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Preclinical evaluation of lestaurtinib (CEP-701) in combination with retinoids for neuroblastoma

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Abstract

Purpose—Lestaurtinib (CEP-701), a multi-kinase inhibitor with potent activity against the Trk family of receptor tyrosine kinases, has undergone early phase clinical evaluation in children with relapsed neuroblastoma. We studied the interaction of CEP-701 with isotretinoin (13cRA) and fenretinide (4HPR), two retinoids that have been studied in children with high-risk neuroblastoma.

Methods—In vitro growth inhibition was assessed following a 72-hour drug exposure using the sulforhodamine B (SRB) assay in eight neuroblastoma cell lines with variable TrkB expression. When appropriate, the combination index (CI) of Chou-Talalay was used to characterize the interaction of 13cRA (non-constant ratio) or 4HPR (constant ratio) with CEP-701.

Results—The median (range) IC_{50} of single-agent CEP-701 across all cell lines was 0.09 (0.08–0.3) μM. The combination of 13cRA and CEP-701 resulted in additive to synergistic interactions in four of the five cell lines studied. Addition of 1 or 5 μM of 13cRA decreased the median (range) CEP-701 IC_{50} 1.5-fold (1.1–2.8-fold) and 1.7-fold (1.5–1.8-fold), respectively. With 10 μM 13cRA, less than 50% of cells survived when combined with various concentrations of CEP-701.

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The combination of 4HPR and CEP-701 trended toward being antagonistic, with a median (range) CI at the ED_{50} of 1.3 (1.1–1.5).

Conclusions—The combination of 13cRA and CEP-701 was additive or synergistic in a spectrum of neuroblastoma cell lines, suggesting that these agents can be potentially studied together in the setting of minimal residual disease following intensive chemoradiotherapy for children with high-risk neuroblastoma.

Keywords

Neuroblastoma; Retinoid; Lestaurtinib (CEP-701); In vitro

Introduction

Neuroblastoma is the most common extra-cranial childhood solid tumor, accounting for 8–10% of all pediatric cancers [2]. It is derived from the sympaticoadrenal lineage of the neural crest and can develop anywhere along the sympathetic chain [20]. The prognosis of children diagnosed with neuroblastoma varies widely with age, stage of tumor, biology, and histology. Children less than 1 year of age at diagnosis with a stage 1 tumor with favorable features have an estimated survival of 95% [19]. However, children diagnosed at greater than 18 months of age with stage IV tumors with unfavorable features have an estimated survival of 25–40% and experience significant short and long-term toxicities associated with intensive therapy [6].

A feature of unfavorable neuroblastomas is the expression of the neurotrophin receptor, TrkB, and its ligand, brain derived neurotrophic factor (BDNF) [24]. The BDNF/TrkB loop may up-regulate growth and promote survival of unfavorable neuroblastoma through autocrine or paracrine pathways [1, 24]. In addition, unfavorable neuroblastoma cell lines with high expression of TrkB have been shown to be more resistant to chemotherapy than neuroblastoma cell lines with lower expression of TrkB [13].

Lestaurtinib (CEP-701), a novel tyrosine kinase inhibitor, is a potent inhibitor of the Trk tyrosine kinase receptor family. Like its parent compound, CEP-751, CEP-701 is a synthetic derivative of K252a, but CEP-701 can be given orally. While CEP-701 has been shown to inhibit multiple kinases [33], its ability to inhibit TrkB makes it potentially relevant for the treatment of children with neuroblastoma. CEP-701 has been studied in a spectrum of adult cancers [4, 17, 22, 23, 28, 29, 32] and has undergone investigation in acute myelogenous leukemia (AML) because of its ability to inhibit FLT3, inducing apoptosis in FLT3/ITD-expressing cell lines and leukemic blasts [3, 16, 17, 28]. CEP-701 is currently undergoing evaluation in combination with conventional cytotoxic therapy for infants with leukemia. A phase 1 study of CEP-701 is also being conducted in children with refractory/relapsed neuroblastoma.

Isotretinoin (13cis retinoic acid, 13cRA) is a synthetic retinoid that causes differentiation and decreased proliferation of neuroblastoma cells [25, 26, 30]. When incorporated into treatment following myeloablative therapy with autologous stem cell transplantation, 13cRA increases 3-year event-free survival in children with high-risk neuroblastoma [21] and is

now a standard component of treatment for patients with high-risk neuroblastoma. Fenretinide (4HPR) is a synthetic retinoid that triggers apoptosis in neuroblastoma cells [7, 18] that has completed early phase clinical trials for patients with relapsed/refractory neuroblastoma [10, 11, 31].

We evaluated the in vitro profiles of CEP-701, 13cRA, and 4HPR when given as single agents and in combination in a panel of neuroblastoma cell lines. Our goal was to explore the integration of CEP-701 for potential clinical use following chemoradiotherapy in children with high-risk neuroblastoma. Based on prior preclinical evaluations of CEP-701 and CEP-751 [8, 9, 14, 22, 23], we hypothesized that the single-agent efficacy of CEP-701 would be related to TrkB expression status.

Materials and methods

A panel of five neuroblastoma cell lines was initially used: CHP-134, IMR-5, E6-NBLS, SH-SY5Y, and the SH-SY5Y-BR6. The SH-SY5Y-BR6 line has been engineered from SH-SY5Y to express high levels of full-length, functional TrkB receptors [13, 14]. Based on our initial results, we expanded our analysis to include three additional neuroblastoma cell lines: NLF, NLF-A, and NLF-B. The NLF-A and NLF-B lines have been engineered from NLF to express high levels of functional TrkA and TrkB levels, respectively. CHP-134, IMR-5, E6-NBLS, SH-SY5Y, and NLF cell lines were grown in RPMI-1640 containing 10% fetal bovine serum, 1% glutamine, and 1 mM oxaloacetate, 0.45 mM pyruvate, and 0.2 U/ml insulin. The SH-SY5Y-BR6, NLF-A, and NLF-B lines were grown the same media with the addition of 0.3 mg/mL G418. All cell lines were tested for mycoplasma contamination using MycoAlert® mycoplasma detection assay from Cambrex (East Rutherford, NJ). 13cRA and 4HPR were purchased from Sigma (St. Louis, MO). CEP-701 was provided by Cephalon (Frazer, PA).

Growth inhibition assays

Each cell line was studied in growth inhibition experiments using 96-well microtiter plates. Twenty-four hours after cell plating, cell lines were exposed to CEP-701, 13cRA, 4HPR or their combination for 72 h (three replicates per experiment). Cells were then washed in the appropriate media and grown for an additional 72 h. To ensure that a complete sigmoidal survival-concentration curve could be observed, the following drug concentrations were studied: CEP-701 (0.01–0.5 μM), 13cRA (0.001–200 μM), and 4HPR (0.1–10 μM). Experiments were repeated at least twice.

Survival-concentration curves were generated using the SRB assay [15, 27]. Following 72-hour drug exposure and subsequent 72-hour growth, 50 μl of cold 50% trichloroacetic acid (TCA) (4°C) was added to the wells at the liquid air interface of each well to produce a final TCA concentration of 10%. The cell culture plates were incubated for 30 min at 4°C and then washed three times with distilled water. Once plates were air dried, 100 μl of 0.4% SRB stain containing 1% acetic acid was added to each well. The plates were then incubated for 30 min at room temperature and washed with 1% acetic acid. Once the stained plates were air dried, 100 μl of 10 mM Tris Base was added to each well and plates were gently agitated for 5 min. The optical density was then read using a Molecular Devices VERSAmax

(Sunnyvale, CA) microplate reader at 520 nm. The background signal from media-alone controls was subtracted and data were normalized to untreated cells. The 50% growth inhibitory concentration (IC_{50}) was determined by fitting a four parameter logistic equation to the data:

$$\% \quad \text{survival} = \left[(E_{max} - E_{min}) / \left(1 + (X/EC_{50})^{slope} \right) \right] + E_{min}$$

where X is the drug concentration, E_{max} and E_{min} are the concentrations at which maximum and minimum cytotoxicity are observed, respectively, and EC_{50} is the concentration at which 50% of the maximum cytotoxicity is attained ($E_{min} + E_{max}$)/2. The IC_{50} was then calculated using the fitted parameters and solving for X with survival set to 50%.

Determination of synergy

The interaction between CEP-701 and 4HPR was characterized using a constant drug ratio (based on IC_{50}) and analyzed with the combination index (CI) method [5]. The CI method is based on the median effect equation, and determination of synergy using this method is independent of the mechanism of inhibition of the two drugs. This analysis yields two parameters that describe the interactions among drugs in a given combination: the combination index (CI) and the dose reduction index (DRI). The combination index is calculated by the equation:

$$CI = \sum_{n=1}^{2,3} \frac{(C)_n}{(C_x)_n} = \sum_{n=1}^{2,3} \frac{1}{(DRI)_n}$$

A CI of<1 indicates synergism, a CI of 1 indicates additive effects, and a CI of >1 indicates antagonism.

Because the IC_{50} of 13cRA is not clinically achievable, pharmacologically relevant fixed concentrations of 13cRA (previously determined to result in ≤25% growth inhibition) were added to cells exposed to CEP-701 in order to evaluate the interaction between 13cRA and CEP-701.

Results

We initially conducted our experiments in five cell lines (SH-SY5Y, SH-SY5Y-BR6, IMR-5, CHP-134, E6-NBLS). TrkA and B expression were determined using Illumina expression, semi-quantitative PCR, or TaqMan® gene expression assays. Qualitative expression of TrkA and TrkB in each cell line is presented in Table 1. Because of the finding that expression of TrkB did not appear to predict for in vitro drug sensitivity, we expanded our study to include 3 additional cell lines (NLF, NLF-A, NLF-B). The individual cytotoxicity profiles for each agent are shown in Table 1. The median (range) IC_{50} of single-agent CEP-701 across all cell lines was 0.09 (0.08–0.3) μM. The median (range) IC_{50} of 13cRA and 4HPR were 25 (2 to > 100) and 1.0 (0.4–2.4) μM, respectively.

The combination of 4HPR and CEP-701 trended toward being antagonistic, with a median (range) CI at the ED_{50} of 1.3 (1.1–1.5) (Table 1). Combination plots illustrating the negative interaction between CEP-701 and 4HPR are shown in Fig. 1.

The combination of 13cRA and CEP-701, however, resulted in additive to synergistic interactions in four of the five cell lines studied. Addition of 1 and 5 μM of 13cRA decreased the median (range) CEP-701 IC_{50} 1.5-fold (1.1–2.8-fold) and 1.7-fold (1.5–1.8-fold), respectively (Figs. 2, 3, and Supplemental Table). With 10 μM 13cRA, less than 50% of cells survived when combined with various concentrations of CEP-701. Due to the increased sensitivity of SH-SY5Y-BR6 and SH-SY5Y lines to 13cRA, 0.05, 0.1, and 0.5 μM of 13cRA was added to CEP-701. These combinations also demonstrated additive to synergistic interactions (Fig. 3).

Discussion

We evaluated the in vitro cytotoxicity of CEP-701, 13cRA, and 4HPR as single agents and when used in combination in a panel of neuroblastoma cell lines. Evaluation of CEP-701 as a single agent demonstrated that neither TrkA nor TrkB expression appeared to correlate with CEP-701 single-agent growth inhibition. While CEP-701 is a promiscuous tyrosine kinase inhibitor, its activity in neuroblastoma has been thought to be through inhibition of the Trk family of kinases. For this reason, we hypothesized that CEP-701 would have similar activity to its parent compound, CEP-751, in competitively inhibiting the ATP-binding sites of the Trk family of tyrosine kinases. Evans et al. [9] evaluated the in vivo antitumor activity of CEP-751 in xenograft models using IMR-5, CHP-134, E6-NBLS, SH-SY5Y, and SH-SY5Y-TrkB(G12) cell lines. The greatest activity of CEP-751 was seen in the SH-SY5Y-TrkB(G12) cell line, the line with the highest TrkB expression. Subsequent evaluations confirmed that activation of TrkB by BDNF in SH-SY5Y-TrkB(G12) cells was inhibited by CEP-751 in a dose-dependent fashion [8]. A recent publication by Iyer et al. [14] confirmed that CEP-701 can inhibit the phosphorylation of TrkB in the SH-SY5Y-BR6 cell line at concentrations of 10–250 nM in the presence of exogenous BDNF, however, complete inhibition of phosphorylation was not demonstrated. This study also demonstrated that there was no TrkB phosphorylation in the SH-SY5Y-BR6 line in the absence of exogenous BDNF. Therefore, one possible reason that our results did not demonstrate that activity of CEP-701 could be predicted based on expression of TrkB is that exogenous BDNF is needed in in vitro models to activate the TrkB pathway. It cannot be excluded, however, that the activity of CEP-701 on the Trk B pathway may be different than its parent compound CEP-751 or that the antitumor effects of CEP-701 in neuroblastoma are due to interactions with other tyrosine kinases.

Our analysis demonstrates complementary approaches that can be used to evaluate interaction (antagonism, additivity, synergy) of chemotherapeutic agents. We evaluated the interaction between CEP-701 and 4HPR using a fixed ratio of the compounds based on their IC_{50}. We then characterized the interaction using the CI as proposed by Chou and Talalay. Using this approach, we found that the interaction of CEP-701 and 4HPR trended toward antagonistic in all of the cell lines studied. These results are supported by the recent evaluation of the combination of CEP-701 and 4HPR in an SH-SY5Y-BR6 xenograft model

of neuroblastoma [14]. This study found that while both 4HPR and CEP-701 demonstrated activity as single agents compared to vehicle control, when combined their activity was no greater than that of CEP-701 alone.

Because the IC_{50} of 13cRA in each cell line is not clinically achievable, we used fixed concentrations of 13cRA that result in ≤25% cytotoxicity to determine the interaction between CEP-701 and 13cRA. The single-agent cytotoxicity of 13cRA presented in Table 1 demonstrates a high degree of variability in each of the cell lines studied. It should be noted that in a subset of experiments at high 13cRA concentrations, the method to accurately estimate E_{max} and hence IC_{50} is limited; these values should be interpreted with caution. Overall the addition of 13cRA to CEP-701 resulted in at least additive interactions in four of the five cell lines studied with the greatest effect seen in the cell line with the highest expression of TrkB. 13cRA likely exerts its effects on neuroblastoma cells via isomerization to all-trans retinoic acid or 9-cis retinoic acid, retinoids that have both been shown to increase the expression of TrkB [12]. Therefore, one possible mechanism for the observed synergy of these two compounds is that 13cRA up-regulates the TrkB pathway leading to increased sensitivity to CEP-701, even in the absence of exogenous BDNF. Because of the promiscuity of CEP-701, further testing of 13cRA with a potent and selective inhibitor of TrkB is needed to confirm the hypothesis that the observed favorable interaction of CEP-701 and 13cRA is via inhibition of the TrkB pathway. Nonetheless, our results suggest that the combination of CEP-701 and 13cRA may have the potential to further eliminate minimal residual disease following intensive chemoradiotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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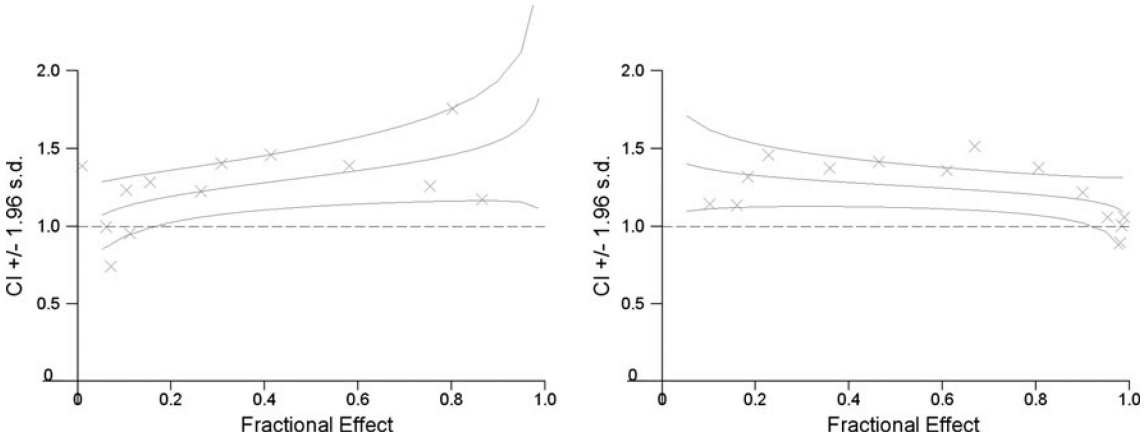

Fig. 1.

Combination index plots illustrating the interaction between CEP-701 and 4HPR in SH-SY5Y (*right*) and SH-SY5Y-BR6 (*left*) cell lines. The *points* on the graph delineate the CI for each experimental value. The *middle line* represents the estimated CI associated with the fractional effect and the *upper* and *lower lines* represent the bounds of the 95% confidence interval for the estimated CI. Both graphs demonstrate that the interaction trends toward antagonistic (CI >1)

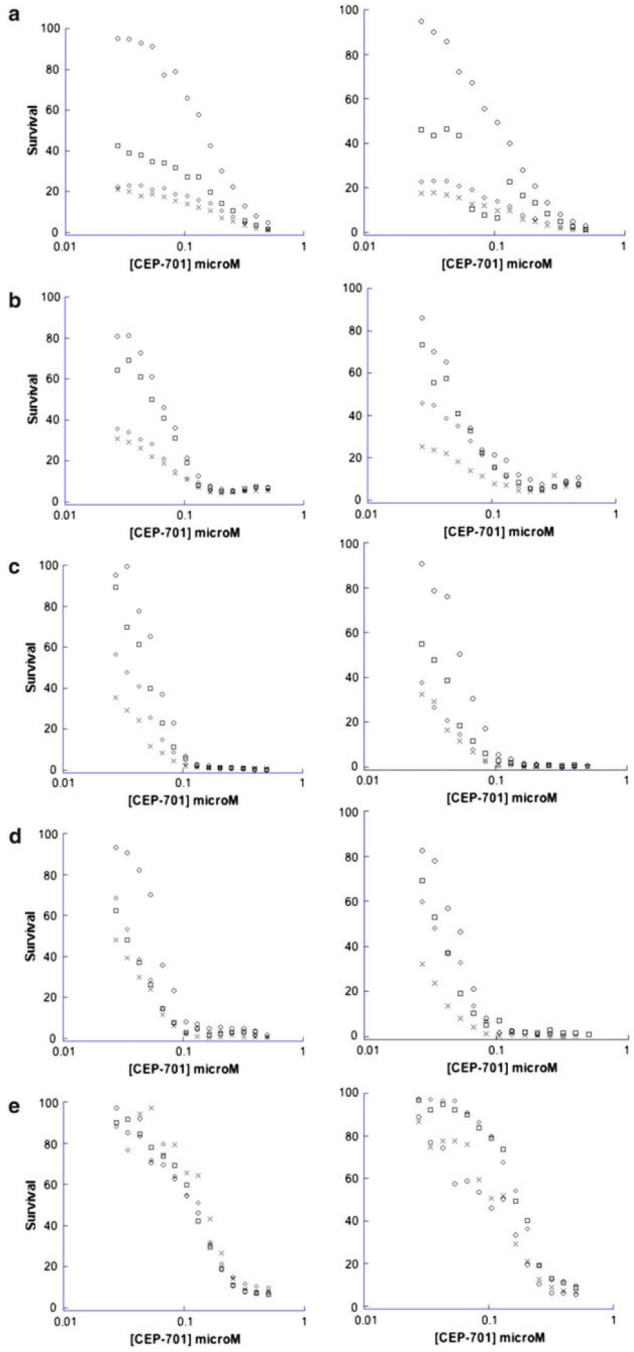

Fig. 2.

Dose-effect plots illustrating the interaction between CEP-701 and 13cRA in the **a** SH-SY5Y-BR6, **b** SH-SY5Y, **c** IMR-5, **d** CHP-134, and **e** E6-NBLS cell lines. ○ CEP-701, □ CEP-701 and 1 microM 13cRA, ◇ CEP-701 and 5 microM 13cRA, × CEP-701 and 10 microM 13cRA

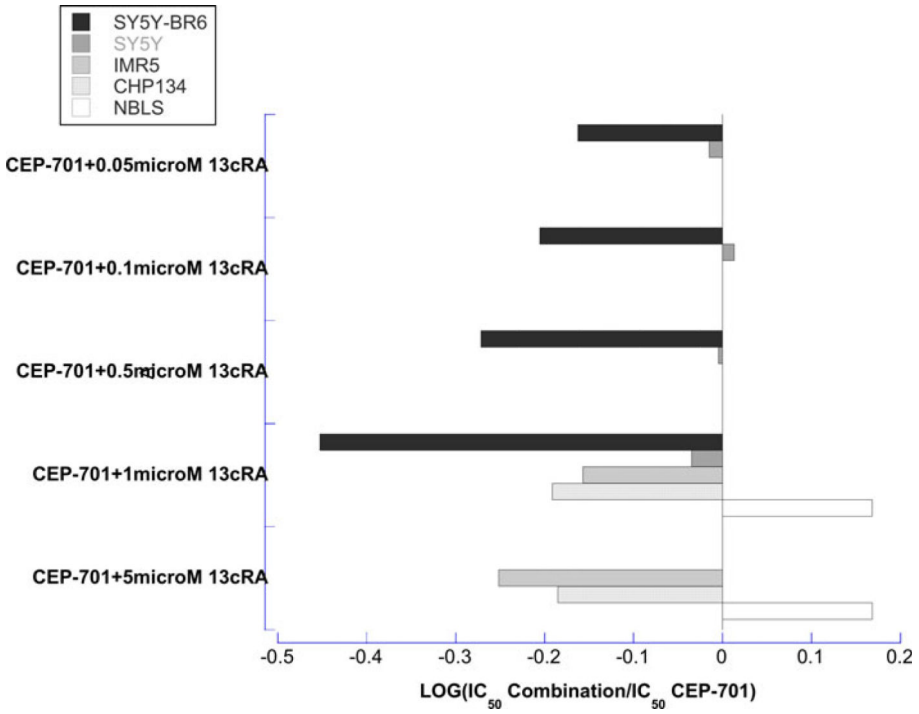

Fig. 3.

Bar graph illustrating the effect of 13cRA on the cytotoxicity (as measured by the IC_{50}) of CEP-701. In the SH-SY5Y and SH-SY5Y-BR6 lines, less than 50% of cells survived when 5 μM was added to various concentrations of CEP-701. Due to observed increased sensitivity of SH-SY5Y and SH-SY5Y-BR6 lines to 13cRA, the interaction was also assessed using 0.5, 0.1, and 0.05 μM 13cRA

Table 1

TrkA and B status for each cell line studied (Ø no expression, ⇑⇑ high expression, ⇑ moderate expression, ⇓ low expression). Single- agent IC_{50} data for CEP-701, 13cRA, and 4HPR. CI data for the CEP-701 and 4HPR combination

Cell line	Trk status	IC_{50}μM (SD)			Combination index [CI (range)] 4HPR + CEP-701
		CEP-701	13cRA	4HPR	
SH-SY5Y-BR6	Ø TrkA ⇑⇑TrkB	0.17 (0.03)	2.3 (1.2)	2.0 (0.4)	1.1 (0.9–1.3)
SH-SY5Y	ØTrkA ØTrkB	0.08 (0.02)	2.1 (1.9)	1.0 (0.3)	1.3 (1.2–1.7)
IMR-5	⇑TrkA ⇑TrkB	0.08 (0.02)	25 (13)	0.4 (0.04)	1.3 (1.2–1.4)
CHP-134	⇑TrkA ⇑TrkB	0.09 (0.03)	25 (10)	0.5 (0.1)	1.1 (1.1–1.1)
E6-NBLS	⇓TrkA ØTrkB	0.16 (0.01)	99 (10)	2.4 (0.5)	1.5 (1.3–1.7)
NLF-A	⇑⇑TrkA ØTrkB	0.3 (0.003)	>100	-	-
NLF-B	ØTrkA ⇑⇑TrkB	0.08 (0.01)	18 (4)	-	-
NLF	ØTrkA ØTrkB	0.09 (0.02)	14 (3)	-	-