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CEP-701 and CEP-751 Inhibit Constitutively Activated RET Tyrosine Kinase Activity and Block Medullary Thyroid Carcinoma Cell Growth[1]

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ABSTRACT

All of the cases of medullary thyroid carcinoma (MTC) express the RET receptor tyrosine kinase. In essentially all of the hereditary cases and ~40% of the sporadic cases of MTC, the RET kinase is constitutively activated by mutation. This suggests that RET may be an effective therapeutic target for treatment of MTC. We show that the indolocarbazole derivatives, CEP-701 and CEP-751, inhibit RET in MTC cells. These compounds effectively inhibit RET phosphorylation in a dose-dependent manner at concentrations <100 nM in 0.5% serum and at somewhat higher concentrations in the presence of 16% serum. They also blocked the growth of these MTC cells in culture. CEP-751 and its prodrug, CEP-2563, also inhibited tumor growth in MTC cell xenografts. These results show that inhibiting RET can block the growth of MTC cells and may have a therapeutic benefit in MTC.

INTRODUCTION

MTC,[3] a cancer of the parafollicular C cells of the thyroid, accounts for approximately 5–10% of all cases of thyroid cancer and a disproportionate percentage of the 1100–1300 thyroid cancer deaths per year in the United States (1, 2). In ~20% of the cases, MTC occurs as an autosomal dominantly inherited cancer as part of several closely related MEN 2 syndromes (3). In the first syndrome, MEN 2A, MTC is often accompanied by adrenal pheochromocytomas and hyperparathyroidism. In MEN 2B, MTC is accompanied by adrenal pheochromocytomas and multiple enteric ganglionic abnormalities. Familial MTC is characterized by MTC without other endocrine abnormalities (4). In all of these syndromes, affected individuals harbor a germ-line activating mutation in the *RET* receptor tyrosine kinase gene. In MEN 2A and familial MTC, this mutation is most commonly found in one of six cysteines located just outside the cellular membrane, although other activating mutations in the intracellular kinase domain have been reported (5, 6). In MEN 2B, the activating mutation is almost always M918T, which is in the substrate binding moiety of the kinase domain (7). This activating mutation is also seen in ~40% of sporadic MTC, which indicates that *RET* activation is also important for tumorigenesis in a significant portion of sporadic cases (8).

Treatment of sporadic MTC typically consists of a total thyroidectomy in combination with central compartment and modified lateral neck dissections (9–11). Both chemotherapy and radiotherapy have had only limited effectiveness in MTC, with few, if any, complete responses, and partial response rates limited to 15–30% for brief durations.

Therapy of the 20% of the patients with hereditary MTC has been markedly improved by presymptomatic detection of germ-line *RET* mutations and prophylactic thyroidectomy in childhood (12–14). However, when adjusted for clinical stage, the overall aggressiveness of hereditary MTC parallels that of the sporadic form (15). This lack of any effective treatment demonstrates a pressing need for new approaches to effective systemic therapy of MTC.

The association of *RET* mutations with both familial and sporadic MTC, and the demonstration that activated RET is oncogenic in both cell culture and transgenic animals, suggests that RET may be an excellent therapeutic target for the treatment of MTC (16–20). The potential of RET as a target has been additionally validated by the recent demonstration that expression of a dominant-negative form of RET inhibited growth of MTC cells (21). In this report, we show that the indolocarbazole compounds, CEP-701 and CEP-751, inhibit RET at nanomolar levels. This results in growth arrest and apoptosis in the human MTC cell line, TT, which harbors a mutationally activated *RET* allele. We also show that CEP-751 and its prodrug, CEP-2563, have growth-inhibitory effects in TT cell xenografts in nude mice. Our results demonstrate that the inhibition of RET may be an effective strategy for the treatment of MTC.

MATERIALS AND METHODS

Reagents. CEP-701, CEP-751, and CEP-2563 were supplied by Cephalon, Inc. (West Chester, PA). These agents were dissolved in DMSO at a concentration of 4 mM for dilution in cell culture experiments. CEP-701 and CEP-751 were formulated in a 40% polyethylene glycol 1000, 10% povidone C30, and 2% benzyl alcohol in sterile water for use in nude mouse experiments. CEP-2563 was formulated in solutol (polyethylene glycol 660 12-hydroxystearate). RET antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphotyrosine antibody 4G10 was obtained from Upstate Biotechnology (Lake Placid, NY). The Phospho-RET antibody was purchased from Cell Signaling Technology (Beverly, MA). The poly(ADP-ribose) polymerase antibody was obtained from Becton Dickinson PharMingen (San Diego, CA). The glyceraldehyde-3-phosphate dehydrogenase antibody was purchased from Trevigen (Gaithersburg, MD). The secondary antibodies were purchased from Santa Cruz Biotechnology. Phosphatase inhibitor and protease inhibitor mixtures were purchased from Sigma (St. Louis, MO).

Cell Culture. TT cells, a human MTC cell line, are available from American Type Culture Collection (Manassas, VA). TT RET2B cells, which express a *RET* allele with the constitutively active M918T mutation, have been described previously (22). Cells were maintained in RPMI 1640 supplemented with 16% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin in a 37°C incubator with 5% CO_2.

Immunoprecipitation. The cells were treated with CEP-701 and CEP-751 for 2 h in the presence of normal (16%) or low (0.5%) concentrations of FBS. The cells were then washed with PBS and harvested with radioimmunoprecipitation assay buffer (1× PBS, 1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS) + protease inhibitors and phosphatase inhibitors. Five-hundred μg of clarified lysate total protein were incubated overnight in the presence of RET antibody and protein agarose G beads (Santa Cruz Biotechnology). The lysate was then spun down and washed four times with radioimmunoprecipitation assay buffer. The samples were electrophoresed on a 6% polyacrylamide gel and transferred to nitrocellulose (Bio-Rad, Hercules, CA). The blots were then probed with antiphosphotyrosine antibody 4G10 and visualized using Supersignal Pico chemiluminescence (Pierce, Rockford, IL).

Western Blotting. Cells were treated with drug for 2 h as described above. They were then washed with PBS and harvested by scraping the cells with 1×

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[3] The abbreviations used are: MTC, medullary thyroid cancer; MEN, multiple endocrine neoplasia; FBS, fetal bovine serum; AGP, acidic glycoprotein.

SDS lysis buffer [2% SDS and 62.5 mM Tris (pH 6.8)]. Lysates were electrophoresed on 6% polyacrylamide gel and transferred onto nitrocellulose. Blots were probed at 4°C overnight with primary antibodies diluted 1:1000 in 5% milk. Secondary antibodies were diluted 1:5000, and blots were visualized using Pierce Supersignal Pico Chemiluminescence.

Growth Curves. Growth curves were performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Sigma). Cells were seeded in 24-well plates using phenol red-free RPMI 1640 with 16% FBS. After 48 h, the medium was replaced with either low serum medium (0.5% FBS) or normal serum medium (16% FBS) containing CEP-701 and CEP-751 at appropriate concentrations or DMSO as a vehicle control. Each concentration was done in quadruplicate, and the medium containing drug was replaced daily. The assay was performed as described previously (23).

Cell Cycle Analysis. Cells (10^6) were treated overnight with CEP-701 and CEP-751 under normal and low serum conditions at 100 nM concentrations of drug. The nuclei were stained with propidium iodide (24). The nuclei were then analyzed by an LSR Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ) gated for single nuclei. The cell cycle profile was determined from 10,000 gated nuclei using CellQuest software.

***In Vivo* Tumor Growth in Nude Mice.** TT cells suspended in HBSS (10^7 cells/100 μl) were inoculated s.c. into the right flank of 4–6-week-old male athymic nude (ν/ν) mice (Harlan Labs, Indianapolis, IN). Once palpable, tumors were measured at indicated intervals with vernier calipers. Tumor volumes were calculated using the formula length × width × height × 0.5236. After a 4–5-week period tumors reached ~0.1 ml average size, and animals were sorted into groups of 10 to achieve equal distribution of tumor size in all of the treatment groups. Animals received s.c. injections twice daily for 5 consecutive days/cycle of either vehicle control (40% polyethylene glycol 1000, 10% povidone C30, and 2% benzyl alcohol in sterile water), CEP-701 (10 mg/kg/dose), or CEP-751 (10 mg/kg/dose). CEP-2563 was delivered via an osmotic minipump (Alzet, Cupertino, CA) with a capacity of 225 μl and a pump rate of 0.5 μl/h such that animals received a total of 24 mg/kg/day over a 14-day period. At the end of the experiments, animals were sacrificed by CO_2 overdose. Statistical analysis of differences in tumor volumes were performed using Student's *t* test and *P*s <0.05 reported in text. All of the animal studies were performed according to protocols approved by the Johns Hopkins Animal Care and Use Committee.

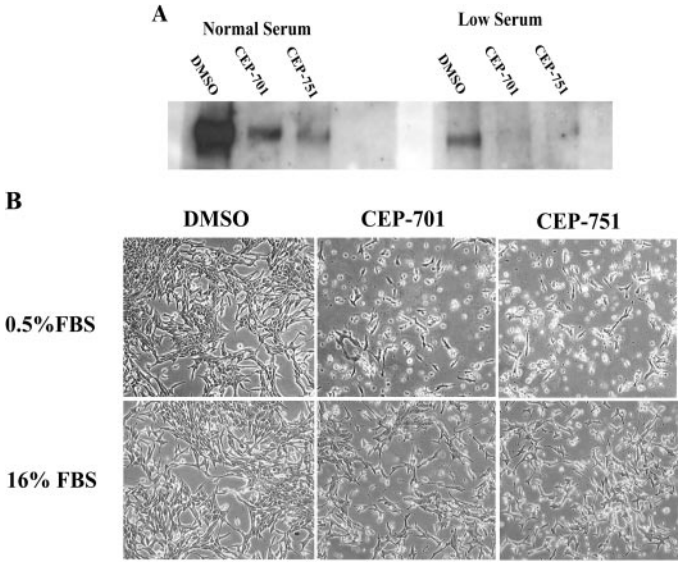


Fig. 1. Inhibition of TT cell growth and RET autophosphorylation. *A*, TT cells were treated for 2 h with 100 nM CEP-701 or CEP-751 in either low or normal serum. CEP-701 and CEP-751 completely block RET phosphorylation in low serum medium. There is incomplete inhibition in the presence of normal serum. *B*, TT cells were treated with 100 nM CEP-701 or CEP-751 in the presence of low serum (0.5% FBS) or normal serum (16% FBS) for 3 days. Medium was replaced daily with fresh drug. Magnification is ×100.

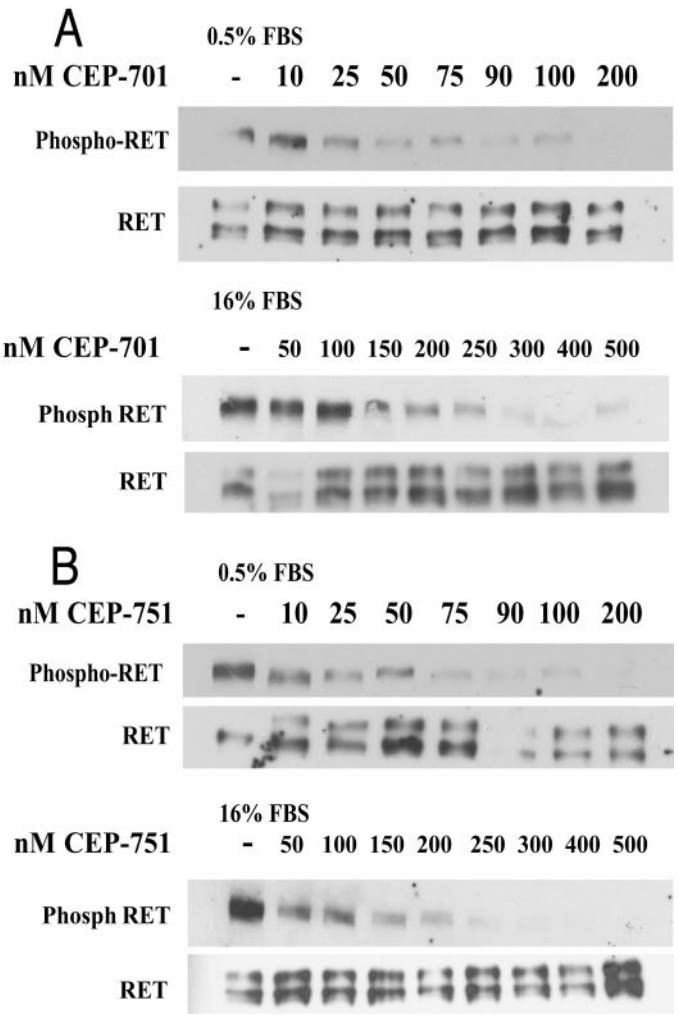


Fig. 2. Dose-dependent inhibition of RET autophosphorylation. *A*, TT cells were treated for 2 h at the indicated concentrations of CEP-701 in 0.5% FBS or 16% FBS. *B*, TT cells were treated with CEP-751 at the relevant concentrations for 2 h in both 0.5% FBS and 16% FBS. Each lane contains 50 μg of protein. Blots were probed with the antiphospho-RET antibody. Loading controls were done by probing for total RET.

RESULTS

Inhibition of RET Phosphorylation and Cell Growth. We evaluated the ability of CEP-701 and CEP-751 to inhibit RET kinase activity in a cell-based assay using the human MTC cell line, TT. TT cells were derived from a tumor from a patient with sporadic MTC. These cells contain a *RET* allele constitutively activated by a C634W mutation in the extracellular domain (25). For the initial assay, the TT cells were treated for 2 h with 100 nM of either compound. RET autophosphorylation was then determined by immunoprecipitation followed by Western blotting for phosphotyrosine. The results in Fig. 1*A* demonstrate that both CEP-701 and CEP-751 effectively inhibit RET phosphorylation in TT cells in low (0.5% FBS) serum. However, there is only partial inhibition of RET phosphorylation in the presence of normal (16% FBS) serum. This differential may be because of the efficient sequestration of CEP-701 and CEP-751 by the α1 AGP present in serum ($K_d = 1 \times 10^{-6}$ M).[4] Such binding has been reported previously for other indolocarbazole compounds such as UCN-01 (7-hydroxy staurosporine; Ref. 26).

Fig. 1*B* shows cells treated with 100 nM of either CEP-701 or CEP-751 in 16% FBS and 0.5% FBS after 3 days. In the presence of

[4] B. R., unpublished observations.

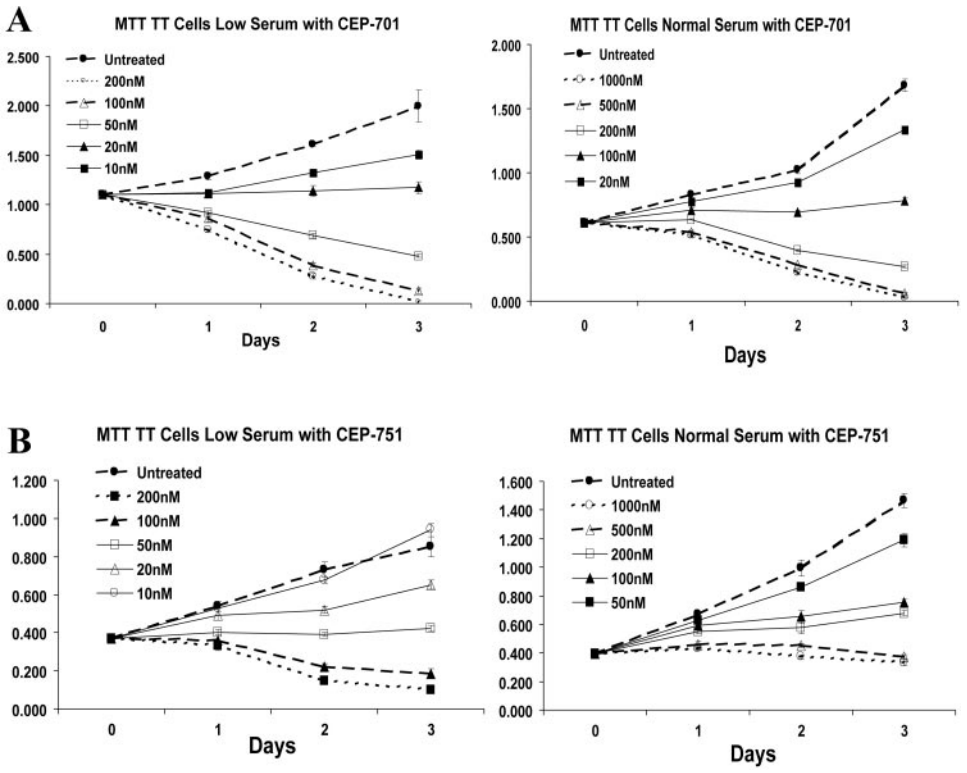


Fig. 3. CEP-701 and CEP-751 block proliferation of TT cells. Cells were treated with either CEP-701 (*A*) or CEP-751 (*B*) daily in normal serum (16% FBS) or low serum (0.5% FBS) medium. Cell growth was measured daily by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. All points were done in quadruplicate.

0.5% FBS the cells treated with either drug ceased growing and died. In the presence of 16% FBS the effect of treatment seems to be mainly cytostatic.

RET Autophosphorylation Is Inhibited in a Dose-dependent Manner. The concentration of CEP-701 and CEP-751 to inhibit RET autophosphorylation was examined by Western blotting with an antibody specific for phosphorylated RET. Fig. 2*A* shows that CEP-701 significantly inhibits RET phosphorylation in low serum at 50 nM, with complete inhibition evident at 100–200 nM. In the presence of 16% FBS, the inhibition is shifted significantly with very little inhibition occurring at 100 nM levels of drug. Above 200 nM CEP-701, RET phosphorylation decreased substantially with complete inhibition shifted 5-fold to 500 nM in 16% FBS.

The ability of CEP-751 to inhibit RET autophosphorylation was also measured. The results in Fig. 2*B* show that in the presence of low serum, the inhibition curve is roughly equivalent to the CEP-701 curve. In the presence of normal serum (16% FBS), CEP-751 is more active than CEP-701 against RET phosphorylation. Under these higher serum conditions, CEP-751 inhibits RET phosphorylation significantly even at 100 nM with complete inhibition occurring between 250 and 300 nM. Nevertheless, these results indicate that FBS may interfere with the ability of both CEP-701 and CEP-751 to block RET kinase activity.

CEP-701 and CEP-751 Block Proliferation of TT Cells. Fig. 3 shows the growth curves of TT cells treated with CEP-701 (Fig. 3*A*) and CEP-751 (Fig. 3*B*) in the presence of 16% FBS (normal serum) or 0.5% FBS (low serum). These data show that in the presence of low serum, TT cell growth was inhibited at all concentrations of drug compared with vehicle control. Complete growth cessation and significant cell loss occur at 100 nM levels and above with both drugs. In 16% serum, ~3-fold higher concentrations of both CEP-701 and CEP-751 were required to block cell growth. These results were consistent with what was observed in the inhibition curves of RET phosphorylation (Fig. 2).

Cell Cycle. Treatment with either CEP-701 or CEP-751 resulted in cell cycle arrest and apoptosis. Fig. 4 shows the cell cycle analysis of the TT cells treated with 100 nM CEP-701 and CEP-751 in the presence or absence of serum. In the presence of either drug in low serum, TT cells arrest in G_2/M and to a lesser extent G_1. In 16%

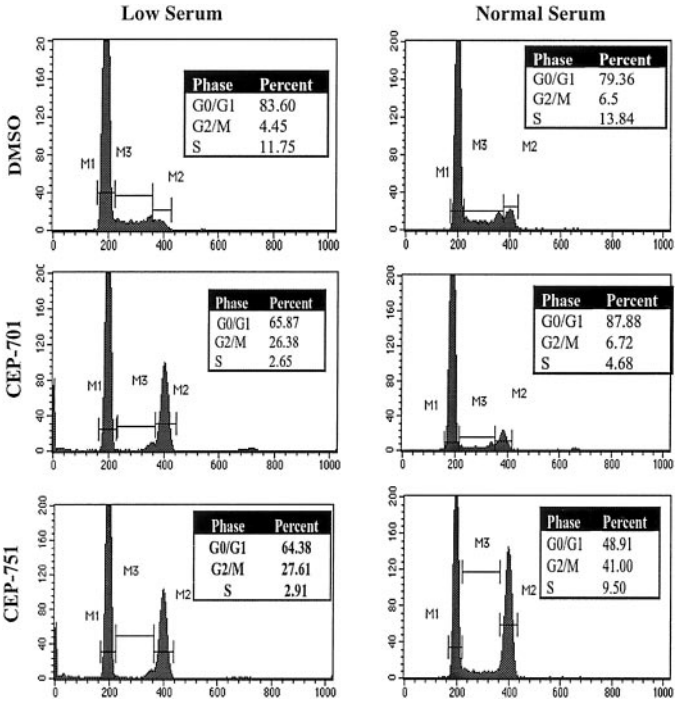


Fig. 4. Cell cycle effects of CEP-701 and CEP-751. TT cells were harvested 24 h after treatment with either CEP-701 or CEP-751, and the nuclei were stained with propidium iodide for cell cycle analysis. *M1* represents G_0/G_1, *M2* represents G_2/M, and *M3* represents S phase.

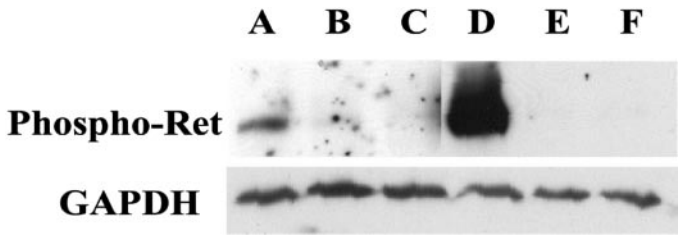


Fig. 5. CEP-701 and CEP-751 inhibit phosphorylation of the RET MEN2B mutation. TT cells containing the MEN2B mutation, C634W, were treated with CEP-701 and CEP-751 for 2 h in medium supplemented with 0.5% FBS. Each lane contains 50 μg of protein. GADPH acts as a loading control. *Lane A* is TT +DMSO, *B* is TT + CEP-701, *C* is TT + CEP-751, *D* is TT MEN2B + DMSO, *E* is TT MEN2B + CEP-701, and *F* is TT MEN2B + CEP-751.

serum, the cells treated with 100 nM CEP-701 marginally accumulate in G_0/G_1 with no accumulation in G_2/M. Alternatively, 100 nM CEP-751 treatment in full serum results in extensive accumulation in G_2/M. This effect appears to be an incomplete block because there is still significant S phase present. Although the cell cycle effects in low serum for the two drugs seem to be identical, normal serum levels (16% FBS) seem to interfere with the G_2/M effects in the CEP-701-treated TT cells.

For both agents, there is no accumulation of a sub-G_0 population that is indicative of apoptosis. However, 4′,6-diamidino-2-phenylindole staining of cells treated with either drug under the low serum conditions does show significant nuclear condensation, which is consistent with induction of apoptosis (data not shown).

Inhibition of RET MEN 2B Mutations by CEP-701 and CEP-751. TT cells have an activating mutation in RET at C634W. Such mutations in the extracellular cysteines are common in the hereditary MEN 2A syndrome. However, the MEN 2B syndrome and sporadic cases of MTC commonly have the mutation M918T. This mutation is located in the substrate recognition domain (Hanks' Domain VIII) of the kinase. Because the M918T mutation is common in MTC, it was important to show that CEP-701 and CEP-751 could inhibit RET activated by this mutation. TT cells expressing RET-M918T were treated with 100 nM CEP-701 and CEP-751. Results in Fig. 5 demonstrate that the compounds are effective against the MEN2B-mutated *RET*. Although the basal levels of RET and phosphorylated RET are significantly higher in the TT cells expressing RET M918T, 100 nM of either compound completely blocked phosphorylation. Thus, CEP-701 and CEP-751 inhibit RET kinase activated by the predominate types of mutations found in sporadic and hereditary forms of MTC.

Effect on TT Cell Growth in a Nude Mouse Model. The *in vivo* effectiveness of CEP-701 and CEP-751 was tested in nude mice bearing s.c. TT cell xenografts. In these studies, we also examined the effect of continuous infusion of CEP-2563 on the tumors. CEP-2563 is a lysinyl-β-alanyl ester prodrug of CEP-751, which was selected for its enhanced solubility and is rapidly converted to CEP-751 in serum (27). CEP-701 was found to be ineffective against the growth of these tumors (data not shown). In contrast, CEP-751 and its prodrug CEP-2563 were found to suppress the growth of the tumors. The results in Fig. 6 show that CEP-751 and CEP-2563 both inhibit growth to ~50% of the control vehicle-treated mouse over a period of 26 days for CEP-751 and 17 days for CEP-2563. The table containing the *P*s also shows that this inhibition is statistically significant. These results show that the strategy of inhibiting RET may be an effective method to treat MTC tumors.

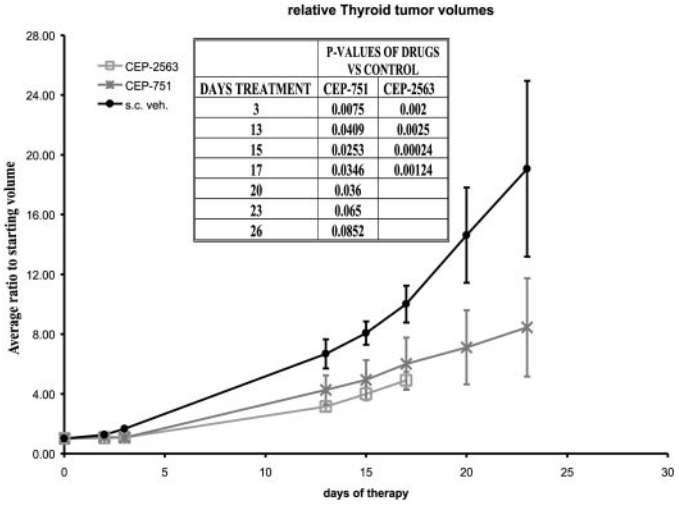
DISCUSSION

In this report, we show that the indolocarbazole compounds CEP-701 and CEP-751 are potent inhibitors of RET, and are cytostatic or cytotoxic to MTC cells both in cell culture and in an *in vivo* xenograft model. CEP-701 and CEP-751 are derivatives of the natural product kinase inhibitor K252a and were originally selected for their relative specificity for the Trk family of receptor tyrosine kinases (28–30). Subsequently, it has been shown that CEP-701 and CEP-751 also inhibit FLT3, VEGFR2, and to a lesser extent protein kinase C (31, 32). It is possible that the activity of CEP-701 and CEP-751 against these or other kinases may contribute to the effects we have shown in MTC cells.

RET has been reported to be expressed in the adult, primarily in cells within the central, peripheral, and enteric nervous system (33–35). Nevertheless, extended exposure of rats (2–22 months) to CEP-701 or CEP-751 resulted in no neuronal damage or other side effects (36). CEP-701 and CEP-2563 have entered Phase I clinical trials, and CEP-701 has now advanced to Phase II trials for several types of cancer.

RET is a well-validated target in the hereditary, MEN-2 associated, forms of MTC. The presence of activating RET mutations in sporadic forms of MTC suggests that RET may be a good target in these cases as well. However, it has been reported that the presence of RET mutations in sporadic MTC is heterogeneous within the tumors, suggesting that such tumors may arise late in tumorigenesis. This raises the possibility that some of these cases of sporadic MTC may not depend on RET for growth and, consequently, RET inhibitors may be ineffective in such tumors. In clinical trials of RET inhibitors for MTC, it will be important to monitor the heterogeneity of *RET* mutations in patients with sporadic cases of MTC.

A few reports have indicated that tyrosine kinase inhibitors can interfere with RET function in cultured cells (37–39). Some of these inhibitors, including clavilactone D and a series of indolinone derivatives, have relatively high IC_{50}s for inhibition of RET kinase activity (38, 40). The src family inhibitor PP1 has been shown recently to inhibit RET kinase with an IC_{50} of 80 nM, and it also blocked growth of RET-transformed NIH 3T3 cells *in vivo* (41). The tyrosine kinase inhibitor ZD6474 was shown to have similar activity with an IC_{50} of 100 nM (37). We have shown here that CEP-701 and CEP-751 are direct inhibitors of RET at nanomolar levels. We have also shown that these agents inhibit the growth of MTC cells both *in vitro* and *in vivo*. Nevertheless, we cannot exclude the possibility that other kinase targets of CEP-701 and CEP-751, in addition to RET, contribute to the MTC cell growth inhibition and cytotoxicity we have observed. However, our results suggest that the concentrations of CEP-701 and CEP-751 required for growth inhibition parallel the concentrations needed for inhibition of RET.



	P-VALUES OF DRUGS VS CONTROL	
DAYS TREATMENT	CEP-751	CEP-2563
3	0.0075	0.002
13	0.0409	0.0025
15	0.0253	0.00024
17	0.0346	0.00124
20	0.036	
23	0.065	
26	0.0852	

Fig. 6. CEP-751 and CEP-2563 inhibit TT growth in nude mice. TT cells (10^7) were s.c. injected into the flank of nude mice. The nude mice were injected with CEP-751 or vehicle in the opposite flank twice a day, 5 days a week. A pump containing CEP-2563 was inserted s.c. in other nude mice to release drug continuously. Tumors were measured at the shown time points; *bars*, ±SE.

In future studies, it will be important to optimize the effect of the indolocarbazole compounds for MTC *in vivo*. It is possible that related compounds may inhibit RET at even lower concentrations than these current drugs. In addition, as mentioned previously, CEP-701 and CEP-751 may be sequestered in serum by α 1 AGP (26). This binding likely accounts for the reduced efficacy we observed for CEP-701 and CEP-751 in the presence of high concentrations of serum. AGP limits the availability of a wide spectrum of drugs, and methods being developed to circumvent AGP binding may increase the activity of CEP-701 and CEP-751 in MTC. Finally, many tyrosine kinase inhibitors are most effective in combination with standard chemotherapeutic agents. As noted above, MTC has been particularly refractory to chemotherapy. Therefore, it would be of particular interest to examine whether CEP-701 or CEP-751 may sensitize MTC to these chemotherapeutic agents.

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