Characterization of compound 584, an Abl kinase inhibitor with lasting effects

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Acknowledgments: we thank Sir Philip Cohen, Jennifer Bain, James Hastie and Hilary McLaughlan at the Protein Production and Assay Development team of the Division of Transduction Therapy, University of Dundee for providing the protein kinases and performing the specificity profile experiment; Oriano Marin for peptide synthesis; G. Nilsson and J. Butterfield for kindly providing the HMC-1560 human mastocytosis cell line; J. Fletcher for providing the human GIST882 cell line, and Paolo Vigneri for the GST-Crk expression plasmid. We also thank Malù Coluccia for helpful discussion of the manuscript.

Funding: this research was supported by grants from the associazione Italiana per la Ricerca sul Cancro, Min. San. Ricerca Finalizzata (2003); Centre National de la Recherche Scientifique and Minister of Education, University and Research-COFIN and PRIN programs (2003 and 2004); European Union (Prokinase network #503467); Canadian Fund for Innovation (CFI); and the NCI of Canada.

Manuscript received September 21, 2007. Revised version arrived on November 19, 2007. Manuscript accepted December 5, 2007.

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The online version of this article contains a supplemental appendix.

ABSTRACT

Background

Resistance to imatinib is an important clinical issue in the treatment of Philadelphia chromosome-positive leukemias which is being tackled by the development of new, more potent drugs, such as the dual Src/Abl tyrosine kinase inhibitors dasatinib and bosutinib and the imatinib analog nilotinib. In the current study we describe the design, synthesis and biological properties of an imatinib analog with a chlorine-substituted benzamide, namely compound 584 (cmp-584).

Design and Methods

To increase the potency, we rationally designed cmp-584, a compound with enhanced shape complementarity with the kinase domain of Abl. cmp-584 was synthesized and characterized *in vitro* against a panel of 67 serine/threonine and tyrosine kinases using radioactive and enzyme-linked immunosorbent kinase assays. We studied inhibitory cellular activity using Bcr/Abl-positive human cell lines, murine transfectants in proliferation experiments, and a murine xenotransplanted model. Kinase assays on isolated Bcr/Abl protein were also performed. Finally, we used a wash-out approach on whole cells to study the binding kinetics of the inhibitor.

Results

cmp-584 showed potent anti-Abl activity both on recombinant protein (IC_{50}: 8 nM) and in cell-based assays (IC_{50}: 0.1-10 nM). The drug maintained inhibitory activity against platelet-derived growth factor receptors and c-KIT and was also active against Lyn (IC_{50}: 301 nM). No other kinase of the panel was inhibited at nanomolar doses. cmp-584 was 20- to 300-fold more active than imatinib in cells. This superior activity was evident in intact cells, in which full-length Bcr-Abl is present. *In vivo* experiments confirmed the activity of cmp-584. Wash-out experiments showed that short exposure to the drug impaired cell proliferation and Bcr-Abl phosphorylation for a substantially longer period of time than imatinib.

Conclusions

The present results suggest a slower off-rate (dissociation rate) of cmp-584 compared to imatinib as an explanation for the increased cellular activity of the former.

Key words: Abl, tyrosine kinase inhibitor, off-rate, imatinib analog.

Citation: Puttini M, Redaelli S, Moretti L, Brussolo S, Gunby RH, Mologni L, Marchesi E, Cleris L, Donella-Deana A, Drueckes P, Sala E, Lucchini V, Kubbutat M, Formelli F, Zambon A, Scapozza L, and Gambacorti-Passerini C. Characterization of compound 584, an Abl kinase inhibitor with lasting effects. Haematologica 2008 May; 93(5):653-661. doi: 10.3324/haematol.12212

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Introduction

The molecular signature of chronic myeloid leukemia (CML) is the *BCR-ABL* fusion gene, originating from a reciprocal t(9;22) chromosomal translocation in a pluripotent hematopoietic stem cell and driving the expression of a deregulated tyrosine kinase, Bcr-Abl.[1] Imatinib (gleevec/glivec, STI-571) is the first line treatment for CML[2]: it induces a rapid remission in Bcr-Abl+ patients in chronic phase with minimal toxicities.[3] Imatinib was described to target the CML-specific tyrosine kinase Bcr-Abl, but has also shown potency against platelet-derived growth factor receptors (PDGFR), and stem cell factor receptor (c-KIT),[4] and identifies the receptor tyrosine kinase discoidin domain receptor 1 (DDR1),[5] the Abl-related protein ARG and c-Fms.[6,7] Imatinib has been successfully tested in phase II clinical trials of KIT- and PDGFRα-positive gastrointestinal stromal tumors[8] and has shown promising efficacy in a number of diseases with known deregulation of PDGFR kinase activity, including dermatofibrosarcoma protuberans,[9,10] hypereosinophilic syndrome,[11] and chronic myelomonocytic leukemia.[12] However, secondary resistance is frequent, as observed in CML patients not treated at diagnosis, and is usually due to the selection of clones with mutations in the catalytic site or in nearby regions of Bcr-Abl[13], *BCR-ABL* gene amplification[14] or additional mechanisms. Crystallographic studies have revealed that imatinib binds to the kinase domain of c-Abl only when the domain adopts the inactive *closed* conformation.[15,16] This fact epitomizes the importance of conformation-specific inhibitors and mutations in determining the activity of a given inhibitor. Classes of new conformation-specific inhibitors include selective Abl inhibitors, inhibitors of both Abl and Src-family kinases, aurora kinase inhibitors and non-ATP competitive inhibitors of Bcr-Abl. Some of them were developed starting from the imatinib scaffold introducing substitutions that improve Abl activity (nilotinib)[17] or provide selectivity against the Src-family kinase Lyn (NS-187).[18] These drugs are now in phase III and I clinical trials, respectively. We report here an extensive characterization of a chloro-derivative of imatinib[19], 3-chloro-4-(4-methyl-piperazin-1-ylmethyl)-N-[4-methyl-3-(4-pyridin-3-yl-pyrimidin-2-yl amino)-phenyl]-benzamide, named compound 584 (cmp-584).

Design and Methods

Molecular modeling

cmp-584 was designed based on the crystallographic structure of the kinase domain of Abl in complex with imatinib (PDB code: 1IEP). The atomic coordinates of this X-ray-solved structure were visualized and studied with SYBYL 7.1 (Tripos Inc., St. Louis, MO, USA). To guide the design, the molecular surface, electrostatic potential and liphophilic properties of the protein were calculated and represented by means of MOLCAD of SYBYL 7.1.

Chemistry

3-chloro-4-(4-methyl-piperazin-1-ylmethyl)-N-[4-methyl-3-(4-pyridin-3-yl-pyrimidin-2-ylamino)-phenyl]-benzamide (cmp-584) is shown in Figure 1A and the scheme of synthesis is described in the *Online Supplementary Appendix*. The synthetic route used resulted in the production of highly pure cmp-584 with a mass of 528.2263 (m/z ($M+H)^+$), as measured by high resolution mass spectrometry. The identity of cmp-584 and all intermediates was verified by mass spectrometry, ^1H nuclear magnetic resonance, ^{13}C nuclear magnetic resonance, nuclear Overhauser effect spectroscopy (NOESY) and heteronuclear quantum correlation (HMQC) (details and spectra are given in the *Online Supplementary Appendix*). Imatinib mesylate was synthesized as described elsewhere.[20]

In vitro *and* in vivo *testing*

All reagents used are described in the *Online Supplementary Appendix.*

Preparation of recombinant and native protein kinases

ABL, human FLT-3 and RET and Alk kinase domain were expressed as recombinant proteins.[21-23] The non-receptor tyrosine kinases Lyn, c-Fgr, Syk and Csk were purified from rat spleen to near homogeneity[22] (details are given in the *Online Supplementary Appendix*).

Radioactive kinase assay using the isolated enzymes

Tyrosine kinase assays were performed using ARDIYRASFFRKGGCAMLPVK as the peptide-substrate of Abl, Ret, Alk, and Flt-3, and R-angiotensin as the peptide-substrate of the other tyrosine kinase as described elsewhere.[24] One unit was defined as the amount of tyrosine kinase which transferred 1 pmol phosphate per minute to 0.1 mg/mL polyGlu4/Tyr under standard conditions (details are given in the *Online Supplementary Appendix*).

Inhibitor specificity profiling

Tyrosine kinase profile. A radioisotopic protein kinase assay (33PanQinase® Activity Assay, ProQinase GmbH, Freiburg, Germany) was used to measure the kinase activity of the protein kinases. The Abl construct spans amino acids 118-535 and contains the SH2 domain (details are given in the *Online Supplementary Appendix*).

Ser-Thr kinase profile. Radioactive kinase assays were performed at room temperature in the presence of substrate peptides, [γ-^{33}P]ATP (ATP was used at concentrations corresponding to the Km values of the tested proteins), and 1 μM cmp-584, using recombinant kinases obtained from various expression systems. The procedures for purifying and assaying 30 of the 52 kinases have been described previously.[23,25]

Cell lines

The leukemic Bcr-Abl-positive cell lines (KCL22, K562, KU812 and Lama 84) and the neoplastic Bcr-Abl-negative line (U937) have been described previously.[26] Stably transfected Ba/F3 (murine pro-B) cell lines expressing NPM/ALK (BaF3-N/A) and RET kinases

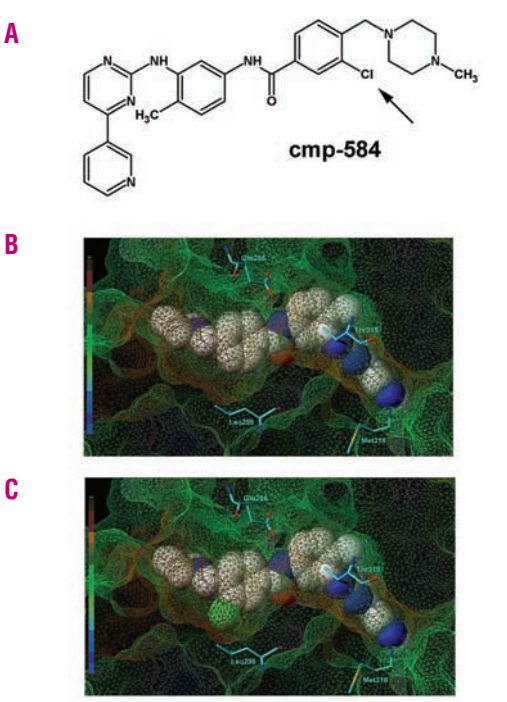

Figure 1. cmp-584 structural formula and modeling. (A) Structural formula of cmp-584. (B and C). Binding modes of imatinib and cmp-584 in Abl tyrosine kinase (PDB code: 1IEP). The atoms of the ligands are in CPK representation, cmp-584 (C) with the additional chlorine atom (in green) compared to imatinib (B). The three residues forming H-bonds (Glu286, Met318 and Thr315) as well as Leu298 located at the bottom of the targeted hydrophobic pocket are shown as sticks and color-coded (oxygen in red, nitrogen in blue, sulphur in yellow and carbon in cyan). The Connolly surface of the protein is represented by lines and color-coded according to lipophilicity (with the color scale on the left, ranging from highly lipophilic [brown] to highly hydrophilic [blue]).

(Ba/F3-PTC) have also been described previously.[27,28] Ba/F3 cells expressing Tel-PDGFRβ kinase, the human mast cell subline HMC-1[560], harboring a point mutation (G560V) in the juxtamembrane domain of c-KIT and the human gastrointestinal stromal tumor cell line, GIST882, expressing an activating c-KIT mutation (exon 13, K642E) have been described elsewhere.[21,29-31] The imatinib resistant cell lines K562R and KCL22R were derived as previously described for Lama 84R.[21,32]

Immunoprecipitation experiments on Bcr-Abl

A lysate of Lama 84 cells was obtained as described in the *Online Supplementary Appendix*. Bcr-Abl was immunoprecipitated by incubating total proteins (100 μg) with 2 μg of anti-c-Abl antibody for 1 h at 4°C, followed by incubation for 1 h with 30 μg protein A-Sepharose (Pharmacia Biotech, St. Alban, UK). Immunocomplexes were washed twice in lysis buffer and in kinase buffer before performing *in vitro* kinase assays.

Radioactive kinase assay. Bcr-Abl immunocomplexes derived from 10^7 Lama 84 cells/sample (approx. 2.5 mg total protein) were pre-incubated with indicated inhibitors at different concentrations for 10 min in reaction buffer containing 25 mM Hepes pH 7.0, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT, and 50 μM Abl peptide substrate (EAIYAAPFAKKK). The kinase reaction was initiated by the addition of 30 μM ATP, 1 μCi [γ^{33}]P-ATP. After 15 min at 30°C the reaction was terminated by spotting onto P81 phosphocellulose paper, which was then processed as described elsewhere.[24]

Cold kinase assay. A GST-Crk phosphorylation assay was performed as described elsewhere, with minor modifications.[33] GST-Crk phosphorylation was visualized by immunoblotting with the anti-phosphotyrosine (4G10, Upstate Biotechnology) antibody. An anti-GST antibody (Amersham Pharmacia Biotech, Freiburg, Germany) was used to control protein loading (details are given in the *Online Supplementary Appendix*).

Proliferation assay

Ba/F3 cells stably transfected with wild type Bcr/Abl (BaF3-BA) and the Bcr/Abl mutants D276G, Y253F, E255K and T315I mutants were generated as described elsewhere.[21] Cell proliferation was assessed with a tritiated thymidine uptake assay as described previously.[34]

Wash-out assay

To determine the persistence of the effects of drug exposure, Lama 84 cells (10^6/mL) were incubated in medium containing 1 μM cmp-584 or 10 μM imatinib or left untreated. At various time points (1 or 6 hours), cells were collected, washed twice in phosphate-buffered saline (PBS) and resuspended in complete medium at a cell density of 10^5/mL. The cells were then transferred to a 96-microtiter plate (10^4 cells/well, six replicates). Proliferation was measured at different times (0, 24, 48 and 72 hours) using CellTiter96™ AQueous Assay (MTS assay; Promega Corporation, Madison, WI, USA).

Cellular Bcr-Abl phosphorylation assay

Lama 84 cells were treated for 1 h with 1 μM cmp-584, 10 μM imatinib or left untreated, as detailed in Figure 2. Cells were washed twice in PBS and resuspended in complete medium without inhibitors. Cells (2.5×10^7 cells) were harvested at the time of wash-out or at 1, 2, 3, or 4 h after the wash-out. Cells were lysed in complete lysis buffer and equal amounts (30 μg) of total protein were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (7.5%), transferred to a nitrocellulose membrane and probed with anti-phospho-c-Abl [pTyr412] (Biosource International, Camarillo, CA, USA) and anti-c-Abl antibody (K-12, Santa Cruz Biotechnology, Santa Cruz, CA, USA). As a control of protein loading, an anti-actin antibody (Sigma-Aldrich Inc., Saint Louis, MO, USA) was used.

In vivo studies

Five-to-seven week-old female CD1 nu/nu mice purchased from Charles River Breeding Laboratories (Calco, Italy) were kept under standard laboratory conditions according to the guidelines of the National Cancer Institute, Milan, Italy. This study was approved by the institutional ethics committee for laboratory animals used in experimental research of the National Cancer Institute. Human Bcr-Abl$^+$ KU812 cells (5×10^7) and Ba/F3-BA cells (1×10^7) were suspended in 0.5 and 0.2 mL of PBS and injected subcutaneously (s.c.) in the

left flank of each animal. Tumor weight and body weight were monitored every 3 or 4 days. Tumor weight was calculated as described elsewhere.[21] cmp-584 was administered by oral gavage 1 day after cell injection, or at 7 days after cell-injection when tumors entered the growth phase, as described in the legend to Figure 3. Placebo-treated animals received the same regimen using vehicle alone (0.5% methylcellulose-0.4% Tween 80).

Statistical analysis

Statistical analysis of tumor weights was performed by Student's *t* test using the GraphPad software analysis program (Prism, San Diego, CA, USA). For tumor-free survival analysis, data were compared by the log-rank test.[35] p values <0.05 were considered statistically significant and were derived from two-sided statistical test.

Results

cmp-584 rational design

cmp-584 is an imatinib derivative with a chlorine substitute replacing a hydrogen atom in the ortho position of the phenyl ring bound to the piperazinylmethyl group (Figure 1A). The rationale for this modification was the following. The crystal structure of the kinase domain of Abl in complex with imatinib showed that the determinants for the binding of imatinib to Abl are the targeted inactive conformation of the enzyme, some hydrogen bonds and a large hydrophobic interaction pattern. The lipophilic property of the protein projected on its molecular surface representation reveals a large hydrophobic pocket accommodating the hydrogen atom in the ortho position to the N-methylpiperazine group (Figure 1B). In particular, unoccupied space is present between the ligand atoms and the protein surface lined by the residues Ile293, Leu298 (shown in Figure 1B), Leu354, Ala380 and His381. Thus, the substitution of the hydrogen atom with chlorine was suggested in order to increase Van der Waals' contacts by filling the available space (Figure 1C) under the assumption that cmp-584 shares the same binding mode as imatinib and that the increased contacts favor binding as recently reported for a series of pyrrolopyridine-based inhibitors overriding the T315I Abl mutation.[36]

In vitro *profiling of cmp-584*

In an *in vitro* radioactive kinase assay using purified recombinant proteins, cmp-584 inhibited Abl kinase activity on an exogenous peptide substrate with an IC_{50} value of 8 nM , which was lower than the imatinib IC_{50} (21 nM). In order to test the selectivity of cmp-584, three different assays were performed (details are given in the *Online Supplementary Appendix*). The *in vitro* radioactive assay (*Online Supplementary Table S1*) showed that cmp-584, like imatinib, was poorly active against most of the tested tyrosine kinases, and inhibited only Lyn at submicromolar concentrations (IC_{50} 301 nM). The 33PanQinase® Activity Assay (ProQinase) on a panel of 15 tyrosine kinase, and the radioactive kinase assay on a panel of 52 protein kinases belonging to the Ser/Thr and Tyr kinase families used to assess the inhibitor specificity profile gave comparable results (*Online Supplementary Figures S1A and S1B*). These indicated that, in comparison with imatinib, cmp-584 is a more potent inhibitor of Abl and gains activity against Src-related tyrosine kinase. In addition, cmp-584 retains the ability to inhibit PDGFR α/β and c-KIT receptors. It is interesting to note that utilizing an Abl construct that includes its SH2 domain, cmp-584 was approximately 30 times more potent than imatinib (*M. Kubbutat, personal communication*).

The IC_{50} values for cmp-584 and imatinib were 12 and 303 nM, respectively when inhibition activity was tested using Lama84 immunoprecipitated native Bcr-Abl kinase (*Online Supplementary Figure S1C*). This shows that cmp-584 was approximately 25-fold more potent than imatinib. These results were confirmed by the non-radioactive kinase assay using the physiological substrate Crk, which indicated that cmp-584 was approximately 1 log more potent than imatinib in inhibiting Bcr-Abl kinase (*Online Supplementary Figure S1D*) as well as Lyn kinase activity (*data not shown*).

Anti-proliferative activity of cmp-584

The anti-proliferative activity of cmp-584 and imatinib was studied in several human CML-derived cell lines (Table 1). cmp-584 showed greater potency than imatinib against all Bcr-Abl$^+$ cell lines tested, ranging from picomolar IC_{50} values in KU812 and Lama 84 (IC_{50}: 80 pM and 170 pM, respectively), to nanomolar IC_{50} values in K562 and KCL22 (IC_{50}: 3.5 and 5.9 nM, respectively). In contrast, imatinib was active only in the nanomolar range (50-220 nM) in these cell lines. Thus, cmp-584 is a more potent inhibitor of proliferation in CML-derived Bcr-Abl-expressing cell lines than imatinib with 1 to 3 log improved efficacy.

Subsequently cmp-584 activity was evaluated against two additional targets of imatinib, PDGFRβ and c-KIT. In PDGFRβ-transformed murine Ba/F3 cells (Table 1), a 50% inhibition of proliferation was observed at 2.4 nM of cmp-584, a concentration similar to the 3 nM of imatinib required. In addition, the mastocytosis HMC-1^{560} cell line and the GIST-882 cell line, harboring activating mutations of c-KIT, were inhibited by cmp-584 in the same nanomolar range (93 and 15.5 nM, respectively), as that with imatinib (19 and 29.5 nM, respectively) (Table 1). Taken together, these data suggest that cmp-584 is more active than imatinib against Bcr-Abl tyrosine kinase and retains the same potency against the two other imatinib-targeted proteins, c-KIT and PDGFRβ, confirming data obtained using purified proteins (*Online Supplementary Figure S1*). Furthermore, cmp-584, like imatinib, had no significant effect on the viability or proliferation of Ba/F3 cells rendered growth factor-independent through expression of activated forms of RET or ALK tyrosine kinase, parental Ba/F3 cells cultured in the presence of interleukin-3, or U937 cells (Table 1).

cmp-584 was also tested against different imatinib-resistant Bcr-Abl$^+$ cells. Imatinib and cmp-584 were first

compared for their effects on Lama 84R, KCL22R and K562R human cell lines (Table 1). The mechanism of imatinib-resistance of Lama 84R cells is amplification of the *BCR-ABL* fusion gene,[32] while the underlying mechanisms of KCL22R and K562R resistance have not yet been defined. cmp-584 was significantly more active than imatinib in reducing the proliferation rate of Lama 84R (7.6 *vs.* 700 nM), KCL22R (80 *vs*. 2000 nM), and K562R (91 *vs.* 3000 nM). The effect of cmp-584 on mutated Bcr-Abl, a second known mechanism of imatinib resistance, was assessed using murine pro-B Ba/F3 cells stably transformed by four kinase domain point mutants (D276G, Y253F, E255K and T315I) and wild-type p210 Bcr-Abl protein as a control. cmp-584 inhibited the proliferation rate of wild-type and D276G mutant transfectants in the low nanomolar range with IC_{50} values of 7.2 and 24 nM, respectively (Table 1). Y253F and E255K mutants were inhibited by intermediate concentrations of cmp-584 (IC_{50}: 120 nM and 688 nM, respectively),while no inhibition was observed for the T315I mutant (IC_{50} value: >5000 nM). cmp-584, therefore, shows greater potency than imatinib against both imatinib-sensitive and resistant cell lines. The pattern of effectiveness against cell lines is similar for both cmp-584 and imatinib, supporting the hypothesis that they share the same binding mode in the Abl kinase domain (Figure 1).

Persistent inhibition of Abl exerted by cmp-584

It was previously shown that 3-halogenated and 3-trifluoromethylated substitutes of the phenyl moiety linked to the piperazinylmethyl group of imatinib conferied significantly increased activity compared to the parent compound.[19] In order to investigate the acquired potency of cmp-584 over time, a series of wash-out experiments was performed monitoring the autophosphorylation of Abl. Lama 84 cells were treated for 1 h with either 1 μM cmp-584 or 10 μM imatinib or left untreated. These concentrations were chosen based on the 1-log difference in potency observed in the *in vitro* kinase assay. Subsequently the inhibitors were washed-out, Bcr-Abl protein was immunoprecipitated for 2 h and activity was assessed in an autokinase assay (Figure 2A). Bcr-Abl derived from cmp-584 treated cells remained almost completely unphosphorylated, even without the addition of fresh inhibitor (compare lanes 2 and 3). In contrast, Bcr-Abl from imatinib-treated cells displayed autophosphorylation activity that was inhibited by the re-addition of inhibitor (lanes 4 and 5). The persistence of the inhibitory activity of cmp-584 in cells was determined using a proliferation assay. In this assay, the ability of exposure to 1 to 6 h to cmp-584 (1 μM) or imatinib (10 μM) to cause prolonged inhibition of proliferation of Lama 84 cells was tested. The results of a representative assay are shown in Figure 2B. Short treatment (1 h) with cmp-584 was sufficient to reduce the growth of Lama 84 cells at day 3 by about 40% compared to that of untreated control cells (Figure 2B, left panel; p value: <0.0001). This effect was more evident with treatment for 6 h when the cell growth was completely arrested (Figure 2B, right panel; p value: <0.0001). In contrast, 1 h of exposure to imatinib had little effect on Lama 84 proliferation, while after 6 h of treatment, cell growth at day 3 was inhibited by less than 50% compared to that of untreated cells. Statistical analysis comparing the effects of cmp-584 and imatinib showed that cmp-584 was significantly more effective in both cases (p value: <0.0001). These results are consistent with those of a previous study in which prolonged exposure to 1 μM imatinib (20 h) was necessary to affect the proliferation rate of CML cells,[34] and suggest that brief exposure to cmp-584 could be effective due to a slower off-rate (dissociation rate) of the drug.

To understand the basis of the lasting inhibitory activity of cmp-584 in cells, the kinetics of Bcr-Abl inhibition was investigated. As shown in Figure 2C, Lama 84 cells were pre-treated with inhibitors for 1 h, followed by wash-out, and whole cell lysates were analyzed using an anti-phosphotyrosine antibody detecting the phosphorylated tyrosine of the autophosphorylation site of the Bcr-Abl kinase domain (P-Tyr^{412}-Abl). Pre-treatment with 1 μM cmp-584 was sufficient to inhibit Bcr-Abl phosphorylation completely and this effect was also evident 4 h after wash-out. In contrast, while 10 μM imatinib pre-treatment induced complete dephosphorylation of Bcr-Abl, 1 h after the inhibitor wash-out, partial recovery of activity was already evident (Figure 2B). These findings support the hypothesis that the dissociation rate from Bcr-Abl protein could be slower for cmp-584 than for imatinib.

These data indicate that cmp-584 has a durable inhibitory effect on Bcr-Abl, which is in agreement with the hypothesis of an enhanced interaction with Bcr-Abl kinase domain mediated by the chlorine atom.

The effect of cmp-584 on in vivo *tumor growth*

A sustained inhibition of Bcr-Abl protein, obtained with imatinib 160 mg/kg three times a day, is necessary to completely eradicate measurable tumors in xenograft CD1 nu/nu mice.[34] In order to assess the *in vivo* antitumor efficacy of cmp-584 directly, the molecule was first tested in a human tumor xenograft CML model using KU812 cells. Mice bearing measurable tumors were treated with cmp-584, 7 days after leukemic cell injection, for 5 days using different doses (25, 37.5, 50, 75 mg/kg twice a day). As shown in Figure 3A, tumors started to regress 48 h after treatment and showed a dose-dependent anti-tumor response; the reduction compared with that of the control group averaged between 80% and 96% at the end of treatment (day 11; p value: 0.025 and 0.01, respectively). Notably, in every group of cmp-584-treated mice complete tumor regression was obtained in 100% of animals (days 18-21) and mice remained tumor-free up to day 100. No deaths occurred and no weight loss was observed in animals treated with the highest dose tested (75 mg/kg twice a day). Imatinib was less effective in these experimental conditions[34] in mice treated with imatinib 160 mg/kg three times a day for 11 days[34] the relapse rate was 33%. These data demonstrate the improved efficacy of cmp-584 in mice bearing CML-derived tumors, even at the lowest dose tested (25 mg/kg twice a day) and using short treatment regimens

(5-day schedule).

In order to test the efficacy of cmp-584 in a syngeneic murine xenograft model, p210 wild-type transfected Ba/F3 cells were injected subcutaneously in CD1 nu/nu mice. cmp-584 was used at a dose of 110 mg/kg twice a day and mice were treated for 11 days starting on day 1 after tumor inoculation. All four untreated mice developed measurable tumors by day 11, whereas four out of five cmp-584-treated mice developed measurable tumors by day 28, with a statistically significant reduced tumor weight (p value <0.01, Figure 3B) and growth delay (p value=0.0027, Figure 3C). The 11-day treatment schedule of 110 mg/kg twice a day did not show overall toxicity (in tems of weight loss or death), demonstrating a good safety profile of this compound.

Table 1. Comparison of the effects of cmp-584 and imatinib on cell proliferation.

Kinase	*cmp-584 [nM] IC_{50}±SEM (n. of repeats)*	*Imatinib [nM] IC_{50}±SEM (n. of repeats)*
wt-Ba/F3 +IL3	6020±487 (n=4)	5660±2760 (n=3)
Bcr-Abl (K562)	3.5±2 (n=4)	221±20 (n=2)
Bcr-Abl (Lama 84)	0.17±0.09 (n=6)	86±8 (n=11)
Bcr-Abl (Ku812)	0.08±0.02 (n=2)	51±5 (n=2)
Bcr-Abl (KCL22)	5.9±3.7 (n=2)	70.5 (n=1)
Bcr-Abl (K562R, IM resistant)	91±33 (n=4)	3190 (n=1)
Bcr-Abl (Lama 84R, IM resistant)	7.6±0.5 (n=2)	735±380 (n=2)
Bcr-Abl (KCL22R, IM resistant)	80±27.5 (n=2)	2170±460 (n=2)
p210 Bcr-Abl (Ba/F3)	7.2±3.1 (n=9)	401±70 (n=11)
D276G Bcr-Abl (Ba/F3)	24±3.3 (n=3)	1147±231 (n=4)
Y253F Bcr-Abl (Ba/F3)	121±36 (n=4)	1888±979 (n=4)
E255K Bcr-Abl (Ba/F3)	688±312 (n=2)	3174±1211 (n=2)
T315I Bcr-Abl (Ba/F3)	>5000 (n=1)	>10000 (n=1)
Tel-PDGFR-β (Ba/F3-T/P)	2.4±1.4 (n=4)	3.4±0.9 (n=5)
c-Kit exon 13 mutant (GIST882)	93 ± 11 (n=2)	29.5 (n=1)
c-Kit G560V mutant (HMC-1)	15.5±0,8 (n=2)	19 (n=1)
Ret (Ba/F3-PTC)	>5000 (n=1)	>10000 (n=1)
NPM-Alk (Ba/F3-N/A)	>5000 (n=1)	>10000 (n=1)
(U937) (Bcr-Abl negative)	>5000 (n=4)	>10000 (n=4)

Discussion

Imatinib was the first targeted therapy developed for CML. Despite its excellent activity in chronic phase-CML, 10% to 15% of newly diagnosed patients fail to achieve complete cytogenetic remission and up to 1% of patients in such a remission, lose it every year.[3] Furthermore, most patients with Philadelphia chromosome-positive acute lymphocytic leukemia do not achieve durable responses with imatinib.[3] These data justify the development of more potent and selective tyrosine kinase inhibitors, such as the dual Src/Abl inhibitors, dasatinib and bosutinib, characterized by less restrictive conformational binding conditions,[21,37] or nilotinib with increased potency and the same selectivity pattern as imatinib.[17] We characterize a yet only reported molecule[19] with increased activity and long-lasting effects, which are potentially explained by increased Van der Waals' contacts with the protein leading to a decreased off-rate.

Analysis of the crystal structure of Abl in complex with imatinib revealed an unoccupied hydrophobic pocket close to the phenyl ring bound to the piperazinylmethyl group. We, therefore, hypothesized that substitution of a phenyl-hydrogen by a chlorine atom should result in more favorable interactions between the inhibitor and the Abl kinase domain. The molecule designed on the basis of this hypothesis, called cmp-584, was synthesized and tested for anti-Abl activity *in vitro* and *in vivo* and showed activity in the nanomolar range against Philadelphia chromosome-positive CML cells.

The proposed binding mode of cmp-584, with the

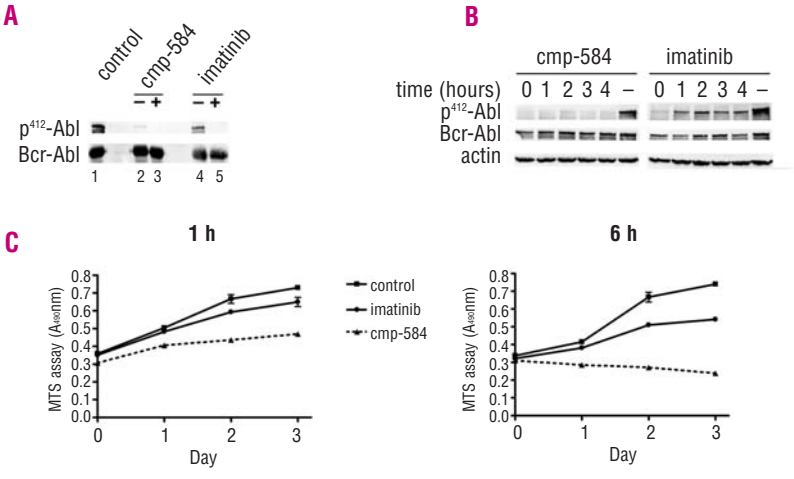

Figure 2. The durability of Bcr-Abl inhibition exerted by cmp-584. (A) Effects of cmp-584 and imatinib inhibitors on autokinase activity. After 1 h of drug exposure, cells were immunoprecipitated with anti-Abl (K12) antibody and 30 µg of protein A sepharose. Immunocomplexes were subjected to cold autokinase assay with the addition of 100 µM ATP, with (+) or without (-) 1 µM cmp-584 or 10 µM imatinib. Each sample was divided into two aliquots, separated by 10% SDS-PAGE, and subjected to immunoblotting with anti-P^{412}-Abl antibody or with anti-c-Abl polyclonal antibody. (B) Lama 84 cells were treated with 1 µM cmp-584, 10 µM imatinib or left untreated (control). Cells were collected 1h (left panel) or 6 h (right panel) later, washed twice in PBS, resuspended in complete medium and further cultured. The number of viable cells was quantified using a colorimetric MTS assay every day. Data from a representative experiment are shown.

(C) Effects of cmp-584 and imatinib on the autophosphorylation of Bcr-Abl kinase. Lama 84 cells were treated for 1 h with 1 µM cmp-584, 10 µM imatinib or left untreated (control). After drug-exposure, cells were collected, washed twice in PBS, reseeded in complete medium and lysed at different time points after the wash-out, as indicated. Cell lysates were subjected to western blot analysis to detect phosphorylated Bcr-Abl (P-Tyr^{412}-Abl), and total Bcr-Abl protein. Actin was used as a loading control.

consequent enhanced interactions, is compatible with the lower IC_{50} values for this compound compared with imatinib. The increased van der Waals' contacts of cmp-584 could also lead to a higher constant for the ligand off-rate. This possibility was hypothesized for hydrophobic 3-substitutes of the imatinib scaffold with increased activity compared to imatinib, indicating the existence of better van der Waals' contacts between the 3-substituent and hydrophobic side chains of Ile-293, Leu-298, Leu-354, and Val-379of Abl.[19] The positive effect of increasing the contacts was recently shown for a series of pyrrolopyridine-based inhibitors of the T315I mutant of Abl.[36] In addition, the ortho substitution of the phenyl moiety may favorably influence the entropic term of the free energy of binding by restricting the rotation of the methylpiperazine group, thereby increasing its activity.[19] The results of the reported wash-out experiments are compatible with the interpretation that the binding of the inhibitor in more persistent than that of imatinib, leading to a higher activity also in cell-based assays against Bcr-Abl$^+$ CML cells. To our knowledge these data represent the first experimental evidence that the chlorine-substituted benzamide of imatinib possesses such a property. Other tyrosine kinase inhibitors, for example those targeting vascular endothelial growth factor receptor, have been described to have similar properties.[38]

Nilotinib, another second generation Abl inhibitor, derives its greater affinity from a better topological fit of the drug into the inactive conformation of Abl.[17] Chemical modifications of imatinib made with the guidance of molecular modeling have yielded several promising compounds that could override imatinib resistance.[19] Among them, the compound selected for further development denoted NS-187[19] has a trifluoromethyl group that interacts well with the same hydrophobic pocket targeted by the clorine atom of cmp-584 (Ile-293, Leu-298, Leu-354, and Val-379). In the panel of imatinib derivatives published by Asaki *et al.*,[19] compound 5b corresponded to our cmp-584. Whether NS-187 or other compounds presented in that panel possess the same slow off-rate as cmp-584 remains to be elucidated.

The data presented here also point to the importance of assessing the activity of inhibitors, e.g. cmp-584 and imatinib, on the full-length Bcr-Abl protein. The activity ratio between cmp-584 and imatinib was only 3 when the isolated kinase domain of Abl was used, while it increased to 20-300 when the full-length Bcr-Abl protein, either immunoprecipitated or present in intact cells, was targeted (Table 1 and *Online Supplementary Figure S1*). Similar results were obtained when a construct including the kinase and the SH2 domains of Abl was used. These differences in activity ratio between cmp-584 and imatinib suggest that cmp-584 enhances the inhibitory effect of SH2 on the kinase domain by stabilizing its inactive conformation, favoring tight packing between Y245 of the SH2-kinase linker and K313/P315 of the kinase domain as seen in the X-ray structure of c-Abl$^{46\text{-}534}$ complexed to PD166326 and myristate.[39]

cmp-584 was also tested in two models of imatinib resistance *in vivo*. No overall toxicity was observed using cmp-584, confirming the good safety profile of imatinib-like drugs. In the first model, when tested in a xenograft mouse model against Philadelphia chromosome-positive KU812 tumors, cmp-584 showed complete eradication of the tumor without relapses, even in a setting in which imatinib was known not to be curative.[34] In the same model, complete eradication was also observed using NS-187.[18] In the second model, in which Ba/F3 p210 wild-type transfectants were injected into nude mice, pronounced growth retardation was evident, as observed previously using other inhibitors,[18,21] but not with imatinib.[18] Despite the submicromolar activity against Abl kinase mutants shown in proliferation assays, only minor activity was found when using the same mutants *in vivo* (*data not shown*).

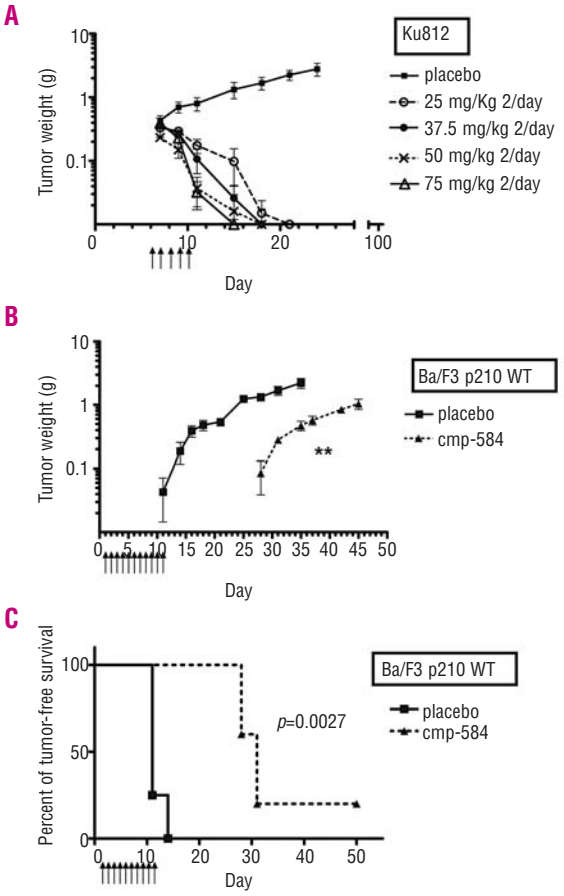

Figure 3. ***In vivo*** **effects of cmp-584 on tumor growth. (A) CD1 nu/nu mice were subcutaneously transplanted with KU812 cells (day 0). cmp-584 was administered orally twice a day, from day 7 to day 11, at different doses (25-37.5-50-75 mg/kg) to a group of four animals with tumors already weighing 230-400 mg. Data points represent the mean ± standard deviation calculated for at least four mice. (B and C) Effect of cmp-584 in a syngeneic Ba/F3 xenograft model. CD1 nu/nu mice were injected with Ba/F3 murine cells transfected with p210 Bcr-Abl wild-type (WT) kinase and treated the day after inoculation of tumor cells with cmp-584 at 110 mg/kg twice a day for 11 days. Tumor weight growth curves (B) and Kaplan-Meier plots of tumor-free survival (C) of control mice given placebo (solid line) or cmp-584 (dashed line) are presented. *****p*** **value <0.01 using Student's *t* test. Treatment days are marked by arrows.**

One potential clinical advantage of cmp-584 could, therefore, lie in the increased Bcr-Abl inhibition when used as first line treatment; in this setting cmp-584 could prevent the occurrence of Bcr-Abl mutations more effectively.

cmp-584 showed some activity against the Src-family tyrosine kinase Lyn. However, this activity was at least 1 log lower than against Abl. The recently solved structure of c-Src in complex with imatinib showed that imatinib binds to an inactive Abl/c-Kit-like conformation of c-Src in the same orientation as in Abl but with a thermodynamic penalty which causes a 3-log lower inhibitory efficacy of imatinib towards c-Src.[40] Our model predicts binding of cmp-584 to the inactive conformation of the Abl kinase domain, and not to the intermediate or active conformations, as seen with both dasatinib and bosutinib.[21,37] Thus, the data suggest that tyrosine kinase Lyn may also adopt an inactive Abl/c-Kit-like conformation to which cmp-584 binds, similarly to the predicted binding mode for Abl. In this case the thermodynamic penalty would be partially compensated by the additional chlorine atom. Further studies will be required to assess whether cmp-584 can truly be considered a dual Src/Abl inhibitor.

In conclusion, this paper describes an Abl inhibitor with the ability to remain bound to Abl for a longer time than imatinib. To our knowledge, this represents the first evidence of higher anti-Abl activity potentially caused by a slower off-rate. Further studies will be required to determine whether the increased activity of cmp-584 also results from enhanced cellular retention of the compound.

Authorship and Disclosures

MP performed experiments and wrote paper, SR performed immunoprecipitation experiments and wrote the paper, LoMo designed the compound and wrote the paper, SB synthesized the compound, RHG performed radioactive experiments, LuMo performed radioactive experiments, EM cultured the cells, LC performed *in vivo* experiments, AD-D performed experiments with Lyn kinase, PD performed *in vitro* kinase profile experiment, ES performed immunoprecipitation experiments, VL supervised the chemical synthesis, MK supervised the *in vitro* kinase profile experiment, FF supervised the *in vivo* experiments, AZ performed and supervised synthesis, LS supervised the molecular modeling part and wrote the paper, CG-P supervised the entire work and wrote the paper. The authors reported no potential conflicts of interest.

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