cDNA was made from 2–3  $\mu\text{g}$  of RNA using oligo(dT) and reverse transcriptase in kit form (Life Technologies, Inc.). The PCR cycle consisted of 1 min at 95°C followed by 1 min at 63°C and 1 min at 72°C for 35 cycles. For human samples, 10  $\mu\text{l}$  of each PCR were electrophoresed through a 1% agarose gel, stained with ethidium bromide for 10 min, and imaged on the Eagle Eye II still video system (Stratagene, Inc.). Rat samples were electrophoresed on a 5% acrylamide gel; the PCR mixture included 0.5  $\mu\text{Ci}$  of [ $^{32}\text{P}$ ]CTP (DuPont NEN, Wilmington, DE), and incorporation of label was detected using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

For human trk A, the sense primer (5'-TCCGCTCCATCATGGCTGCCTT-3') and antisense primer (5'-CCCAAACCTGTTTCTCCGTCCACA-3') corresponded to nucleotides 1231–1253 and 1426–1449, respectively, of the human trk proto-oncogene insert of pLM6; for trk B, the sense primer (5'-CTGACGCAGTCGCAGATGCTGCA-3') and antisense primer (5'-AATGCAGAAGCCAGATTGATTCAAAGA-3') corresponded to nucleotides 2055–2077 and 2889–2916, respectively, of the human trkB cDNA; and the trk C sense primer (5'-TTCGCATGAACATCAGTCAGTGTG-3') and antisense primer (5'-CTCACCACACGTGGGGGATAGTAGA-3') corresponded to nucleotides 753–777 and 1057–1881, respectively, of the human trk C cDNA.

The rat oligonucleotide primers used for detection of rat trkA were 5'-ATGGCTGCCTTTATGGACAACC and 5'-GACCCAAAAGGTGTTTCGTCC (reverse primer), corre-

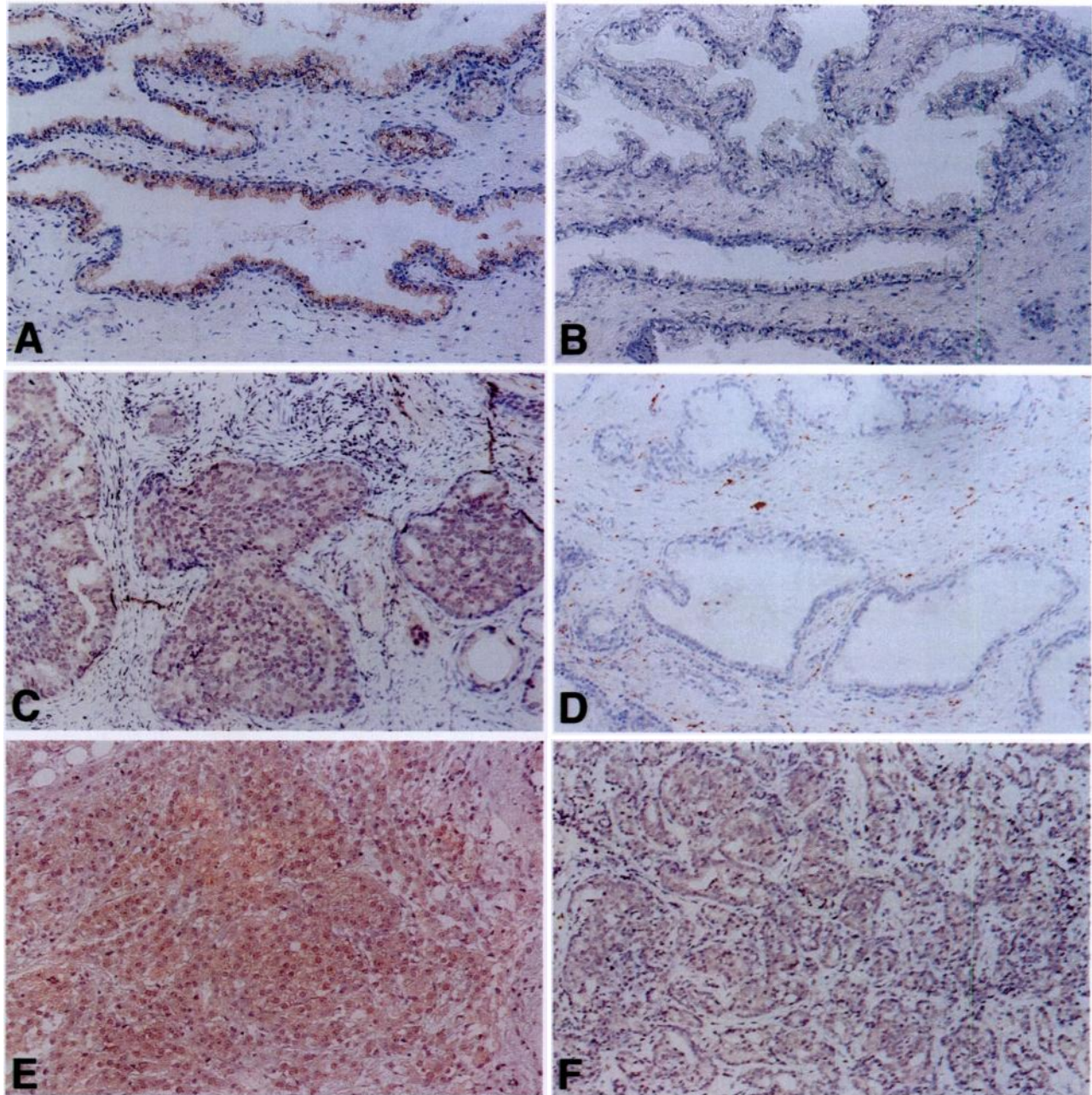
sponding to residues 1193–1214 and 1303–1324, respectively, in the sequence described by GenBank accession no. M85214. This set of primers is known to recognize alternatively spliced forms of trkA (22). The rat trkB oligonucleotide primers consisted of the sequences 5'-AAGTCCTCTATGAAGACTG-GACC and 5'-TGCCAAACTTGGAAATGTCTCGCCA (reverse primer), corresponding to residues 1845–1867 and 2028–2051, respectively, in the sequence described by GenBank accession no. M55291. The rat trkC oligonucleotide primers consisted of the sequences 5'-TTGGCCTCCAGCACTTTGT and 5'-GC-CAAGAATGTCCAGGTAGA (reverse primer), corresponding to residues 2059–2078 and 2507–2526, respectively, in the sequence described by GenBank accession no. L03813. This set of primers is known to recognize alternatively spliced forms for trkC (23). Oligonucleotides for each of the trk cDNAs were synthesized by Life Technologies, Inc.

The specificity of each set of rat trk primers was verified using PC-12 cells for trkA and transfected NIH-3T3 cells expressing rat trkB and rat brain for trkC. All primers used in these experiments are known to cross an intron-exon boundary. As a further control for contamination of RNA samples with genomic DNA, RT-PCRs were performed using cDNA made in the absence of reverse transcriptase (data not shown).

#### Determination of Percentage of H Prostate Cancer Cells in S-Phase *versus* Cells Undergoing Apoptotic Death.

To label H prostate cancer cells in the S-phase of the proliferative cell cycles, tumor-bearing rats that had been dosed with CEP-751 10 mg/kg s.c. qd for various times *versus* H tumor-bearing rats injected with vehicle were injected i.p. with BrdUrd 50 mg/kg. One h later, tumor tissue was harvested, fixed in 10% buffered formalin, and paraffin embedded, and 5- $\mu\text{m}$  histological sections were prepared. These histological sections were deparaffinated and used for immunocytochemical staining using a mouse anti-BrdUrd monoclonal antibody to detect H prostate cancer cells in the S phase based upon the incorporation of BrdUrd into the nuclear DNA as described previously (24). Additional step sections were deparaffinated and incubated with biotinylated dUTP and purified terminal transferase to detect H tumor cells undergoing cell death based upon end-labeling the 3'-free hydroxyl groups of the fragmented nuclear DNA of dying cells (TUNEL labeling) as described previously (3). The results are expressed as the percentage of H tumor cells either in the S phase or undergoing apoptosis based upon random sampling of 2000 H tumor cells, as described previously (24).

**Androgen-dependent Regrowth Experiments.** Adult male Copenhagen rats were castrated, and 2 weeks later, they were divided into three groups of eight rats each. One group was treated daily with s.c. injections of vehicle alone to serve as a castration control. The second group was implanted s.c. with a 2-cm silastic tube filled with testosterone to restore and maintain the serum testosterone within the physiological range (*i.e.*, 2–3 ng/ml) and injected s.c. daily with vehicle alone to serve as maximal restoration control. The third group was implanted s.c. with a 2-cm testosterone-filled silastic tube and injected with 10 mg/kg s.c. qd of CEP-751. After 10 days, the animals were sacrificed, and the sex accessory tissues (*i.e.*, ventral, dorsal, and anterior prostates and seminal vesicles) were removed, separated from fat and mesentery, and weighed, and DNA content was determined as described previously (25).



**Fig. 1** Immunocytochemical detection of trk proteins in normal and malignant human prostate tissues. *A*, normal prostatic tissue stained with anti-trkA antibody demonstrates trkA protein expression in the prostatic glandular epithelial cells. *B*, normal prostatic tissue stained with anti-trkA antibody preincubated with trkA peptide used to generate the rabbit polyclonal antiserum demonstrating antibody specificity. *C*, primary prostatic cancer stained with anti-trkA antibody. *D*, normal prostatic tissue stained with anti-trkB antibody. *E*, metastatic prostate cancer in bone stained with anti-trkB antibody. *F*, metastatic prostate cancer in bone stained with anti-trkC antibody.

**Statistical Analysis.** Statistical analysis of the effect of treatment was done using one-way ANOVA. Multiple comparisons *versus* a control were performed using Dunn's method for all models except the Dunning H and androgen-dependent re-growth experiments, in which the Neumann-Keuls test was used. All analyses were performed using SigmaStat for Windows (Jandel Scientific, San Rafael, CA).

## RESULTS

**trk Expression in Human and Rat Prostate.** To investigate the possibility that trks might contribute to prostate cancer cell growth, trk expression in normal and cancerous human prostate tissue was first examined. Using immunocytochemical analysis, trkA was found to be expressed in epithelial cells in samples obtained from both normal human prostates (Fig. 1A)

Table 1 Expression of trks in human prostate cancer

A series of primary human prostatic cancers and bone metastases were examined for expression of trk isoforms using immunocytochemical staining. trk expression was labeled homogeneous if >80% of the cancer cells stained positive or heterogeneous if <80% but  $\geq$ 20% of the cells were positive. "None" means that <20% of the cells stained positive for trk.

	Immunocytochemical staining in primary tumors, % (n = 32)			Immunocytochemical staining in bone metastases, % (n = 10)		
	Homogeneous	Heterogeneous	None	Homogeneous	Heterogeneous	None
trkA	60	40	0	80	20	0
trkB	30	40	30	30	30	40
trkC	40	30	30	30	50	20

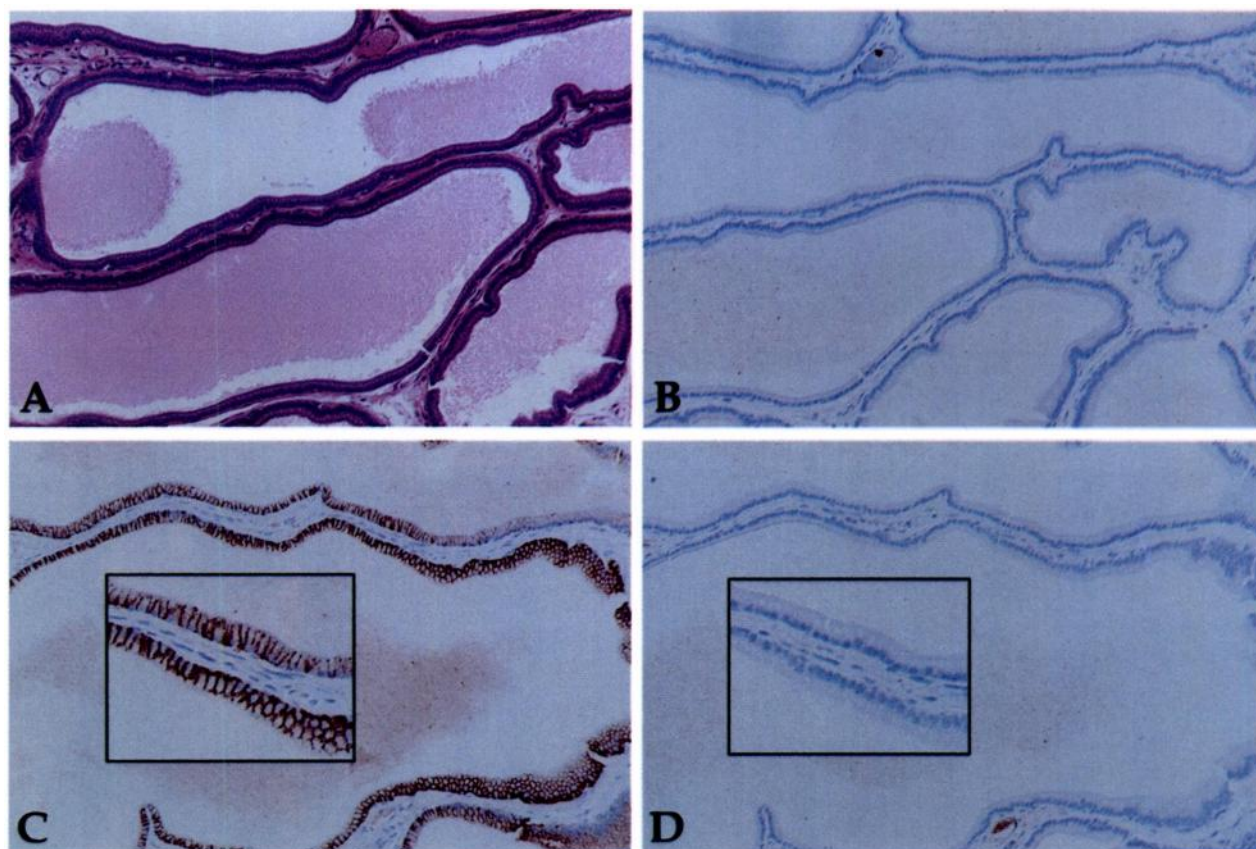


Fig. 2 Immunohistochemical detection of trk proteins in normal rat prostate tissues. A, H&E histology of the ventral prostate from an adult male Copenhagen rat. B–D, normal ventral prostate stained with antibodies specific for trkB (B), trkA (C; inset is at a higher magnification), or trkC (D).

and primary prostatic cancer tissue (Fig. 1C). If tissue was stained with anti-trkA antibody preincubated with the trkA peptide used to generate the rabbit polyclonal antiserum, trkA expression was not observed, thus demonstrating the specificity of the trkA antibody used in these studies (Fig. 1B). Unlike trkA, neither trkB (Fig. 1D) nor trkC (data not shown) protein expression was immunocytochemically detectable in normal human prostatic epithelium. trkB was expressed, however, by macrophages scattered throughout the stromal compartment of the prostate. Interestingly, both trkB and trkC expression was clearly detectable in cells derived from bone metastases (Fig. 1, E and F).

These data suggest that prostate cancer cells continue to express trkA while up-regulating the expression of trkB and trkC compared to normal prostate epithelial cells. This conclusion is supported by analysis of the frequency of trk expression in a series of primary human prostatic cancers and bone metastases (Table 1). trkA was found to be homogeneously expressed (*i.e.*, expressed in  $\geq$ 80% of the cancer cells examined) by 60% of the primary tumors and 80% of the metastases. All of the remaining tumors had at least heterogeneous expression (*i.e.*,  $\geq$ 20% of cancer cells positive) of trkA. trkB and trkC were also expressed in a significant number of both primary and metastatic prostate cancers. It was determined in additional calcula-

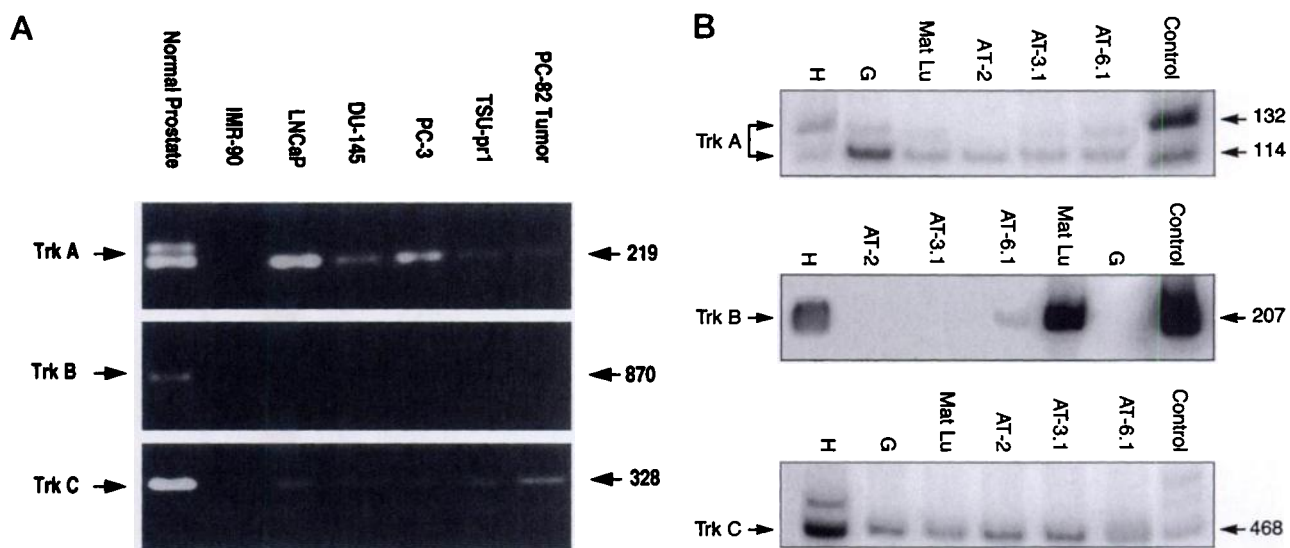


Fig. 3 RT-PCR analysis of mRNA expression of trks by normal and cancerous prostatic cell lines and tissues. RNA was isolated from human (A) or rat (B) prostate cell lines and tissues and subjected to RT-PCR amplification to identify transcripts corresponding to trkA, trkB, or trkC. Controls for the utility of the primers consisted of IMR-90 human fibroblasts (A) and rat PC12 cells for trkA and rat brain for trkB and trkC (B).

tions that human prostatic cancer cells in 90% of the primary tumors and metastases homogeneously express detectable levels of at least one of the trk receptor proteins.

Because the Dunning series of serially transplantable rat prostatic cancers arose from and are maintained in Copenhagen rats, trk expression in normal Copenhagen rat prostate was examined by immunocytochemical analysis. Histology of the normal rat prostate is shown in Fig. 2A for comparison. trkA expression was clearly noted in ventral prostate and was found to be localized to the plasma membrane (Fig. 2C). No expression of trkB or trkC was observed (Fig. 2, B and D). These data are similar to those gathered from the human tissues examined, suggesting that trk expression is regulated in similar ways in normal rat and human prostates.

Immunocytochemistry is specific but limited in its ability to detect trk expression by the avidity of the antibodies used. To confirm and extend immunocytochemical results, RT-PCR methods were used in a series of four human (*i.e.*, LNCaP, DU145, PC-3, and TSU-Pr1) and five rat (*i.e.*, Dunning G, MatLu, AT-2, AT-3.1, and AT-6.1) prostate cancer cell lines maintained as pure populations of malignant cells in tissue culture (Fig. 3). IMR90 human fibroblast cells were used as negative controls in the human cell line experiments. trk expression was also examined in a human xenograft (PC-82) and a syngeneic rat tumor (Dunning H) grown *in vivo* (Fig. 3). Each of these prostate cancer lines or tissues expressed both trkA and trkC mRNA transcripts, and three rat samples (Dunning H, AT-6.1, and MatLu) also expressed trkB mRNA transcripts. Alternatively spliced forms of trkA and trkC were apparent in some of the rat cell lines and tissues examined; alternative splicing of trk genes has been described previously (26, 27). These data indicate that trk expression is maintained in all established tumors and cell lines derived from spontaneous human and rodent prostate cancers.

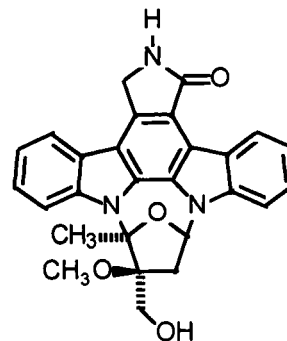


Fig. 4 Structure of CEP-751

#### The trk Inhibitor CEP-751 Inhibits Prostate Tumor Growth *in Vivo*.

If a functional trk signaling cascade is vital to either the survival or growth of prostate cancer cells, then agents that interfere with trk signaling should have therapeutic benefit. The trk tyrosine kinase inhibitor, CEP-751 (Fig. 4), was selected from a library of K-252a indolocarbazole derivatives and has been shown to inhibit neurotrophin/trk signaling at low nanomolar concentrations *in vitro* and at low mg/kg doses *in vivo* (14). Initial *in vivo* studies demonstrated that CEP-751 could be given s.c. to nude mice at a dose of up to 24 mg/kg BID for at least 4 weeks and at a dose of 14 mg/kg BID for 8 weeks with no mortality or morbidity (*i.e.*, defined as >10% loss in body weight). For rats, the maximum tolerated dose in a s.c. dosing regimen is 10 mg/kg qd.

CEP-751 was tested for antitumor efficacy using s.c. dosing regimens in nine different models of human and rodent prostate cancer. CEP-751 inhibited the growth (*i.e.*, decreased the T/C ratio) of all of the prostatic cancer sublines tested.

Table 2 Response of human and rodent prostatic cancers to daily treatment with CEP-751

CEP-751 was administered s.c. to adult male rodents as indicated below beginning after the formation of measurable tumors. Animals were sacrificed 1 day after the last day of treatment in all experiments. Human cell lines were grown as xenografts in nude mice; Copenhagen rats served as hosts for the rat tumors. T/C ratio in Dunning H tumors reflects tumor regression.

Prostate cancer subline	Histology	Androgen sensitivity	Metastatic ability	Growth rate (volume doubling times), days	Ratio of tumor volume in CEP-751-treated versus vehicle control hosts
Human LNCaP	PD <sup>a</sup>	Yes	No	7 ± 1	0.63 <sup>b</sup>
Human DU145	PD	No	Yes	12 ± 2	0.40 <sup>c</sup>
Human PC-3	PD	No	Yes	6 ± 1	0.56 <sup>c</sup>
Human TSU-Prl	PD	No	Yes	6 ± 1	0.37 <sup>d</sup>
Human PC-82	MD	Yes	No	22 ± 4	0.45 <sup>c</sup>
Rat R-3327 G	PD	Yes	No	4 ± 1	0.19 <sup>f</sup>
Rat R-3327 Mat-Lu	A	No	Yes	4 ± 1	0.38 <sup>e</sup>
Rat R-3327 AT-2	A	No	No	3 ± 1	0.36 <sup>e</sup>
Rat R-3327 H	WD	Yes	No	24 ± 4	0.20 <sup>e</sup>

<sup>a</sup> PD, poorly differentiated; MD, moderately differentiated; WD, well-differentiated; A, anaplastic.

<sup>b</sup> 6 days of treatment, 10 mg/kg/BID.

<sup>c</sup> 28 days of treatment, 24 mg/kg/BID.

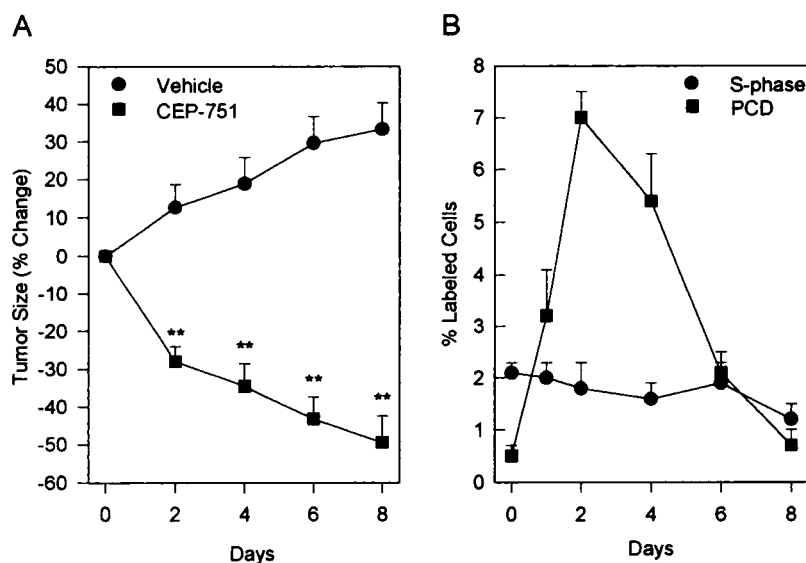
<sup>d</sup> 17 days of treatment, 10 mg/kg/BID.

<sup>e</sup> 52 days of treatment, 14 mg/kg/BID.

<sup>f</sup> 14 days of treatment, 10 mg/kg/qd.

<sup>g</sup> 10 days of treatment, 10 mg/kg/qd.

Fig. 5 Effect of CEP-751 on the growth and cell kinetics of Dunning R-3327 H tumors *in vivo*. A, kinetics of tumor volume regression induced by CEP-751. Adult male rats implanted s.c. with testosterone-filled silastic capsules and bearing an established H tumor were injected with 10 mg/kg s.c. qd CEP-751. The data are expressed as percentage of change in tumor volume normalized to the starting tumor volume on day 0 (*i.e.*, 5–7 cm<sup>3</sup>). B, percentage of H-prostatic cancer cells in S phase versus cells undergoing programmed death at various days following CEP-751 treatment. A and B, results are presented as mean ± SE. \*\*, *P* < 0.01 relative to vehicle control by Neumann-Keul's test. PCD, programmed cell death.



independent of their state of differentiation, androgen sensitivity, metastatic ability, or growth rate (Table 2). In all experiments except those involving Dunning H tumors, inhibition of the T/C ratio was due to a slowing of the net tumor growth rate with no tumor regression.

When adult rats harboring well-established (*i.e.*, 5–7 cm<sup>3</sup>) Dunning H tumors were treated with 10 mg/kg s.c. qd CEP-751, a regression in tumor volume of 28% was observed within 2 days of dosing, reaching a maximum of 45% tumor volume decrease within 8 days of dosing (Fig. 5A). In contrast, vehicle-treated rats exhibited a 31% increase in tumor volume during this period (Fig. 5A). The H tumor regression induced by CEP-751 was not due to an effect on androgen levels because the experiments were performed in hosts bearing testosterone-releasing silastic implants that maintain circulating testosterone

at physiological levels. Serum testosterone levels measured at the end of the experiment confirmed that testosterone was  $\geq 1$ –2 ng/ml. A reduction in volume of well-established (*i.e.*, 5–7 cm<sup>3</sup>) Dunning H tumors was not observed following treatment with other standard chemotherapeutic agents; cyclophosphamide, methotrexate, 5-fluorodeoxyuridine, or etoposide given at maximally tolerated doses produced a  $\leq 10\%$  reduction in tumor volume when H tumors were  $> 2$  cm<sup>3</sup> in size at initiation of treatment (15).<sup>4</sup>

<sup>4</sup> J. Isaacs, unpublished observations.

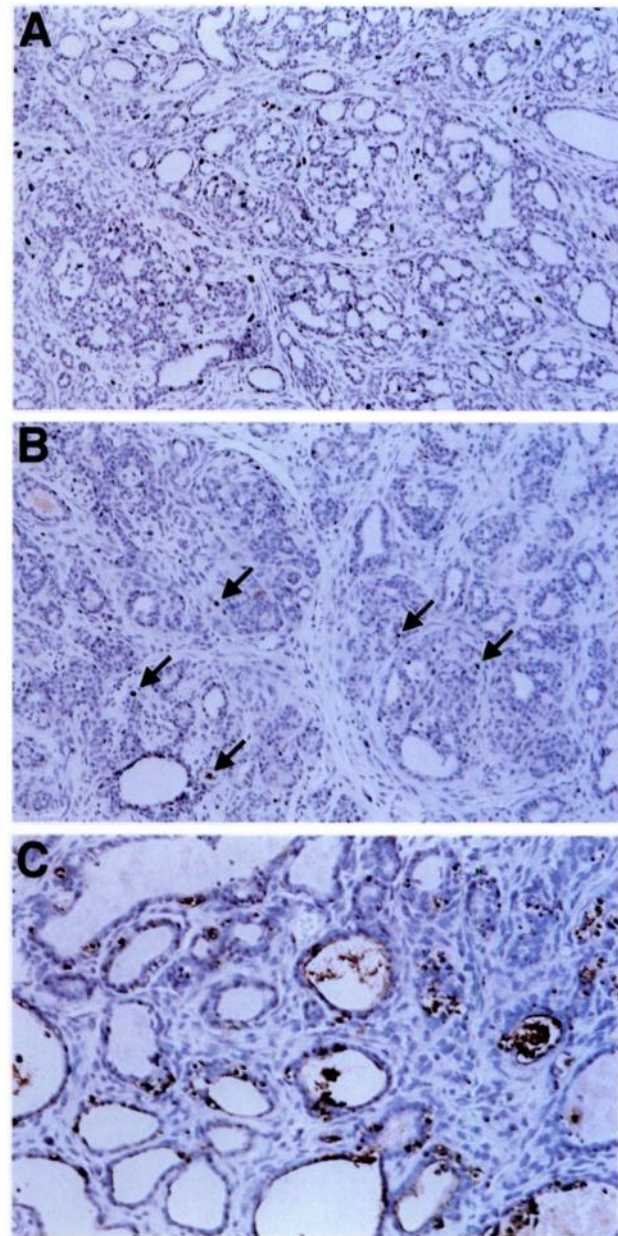
### CEP-751 Induces Cell Death in Prostate Cancer Cells.

Experiments were next undertaken to determine whether the antiprostate cancer effects of CEP-751 are mediated via effects on cell proliferation and/or cell death. Animals bearing Dunning R-3327 H rat prostate tumors were treated daily for 1, 2, 4, 6, or 8 days with 10 mg/kg s.c. qd CEP-751. At each time point all animals to be sacrificed were injected with BrdUrd to label cells undergoing DNA synthesis (*i.e.*, in the S phase of the cell cycle). One h after injection of BrdUrd, the animals were sacrificed, and tumors were removed, fixed, sectioned, and then stained either with anti-BrdUrd (to identify proliferating cells) or by the TUNEL method (to identify cells undergoing cell death).

On the first day of experimentation, before initiation of treatment,  $2.1 \pm 0.2\%$  of prostate epithelial and stromal cells were in S phase, as indicated by labeling with BrdUrd (Fig. 6A). A small percentage of cells,  $0.5 \pm 0.2\%$ , was undergoing apoptotic death as indicated by TUNEL labeling (Fig. 6B, arrows indicate TUNEL-labeled cells). Increased TUNEL labeling was clearly observed after 2 days of administration of 10 mg/kg s.c. qd CEP-751 (Fig. 6C). Interestingly, CEP-751-induced cell death appeared to be confined to the transformed epithelial cell component of the tumor, with apparent sparing of the stromal cell component (Fig. 6C).

The percentage of cancer cells labeled with BrdUrd was compared to the percentage of cells labeled by the TUNEL method during CEP-751 treatment of animals bearing Dunning H prostate tumors. Treatment with CEP-751 both inhibited the rate of proliferation and enhanced the rate of death of Dunning H rat prostatic cancers (Fig. 5B). By 8 days of 10 mg/kg s.c. qd CEP-751 treatment, the number of Dunning H tumor cells in S-phase decreased by ~2-fold (*i.e.*, from  $2.1 \pm 0.2\%$  on day 0 to  $1.2 \pm 0.3\%$  after 8 days of CEP-751;  $P < 0.05$  by the Neumann-Keul's test). No significant effect of CEP-751 on the number of cells in S phase was observed prior to day 8. In contrast to the modest decrease in proliferation, CEP-751 treatment caused a pronounced 14-fold increase in the number of cells labeled by the TUNEL method after only two daily doses of drug, (*i.e.*,  $0.5 \pm 0.2\%$  on day 0 versus  $7.1 \pm 0.5\%$  after 2 days of CEP-751; Fig. 5B).

**CEP-751 Does Not Affect *in Vivo* Growth of Normal Prostate Cells.** Because *trkA* is expressed in normal human and rat prostate epithelial cells (Figs. 1 and 2), it was of interest to determine whether the growth inhibitory effects of CEP-751 were selective for transformed *versus* normal prostate epithelial cells. To address this question, castrated rats were treated without or with CEP-751, and androgen-mediated regrowth of the prostate gland was measured. Castration caused profound regression of prostate tissues; in castrated animals bearing a testosterone implant, weight of ventral, dorsolateral, and anterior prostates and of the seminal vesicles was significantly increased compared to untreated castrated controls (Table 3). Daily treatment with 10 mg/kg s.c. qd CEP-751 did not affect the ability of normal prostate cells to regrow in response to testosterone following castration (Table 3). In addition, no differences in DNA content or cell morphology could be detected in testosterone-treated animals regardless of the presence or absence of CEP-751 (data not shown). These data suggest that CEP-751 actions on prostatic cell growth are selective for cancerous cells.



**Fig. 6** Histological detection of cell proliferation and death in Dunning R-3327 H rat prostatic cancers in control and CEP-751 treated hosts. **A**, detection of H prostate cancer cells that have incorporated BrdUrd (*i.e.*, S-phase) using anti-BrdUrd antibody on tumor sections from untreated host. **B**, detection of H prostate cancer cells that have fragmented their DNA (*i.e.*, are undergoing death) using TUNEL on tumor sections from untreated hosts (arrow, TUNEL-positive cells). **C**, TUNEL labeling in hosts treated for 2 days with 10 mg/kg s.c. qd CEP-751. **A** and **B**,  $\times 100$ ; **C**,  $\times 250$ .

**CEP-751 Exhibits Selective Antitumor Efficacy.** To determine whether the antitumor effect of CEP-751 was specific to tumors of prostatic origin, CEP-751 was administered in mouse models of ovarian, skin, breast, and lung cancer (Table 4). CEP-751 reduced tumor growth in OVCAR-3 and SK-Mel-5 human tumor xenograft models of ovarian cancer and mela-



Table 3 Effect of CEP-751 on androgen-dependent regrowth of male sex accessory tissues in castrated adult rats

Treatment (10 days) <sup>a</sup>	Body weight (g), starting	Body weight (g), ending	Ventral prostate <sup>b</sup>	Dorsolateral prostate <sup>b</sup>	Anterior prostate <sup>b</sup>	Seminal vesicles <sup>b</sup>
Castrated control	286 ± 7	272 ± 8	36 ± 2	87 ± 11	11 ± 1	70 ± 7
Castrated + testosterone implant	300 ± 8	293 ± 7	254 ± 10 <sup>c</sup>	334 ± 36 <sup>c</sup>	97 ± 15 <sup>c</sup>	286 ± 15 <sup>c</sup>
Castrated + testosterone implant + CEP-751 (10 mg/kg s.c. qd)	301 ± 3	279 ± 3	269 ± 18 <sup>c</sup>	352 ± 26 <sup>c</sup>	95 ± 8 <sup>c</sup>	339 ± 18 <sup>c</sup>

<sup>a</sup> Animals castrated for ~1 month. *n* = 8 except castrated + testosterone + CEP-751 (*n* = 7; 1 animal lost testosterone implant).

<sup>b</sup> Wet weight in mg/gland.

<sup>c</sup> *P* < 0.05 versus castrated control by the Neumann-Keul's test.

Table 4 CEP-751 exhibits selective antitumor activity

CEP-751 was administered s.c. to adult rodents as indicated below, beginning after the formation of palpable tumors for all experiments except those involving B16 tumors. For B16 experiments, administration of CEP-751 was begun 1 day after tumor implantation. Human cell lines were grown as xenografts in nude mice; B6C3F1 mice served as hosts for B16 and Lewis lung tumors.

Tumor type	Tumor growth inhibition
SK-OV-3 human ovarian cancer	No effect <sup>a</sup>
OVCAR-3 human ovarian cancer	60% <sup>a</sup>
B16 mouse melanoma	No effect <sup>b</sup>
SK-Mel-5 human melanoma	75% <sup>c</sup>
MCF-7 human breast cancer	No effect <sup>d</sup>
MX-1 human breast cancer	No effect <sup>e</sup>
Lewis mouse lung cancer	No effect <sup>f</sup>

<sup>a</sup> 29 days of treatment, 21 mg/kg/BID.

<sup>b</sup> 19 days of treatment, 10.5 mg/kg/BID.

<sup>c</sup> 31 days of treatment, 21 mg/kg/BID.

<sup>d</sup> 25 days of treatment, 21 mg/kg/BID.

<sup>e</sup> 18 days of treatment, 21 mg/kg/qd.

<sup>f</sup> 10 days of treatment, 9.3 mg/kg/qd.

noma, respectively. CEP-751 did not affect tumor growth in SK-OV-3 human ovarian cancer xenografts, B16 mouse melanomas, MCF-7 or MX1 human breast cancer xenografts, or syngeneic Lewis lung tumors. *trk* expression was examined by RT-PCR in the CEP-751-sensitive OVCAR-3 and SK-Mel-5 tumors. OVCAR-3 tumors expressed *trkA* and *trkB* but not *trkC*; SK-Mel-5 tumors expressed *trkA*, *trkB*, and *trkC* (data not shown). *trk* expression was not fully characterized in the tumors that were not responsive to CEP-751. The time course of CEP-751 treatment of SK-OV-3 tumors has been presented previously (14). Differential responsiveness of ovarian tumor xenografts to CEP-751 was not attributable to pharmacokinetic parameters because plasma and tumor drug levels were comparable in OVCAR-3- and SK-OV-3-bearing animals (data not shown). These data suggest that, unlike many chemotherapeutic agents currently used in cancer therapy, CEP-751 exhibits selective antitumor activity.

## DISCUSSION

We examined the hypothesis that *trk* signaling is an appropriate target for the development of therapeutic antiprostata cancer drugs by (a) characterizing the expression of *trks* in primary and metastatic prostate cancer, and (b) experimentally determining the therapeutic potential of a *trk* inhibitor in mul-

tipule animal models of prostate cancer. Immunohistochemical methods were used to demonstrate that 90% of prostate cancers homogeneously express at least one of the three *trk* receptors and that four human and five rat prostate cancer cell lines express *trk* isoforms detectable by RT-PCR methods. Consistent with a role for *trk* in prostate cancer growth, the *trk* inhibitor CEP-751 was shown to exert antitumor efficacy against androgen-dependent and androgen-independent prostate tumors of both human and rat origin. These data support the concept that *trk* activity can contribute to growth of prostate cancer cells and suggest that CEP-751 constitutes a potential new antiprostata cancer therapy.

Anticancer efficacy of CEP-751 was observed in animals bearing tumors derived from LNCaP, DU145, PC-3, TSU-Pr1, PC-82, G, Mat-Lu, AT2, or H prostatic cancer sublines. These nine models vary considerably in their state of differentiation, androgen sensitivity, metastatic ability, and growth rate; in addition, hosts included both nude mice and Copenhagen rats. In fact, an animal model of prostate cancer has not yet been identified that is insensitive to the antitumor efficacy of CEP-751. Inhibition of tumor growth in all of these models provides strong evidence that CEP-751 may possess antiprostata cancer activity against human clinical disease.

The Dunning H tumor was originally derived from a spontaneous prostate tumor in an aged Copenhagen rat. Its androgen sensitivity, coupled with its very slow growth rate, has led to the suggestion that the H tumor may be a better mimic of human disease than other, faster-growing prostate tumor lines (15). CEP-751 was shown to induce tumor regression in animals bearing established (*i.e.*, 5–7 cm<sup>3</sup>) Dunning H tumors. The antitumor effect of CEP-751 in H tumors was coincident with an increase in the number of cells undergoing apoptotic cell death, as indicated by TUNEL labeling. After 2 days of treatment with CEP-751, there was a 14-fold increase in the percentage of rat prostate cells labeled by the TUNEL method, from 0.5% on day 0 of treatment to 7% on day 2. It has been determined that rat prostate cells induced to undergo death via androgen ablation are detectable by TUNEL labeling for 4 h (28). Using this value for the TUNEL-detectable phase of Dunning H tumor cell death, it is estimated that on day 2, the daily rate of CEP-751 induced prostate cancer cell death was 42%. It is likely that the apparent decrease in the percentage of apoptotic cells observed between days 2 and 8 of treatment is related to a decrease in the absolute number of prostate cancer cells remaining after initial treatment with CEP-751.

In contrast to its dramatic effect on cell death, no effect of CEP-751 on the number of cells in S phase was observed on day 2 of treatment. The length of the S phase for rat prostatic glandular cells has been determined to be 8 h (29). Using this value and the percentage of cells in S phase (*i.e.*, ~2%), it can be calculated (3) that approximately 6% of H cancer cells proliferate per day. It is clear from these calculations that after 2 days of treatment with CEP-751, the normalized daily rate of prostate cancer cell death greatly exceeded the daily rate of proliferation (*i.e.*, 42% versus 6%). CEP-751 induces death of prostate cancer cells *in vivo*, independent of effects on cell cycle. These data suggest that CEP-751 is targeting a fundamental survival pathway of prostate cancer cells and is acting as a selective cytotoxic agent rather than inducing cytostasis or differentiation.

The antitumor effects of CEP-751 compare quite favorably with other compounds that have been examined in similar preclinical models. The degree of tumor growth inhibition observed in AT-2 and MAT-Lu rat prostate tumor models is similar to that elicited by the antiangiogenic factor linomide (30, 31), the growth factor antagonist suramin (25), and the retinoic acid metabolism inhibitor liarozole (32, 33), the latter two of which have advanced to late stage clinical evaluation. The effects of CEP-751 appear superior to those of liarozole in the Dunning H model, in which tumor regression rather than inhibition of growth is observed.

CEP-751 was identified based on its inhibition of the trk signal transduction pathway (14). Because signal transduction pathways function in both normal and transformed cells, these putative therapeutics have the potential to affect physiological as well as pathological function of cells. It was important, therefore, to determine the selectivity of CEP-751 for transformed prostate cells. A common approach for evaluating normal prostatic epithelial cell growth is to monitor androgen-dependent regrowth of the prostate in castrated adult rats (25, 34). Using this model, there was no difference in prostate weights or DNA content between castrated rats given testosterone alone and those given testosterone plus CEP-751. These results indicate that the growth inhibitory effects of CEP-751 are selective for cancerous prostate epithelial cells. It is interesting to note that the commonly used anticancer therapeutics 5-fluorouracil and Adriamycin prevented cell growth in this model (34). The lack of efficacy exhibited by CEP-751 clearly distinguishes this compound from nonspecific inhibitors of cell growth.

The lack of effect of CEP-751 on normal prostate cell growth has interesting implications regarding the role of trk in prostate cell growth. Normal human prostate epithelial cells clearly express trkA, trkC expression is detectable at the level of RT-PCR but not by immunocytochemical means, and trkB expression is undetectable by either technique. All three trks are expressed in a significant proportion of transformed prostate epithelial cells, both in primary sites and in bony metastases. It is important to note that the antibodies used in our immunocytochemical analyses recognize epitopes at or near the carboxyl termini of the proteins, so it is likely that the detected proteins have full-length kinase domains capable of generating tyrosine kinase activity. Taken together, the tumor regression, androgen-dependent regrowth, and trk expression data suggest that in the

process of transformation from normal to malignant growth, prostate epithelial cells acquire a dependency on a neurotrophin/trk signaling pathway for survival.

The exact mechanism by which trk affects cancerous cell growth is not completely understood. Although naturally occurring, activating, genetic rearrangements of trkA have been identified in a sporadic colon carcinoma (35) and in papillary thyroid carcinomas (36), no evidence was obtained to suggest that trkA mutants were present in our panel of human prostate cancer specimens (37). There is a difference in trkB and trkC expression between normal and transformed prostate epithelial cells, but we have not discovered further changes in the expression of the three trks during tumor progression (data not shown). Significant amounts of neurotrophins, particularly NGF, are synthesized in normal prostate (7–9), suggesting that primary prostate cancers can be driven in a paracrine fashion. Alternatively, expression of trk mutants or coexpression of wild-type trk isoforms with their cognate ligands have clearly established that autocrine trk signaling can lead to cellular transformation (38–41). Preliminary evaluation of neurotrophin expression in our panel of human prostate tissues indicates significant NGF and NT-3 expression in the bony metastases (data not shown), suggesting that NGF/trkA and NT-3/trkC autocrine loops are potentially operational in these samples.

Interestingly, CEP-751 did not block tumor growth in every model studied. Although the anticancer efficacy of CEP-751 was reproducibly observed in nine prostatic cancer sublines tested *in vivo*, several other cancer models using human and rodent ovarian, melanoma, breast cancer, and lung cancer cell lines were nonresponsive to CEP-751 anticancer activity *in vivo*. CEP-751 did reduce growth in OVCAR-3 and SK-Mel-5 human tumor xenografts, suggesting that this compound may be therapeutically useful for ovarian cancer and melanoma; additional studies are currently under way to further evaluate this hypothesis. Because responsive and nonresponsive tumor types were grown in the same type of host (*i.e.*, mice), these results demonstrate that it is the phenotype of the cancer cells themselves that determines responsiveness to CEP-751. The lack of generalized responsiveness to CEP-751 negates the suggestion that CEP-751 is acting as a nonspecific inhibitor of cell proliferation and intimates that this compound may provide targeted anticancer therapy.

All of the tumors, both prostate and nonprostate, that are sensitive to growth inhibition by CEP-751 have been found to express at least one isoform of trk. Although these data are consistent with a mechanism of action that includes inhibition of trk, CEP-751 has also been shown to potently inhibit other kinases, including PKC and PDGF receptor (14), which have been suggested to play a role in tumor growth (42–44). CEP-751 analogues in which the inhibitory activity toward PKC and PDGF receptor was greatly reduced or eliminated exhibited antitumor activity in prostate cancer models similar to that of CEP-751 (data not shown), suggesting that inhibition of these kinases is not likely to contribute to the antitumor activity of CEP-751 in prostate tumors. It must be considered a possibility, however, that inhibition of kinases other than trk may play a role in the antitumor activity of CEP-751.

In summary, CEP-751 was found to exert antitumor efficacy in nine different animal models of prostate cancer, includ-

ing Dunning H tumors. A clinically effective chemotherapeutic agent for prostate cancer will likely target the death or differentiation of prostate cancer cells in a cell proliferation-independent fashion; CEP-751 appears to fulfill this critical criterion. CEP-751 is selective for cancerous *versus* normal prostate epithelial cells and does not exert a generalized antitumor effect, thereby distinguishing this molecule from existing antiprostata cancer therapeutics. Because it is a small organic molecule, CEP-751 represents a molecular platform upon which to build a new generation of therapeutic agents. Indeed, the lysinyl- $\beta$ -alanyl ester of CEP-751, CEP-2563, is now undergoing clinical evaluation as an anticancer therapeutic in the United States.

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