Research Article

Anti-tumor Activity of Novel Compounds Targeting BCR-ABL, c-SRC and BCR-ABL^T315I in Chronic Myelogenous Leukemia

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Abstract

Chronic myelogenous leukemia (CML) is a hematological stem-cell disorder characterized by the expression of the BCR-ABL fusion protein, a constitutively active tyrosine kinase that causes pathogenesis. The development of tyrosine kinase inhibitors (TKIs) targeting the BCR-ABL oncogene has proven an effective approach to treat CML, but a non-negligible proportion of patients develop a resistance to this class of drugs. Of note, the T315I mutant of BCR-ABL is resistant to all known TKIs, with the noticeable exception of ponatinib. To address this unmet medical need, a new series of compounds was designed and tested for anti-tumor effects against BCR-ABL^T315I CML. The effects of three OriBase Pharma compounds (OR1001, OR1002 and OR1003) on the kinase activity of wild-type and mutant BCR-ABL proteins, on cell proliferation and on the growth of subcutaneous xenografts of CML cells in athymic mice were investigated. *In vitro*, the three compounds were potent inhibitors of both ABL and c-SRC kinases and of the main mutants of ABL, including T315I. The three compounds inhibited the proliferation of cell lines expressing the wild-type and several mutated forms of BCR-ABL, including T315I. Finally, in a mouse xenograft model, OR1001, was found to significantly reduce tumor growth. These data support the potential of OR1001 as an effective therapy for the treatment of *de novo* and TKI-resistant patients.

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Introduction

Chronic myelogenous leukemia (CML) is a myeloproliferative disorder resulting from a reciprocal translocation between chromosomes 9 and 22 (t(9;22) or Philadelphia chromosome) that results in the coding of an abnormal fusion protein (BCR-ABL) with deregulated tyrosine kinase activity [1]. Without

therapy, CML progresses from a chronic phase (CP) to an accelerated phase (AP) and then to a blast phase (BP). Although it is a rare disease, CML was the focus of intensive investigations as a disease model against which to design tyrosine kinase inhibitors (TKIs). Three TKIs have thus received approval for the treatment of CML. Imatinib was the first TKI to be approved for CML patients, and it revolutionized the standard of care [2]. Dasatinib and nilotinib were developed as second-line treatments for cases of imatinib failure [3]. They are active against most, but not all, ABL tyrosine kinase domain mutations that impart resistance to imatinib. As expected, second-generation TKIs were found to be effective in the first-line setting and have received regulatory approval for that indication.

Although imatinib, nilotinib and dasatinib share a common mechanism of action, namely tyrosine kinase inhibition, they have important differences. From a mechanistic standpoint, both imatinib and nilotinib bind to the inactive conformation of the ABL kinase, whereas dasatinib binds to multiple conformations [3]. Nilotinib is up to 50 times more potent than imatinib in inhibiting BCR-ABL in cell proliferation assays, whereas dasatinib is up to 325 times more potent [4,5]. Of the three agents, dasatinib is a dual BCR-ABL and SRC inhibitor, and nilotinib is the most selective inhibitor. ABL kinase domain mutations constitute the most common mechanism of resistance to imatinib (BCR-ABL-dependent imatinib resistance) [6]. More than 50 of these mutations have been described [7–10]. Both dasatinib and nilotinib are active against all known imatinib-resistant mutations except the T315I mutation, which imparts resistance to all three agents. However, dasatinib and nilotinib have differences in their potency against specific non-T315I mutations [5]. Other mechanisms may contribute to imatinib resistance, and reports have demonstrated a requirement for SRC kinase activity in BCR-ABL transformation and oncogenic signal transduction [11,12]. Some members of the SRC family of kinases (SFKs) are overexpressed in isolated imatinib-resistant patient cell lines, suggesting that SFKs may be involved in BCR-ABL-independent imatinib resistance [13,14].

To date, there are not yet any clinically approved T315I inhibitors on the market. Thus, alternative approaches for the inhibition of the T315I mutant,

which represents 15-20% of all clinically observed mutants, are of great pharmacological and medical interest [15,16]. As an example, ponatinib, a pan-BCR-ABL TKI still under investigation, was developed to overcome the T315I mutation in resistant CML patients. The development of new compounds that are able to counteract imatinib resistance in inhibiting the SRC kinase pathways and all known BCR-ABL mutations, including T315I, remains a pharmacological challenge for many researchers. In this context, we have designed new optimized series of ABL and c-SRC kinase inhibitors with enhanced affinity and potency against the main ABL kinase domain mutations, including T315I. These inhibitors are potential new therapies for CML, Philadelphia chromosome (Ph)-positive acute lymphoblastic leukemia (ALL) and patients that do not respond or become resistant to currently approved agents.

Materials and Methods

Inhibitors and reagents

Inhibitor stock solutions in DMSO (10 mM) were stored at -20°C, and diluted just before use for *in vitro* assays. Imatinib and dasatinib were purchased from LC Laboratories (Woburn, MA, USA). OR1001, OR1002 and OR1003 were designed and synthesized at OriBase Pharma laboratories. Cell reagents and MTT reagent (thiazolyl blue tetrazolium bromide) were purchased from Sigma-Aldrich (Saint Louis, MO, USA).

Cell lines

Murine Ba/F3 BCR-ABL^{WT}, Ba/F3 BCR-ABL^{T315I}, Ba/F3 BCR-ABL^{G250A}, Ba/F3 BCR-ABL^{G250E}, Ba/F3 BCR-ABL$^{E255K+M351T}$ and Ba/F3 BCR-ABL$^{G250A+E279N}$, a kind gift of Dr. JM Pasquet, were grown in RPMI-1640 medium. Da1-3b BCR-ABL^{WT} and Da1-3b BCR-ABL$^{E255K+T315I}$, provided by Dr. Bruno Quesnel [17], were grown in DMEM medium (high glucose) supplemented with 1% pyruvate sodium and 1% non-essential amino acids. All media were supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin. Cells were

maintained in a humidified incubator at 37°C with 5% CO_2.

In vitro kinase assays

The biochemical activity of compounds was measured using Invitrogen's SelectScreen® Biochemical Kinase Profiling Service (Life Technologies Ltd., Paisley, UK). The in vitro IC_{50} values (IC_{50} represents the concentration of a compound that is required for 50% inhibition in vitro of the kinase activity) were determined at the ATP Km concentration.

Cell proliferation assays

Murine Ba/F3 BCR-ABL^WT, Ba/F3 BCR-ABL^T315I, Ba/F3 BCR-ABL^G250A, Ba/F3 BCR-ABL^E255K+M351T (1 x 10^4 cells/well), Ba/F3 BCR-ABL^G250E, Ba/F3 BCR-ABL^G250A+E279N (2 x 10^4 cells/well), Da1-3b BCR-ABL^WT and Da1-3b BCR-ABL^E255K+T315I (5 x 10^3 cells/well) cell lines were distributed in 96-well plates and incubating in duplicate with escalating concentrations (1 pM to 10 µM) of compounds for 72 hr. Cell proliferation was measured using MTT (3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). The EC_{50} values (EC_{50} represents the concentration of a compound that is required for 50% inhibition of the cellular proliferation and/or cellular viability) were calculated from sigmoidal dose-response curves utilizing Prism 5.0 from Graph-Pad Software (GraphPad Software, La Jolla, CA, USA), with values normalized to those of DMSO-treated control wells (0%) and 1% SDS control wells (100%).

In vivo efficacy studies

The xenograft mouse model of imatinib-resistant leukemia was prepared by subcutaneously injecting Ba/F3 BCR-ABL^T315I (1 x 10^8 cell/mL sterile PBS) into the right flank of athymic nude male mice (HSD, 6-7 weeks old). When the tumor volume reached approximately 50 mm^3, mice were assigned randomly to either vehicle alone or OR1001, OR1002 or OR1003 treatment groups (five mice per group). Mice were treated with either vehicle (DMSO) or compounds (40 mg/kg q.d.; per os for 11 consecutive days). Tumor volumes in mm^3 were determined three times a week with a digital caliper and calculated using the following formula: Tumor Volume (mm^3) = length (mm) x width (mm) x width (mm) x ½. Body weight was measured three times a week, and mice were observed daily for monitoring signs of stress to detect possible toxicities. One-way ANOVA was used for statistical comparisons. Data were analyzed with Prism 5.0b (GraphPad Software) by one-way ANOVA with Bonferroni post hoc.

Pharmacokinetics

The pharmacokinetic profile of OR1001 and OR1003 was assessed in Swiss male mice around 5 weeks old after single intravenous (IV) or oral (PO) dosing. For the intravenous route of administration, the compounds were dosed at 2 mg/kg using a PEG400/DMA (polyethylene glycol 400 / dimethyl acetamide) 2/1 w/w solution. For oral gavage, the compounds were dosed at 20 mg/kg in a PEG400 suspension. Blood samples were collected at various time points and the compound concentrations in plasma determined by an internal standard LC/MS/MS method using protein precipitation and calibration standards prepared in blank mouse plasma. Reported concentrations are average values from 2-mice/time point/dose group.

Results

Inhibitory effect of compounds on ABL and c-SRC kinases activities in vitro

We have previously reported the use of a rational fragment-based drug design approach to design new dual inhibitors of ABL and c-SRC kinases [18]. Three optimized compounds (OR1001, OR1002 and OR1003) potently inhibited native ABL and c-SRC kinase activities in biochemical assays (Table 1). All three inhibitors reduced the enzymatic activity of wild-type ABL and were also effective against the ABL^T315I mutant with IC_{50} values in the nanomolar range. The three compounds also had potent inhibitory effects on the other imatinib-resistant ABL mutants

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tested, including ABL^{G250E}, ABL^{Y253F} and ABL^{E255K}, establishing that they target directly native ABL and c-SRC kinases and also mutants of ABL kinase, including T315I.

Table 1 Inhibitory activity (IC50 in nM) of OR1001, OR1002 and OR1003 on isolated tyrosine kinases

Kinase	OR1001	OR1002	OR1003
ABL	2.9	5.7	0.7
c-SRC	7.2	40.3	17.9
ABL^{E255K}	7.6	73.0	8.6
ABL^{G250E}	7.6	46.1	6.9
ABL^{Y253F}	3.4	51.1	4.8
ABL^{T315I}	110.0	384.0	62.3

Growth inhibition of cells expressing native or mutant BCR-ABL

Cellular proliferation assays were performed with murine Ba/F3 cells expressing native BCR-ABL or mutated forms of BCR-ABL, and murine Da1-3b cells expressing native BCR-ABL or the E255K+T315I double mutated form of BCR-ABL (Figure 1). All compounds inhibited the proliferation of Ba/F3 and Da1-3b cells expressing wild-type BCR-ABL in a dose-dependent manner. In all cell lines and all BCR-ABL mutants, the compounds exhibited an antiproliferative activity at least 10 times more potent than that of imatinib. The EC_{50} values for OR1001, OR1002 and OR1003 were very close to that of dasatinib in each cell line, except in the $BCR-ABL^{G250E}$, $BCR-ABL^{T315I}$ and $BCR-ABL^{E255K+T315I}$ cell lines for which the compounds exhibited a better antiproliferative effect than dasatinib (EC_{50} between 2 to 200 times lower than the EC_{50} of dasatinib). In the Ba/F3 $BCR-ABL^{T315I}$ cell line, OR1001, OR1002 and OR1003 were, respectively, 10, 150 and 80 times more potent than dasatinib (Table 2).
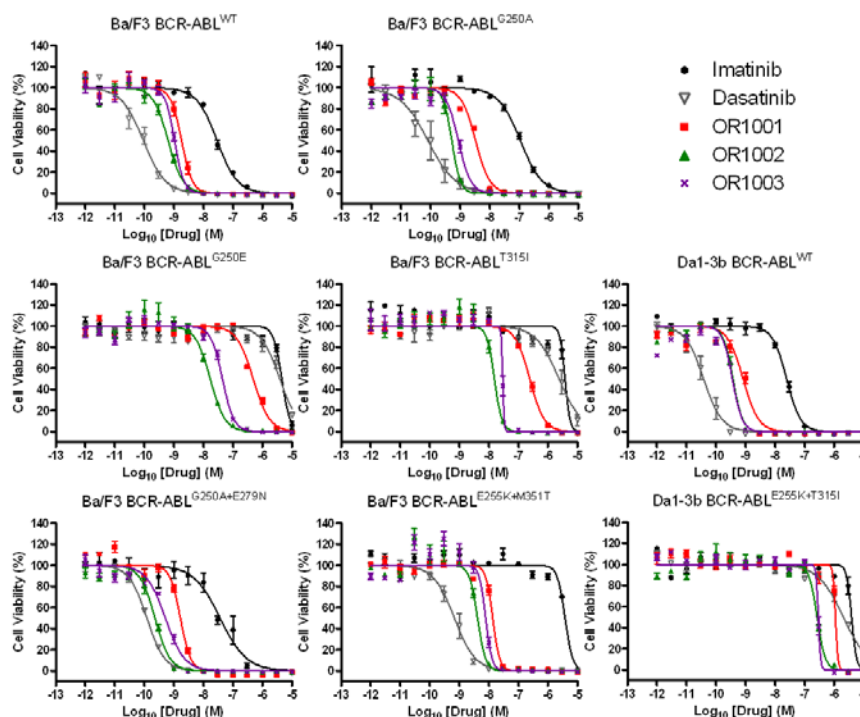

Figure 1 Inhibitory activity of OR1001, OR1002 and OR1003 on cell line proliferation using Ba/F3 or Da1-3b cells expressing BCR-ABL wild-type or carrying either a single or double mutation. Cell viability was assessed by tetrazolium dye assay 72 h after drug treatment.

Table 2 EC50 values (nM) of OR1001, OR1002 and OR1003 on cell line proliferation.

EC50 values are the mean of two experiments using duplicate wells.

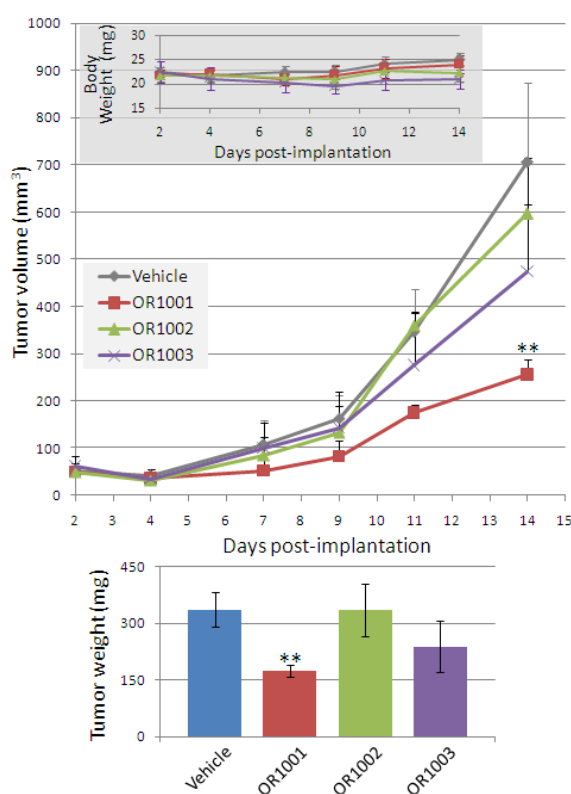| Cell line	OR1001	OR1002	OR1003	Imatinib	Dasatinib
Ba/F3 BCR-ABL^{WT}	1.8	0.6	1.1	29.0	0.1
Ba/F3 BCR-ABL^{G250A}	3.3	0.5	0.9	109.5	0.1
Ba/F3 BCR-ABL^{G250E}	484.1	17.0	44.1	4510.0	3870.0
Ba/F3 BCR-ABL^{T315I}	244.0	15.4	29.5	3798.0	2388.0
Ba/F3 BCR-ABL$^{G250A+E279N}$	1.7	0.2	0.5	36.7	0.1
Ba/F3 BCR-ABL$^{E255K+M351T}$	13.6	4.1	7.8	3815.0	0.7
Da1-3b BCR-ABL^{WT}	0.8	0.4	0.4	24.6	0.1
Da1-3b BCR-ABL$^{E255K+T315I}$	1109.0	265.6	291.3	3895.0	2054.0

Figure 2 Anti-tumor activity of OR1001, OR1002 and OR1003 in mice bearing Ba/F3 BCR-ABL^{T315I} subcutaneous tumors. Mice groups (n=5) were orally and daily dosed with either vehicle alone (DMSO) or compounds at 40 mg/kg for 11 days. Body weight and tumor volume were measured three times a week; tumors were weighed at the end of the study. **P<0.01 *versus* vehicle-treated group.

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Oral *in vivo* anticancer activity

Based on their high biochemical inhibitory effect and good antiproliferative activity *in vitro*, OR1001, OR1002 and OR1003 were assessed for anticancer activity *in vivo* in a Ba/F3 BCR-ABL[T315I] tumor model in athymic mice. Three days after implantation (when tumors reached approximately 50 mm^3), mice were treated daily with an oral dose of 40 mg/kg of OR1001, OR1002 or OR1003 for 11 days. Mice bearing Ba/F3 BCR-ABL[T315I] cells tolerated daily administration of all compounds with no obvious evidence of toxicity as shown by no evident loss of weight during the study (Figure 2). Treatment with OR1001 led to significant tumor growth inhibition (TGI) close to 65% when compared to the vehicle-treated mouse group. As a consequence of the *in vivo* activity of OR1001, macroscopic examination at the end of the study indicated that the tumors of OR1001-treated mice were not vascularized (not shown). Importantly, in contrast to the mice treated with vehicle alone, no evidence of splenomegaly was detected in OR1001-treated mice (data not shown). All animals were necropsied and inspected and no gross anatomical abnormalities were observed (liver, kidney, pancreas, heart, intestine …).

Pharmacokinetics

In order to compare the pharmacokinetic properties of OR1001 and OR1003, their main pharmacokinetic (PK) parameters were evaluated in mice following intravenous (IV) or oral (PO) dosing (Table 3). In mice orally administered at a dose of 20 mg/kg, the maximum concentrations (C_{max}) of OR1001 and OR1003 were obtained 30 minutes after dosing and reached 127 ng/mL (209 nM) and 239 ng/mL (402 nM) respectively. Area under the curve (AUC)$_{0-\infty}$ was 1125 ng·h/mL for OR1001 and 816 ng·h/mL for OR1003 corresponding to an estimated total exposure of 1.9 µM·h and 1.4 µM·h respectively. The half-lives ($T_{1/2}$) and the bioavailability values (F) were similar for both compounds (3.5 hours and 12 % respectively).

Table 3 Mouse PK parameters following oral dosing at 20 mg/kg

Compound	C_{max}	T_{max}	AUC$_{0-\infty}$	$T_{1/2}$	F
OR1001	127	0.5	1125	3.6	12
OR1003	239	0.5	816	3.5	12

These results demonstrate that the animal exposure represented by the AUC values exceeding by more than 15 times the *in vitro* IC50 values for SRC and all tested ABL mutants is sustained in mice at the oral dose of 20 mg/kg. According to these results, OR1001 and OR1003 present similar pharmacokinetic profiles in Swiss mice.

Discussion

Resistance to imatinib in CML patients is classified as primary (i.e., no response from the onset of therapy) or secondary (i.e., following achievement of an objective response). Secondary resistances are usually attributed to point mutations in the kinase domain of BCR-ABL [19]. Numerous mutations have been identified throughout the ABL sequence and led to the development of new generations of inhibitors. However, none of the currently approved TKIs (i.e., imatinib, dasatinib and nilotinib) are active against the particularly aggressive T315I BCR-ABL mutation [15]. Although ponatinib, a third-generation BCR-ABL inhibitor, demonstrated significant clinical activity against the T315I mutation, important challenges remain. Further efforts are still needed to manage TKI side effects, improve the depth of cytogenetic and molecular responses and time to treatment response and then understand and override BCR-ABL-independent mechanisms of resistance. As an example of the latter type of resistance, many results indicate that BCR-ABL and SRC kinases interact with each other [20,21] and that SFKs play important roles in both CML progression and imatinib resistance [22,23]. In this context, the objective of this

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study was to develop newer and more potent pan-BCR-ABL and c-SRC inhibitors able to target the native and mutated forms of BCR-ABL.

In this study, we investigated the *in vitro* and *in vivo* effects of three synthetic small-molecule inhibitors of BCR-ABL and c-SRC kinases [18]. All OriBase Pharma compounds inhibited native ABL, native c-SRC kinase and a panel of clinically relevant mutants of ABL, including T315I, *in vitro* with IC$_{50}$ values in the nanomolar range. All inhibitors exhibited anti-leukemic effects against cell lines expressing wild-type BCR-ABL and single or double mutants of BCR-ABL. *In vivo*, only OR1001 significantly induced a decrease of tumor growth in xenografted mice bearing BCR-ABL^{T315I} tumors while similar pharmacokinetic profiles for OR1001 and OR1003 were obtained in Swiss mice. This unexpected greater *in vivo* activity of OR1001 compared to OR1002 and OR1003 was not consistent with the anti-proliferative activities and several hypotheses can be proposed. Firstly, the mice strain and the dose used to evaluate the PK parameters of OR1001 and OR1003 are different from those used for the efficacy studies. Possible strain differences between Swiss and athymic nude mice may affect the drug disposition and, the dose proportionality of the pharmacokinetics of compounds remains to define. A second hypothesis is that OR1001 could exhibit better ADME (Absorption, Distribution, Metabolism and Excretion) properties, such as a higher level of distribution to the tumor or the formation of one or several highly active metabolite(s), which give OR1001 the best anti-tumor effect. Another hypothesis is that OR1001 could present a secondary pharmacodynamic activity with a high activity against other anti-tumor targets of interest, other kinases as well as other molecular targets, conferring an added *in vivo* anti-tumor activity to OR1001.

Additional studies are ongoing to investigate the pharmacokinetic properties, dose proportionality, in the mouse strain used in efficacy studies and, the *in vivo* dose-dependent response on different CML models to OR1001. However, our data have already validated the rational approach to design new compounds active against both c-SRC kinase and mutated forms of ABL to counteract TKI-resistance in CML. Our findings strongly suggest that OR1001 could be a good therapeutic option for TKI-resistant CML patients.

Acknowledgements

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