Entrectinib, a Pan–TRK, ROS1, and ALK Inhibitor with Activity in Multiple Molecularly Defined Cancer Indications

Elena Ardini1, Maria Menichincheri1, Patrizia Banfi1, Roberta Bosotti1, Cristina De Ponti1, Romana Pulci2, Dario Ballinari1, Marina Ciomei1, Gemma Texido1, Anna Degrassi1, Nilla Avanzi1, Nadia Amboldi1, Maria Beatrice Saccardo1, Daniele Casero1, Paolo Orsini1, Tiziano Bandiera1, Luca Mologni3, David Anderson4, Ge Wei4, Jason Harris4, Jean-Michel Vernier4, Gang Li4, Eduard Felder1, Daniele Donati1, Antonella Isacchi1, Enrico Pesenti2, Paola Magnaghi1, and Arturo Galvani1

Abstract

Activated ALK and ROS1 tyrosine kinases, resulting from chromosomal rearrangements, occur in a subset of non–small cell lung cancers (NSCLC) as well as other tumor types and their oncogenic relevance as actionable targets has been demonstrated by the efficacy of selective kinase inhibitors such as crizotinib, ceritinib, and alectinib. More recently, low-frequency rearrangements of TRK kinases have been described in NSCLC, colorectal carcinoma, glioblastoma, and Spitzoid melanoma. Entrectinib, whose discovery and preclinical characterization are reported herein, is a novel, potent inhibitor of ALK, ROS1, and, importantly, of TRK family kinases, which shows promise for therapy of tumors bearing oncogenic forms of these proteins. Proliferation profiling against over 200 human tumor cell lines revealed that entrectinib is exquisitely potent in vitro against lines that are dependent on the drug’s pharmacologic targets. Oral administration of entrectinib to tumor-bearing mice induced regression in relevant human xenograft tumors, including the TRKA-dependent colorectal carcinoma KM12, ROS1-driven tumors, and several ALK-dependent models of different tissue origins, including a model of brain-localized lung cancer metastasis. Entrectinib is currently showing great promise in phase I/II clinical trials, including the first documented objective responses to a TRK inhibitor in colorectal carcinoma and in NSCLC. The drug is, thus, potentially suited to the therapy of several molecularly defined cancer settings, especially that of TRK-dependent tumors, for which no approved drugs are currently available. Mol Cancer Ther; 15(4); 628–39. ©2016 AACR.

Introduction

In 2007, two independent studies reported that the EML4 and ALK genes were rearranged in a small subset of lung cancers to produce the EML4–ALK fusion protein, bearing an activated ALK kinase domain (1,2). Even though the occurrence of this event was restricted to 3% to 7% of non–small cell lung cancer (NSCLC) patients, this finding was highly significant because it represented the first description of a recurring gene translocation leading to the generation of an oncogenic kinase fusion protein in a major epithelial malignancy. Thus ALK, whose oncogenic role in the niche indication of anaplastic large cell lymphoma (ALCL) had long been established (3) but which had been largely overlooked as a drug target, suddenly stirred strong interest within the pharmaceutical industry, spurring the clinical development of several ALK kinase inhibitors.

A similar story is also unfolding for TRKA, the high-affinity receptor for nerve growth factor (NGF) that, together with the highly related TRKB and TRKC, constitutes the TRK subfamily of receptor tyrosine kinases (RTK). Whereas the physiologic role of TRKA is relatively well elucidated (4), its involvement in neoplastic transformation and tumor progression was until very recently limited to identification of rearrangements in papillary thyroid carcinoma (PTC), a tumor type characterized by a relatively good prognosis and reports of activation due to presence of putative autocrine loops in neuroblastoma, prostate, pancreatic, and breast cancer (5–10). However, chromosomal rearrangements involving the NTRK1 gene, resulting in the expression of different TRKA fusion proteins were recently reported by Vaishnavi and colleagues (11) in NSCLC, along with robust preclinical demonstration of the oncogenic potential of the predicted chimeric proteins. In addition, we very recently reported the presence of the recurring rearrangement TPM3-NTRK1 in colorectal carcinoma patients, together with preclinical validation data supporting the role of TPM3–TRKA fusion protein as a driver for proliferation and survival of TRKA-positive tumors (12).
Although an accurate estimation of the frequency of translocation events involving TRKA in either NSCLC or colorectal carcinoma is not yet available due to the low number of cases reported to date, the identification of additional chromosomal rearrangements involving NTRK1 as well as NTRK2 and NTRK3, the genes which respectively encode the TRKA, TRKB, and TRKC proteins, in further tumor types (14–17). These findings provide a strong rationale for the development of TRK kinase inhibitors in several distinct selected patient populations.

An additional RTK recently identified to be involved in recurring gene rearrangements in NSCLC is ROS1, an orphan receptor whose physiologic functions are still poorly understood (18). ROS1-positive patients, representing approximately 1% of NSCLC cases, tend to possess typical clinicopathologic features similar to those described for ALK-positive NSCLC patients (18,19).

The identification of recurring chromosomal rearrangements that result in the constitutive activation of ALK, TRKA, and ROS1 kinases in different clinical indications, together with the preclinical validation of the driving role of these oncogenes in the tumors, has led to the clinical exploration of inhibitors of these kinases as novel therapeutic opportunities. The clinical proof of concept that the EML4–ALK fusion protein found in NSCLC is indeed a driver oncogene in this disease was achieved in a very short time after the initial description of this aberration. A phase I/II study of crizotinib in selected ALK-positive NSCLC patients demonstrated striking clinical efficacy of the drug in this subset, with a 60% objective response rate, resulting in accelerated approval of crizotinib in 2011 by the FDA for the treatment of ALK-positive NSCLC patients (20). However, the observation that about one third of ALK-positive NSCLC patients progresses after initial clinical response to the drug due to the acquisition of secondary mutations (21–23), together with the apparently poor ability of crizotinib to reach effective concentrations beyond the blood–brain barrier (BBB; ref. 24), have prompted the clinical development of several “second-generation” ALK inhibitors, such as ceritinib (25) and alectinib (26).

Interestingly, some of the ALK inhibitors mentioned above, most notably ceritinib itself, are also active against ROS1 (27). On the basis of this cross-reactivity and supported by the preclinical activity observed in ROS1-driven tumor models, a total of 50 ROS1-positive NSCLC patients were enrolled in an expansion cohort of the phase I study of crizotinib. The results of this study showed marked antitumor activity in this patient population and prompted the FDA to assign breakthrough therapy designation for crizotinib to support its intensive clinical development in ROS1-positive NSCLC (19,28).

Although it has long been known that TRKA is of oncogenic relevance in PTC (5), there are as of today very few selective inhibitors of this kinase in clinical development. The recent description of recurring rearrangements of the NTRK1 gene, which encodes this kinase, in subsets of major indications such as NSCLC, colorectal carcinoma together with the identification of NTRK2 and NTRK3 rearrangements in glial tumors and inflammatory breast cancer, respectively, and perhaps additional tumor types (11,12,14,16,17), has highlighted the relevance of these kinases as pharmacologic targets and the clinical need for effective inhibitors.

Here, we disclose the structure and preclinical activity profile of entrectinib (RXDX-101, also formerly known as NMS-6628, NMS-0191372), a potent, orally available inhibitor of the TRK kinases invented and initially developed at NMS, and report the characterization of its biologic activity in TRKA-driven tumor models. The studies described herein provide the scientific rationale supporting the enrollment of patients harboring TRKA-dependent tumors in clinical trials of entrectinib, which are currently ongoing (EudraCT Number:2012-000148-88, NCT02097810; refs. 29–31). Being also a potent ROS1 and ALK inhibitor, entrectinib additionally possesses excellent in vitro and in vivo antitumor activity in models derived from different tumor types that are variously dependent upon ROS1 or ALK fusion proteins, supporting the rationale for exploring its clinical activity in ROS1- and ALK-dependent tumor settings.

Materials and Methods

Compound

Entrectinib was synthesized at Nerviano Medical Sciences srl as previously reported (WO2009/013126). Crizotinib was purchased from Selleck Chemicals.

Kinase biochemical profiling

Evaluation of the selectivity of inhibitors (IC50s) against a panel of kinases was performed using a radiometric assay format as previously described (32). All assays were performed in house, with the exception of TRKB and TRKC, for which IC50s were extrapolated from percentage inhibition values at 10 and 100 nmol/L of inhibitor in duplicate obtained using the SelectScreen Kinase Profiling Services from Life Technologies using the equation: IC50 extrapol = ([H] × 100/% inhibition) − [I], with the assumption that Hill Slope = 1. The IC50 value reported in Table 1 is the average of the IC50s calculated at each concentration.

Cell culture and cell proliferation analysis

Human cancer cell lines were obtained from the ATCC, ECACC, Interlab Cell Line Collection (ICLC) and from NCI tumor cell line repository (see Supplementary Table S1). Cells were maintained in the media recommended by the suppliers, in a humidified 37°C incubator with 5% CO2. The identity of all cell lines used in this study was verified using DNA fingerprinting technology (AmpFISTR Identifier Plus PCR Amplification kit, Applied Biosystems; ref. 33). Oncogene-driven Ba/F3 cells were generated as detailed in Supplementary Data. The antiproliferative activity of test compounds and the effect of entrectinib on cell cycle were evaluated as previously described (12).

| Table 1. Enzymatic profile of entrectinib against a panel of selected kinases |
|-----------------------------|------------------|-----------------|------------------|
| **Kinase**                  | **IC50 (nmol/L)**| **IC50 (nmol/L)**|
| TRKA                       | 1                | 122              |
| TRKB                       | 3                | 140              |
| TRKC                       | 5                | 164              |
| ROS1                       | 7                | 195              |
| ALK                        | 12               | 209              |
| JAK2                       | 40               | 215              |
| ACK1                       | 70               | 349              |
| JAK1                       | 112              | 395              |

NOTE: IC50 > 1 μmol/L: FGFR1, VEGFR2, VEGFR3, LCK, KIT, AUR1, ABL, PKCß, CDK2/CyclA, SYK.

IC50 > 10 μmol/L: AKT1, CDC7, CHK1, CK2, EEF2K, EGFR1, ERK2, GSK3β, IKK2, MAPKAPK2, MELK, MET, MPS1, MST4, NEK6, p53, PAK4, PDGFRβ, PDK1, PERK, PKM1, PKAα, PLK1, SULT1, ZAP70.
Western blot analysis

Cellular mechanism of action of entrectinib was investigated as described (12). The following antibodies were used for Western blot analysis: Phospho-TRKA-Tyr490 (#625725) and TRKA (#PC31) from Calbiochem, ALK (#35-4300) from Invitrogen, Phospho-ALK-Tyr1604 (#3341), Phospho-ROS1-Tyr2274 (#3078), ROS1 (#3266), Phospho-PLCγ1-Tyr783 (#2821), PLCγ1 (#2822), Phospho-STAT3-Tyr705 (#9131), Phospho-AKT-Ser473 (#4060), AKT (#9272), Phospho-ALK-Tyr1604 (#3341), Phospho-ROS1-Tyr1278 (#9549), and PARP (#9542) from Cell Signaling Technology, STAT3 (#610189), p21 (#556431) and p27 (#610242) from BD Biosciences, actin (#A5316) from SIGMA, GAPDH (#sc-25778) from Santa Cruz Biotechnology.

Efficacy studies and ex vivo target modulation in xenograft models

All procedures adopted for housing and handling of animals were in strict compliance with Italian and European guidelines for Laboratory Animal Welfare. For epithelial models, a total of 10^7 NCI-H2228 NSCLC cells or 5 x 10^5 KM-12 colorectal carcinoma cells were transplanted subcutaneously in athymic nu/nu mice (Harlan). For the ALCL model, a total of 10^7 Karpas-299 cells were transplanted subcutaneously in SCID mice (Harlan) previously pre-treated with total body irradiation of 2 Gy. For the ROS1 model, 5 x 10^6 Ba/F3-TEL-ROS1 cells were transplanted subcutaneously in SCID mice (Harlan). Mice bearing a minimal tumor mass (150–250 mm^3) were randomized into vehicle and treated groups made of 6 to 7 animals each. Oral treatments started the day after randomization, with different doses as described. Tumor dimensions were measured regularly using Vernier calipers and tumor volume was calculated according to the following formula: \( V = \frac{1}{2} \times \text{length} \times \text{width}^2 \). The percentage of tumor inhibition (%TI) was calculated as follows: %TI = 100 – (mean tumor volume of treated group/mean tumor volume of control group) x 100.

Analysis of statistical significance was performed using the Student t test (P values of less than 0.05 were considered to be statistically significant).

Toxicity was evaluated on the basis of body weight reduction. At the end of the experiment, mice were sacrificed and gross autopsy findings were reported.

Generation of the NPM-ALK transgenic mouse model is described in Supplementary Data.

For ex vivo target modulation analysis xenograft tumor samples were snap frozen in liquid nitrogen immediately after excision and stored at −80°C until analyzed. The frozen samples were homogenized using an Ultra Turrax T25 potter (Janke & Kunkel) at a 5:1 ratio (v/w) in a lysis buffer containing 100 mmol/L Tris-HCl pH 7.4, 2% SDS, 1 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 1 mmol/L DTT and immediately boiled. Tumor lysates were briefly sonicated, clarified by centrifugation, assayed for protein content and used for Western blot analysis.

Efficacy study in an intracranial growth model

Twenty Balb/c male nude mice, ages 6 to 8 weeks, were used for the experiment. For intracranial injection, mice were anesthetized and stereotactically implanted in the caudate nuclei region with 2 x 10^5 NCI-H2228 cells in 2 μL. With a microdrill, a small hole was done at the chosen coordinates and cells were injected at the speed of 1 μL every minute. Hole was closed using bone wax and the wound with sterile autoclips. After the end of surgery, mice were monitored for recovery until complete waking. Two weeks after cell implantation, all mice were examined by MRI as described in Supplementary Data and randomized into four groups (n = 6 controls; n = 7 Entrectinib 120 mg/kg; n = 7 Entrectinib 60 mg/kg; n = 7 Crizotinib 100 mg/kg). At the end of treatment (day 11) all mice were reexamined by MRI.

Results

Entrectinib is a potent inhibitor of TRK, ROS1, and ALK kinase activities

A high-throughput screening (HTS) campaign of the Nerviano Medical Sciences (NMS) collection of kinase-targeted chemical libraries performed against a series of potentially cancer-related tyrosine kinases, including ALK and TRKA, resulted in the identification of several series of hit compounds belonging to different chemical classes with intriguing activity on both ALK and TRKA tyrosine kinases. Among these, subsequent medicinal chemistry efforts within the optimization program of a 3-amino-5-substituted-indazole chemical class led to the identification of entrectinib (Fig. 1A), which was found to be a highly potent inhibitor of all three members of the TRK subfamily of tyrosine kinases TRKA, TRKB, and TRKC and of ALK as well as of ROS1 kinases and which was endowed with favorable administration/metabolism (ADME) and toxicologic properties.

In biochemical assay, entrectinib inhibits the kinase activity of TRK family members with IC_{50} values of 1, 3, and 5 nmol/L for TRKA, TRKB, and TRKC, and of ALK and ROS1 with IC_{50} values of 12 and 7 nmol/L respectively. Steady-state kinetic experiments performed on the ALK kinase enzyme demonstrated that entrectinib is a competitive inhibitor with respect to ATP and noncompetitive with respect to peptide substrate, thus behaving as a pure ATP competitor (Fig. 1B). When profiled against the Kinase Selectivity Screen (KSS), a panel of kinases representative of the human kinome that is used internally within NMS to profile inhibitor selectivity (34), entrectinib showed limited kinase cross-reactivity with other tyrosine kinases (Table 1). To evaluate the cellular potency and selectivity of entrectinib, the in vitro antiproliferative activity of the compound was profiled against a collection of more than 200 human tumor cell lines following 72 hours of continuous exposure (Fig. 1C and Supplementary Table S1). Entrectinib was found to be exquisitely active in inhibiting the proliferation of a limited number of cell lines: although mean IC_{50} of the compound across the entire panel was approximately 2 μmol/L only seven cell lines were inhibited with IC_{50} lower than 100 nmol/L. These were: the TRKA-driven colorectal carcinoma cell line KM12 with an IC_{50} of 17 nmol/L, the ALK-dependent ALCL cell lines SU-DHL-1, Karpas-299, SUP-M2 and SR-786 which exhibited IC_{50}s of 20, 31, 41, and 81 nmol/L respectively, the ALK-dependent N82/MRC cell line NCI-H2228 with an IC_{50} of 68 nmol/L and the FLT3-dependent AML cell line MV-4-11, with an IC_{50} of 81 nmol/L.

Because we also wished to explore the biologic activity of entrectinib in cellular settings dependent upon TRKB, TRKC, or ROS1 kinases, we used engineered variants of the murine pro-B cell line Ba/F3, which is normally dependent on interleukin-3 (IL3) for growth, but can be transformed to growth factor independence by a wide range of oncogenic kinases, and...
therefore represents an excellent model system for the cellular assessment of kinase inhibitor potency and selectivity (35). We generated diverse Ba/F3 cell lines exogenously expressing the ALK, TRKA, TRKB, TRKC, or ROS1 intracellular domains as ETV6/TEL or EML4 fusions. Entrectinib potently blocked proliferation of Ba/F3-TEL-TRKB (IC_{50}: 2.9 nmol/L), Ba/F3-TEL-TRKC (IC_{50}: 3.3 nmol/L), and Ba/F3-TEL-ROS1 (IC_{50}: 5.3 nmol/L) cells, with a high degree of selectivity versus parental Ba/F3 cells or those transformed by nontargeted kinases such as ABL and RET, which were inhibited with IC_{50}s in the range of 2 to 3 μmol/L. As expected from the high antiproliferative activity observed for entrectinib in KM12 (IC_{50}, 17 nmol/L) is related to its ability to inhibit signaling activity of TRKA, we treated cells with a range of drug concentrations and performed Western Blot analysis to assess levels of kinase domain autophosphorylation and activation status of TRKA downstream signaling components. Entrectinib abolishes autophosphorylation of TPM3–TRKA after 2 hours treatment, concomitant with complete inhibition of the phosphorylation of PLCγ1, AKT, and MAPK (Fig. 2A). Evaluation of the cell-cycle effects of entrectinib in this tumor cell line showed that a dose as low as 10 nmol/L is able to induce accumulation of cells in the G1 phase of the cell cycle at 24 hours treatment, followed by apoptosis induction at 48 hours, as assessed by subG1 DNA content and PARP cleavage (Fig. 2B and C).

The preliminary pharmacokinetic characteristics of entrectinib in the mouse (Supplementary Table S2) suggested that the drug is highly suited to oral administration and that by this route sustained plasma levels at concentrations compatible with predicted somal rearrangement that leads to the expression of oncogenic fusion protein TPM3–TRKA (12) was selected as an in vitro and in vivo model for characterization of entrectinib preclinical activity in a TRKA-driven setting. To confirm that the high antiproliferative activity observed for entrectinib in KM12 (IC_{50}, 17 nmol/L) is related to its ability to inhibit signaling activity of TRKA, we treated cells with a range of drug concentrations and performed Western Blot analysis to assess levels of kinase domain autophosphorylation and activation status of TRKA downstream signaling components. Entrectinib abolishes autophosphorylation of TPM3–TRKA after 2 hours treatment, concomitant with complete inhibition of the phosphorylation of PLCγ1, AKT, and MAPK (Fig. 2A). Evaluation of the cell-cycle effects of entrectinib in this tumor cell line showed that a dose as low as 10 nmol/L is able to induce accumulation of cells in the G1 phase of the cell cycle at 24 hours treatment, followed by apoptosis induction at 48 hours, as assessed by subG1 DNA content and PARP cleavage (Fig. 2B and C).

The preliminary pharmacokinetic characteristics of entrectinib in the mouse (Supplementary Table S2) suggested that the drug is highly suited to oral administration and that by this route sustained plasma levels at concentrations compatible with predicted somal rearrangement that leads to the expression of oncogenic fusion protein TPM3–TRKA (12) was selected as an in vitro and in vivo model for characterization of entrectinib preclinical activity in a TRKA-driven setting. To confirm that the high antiproliferative activity observed for entrectinib in KM12 (IC_{50}, 17 nmol/L) is related to its ability to inhibit signaling activity of TRKA, we treated cells with a range of drug concentrations and performed Western Blot analysis to assess levels of kinase domain autophosphorylation and activation status of TRKA downstream signaling components. Entrectinib abolishes autophosphorylation of TPM3–TRKA after 2 hours treatment, concomitant with complete inhibition of the phosphorylation of PLCγ1, AKT, and MAPK (Fig. 2A). Evaluation of the cell-cycle effects of entrectinib in this tumor cell line showed that a dose as low as 10 nmol/L is able to induce accumulation of cells in the G1 phase of the cell cycle at 24 hours treatment, followed by apoptosis induction at 48 hours, as assessed by subG1 DNA content and PARP cleavage (Fig. 2B and C).
Preclinical Characterization of Entrectinib

Additionally, characterization of the pharmacologic activity of entrectinib against TRKA was performed in a TRKA-driven Ba/F3 model. Consistent with that observed for KM12 cells, entrectinib inhibits proliferation and completely suppresses TRKA phosphorylation in Ba/F3-TEL-TRKA cells with low nanomolar potency (Fig. 2F) whereas in vivo, the treatment of mice bearing Ba/F3-TEL-TRKA tumors with 30 mg/kg of drug per os twice daily for 10 days induced complete tumor regression (Fig. 2G).

As mentioned above, entrectinib resulted to be well tolerated, with no signs of overt toxicity at any of the doses and regimens tested in efficacy studies. Within the context of an exploratory repeated-dose toxicity study in the mouse, the compound was administered to animals by the oral route for 14 consecutive days up to 240 mg/kg/d, which was found to be a NOAEL (No Observed Adverse Effect Level). In this study, all animals showed good general condition at the end of the treatment period at all the doses tested. No changes in blood counts or in pathology of all the organs examined were reported: these included liver, lungs, kidneys, and brain. Interestingly, in this study no neurologic effects or abnormal behavior were observed up to maximal tested dose of 240 mg/kg/d (corresponding to an entrectinib level in the brain of 1.94 μmol/L).

**Efficacy of entrectinib in a ROS1-driven model**

To evaluate the activity of entrectinib against ROS1-driven tumors, we generated IL3-independent Ba/F3 cells through the expression of TEL–ROS1 fusion protein, which bears constitutively active ROS1 kinase. Entrectinib potently blocks proliferation of these Ba/F3-TEL-ROS1 cells with an IC50 of 5 nmol/L, whereas crizotinib, tested in parallel, was found to be 40-fold less potent (Table 2). As expected, the inclusion of IL3 in culture medium, which allows cells to bypass reliance on TEL-ROS1, abrogates the growth inhibitory activity of entrectinib, confirming that its antiproliferative activity against this line is selectively dependent on the ability of the drug to inhibit ROS1 (Table 2). The higher potency of entrectinib against ROS1 with respect to crizotinib was also confirmed by Western blot analysis of target modulation in lysates of drug-treated cells (Fig. 3A). As further validation, entrectinib and crizotinib were tested in parallel also

---

**Figure 2.**

*In vitro and in vivo activity of entrectinib in TRKA-driven models. A. KM12 cells were treated with entrectinib for 2 hours at the indicated concentrations. Levels of phosphorylated and total TPM3–TRKA, PLCγ1, AKT, and MAPK were evaluated by Western Blot analysis using specific antibodies. B. Cell-cycle distribution was evaluated by PI staining and cytofluorimetric analysis after treatment of KM12 cells with entrectinib for 24 or 48 hours. C. KM12 cells were treated for 24 hours with the indicated concentrations of entrectinib. Cell lysates were analyzed by Western blot analysis with specific antibodies to detect caspase-3 and PARP cleavage. D. nu/nu mice bearing established KM12 xenografts were administered entrectinib per os twice daily at the indicated doses or vehicle daily for 21 consecutive days. Tumor volume for each group was measured. Data are expressed as mean ± SD (n = 7). P < 0.0001 for treated versus control group at day 20. See also Supplementary Fig. S1. E. KM12 tumor-bearing mice were treated with the drug per os twice a day for 3 consecutive days at 15 mg/kg. Tumors were harvested at the indicated time points after the last administration and phosphorylation of TRKA and PLCγ1 in tumor lysates were evaluated by Western blot analysis. F. TRKA-dependent Ba/F3 cells were treated for 2 hours with different concentrations of entrectinib and analyzed by Western blot using the indicated antibodies. G. SCID mice bearing Ba/F3-TEL-TRKA tumors were orally administered vehicle or entrectinib at 30 mg/kg twice daily for 10 consecutive days. Tumor volume for each group was measured. Data are expressed as mean ± SD (n = 7). P < 0.0001 for treated versus control group at day 19.

---

**Table 2.**

<table>
<thead>
<tr>
<th>Entrectinib</th>
<th>Crizotinib</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ba/F3</td>
<td>Ba/F3-TEL-ROS1</td>
</tr>
<tr>
<td>IC50 (μmol/L)</td>
<td>IC50 (μmol/L)</td>
</tr>
<tr>
<td>7.8</td>
<td>30.7</td>
</tr>
<tr>
<td>0.12</td>
<td>0.30</td>
</tr>
<tr>
<td>0.005</td>
<td>0.005</td>
</tr>
<tr>
<td>0.005</td>
<td>0.005</td>
</tr>
<tr>
<td>0.10</td>
<td>0.90</td>
</tr>
<tr>
<td>0.005</td>
<td>0.005</td>
</tr>
</tbody>
</table>

**Figure 3.**

*Activity of entrectinib in ROS1-transformed Ba/F3 cells. A. Ba/F3 cells expressing TEL-ROS1 were treated with the indicated concentrations of entrectinib or crizotinib for 2 hours. Levels of ROS1 and phospho-ROS1 were detected by immunoblot analysis using specific antibodies. B. mice bearing Ba/F3-TEL-ROS1 tumors were administered vehicle or entrectinib orally twice daily at the dose of 60 mg/kg for 10 consecutive days. Tumor volume for each group was measured. Data are expressed as mean ± SD (n = 7). P < 0.0001 for treated versus control group at day 28.
on TEL–ALK-driven Ba/F3 cells confirming the higher potency of entrectinib also on this target (Supplementary Fig. S2). The ROS1-driven engineered model was also used for efficacy studies: in SCID mice bearing tumors generated by s.c. injection of Ba/F3-TEL-ROS1 cells, treatment with entrectinib at 60 mg/kg per os twice daily for 10 consecutive days resulted in complete tumor regression in all animals (Fig. 3B).

**In vitro and in vivo activity of entrectinib in ALK-dependent tumors**

To confirm that the high antiproliferative activity of entrectinib in ALK-dependent tumors is related to its ability to inhibit signaling activity of this kinase, we treated various ALCL cell lines for 2 hours with a range of drug concentrations and performed Western Blot analysis, as well as cell-cycle analysis by flow cytometry (Fig. 4 and Supplementary Fig. S3). In the several cellular models tested, inhibition of *in vitro* growth correlated with modulation of driver kinase activation. In Karpas-299 cells (Fig. 4A), for example, almost complete inhibition of NPM-ALK phosphorylation can be observed at 2 hours after treatment starting from the dose of 10 nmol/L, with concomitant modulation of phospho-STAT3, which is an ALK transducer in ALCL (36). At the same doses, the drug also induced a concomitant block of the cell cycle in G1 phase, as judged by flow cytometric evaluation of DNA content (Supplementary Fig. S3A). Similar results were obtained for the other ALK-dependent ALCL cell lines examined, SR-786 (Fig. 4B and Supplementary Fig. S3B), SU-DHL-1, and SUP-M2 (Supplementary Fig. S4A and S4B).
Both dose levels were efficacious, inducing regression of all tumors to nonpalpable dimensions. During the observation period following cessation of treatment at 60 mg/kg, tumor eradication persisted in 4 of 7 mice at day 80 after end of treatment, whereas tumor regrowth was observed in all animals treated at the dose of 30 mg/kg. Excellent efficacy was also observed in SR-786 ALCL tumor xenografts, where the dose of 30 mg/kg was sufficient to induce tumor eradication in 6 out of 7 mice at the end of the observation period (Fig. 4D).

To confirm in vivo mechanism of action of entrectinib against ALK, a study was performed in which Karpas-299 tumor-bearing animals were treated with a single oral administration of entrectinib at the doses of 30 and 60 mg/kg, followed by tumor collection at 6, 12, 18, or 24 hours following treatment (Fig. 4E). Six hours after administration of 30 mg/kg entrectinib, complete inhibition of ALK and STAT3 phosphorylation was achieved, but recovery of both signals was observed within 12 hours, whereas at 60 mg/kg complete inhibition of signaling is maintained for at least 12 hours, consistent with the greater degree of activity seen at this dose over extended observation period.

To further characterize efficacy of entrectinib in the setting of ALCL, we tested the compound against a transgenic mouse model in which the NPM-ALK oncogene (a common form of oncogenic ALK in human ALCL) is expressed in T cells under the control of the T-cell–specific Lck tyrosine kinase gene promoter. This in vivo model faithfully recapitulates the human disease, with the development of thymic lymphomas as well as tumor masses at the level of peripheral lymph nodes appearing in all transgenic animals with a latency of about 20 weeks. For the study, animals were monitored by MRI and treated with entrectinib when the tumors were well established. Figure 5 depicts the MRI scans of a representative animal bearing an extensive thymic lymphoma that invades the thoracic cavity. Treatment with entrectinib at the dose of 60 mg/kg twice daily for 2 days induced complete tumor regression by day 2. Similar results were achieved in a series of additional animals, confirming that this ALK-driven tumor is highly sensitive to treatment with entrectinib.

Because ALK is a clinically validated target in a subset of NSCLC patients whose tumors harbor EML4–ALK, the in vitro and in vivo activity of entrectinib was then evaluated in NCI-H2228, a human cell line derived from a lung adenocarcinoma patient bearing this rearrangement (Fig. 6). As observed for the ALCL lines described above, strong inhibition of ALK phosphorylation is observed following 2 hours of drug exposure, with concomitant inhibition of phosphorylation of downstream signaling components AKT and p42/44MAPK (Fig. 6A). Analysis of cell cycle distribution in cells treated with entrectinib showed an accumulation of cells in G1 phase at 24 hours after treatment and increased subG1 fraction, an indicator of apoptosis induction, at 96 hours (Fig. 6B). Consistent with this observation, Western blot analysis performed in parallel with flow cytometry showed increased levels of p21 and p27 and of PARP cleavage.

We then tested the in vivo efficacy of entrectinib in NCI-H2228 tumor xenografts. NCI-H2228 cells were injected s.c. in left flanks of nude mice and, when tumors were established (volume ca. 200 mm3), entrectinib was administered orally at the dose of 60 mg/kg twice daily for 10 consecutive days. Tumors began to regress within a few days of compound administration, and complete regression was observed in all animals at the end of the treatment period. At 97 days after the end of treatment, the last observation made for the study, 3 of 7 mice remained tumor free (Fig. 6C). Analogously, crizotinib, used as reference in the same experiment and administered for 10 consecutive days at the dose of 100 mg/kg, induced complete tumor regression but tumor regrowth was observed in all treated mice few weeks after the end of treatment.

To confirm in vivo mechanism of action of entrectinib in this model, biomarker analysis by Western Blot of tumor lysates was performed on tumors excised at different time points after a single oral administration of entrectinib. This analysis confirmed strong dose-dependent modulation of ALK phosphorylation as well as of the downstream signaling component AKT consistent with the efficacy observed (Fig. 6D). Additional efficacy studies were performed in NCI-H2228 tumor-bearing animals to explore dose/scheduling of the drug: Similarly, high efficacy was maintained at the doses of 15 and 30 mg/kg per os twice daily for 10

![Figure 5.](image-url)
consecutive days (data not shown). Interestingly, also in this model strong efficacy was observed after repeated cycles of administration with the 4 days on/3 days off scheduling currently used in the clinical development of entrectinib (Supplementary Fig. S5).

As mentioned above, entrectinib resulted to be well tolerated, with no signs of overt toxicity at any of the doses tested in efficacy studies. Interestingly, toxicologic studies revealed that entrectinib efficiently crosses the BBB (i.e., with drug levels within the brain comparable with or exceeding plasma levels) in all three preclinical species tested (data not shown). Because metastasis to the brain represents a major problem in the clinical care of NSCLC and is the cause of relapse in many patients treated with the ALK inhibitor crizotinib (24), this finding prompted us to evaluate the efficacy of entrectinib in a murine xenograft model of brain metastasis, in which human NCI-H2228 cells were injected intracranially in nude mice, with animals subsequently monitored by MRI until tumor masses could be clearly detected in the brain. Animals were then treated with entrectinib per os twice daily for 10 consecutive days. Because in mouse the drug distributes to brain at approximately 50% of concurrent plasma concentrations (Supplementary Table S2), a dosing of 120 mg/kg/d was initially selected for this study and crizotinib was used as comparator. Treatment with entrectinib resulted in a favorable disease control rate compared with vehicle treated animals (Fig. 6E and Supplementary Fig. S6A and S6B), that translated into a significant increase in overall survival (Fig. 6F). In addition a lower dose of entrectinib corresponding to 60 mg/kg/d was also tested with partial but still significant activity achieved (Supplementary Fig. S6C). As expected due to its well-known low brain penetration, crizotinib was poorly active in this model (Supplementary Fig. S6D).

Clinical experience with crizotinib as well as with second-generation ALK inhibitors reveals that a frequent cause of relapse in patients after initial clinical efficacy is the acquired resistance associated with the introduction of single point mutations that impair the binding of the inhibitor to the active site of ALK kinase. Interestingly, clinical data demonstrated that patients who relapsed due to appearance of such mutations might still benefit from treatment with an ALK inhibitor with a different binding mode (22,26,37).

Table 3. Antiproliferative activity of entrectinib on Ba/F3 cells expressing different EML4-ALK mutants

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>IC50 (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ba/F3</td>
<td>2.650</td>
</tr>
<tr>
<td>Ba/F3–EML4–ALK-wt</td>
<td>0.028</td>
</tr>
<tr>
<td>Ba/F3–EML4–ALK–G1269Y</td>
<td>1.490</td>
</tr>
<tr>
<td>Ba/F3–EML4–ALK–L1196M</td>
<td>0.029</td>
</tr>
<tr>
<td>Ba/F3–EML4–ALK–G1202R</td>
<td>1.070</td>
</tr>
<tr>
<td>Ba/F3–EML4–ALK–G1269A</td>
<td>0.390</td>
</tr>
</tbody>
</table>

With the aim of testing the activity of entrectinib on different crizotinib or ceritinib-resistant mutated forms of ALK, Ba/F3 cells were made ALK-dependent upon the expression of activated EML4–ALK wt or harboring the most relevant mutations described to date. Entrectinib showed good antiproliferative activity on ALK-driven Ba/F3 cells (Table 3), which, despite a slight loss of potency with respect to wt ALK for the latter one, is maintained in the presence of C1156Y and L1196M mutations. More than 10-fold lower activity was found when Ba/F3 cells driven by G1269A-mutated form of ALK were tested. As expected, on the basis of predicted binding mode of entrectinib, very low activity was found on G1202R mutant, a feature that is in common with most of the second-generation ALK inhibitors such as ceritinib and alectinib. This finding is particularly relevant as the G1202R mutation in ALK is predicted to correspond to the G2032R mutation in ROS1 kinase, the most important acquired resistance mechanism that has been described to date in ROS1-positive NSCLC, and so based on these data we would not predict significant activity of entrectinib in such clinical cases. Nevertheless, G2032 is, as of today, the only acquired resistance mutant reported for ROS1-positive patients treated with crizotinib and we cannot exclude, based on previous experience on ALK that additional, entrectinib-sensitive ROS1 mutations will be identified.

Discussion

In the present study, we disclose for the first time the structure of entrectinib and describe the preclinical characterization of this novel, orally available, small-molecule inhibitor, which is currently being investigated in two clinical trials in adult patients with TRK-positive tumors (29–31). Its preclinical activity on ROS1- and ALK-positive tumor models additionally supports the enrollment of ROS1- and ALK-positive patients in the clinical trial. In the initial characterization of the molecule, the high biochemical potency observed against TRKA, ROS1, and ALK was reflected by remarkable activity and selectivity in cellular models. When profiled on a panel of more than 200 human tumor cell lines, entrectinib selectively inhibited proliferation of tumor cells dependent upon endogenous expression of these kinases with sub 100 nmol/L activity, with negligible activity against unrelated cell lines. Subsequent studies were conducted to confirm mechanism of action of entrectinib in TRK, ROS1- and ALK-dependent models, and to explore in vivo antitumor activity.

With regard to these distinct oncogenic events, it is fair to say that until recently, TRKA was not considered by the pharmaceutical industry as a high priority therapeutic target in the cancer field, because the best characterized oncogenic rearrangements of its gene, NTRK1, were primarily associated with a subset of PTC, a tumor type that has a good prognosis, with thyroidectomy generally being a curative surgical procedure. No TRKA inhibitors were explored in clinical trials with the possible exception of leustaurtinib (CEP-701), a staurosporine-derivative multikinase inhibitor that was investigated in clinical settings with reported TRKA overexpression, without evidence of efficacy (7,38). However, recent studies that have identified recurring oncogenic NTRK1 rearrangements in subsets of NSCLC and colorectal carcinoma patients (11,12), as well as other studies suggesting a potential role of activated TRKA in other tumor types, including glioblastoma and Spitz melanoma (14–16,39), have created much renewed interest in the identification and clinical investigation of effective TRKA inhibitors (13). In a rapidly evolving scenario, these findings indicate that TRKA inhibition may represent an innovative opportunity for the therapy of selected patients whose tumors harbor NTRK1 gene rearrangements and this has supported the enrollment of TRKA-positive patients in ongoing clinical trials of entrectinib. Here, we report that
Figure 6.
Activity of entrectinib against NCI-H2228 NSCLC tumors. A, NCI-H2228 cells were treated with entrectinib at different concentrations for 2 or 96 hours. Levels of proteins and phospho-proteins were detected by immunoblot analysis using specific antibodies. B, NCI-H2228 cells were treated with 50 nmol/L entrectinib for 24 and 96 hours and cell-cycle distribution was evaluated by PI staining and cytofluorimetric analysis (top). Induction of apoptosis was confirmed by Western Blot analysis of PARP cleavage in treated cells (bottom). C, nu/nu mice bearing established NCI-H2228 xenografts were administered entrectinib per os twice daily at 60 mg/kg or crizotinib per os die at 100 mg/kg or vehicle for 10 consecutive days. Tumor volume for each group was measured. Data are expressed as mean ± SD (n = 7). P < 0.0001 for treated versus control group at day 70. See also Supplementary Fig. S5. D, mice bearing established NCI-H2228 xenografts were administered orally a single dose of entrectinib per os and animals were sacrificed 6, 12, or 24 hours after treatment. Tumors were resected, snap frozen, and protein lysates were analyzed by Western Blot analysis with the indicated antibodies. E, mice bearing intracranial tumors were treated with vehicle or with entrectinib orally at the dose of 120 mg/kg twice daily for 10 consecutive days. Representative magnetic resonance images of vehicle or entrectinib-treated mice are shown. To gain insight into viability and perfusion of tumors, a Dynamic Contrast Enhanced (DCE)-MRI experiment was also performed by acquiring images before and after i.v. injection of a Gd-based contrast agent (see also Supplementary Figure S6). F, survival curves of vehicle- or entrectinib-treated mice used in the experiment (E) are reported (n = 6).
entrectinib is extremely effective against the human TRKA-dependent colorectal carcinoma cell line KM12, with efficacy studies showing that oral administration yields potent tumor growth inhibition at well-tolerated doses. In vivo target modulation analysis showed strong correlation between in vivo antitumor activity and target inhibition, as assessed by the phosphorylation status of TRKA, as well as of the downstream signaling transducer PLCγ1, thus providing strong evidence of on-target mechanism of action. These data support the rationale for clinical development of entrectinib in TRKA-dependent indications. As entrectinib was found to be also a potent inhibitor of the other members of the TRK family, the drug is currently being explored in TRKB- and TRKC-dependent clinical settings.

While being an extremely potent TRK inhibitor, we found that entrectinib also possesses excellent biochemical activity against ROS1 kinase, which translates into potent in vitro and in vivo efficacy in a ROS1-driven tumor model. Although detailed data on remarkable clinical benefit observed in ROS1-positive patient population enrolled in an expansion cohort of the phase I clinical trial with crizotinib have been recently reported (28), we believe that our report of the high in vitro and in vivo activity of entrectinib against ROS1-driven tumor models is an additional significant finding. In particular, cellular proliferation and mechanism of action data indicate that entrectinib is in the range of 10- to 100-fold more potent against ROS1 than crizotinib, and we thus propose that it holds potential for exciting clinical activity in specific subsets of NSCLC, as well as additional settings such as cholangiocarcinoma and inflammatory myofibroblastic tumors, which harbor genetic aberrancies in ROS1 (40).

Regarding the ALK-dependent tumors examined in this study, the correlation between in vitro and in vivo antitumor activity and target inhibition, as assessed by the phosphorylation status of NPM-ALK in ALCI cell lines and of EML4-ALK in the NSCLC cell line NCI-H2228, as well as detailed pharmacokinetic/pharmacodynamic studies provide confirmation of target-dependent activity of entrectinib in these models. In ALK-driven xenograft models, oral treatment with entrectinib at extremely well-tolerated doses induced remarkable tumor regression with all treated animals being tumor free by the end of a 10 days treatment period and again with persistent complete tumor eradication observed in the majority of animals. Because the development of brain metastases is a major cause of relapse after initial clinical benefit with crizotinib (24), we believe that a highly significant feature of entrectinib is its favorable BBB penetration in all preclinical species tested. To explore this feature of entrectinib’s profile, the drug was tested against an intracranial growth model of NSCLC in the mouse, showing an excellent tumor growth control rate, and suggesting that the drug may be of particular clinical benefit in patients with brain metastases. This finding is particularly relevant in the perspective of potential clinical development in indications such as TRKA-positive glioblastoma and in clinical settings where brain metastases are commonly encountered, as occurs in fusion-driven NSCLC.

In conclusion, the studies described herein provide a scientific rationale supporting the development of entrectinib in TRK-, ROS1-, and ALK-dependent tumor settings. Clinical trials with the compound are currently ongoing, with promising early signals of activity (29–31). Interestingly, the active recruitment of TRKA-positive patients already in phase I trial resulted in successful treatment of a colorectal carcinoma patient and a NSCLC patient harboring TRKA-rearranged tumors, with remarkable activity observed after the first cycle of administration, including complete regression of a brain metastasis in the latter case (30,31). In addition, durable objective responses were observed in several NSCLC patients whose tumors harbored ROS1 or ALK fusions and in ALK-driven neuroblastoma (29). These exciting preliminary clinical data are fully coherent with the preclinical studies described herein, and strongly support further investigation of the drug in the patient subsets described above.

Disclosure of Potential Conflicts of Interest

D. Anderson has ownership interest (including patents) in and is a consultant/advisee for Ignyta. G. Li has ownership interest (including patents) in Ignyta. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: E. Ardini, M. Menichincheri, M.B. Saccardo, D. Anderson, J. Harris, G. Li, A. Isacchi, P. Magnaghi, A. Galvani
Development of methodology: C. De Ponti, D. Ballinari, G. Texido, N. Amboldi, M.B. Saccardo, P. Orsini, D. Anderson, J. Harris, E. Felder, D. Donati
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P. Banfi, D. Ballinari, M. Ciometi, G. Texido, A. Degrassi, N. Avanzi, N. Amboldi, M.B. Saccardo, D. Casero, L. Mologni, G. Wei, G. Li
Writing, review, and/or revision of the manuscript: E. Ardini, R. Bosotti, R. Pulici, G. Texido, A. Degrassi, M.B. Saccardo, D. Anderson, G. Wei, J.-M. Vernier, G. Li, D. Donati, A. Isacchi, A. Galvani
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Bosotti, G. Wei
Study supervision: E. Ardini, A. Isacchi, E. Pesenti, P. Magnaghi, A. Galvani
Other (participated in the chemistry program that led to entrectinib): T. Bandiera

Acknowledgments

The authors thank the NMS Biochemical Screening and Experimental Therapy Groups, as well as our colleagues in various support units for their contribution to the experimental activities. We are indebted to Ettore Perrone for skilled synthetic chemistry support and to Valter Croci, colleague and friend, for his dedicated work. Finally, the authors thank Zachary Hornby and Robert Wild for critical reading of the article.

Grant Support

This work was financially supported by Nerviano Medical Sciences srl. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 11, 2015; revised January 20, 2016; accepted January 21, 2016; published OnlineFirst March 3, 2016.

References


638 Mol Cancer Ther; 15(4) April 2016 Molecular Cancer Therapeutics


Molecular Cancer Therapeutics

Entrectinib, a Pan–TRK, ROS1, and ALK Inhibitor with Activity in Multiple Molecularly Defined Cancer Indications

Elena Ardini, Maria Menichincheri, Patrizia Banfi, et al.