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Original Articles

Entrectinib is a potent inhibitor of Trk-driven neuroblastomas in a xenograft mouse model

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ABSTRACT

Neuroblastoma (NB) is one of the most common and deadly childhood solid tumors. These tumors are characterized by clinical heterogeneity, from spontaneous regression to relentless progression, and the Trk family of neurotrophin receptors plays an important role in this heterogeneous behavior. We wanted to determine if entrectinib (RXDX-101, Ignyta, Inc.), an oral Pan-Trk, Alk and Ros1 inhibitor, was effective in our NB model. *In vitro* effects of entrectinib, either as a single agent or in combination with the chemotherapeutic agents Irinotecan (Irino) and Temozolomide (TMZ), were studied on an SH-SY5Y cell line stably transfected with TrkB. *In vivo* growth inhibition activity was studied in NB xenografts, again as a single agent or in combination with Irino-TMZ. Entrectinib significantly inhibited the growth of TrkB-expressing NB cells *in vitro*, and it significantly enhanced the growth inhibition of Irino-TMZ when used in combination. Single agent therapy resulted in significant tumor growth inhibition in animals treated with entrectinib compared to control animals [$p < 0.0001$ for event-free survival (EFS)]. Addition of entrectinib to Irino-TMZ also significantly improved the EFS of animals compared to vehicle or Irino-TMZ treated animals [$p < 0.0001$ for combination vs. control, $p = 0.0012$ for combination vs. Irino-TMZ]. We show that entrectinib inhibits growth of TrkB expressing NB cells *in vitro* and *in vivo*, and that it enhances the efficacy of conventional chemotherapy in *in vivo* models. Our data suggest that entrectinib is a potent Trk inhibitor and should be tested in clinical trials for NBs and other Trk-expressing tumors.

Introduction

Receptor tyrosine kinases (RTKs) play important roles in normal development [1], and they are frequent targets of activating mutations, rearrangements or overexpression in human cancers [2]. Thus, there is considerable interest in identifying the RTKs that contribute to tumorigenesis in specific tumors, as these present attractive targets for biologically-based therapy. Neuroblastoma (NB) is a tumor of the peripheral nervous system in children, and several RTK genes have been implicated in malignant transformation or progression of these tumors, including *ALK*, *NTRKs*, *RET*, *EGFR* and *IGFR* [reviewed in Ref. [3]]. We have focused primarily on the Trk family of neurotrophin receptors (TrkA encoded by *NTRK1*, TrkB encoded by *NTRK2*, TrkC encoded by *NTRK3*) because of the critical role they play in regulating both favorable and unfavorable clinical behavior in NBs [4,5].

TrkA is the receptor for nerve growth factor (NGF), and high TrkA expression is associated with clinically and biologically favorable tumors that have a propensity to undergo spontaneous regression or differentiation [6–11]. Conversely, TrkB is the cognate receptor for brain-derived neurotrophic factor (BDNF), and high expression of TrkB plus BDNF is found in the majority of high-risk NBs, especially those with *MYCN* amplification [8]. Tumors that co-express TrkB and BDNF are more likely to be invasive, metastatic, angiogenic and drug resistant [12–18]. TrkC is also expressed in primary NBs, but these tumors appear to be a subset of TrkA-expressing tumors [19–21]. Thus, targeting Trk receptors, especially TrkB, should be an effective therapeutic strategy in NBs [3–5]. Furthermore, Trks are activated by translocation or autocrine overexpression in a number of common pediatric and adult cancers [22,23], so a potent and selective Trk inhibitor would be of interest for the treatment of a variety of cancers.

We have shown previously that inhibition of the Trk signaling pathway with lestaurtinib (CEP-101, Cephalon, Inc.), a pan-Trk inhibitor, resulted in inhibition of growth of TrkB-expressing NB cells *in vitro* and *in vivo*, and cotreatment with lestaurtinib and

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chemotherapy resulted in enhanced anti-tumor efficacy [13,24–26]. Indeed, this agent was used in a phase 1 clinical trial, and half the patients treated at a biologically effective dose had durable clinical benefit (partial responses, stable disease) for a mean of over 10 months [27]. However, support for clinical development of this agent was discontinued after a corporate takeover. We tested additional pan-Trk inhibitors, including AZ64 (AstraZeneca, Inc.) and GNF-4256 (Genomics Institute of the Novartis Research Foundation) *in vitro* and in our NB xenograft model [28]. Both were effective inhibitors of TrkA/B/C activation in the low nanomolar range, and both inhibited growth of NB xenografts as single agents. Also, both enhanced the efficacy of chemotherapy with Irinotecan (Irino) and Temozolomide (TMZ), without additional toxicity [28,29]. However, support for clinical development of these agents was also discontinued.

In this study, we tested the efficacy of entrectinib (RXDX-101; Ignyta, Inc.), a selective pan-Trk, Alk and Ros tyrosine kinase inhibitor, to inhibit the growth of TrkB-expressing NB cells *in vitro* and *in vivo*. The compound was tested both as a single agent and in combination therapy with the relapsed NB chemotherapy regimen, Irino-TMZ. We saw significant inhibition of NB growth both *in vitro* and *in vivo* with entrectinib as a single agent. In fact, this agent was more potent than lestaurtinib, which served as a positive control for these studies. Furthermore, the combination of entrectinib with Irino-TMZ resulted in significantly increased EFS compared to the group receiving chemotherapy alone. Therefore, entrectinib is a promising agent that inhibits activated TRK receptors, and we are moving this agent forward to phase 1 clinical trials.

Materials and methods

Compounds

Entrectinib (RXDX-101, Ignyta, Inc.) is an orally available small molecule inhibitor of pan-Trk, Alk and Ros1 tyrosine kinases. It was dissolved in DMSO to obtain stocks for *in vitro* studies. For *in vivo* experiments, it was reconstituted in 0.5% methylcellulose (Sigma-Aldrich, viscosity 400 cP, 2% in H_2O) containing 1% Tween 80 at a final dosing volume of 10 ml/kg (e.g., 0.2 ml for a 20 g mouse). Entrectinib solution was stirred at RT for 30 min and then sonicated in a water bath sonicator for 20 min. This formulation was made fresh every week. Animals were dosed BID, 7 days/week at 60 mg/kg.

Temozolomide (Temodar – TMZ, Teva, 20 mg/capsule) was obtained from the pharmacy at The Children's Hospital of Philadelphia (CHOP). The compound was reconstituted in saline at a concentration of 1 mg/ml. Animals were dosed once a day PO at 7.5 mg/kg Monday through Friday of each week (except for the groups that received the compound every other week). Irinotecan (Camptosar – Irino, Novaplus, 20 mg/ml) was diluted in saline and dosed once a day PO at 0.63 mg/kg Monday through Friday of each week.

Cell lines and authentication

Parental NLF and SH-SY5Y cells were obtained from ATCC and cultured as per ATCC guidelines and instructions. Trk-null SH-SY5Y cells (ALK-mutated, F1174L) were stably transfected with TrkB (SY5Y-TrkB, clone BR6) and NLF cells (ALK-WT) stably transfected with TrkB (NLF-TrkB, clone #6). We tested the integrity and authenticity of these cell lines for endotoxins, mycoplasmas, bacterial and other viral contaminations as well as genetic variations by multiplex PCR techniques. These tests were performed on an annual basis at the cell center services facility of University of Pennsylvania. These cell lines were used for *in vitro* and *in vivo* experiments to determine the effect of entrectinib on TrkB phosphorylation. SH-SY5Y cells were used as Trk-null controls. Cells were grown in RPMI-1640 medium (Gibco) containing 10% fetal bovine serum (Cellgro), 0.4 mg/ml Penicillin/Streptomycin (Gibco), and maintained in 150 cm^3 Costar culture flasks in a humidified atmosphere of 95% air and 5% CO_2. Transfected cells were maintained in media containing 0.3 mg/ml G418 sulfate (stock solution: 50 mg/ml; Corning). Cells were harvested using 0.2% tetrasodium EDTA in phosphate buffered saline (PBS). Xenograft studies were conducted with SH-SY5Y cells stably transfected with TrkB.

Animals

Six-week-old athymic nu/nu mice were obtained from Jackson Laboratories. Mice were maintained at five per cage under humidity- and temperature-controlled conditions in a light/dark cycle that was set at 12-hr intervals. The Institutional Animal Care Committee of the Children's Hospital of Philadelphia (CHOP) Research Institute approved the animal studies described herein.

In vitro *experiments and Western blot analysis*

To determine the inhibitory effect of entrectinib on TrkB phosphorylation, cells were grown in 10 cm^3 dishes to 70–80% confluence under standard culture conditions. Cells were serum starved in 2% FBS medium for 2 hr before being exposing to different concentrations of entrectinib (10–200 nM) for 1 hr. Cells were stimulated with 100 ng/ml of the TrkB ligand, BDNF (PeproTech, Rocky Hill, NJ) for 15 minutes before total protein was harvested for analysis by Western blots. Trk expression was confirmed using anti-Phospho Trk antibody (p-Trk, Tyr-490, Cell Signaling Technology, Danvers, MA) or anti-Pan-Trk antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Downstream signaling inhibition was analyzed using anti-phospho-Akt, anti-phospho-Erk1/2 antibodies, total Akt and anti-Erk1/2 (Cell Signaling Technology, Danvers, MA), and actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used as loading control.

Sulforhodamine B (SRB) assay

Sulforhodamine B (SRB) assays were performed to determine the effect of entrectinib as a single agent and in combination with Irino-TMZ on the survival and growth of TrkB-expressing NB cells. NLF, NLF-TrkB, SY5Y or SY5Y-TrkB cells (5×10^3/well) were plated in 96 well plates, and they were exposed to drug at different concentrations (1, 5, 10, 20, 30, 50 and 100 nM of entrectinib, 1.5 μM Irino and 50 μM TMZ, respectively) for one hour followed by addition of 100 ng/ml of BDNF. Plates were harvested at 24, 48, and 72 hr following the addition of drug. The plates were processed and cell viability was analyzed using a standard SRB assay protocol [30]. All *in vitro* experiments were performed in triplicate and repeated at least 3 times.

In vivo *experiments*

For the xenograft studies, animals were injected subcutaneously in the flank with 1×10^7 SY5Y-TrkB cells in 0.1 ml of Matrigel (BD Bioscience, Palo Alto, CA). Tumors were measured 2 times per week in 3 dimensions, and the volume was calculated as follows: $[(0.523 \times L \times W \times W)/1000]$. Body weights were measured at least twice a week, and the dose of compound was adjusted accordingly. Treatment with entrectinib, Irino and TMZ started about 15–17 days after tumor inoculation when the average tumor size was 0.2 cm^3. Mice were sacrificed when tumor volume reached 3 cm^3. Tumors were harvested and flash frozen on dry ice for analysis of protein expression using Western blot. Tumor lysates were obtained using Fast Prep 24 System (MP Biomedicals) in the presence of a protease inhibitor cocktail (EMD Millipore) and phosphatase inhibitor cocktail (EMD Millipore). The following antibodies were used for the Western blot (all were from Cell Signaling Technology, unless otherwise specified): anti-TrkB (Abcam), anti-phospho-TrkB (Tyr816); anti-Trk (pan-Trk); anti-phospho-Akt (Ser473); anti-Akt; anti-phospho-p44/42 Erk (Thr202/Tyr204); anti-p44/42 Erk; anti-Phospho-PLCγ1 (Tyr783) and anti- PLCγ1. Plasma was obtained at different time points after dosing for PK/PD studies.

Pharmacokinetic studies

Entrectinib was dosed at 60 mg/kg BID for the entire duration of the study. After the final dose was given, the blood samples were drawn from 4 mice per time point via retro-orbital bleeding and collected in heparinized tubes on wet ice. The plasma was then separated by centrifugation at $1200 \times g$ for 10 minutes at 4 °C. The concentration of entrectinib (free base) was measured by LC–MS–MS. The pharmacokinetic analysis was performed using the Watson system (v. 7.4, Thermo Fisher Scientific, Waltham, MA, USA), and plotted using GraphPad Prism (mean ± SD).

Statistical analysis

Linear mixed effects model was used to test the difference in the rate of tumor volume changing over time between different groups. The model included group, day, and group-by-day interaction as fixed effects, and included a random intercept and a random slope for each mouse. A significant group-by-day interaction would suggest that the tumor volume changes at different rates for the two comparison groups. The model used the control group as the reference group and created separate group indicators and interaction terms for other groups. Appropriate contrast statements were created to compare the two groups other than control group. Event free survival (EFS) curves were estimated using Kaplan–Meier method and compared using log-rank test. Event includes death and sacrifice of mice due to tumor burden. Statistical analyses on the western blot images were performed using the Prism two-way ANOVA method followed by a Sidak post-test. Each experiment was performed at least three times either in triplicate or quadruplicate sets. Results for phosphoprotein expression were normalized against each total protein detection antibody.