Identification and preclinical characterization of AZ-23, a novel, selective, and orally bioavailable inhibitor of the Trk kinase pathway

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Abstract
Tropomyosin-related kinases (TrkA, TrkB, and TrkC) are receptor tyrosine kinases that, along with their ligands, the neurotrophins, are involved in neuronal cell growth, development, and survival. The Trk-neurotrophin pathway may also play a role in tumorigenesis through oncogenic fusions, mutations, and autocrine signaling, prompting the development of novel Trk inhibitors as agents for cancer therapy. This report describes the identification of AZ-23, a novel, potent, and selective Trk kinase inhibitor. In vitro studies with AZ-23 showed improved selectivity over previous compounds and inhibition of Trk kinase activity in cells at low nanomolar concentrations. AZ-23 showed in vivo TrkA kinase inhibition and efficacy in mice following oral administration in a TrkA-driven allograft model and significant tumor growth inhibition in a Trk-expressing xenograft model of neuroblastoma. AZ-23 represents a potent and selective Trk kinase inhibitor from a novel series with the potential for use as a treatment for cancer. [Mol Cancer Ther 2009;8(7):1818–27]

Introduction
The NTRK1-3 gene family encodes three high-affinity growth factor receptors for the neurotrophin family of soluble ligands that include nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF). The three Trk receptor isoforms, TrkA, TrkB, and TrkC, are associated with neuronal maintenance and survival during development (1, 2). Trk receptors have also been shown to be potent oncogenes with roles in malignant transformation, metastasis, and survival signaling in human tumors (3–5). Independent mechanisms of Trk pathway activation, including constitutive oncogenic fusions, autocrine signaling, and point mutations, have been described in medulloblastoma; neuroblastoma; acute myelogenous leukemia (AML); and thyroid, pancreatic, breast, lung, and prostate cancers, suggesting that the Trk pathway may be more broadly involved in carcinogenesis and tumor cell survival (6–12).

Translocations leading to TrkA or TrkC fusion proteins with constitutive kinase activity have been identified in primary human tumors. A t(12;15) (p13, q25) translocation leads to expression of a fusion protein derived from the NH2 terminus of the ETV6 transcription factor and the COOH-terminal kinase domain of TrkC (13). ETV6-TrkC fusions are oncogenic in vitro, appear to be causative in secretory breast carcinoma, and have been found in AML, congenital fibrosarcoma, and congenital mesoblastic nephroma (14–17). Additionally, constitutively active TrkA fusions occur in a subset of papillary thyroid cancers and colon carcinomas (9, 18). Autocrine signaling pathway activation involving NGF and TrkA or BDNF and TrkB have been described in subpopulations of mesothelioma and breast and ovarian cancers, and activated Trk expression has been shown to correlate with poor prognosis in these settings (19–22). Recent data also suggest a role for TrkB and BDNF expression in the survival of multiple myeloma cells (23, 24). The most clearly defined cancer linkage for the Trk/neurotrophin axis exists in neuroblastoma, where TrkB expression (along with MYCN gene amplification) is a strong predictor of aggressive tumor growth and poor prognosis (reviewed in ref. 25). Moreover, TrkB overexpression is associated with increased resistance to chemotherapy in neuroblastoma tumor cells in vitro (26, 27). In this context, it has been shown that TrkB, signaling through the phosphatidylinositol-3-kinase/AKT signaling axis, protects cancer cells from chemotherapy-induced apoptosis. Collectively, these data suggest that Trk receptor and associated ligand expression may play an important role in regulating malignant transformation and survival.

Inhibition of the Trk signal transduction pathway for therapeutic purposes has been attempted using several
strategies. Both small-molecule kinase inhibitors and monoclonal antibody-based therapeutic approaches have been developed for clinical inhibition of peripheral pain and central nervous system disorders as well as for cancer therapy. The small-molecule Trk kinase inhibitors that have been most widely described are indolocarbazole analogues and include k252a and CEP-701 (lestaurtinib). While these agents are potent Trk kinase inhibitors, they also have activity against multiple kinase targets. CEP-701 has been shown to inhibit platelet-derived growth factor receptor, Glial cell line neurotrophic factor receptor (RET), vascular endothelial growth factor receptor, and protein kinase C (PKC) and is currently in clinical trials as a Flt3 inhibitor for the treatment of AML and as a JAK2 kinase inhibitor in myeloproliferative disorders (28–32). Although multikinase inhibition may be an effective anticancer strategy in some circumstances, the likelihood of toxicity is increased. Hence, the development of selective inhibitors is needed to target specific kinases, e.g., Trks, in particular for use in the setting of combination chemotherapy.

Here, we describe the characterization of AZ-23, a selective small-molecule inhibitor of the Trk tyrosine kinase family. AZ-23 is a potent ATP-competitive inhibitor of the three Trk isoforms (A/B/C) and seems to be at least 30-fold specific for Trk in cell-based assays when tested against a wide range of other kinases. AZ-23 is orally bioavailable in rodents and effectively blocks tumor growth in both an engineered TrkA-driven allograft model and in a Trk-expressing xenograft model of neuroblastoma. AZ-23 is based on a novel chemical scaffold and represents a potent and selective Trk kinase inhibitor with potential for use in clinical settings, including the treatment of cancer.

**Materials and Methods**

**Synthesis of Compound**

AZ-23 was prepared as described previously (33).

**In vitro TrkA Kinase Assays**

The intracellular kinase domain (amino acids 441-796) of human TrkA was expressed as an NH2-terminal His6-tagged protein in Sf9 cells and purified through Ni-affinity and size-exclusion chromatography. A kinase reaction mixture (0.838 ng/mL Trk A, 9 mmol/L HEPES, 45 μg/mL...
bovine serum albumin, 10 mmol/L MnCl₂, 5 nmol/L poly EY, 0.01% Triton X-100, 70 μmol/L ATP) was incubated for 20 min at room temperature and the reaction was stopped by the addition of 30 mmol/L EDTA. The products were detected with streptavidin and phosphotyrosine-specific antibody-coated beads. Plates were read using the EnVision Multilabel Plate Reader. Compounds were also tested using the Kinase Profiler platform as per the provider’s methods (Millipore).

**Kinetic Analysis of AZ-23**

The mode of inhibition and inhibition constant (Ki) of AZ-23 against the TrkA kinase were evaluated by inhibition kinetics. A series of TrkA-catalyzed reactions were set up in HDT buffer with a fixed concentration of FAM-Blk/Lyntide

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**Figure 2.** AZ-23 potently and selectively inhibits Trk phosphorylation in cells. **A,** multiple phosphospecific cellular end point assays (ELISA-based) were used to screen for AZ-23 activity against TrkA and a range of other cellular targets. Fold selectivity versus cellular phospho-TrkA inhibition was determined (bottom). Note that MDA-MB-468 cells are PTEN null with constitutive AKT phosphorylation. Identical results are obtained in PTEN wild-type cell lines (data not shown). **B,** effect of varying concentrations of AZ-23 on ligand-induced Trk phosphorylation in NIH-3T3 cells overexpressing human TrkA, TrkB, or TrkC. Cells grown in 10% FBS were treated for 1 h with the indicated concentration of AZ-23 and subsequently stimulated with the appropriate ligand for 10 min; NGF (left), BDNF (middle), or NT-3 (right). Cell lysates were immunoblotted with both phospho-Trk and total Trk antibodies. **C,** effect of varying concentrations of AZ-23 on NGF-induced Trk phosphorylation in human K562 cells endogenously expressing TrkA. Cells grown in 10% FBS were treated for 1 h with the indicated concentration of AZ-23 and subsequently stimulated with NGF for 10 min. Cell lysates were immunoblotted with both phospho-Trk and total Trk antibodies. **D,** EC₅₀ determination of AZ-23 activity against a panel of self-Ba3 survival assays driven by the indicated target using a commercially available screen (ACD). Five distinct concentrations (run in quadruplicate) of AZ-23 were tested, ranging from 1 to 500 nmol/L. NGF, nerve growth factor; NT-3, neurotrophin 3; BDNF, brain-derived neurotrophic factor.
All EC50 data are a mean of at least three assays run in triplicate wells with a 10-point dose response.

NOTE: The indicated cell lines were dosed with AZ-23 and the viability of the cells was measured 72 h later using the CellTiter 96 AQuesous MTS system. All EC50 data are a mean of at least three assays run in triplicate wells with a 10-point dose response.

(Molecular Devices) and varied concentrations of ATP and AZ-23. The reaction progresses were monitored by Caliper LC3000 system (Caliper Life Sciences) and the initial velocity of each reaction was extracted from the corresponding reaction time course.

Cell Culture
All parental human cell lines were obtained from the American Type Culture Collection and cultured in medium with fetal bovine serum (FBS) as prescribed by the American Type Culture Collection, with the following exceptions: MCF10A cells were grown in DMEM-F12 plus 4 mmol/L L-glutamine, 20 mmol/L HEPES, 5% horse serum, 10 μg/mL insulin, 0.5 μg/mL hydrocortisone, 20 ng/mL epidermal growth factor (EGF), whereas the MCF10A-TrkA-Δ cells were grown in identical medium minus the insulin, hydrocortisone, and EGF (“factor-free medium”). TF-1 cells were grown in either “growth medium” [RPMI 1640 + 10% FBS + 2 mmol/L L-glutamine + 1.5 g/L NaHCO3 +4.5 g/L glucose + 10 mmol/L HEPES + 1 mmol/L sodium pyruvate, and 2 ng/mL recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF); R&D Systems] or growth medium minus GM-CSF (“basal medium”) for NGF-stimulated assays.

Phospho-TrkA ELISA
A MCF10A cell line was engineered to stably express a constitutively active form of human TrkA containing a 75-amino-acid extracellular domain deletion (MCF10A-TrkA-Δ) originally isolated from an AML patient (7). The phospho-TrkA ELISA was run from a modified version of a published protocol (34). The MCF10A-TrkA-Δ cells were seeded at a density of 74,000 cells per well and compound treatment was carried out in 10% serum conditions. The ELISA capture antibody was TrkA C-14 (SCBT, Santa Cruz Biotechnology) used at a 1:1,000 dilution and the detection antibody was LANCE Eu-W1024 anti-phosphotyrosine (Perkin-Elmer) used at 1:2,000. The ELISA plates were read on the Victor2 using time-resolved fluorometry, 340-nm excitation and 615-nm emission with 400-μs delay, 400-μs window, and 1,000-μs cycle.

Immunoblot Analyses for Trk
Cells were grown in six-well plates and incubated in standard culture conditions for 18 h before compound exposure. Subconfluent cells were exposed to AZ-23 at indicated concentrations for 1 h before ligand stimulation at 37°C (100 ng/mL recombinant NGF, BDNF, or NT3; R&D Systems) for 10 min. Cells were washed with PBS before direct lysis with 4× SDS loading buffer. All samples were heated to 100°C for 10 min, then centrifuged for 5 min at top speed in a microcentrifuge. After resolution on 4% to 12% NuPage Gradient Gels (Invitrogen), the resultant membranes were incubated overnight with either phospho-Trk antibody (Cell Signaling Technology at a 1:1,000 dilution) or total Trk antibody C-14 (Santa Cruz Biotechnology at a 1:1,000 dilution). The blots were then processed as per standard procedure.

MCF10 Proliferation Assay
On day 1, 90 μL/well of EGF-dependent MCF10 parental lines or TrkA-dependent MCF10A-TrkA-Δ cells were plated onto 96-well flat-bottomed plates at concentrations of 16,667 and 22,222 cells/mL, respectively, and allowed to adhere overnight at 37°C. On day 2, cells were treated with AZ-23 and incubated at 37°C for an additional 72 h. CellTiter 96 AQuesous MTS solution (20 μL; Promega) was added to each well and plates incubated for 2 h at 37°C. Absorbance was then read at 490 nm/L.

TF-1 Proliferation Assay
On day 1, exponentially growing TF-1 cells in growth medium were washed thrice with basal medium and cells were starved overnight in basal medium at 37°C. On day 2, 15,000 cells per well were plated onto 96-well flat-bottomed plates in a total volume of 80 μL basal medium per well. Cells were treated with various concentrations of AZ-23 and then incubated for an additional 72 h at 37°C in either growth or basal medium plus 100 ng/mL NGF. CellTiter 96 AQuesous MTS solution (20 μL; Promega) was added to each well and plates were incubated for 2 h at 37°C before reading.

Kinase-Driven Baf3 Survival Assays
AZ-23 was tested in a five-point dose response (0, 1, 10, 50, 250, and 500 nm) against the indicated kinases using the cellular platform provided by Advanced Cellular Dynamics, as per the provider’s methods (ACD).

Animals for In vivo Studies
Six- to 8-wk-old, athymic nude male mice (NCR-nude, Taconic Farms) were used for 3T3-TrkA-Δ in vivo studies and 6- to 8-wk-old NOD/SCID/IL2R-gamma mouse (The Jackson Laboratory) were used for SK-N-SH in vivo studies. Mice were housed and treated in accordance with guidelines of the local institutions and the Association for the Assessment and Accreditation for Laboratory Animal Care.

3T3-TrkA-Δ Phamacodynamic Assay
3T3-TrkA-Δ cells (1 × 106) were inoculated s.c. into the right flank of male athymic mice on day 1. On day 15, when tumors were ~300 mm3, the mice received compound or vehicle (0.5% hydroxy propyl-methyl cellulose/0.1%Tween 80 v/v) orally at 10 mL/kg. For pharmacokinetic/pharmacodynamic (PK-PD) analysis, blood and tumor samples were collected from each animal (two mice per group per time point) at the indicated times postdose. Plasma concentrations of AZ-23 were determined using reverse-phase high-performance liquid chromatography with mass spectrometric detection. Tumors were homogenized in 1 mL lysis buffer (20 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 1%
NP40 substitution, 1 mmol/L sodium vanadate] and spun for 10 min at 14,000 rpm. Total protein concentration was determined using bicinchoninic acid protein assay (Pierce). Eighty micrograms of supernatants were then processed in the phospho-TrkA ELISA assay as indicated above.

**3T3-TrkA-Δ Allograft Tumor Studies**

Mice bearing 3T3-TrkA-Δ tumors of ~200 mm³ in size were randomized into treatment groups of either vehicle or AZ-23 and dosed by oral gavage. Animal body weights and tumor measurements were recorded two to three times a week in the Labcat Tumor Analysis and Tracking System. For all tumor growth inhibition experiments, 10 to 15 mice per treatment group were used. A Student's t test was used to determine the P value.

**SK-N-SH Xenograft Tumor Studies**

Mice were inoculated s.c. into the right flank with 5 × 10⁷ SK-N-SH neuroblastoma tumor cells. Once tumors were palpable (~125 mm³), mice were treated twice daily by oral gavage feeding with AZ-23 at 100 mg/kg. A total of seven mice were treated in each group. Tumor measurements and mouse weights were obtained three times per week by the same investigator (L.Z.) using vernier calipers and tumor volumes were calculated. Animals were sacrificed 18 d after the start of treatment in all cases. Blood and tumor samples were collected from a cohort of animals (two mice per group per time point) at the indicated times postdose. Drug concentrations were determined through mass spectrometry as described above.

**Results**

**Identification of Novel Small-Molecule Trk Kinase Inhibitors**

High-throughput screening of internal compound collections yielded a diverse range of molecules capable of inhibiting in vitro Trk kinase activity. A medicinal chemistry program was initiated in an effort to optimize the potency and selectivity of several chemical series, resulting in focused efforts on lead compounds from the 4-aminopyrazolylpyrimidine chemistry class, exemplified by AZ-23 (Fig. 1A; ref. 33). Kinetic analysis of TrkA kinase activity in the presence of varied concentrations of both ATP and compound

![Figure 3. In vivo activity of AZ-23 in the 3T3-TrkA-Δ tumor model. A, PD-PK relationship of AZ-23 plasma concentration and phospho-TrkA inhibition. Tumor-bearing mice were given a single oral dose of AZ-23 at multiple concentrations over several experiments. At varying time points postdose (2–24 h), plasma and tumor were recovered for AZ-23 and phospho-TrkA activity quantification, respectively. The plasma concentration of AZ-23 required to inhibit 80% of tumor-resident TrkA phosphorylation (EC₈₀) was found to be 83 ng/mL. B, tumor growth inhibition following dosing of AZ-23 at the indicated concentrations once (QD) or twice (BID) daily for 4 d by oral gavage. Dosing of AZ-23 was stopped after day 17 and tumor regrowth occurred in a dosedependent manner. Vehicle treated (□), 50 mg/kg twice daily (127.6 μmol/kg; ○), 10 mg/kg twice daily (25.5 μmol/kg; △), and 50 mg/kg once daily (127.6 μmol/kg; ×). Bars, SE; P < 0.001 for all AZ-23 dose groups relative to vehicle treated. C, quantification of tumor growth inhibition. Growth delay is the time delay (in days) for the AZ-23–treated groups to return to the average vehicle tumor volume initially measured on day 1 of dosing. T/C is a ratio of tumor growth between treated and control groups, expressed in percent: T/C = (T₂ − T₁) / (C₂ − C₁) × 100, where T₁ is AZ-23 average initial tumor volume, T₂ is AZ-23 average final tumor volumes, and C₁ is vehicle average initial tumor volume and C₂ is vehicle average final tumor volume. Log cell kill (LCK) is the growth delay (GD) normalized to the tumor doubling time of the vehicle group where LCK = GD / (3.32 × TD).**
showed AZ-23 as an ATP-competitive, tight-binding kinase inhibitor of TrkA with a $K_i$ of 0.84 ± 0.19 nmol/L (Fig. 1B). In vitro selectivity profiling across a broad panel of 177 kinases (Millipore’s Kinase Profiler) showed that 500 nmol/L AZ-23 significantly inhibited (>75% inhibition) only a small subset of these enzymes (Supplementary Table S1).3 Sensitivity kinases identified through this single concentration screening were further evaluated and IC$_{50}$ values were determined. These data confirmed at least 10-fold specificity for TrkA versus fibroblast growth factor receptor 1 (FGFR1) and 25-fold or higher selectivity versus all other kinases tested (Fig. 1C).

**AZ-23 Potently and Selectively Inhibits Trk Receptor Phosphorylation in Cells**

The cellular potency of AZ-23 against Trk phosphorylation was determined using a phospho-TrkA ELISA assay. A stably transfected human MCF10A cell line was engineered to express a constitutively active form of human TrkA containing a 75-amino-acid extracellular deletion (MCF10A-TrkAΔ) originally isolated from an AML patient (7). A cellular EC$_{50}$ value of 1.2 ± 0.7 nmol/L was determined for AZ-23, a result corroborated through immunoblotting for phosphorylated TrkA protein (Fig. 2A, top).

To test the activity of AZ-23 on wild-type forms of Trk receptors, the cell-based activity of AZ-23 was determined against the three Trk isoforms. Stably transfected murine 3T3 fibroblast cell lines were generated expressing wild-type, full-length forms of human TrkA, TrkB, or TrkC. AZ-23 was found to completely inhibit NGF-induced TrkA phosphorylation (Fig. 2B) in 10% serum conditions at concentrations of ≥5 nmol/L, with a similar profile seen for ligand-induced TrkB and TrkC phosphorylation, indicating AZ-23 as an equipotent inhibitor of all three Trk receptor isoforms in cells. AZ-23 was also tested for inhibition of NGF-induced TrkA phosphorylation in the endogenously expressing K562 cancer cell line. As shown in Fig. 2C, ligand-stimulated Trk activity was fully inhibited by AZ-23 at subnanomolar concentrations in K562 cells.

**Additional Cell-based Kinase Selectivity Assays**

Additional cell-based kinase selectivity assays were done to further characterize AZ-23. Based on the biochemical activity profile detailed in Fig. 1C, functional follow-up studies were first focused on quantifying the cellular activity of AZ-23 against the FGFR, Flt3, Ret, and Lck kinases. AZ-23 was tested in a five-point dose response against these kinases (alongside of TrkA, TrkB, and TrkC for comparison) using a commercially available profiling service (Advanced Cellular Dynamics). This cell panel uses engineered Baf3 cells where cell survival is dependent on maintenance of the activity of an introduced kinase. As can be seen in Fig. 2D, AZ-23 potently inhibited Trk-mediated survival (EC$_{50}$ ∼2 nmol/L) while having at least a 25-fold window of selectivity versus the other tested assays. In addition to this downstream, phenotypic readout of cell survival, the profiling of AZ-23 was extended to examine the potential modulation of eight specific cellular kinases using phosphodirected end points. Again, a substantial degree of cell-based selectivity was indicated (Fig. 2A, bottom). These data, gathered through the testing of AZ-23 in engineered cell lines, indicate the compound shows the most evident (non-Trk related) cellular activity against FGFR family kinases. We further evaluated this FGFR activity in a tube formation assay where addition of exogenous β-FGF ligand, acting through endogenous FGFR1+2 receptors of endothelial
cells, drives tube formation in vitro. AZ-23 showed an EC$_{50}$ of $>$500 nmol/L in this FGFR-driven tube assay, whereas PD173034, a selective and potent FGFR kinase inhibitor (IC$_{50}$ $\sim$ 4 nmol/L), was substantially more active (see Supplementary Table S2; ref. 35).$^3$ Taken together, these data provide evidence in support of AZ-23 as a Trk selective kinase inhibitor in cell-based assays at doses that are likely to be physiologically relevant.

**AZ-23 Inhibits Trk-Dependent Survival in MCF10A-TrkA-$\Delta$ and TF-1 Cell Lines**

To further characterize the effects of AZ-23 on cell viability, a cell-based in vitro assay was developed using engineered MCF10A-TrkA-$\Delta$ and MCF10A parental cell lines. MCF10A cells require EGF addition to the medium to enable growth, whereas the MCF10A TrkA-$\Delta$-transfected cells are EGF independent due to the constitutive TrkA signaling. This system provides the opportunity to address compound selectivity in a near-isogenic cellular background. As shown in Table 1, AZ-23 was found to potentely inhibit Trk-dependent survival in the MCF10A-TrkA-$\Delta$ cells (EC$_{50}$ 1.51 nmol/L) with a >1,600-fold window over EGF-driven survival in the parental MCF10A cells (EC$_{50}$ 2,430 nmol/L). A similar selectivity assay was done using the erythroid leukemia TF-1 cell line. TF-1 cells were originally derived from a patient with AML and express endogenous levels of wild-type TrkA. We and other groups have shown that whereas growth of TF-1 cells in culture is typically dependent on exogenous GM-CSF, NGF can substitute for GM-CSF and subsequently drive TF-1 proliferation to a similar degree (36). Using this assay, AZ-23 was found to inhibit NGF-mediated survival (EC$_{50}$ 1.01 nmol/L), a result similar to that seen in the engineered MCF10A-TrkA-$\Delta$ cells (Table 1). AZ-23 had no effect on GM-CSF driven proliferation at concentrations up to 1 $\mu$mol/L. Together, these data suggest that AZ-23 is a potent and selective inhibitor of survival in cell lines whose growth is mediated by activation through the Trk-NGF kinase pathway.

**AZ-23 Inhibits TrkA Phosphorylation in a Murine Pharmacodynamic Assay**

To determine the in vitro effects of AZ-23 and evaluate time- and dose-dependent effects on pTrkA inhibition, a murine pharmacodynamic model was developed. The constitutively active form of TrkA described above was used to transform 3T3 cells (3T3-TrkA-$\Delta$) and s.c. injection of these cells led to tumor formation in nude mice. Tumor-bearing mice were given a single, oral dose of compound and individual mice were sacrificed at various time points postdose (2, 6, 16, or 24 hours). Tumors were excised and homogenized and the resulting tumor lysates were analyzed using an ELISA for pTrkA. Plasma samples were also collected at the time of tumor collection and drug concentrations were determined. From these studies, the relationship between AZ-23 plasma concentration and pTrkA inhibition was determined and represented as drug plasma concentration required to reach 80% intratumoral p-TrkA inhibition (pTrkA EC$_{80}$), a value found to be $\sim$83 ng/mL ($\sim$210 nmol/L total, $\sim$3.7 nmol/L free; Fig. 3A).

**AZ-23 Shows Potent In vivo Activity in TrkA-Driven Allograft Models**

The in vivo efficacy of AZ-23 was initially determined using the 3T3-TrkA-$\Delta$ allograft model. In these studies, nude mice were s.c. implanted with 3T3-TrkA-$\Delta$ cells and mice were randomized into treatment groups once tumors reached an average size of $\sim$200 mm$^3$. Mice were dosed once or twice daily, by oral gavage, for $\sim$4 days at 10 or 50 mg/kg. These doses were chosen based on extended pharmacodynamic studies showing that a single oral dose of 50 mg/kg AZ-23 maintained a $>$80% suppression of TrkA activity for at least 16 hours with recovery of activity to $\sim$50% of control by 24 hours postdose. As seen in Fig. 3B and C, statistically significant, dose-dependent inhibition of tumor growth and growth delay after treatment cessation was observed. All doses/schedules of AZ-23 caused regressions during treatment. At the higher dose level, both schedules caused tumor regressions in $\sim$80% of animals and prevented tumor regrowth after the last dose for $\sim$3 to 4 days. AZ-23 was well tolerated at doses up to 100 mg/kg twice daily for 14 days with minimal body weight loss (<5%) seen during the dosing period in all treatment groups, including vehicle, and no signs of clinical distress. At high doses (50–100 mg/kg, twice daily), some reversible weight gain was observed.

**AZ-23 Potently Inhibits SK-N-SH Neuroblastoma Xenograft Tumors In vivo**

The well-documented clinical association between pediatric neuroblastomas and Trk pathway signaling prompted us to evaluate AZ-23 in a more disease-relevant model. SK-N-SH neuroblastoma cells were initially evaluated in vitro to confirm Trk receptor expression and selective sensitivity to AZ-23 (Supplementary Fig. S3).$^3$ NOD/SCID/IL2R-gamma immunocompromised mice were injected s.c. with SK-N-SH cells and palpable tumors were allowed to develop. Mice were then dosed by oral gavage twice daily with either vehicle alone or 100 mg/kg AZ-23. Mice were sacrificed and tumors were harvested 12 days after the start of treatment or when tumor growth reached institutional limits. AZ-23 treatment resulted in significantly reduced tumor growth [$P < 0.01$, % treated versus control (%T/C) = 2.6; Fig. 4A] and significantly reduced final tumor weight ($P < 0.01$; Fig. 4B), with no relevant body weight loss. Plasma and intratumoral concentrations of AZ-23 were determined in a satellite cohort of animals at 1, 2, and 6 hours following the final dose of AZ-23. Time points were chosen based on previous PK studies in non–tumor-bearing mice and were meant to ensure capture of C$_{max}$ drug levels ($\sim$1 hour). After adjusting for predicted levels of protein binding, the intratumoral, free (unbound) concentration of AZ-23 was found to significantly exceed the measured cellular TrkA EC$_{50}$ of $\sim$1.2 nmol/L at least through 6 hours postdose (Fig. 4C). Importantly, even at approximately C$_{max}$ (1–2 hours), tumor levels of AZ-23 never significantly exceeded the cellular EC$_{50}$ of AZ-23 against FGFR1/2 ($\sim$40–50 nmol/L, dependent on assay), suggesting that the antitumor effects of AZ-23 in this SK-N-SH tumor model are not mediated by FGFR inhibition or inhibition of other kinases that have been tested.

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Discussion

The Trk-neurotrophin pathway is an example of an increasing number of growth factor signaling cascades with a range of cellular functions dependent on molecular, cellular, and disease context. The involvement of various Trk pathway components in neuronal cell survival and signaling is well defined. However, despite the fact that NGF was originally purified from a sarcoma and that TrkA was one of the first transforming oncoproteins identified, only recently has the potential for Trk kinase inhibition as a targeted approach to cancer treatment been seriously considered (18, 37).

Similar to EGFR, platelet-derived growth factor receptor, and FGFR, the Trk receptor tyrosine kinases have been found to become activated in tumors through three primary mechanisms: oncogenic fusions/translocations, mutational activation, and autocrine/paracrine pathway signaling. Expression studies (RNA and protein) of clinical tissue have shown the presence of both Trk receptors and their corresponding ligands in tumor types of diverse origin, including breast, ovarian, neuroblastoma, and lung. The use of phosphospecific Trk antibodies has shown that the receptors are activated in a majority of some of these tumors and that their presence on specific tumors correlates with poor patient survival. Apart from the coexpression of ligand and receptor, molecular analyses of the tumor-resident NTRK genes have documented alternative paths to kinase activation and phosphorylation. Activating genetic translocations involving the linkage of NTRK1 and NTRK3 to various partner proteins have been described in several cancers, most notably in thyroid and secretory breast carcinomas. A growing list of intriguing point mutations in the Trk genes have also been recently reported in primary tumors of various origin (5, 6). Whereas the functional consequences of these mutations have yet to be elucidated in most cases, the localization of many of the mutations to key residues of the kinase domain suggests that they could be activating in nature.

As summarized above, the data accumulated over the past decade have clearly established Trk receptors as viable targets for anticancer therapeutic intervention. Small-molecular-weight kinase inhibitors offer the most straightforward approach to clinical targeting of the Trk enzymes and the most advanced agent of this type is Cephalon’s CEP-701 (Lestaurtinib). Preclinically, CEP-701 is a potent pan-Trk kinase inhibitor that has shown activity against a range of in vitro models, including prostate, pancreatic, and neuroblastoma xenografts, prompting further investigation in clinical trials. In addition to Trk, however, Lestaurtinib displays multiple pharmacologic activities at clinically relevant doses. Whereas it remains active in trials as a “multitargeted” kinase inhibitor, these are in clinical settings where Trk kinases are not thought to be the key drivers of disease (Flt3+ AML and Jak2 mutant MPD). Therefore, the development of potent and selective Trk kinase inhibitors remains critical to adequately test the role of the Trk pathway in the development and progression of cancer.

This article describes the discovery and pharmacologic characterization of AZ-23, a novel, potent, and selective Trk kinase inhibitor. AZ-23 inhibits the three Trk isoforms (A/B/C) at low nanomolar concentrations in cellular assays but shows significant selectivity versus a wide range of other kinases. In vitro screening of AZ-23 against a large panel of kinases revealed only minimal activity. Moreover, the kinase inhibitory profile of AZ-23 was found to be distinct from that of Lestaurtinib and other indolocarbazole analogues with, for example, no detectable activity against platelet-derived growth factor receptor or any of the FcR isoforms, suggesting likely differences in clinical activity and toxicity. Because in vitro enzyme activity does not always correlate with cellular potency, extensive profiling was done in cellular assays to examine both specific kinases of interest as well as more general measures of cell selectivity. These latter assays have the potential to reflect diverse pharmacology of the compound, not just cross-kinase liability. AZ-23 showed little activity in the TF-1 and MCF10A Trk-independent cell growth assays even at compound concentrations 1,000-fold above that required for complete inhibition of Trk receptor phosphorylation. In fact, more than 50 cancer cell lines have been profiled for their sensitivity to AZ-23 and the vast majority have been found to be relatively insensitive to the compound. These results are consistent with the observation that the majority of the cell lines have little or no detectable Trk receptor expression (data not shown). Although AZ-23 is a substantially selective agent, it is not an absolutely specific inhibitor of the Trk receptors. It carries activity against other kinase enzymes, including the FGF and Flt3 receptor tyrosine kinases, which may or may not represent important off-target activities. Profiling of AZ-23 in both engineered and native cellular systems was completed in an attempt to quantify these additional activities. The combined data show that there is a substantial window of selectivity for Trk versus other evaluated kinases.

The cell potency, selectivity, and rodent pharmacokinetic properties of AZ-23 prompted selection of this compound for further testing in animal models. Activity against the TrkA receptor in vivo was thoroughly evaluated using the 3T3-TrkA-Δ tumor allografts, which express high levels of constitutively active TrkA. The advantage of this model is that it provides a robust, quantitative ELISA-based measure of phosphorylated TrkA in vivo, allowing for rapid PD evaluation of a series of compounds. Extended PD studies showed that a single 50 mg/kg oral dose of AZ-23 was sufficient to ensure plasma concentrations in excess of the phospho-Trk EC80 of 83 ng/mL (∼3-4 nmol/L free drug) for at least 16 hours, suggesting that AZ-23 may be amenable to a once or twice daily dosing regimen. Tumor growth inhibition studies first using the same 3T3-TrkA-Δ allograft model were evaluated using a range of AZ-23 dosages. All dosing schedules resulted in tumor regressions during the 4-day dosing period to the point where no palpable tumor remained at the end of day 4. Drug cessation resulted in a dose-dependent recovery of the tumors. Notably, despite PD data indicating that a single 50 mg/kg dose would likely not supply 24-hour plasma coverage above the EC80 level, the tumors regressed.
These data suggest that continuous dosing and sustained Trk inhibition may not be required for antitumor activity in vivo. Dosing of AZ-23 to mice twice daily for 14 days, even at doses up to 100 mg/kg, was well tolerated with no weight loss or other clinical signs of distress. Interestingly, high doses of AZ-23 (50 and 100 mg/kg) caused reversible weight gain in mice. Although there are reports implicating the TrkB axis in hyperphagia where inhibition results in overeating and obesity (38–40), whether AZ-23 im-pinges directly on this axis have not thus far been inves-tigated further.

The 313-TrkA-Δ allograft is an engineered subcutaneous model designed to be dependent on TrkA signaling and is therefore unlikely to represent the true molecular context of Trk in human disease. To assess AZ-23 activity in a more disease-relevant in vivo model, we evaluated the compound in SK-N-SH xenografts, a neuroblastoma model reported to endogenously express functional Trk receptors (41–43). Before in vivo experimentation, the SK-N-SH cell line was char-acterized in our hands for both Trk expression and for in vitro sensitivity to AZ-23 (Supplementary Fig. S3). Upon oral dosing to tumor-bearing animals, AZ-23 was found to significantly reduce SK-N-SH xenograft growth and final tumor weight, demonstrating the potential efficacy of Trk inhibition in a neuroblastoma setting. PK determination confirmed the presence of intratumoral AZ-23 at levels far above those required for Trk inhibition in cellular assays. Even 6 hours after oral dosing, free concentrations of AZ-23 were, on average, 12-fold above the cellular EC50, suggesting that the twice-daily dosing regimen was likely sufficient to allow for near-continuous inhibition of Trk kin-ase activity. Attempts were made to directly measure Trk phosphorylation in SK-N-SH cells (in vivo and in vitro) but we were unable to unequivocally show kinase modulation. Possible explanations for this inconsistency may be due to the relatively low levels of Trk protein and sensitivity of detec-tion of our antibody reagents or that the Trk phosphorylation site may be rapidly turned over, resulting in only transient signaling events. Nonetheless, several lines of evidence indicate that the antitumor activity of AZ-23 in SK-N-SH xenografts is mediated through selective Trk inhibi-tion. The extensive cellular profiling of AZ-23 shows that the compound has a substantial window of selectivity ver-sus other “off-target” kinases. Moreover, the tumor levels of AZ-23 achieved in the xenograft studies were likely insuffi-cient to inhibit non-Trk kinases for any biologically relevant length of time. For example, FGFFR seems to be the non-Trk kinase most sensitive to AZ-23, but tumor drug levels never significantly surpassed the cellular EC50 levels for FGFR in-hibition. That, combined with the inability of the anti-FGFR compound PD173074 to inhibit SK-N-SH growth in vitro, suggest that the antitumor effects of AZ-23 are Trk me-diated. With that said, limited off-target effects of AZ-23 are possible and further study of these pathways is still warranted.

AZ-23 is a novel and potent small-molecule Trk inhibitor with a selectivity profile distinct from other reported agents. Whereas clinical studies with multitargeted kinase inhibi-tors that can block Trk kinase activity have been described, the work described here with AZ-23 suggests that selective Trk inhibition may be sufficient to inhibit tumor growth in in vivo systems. A growing body of evidence suggests that the Trk/neurotrophin axis may play a broader role in tu-morigenesis than originally appreciated. AZ-23 represents a novel agent with the potential for therapeutic utility in neuroblastoma and multiple other cancer indications.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Identification and preclinical characterization of AZ-23, a novel, selective, and orally bioavailable inhibitor of the Trk kinase pathway

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