Pan-trk Inhibition Decreases Metastasis and Enhances Host Survival in Experimental Models as a Result of Its Selective Induction of Apoptosis of Prostate Cancer Cells[1]

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ABSTRACT

During the progression of prostate cancer, molecular changes occur resulting in the autocrine production of a series of neurotrophins by the malignant cells. This is coupled with expression of high-affinity cognate receptors for these ligands, termed trk receptors, by these cancer cells. The binding of the neurotrophins to their trk receptors activates the receptor's latent tyrosine kinase activity inducing a series of signal transduction pathways within these prostate cancer cells. These molecular changes result in the acquisition by prostate cancer cells of a restricted requirement for these trk signaling pathways for optimal survival. CEP-701 is an indolocarbazole compound specifically designed as a potent inhibitor (IC_{50}, 4 nM) of the tyrosine kinase activity of the trk receptors required for initiation of these survival pathways. In the present studies, the consequences of CEP-701 inhibition of these trk signaling survival pathways were tested *in vivo* using both rat (R3327 AT 6.3 and H) and human (TSU-pr1 and CWR-22Rv1) prostatic cancer models.

These *in vivo* studies demonstrated that treatment with CEP-701 inhibits the growth of both rodent and human prostate cancers, without being toxic to the normal tissue including the host prostate. Because of this selective effect, CEP-701 inhibits metastasis and growth of both primary and metastatic sites of prostate cancer. Based upon this profile, long-term survival studies were performed using the slow-growing Dunning H rat prostate cancer model. For these latter studies, the dosing regimen was 10 mg CEP-701/kg/dose twice a day via gavage 5 days a week. This regimen maintains CEP-701 tumor tissue concentrations of 25–50 nM. Such chronic dosing increased ($P < 0.001$) the median survival of rats bearing the slow growing H prostate cancers from 408 days (395–432 days, 95% confidence interval) for the vehicle group ($n = 18$) to 566 days (497–598 days, 95% confidence interval) for the CEP-701-treated group ($n = 24$).

INTRODUCTION

Androgen-dependent prostate cancer cells require a critical supply of androgen to maintain their survival (1, 2). These cells undergo apoptotic elimination after androgen ablation therapy (1, 2). Metastatic prostate cancers are lethal because they are heterogeneously composed of both androgen-dependent and non-androgen-dependent prostate cancer cells (3–6). Androgen ablation does not induce the apoptotic death of the non-androgen-dependent cells because these latter cells activate survival pathways that do not require androgenic stimulation (7–10). It is the continuing survival and proliferation of these non-androgen-dependent prostate cancer cells that eventually kills, no matter how complete the androgen ablation is within the prostate cancer patient (11). Therefore, therapies that can eliminate these non-androgen-dependent cancer cells are urgently needed.

There are a large variety of cytotoxic chemotherapeutic agents presently available. There is essentially no therapeutic index, however, for the cytotoxic response induced by these agents between prostate cancer cells and rapidly proliferating normal gut, skin, and blood cells (12). This lack of cancer cell specificity leads to host toxicity, which limits both the dose and total length of treatment with such nontargeted cytotoxic agents. Therefore, what is needed is a method for selectively targeting the apoptotic death of non-androgen-dependent prostate cancer cells without inducing such death in host normal cells. One method to accomplish this goal is to inhibit signal transduction pathways to which the survival of these prostate cancer cells are restrictively sensitive.

Our recent studies have shown that signal transduction induced by neurotrophin binding to its high-affinity trk receptors initiates such restricted survival pathways for malignant prostate cells but not for normal host cells (10, 13, 14). The neurotrophins include NGF,[3] brain-derived neurotrophic factor

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[3] The abbreviations used are: NGF, nerve growth factor; IGF, insulin-like growth factor; PDGF, platelet-derived growth factor; BID, twice daily dosing; IC_{50}, concentration of test compound that inhibits 50% of

(BDNF), neurotrophin 3 (NT-3) and neurotrophin 4/5 (15). Each of these peptide factors binds with high affinity to a specific subtype of the M_r 140,000 neurotrophin receptor family known as the trk receptors, each of which is encoded by a separate gene. For NGF, the specific receptor is trk A; for BDNF and neurotrophin-4/5, it is trk B; and for NT-3, it is trk C (15). Normal prostatic epithelial, but not prostatic stromal cells, express trk A but not trk B or C (13). We have demonstrated that 60% of the primary prostate cancers and 80% of metastatic sites in untreated patients retain the expression of trk A (13). In ~1/3 of these lesions, trk A expression is actually higher than in normal prostate epithelial cells (13). These studies also demonstrated that 60–70% of primary and metastatic sites ectopically express trk B and C receptor (13). Combining these results, more than 90% of human prostate cancers express at least one of the trk receptors (13).

Additional studies have demonstrated that during the progression of prostate cancer to a metastatic state, these malignant cells acquire the ability to synthesize and secrete various neurotrophin ligands as well as expressing their cognate trk receptors (10). This results in an autocrine survival pathway that is initiated by the binding of these neurotrophins to their cell surface cognate receptors inducing their dimerization (10). This dimerization activates the intracellular tyrosine kinase domain of each receptor monomer allowing it to *trans*-phosphorylate specific sites in the intracellular domain of the other receptor monomers in the complex. This tyrosine *trans*-autophosphorylation between dimerized receptors functions to recruit intracellular signaling proteins to bind via their src homology (*i.e.,* SH_2) domains to specific phosphorylated tyrosines in the ligand-occupied dimeric receptor complex (16). This autophosphorylation initiates a variety of kinase-dependent signaling cascades that regulate cell survival (17–19).

This raises the issue of what would happen to these prostate cancer cells if the trk receptor tyrosine kinase, activated by neurotrophin binding, were inhibited, thus preventing down stream signaling. For these initial studies, the pan-trk tyrosine kinase inhibitor, CEP-751, was selected from a library of K-252a indolocarbazole derivatives Fig. 1. We have documented that CEP-751, via its ability to compete at the ATP binding site, inhibits neurotrophin-dependent kinase signaling of all three of the trk receptor subtypes (*i.e.,* trk A, B, and C) at low nanomolar concentrations *in vitro* and at low mg/kg doses *in vivo* (20). Using CEP-751, we demonstrated that such pan-trk inhibition had no discernable effect on the trk A-expressing normal rat prostate epithelial cells (13). This is not unreasonable because inhibiting trk receptors does not inhibit the activation of survival pathways induced by the binding of the other growth factors present (*i.e.,* IGF I and II, PDGF, etc.) to their cognate receptors in the normal prostate cells. What was unexpected was the observation that CEP-751 treatment of five different models of human (*i.e.,* LNCaP, DU145, PC-3, TSU, PC-82) and four rodent (Dunning G, MATLu, AT-2, and H) prostate cancers inhibited the *in vivo* growth by 40–80% in all of the prostatic
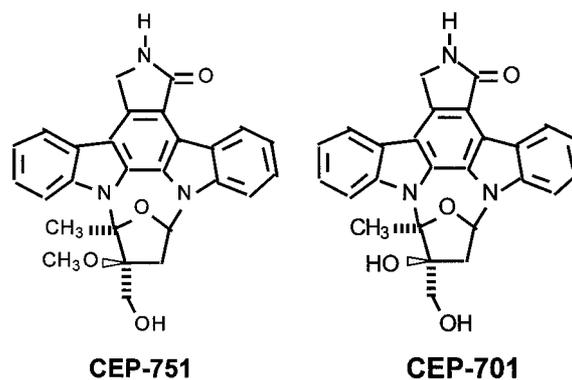
Fig. 1 The structure of CEP-701 and CEP-751.

cancer sublines tested, independent of their state of differentiation, androgen sensitivity, metastatic ability, or growth rate (13, 14). In the H rat prostate cancer model, CEP-751 treatment induced tumor regression, not simply inhibition of the rate of net continuous growth. Using TUNEL to quantitate the percentage of H rat prostate cancer cells dying, and bromodeoxyuridine incorporation to determine the percentage of cells in cycle, we documented that the major effect of 751 treatment on H prostate cancer cells was to enhance the apoptotic death and not simply to inhibit the proliferation of these cancer cells (13, 14). This was confirmed using *in vitro* clonogenic survival assays that demonstrated that CEP-751 directly induces the apoptotic death of all of the prostate cancer cells tested (10). This occurred even in the presence of FCS, which contains biologically active levels of IGF-I and -II and of PDGF (10). Thus, unlike normal prostate cells, in which survival is regulated by a redundant series of signal transduction, malignant prostatic cells are much more restricted in their requirement for neurotrophin/trk signaling for their survival (10).

CEP-701 is the *O*-desmethyl metabolite of CEP-751, Fig. 1. CEP-701 has an IC_{50} value of 4 nM for the inhibition of trk A, B, and C and is a p.o.-active compound (14). In the present study, the antitumor efficacy *versus* host toxicity of CEP-701, given both s.c. and by gavage, was determined against a variety of rat and human prostate cancers *in vivo*.

MATERIALS AND METHODS

In Vitro **Cell Lines and Culture Conditions.** The characteristics and history of the Dunning R-3327 rat prostatic cancer sublines used have been described previously (21, 22); and the characteristics and history of the human prostate cancer lines used have been described previously (13, 23). WI-38 human fibroblasts were obtained from the American Type Culture Collection (Rockville, MD). For *in vitro* passing, the cell lines were maintained in RPMI 1640 containing 10% FCS. For AT6.3 cells only, 200 μM dexamethasone were additionally supplied to the medium.

Determination of Proliferation and Apoptotic Rate. The daily rate of cell proliferation was determined by multiplying the growth fraction (*i.e.,* percent of cells in proliferative cell cycle) by the cell cycle (times in days) as previously described (12). The cell cycle times were determined to be 1 day for rodent and 2 days

for human prostatic cancer cells as described previously (12). The growth fractions were determined using immunocytochemically stained MIB5 monoclonal antibody (Immunotech, Marseille Cedex, France), which recognizes the Ki-67 antigen of rat and human tissues (24). The daily rate of apoptotic cell death was determined by multiplying the apoptotic index by the half-life of apoptotic cells (*i.e.,* 0.5 days) as determined previously (12). The apoptotic index was determined using H&E-stained histological sections based on morphological criteria as described previously (1). In addition, TUNEL assay was performed on indicated samples as described previously (13). Determination of the apoptotic index based on morphological criteria is consistently more sensitive than using TUNEL staining (25).

Drugs. Cephalon Inc. (West Chester, PA) synthesized CEP-701 and CEP-751, Fig. 1. For s.c. dosing at 10 mg/kg/dose, CEP-701 and CEP-751 were formulated in a vehicle containing 40% polyethylene glycol (Spectrum, Los Angeles, CA) 10% Providone C-30 (ISP, Bound Brook, NJ) 2% benzyl alcohol (Spectrum) and water (milli-Q or high-performance liquid chromatography grade; Fisher Scientific, Malvern, PA). For p.o. BID, at 10 mg/kg/dose, CEP-701 was formulated in a vehicle of 50% Tween 80, and 50% propylene glycol (Spectrum).

Animals. Four-to-6-week-old nude mice and two-month-old 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 of the animals were given a commercial diet (TekLab 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 of the animal studies were performed according to the animal protocols approved by Cephalon and the Johns Hopkins School of Medicine Animal Care and Use committees.

In Vivo **Experiments.** Tumors were measured in unanesthesized animals at indicated intervals using a Vernier caliper as described previously (13). Body weights were also obtained at these times. Dosing volumes were adjusted as necessary twice a week (*i.e.,* 1 ml/kg rats p.o.; 1 ml/kg, rats s.c.; 5 ml/kg, mice s.c.). The *in vivo* histology, androgen sensitivity, metastatic ability, and growth rate were determined as described previously (21). For the experiments using rat AT6.3 prostate cancers, male Copenhagen rats were inoculated s.c. in the leg with 1×10^6 viable cells, obtained from exponentially growing *in vitro* cultures. All of the animals eventually had their tumor-bearing legs removed under anesthesia [i.m. injection of ketamine (4.2 mg/100 g body weight) plus Xylazine (0.85 mg/100 g body weight) at the indicated time] using sterile surgery as described previously (26). The androgen-responsive Dunning H rat prostate cancers were established by s.c. implantation of ~20 mg of minced tumor tissue in 0.2 ml of Matrigel (Collaborative Research, Beltsville, MD) in the flank of Copenhagen male rats as described (14). These animals also had implanted in the opposite flank a 2-cm long testosterone filled-capsule fabricated from sealed silastic tubing to maintain the serum testosterone within the physiological range for rats (*i.e.,* 1–2 ng/ml). The silastic capsules were formulated and the serum testosterone was determined as described previously (27). The capsules were replaced every 6 months.

RT-PCR Detection of trk Receptors in Prostate Cancer Cells. TSU-pr1, CWR-22Rv1, and WI-38 cells were harvested from *in vitro* culture. RNA was isolated from these samples using TRIzol reagent (Life Technologies, Inc., Bethesda, MD). cDNA was transcribed from 2–3 μg of RNA using MuMLV reverse transcriptase (Ambion). RT-PCR was performed using oligonucleotide primers, which were specific for human trk A as described previously (13).

Detection of the Phosphorylation Status of the trk A Receptor in Prostate Cancer Cells. Antibody to the phospho-trk A receptor was obtained from Cell Signaling Technology (Beverly, MA). Cells were grown to 80% confluency and then treated with 100 nM of CEP 701 or vehicle control for 24 h, and then were harvested according to the manufacturer's protocol, *i.e.,* on ice using cell lysis buffer [1% Triton X-100, 50 mM Tris (pH 7.5), 10 mM EDTA, 0.02% sodium azide, 1:20 dilution of protease inhibitor cocktail (Boehringer Mannheim), and 1 mM sodium ortho-vanadate] and 0.5 M NaCl. After a 30-min incubation on ice, cells were centrifuged at 12,000 rpm for 30 min, at 4°C. Four volumes of cell lysis buffer without NaCl were added. The amount of protein in the supernatant was quantitated using the Pierce BCA protein quantitation assay. For immunoprecipitation, 1 mg of lysate was incubated overnight with the antibody to trk receptor phosphorylated at residues 674/675, at 4°C with rocking, and then bound to protein A agarose for four h the following day. The slurry was centrifuged and washed several times and the antigen released from the beads by heating in SDS sample buffer. The resulting immunoprecipitant was run out on SDS-PAGE 10% Tris-glycine ready-gels (Bio-Rad) and transferred onto 0.2 μm polyvinylidene difluoride membrane from Bio-Rad (Hercules, CA). The membranes were probed with the phospho-trk 674/675 antibody and then visualized using the ECL system (Amersham).

Serum PSA Analysis. The serum PSA was determined as described previously (28).

CEP 701 Blood and Tissue Determinations. One hundred μl of an internal standard was added to each plasma sample, which was then extracted in methanol, and the supernatants analyzed by liquid chromatography-mass spectrometry equipped with an automatic solid-phase extractor. Similarly, analyzed tumor samples, snap-frozen in liquid nitrogen were ground to a coarse powder under liquid nitrogen in a mortar and pestle and homogenized in neutral PBS; 100 μl of an internal standard was added, and combined fractions were extracted with methanol as for plasma sample and analyzed by liquid chromatography-mass spectrometry. Results were corrected for procedural losses based on the recovery of the internal standard and were compared with known CEP-701 standards ranging from 5 to 4500 nM. For tissue samples, 1 g was equated to 1 ml of volume.

Statistical Analysis. Results are expressed as the mean ± SE. The long-term survival data were analyzed using the log-rank statistic nonparametric test. All of the other statistical analyses were performed as described previously (13).

RESULTS

Selective Inhibition of Growth *in Vivo* by CEP-751 Involves Induction of Apoptosis of Prostatic Cancer Cells. In the majority of models tested, CEP-751 inhibited the growth of the prostate cancer without inducing a regression in tumor volume (13, 14). For example, the growth rate of the AT-2 rat

Table 1 Effect of CEP-751 treatment on prostate cancer volume, proliferation, and death rate

Tumor type	host	Treatment (time)	Cancer volume[a]	Percentage of Cancer Cells	
				Proliferation day	Dying day
AT-2	Rats	Vehicle (10 days)	15.2 ± 1.1	49.3 ± 3.2	24.5 ± 1.8
		CEP-751[b] (10 days)	6.1 ± 0.5[c]	57.6 ± 1.4	37.2 ± 4.1[c]
LNCaP	Nude mice	Vehicle (21 days)	4.2 ± 0.3	23.4 ± 5.1	14.0 ± 2.1
		CEP-751[b] (21 days)	1.7 ± 0.2[c]	25.2 ± 6.3	21.8 ± 2.5[c]

[a] Cancer volume expressed as fold increase from initiation of treatment.
[b] CEP-751 given s.c. at 10 mg/kg/dose. For AT-2, rats were given one dose/day; for LNCaP and TSU-pr1, nude mice were given two doses/day.
[c] $P < 0.01$ *versus* vehicle control.

and LNCaP human prostatic cancer models was inhibited more than 50% by CEP-751 treatment (Table 1). The inhibition in the growth of these nonregressing prostate cancer models could be caused by either suppression of proliferation and/or induction of apoptosis. To resolve this issue, the daily rate of proliferation and apoptosis was determined in those two nonregressing prostatic cancer lines from vehicle *versus* CEP-751 treated animals (Table 1). These data demonstrate that there was no statistically significant decrease in the rate of proliferation, although there was significant increase in the rate of cell death in these prostatic cancer models. The reason tumor regression does not occur in these particular models is that, although cancer cell death is increased, this increase is not large enough to overcome the extremely high rates of cell proliferation by these cancers.

CEP-701 Inhibits the Metastatic Ability and Growth of the Primary Prostate Cancer Cells. Previously we have demonstrated that all of the 5 rat prostate cancer lines tested (*i.e.,* AT2, AT3, G, MAT-Lu, and H) are significantly growth-inhibited by a daily s.c. injection with 10 mg/kg CEP-751 (13). In these earlier experiments, the ability of CEP-751 to inhibit metastasis was not tested definitively. To resolve this and to confirm that CEP-701 is active *in vivo*, like CEP-751, the Dunning R-3327 AT6.3 subline was used as a model system. This subline was chosen because it is completely androgen independent and has a very high rate of lung metastasis when grown s.c. in syngeneic rats (22). To test the ability of CEP-701 to inhibit the growth of the primary tumor and its ability to mestastasize to the lung tissue, rats were inoculated s.c. in the leg with AT6.3 cells and immediately randomized into groups of CEP-701 treated (*i.e.,* 10 mg/kg/s.c. once daily) or vehicle-alone injected animals. Tumor volumes were measured longitudinally in each animal, and these data demonstrated that CEP-701 inhibited primary tumor growth by ~70%. Because of this inhibition, the AT6.3 tumors were 0.8 ± 0.1 cm^3 in the CEP-701-treated groups ($n = 5$) *versus* 2.2 ± 0.3 cm^3 ($P < 0.01$) in the vehicle-treated group ($n = 8$) on day 16 postinoculation. On day 16 post-tumor inoculation for the vehicle-only treated group, the tumor-bearing leg was surgically removed, and the primary cancer processed for histological analysis. Because CEP-701 treatment inhibited the growth of the primary cancer, 10 additional days were allowed before surgical removal of the tumor-bearing leg on day 26 in the CEP-70-treated animals. This was done so that the size of the primary cancer was at least as large as that of the vehicle-only group. On day 26 for the CEP-701 treated group, when the tumor was 3.9 ± 1.19 cm^3 in size, the tumor-bearing legs were surgically removed and pro-

cessed for histological analysis. Histological analysis of the primary cancer in the surgically removed leg demonstrated that there was no difference in the daily rate of cell proliferation in the CEP-701 treated *versus* vehicle-control groups (*i.e.,* 24.5 ± 4.2% *versus* 23.2 ± 5.1%, respectively), whereas there was an enhanced ($P < 0.01$) rate of cell death (*i.e.,* 7.1 ± 1.8% *versus* 4.0 ± 0.6, respectively). Thus, the overall 70% inhibition of the growth of the AT6.3 primary tumors induced by CEP-701 is consistent with enhanced apoptosis of the cancer cells and not an inhibition of their proliferation.

After the tumor-bearing legs in both groups were surgically removed, the animals were allowed to go untreated for 3 weeks before being killed. At death, the lungs were removed and weighed. Because sufficient time (*i.e.,* 3 weeks) was allowed without any further CEP-701 treatment, any difference in the weight of lung metastases after this 3-week drug free period is attributable to the ability of CEP-701 to inhibit the number of initial lung metastases established before removal of the primary tumor. A series of age-matched non-tumor-bearing rats were also killed, and the lung weights determined. By subtracting the mean lung weights for these non-tumor-bearing rats from the mean lung weights of the vehicle- and CEP-701-treated rats, the mean weight of the lung metastases for the two groups was determined (*i.e.,* 2.70 ± 0.98 g of lung metastases for the vehicle *versus* 0.88 ± 0.52 g of lung metastases for the CEP-701 group). CEP-701 greatly reduced the total metastatic tumor burden, most likely by reducing the number of metastases established during the treatment period. This explanation is consistent with the observation that metastatic burden was lower in treated animals even when normalized for primary tumor mass.

Effect of CEP-701 on the Growth of Metastatic Prostate Cancer *versus* Normal Prostate Cells. The previous studies demonstrated that CEP-701 could inhibit both the metastatic ability and growth of primary AT 6.3 prostate cancers. These studies did not resolve, however, whether CEP-701 could also inhibit the growth of the lung metastases themselves. To resolve this, rats were inoculated with AT6.3 cells and allowed to go untreated for 3 weeks, at which time the primary tumors were 4–6 cm^3 in size. This time period was chosen based on the study just presented, which demonstrated that AT6.3 prostate cancer cells disseminate from the primary tumor site in untreated animals and establish micrometastases in the lungs within 2 weeks post-tumor inoculation. Therefore, after 3 weeks, the tumor-bearing leg was removed from each animal, and the animals with micrometastatic disease were randomized into two groups. One group was given injections s.c. of 10 mg

of CEP-701/kg/day for 17 days and then not given the drug for 6 days before reinitiating CEP-701 s.c. daily dosing on day 23. The second group received vehicle only, using the same dosing regimen. On day 29, after removal of the primary tumor, all of the animals were killed, and their ventral prostate was removed, weighed, and then processed for histological analysis. The lungs were fixed with Bouin's solution, and the number of macroscopically detectable surface lung metastases counted before the lungs were processed for histological analysis.

These studies demonstrate that there was no difference in the ventral prostate wet weight in the CEP-701 treated *versus* vehicle controls (*i.e.,* 250 ± 17 mg *versus* 235 ± 11 mg wet weight, respectively). In addition, histological examination demonstrated no significant changes in the ventral prostate between the CEP-701- *versus* vehicle-treated rats (*i.e.,* no differences in the percentage of Ki-67-positive proliferating cells or terminal transferase-positive dying cells, both percentages being <0.5%). These studies also demonstrated that the number of macroscopically detectable surface metastases was decreased ($P < 0.01$) from a value of 48 ± 5 in the vehicle control group to 23 ± 4 in the CEP-701-treated group. Because the tumor-bearing leg was removed, no further establishment of micrometastases can occur before the initiation of CEP-701 treatment. Thus, the inhibition of the number of lung metastases that grew to a macroscopically detectable size documents that CEP-701 inhibited the growth of these preestablished lung micrometastases during the subsequent treatment. Microscopic analysis of the lung metastases demonstrated no difference in the rate of cancer cell proliferation (*i.e.,* 29.4 ± 6.1% *versus* 30.3 ± 7.2% in CEP-701 *versus* vehicle groups, respectively), whereas there was enhanced ($P < 0.01$) rate of apoptosis (5.6 ± 0.9% *versus* 2.4 ± 0.2%, respectively). Thus, inhibition of the growth of prostate cancer micrometastases to a macroscopically detectable size is consistent with an enhanced apoptotic effect, not an inhibition of proliferation. This occurred, although CEP-701 had no apoptotic effect on normal prostate cells.

Effects of Chronic Oral Treatment with CEP 701 on Survival of Prostate Cancer-bearing Rats. Although useful for studies of metastatic ability, the AT6.3 model has limitations, however, in that its growth rate is much faster (*i.e.,* tumor volume doubling time is ~5 days in untreated hosts) than the typical 2–3-month doubling times for human prostate cancers (12). This fast growth rate is primarily driven by a high rate of cell proliferation of the AT6.3 cells (*i.e.,* ~25% growth fraction for AT6.3 cells *in vivo*). Within the Dunning R3327 system of prostate cancers, there is a more appropriate slow-growing model known as the H subline. The H subline is a well differentiated, slow growing, androgen responsive, prostate adenocarcinoma. This H cancer has a tumor volume doubling time of ~1 month in the presence of physiological levels of androgen (26). The H cancer is heterogeneously composed of androgen-dependent, -sensitive, and -independent prostate cancer cells (3, 4, 26, 29). The malignant acinar cells in these H cancers express trk (13, 14) and neurotrophins (30). These cells undergo apoptosis when H tumor-bearing rats are treated with CEP 751 or CEP 701 (13, 14). Because of its highly selective ability to induce apoptosis, the effect of chronic (*i.e.,* >1 year) oral CEP-701 treatment on the survival of H cancer-bearing rats was tested.

To do this, rats were inoculated s.c. in the flank with H

Table 2 Concentration of CEP-701 in blood plasma and H rat prostate cancer tissue in animals given two cycles of 10 mg p.o. of CEP-701/kg body weight BID for 5 days/week/cycle

Time after last oral dose, h	CEP-701 concentration in	
	Plasma (nM)	H tumor (nM)
1 (*n* = 3)	4.5 ± 2.3	35.6 ± 6.6
2 (*n* = 3)	49.2 ± 30.0	26.6 ± 18.6
4 (*n* = 3)	6.8 ± 2.3	30.2 ± 15.0
8–12 (*n* = 5)	<2	50.4 ± 39.7

prostate cancer tissue, and the other flank was implanted with a 2-cm-long silastic capsule filled with testosterone. Previously, we have demonstrated that this size of testosterone implants maintains the serum testosterone levels within the normal physiological level of 1–2 ng/ml of testosterone for more than 6 months (27). These testosterone implants were replaced every 6 months during the entire period of the long-term study. The reason for the testosterone implantation is that, in preliminary studies, we demonstrated that the serum testosterone level is 1.5 ± 0.2 ng/ml in H tumor-bearing male Copenhagen rats younger than 10 months of age. After this age, the serum testosterone levels decrease so that by 1 year of age, the serum testosterone level is 0.9 ± 0.3 ng/ml, and by 16 months, it is 0.5 ± 0.2 ng/ml (*i.e.,* the serum testosterone level in surgically castrated rats is less than 0.1ng/ml). In previous studies, we have demonstrated that for the optimal growth of the H cancer, the serum testosterone level must be at least 1ng/ml (26). Therefore for long-term experiments of more than 1-year duration, it is vital to supplement the rats with exogenous testosterone. In this way, the non-androgen ablation effects of CEP-701 can be evaluated appropriately.

The testosterone-supplemented H tumor-bearing rats were allowed to go untreated until the cancers were ~2–3 cm³ in size (*i.e.,* ~6 months postinoculation and, thus, at a time when the hosts were 9–10 months of age). Tumor-bearing rats were then randomized into two groups. One group received CEP-701 given by gavage at 10 mg/kg/dose BID, 5 days per week. The second group received vehicle p.o. using the same dosing regimen. In preliminary studies, blood and tissue were collected over a course of 12 h from H-tumor-bearing Copenhagen rats that had been treated with two cycles of 10 mg/kg BID, 5 days a week. These results (Table 2) demonstrate that peak plasma levels of CEP-701 (*i.e.,* 50 nM) are achieved within 2 h of oral dosing. In contrast to the large time-dependent variation in plasma CEP-701 levels using the multicycle regimen, the H tumor levels of CEP-701 remained reasonably constant in the 25–50 nM range.

On the basis of these results, larger groups of rats bearing 2–3-cm³ size H tumors were begun on vehicle *versus* CEP-701 treatment chronically using the 5-day oral dosing schedule described. The results of these long-term studies demonstrated that median survival from the initiation of vehicle-only treatment (*n* = 18) was 408 days (95% confidence interval, 395–432 days) *versus* 566 days (95% confidence interval, 497–598 days) for the rats gavaged with CEP-701 (*n* = 24; Fig. 2). This 160-day increase in survival in the CEP 701-treated group was statistically significant ($P < 0.0001$). The mean H cancer size at
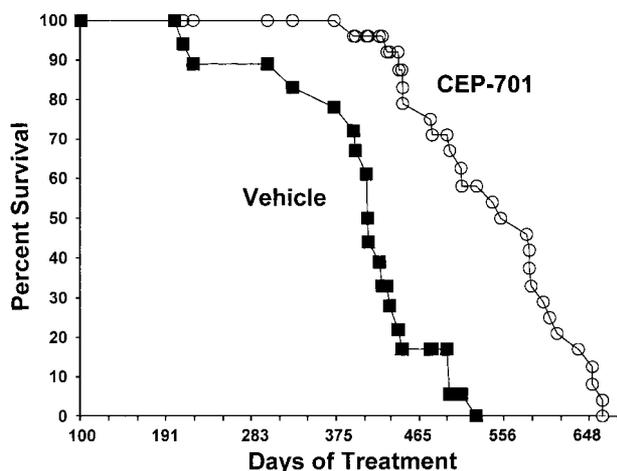
Fig. 2 Long-term survival of rats bearing the R3327H rat prostatic cancer given p.o. BID dosing with 10 mg of CEP-701/kg/dose ($n = 24$) *versus* vehicle only ($n = 18$; $P < 0.01$ for all of the time points after 366 days for CEP-701 *versus* corresponding time point for vehicle group).

Fig. 3 *A*, RT-PCR analysis of trk A expression in the CWR-22Rv1 and TSU-Pr1 human prostate cancer cell lines and in the WI38 human fibroblast line. The WI38 cells were analyzed as a known negative control. *B*, Western blot analysis of the phosphorylation status of trk A receptor in vehicle-treated TSU-Pr1 human prostatic cancer cells *versus* cells treated *in vitro* for 24 h with 100 nm CEP-701.

death was 158 ± 22 cm^3 for the vehicle-only group *versus* 139 ± 14 cm^3 for the CEP-701-treated animals. The demonstration that H tumor-bearing rats died with similar-sized cancers documents that the death of the CEP-701 animals is attributable to total tumor burden and not to accumulated host toxicity to CEP-701 treatment.

CEP-701 Inhibits Human Prostate Cancer Growth and Lowers Serum PSA in Xenograft Models.   To validate that the therapeutic response to CEP 701 of AT6.3 and H prostate cancers is not rat prostate cancer-specific, the response of the androgen-independent TSU-pr1 and CWR-22Rv1 human prostate cancer xenografts in nude mice was also tested. The TSU-Pr1 cells express trk A (Fig. 3*A*) and trk C (13) and secretes neurotrophins in an autocrine fashion (10). In addition, CEP-751 treatment *in vitro* induces the apoptotic death of these cells (10). To document that this apoptotic death is attributable to trk inhibition, Western blot analysis using a phospho-trk-specific antibody was performed on trk immunoprecipitates from vehicle *versus* CEP-701-treated TSU-pr1 cells (Fig. 3*B*). These results demonstrate that the phosphorylated trk (*i.e.,* activated) receptor is depleted by CEP-701 treatment. On the basis of these results, 10 nude mice were inoculated with TSU-pr1 cells and allowed to go untreated until the tumors were $0.1–0.5$ cm^3 in size. At this time, the tumor-bearing mice were randomized into two groups of five. One group received two 5-days-per-week cycles, consisting of BID injections s.c. with 10 mg/kg/injection of CEP-701. The other group received the same dosing regimen with vehicle alone. The tumor volume data demonstrate that CEP-701 treatment completely inhibited the continuous growth of TSU pr1 prostate cancers that was observed in the vehicle control group (Fig. 4).

Recently, a cell line has been established from the androgen-independent CWR-22R human prostate cancer xenograft line, termed the CWR-22Rv1 (23). The advantage of this line is that its growth *in vivo* in nude mice is androgen independent and
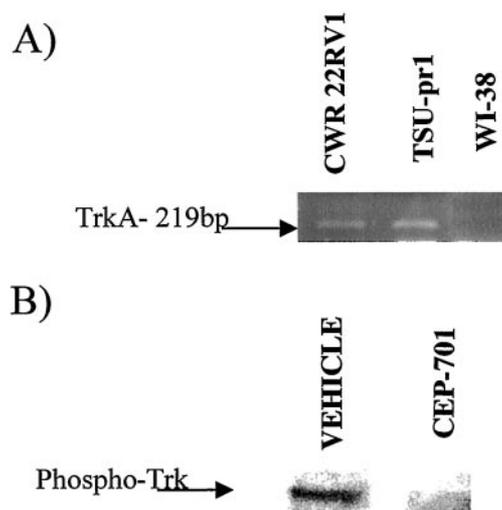
yet these cancers, unlike the TSU-pr1 cancers, still produce PSA. Using RT-PCR, it was determined that the CWR-22Rv1 cells express trk A (Fig. 3*A*). Previously, we have documented that these cells secrete NGF in an autocrine manner (10). Thus, this line was used to test the effect of pan-trk inhibition by CEP 701 not only on the *in vivo* growth of CWR-22Rv1 cells but also on serum PSA values. To do this, nude mice were inoculated s.c. with CWR-22Rv1 cells, and the mice were untreated until the tumors were $0.4–0.6$ cm^3 in size. Tumor-bearing mice were then randomized into two groups: one received s.c. injections BID of vehicle alone and the other BID injections of 10 mg/kg/dose of CEP701.

The tumor volume data demonstrated that there was no growth of the CWR-22Rv1 prostate cancers in the CEP-701-treatment group as compared with a doubling of the tumor volume in the vehicle control mice (Fig. 5). After 15 days of treatment, serum was collected for PSA measurements, and the tumor was harvested and weighed. These results demonstrated that the serum PSA levels in the CEP-701 treated mice (*i.e.,*1.9 ± 0.4 ng/ml) were significantly lower ($P < 0.001$) than in the vehicle control group (*i.e.,*7.1 ± 0.4 ng/ml). When these raw values were normalized to the tumor weight, however, the results demonstrated that there was no statistically significant difference in the normalized serum PSA output values (*i.e.,*7.9 ± 0.9 ng of PSA/ml serum/gram CWR-22Rv1 cancer in CEP-701-treated animals *versus* 15.7 ± 4.8 ng of PSA/ml serum/gram CWR-22Rv1 cancer in vehicle-only treated animals). These results demonstrate that CEP-701 does not directly inhibit the secretion of PSA by CWR-22Rv1 human prostate cancer cells. These results validate that for this model, serum PSA levels can be used as a prognostic indicator of tumor burden to follow overall response to CEP-701.
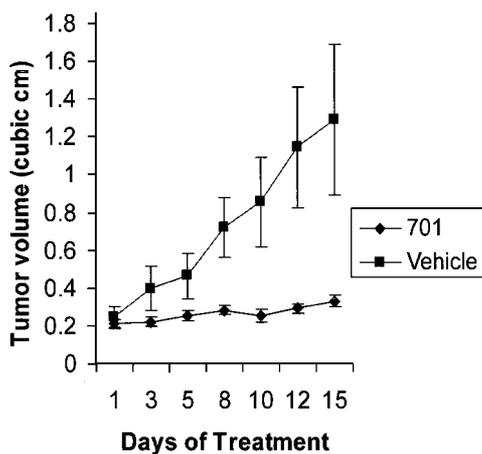
Fig. 4 Response of the TSU-Pr1 human prostate cancers growing in nude mice treated 5 days a week BID with s.c. injections of either 10 mg of CEP-701/kg/dose or vehicle alone. ($P < 0.01$ for all time points of CEP-701 *versus* corresponding time points for vehicle group.)

DISCUSSION

CEP-701 inhibits the tyrosine kinase activity of all three subtypes of trk receptors (*i.e.,* trk A, B, and C) via competition at the ATP-binding site and thus blocks the signaling ability of the ligand (*i.e.,* neurotrophin)-occupied trk receptor complexes. An advantage of this approach is that once the tyrosine kinase of the trk receptor is inhibited by CEP-701, no amount of neurotrophin can overcome this inhibition, and, thus, downstream signals cannot be generated. Because of the molecular changes during prostate carcinogenesis, prostate cancer cells become sensitive to these neurotrophin/trk signaling pathways for their survival to an extent both unexpected and restricted with respect to other normal host tissues including nerve and brain cells (10). Because of this restricted sensitivity, CEP-701 can be given chronically using an oral dose regimen that maintains the prostate cancer tissue level of CEP-701 in the range of 25–50 nM without producing host toxicity. The concentration of CEP-701 needed to inhibit 50% of the tyrosine kinase activity (*i.e.,* IC_{50}) of the trk receptors (*i.e.,* trk A, B, or C) is 4 nM in soluble enzyme assays and 25 nM in intact cells (14). In contrast, in soluble enzyme assays, the IC_{50} values for CEP-701 inhibition of insulin receptors, IGF receptors, and epidermal growth factor receptors are >1000 nM; for PDGF receptors, fibroblast growth factor receptors, and protein kinase C, the IC_{50}s are >200 nM; and for vascular endothelial growth factor receptors, the IC_{50}s are >50 nM (14). Therefore, at a tissue concentration of 25- 50 nM, CEP-701 should be a highly specific and potent inhibitor of the tyrosine kinase activity of all of the trk receptors subtypes, with little inhibition of the tyrosine kinase activity of other plasma membrane receptors (20). Such a targeted pan-trk inhibition provides a rationale for the observations that when CEP-701 is given to prostate cancer-bearing rats chronically (*i.e.,* for >1 year), significant inhibition of the prostate cancer growth occurs without producing host toxicity even in the normal prostate. Thus, such chronic CEP-701 treatment extends host survival.

CEP-701 also inhibits the ability of prostate cancer cells to
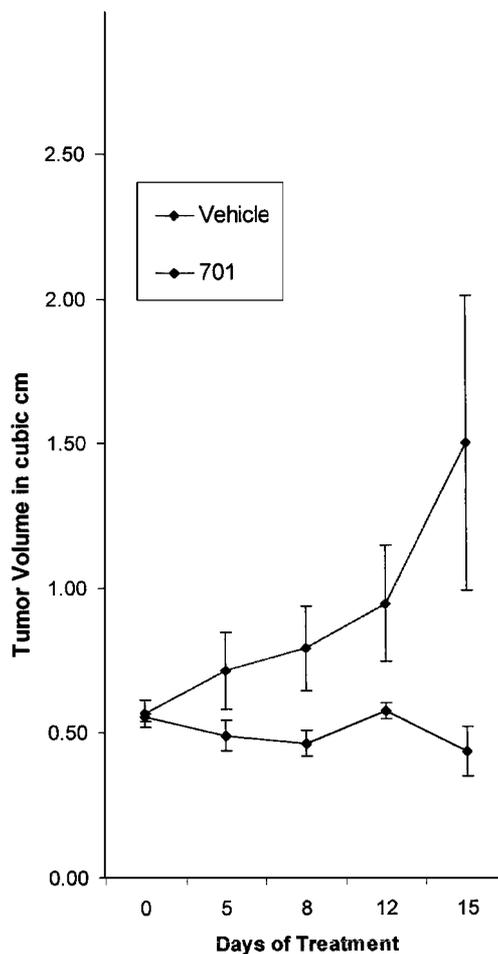

Fig. 5 Response of the CWR-22Rv1 human prostate cancers growing in the nude mice treated continuously BID with s.c. injections of either 10 mg of CEP-701/kg/dose or vehicle. ($P < 0.01$ for all time points of CEP-701 *versus* corresponding time points for vehicle group.)

metastasize and inhibits the growth of these cells in micrometastases as well as in primary prostate cancers. Antitumor effects of CEP-701 are not rodent prostate cancer-specific, because there is pronounced inhibition of growth of human prostate cancer xenografts induced by treatment with this drug. In part on the basis of these preclinical data, CEP-701 has entered clinical development and is currently undergoing Phase II evaluation in hormone-refractory prostate cancer patients. In a separate clinical study, patients with localized prostate cancer have been administered CEP-701 p.o. for 5 days before undergoing radical prostatectomy. These prostatectomy tissues are being assayed for tissue-drug concentrations and biological markers of drug activity to ascertain whether preclinical observations of CEP-701-induced biochemical and cellular events extend to the human patient population.

Prostate cancers are not the only types of malignancies that are responsive to CEP-701. Preclinical studies have documented that both pancreatic cancers as well as neuroblastomas and medulloblastoma consistently respond to CEP-751- and CEP-701-induced trk inhibition (13, 30, 31). In

contrast, ovarian cancers, colon cancers, renal cancers, breast cancers, lung cancers, and melanomas are only occasionally responsive *in vivo* to CEP-751 and CEP-701, with the responsive variants expressing at least one of the trk receptors (13, and unpublished data).[4] These results are consistent with CEP-751/CEP-701 having a selective mechanism of antitumor efficacy. Presently, the specific signaling pathways activated during the apoptosis induced by these agents are being identified. This information is critical to developing rational combinational approaches using CEP-701. This is significant because the therapeutic response to CEP-701 can be enhanced. For example, additional animal studies have confirmed that CEP-701 can be combined with androgen ablation to enhance the anti-prostate cancer effect beyond that produced by either of the monotherapies (14). Thus, additional studies are under way to identify additional modalities (*e.g.,* antiangiogenic agents) that can be combined with CEP-701 to produce a more curative approach without unacceptable host toxicity in both prostatic and pancreatic cancer patients.

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