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14. ABSTRACT

We have developed several novel small molecule inhibitors of BCR-ABL that inhibit the proliferation and induce apoptosis of CML cell lines that express the WT or the T315I mutant form of BCR-ABL. These compounds readily induced the down-regulation of BCR-ABL auto-phosphorylation and STAT-5 phosphorylation. Using ON044580 as the lead compound, we have carried out detailed structure/function studies which demonstrate that ON044580 inhibits the kinase activity of both the wild-type and T315I mutant form of BCR-ABL. In addition, this compound inhibited the kinase activity of WT and V617F mutant forms of JAK2 and induce apoptosis of leukemic cell lines that express the V617F mutant form of JAK2. We show that ON044850 destroys the Bcr-Abl/Jak2 protein Network, which is a large multi-component signaling structure maintained in an active state by members of the HSP90 chaperone complex. ON044850 causes reduction of STAT3 levels leading to reduced expression of HSP90. Thus, our results suggest that targeting Jak2 and Bcr-Abl kinases is an effective way to destabilize Bcr-Abl and its network complex, which leads to the onset of apoptosis in IM-sensitive and -resistant Bcr-Abl+ cells.

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BCR-ABL, Imatinib Resistance

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Table of Contents

Introduction

The recent surge in the development of targeted kinase inhibitors as therapies for cancer was spurred from the success of imatinib (Gleevec®, STI571) for the treatment of Philadelphia chromosome-positive chronic meylogenous leukemia. This drug, for the first time, showed that a small molecule could be designed to inhibit an oncogenic tyrosine kinase (BCR-ABL) that was responsible for inducing malignant transformation of a particular cell type. In spite of the fact that the majority of patients receiving imatinib respond to treatment at both the hematological and cytogenetic levels, relapse occurs in a subset of patients with chronic disease, and this number jumps significantly to nearly 100% in those patients whose disease is in the advanced stages (reviewed in 1). Because of the frequency of mutations, it has become important to develop novel inhibitors that are active against imatinib resistant mutants of BCR-ABL. In response to this demand, two different promising compounds have recently been approved for the treatment of CML. These compounds known as BMS-354825 or Dasatinib, (2) and AMN107 or Nilotinib (3) were found to be inhibitory to nearly all imatinib-resistant forms of BCR-ABL with the exception of one mutation at position 315 where Threonine is replaced by Isoleucine (T315I). As this particular mutation is the most resistant to imatinib and emerges in the largest percentage of patients who develop resistance (reviewed in 4), there is an urgency to develop alternative compounds that are capable of inhibiting this particular (as well as other) amino acid substitution. A major goal of our team was to generate a potent inhibitor of BCR-ABL by targeting regions outside the ATP binding site of this enzyme as these compounds offer the potential to be unaffected by mutations that make CML cells resistant to imatinib, Dasatinib and Nilatinib.

Body

Work accomplished by Dr. Reddy's Group

1. Screening Assay Development

Because it is now apparent that a significant proportion of patients chronically treated with imatinib develop resistance due to the acquisition of mutations in the kinase domain of BCR-ABL, our aim was to generate a potent inhibitor of BCR-ABL by targeting

regions outside the ATP binding site of this enzyme as these compounds offer the potential to be unaffected by mutations that make CML cells resistant to imatinib. Studies with kinase inhibitors have identified three general mechanisms for pharmacological inhibition of kinase activity: (i) Direct binding in the ATP binding site, (ii) binding in the substrate-binding site, and (iii) engagement of an allosteric site resulting in the altered conformation and activity of the kinase.

One of the important facts that has emerged in the past four years is the realization that non-ATP competitive inhibitors often show little or no kinase inhibitory activity in *in vitro* assays (5). An explanation for this lack of *in vitro* activity could be the absence of kinase-associated proteins in *in vitro* reactions which appear to dictate the specificity of kinase reactions *in vivo*. This explanation appears to be supported by the fact that many of the kinases phosphorylate artifical substrates such as casein, IgG and synthetic peptide substrates, which are not normally the targets for these enzymes. In addition, the *in vitro* kinase reactions are usually carried out in the presence of an excess amount of substrate which does not favor inhibition by substrate-competitive inhibitors.

In order to more efficiently screen our library of compounds for BCR-ABL inhibitors in a cell-based assay, we set up a high thru put screen employing a 96 well format in combination with spot blot hybridization using infrared technology developed by Li-Cor, Biosciences, NE. The power of this screen lies in the increased sensitivity, high signal-to-noise ratio and the ability to use two-color detection with little bleed-through between detection channels. This high thru put assay for cell-based enzyme inhibition was found to be very sensitive for non-ATP competitive inhibitors. Briefly, we plated 2.5×10^4 K562 cells per well in a 96 well plate. The cells were treated in duplicate for 2 hours with 10 μM concentration of each compound or 10 μM of imatinib, which was used as a positive control. DMSO alone was used as the vehicle control. Following the incubation, the plate was centrifuged for 20 minutes to pellet the cells. The cell pellets were then lysed in the plate and the cellular debris was removed by high speed centrifugation. The cell lysates were then spotted onto nitrocellulose (Millipore) and the membrane was processed for hybridization according to the manufacture's instructions.

The blot is hybridized using two primary antibodies simultaneously. One antibody is specific for phospho-bcr-abl (Cell Signaling Rabbit polyclonal:CS-390) and the second is specific for Actin (Sigma monoclonal:A2228, loading control). The blot was then hybridized with two secondary antibodies that are specific for either murine or rabbit IgG molecules. These antibodies were conjugated with infrared dyes that excite at either 685 or 785 nm excitation wavelengths. The blots were scanned using two diode lasers (Odyssey scanner) where the detection is based on filtration by two independent detection channels. The image was then stored digitally and analyzed by many different parameters, including direct quantification using software provided by the manufacturer. Compounds that inhibit BCR-ABL kinase activity were identified by their ability to inhibit the autophosphorylation of BCR-ABL (as measured by reduction in the 700 nm fluorescence signal), and were compared to the reduction caused by imatinib, which was used as a positive control. This assay allowed us to screen up to 44 compounds in duplicate at a time using very limited number of man-power hours.

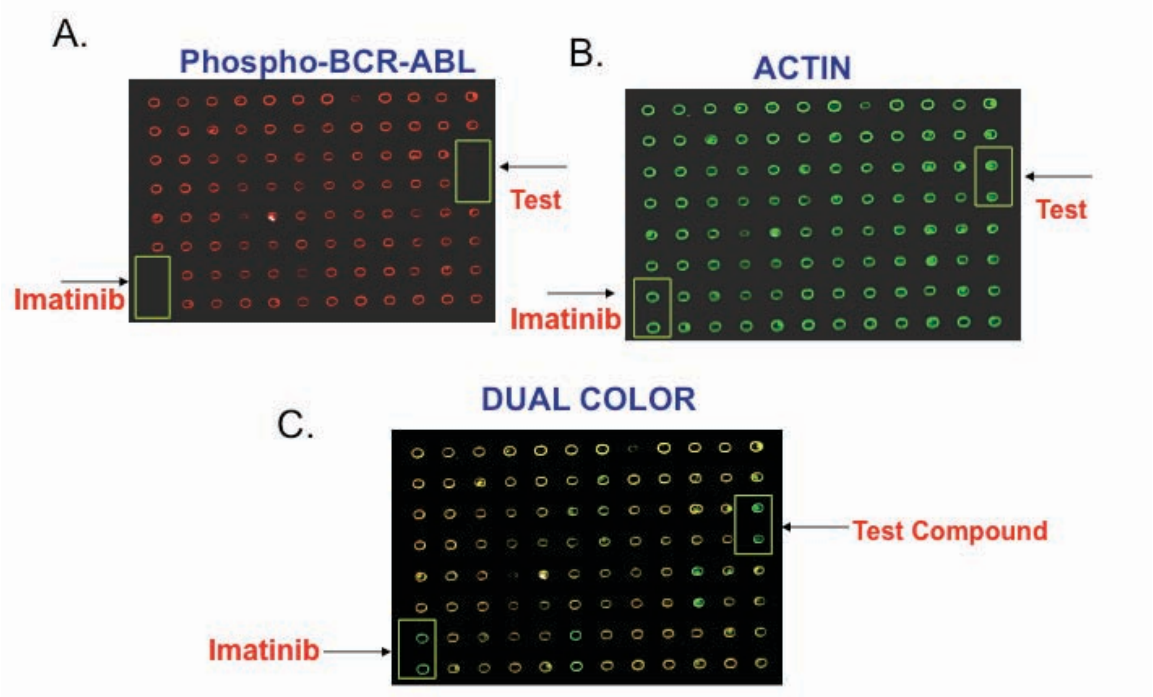

Fig. 1. Dual Infrared Based High Thru Put Screen. K562 cells were plated in a 96 well plate and treated with 10 uM of each test compound or imatinib for 2 hours. The plate were centrifuged, cells were lysed and total cell lysate was spotted onto a nitrocellulose filter paper. The blot was hybridized simultaneously with anti phosphospecific BCR-ABL (red:polyclonal) and anti actin (green:monoclonal) antibodies. The blot was then treated with secondary antibodies conjugated with infrared dyes and the final blot was scanned and imaged using the Odyssey scanner (Li-Cor).

Figure 1 is an example of a typical 96 well assay screen. We treated K562 cells with 44 new compounds from our compound library, at a final concentration of 10 uM in duplicate. The top panels show images derived from each independent channel. Panel A shows the detection of phosphorlated form of BCR-ABL. Duplicate wells located in positions 12:CD were completely negative for 700 nm signal but had normal levels of total protein as determined by the 700 nm signal shown in panel B. Panel C shows the dual color image when the red and green channels are super imposed upon one another. Here one can clearly see one positive compound. The final scan identified one compound in position 12:CD that had the ability to completely inhibit the kinase activity of BCR-ABL. The amount of inhibition was equal to or better than that found for 10 uM of imatinib, located in position 1:FG.

Following the identification of inhibitors of BCR-ABL, we next performed secondary assays to reproduce the primary screen and to more closely determine the IC_{50} value of the compound. This was performed by treating K562 cells with increasing concentrations of the compound (dose response) and then performing a typical western blot assay using the infrared based screening protocol described for western blotting. The screening of a novel library of small molecule kinase inhibitors which are unrelated to ATP or other purine and pyrimidine nucleosides (provided to us by Onconova Therapeutics) using the high thru put assay described above in combination with kinase assays led to the identification of five new compound series which include the ON27 series (represented by ON271300), the ON01 series (represented by ON015290), the ON04 series (represented by ON044580 and ON045000), the ON88 series (represented by ON88320) and a new series of compounds represented by ON96030. Of these, ON044580 and ON045000 were found to be most active against all of the imatinib-resistant forms of BCR-ABL including the T315I mutation. In addition, ON044580, ON045000 and ON96030 were found to be dual inhibitors of BCR-ABL and JAK-2,

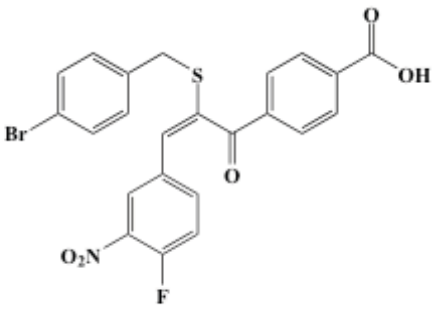

Figure 2. Structure of ON044580

which makes them ideal agents for the treatment of other myeloproliferative diseases in addition to CML. Of these ON044580 (Figure 2) was found to be most active and hence we focused our efforts on the pre-clinical development and delineation of mechanism of action of this compound.

2. Biochemical and [illegible]

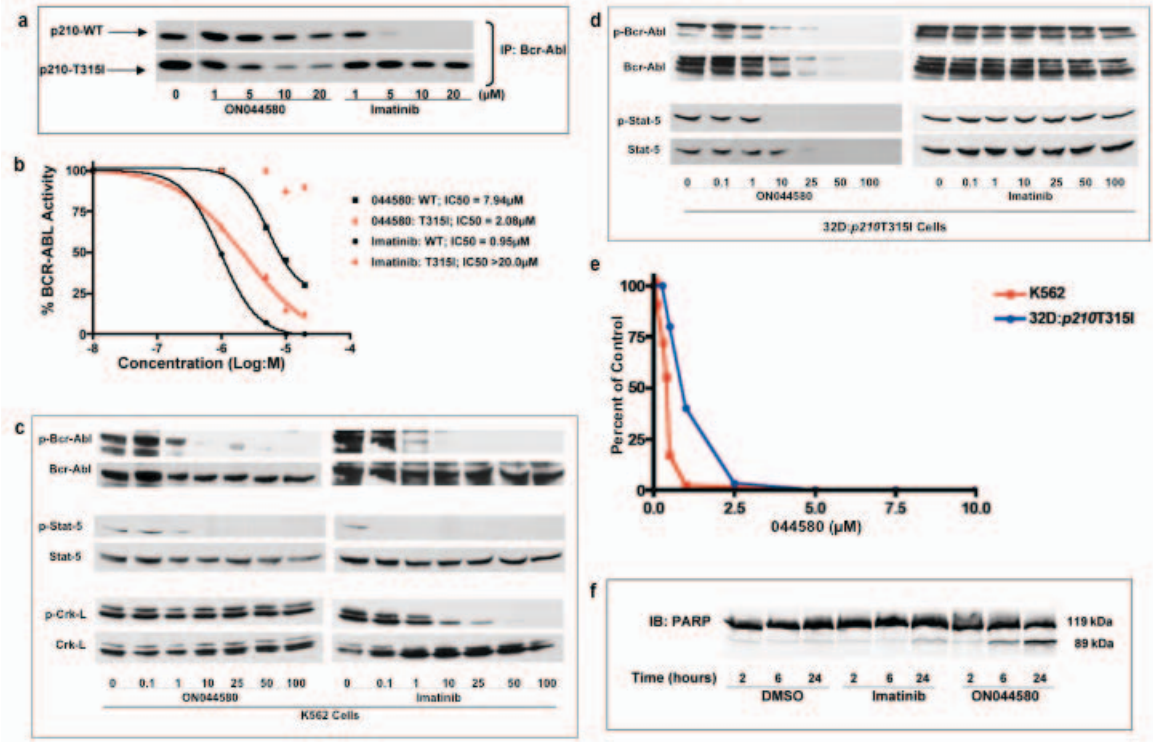

Figure 3. ON044580 inhibits Imatinib sensitive and resistant forms of BCR-ABL (a): BCR-ABL was immunoprecipitated from K562 and 32D:*p210*T315I cells and the immunoprecipitates were used to perform kinase assays. Imatinib was used as a control to inhibit wild-type BCR-ABL. (b): Determination of IC_{50} values. From the autoradiograms presented in a, the values of individual bands corresponding to phosphorylatoin of GST-Abltide were analyzed using MacBas software and plotted as a function of drug concentration using Prism 4 Graphpad software. (c): Inhibition of BCR-ABL and STAT-5 phosphorylation by ON044580 in K562 cells. Exponentially growing K562 cells were treated for 2 hours with indicated concentrations of ON044580. Washed cells were lysed and the clarified lysates were subjected to SDS-PAGE followed by Western blotting and immunodetection with indicated antibodies. (d): Inhibition of BCR-ABL and STAT-5 phosphorylation by ON044580 in imatinib resistant 32D:*p210*T315I cells. Exponentially growing 32D:*p210*T315I cells were treated for 2 hours with indicated concentrations of ON044580. Washed cells were and the clarified lysates were subjected to SDS-PAGE followed by Western blotting. (e): ON044580 inhibits growth of cells expressing Imatinib sensitive and resistant forms of BCR-ABL. K562 and 32D:*p210*T315I cells were grown in the presence of varying concentrations of ON044580 for 72 hours. Cell viability was measured by trypan blue exclusion. (f): ON044580 induces apoptosis in CML cells. K562 cells were treated with 1 μM ON044580 or Imatinib for 2, 6 and 24 hours. Thereafter the cells were harvested, washed, lysed and immunoblotted to ascertain PARP status.

***In vitro* inhibition of wild-type and T315 mutant forms of BCR-ABL kinase by ON044580.** We evaluated the effect of ON044580 on the *in vitro* kinase activity of mammalian BCR-ABL proteins immunoprecipitated from cultured cells. Lysates

prepared from K562 cells expressing the wild type BCR-ABL or 32Dcl3 cells expressing the T315I mutant form were incubated with antibodies directed against the BCR-ABL protein. Kinase assays were performed on the washed immunoprecipitates in the presence of different concentrations of ON044580. Imatinib was used as a control in all of these assays. These studies showed that imatinib readily inhibited the kinase activity of WT BCR-ABL but failed to do so with the T315I-BCR-ABL kinase. On the other hand, ON044580 inhibited both WT and T315I mutant forms of the BCR-ABL kinase (Fig. 3a, b), suggesting that mutations that affect the kinase inhibitory activity of imatinib do not affect the inhibitory activity of ON044580. It is interesting to note that ON044580 was more effective in inhibiting the T315I mutant form compared to wild type BCR-ABL kinase.

Cellular inhibition of the kinase activity of BCR-ABL. Having demonstrated direct biochemical inhibition of wild-type and imatinib resistant BCR-ABL kinase activity, we proceeded to evaluate the *in vivo* inhibition of the BCR-ABL activity by ON044580. We examined the autophosphorylation status of BCR-ABL protein as well as the phosphorylation status of STAT-5 and CrkL in cells treated with increasing concentrations of this compound for 2 hours. Data presented in Figure 3c show that ON044580 inhibited the autophosphorylation of wild type BCR-ABL protein expressed in K562 cells. This compound also inhibited the phosphorylation of STAT-5 but did not affect the phosphorylation status of CrkL. Imatinib (Gleevec®) was used as a positive control in all of these experiments. These results suggest that ON044580 is selective in its inhibitory of activity of BCR-ABL substrates.

Following the establishment of its *in vivo* activity towards WT BCR-ABL kinase, we next examined the ability of ON044580 to inhibit the autophosphorylation of T315I-BCR-ABL kinase and transphosphorylation of STAT-5. For these studies we used the 32D:*p210*T315I cell line that expresses high levels of the T315I-BCR-ABL kinase and is known to be resistant to imatinib. As was done with K562 cells, we treated 32D:*p210*T315I cells with increasing concentrations of ON044580 for 2 hrs followed by western blot analysis of cell lysates to determine the ability of this compound to inhibit

the phosphorylation of BCR-ABL and STAT-5. These studies (Fig. 3d) showed that ON044580 was very effective in inhibiting autophosphorylation of T315I-BCR-ABL and STAT-5 phosphorylation in 32D:*p210*T315I cells while imatinib failed to do so. These results suggest the possibility that ON044580 does not bind to the ATP-binding domain of the BCR-ABL kinase, but acts via binding to the substrate-binding domain (that is specific to STAT-5 but not to CrkL) or to an allosteric domain of the BCR-ABL kinase that results in the impairment of its ability to phosphorylate itself and STAT-5. Interestingly, the steady state levels of BCR-ABL-T315I kinase itself were reduced upon treatment with ON044580 suggesting enhanced degradation of the protein in these murine cells.

***In vitro* tumor cell killing activity of ON044580.** We next examined the ability of ON044580 to inhibit the proliferation of BCR-ABL positive myeloid leukemias. For this study, we first used K562 cells that express WT BCR-ABL kinase and 32D:*p210*T315I cells that express an imatinib-resistant form of BCR-ABL. The results presented in Figure 3e show that ON044580 was an effective inducer of myeloid tumor cell death with a GI_{50} of 300-400 nM in cells sensitive to imatinib. Imatinib, in the same assay system showed a GI_{50} of 100-200 nM (data not shown). Significantly, ON044580 inhibited the proliferation of 32D:*p210*T315I cells with a GI_{50} of 500-900 nM while imatinib showed a GI_{50} of 20-30 μM.

ON044580 induces apoptosis in K562 cells. After demonstrating effective growth inhibition of cells expressing imatinib-sensitive and imatinib-resistant forms of BCR-ABL by ON044580 we tested whether this effect was mediated by apoptotic cell death. K562 cells were treated with 1 μM ON044580 or imatinib for 2, 6 and 24 hours. DMSO was used as a control. We carried out western blot analysis of these cell lysates to evaluate the status of PARP. Proteolytic cleavage of PARP upon ON044580 treatment was evident within 6 hours and became more pronounced in 24 hours (Fig. 3f). PARP cleavage was also observed to a lesser extent in K562 cells treated with imatinib for 24 hours.

Short term ON044580 exposure causes growth inhibition of cells expressing mutated JAK2 and BCR-ABL kinases while normal bone marrow cells remain unaffected. Metabolic and/or excretory elimination of drugs after administration is an important pharmacokinetic criterion. It is therefore pertinent that compounds be tested for their ability to effect changes in target cells within short times of exposure. To test these parameters cells expressing mutated JAK2 (Ba/F3:*JAK2*V617F and HEL cells) and BCR-ABL (K562 cells) were treated with increasing concentrations of ON044580 for 2 hours, washed extensively and returned to drug-free growth medium. Normal mouse bone marrow cells were used as a parallel control in all three sets of experiments. As can be seen in the results presented in Figure 4, short term exposure to ON044580 caused growth inhibition of Ba/F3:*JAK2*V617F cells in a concentration dependent manner while mouse bone marrow cells were unaffected. Similar results were obtained for HEL cells that are homogyzous for the *JAK2*V617F allele and K562 cells expressing the oncogenic BCR-ABL fusion protein. These results are corroborated by those presented in Figure 3b and 3d where the inhibitory effects of ON044580 on JAK/STAT signaling were elicited within 30 minutes of treatment.

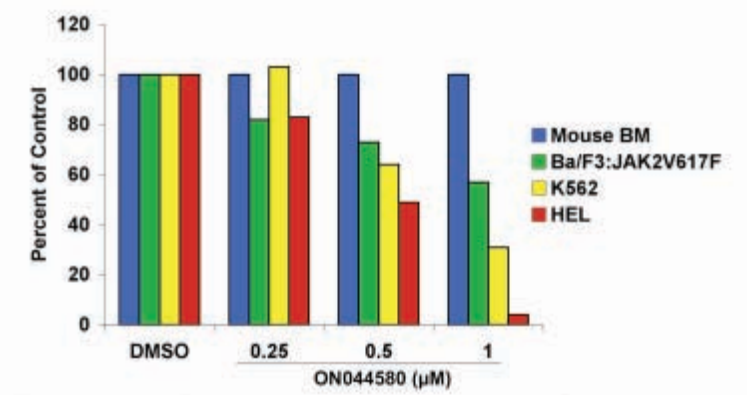

Figure 4. Short-term exposure to ON044580 is cytotoxic to cells expressing oncogenic forms of JAK2 and ABL kinases but not to normal bone marrow cells. Ba/F3:*JAK2*-V617F cells, HEL cells, K562 cells and mononuclear cells isolated from normal mouse bone marrow were treated with increasing concentrations of ON044580 for 2 hours. The cells were then washed and allowed to grow in complete medium in the absence of the compound. The data is plotted as percent viability as compared to DMSO treated controls. Concentration-dependent growth inhibition was observed for all three oncogenic cell lines while mouse bone marrow cells subjected to identical conditions were unaffected.

ON044580 induces apoptosis in primary cells from CML patients refractory to Imatinib. Because ON044580 was able to induce the apoptotic death of 32D:*p210*T315I cells, it was of interest to examine its effects on primary tumor cells derived from patients

that were refractory to imatinib treatment. For this study, we used cells derived from three different patients: a blast crisis CML patient refractory to imatinib treatment, (Fig. 5a), a CML patient in blast crisis (Fig. 5b) and a CML patient in chronic phase (Fig. 5c). Cells were maintained for 48 hr in the absence of cytokines, to enhance the level of BCR-ABL+ cells in the blood cell population and treated for an additional 48 hours with various doses of the drug. Our results show that the blast crisis and chronic phase cells were highly sensitive to apoptosis induction by ON 044580, which suggests that ON 044580 may be useful to treat the unusually resistant blast crisis patients.

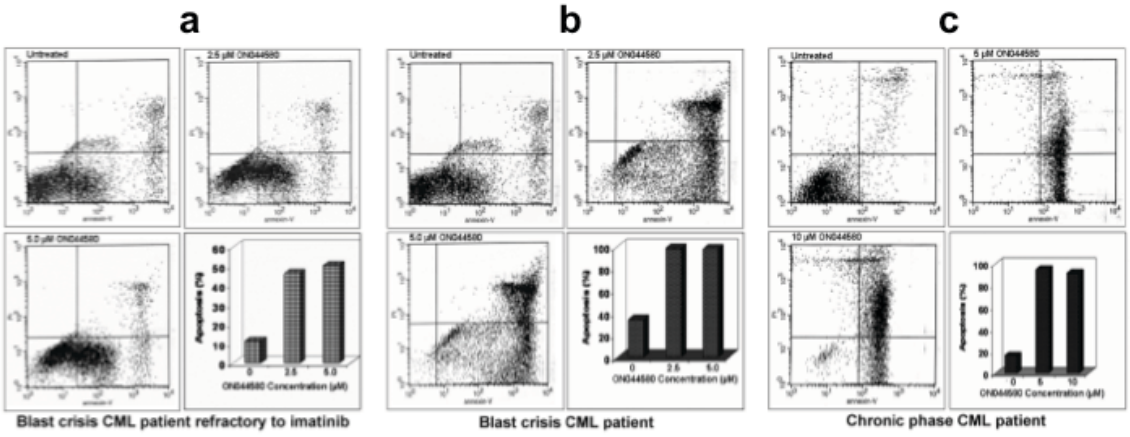

Figure 5. ON044580 induces apoptosis in refractory, chronic phase and blast crisis stages of CML patients. Purified blood samples from a refractory CML patient (a), a CML patient in blast crisis (b) and a chronic phase CML patient (c) were incubated with the indicated concentrations of ON044580 and one untreated sample was kept as a control. After 48 hours the apoptosis was measured by Annexin-V/IP method. Cells accumulated in the lower right and upper right quadrants indicate percentage of cells in early and late stage of apoptosis, respectively.

JAK2 inhibitory activity of ON044580. The studies presented above suggest that the three ON044580 inhibits the BCR-ABL kinase either by binding to the STAT-5 binding domain of BCR-ABL or by binding to an allosteric site which results in altered conformation and inhibition of the kinase activity of the protein. It is now well established that STAT-5 is also a substrate of JAK family of kinases, especially JAK2. The JAK family of tyrosine kinases consist of four members, JAK1, JAK2, JAK3 and Tyk2. Most interleukin-mediated activation of hematopoietic cells appears to result predominantly in the activation of JAK-2, although JAK-1 and TYK-2 have also been found to be activated in certain cell systems (Reviewed in 6). [illegible]

serve as docking sites for other signal transducing proteins, the most important of which are the STAT family of transcription factors. JAK2 is the primary tyrosine kinase activated by erythropoietin (Epo), and is essential for definitive erythropoiesis (6).

The importance of JAKs in human cancer has been highlighted by the discovery of genetic alterations in this family of kinases leading to hyperactivation of the pathways they regulate. These findings include translocations leading to the expression of various forms of JAK2 fusion protein such as TEL/ETV6-JAK2, PCM1-JAK2, BCR-JAK2, RPN1-JAK2, NFE2-JAK2, AML1-JAK2, SSBP2-JAK2 and PAX5-JAK2 which occur in lymphoid/myeloid leukemias and myelodysplasia (MDS) (7-18). In addition, amplification of the JAK2 locus has been shown to occur in Hodgkin's lymphomas (19) and acquired activating mutations in the JAK2 gene have been found in chronic myeloproliferative disorders (CMPD) (20-22), acute lymphoblastic leukemia's (23-26) and myelogenous leukemia's (27-30).

A point mutation in the JAK2 kinase has been suggested as the causative molecular event in most patients with polycythemia vera (PV) as well as in half of the cases of essential thrombocythemia (ET) and chronic idiopathic myelofibrosis (CIMF), all of which are classified as CMPD (31-34). Additionally, it has been reported that around half of refractory anemia ringed sideroblasts with throbocytosis (RARS-T) patients, along with a subset of others with MDS and mixed MDS/CMPD, carry the JAK2 mutation (21,35,36). Remarkably, every sample derived from such patients contained the same amino acid substitution (V617F). Based on predicted JAK2 structure and atomic level simulations, this substitution is believed to disrupt an autoinhibitory interaction between the pseudokinase (JH2) and kinase (JH1) domains of the protein (7,31,37). Studies using Epo receptor mutants have revealed the need for receptor dependant dimerization of the mutant kinase for constitutive activation[35] and a recent report provides biochemical evidence for a regulatory role of the FERM domain in hyperactivation of JAK2 with a V617F substitution[36]. This mutation has been found to confer Epo-independent growth of the mutant cells *in*

vitro due to deregulation of signaling pathways downstream of JAK2 (31). Small interfering RNA-mediated knock-down of JAK2 has also been found to impair EEC formation from PV bone marrow (32). Furthermore, PV patients who lacked the V617F point mutation were found to harbor other activating exon 12 mutations in JAK2[37] making mutations of JAK2 the causative genetic lesion in all cases of this disease.

Activation of the JAK-STAT pathway has also been observed in diseases with signaling defects in proteins upstream of the Janus kinases. One such example is the constitutive activation of JAK2 (41) and STAT1 (42) in cells from monosomy 7 MDS patients, likely due to aberrant cytokine receptor signaling. Monosomy 7 is the second most frequently observed cytogenetic abnormality in MDS, with an incidence of 21% (43). It is the most frequent karyotypic aberration occurring in bone marrow failure patients following immunosuppressive treatment, and is associated with severe cytopenias and a high propensity to develop acute leukemia (44,45). Patients who develop monosomy 7 AML are difficult to treat and often relapse quickly or die of infection (46). Monsomy 7 is especially common in MDS secondary to exposure to alkylating drugs and in pediatric MDS. Monosomy 7 cells show increases in a differentiation-defective GCSFR isoform (IV) that fails to internalize following GCSF binding as normally occurs for the full-length receptor. It is also defective in facilitating phosphorylation of STAT-3, but its ability to signal phosphorylation of STAT-1 and -5 are unimpaired (42,47). As a result, the cell's ability to differentiate is limited while its ability to proliferate via JAK-2 remains intact. These observations suggest an urgent need to develop new JAK-2 inhibitors with greater inhibitory activity and enhanced bio-availability. It was therefore of interest to test ON044580 for JAK2 inhibitory activity since this will not only provide indirect evidence for substrate-competitive nature of our compounds but also could establish their utility for the treatment of MPDs arising due to mutations in JAK2.

In vitro studies using a recombinant JAK-2 protein produced in insect cells (that is commercially available) showed that this compound inhibits the kinase activity of

recombinant JAK2 with an IC_{50} ranging between 0.9 to 1.2 μM (Fig. 6 a, b). Under identical conditions, AG490 was able to inhibit JAK2 kinase activity with an IC_{50} of 36.4

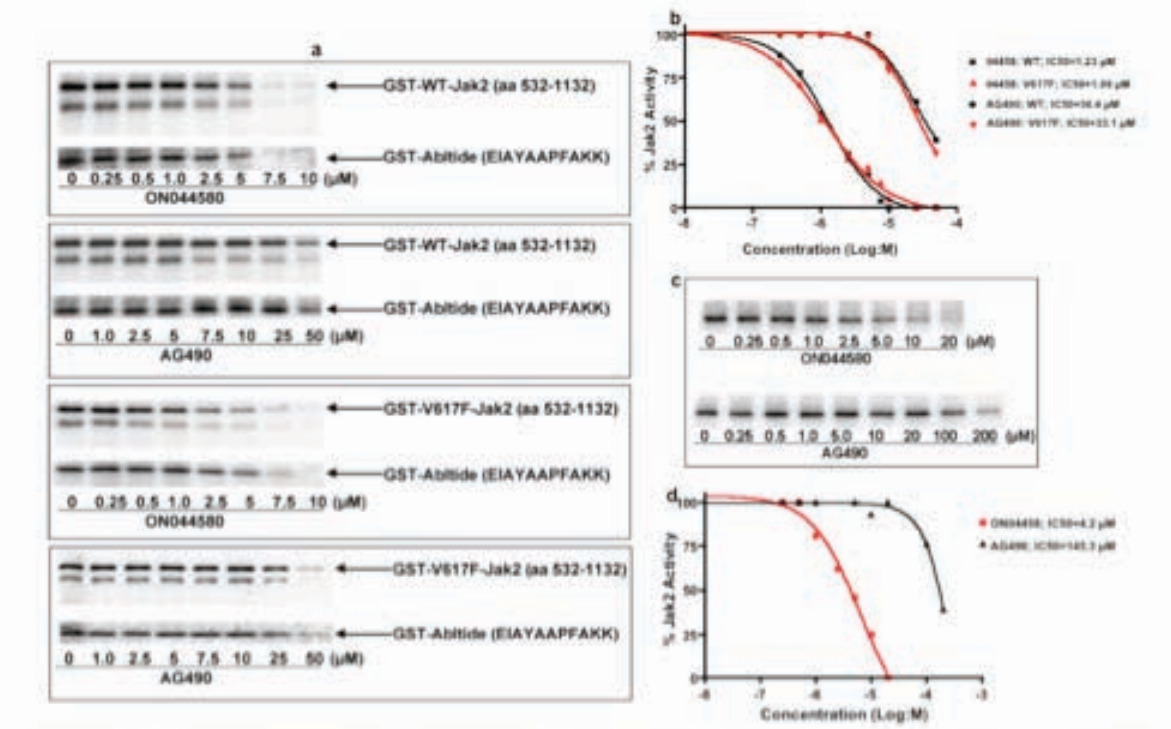

Figure 6: JAK2 inhibitory activity of ON044580. (a) Recombinant wild-type or V617F mutant JAK2 (aa 532-1132) was mixed with increasing concentrations of ON044580 (0.25-10 μM) or AG490 (1-50 μM) and kinase assays performed using recombinant GST-Abltide as substrate. The reaction mixtures were subjected to SDS-PAGE and autoradiography. (b): Determination of IC_{50} values. From the autoradiograms described in a, the values of individual bands corresponding to autophosphorylation of JAK2 were analyzed using MacBas software and plotted as a function of drug concentration using Prism 4 Graphpad software. ON044580 exhibited a 30-fold greater inhibition of wild type and mutated Jak2. (c): Inhibition of mammalian JAK2 activity by ON044580. Full-length JAK2 was immunoprecipitated from clarified lysates of BaF3:*JAK2*V617F cells stimulated with 5 ng/ml recombinant IL-3 for 1 hour. The washed immunocomplexes were incubated for 30 minutes in the presence of increasing concentrations of ON044580 and processed for kinase assays. (d): The samples from c were quantitated and IC_{50} curves were plotted. While ON044580 showed inhibition with IC_{50}=4.2μM, AG490 had an IC_{50} value of 145.3 μM.

μM, which is in agreement with published literature (48). Following these observations, we examined the ability of this compound to inhibit the activated V617F mutant form of JAK2 using commercially available recombinant protein produced in insect cells. The results of this study showed that ON044580 inhibited the kinase activity of mutant JAK2 with a similar IC_{50} (0.8 to 1.1 μM) as that seen with WT JAK2 while AG490 had an IC_{50} of 33.1 μM (Fig. 6 a, b). Because the recombinant preparations of WT and mutant JAK2 proteins are truncated forms of the kinase, we examined the kinase inhibitory activity of these compounds using JAK2 kinase immunoprecipitated from the Ba/F3: *JAK2*V617F

cell line that expresses the full length wild-type and mutant forms of JAK2. These studies again showed that ON044580 inhibited the JAK2 kinase activity with an IC_{50} of approximately 4 µM. Under identical conditions, AG490 inhibited the kinase activity with an IC_{50} of 145 µM (Fig. 6c, d). The observation that ON44580 inhibits recombinant

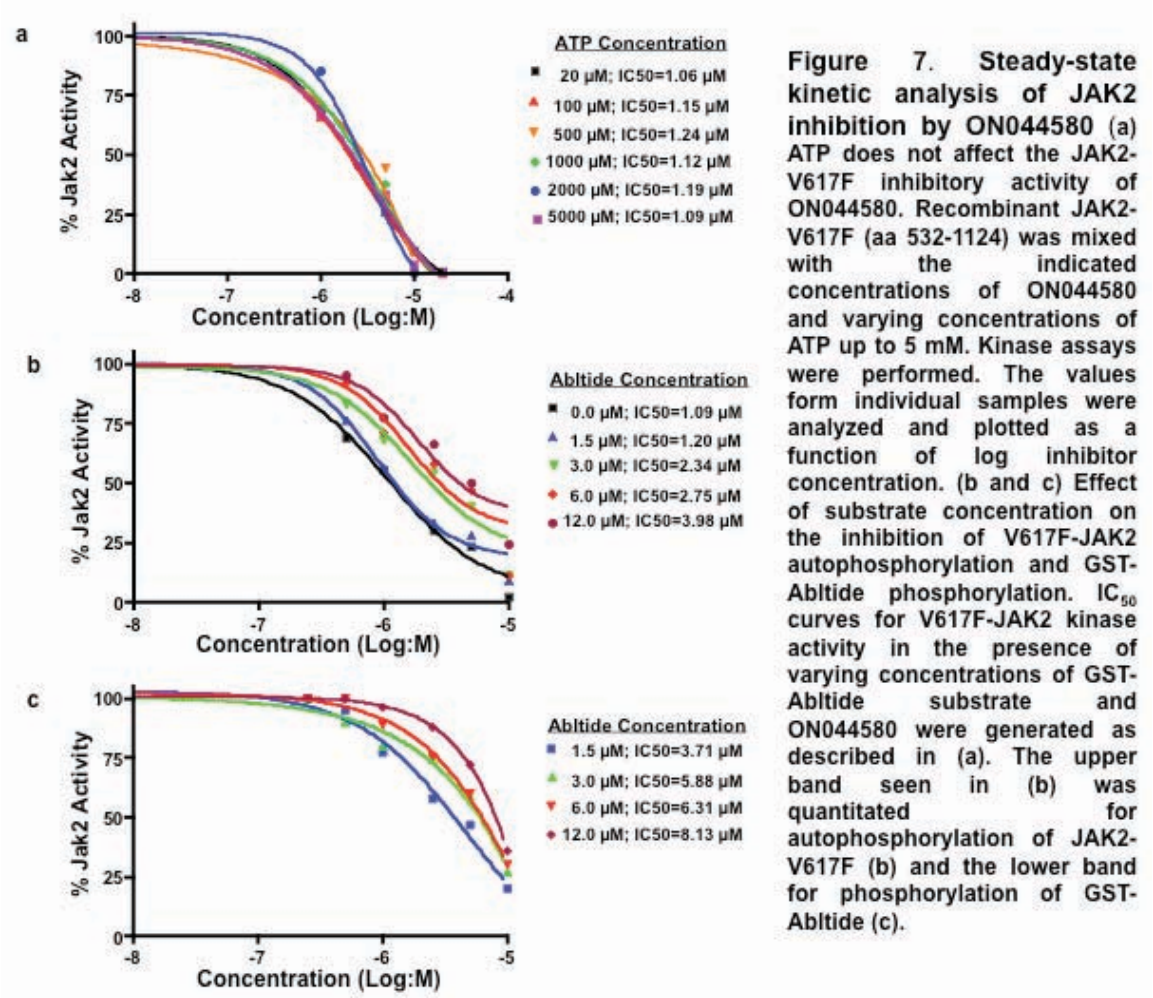

Figure 7. Steady-state kinetic analysis of JAK2 inhibition by ON044580 (a) ATP does not affect the JAK2-V617F inhibitory activity of ON044580. Recombinant JAK2-V617F (aa 532-1124) was mixed with the indicated concentrations of ON044580 and varying concentrations of ATP up to 5 mM. Kinase assays were performed. The values form individual samples were analyzed and plotted as a function of log inhibitor concentration. (b and c) Effect of substrate concentration on the inhibition of V617F-JAK2 autophosphorylation and GST-Abltide phosphorylation. IC_{50} curves for V617F-JAK2 kinase activity in the presence of varying concentrations of GST-Abltide substrate and ON044580 were generated as described in (a). The upper band seen in (b) was quantitated for autophosphorylation of JAK2-V617F (b) and the lower band for phosphorylation of GST-Abltide (c).

JAK2 (aa 532 – 1132) led us to ask whether this compound binds to the catalytic kinase domain (JH1) or the regulatory pseudokinase domain (JH2) of JAK2. To test this, we made use of the commercially available recombinant form of JAK2 containing just the kinase domain (JH1 domain spanning amino acids 808-1132).

Interestingly, ON044580 failed to inhibit JAK2 kinase domain (JH1) activity at 10µM concentration while complete inhibition was observed only at 100 µM (Data not shown). This significant increase in the inhibitory concentration suggests the ATP-binding kinase domain of JAK2 (JH1) is not the primary site of action of ON044580.

ON44580 is a non-ATP competitive inhibitor. Our observation that the pseudokinase domain is required for the kinase inhibitory activity of ON044580 further led us to postulate that it is not an ATP competitive JAK2 inhibitor. We directly tested this hypothesis by carrying out kinase inhibition assays either in the presence of increasing amounts of ATP or in the presence of increasing amounts of substrate. The results of this study, shown in Fig. 7, demonstrate that increasing the ATP concentration in the kinase reaction mixture did not affect the inhibitory activity of ON044580 (Fig. 7a). On the other hand, increasing the substrate concentration in the reaction mixture resulted in a reduction of the kinase inhibitory activity of ON044580. This was seen to be true for both the autophosphorylation of JAK2 kinase itself (Fig. 7b) as well as the transphosphorylation of GST-Abltide substrate (Fig. 7c).

***In vivo* inhibition of Jak2 autophosphorylation and STAT-5 phosphorylation by ON044580 in Ba/F3:*JAK2*V617F cells.** To test the *in vivo* kinase inhibitory activity of ON044580, we treated Ba/F3:*JAK2*V617F cells with increasing concentrations of the compound for 2 hrs in the presence of recombinant IL3 (which enhances the phosphorylation status of JAK2). At the end of the 2 hr incubation period, cells were washed and lysed in detergent containing buffer and the clarified lysates subjected to SDS-PAGE followed by western blotting to detect the phosphorylation status of JAK2. The results of this study (Fig. 8a) showed that ON044580 was able to inhibit the phosphorylation of JAK2 in a concentration dependent manner. AG490, under identical conditions did not inhibit JAK2 phosphorylation, which could be due to the high IC_{50} values seen for full length JAK2 kinase with this compound. As part of this study, we also examined the time course of inhibition where we added 10 μM of ON044580 for periods of time ranging from 15 to 60 minutes and examined the phosphorylation status of JAK2 using western blot analysis. The results of this study presented in Fig. 8b showed that in as little as 15-30 minutes, the compound was able to inhibit IL3-mediated JAK2 phosphorylation.

Using a similar approach, we also examined the phosphorylation status of STAT-5 (a natural substrate of JAK2) in Ba/F3:*JAK2*V617F cells treated with increasing

concentrations of the compound. The results presented in Fig. 8c and d show that ON044580 inhibited STAT-5 phosphorylation in a concentration-dependent and time-

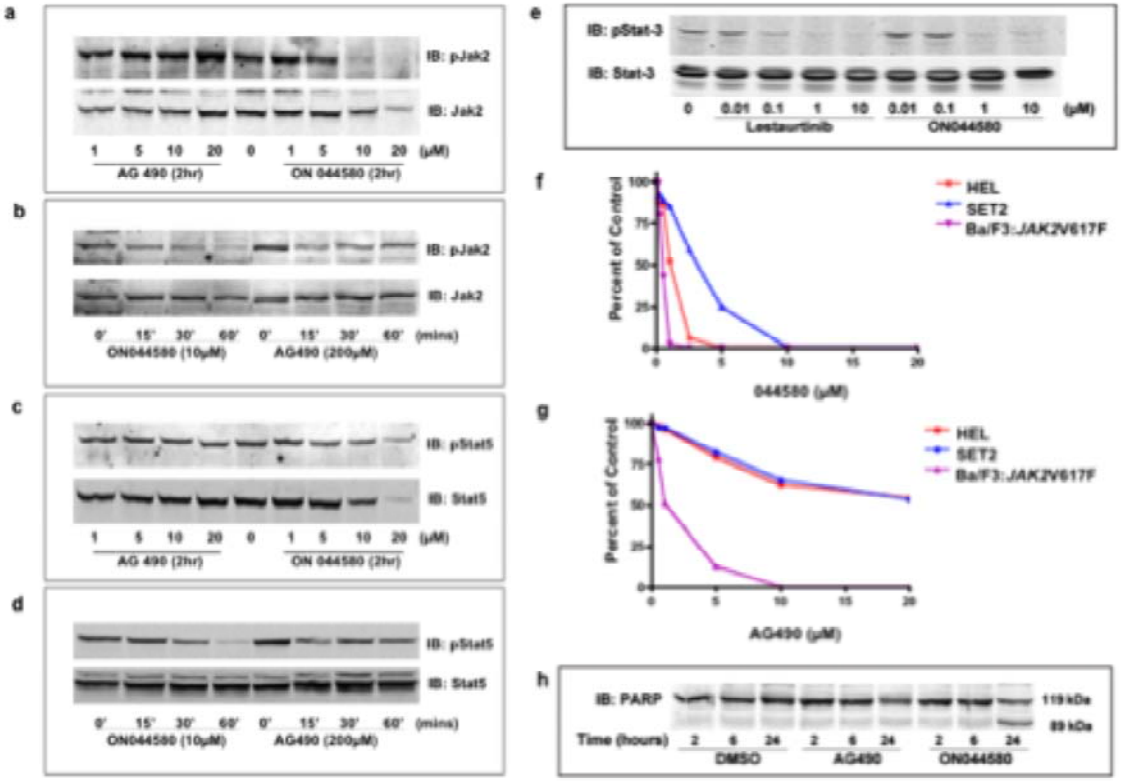

Figure 8. Effects of ON044580 on cellular JAK2-dependent cytokine signaling (a) Inhibition of JAK2 autophosphorylation by ON044580 in IL3-stimulated Ba/F3:*JAK2*V617F cells. Mid-log phase Ba/F3:*JAK2*V617F cells were treated for 2 hours with indicated concentrations of ON044580 and 1 hour with 5 ng/ml recombinant IL-3. Washed cells were lysed and the lysates subjected to SDS-PAGE followed by immunoblotting to detect phosphorylation status of JAK-2. (b) ON044580 inhibits JAK2 autophosphorylation in Ba/F3:*JAK2*V617F cells within 15 minutes. Mid-log phase Ba/F3:*JAK2*V617F cells were treated for the indicated times with 10 μM ON044580 or 200 μM AG490 and processed as above. For all time points the total time for IL-3 stimulation was 60 minutes. (c) Inhibition of STAT-5 phosphorylation by ON044580 in IL-3 stimulated Ba/F3:*JAK2*V617F cells. Ba/F3:*JAK2*V617F cells were treated and processed as in a. Immunoblotting was carried out to assess STAT-5 phosphorylation. (d) ON044580 inhibits STAT-5 phosphorylation in Ba/F3:*JAK2*V617F cells within 30 minutes. Ba/F3:*JAK2*V617F cell treatments were carried out as described in b. Western blot analysis was performed to assay degree of STAT-5 phosphorylation (e) ON044580 inhibits JAK2-dependent STAT-3 phosphorylation in U266 cells. Exponentially growing U266 cells were treated for 2 hours with increasing concentrations of ON044580. The ATP-competitive JAK2 inhibitor, Lestaurtinib was used as a control. Cells were washed, lysed and clarified lysates were resolved by SDS-PAGE for immunoblot analysis to assess phosphorylated STAT-3 levels. (f and g) Growth inhibition of JAK2-V617F expressing cells. Ba/F3:*JAK2*V617F cells (ectopic expression), HEL cells (homozygous *JAK2*V617F) and SET-2 cells (hemizygous *JAK2*V617F) were grown in the presence of varying concentrations of ON044580 (e) or AG490 (f) for 72 hours. Cell viability was measured by Trypan blue exclusion. GI_{50} values were calculated by plotting percent viable cells as a function of drug concentration. (h) ON044580 induces apoptotic cell death in cells expressing JAK2-V617F. Ba/F3:*JAK2*V617F cells were treated with 0.5 μM ON044580 or 2 μM AG490 for 2, 6 and 24 hours. Thereafter the cells were harvested, washed, lysed and probed with anti-PARP antibody following Western blotting.

phosphorylation, these studies suggest that the two events are interrelated.

Cellular inhibition of constitutive JAK/STAT signaling by ON044580. U266 multiple myeloma cell line expresses wild-type JAK2 but has constitutive activation of the IL6 receptor/JAK2/STAT-3 pathway (49). We treated U266 cells with increasing concentrations of ON044580 for 2 hours to test whether this compound could inhibit aberrant JAK/STAT signaling instigated by mechanisms other than genetic alterations in the JAK2 gene itself. The results of this study showed that such a treatment led to a dose-dependent inhibition of STAT-3 phosphorylation at concentrations comparable to a known ATP-competitive JAK2 inhibitor (50) (Fig. 8e).

Growth inhibition of JAK2-V617F expressing cells. To determine whether ON044580 inhibits the proliferation of *JAK2*V617F-positive leukemic cells, we studied its effect on the growth and viability of three different cell lines that express the mutant form of JAK2. These included the Ba/F3:*JAK2*V617F cells that were transfected with an expression vector that encodes the mutant JAK2 and two human leukemic cell lines that were derived from leukemic patients that naturally contained this mutation in their *JAK2* loci. One of them, HEL, is homozygous for V617F mutation while the second cell line, SET2 is hemizygous for the V617F mutation. The results of this study, presented in Fig. 8f, show that ON044580 readily inhibited the proliferation of all the three cell lines at nanomolar or low micromolar concentrations. Thus the GI_{50} for Ba/F3: *JAK2*V617F cells was approximately 250 nM while the GI_{50} for HEL cells was approximately 900 nM. Interestingly, the SET2 cell line that was hemizygous for V617F mutation was more resistant to the cell killing activity of the compound with a GI_{50} value of 3.0 μM. In a similar experiment using AG490, the GI_{50} value for Ba/F3:*JAK2*V617F cells was 1.0 μM while that for HEL and SET-2 cells was greater than 20 μM (Fig. 8g).

ON044580 exerts its antiproliferative effect by inducing apoptosis in Ba/F3:JAK2V617F cells. After demonstrating effective inhibition of myeloproliferation by ON044580, we examined the mechanisms associated with this cytotoxic effect. Upon microscopic observation of cells treated with ON044580 we did not find evidence for

autophagic vacuoles or mitotic arrest. To test if ON044580 activates the apoptotic pathways, we treated Ba/F3:*JAK2*V617F cells for 2, 6 and 24 hours with ON044580 and AG490 at concentrations twice the GI_{50} values obtained in the growth inhibition studies (shown in Figure 3f). DMSO was used as a control. These cells were then harvested and probed for the status of poly-(ADP-ribose) polymerase (PARP), a marker for apoptosis induction (51). The results of this experiment show that treatment with ON044580 did indeed lead to proteolytic cleavage of PARP in 24 hours (Fig. 8h). A similar though less pronounced effect was observed with AG490.

ON044580 effects favorable cytogenetic changes in primary bone marrow cells from Monosomy 7 MDS patients. Monosomy 7 MDS bone marrow mononuclear cells preferentially express the truncated class IV G-CSF receptor, which leads to constitutive signaling through the JAK2 pathway (52). Because ON044580 inhibited the activated IL6 receptor/JAK2/STAT3 pathway in U266 cells (Fig. 8e) and did not show adverse effects on normal bone marrow cells (Fig. 4) we were encouraged to test its effects on Monosomy 7 and diploid hematopoietic colony formation from MDS marrow samples. To assess the effect of ON044580 on these cells with constitutive JAK2 activity, bone marrow aspirate mononuclear cells (BMMNCs) derived from patients with monosomy 7 MDS (confirmed by metaphase karyotyping and FISH) were grown in Mylocult media (Stem Cell Technologies, Vancouver, BC) supplemented with 400 ng/mL G-CSF and growth factor cocktail as previously described (53). Following treatment with ON044580 at 0.1 μM and 0.5 μM, cells were harvested examined for the number of aneuploid and diploid cells by FISH using centromeric probes specific for

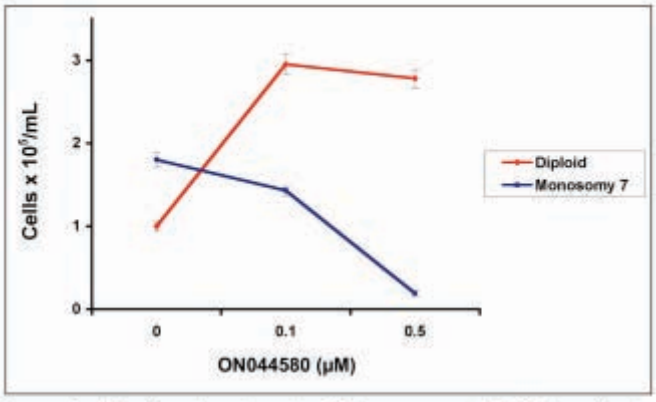

Figure 9. *Ex vivo* treatment of Monosomy 7 MDS patient bone marrow cells with ON044580 causes favorable cytogenetic changes. MDS patient bone marrow mononuclear cells were cultured in colony supporting conditions and treated with 0.5 μM and 1.0 μM ON044580. This led to dose-dependant decrease in the total number of Monosomy 7 cells while maintaining/ increasing the total number of diploid cells.

chromosomes 7 and 8. Results from this study showed that there was a reduction in the number of anueploid cells by 20% and 80% respectively, compared to vehicle treatment control. The effect of ON044580 appeared to be limited to the aneuploid population as the total number of diploid cells in the treatment groups increased (Fig. 9). These preliminary results indicate that ON044580 suppresses monosomy 7 bone marrow cell growth while stimulating growth of normal diploid cells, a finding which may translate into a novel targeted therapy for patients with monosomy 7 MDS.

Nanoparticle delivery of ON044580

In our attempt to assess the efficacy of ON044580 in tumor xenografts models we first attempted to test its toxicity in mice. We found ON044580 dissolved in DMSO to be toxic by tail vein injection and subcutaneous administration at doses of 25mg/kg body weight. This toxicity appears to be partially to be due to DMSO which was used as a solvent. In addition, ON044580 was found to be very insoluble and biologically unavailable. Our attempts at modifying the available side groups of ON044580 invariably led to decrease in its potent kinase inhibitory and/or cytotoxic activities. Hence, we decided to use targeted nanoparticles as a delivery system for administration of ON044580 and its bioactive analogs. We chose to utilize the transferrin-transferrin receptor system to encapsulate the drug because of the biological advantages that this system offers.

Metabolically active cells demand high amounts of iron. Rapidly proliferating malignant cells express higher number of transferrin receptors to facilitate higher iron transport. The use of apotransferrin, transferrin and transferrin receptor antibodies (OX-26) as carriers of drug for targeted delivery to transferrin receptor expressing cells is extensively studied (54).

To reduce the entry of drug into non target cells, several target specific technologies have been developed during the last few years and implemented. Most of these technologies invoke the use of polymeric materials (PEG, PLGA) (55) and proteins (chitins, albumin). These materials though excellent carriers of drug, lack target specificity (56). Thus target

specific ligands like transferrin, antibodies, carbohydrates, signal peptides are widely studied for guiding these delivery systems to specific cells and tissues (57). We reports a target directed nanoparticle drug delivery system comprising of drug loaded transferrin nanoparticles.

Method of preparation of nanoparticles

The schematic below (Figure 10) details the method for preparation of transferring nanoparticles containing ON044580 and Doxorubicin as a control.

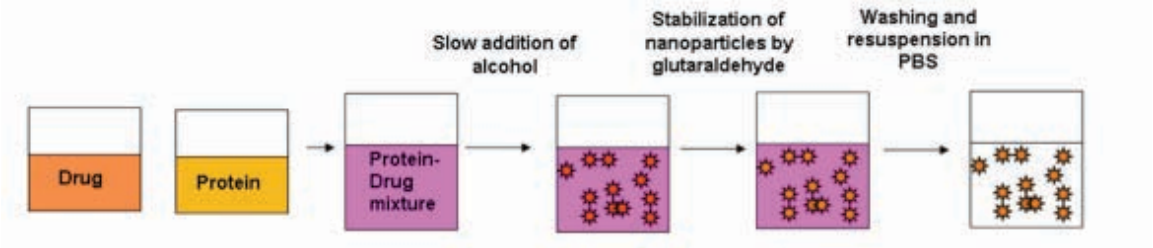

Figure 10. Preparation of drug containing Transferrin nanoparticles. 10 mg of of human holotransferrin was dissolved in sterile PBS (800 ul) with gentle shaking and slow pippeting. 100 ul of drug solution in DMSO was added and incubated at room temperature for 30 min. This was followed by the addition of 600 uL of absolute ethanol in 12 batches of 50 ul with gentle rotation. 1 il pf glutaraldehyde was added and gently rotated for 30 min. The preparation was centrifuged at 4000Xg for 10 min and the pellet dispersed in sterile PBS. This washing step was repeated twice and the final pellet was dispersed in I ml of PBS an used for further analysis.

Size of nanoparticles

The size of the particles was measured by the Beckmann Delsa Nano instrument. The average diameter of drug loaded transferrin nanoparticle was found to be 307nm with a standard deviation of 122nm using the zeta potential sizing method. Our results showed that the majority of nanoparticles were well below 500nm in diameter (Data not shown).

Estimation of drug encapsulation in nanoparticles

In order to be used in biological assays it is imperative that the concentration of drug within the nanoparticle preparation be determined. To that end, 100 µL of the drug-loaded nanoparticles were centrifuged at 4000 x g and the supernatant discarded. The pellet was dispersed in 300 µL acetonitrile and vortexed for 10 minute to release the drug from the nanoparticles. The mixture was centrifuged at 12,000 x g for 10 minutes and the supernatant was analyzed for the presence of ON044580 by high pressure liquid chromatography (HPLC) method using the known concentrations of the drug as standard

references. HPLC analysis of ON044580 released from nanoparticles showed approximately 45% encapsulation efficiency. Similar protocols were employed to make and assess doxorubicin (a known chemotherapeutic with potent anticancer activity) encapsulation in transferring nanoparticles. We found that our preparation of doxorubicin had an encapsulation efficiency of 40% and a molar concentration of 40 μM.

ON044580 loaded transferrin nanoparticles are non-toxic to mice

Having prepared drug containing transferring nanoparticles we tested their toxicity profiles in mice. Female CD-1 mice (25 grams) were injected (IP injections) with a sublethal (24 mg/kg) and lethal dose (64 mg/kg) of ON044580 formulated as nanoparticles. The mice were observed for over 2 weeks with no signs of toxicity or weight loss. However, the injection of these nanoparticles was difficult due to the "stickiness" of the preparations. This could well be due to the size of the nanoparticles (hundereds of nanometer diameter). We are currently working on making finer nanoparticle preparations for greater ease of administration.

Cytotoxicity of ON044580-transferrin nanoparticles

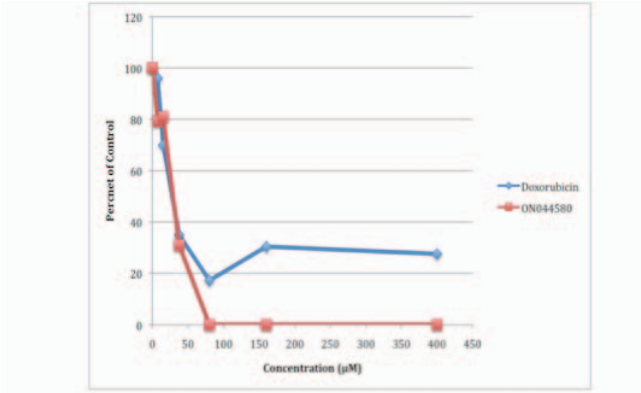

Figure 11. Growth inhibition of 32D:*p210*-T315I cells by ON044580-transferrin nanoparticles

After determining the safety of the nanoparticles in mice we tested their ability to be endocytosed by tumor cells and cause growth inhibition. We carried out cytotoxicity analysis using ON044580 and doxorubicin containing transferring nanoparticles on two cell lines: 32D:*p210*-T315I cells overexpressing the imatinib resistant mutant of BCR-ABL and Ba/F3:V617F-*JAK2* cells that ectopically overexpress the activated JAK-2 mutant.

As can be seen in Figure 11 the nanoparticles of both drugs were excellent inhibitors of tumor cell growth. Against 32D:*p210*-T315I cells both doxorubicin and ON044580 had

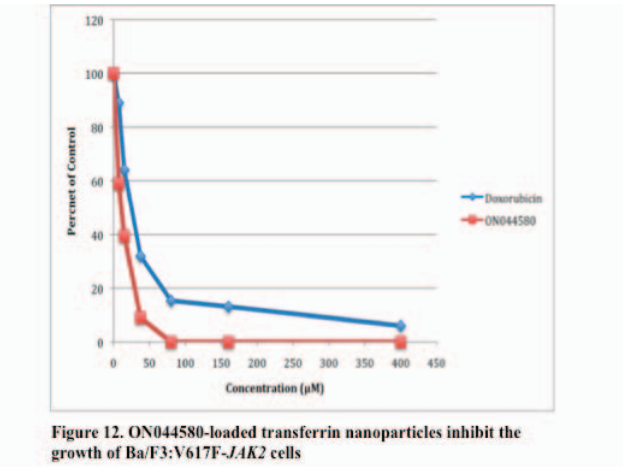

Figure 12. ON044580-loaded transferrin nanoparticles inhibit the growth of Ba/F3:V617F-*JAK2* cells

GI_{50} values of 25μM. However, as can be seen in Figure 12 against Ba/F3:V617F-*JAK2* cells ON044580 had a GI_{50} value of 9 μM as compared to 24 μM of Doxorubicin. This experiment demonstrates that the nanoparticles are endocytosed by the transferrin receptor system of actively dividing tumor cells and also that the drug is released from the nanoparticles leading to their cytotoxic effects. However, these GI_{50} values are inferior (350 nM for Ba/F3:V617F-*JAK2* cells) to those achieved by free ON044580 added directly to culture media. This 25 fold lower activity could be a consequence of the aggregation of these relatively large nanoparticles preventing their efficient uptake. Further improvements of nanoparticle preparation are ongoing with the aim to achieve diameters in the sub-100nm range. We will test the efficacy of these ON044580 nanoparticles and/or those of its bioactive analogs with better pharmacokinetic properties to treat mouse models of chronic myelogenous leukemia (CML) and myeloproliferative disorders (MPD).

Key Research Accomplishments.

1. By employing a high throughput screen, we have identified novel small molecule inhibitors of BCR-ABL that inhibit the proliferation and induce apoptosis of CML cell lines expressing the WT or the T315I mutant forms of BCR-ABL.

2. Of these compounds, ON044580 has been chosen for further investigation. We have found ON044580 to be a non-ATP competitive inhibitor that readily induces the down-regulation of BCR-ABL auto-phosphorylation and STAT-5 phosphorylation.

3. ON044580 has also been found to inhibit the kinase activities of WT and V617F mutant forms of JAK2 and is therefore a dual BCR-ABL/JAK2 kinase inhibitor.

4. ON044580 inhibits the proliferation and induces apoptosis of leukemic cell lines that express the V617F mutant form of JAK2.

5. We have developed a number of bioactive intermediates of ON044580 based on structure activity relationship studies. Further modifications of these intermediates are being developed to obtain an optimized lead molecule with more desirable pharmacokinetic properties and oral bio-availability.

6. We have also developed a transferin nanoparticle strategy for the delivery of ON044580 into tumor cells. Having determined the cytotoxicity of these nanoparticles and their excellent toxicity profile in mice we are working on improving the preparation for treatment of xenograft mouse models of CML and MPD.

Reportable Outcomes:

Publicaions:

1. Reddy MV, Pallela VR, Cosenza SC, Mallireddigari MR, Patti R, Bonagura M, Truongcao M, Akula B, Jatiani SS, Reddy EP. Design, synthesis and evaluation of (E)-alpha-benzylthio chalcones as novel inhibitors of BCR-ABL kinase. Bioorg Med Chem. 2010. **18**:2317-26.

2. Jatiani SS, Cosenza SC, Reddy MVR, Ha JH, Baker SJ, Samanta AK, Olnes MJ, Pfannes L, Sloand EM, Arlinghaus RB, and Reddy EP. A Non–ATP-Competitive Dual Inhibitor of $JAK2^{V617F}$ and $BCR\text{-}ABL^{T315I}$ Kinases: Elucidation of a Novel Therapeutic Spectrum Based on Substrate Competitive Inhibition. Genes & Cancer. *In Press*.

Conferences and presentations:

1. "Identification of a dual JAK-2/BCR-ABL kinase inhibitor for treatment of myeloproliferative disorders." Abstract accepted, poster presentation during the AACR 100th Annual Meeting 2009 in Denver, CO.
2. "Dual inhibition of JAK-2/BCR-ABL kinases for treatment of MPDs". Invited speaker during Fels Research Day 2008 at TUSM. Corresponding abstract published.
3. Poster presented in AACR/EORTC Conference 2007 in San Francisco, CA, USA.
4. Poster presented in International Symposium on Genomic Instability and Cancer at the University of Kashmir, Srinagar, India, 2007.

Conclusions

Our studies reported here allow us to make the following conclusions. Non-ATP competitive inhibitors of BCR-ABL are effective inhibitors of imatinib-resistant forms of BCR-ABL including the T315I-BCR-ABL. Because of their substrate-competitive nature, some of these inhibitors also inhibit JAK2 kinase activity. These compounds are useful therapeutic agents for CML as well as MPDs arising due to mutations in JAK2. Of these, ON044580 was extensively studied and is currently undergoing pre-clinical evaluation in preparation to Phase I human trials. We have also developed a nanoparticle strategy for the delivery of ON044580 into tumor cells.

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Work Accomplished by Dr. Arlinghaus' Group at M.D. Anderson Cancer Center

Background:

In chronic myelogenous leukemia (CML), Bcr-Abl, the fusion protein derived from Philadelphia chromosome, is the constitutively activated protein tyrosine kinase, which is largely unregulated (1-3). It is widely known that Bcr-Abl drives several important signaling pathways- the Ras, PI-3 kinase, STAT5, STAT3 and Jak2 pathways that cause oncogenesis in CML (4-10). Since these important pathways are derived from Bcr-Abl, it is considered to be the critical target molecule for CML therapy. Imatinib mesylate (IM) is an effective inhibitor of the Bcr-Abl tyrosine kinase and is the first-line treatment of CML, since about 75% of early chronic phase CML patients favorably respond to IM treatment. During longer-term treatment with IM, progression of the disease and drug resistance can develop in patients for several reasons (11-18). Continuous targeting of Bcr-Abl can lead to blastic transformation (19) due to activation of other oncogenes and inactivation of tumor suppressor genes. The remission rate of accelerated phase is 50% and for blast crisis phase the remission rate is 20% (17, 20). Alterations of tumor suppressors such as PP2A, mutation of p53, inactivation of tyrosine phosphatases (Shp1), over-expression of new proteins (e.g. SET) leads to the terminal blast crisis stage and ultimately death of the patients. More potent forms of IM (i.e. Nilotinib, NS-187) have been developed for treatment of IM-resistant patients (21) but they fail to kill cells from the blast crisis stage. The dual kinase inhibitor dasatinib (Bcr-Abl and Lyn) is successful in the induction of apoptosis of several IM-resistant Bcr-Abl mutant cells blast crisis patients (22), but dasatinib fails to kill T315I Bcr-Abl mutant cells. Dasatinib-resistant CML has been reported, as 20 out of 21 patients treated with dasatinib developed resistant CML cells containing the T315I mutation (Soverini, S. et al 2006, 2007) (23, 24). Several other second generation drugs were developed for CML therapy, but each drug has its own limitations (25). Although overcoming IM resistance can be achieved for some forms of IM-resistance caused by mutations in BCR-ABL, specific drugs for the T315I BCR-ABL IM-resistant mutant have not yet been developed nor are drugs available to treat blast crisis CML. Untreated chronic phase may last for several years, the accelerated stage lasts for only 4-6 months, and the terminal blast crisis stage, characterized by rapid expansion of either myeloid or lymphoid differentiation-arrested blast cells (blast crisis), lasts for only a few months (17, 18). No successful therapeutic strategy of blast crisis exists at the present time. Allogeneic stem cell transplantation with high chemotherapy has been found to be successful in a small percentage (10%) of patients. New target molecules and specific inhibitor(s) need to be developed to treat advanced stages of CML, particularly blast crisis patients.

Since Bcr-Abl, is considered to be the primary therapeutic target molecule in CML, the stability and regulation of Bcr-Abl in CML cells is one of the critical issues for development of new therapeutic strategies required to overcome drug-resistance. Neviani et al. (26) demonstrated that Bcr-Abl regulates its own stability by inhibiting PP2A-Shp1 phosphatases by inducing expression of tumor suppressor protein SET (26, 27). Our previous studies demonstrated that Jak2 is a major down-stream signaling molecule in CML. It has been shown that Jak2 interacts with Bcr-Abl (9), induces high level c-Myc expression (28), induces tyrosine phosphorylation of Gab2 on YxxM sequences needed for activation of PI-3 kinase (29), is part of a Bcr-Abl network involving proteins such as Akt and GSK3β (29) and regulates SET protein in Bcr-Abl+ cells (30). Jak2 also maintains Lyn kinase in its functionally active form in Bcr-Abl+ cells through a Jak2-SET-PP2A-Shp1 signaling loop where PP2A –Shp1 remained inactive by Jak2 activated SET expression (30). These results indicate that Jak2 is one of the important signaling molecules in Bcr-Abl+ cells.

HSP90, a major molecular chaperone, is known to interact with proteins involved in transcriptional regulation and signal transduction pathways for maintaining the stability and functional conformation of signaling proteins (31-34). HSP90 acts as a biochemical buffer against genetic instability during cancer. HSP90 is responsible for the maturation and functional stability of a plethora of polypeptides called client proteins. HSP90 is over-expressed in leukemia and also in many other cancers and it is assumed that in the cancer, the requirement of HSP90 is critical since most of the client proteins of HSP90s are active participants in signal transduction pathways of cancer cells. These qualities and functional aspects of HSP90 make it a potential target for anti-cancer drugs. Although several small molecules have been identified as anti-HSP90 candidates during past years, none of them have yet been successful in the clinic. Sawyers and colleagues first showed that inhibition of HSP90 expression by 17-AAG caused reduction of wild-type and mutant Bcr-Abl proteins leading to inhibition of growth (14). Later Blagosklonny et al. demonstrated that BCR-ABL+ cells were induced to undergo apoptosis upon treatment with 17-AAG (14, 35). These qualities and functional aspects make HSP90 a potential target for the development of anti-cancer drugs.

Body

In the current study, we have shown that ON044580 showed strong apoptotic activities in Bcr-Abl+ cells and overcomes drug resistance. These apoptotic events were initiated in part due to destabilization of the Bcr-Abl protein from where major signaling pathways originate. We have further demonstrated that ON044580 disrupted a high molecular weight Bcr-Abl/Jak2/HSP90 network structure. These results were obtained due to the unique Jak2 and Bcr-Abl kinase inhibitory properties of ON044580, which make it a novel and potentially useful compound for CML therapy.

Results : ON044580, α–benzoyl styryl benzyl sulfide, is a new compound synthesized by Dr. Reddy' s group (Jatiani, S. et al, Genes and Cancer, 2010) that is not an ATP competitor like many of the tyrosine kinase inhibitors such as IM but inhibits the catalytic activities of Abl (and Bcr-Abl) and Jak2. We present results on the role of ON044580 in modulating Bcr-Abl driven cell signaling pathways and its effects on cell viability, apoptosis and colony formation in soft agar.

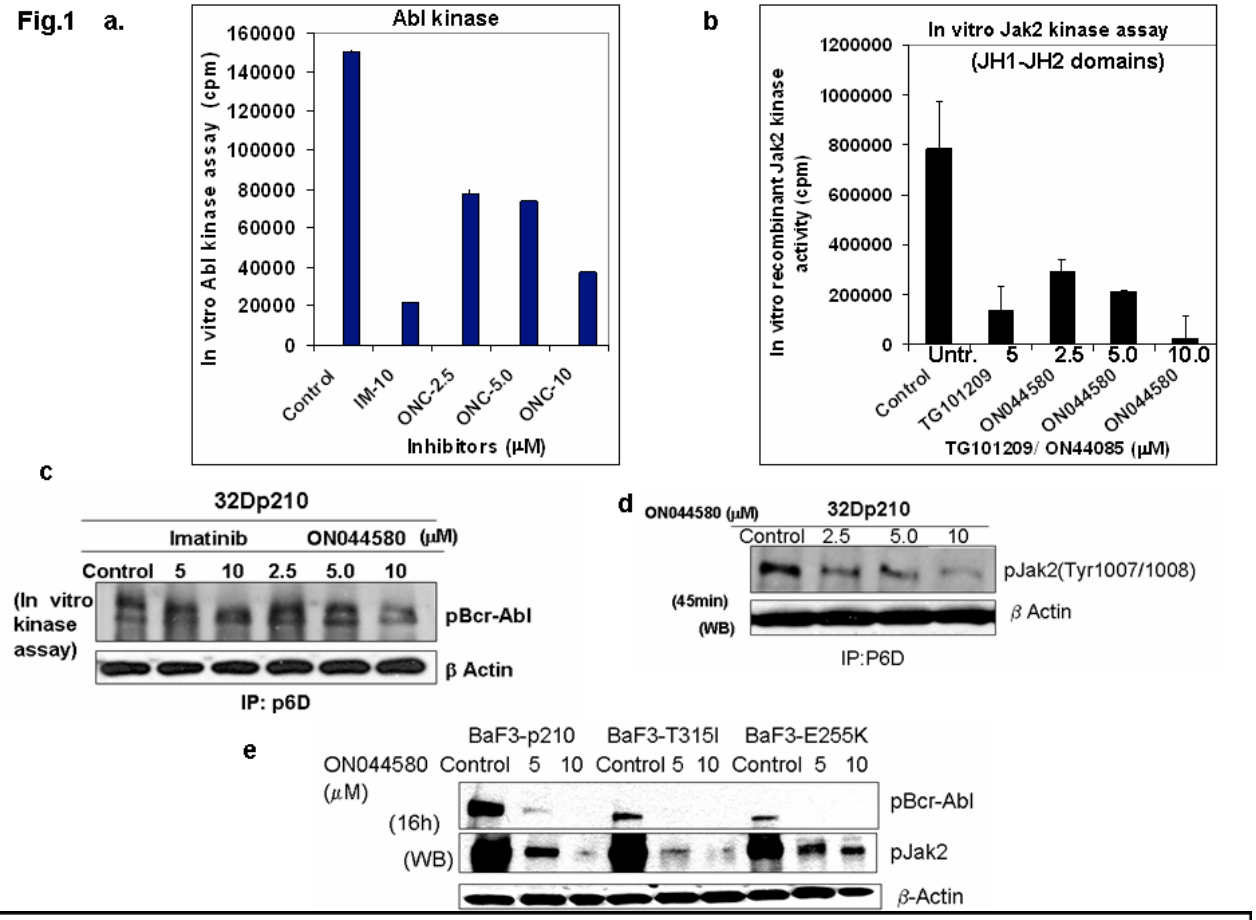


Fig.1. pJak2 and pBcr-Abl are inhibited by ON044580. (a) Inhibition of recombinant Abl kinase by ON044580 during in vitro kinase assay using the Abltide peptide as substrate. . **(b)** Inhibition of recombinant Jak2 kinase (JH1 and JH2 domains) by ON044580 during in vitro kinase assay using a Jak2 peptide as substrate. **(c)** Inhibition of pBcr-Abl by ON044580 during in vitro kinase assay of Bcr-Abl. **(d)** Inhibition of Jak2 by ON044580 during in vitro kinase assay. Detergent extracted Bcr-Abl+ cell lysates were immunoprecipitated with p6D anti-Abl antibody following the standard protocol. **(e)** pBcr-Abl and pJak2 inhibition in Bcr-Abl+ IM-sensitive and -

Recombinant Abl and Jak2 kinase assays. To examine the effects of ON044580 on Abl and Jak2 kinases, we performed in vitro kinase assays with purified recombinant Abl (45 kDa Abl kinase) and Jak2 kinase (JH1-JH2) using Abl tide substrate for assays with Abl kinase and Jak2 peptide containing the Tyr 1007 activation site for the Jak2 kinase, respectively. IM inhibited the phosphorylation of Abl tide by recombinant Abl about 85%, whereas ON044580 at 5μM and 10μM reduced the Abl kinase activity by 50% and 75%, respectively

(Fig. 1a). In the Jak2 kinase assay with JH1-JH2 domains, ON044580 strongly reduced Jak2 kinase activity in a dose-dependent-manner (Fig. 1b). As a positive control TG101209, an authentic Jak2 inhibitor (42) was used which strongly reduced phosphorylation of the Jak2 peptide. These studies indicate that both recombinant Abl kinase and Jak2 kinase are strongly inhibited by ON044580, suggesting that ON044580 is a dual kinase inhibitor (Fig. 1a and b).

ON044580 strongly inhibited Jak2 and Bcr-Abl tyrosine kinase activity in kinase assays performed with immune complexes from Bcr-Abl+ 32D cells. In order to further investigate the effects of ON044850 on the Jak2 kinase, we performed in vitro autophosphorylation assays of Jak2 using Bcr-Abl+ cell lysates. Our previous findings indicate that Jak2 is associated with the C-terminus of Bcr-Abl (9). Based on that observation, for the Jak2 kinase assay, we immunoprecipitated Bcr-Abl from detergent extracted Bcr-Abl+ 32D cell lysates with Abl-specific antibody (P6D). After repeated washing of the immunoprecipitates, the kinase assays were performed using the protocol described for Jak2 kinase (9, 43). The kinase supernatant was analyzed by Western blotting using anti-pTyr (4G10) to detect tyrosine-phosphorylated P210 BCR-ABL (Fig. 1c) and anti-pJak2 (Tyr1007/1008) to detect activated Jak2 (Fig. 1d). We observed that both Bcr-Abl kinase and Jak2 kinase activities were reduced in the presence of ON044580 (Fig.1c and d).

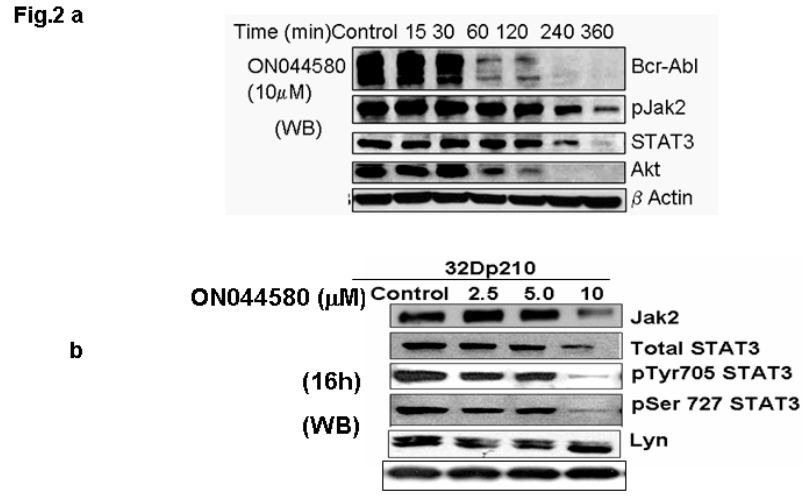
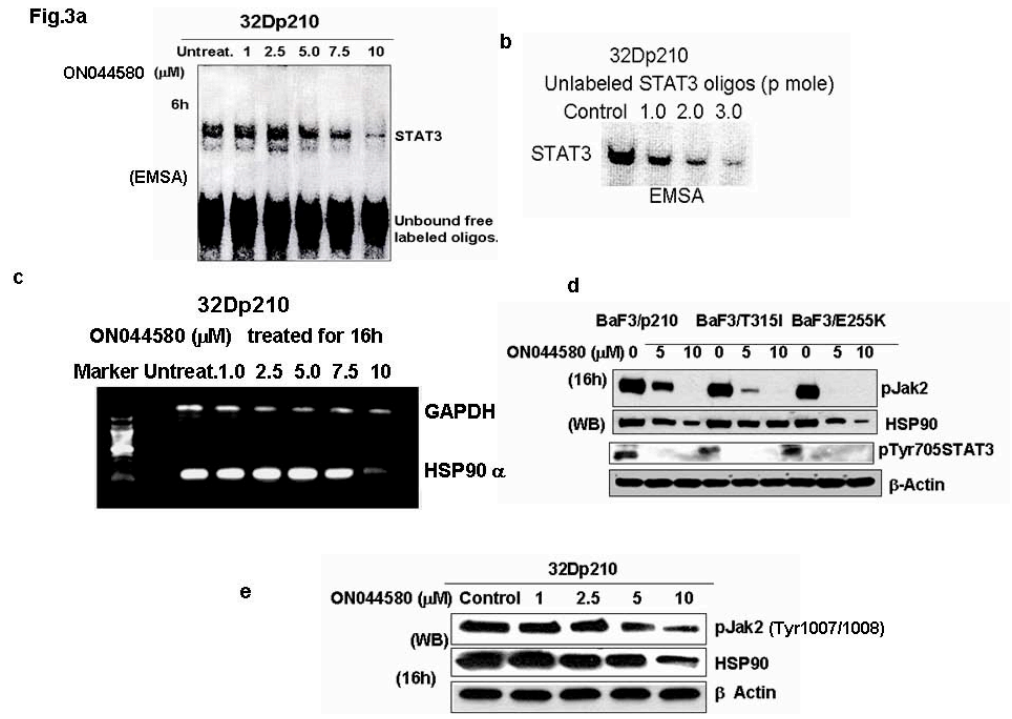


Fig.2. ON044580 mediated inhibition of Jak2 and Bcr-Abl kinases induced reduction of downstream targets of Bcr-Abl signaling molecules. (a) Bcr-Abl+ 32D cells were incubated with 10 μM ON044580 for different time periods as marked. **(b)** shows reduced expression STAT3, pTyr 705 STAT3, pSer 727 STAT3 by different doses of ON044580 for 16h.

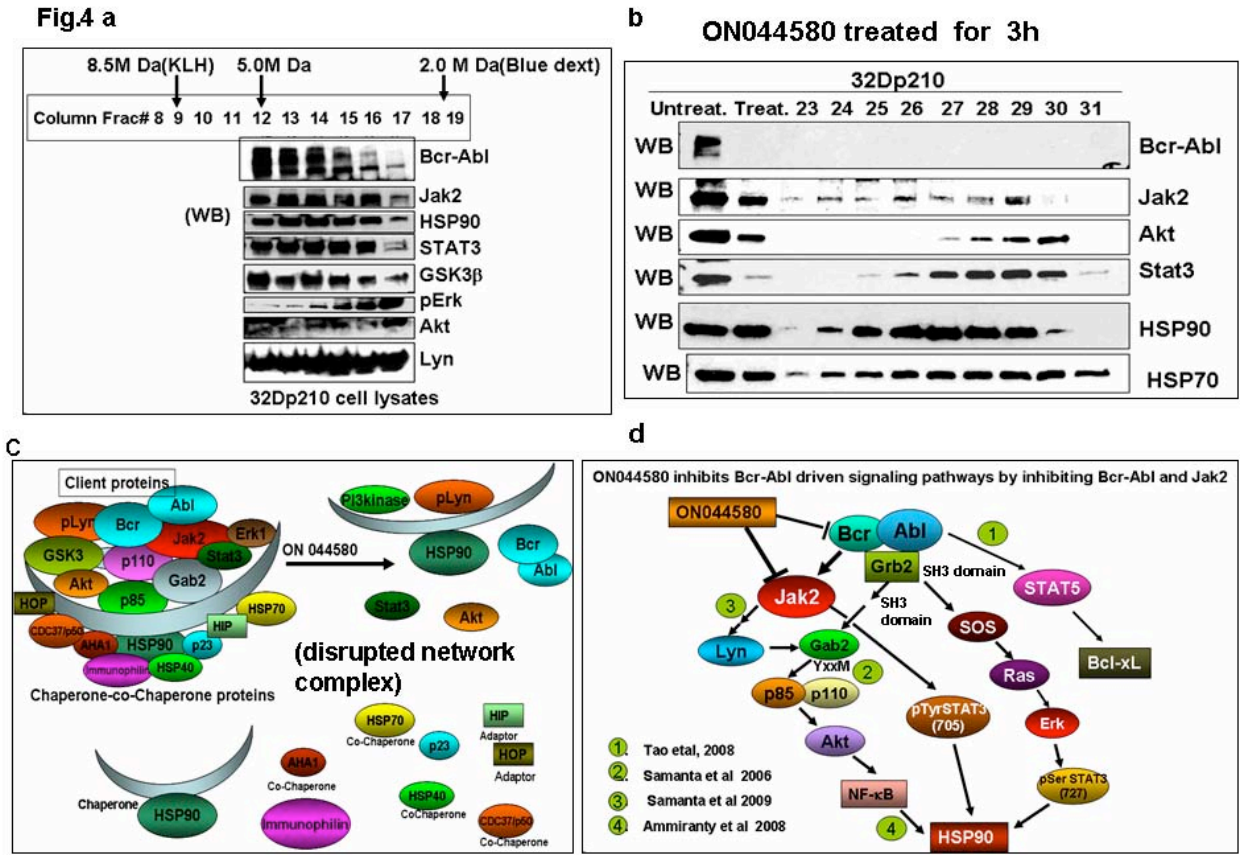


Treatment of IM-resistant cells with ON044580 reduced pTyr Bcr-Abl and pTyr Jak2. We incubated Bcr-Abl+ IM-sensitive (BaF3p210) and IM-resistant cells (BaF3p210 T315I and BaF3p210 E255K cells) with different doses of ON044580 for 16 h. Cell lysates were prepared by detergent extraction and the lysates were analyzed by Western blotting using anti-pTyr antibody (4G10). We observed that the levels of both pTyr Jak2 and pTyr Bcr-Abl were sharply reduced with 16 h incubation (Fig. 1e). However, the Bcr-Abl protein was found to rapidly disappear from the lysate within 2 h of 10 μM ON044580 treatment whereas Jak2 protein levels were not affected during these 2 h treatments. The dose needed to reduce the Bcr-Abl protein levels began at 2.5 μM and was complete at 10μM (Supplement Fig. 1a and b). These studies indicate that treatment of Bcr-Abl+ cells with ON044580 may affect either the stability or solubility of Bcr-Abl.

Fig. 3. ON044580 reduced binding of STAT3 to its consensus sequence and also reduced expression of HSP90 at transcription and translational levels. (a) Binding of STAT3 (obtained from nuclear extract preparation of Bcr-Abl+ 32D cells) to its consensus oligonucleotide radio labeled with 32P ATP was reduced by ON044580 in a dose-dependent manner as elucidated by the electrophoretic mobility shift assay (EMSA). **(b)** The signals for STAT3 binding is specific as increasing amount of unlabeled STAT3 oligos replaced the radiolabeled STAT3 oligos binding to the consensus STAT3 DNA sequence. **(c)** ON044580 reduced HSP90 expression at the transcriptional level. Bcr-Abl+ cells were incubated with ON044580 for 16h and RNA was extracted from the treated cells. RT-PCR was carried out for HSP90 using specific primers for HSP90 and GAPDH (loading control). **(d)** Exposure of ON044580 to the Bcr-Abl+ IM-sensitive and -resistant cells for 16h reduced HSP90 and pJak2 at protein levels. (**e**). ON044580 reduced the levels of pJak2, HSP90 in Bcr-Abl+ cells. After incubation of Bcr-Abl+ cells with ON044580 for 16h, the detergent extracted cell lysates were analyzed by Western blotting and the membrane was probed with pJak2 (Tyr1007/1008) and HSP90 antibodies.

Bcr-Abl, Jak2 and their downstream signaling molecules are reduced in amount by ON044580 in Bcr-Abl+ cells. We addressed the question of whether or not treatment of Bcr-Abl+ cells with ON044580 affected downstream signaling molecules of Bcr-Abl. To examine this possibility, we incubated Bcr-Abl+ 32D cells for 6h using 10μM ON044580 and for 16h with increasing amounts (0 to 10 μM) of the inhibitor. The detergent extracted lysates were analyzed by Western blotting using several antibodies. We observed that in addition to the reduction of Bcr-Abl, pTyr Jak2, STAT3 and Akt levels were also reduced during 6h incubation of Bcr-Abl+ cells with ON044580 (Fig.2a). We further observed that a 16 h incubation of Bcr-Abl+ cells with ON044580 reduced not only Jak2 and STAT3 levels but also pTyr705 and pSer727 STAT3 levels. Interestingly, Lyn was unaffected (Fig. 2b). It is known that Bcr-Abl, Jak2, and STAT3 are the client proteins of HSP90 (44-47), but Lyn has not been reported to be a client protein of HSP90. Thus, our results also suggest that Lyn is not a client protein of HSP90.

ON044580 reduced binding of STAT3 to its consensus sequence in Bcr-Abl+ cells. It is known that tyrosine phosphorylation of STAT3 plays a key role in the dimerization of STAT3, nuclear translocation and



binding to specific DNA consensus sequence of STAT3 whereas serine phosphorylation of STAT3 is essential

for maximum transcriptional activity (48, 49). Since Tyr 705 STAT3 phosphorylation was reduced by

Fig. 4. ON044580 induced disruption of Jak2-Bcr-Abl-STAT3- HSP90 network complex. (a) Detection of a large molecular weight signaling network complex comprised of Bcr-Abl, Jak2 and HSP90 and other proteins (e.g. STAT3, Akt, Erk, GSK3 and Lyn) by gel filtration column chromatography. Proteins from the detergent extracted lysate of Bcr-Abl+ 32D cells were eluted from the column by a detergent-containing buffer. From each eluant a 25µl aliquot was taken and was analyzed by Western blotting and the membrane was probed with different antibodies as indicated. **(b)** Treatment of Bcr-Abl+ 32D cells with 10µM ON044580 for 3h disrupted the Bcr-Abl/Jak2/HSP90 network structure. The procedure for analysis was same as used for (a). **(c)** Diagrammatic representation shows how ON044580 interrupted Bcr-Abl driven signaling pathways affecting Jak2 and Bcr-Abl kinases and disrupted the Bcr-Abl/Jak2/HSP90 network complex. **(d)** Diagrammatic representation to show how ON044580 inhibited Jak2 and Bcr-Abl and how the events of that inhibition affect downstream target molecules in Bcr-Abl+ cells.

ON044580, it was expected that DNA binding of STAT3 to its consensus sequence would be interrupted. Therefore, we examined the binding of STAT3 to its consensus sequence by electrophoretic mobility shift assays (EMSA). STAT3, obtained from nuclear extracts of ON044580-treated Bcr-Abl+ 32D cells (16 h), was allowed to interact with its radiolabeled consensus STAT3 oligonucleotide DNA sequence (50). Bcr-Abl+ cells treated with ON044580 had strongly reduced the STAT3-specific DNA binding activity in a dose-dependent manner (Fig.3a). The assay signal for STAT3 is specific because competition with non-radioactive consensus sequences strongly competed with the radioactive target oligonucleotides in a dose-dependent manner (Fig. 3b, right panel). Similarly, addition of STAT3 antibody to the nuclear lysate caused a mobility shift of the STAT3 complex (not shown), indicating that the signals for STAT3 in EMSA (Fig. 3a) are STAT3 specific.

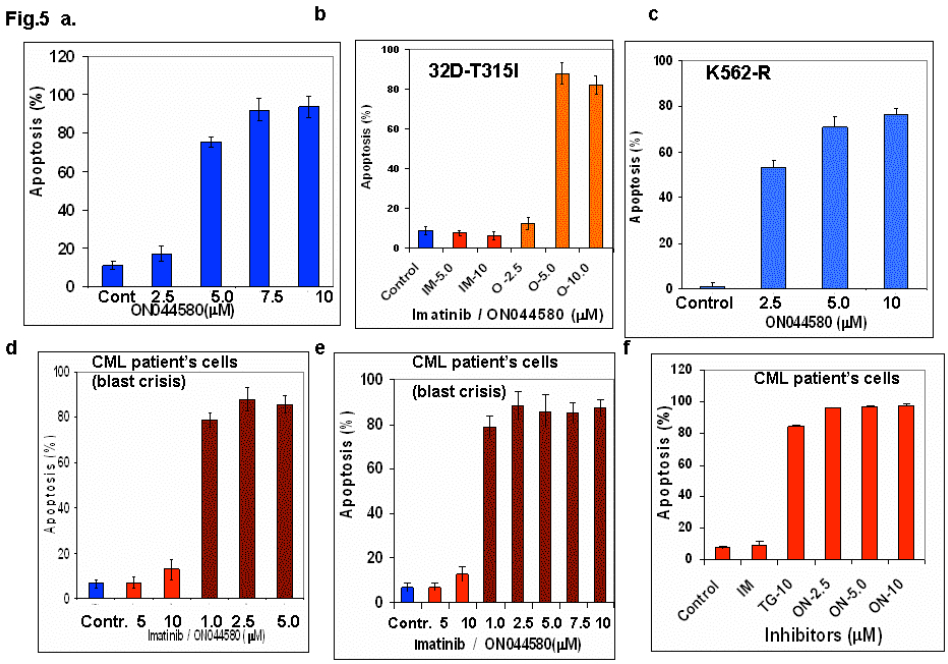
***ON044580 decreased the levels of HSP90 in Bcr-Abl+ cells*.** HSP90 is reported to be a chemotherapeutic target molecule for many cancers including CML (35, 36, 47, 51). Some of the critical signaling molecules in Bcr-Abl+ cells are client proteins of HSP90 (14, 46, 52). We examined whether ON044580 regulated the expression of HSP90 at the transcriptional level. For this we performed RT-PCR assays using HSP90 primers. We treated 32Dp210 cells with ON044580 for 16 h. We note that the HSP90α promoter has a binding site for STAT3 (not shown). Of interest, ON044580 at 10 µM strongly reduced HSP90α transcripts at 16 h of treatment (Fig.3c), which coincides with the amount of ON044580 required to inhibit STAT3 binding to its consensus sequence (Fig. 3a). HSP90α protein levels in IM-sensitive and -resistant cells were also reduced by incubation of cells with 5 and 10µM ON044580 for 16 h. However, T315I cells were partially resistant to HSP90 reduction by ON044580 at 16 h despite the high sensitivity to ON044580 to reduction of activated STAT3 (Fig. 3d). Nevertheless, these results suggest that Jak2 kinase may regulate expression of HSP90α through Jak2's ability to activate STAT3 in Bcr-Abl+ cells Fig. 3a-e).

Fig. 4. ON044580 induced disruption of Jak2-Bcr-Abl-STAT3- HSP90 network complex. (a) Detection of a large molecular weight signaling network complex comprised of Bcr-Abl, Jak2 and HSP90 and other proteins (e.g. STAT3, Akt, Erk, GSK3 and Lyn) by gel filtration column chromatography. Proteins from the detergent extracted lysate of Bcr-Abl+ 32D cells were eluted from the column by a detergent-containing buffer. From each eluant a 25µl aliquot was taken and was analyzed by Western blotting and the membrane was probed with different antibodies as indicated. **(b)** Treatment of Bcr-Abl+ 32D cells with 10µM ON044580 for 3h disrupted the Bcr-Abl/Jak2/HSP90 network structure. The procedure for analysis was same as used for (a). **(c)** Diagrammatic representation shows how ON044580 interrupted Bcr-Abl driven signaling pathways affecting Jak2 and Bcr-Abl kinases and disrupted the Bcr-Abl/Jak2/HSP90 network complex. **(d)** Diagrammatic representation to show how ON044580 inhibited Jak2 and Bcr-Abl and how the events of that inhibition affect downstream target molecules in Bcr-Abl+ cells.

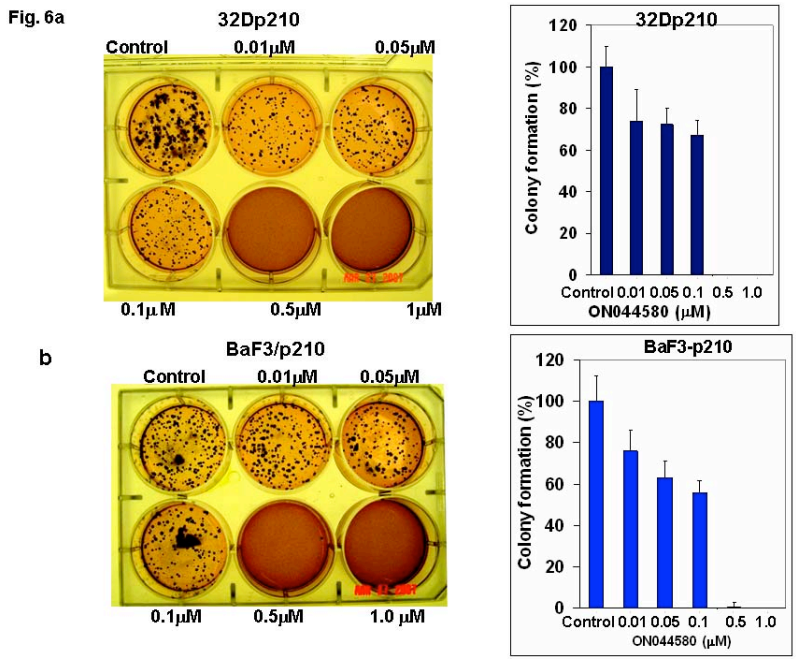
Identification of a large network complex in Bcr-Abl+ cells and disruption of that complex in ON044580 treated cells. From our previous studies with various co-immunoprecipitation experiments, we showed that immunoprecipitation of one member of the Bcr-Abl signaling pathway co-precipitated other members of the pathway. Therefore, we predicted that the presence of a large molecular network complex in Bcr-Abl+ CML cells (31). In order to identify, characterize and estimate the relative size of the Bcr-Abl/Jak2 Network complex, we performed gel filtration column chromatography as a means to determine whether the Bcr-Abl/Jak2 network complex could be detected in a high molecular weight region of the column eluant. In collaboration with our Proteomics Core Facility, we optimized and calibrated the gel filtration column with different marker proteins

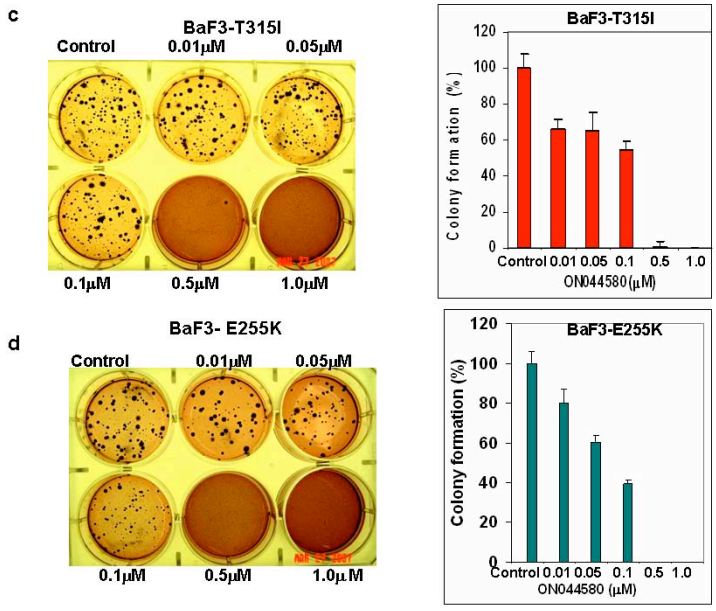
ranging up to eight million molecular weight (Supplement Fig. 1e). Cell lysates of Bcr-Abl+ 32 D cells (32Dp210) were fractionated on the gel filtration column and eluted with a buffer containing NP-40 and glycerol. Fractions were analyzed by Western blotting with various antibodies so as to detect several proteins thought to be present in this network complex (Fig.4a). We detected several signaling proteins including HSP90 in the same fractions of the column eluant (e.g. fraction 12) suggesting the presence of high molecular weight protein complexes (Fig.4a), which were estimated to be in the 4-6 million Dalton molecular size fraction. The Bcr-Abl/ Jak2 network proteins included pTyrJak2 (1007/8), pLyn (Tyr 396), Lyn, Akt, STAT3, GSK3β, pErk and HSP90; several column fractions contained these high molecular weight complexes (Fig. 4a and supplement Fig.1e). Similar results were obtained with lysates of K562 cells (Supplement. Fig1d). The decrease in levels of Bcr-Abl and several other signaling proteins by treatment with ON044580 (Fig. 2a) suggested that this dual kinase inhibitor might disrupt the network structure. To determine whether the elution pattern of the network would be affected by ON044580 treatment, we incubated 32Dp210 cells with 10 µM ON044580 for 3h and the cell lysate was loaded into the column. We observed that Bcr-Abl/Jak2/HSP90 network complex was disrupted, as Bcr-Abl protein was severely reduced in amount and as were other members of the network. Importantly, HSP90 and other the client proteins eluted at a much lower molecular size (Fig. 4b). Although the levels of Jak2, STAT3, Akt were reduced in the column fractions of ON044580-treated lysates, the levels of HSP90 remained almost unchanged but eluted at a much lower molecular size, as the position of the HSP90 protein shifted from elution at the higher molecular weight fractions (e.g. 12-15) to the lower size fractions (Fractions 24-27), indicating that network had been disrupted. These results suggest the following: 1) that the Bcr-Abl/Jak2 network is bound to HSP90; 2) and that decrease in Bcr-Abl and inhibition of both Bcr-Abl and Jak2 kinases lead to disruption of the network structure by separation of Bcr-Abl and Jak2 from its signaling partners. We hypothesize that HSP90 client proteins such as Bcr-Abl are more susceptible to proteolytic degradation when the network structure is disrupted by treatment with ON044580. Under identical conditions lysates of Bcr-Abl+ 32D cells treated with 10 µM imatinib for 6h did not show degradation/ dissociation of signaling molecules (Supplement Fig. 1c). A hypothetical model for disruption of the network by ON044580 is shown in Fig. 4c.

ON044580 induced apoptosis in Bcr-Abl+ cells and overcomes drug resistance in Bcr-Abl+ leukemia cells. Our studies demonstrate that ON044580 strongly inhibits Jak2 and Abl kinase activities, and as a result the levels of downstream signaling molecules are reduced and the large Bcr-Abl/Jak2/HSP90 network complex is disrupted. We next examined how these inhibitory effects on the Bcr-Abl/Jak2/HSP90 network structure affected cell survival. For that purpose we did cell viability/ proliferation assays (MTT), apoptosis assays and colony formation assays. We first assessed the effects of ON044580 on cell viability and proliferation by MTT assays. IM-sensitive Bcr-Abl+ cells (32Dp210) and IM-resistant cells (e.g. K562-R) were inhibited by ON044580, as the viability was reduced in a dose-dependent manner [the IC50 of ON044580 for 32Dp210 and K562R cells were 3-5µ M (Supplement Fig.2a and b)]. Apoptosis assays on several Bcr-Abl+ IM-sensitive and -resistant hematopoietic cell lines (Fig. 5a-c and Supplement Fig.2c and d, 3a and b) was conducted by staining with Annexin and Propidium Iodide followed by Flow Cytometric analysis. Results from this study showed that ON044580 was a potent inducer of apoptosis at concentrations of 1-5 µM. IM-sensitive Bcr-Abl+ cells-32Dp210 and BaF3-p210 cells were very sensitive to ON044580 to apoptosis induction, and 5µM of ON044580 induced >80% apoptosis. IM-resistant cells such as T315I mutant cells and E255K, and K562-R cells although resistant to IM were very sensitive to



apoptosis induction by ON044580 (Fig. 5b). The T315I mutant is termed the gatekeeper mutation (11), and all known kinase inhibitors that target the ATP binding domain of the Bcr-Abl tyrosine kinase fail to induce apoptosis in T315I cells (Fig.5b). Therefore, it is quite significant that ON044580 induced apoptosis in T315I mutant cells. Similar results were obtained with the E255K IM-resistant mutant of Bcr-Abl (not shown).





ON044580 induces apoptosis in primary cells from CML patients. After examination in IM-sensitive and resistant Bcr-Abl+ cell lines, we tested the ability of ON044580 to kill cells from blast crisis CML patients, which are largely resistant to many drugs. As can be seen in Fig. 5d, e and f and Supplement Fig 4a.b and c, white blood cells from the peripheral blood of blast crisis CML patients are quite resistant to IM (5 and 10μM) but are very sensitive to ON044580. Most interestingly, primary CML cells are very sensitive to low doses (1-2 and 0.5μM) of ON044580. We observed that blast crisis patient cells, some of which are resistant to IM, are induced to undergo apoptosis by ON044580 with values ranging from 70-90% (Fig 5d, e and f and supplemental fig. 3c-e).

ON044580 strongly inhibited colony formation at low doses in IM-sensitive and -resistant Bcr-Abl+ cells. Anchorage-independent growth is a cell culture surrogate for tumor behavior in mice. We assessed the ability of ON044580 to inhibit colony formation in soft agar cultures. Cells were seeded into soft agar culture medium at the single cell level. Cultures were allowed to incubate for two weeks in the presence of different doses of ON044580. Colonies were stained, photographed and counted to assess the remaining colony number after the drug treatment. Cells that were both IM-sensitive and IM-resistant were tested (Fig.6a-d). In general, colony formation was completely inhibited at 0.5 μM ON044580. Importantly, IM-resistant forms of Bcr-Abl + cells were also inhibited at similar concentrations (Fig. 6c and d). The results showed that ON044580 severely inhibited colony formation at levels between 0.1 and 0.5 μM (right panels of Fig. 6a-d). These results suggest that oncogenic ability of IM-sensitive and -resistant Bcr-Abl+ cells are inhibited by ON044580 at lower concentrations compared to the concentrations required for apoptosis and MTT assays.

Fig.6. ON044580 reduced soft agar colony formation of IM-sensitive and –resistant Bcr-Abl+ cells in a dose-dependent manner. The experiments were carried out in duplicate plates and the mean counts of colonies in percentage are graphically presented in the right panels. **(a)** and **(b)** represent the reduction of colony formation of IM-sensitive Bcr-Abl+ 32D and BaF3, respectively. **(c)** IM-resistant BaF3-T315I cells. **(d)** IM-resistant Bcr-Abl+ BaF3-E255K cells.

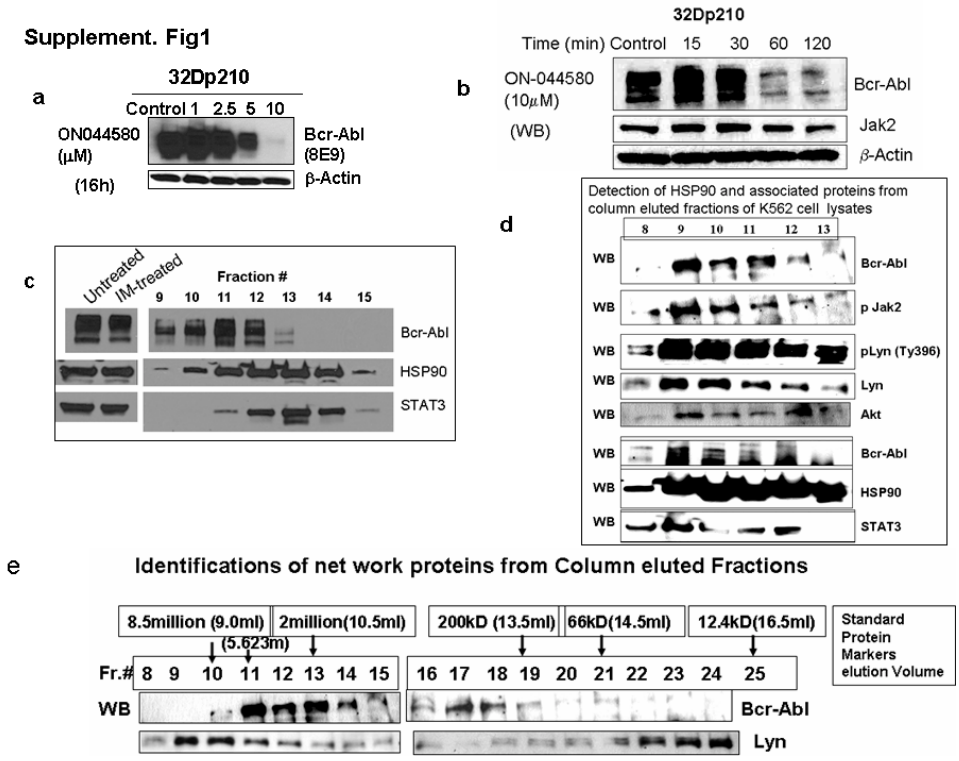


Fig. 1 (a) ON044580 decreases levels of Bcr-Abl in a dose-dependent manner. Bcr-Abl+ cells were treated with various doses of ON044580 for 16h and Western blotting of the cell lysates was performed and the blots were probed with anti-Bcr-Abl antibody (8e9). **(b) ON044580 decreases levels of Bcr-Abl within 60 min.** Bcr-Abl+ cells were treated with 10μM ON044580 in a time-dependent manner for up to 2h and analyzed by Western blotting**. (c) Detection of Bcr-Abl/Jak2/ HSP90 network complex in 32Dp210 cells treated with imatinib.** Bcr-Abl+ cells were treated with 10 μM IM for 6h and the proteins of the detergent-extracted cell lysates were passed through the gel filtration column. The eluants were analyzed by Western blotting. **(d) Detection of the Bcr-Abl/Jak2/HSP90 network in K562 cells.** Fractionation of proteins from a K562 cell lysate on a gel filtration column, as described in Fig. 4a. Detection of signaling molecules in the column eluant was performed by Western blotting. **(e) Detection of Bcr-Abl and Lyn from the Bcr-Abl+ cell lysate fractionated on a gel filtration column.** This column fractionation was carried out following the same method as it is done in Fig.4a. Only identification of the signals for Bcr-Abl and Lyn are presented.

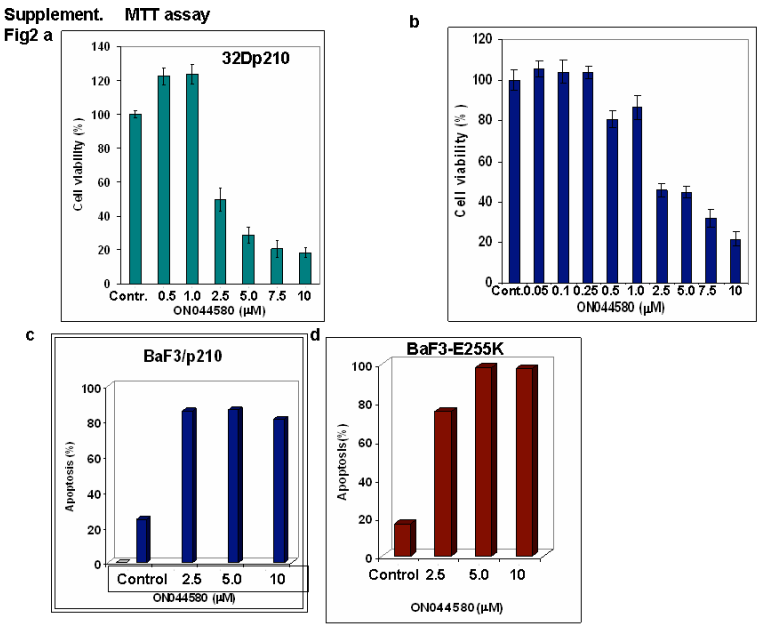
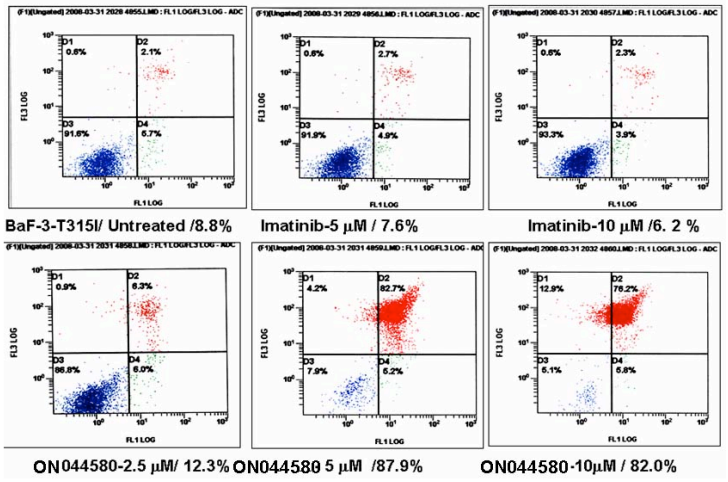
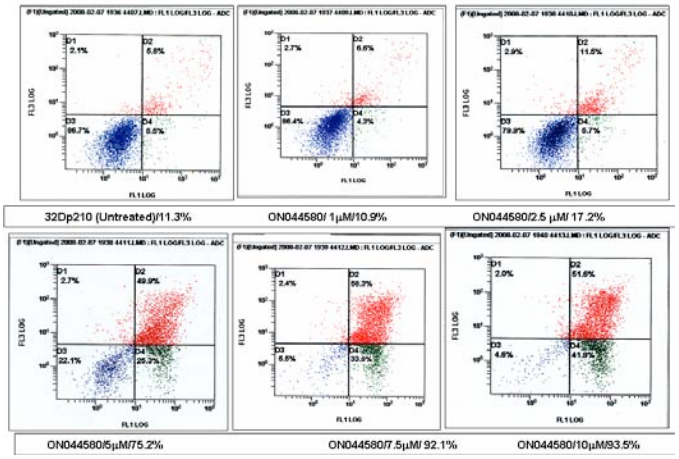


Fig.2. ON044580 decreases cell viability of IM-sensitive Bcr-Abl+ 32D cells and IM-resistant K562-R cells. (a) and (b) Cell viability assays (MTT) of Bcr-Abl+ 32D cells and IM-resistant K562-R cells for 72h in presence of ON044580, respectively. **(c)** Apoptosis values for BaF3p210 and **(d)** IM-resistant BaF3-E255K cells treated with ON044580 for 48h were measured by AnnexinV/PI methods following manufacturer protocol.

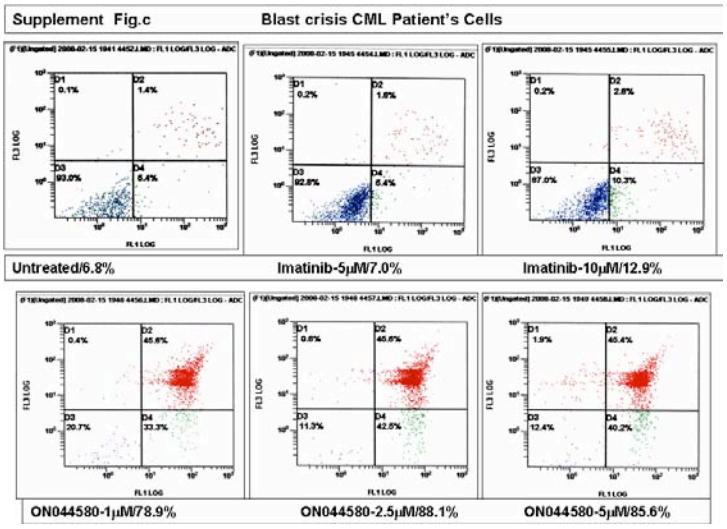
Supplement Fig.3a



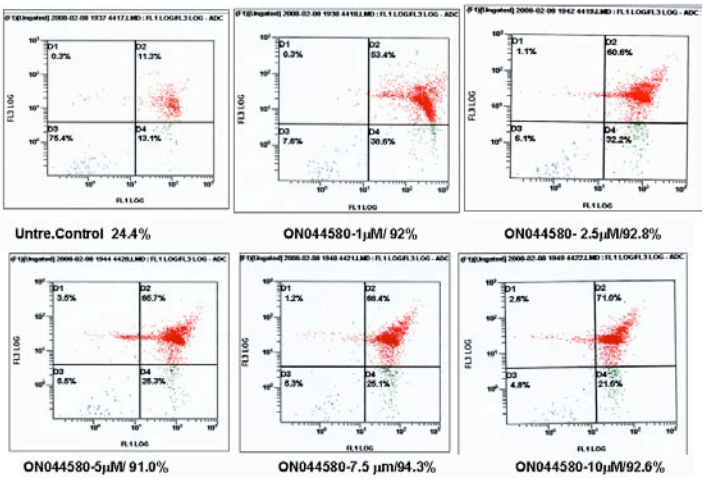
Supplement Fig.3b



Supplement Fig.c Blast crisis CML Patient's Cells



Supple. Fig.d Blast crisis CML patients' cells (81% blasts)



Supplement Fig.e

Blast crisis CML patient's cells

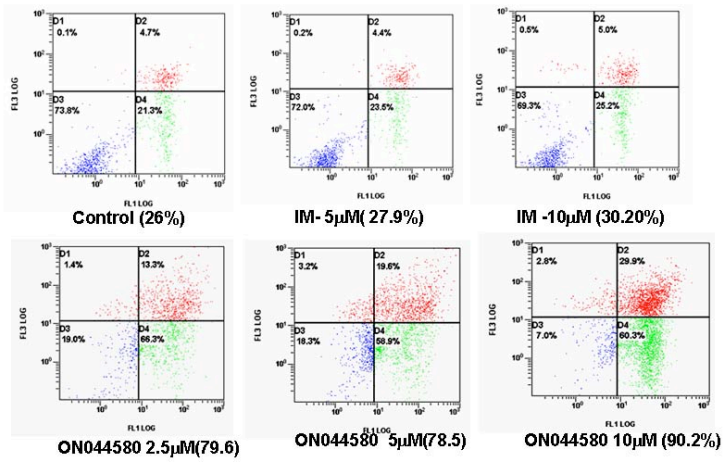


Fig.3. Original apoptosis data obtained from flow cytometry of **(a)** BaF3-T315I**, (b)** 32Dp210 **(c, d** and **e)** for three CML patients cells treated with ON044580 for 48h in a dose-dependant manner. The graphical presentations of these data are shown in Fig.5**.**

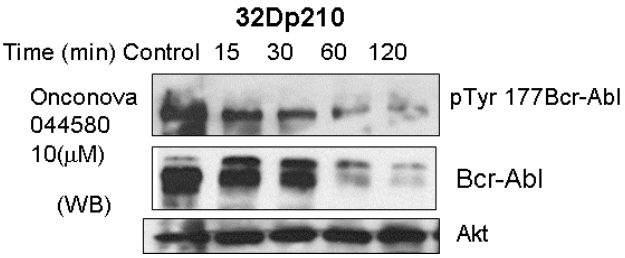


Fig.E1 Incubation of Bcr-Abl+ cells with ON-044580 reduced pTyr177Bcr-Abl as well as Bcr-Abl level as detected by Western blotting probed with respective antibodies. Akt level was not reduced with in this time frame.

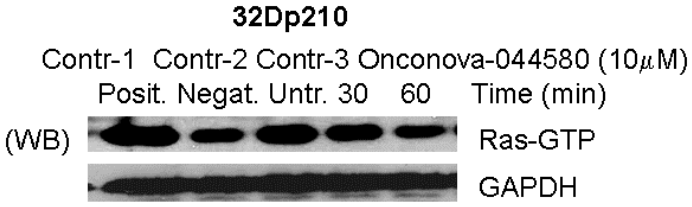


Fig.E2 ON-044580 disrupted the Ras-GTP pathway in Bcr-Abl+ cells. 32Dp210 cells. were treated with 10μM ON-044580 in serum starved (8h) cells for different time periods. Ras-GTP activity was measured following the protocols of the manufacturer (Millipore-Upstate, CA). The Western blots show the positive control(Lane-1), negative control of Ras GTP (Lane-2). Ras GTP level of untreated lysates (Lane-3) and ON-044580 treated lysates with different time points starting from 30 -60 min.(Lane 4 and 5).

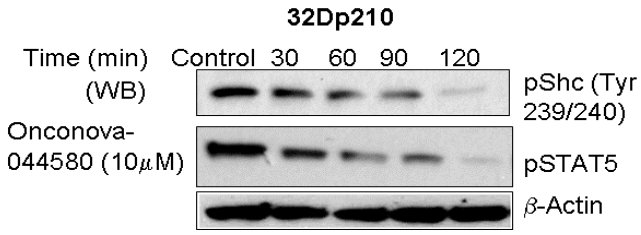


Fig.E3 . Treatment of Bcr-Abl+ cells with 10μM ON-044580 reduced both pShc and pSTAT5 levels in a time dependant manner **(Fig.10)** . Treatment of Bcr-Abl+ cells with 10μM TGAY1 disrupted pGab2 and pSTAT5 pathways (**Fig.11 and 12**).

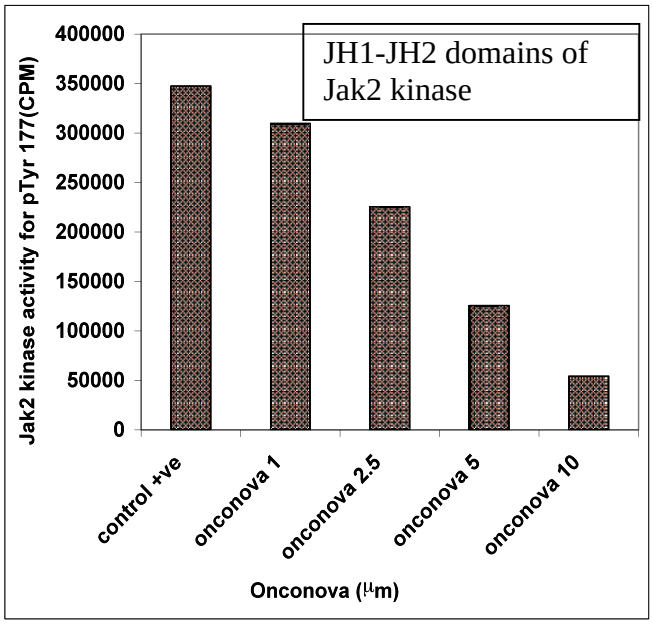
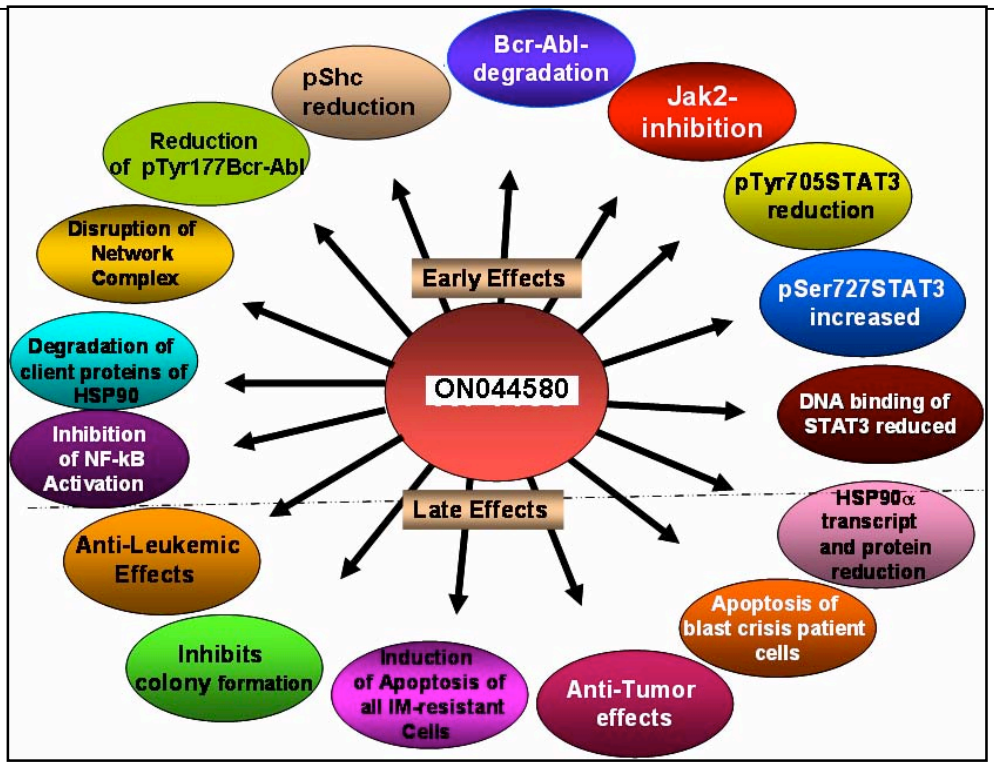


Fig.E4. In vitro Jak2 kinase assay with recombinant Jak2 protein (JH1-JH2 domain) in presence of Onconova -044580**(Fig15).** The peptide substrate was synthesized by Bachem Co and the sequence is as follows: H-Ala-Glu-Lys-Pro-Phe-Tyr-Val-Asn-Val-Glu-Phe-His-His-Glu-Lys-Lys-Lys-OH (Molecular mass 2130.5). Recombinant Jak2 (JH1-JH2 domain) was preincubated with different amount of **Onconova-044580** or Jak2 inhibitor for 10 min. The assay was carried out in presence of 32P gamma ATP, cold ATP, Mg++,Mn++, DTT at 30)C for 15 min and the reaction product was equally dropped on the Whatman filter paper disk (2.3 mm diameter) which was immersed in counting fluid and counted in a Liquid Scintillation counter .

E5. Summary of the multiple functions of ON044580 compound in Bcr-Abl+CML cells



Key Accomplishments

1. ON044580 compound is a dual protein kinase inhibitor. Submicromolar (1-10 µM) concentrations of ON044580 inhibits the Bcr-Abl and Janus 2 kinase activities in vitro.

2. Treatment of Bcr-Abl+ cells with submicromolar concentrations of ON044580 induces a rapid loss of the Bcr-Abl protein from the soluble phase of the leukemia cell.

3. Submicromolar concentrations of ON044580 induces high levels of apoptosis and reduces oncogenic behavior of imatinib mesylate (IM)-resistant Bcr-Abl+ cells and blast crisis chronic myeloid leukemia (CML) cell lines and cells from IM-resistant CML patients.

4. Treatment of Bcr-Abl+ cells with 10 µM ON044580 for three hours causes destruction of the Bcr-Abl/Jak2/HSP90 signaling network, which appears to explain its ability to induce apoptosis in cells from IM-resistant and drug-resistant CML patients.

Reportable Outcomes

We published two papers in the Genes and Cancer journal in collaboration with Dr. E. Premkumar Reddy on the biochemical and biological properties of ON044580 in CML cells.

The references for those papers are:

Samanta AK, Chakraborty SN, Wang Y, Schlette E, Reddy ER, Arlinghaus RB. Destabilization of Bcr-Abl/Jak2 network by a Jak2/Abl kinase inhibitor ON044580 overcomes drug resistance in blast crisis chronic myelogenous leukemia (CML). Genes and Cancer (In Press 2010).

Jatiani SS, Cosenza SC, Reddy MVR, Ha JH, Baker SJ, Samanta AK, Olnes MJ, Pfannes L, Sloand EM, Arlinghaus RB, Reddy EP. A non-ATP-Competitive Dual Inhibitor of JAK2V617F and BCR-AblT3151 Kinases: Elucidation of a novel therapeutic spectrum based on substrate competitive inhibition. Genes & Cancer (In Press 2010)

In addition, another manuscript is in preparation entitled: Dual kinase inhibitor ON044580 disrupts the major signaling pathways in Bcr-Abl+ CML cells. Ajoy. Samanta, Sandip Charkrabarty, Prem kumar Reddy, and Ralph Arlinghaus. In Preparation.

Conclusion from the study:

We investigated the mode of action and functional properties of a new non-ATP competitive kinase inhibitor, ON044580, in Bcr-Abl+ mouse hematopoietic cell lines, IM-resistant cell lines and cells from blast crisis CML patients. Our studies (see Fig. 1) and those of Jatiani et al. (see accompanying paper) indicate that ON044580 is a dual kinase inhibitor that inhibited both Bcr-Abl and Jak2 kinases. Importantly, ON044580 induced apoptosis in IM-sensitive and -resistant cells and cells from late stage of CML patients (Fig. 5a-e). Our findings further showed that ON044580 induced rapid disappearance of Bcr-Abl protein from the detergent-soluble fraction of leukemic cells (Fig. 2a, Supplement Fig. 1a), which affects downstream signaling of Bcr-Abl (Fig. 2a and b) and disrupts the Bcr-Abl/Jak2/HSP90 network complex (Fig. 4a, b, c). The rapid disappearance of Bcr-Abl from Bcr-Abl+ cells caused by ON044580 makes it a novel compound with potential for clinical application in CML. The possible mechanism of rapid decrease of Bcr-Abl protein by ON044580 is not yet established but preliminary experiments with a potent Jak2 inhibitor suggest that Jak2 inhibition only is sufficient for rapid disappearance of Bcr-Abl from the detergent-soluble fraction of cell. Since proteosomal inhibitors (MG132 and lactocyteine) failed to protect the rapid disappearance of Bcr-Abl from the detergent soluble fraction within 2-4 h (data not shown), we predict that upon inhibition with ON044580 Bcr-Abl and Jak2 dissociate from the network complex and Bcr-Abl rapidly migrates to a detergent-insoluble compartment of the cell. How ON044580 treatment may accomplish this is under study in my laboratory. Nevertheless upon dissociation of Bcr-Abl and Jak2 from the network complex, oncogenic signaling would be greatly reduced and the leukemogenic properties of CML cells would similarly be greatly reduced.

The Bcr-Abl/Jak2 dual kinase inhibitory effects of ON044580 are a critical aspect of this compound. Thus unlike IM, where resistant mutations arise in BCR-ABL, ON044580 has the capacity to also inhibit the Jak2 kinase, which induces apoptosis in IM-resistant Bcr-Abl mutant cells including those expressing the gatekeeper mutant T315I (Fig. 5). In addition, signals produced from both Bcr-Abl and Jak2 in IM-sensitive cells will be down-regulated (Fig 2a and b) by treatment with ON044580.

In our previous study we reported that Bcr-Abl is associated with several signaling proteins and forms a signaling network that includes Jak2, Gab2, Akt, and GSH3β (31). In these studies, we showed in co-immunoprecipitation experiments that Bcr-Abl was associated with various members of its down-stream signaling targets. For example, immunoprecipitation of Bcr-Abl+ cells with anti-Jak2 detected Jak2, Akt, GSK-3β and Bcr-Abl. In addition, immunoprecipitation with antibody against GSK-3β co-precipitated Bcr-Abl and immunoprecipitation with an Akt antibody also co-precipitated Bcr-Abl. Normal serum controls established the specificity of these co-immunoprecipitation experiments. We concluded that Bcr-Abl and members of the signaling network described by Samanta et al. (29) were present in a network complex. The gel filtration experiments in Fig. 4 and Supplement Fig. 1 support this conclusion, and indicate that the network is quite large in terms of molecular size, possible more than 6 million daltons.

It is known that HSP90 is a therapeutic target molecule for solid tumor cancers and CML (14, 53). It is also reported that the critical signaling molecules, such as Bcr-Abl, Jak2, Akt, pErk and STAT3, are physically associated with HSP90 (14, 46, 54, 55), which plays a role in their conformational maturation and functional performance and also provides protection from proteases (46, 55, 56). Any disturbance of HSP90 synthesis will eventually lead to proteolytic degradation of the client proteins (57-59). Our studies in Bcr-Abl+ cells

indicate that ON044580 treatment, because of its ability to inhibit both Jak2 and Bcr-Abl kinases (Fig. 1 and 4d), led to inhibition of STAT3, and since STAT3 appears to control HSP90α transcription, this eventually leads to a decrease in HSP90α transcription and decreased HSP90α protein levels (Fig. 3c-e). Whether STAT3 is the direct cause of STAT3 transcription is not known at this point but further experiments are planned to clarify the pathway from STAT3 to HSP90. Importantly, we noticed that the promoter of HSP90α contains binding sites for STAT3 (at -125 bases, our observation) and also for NF-κB (60). Jak2 inhibition also leads to down-regulation of NF-kB (31). We note also that leukemia cells express predominantly the HSP90α form (41, 61). However, the reduction of HSP90α protein levels follows the initial inhibitory event of ON044580, which is the decrease in Bcr-Abl levels within 2-3 h of ON044580 treatment. Our findings also showed that Bcr-Abl, Jak2 and HSP90α exist in a high molecular weight network structure (estimated to be about six million in size) (Fig. 4a) (a similar structure was seen in K562 cells, Supplement Fig.1d) that houses a number of other signaling proteins including Akt, Erk, GSK3β and STAT3 (Fig. 4a). Treatment of Bcr-Abl+ 32D cells with ON044580 for three hours caused destruction of this large network structure (Fig. 4b). It is of interest that IM treatment of Bcr-Abl+ 32D cells had little effect on the Bcr-Abl/Jak2/HSP90 Network complex during a 6 h treatment (Supplement Fig.1c). Thus, these results suggest that Jak2 inhibition by ON044580 is the critical inhibitory activity caused by ON044580 that leads to rapid destruction of the Bcr-Abl/Jak2/HSP90 network complex. However, ON044580, by also decreasing levels of Bcr-Abl might contribute to the rapid destabilization of the network complex. It is likely that this destruction of the Bcr-Abl/Jak2/HSP90 network structure will induce apoptosis and death of the leukemia cell.

Our mechanistic studies (Fig. 4c) regarding ON044580 treatment of Bcr-Abl+ cells is likely to explain why it induces apoptosis in the IM-sensitive and IM-resistant Bcr-Abl + cells such as Bcr-Abl mutant T315I and E255 cells, and IM-resistant CML cell line (K562-R) and drug-resistant CML blast crisis patient cells (Fig. 5a-e). We have proposed a model that describes the roles of Bcr-Abl and Jak2 in signaling pathways that operate in CML cells and the effects of ON044580 (Fig. 4d). In this model, our findings suggest that both Bcr-Abl and Jak2 have important roles in activating STAT3, and importantly, ON044580 treatment of leukemia cells will down-regulate both Bcr-Abl and Jak2 kinase-induced effects. The reduction of STAT3 will lead to reduced transcripts of HSP90α which in turn will reduce HSP90α protein levels. Although these inhibitory effects on HSP90 protein expression probably play an important role in the final apoptotic consequences of ON044580 treatment, we emphasize that the rapid reduction of Bcr-Abl protein levels and the inhibition of the Jak2 kinase are the primary events that initiate the destruction of Bcr-Abl/Jak2/HSP90 signaling complex. We have also presented summary of ON044580 functions in Bcr-Abl+ cells (E5). Based on our findings, we propose that targeting Jak2/Bcr-Abl/HSP90 is an excellent strategy for inducing apoptosis in drug-resistant CML cells of all types including advanced stages of CML like blast crisis, and thus ON044580 may have potential for treatment of many forms of drug resistant CML.

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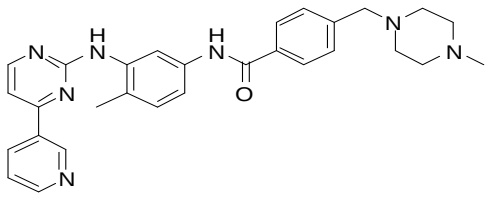
Work Accomplished by Onconova Therapeutics

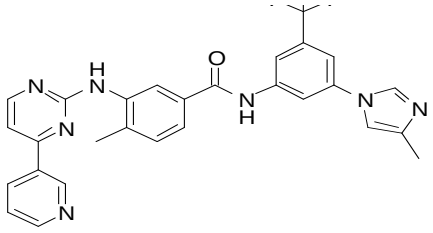
Body

Chemistry – Synthesis and Structure Activity Relationship

Here we report the design, synthesis and evaluation of a new class of kinase inhibitors that are non-ATP mimetic in structure and potently inhibit the kinase activity of mutant JAK2 and BCR-ABL kinases, implicated in myeloproliferative disorders and chronic myeloid leukemia, respectively. These novel (E)-α-benzylthio chalcones are reported with preliminary *in vitro* activity data indicating that several of them are potent inhibitors (comparable to imatinib, the reference compound) of BCR-ABL phosphorylation in leukemic K562 cells, known to express high levels of BCR-ABL. The ability of such compounds to significantly inhibit the proliferation of K562 and Ba/F3:*JAK2*V617F cells suggests that this scaffold could be a promising lead for the development of anticancer agents that are able to block the phosphorylation of oncogenic kinases such as BCR-ABL and activated JAK-2 in leukemic cells. The structure activity relationship (SAR) of this series of compounds for the inhibition of BCR-ABL activity is presented. In the report provided by Ar. Reddy's group, all of the biological experiments were performed with **6c** (ON044580) as a representative of the series.



(STI 571, Imatinib)



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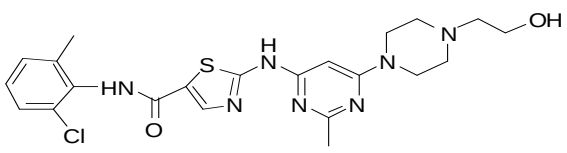

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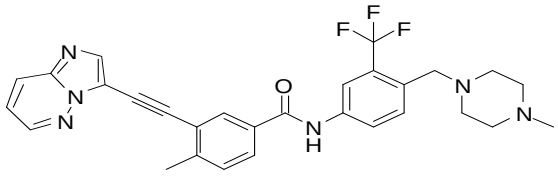
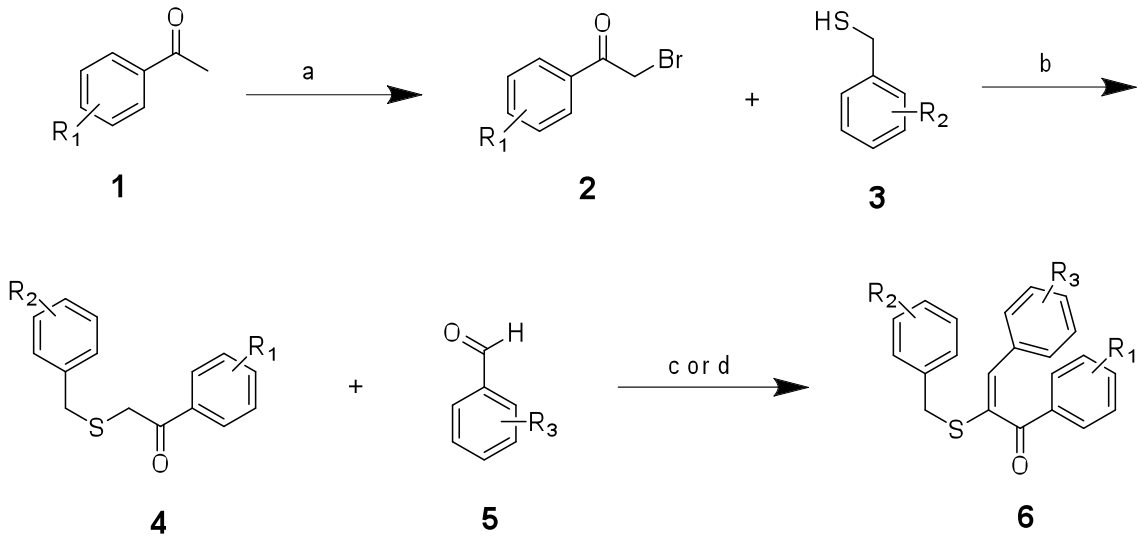


(AP24534)

Chart 1. Structures of known BCR-ABL kinase inhibitors

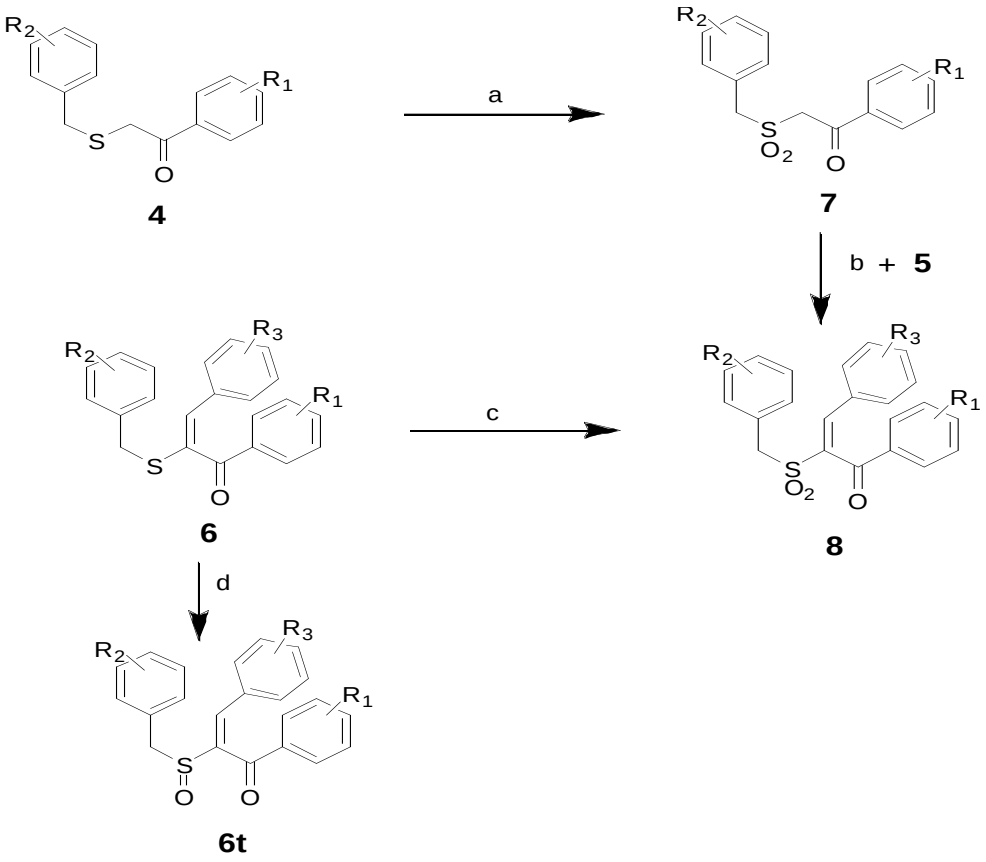
The general method for the synthesis of novel benzylthio chalcones is outlined in Scheme 1. For the synthesis of compounds **6a – 6t** and **6aa - 6ag**, phenacyl bromides **2** were synthesized from acetophenones **1** and then reacted with benzylmercaptans **3** to produce phenacyl benzyl sulfides **4.**[1] Knoevenagel condensation of **4** with aromatic aldehydes **5** in glacial acetic acid in the presence of ammonium acetate yielded α-benzylthio chalcones **6.**[2] Alternatively, the condensation between **4** and **5** was also carried out in glacial acetic acid in the presence of a catalytic amount of benzylamine to obtain **6.**[3]



Scheme 1. General method for the synthesis of (*E*)-α-benzylthio chalcones. Reagents and conditions: (a) Br_2, AcOH, 70 ^{0}C, 3 h; (b) NaOH, MeOH, rt, 2 h; (c) NH_4OAc, AcOH, 70 ^{0}C, 3 h; (d) $PhCH_2NH_2$, AcOH, reflux, 8 h.

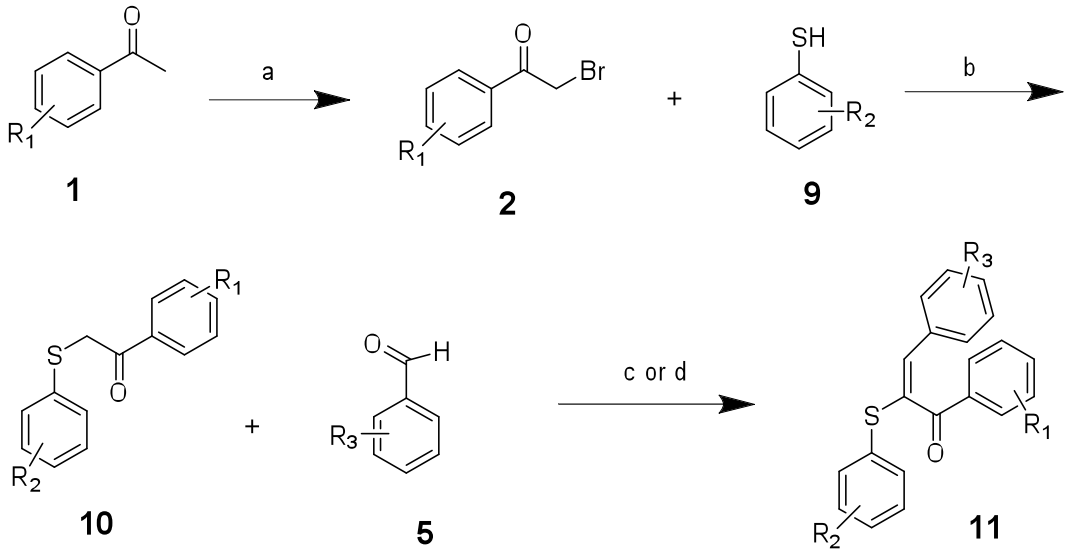
n Ischeme 1, a method for the synthesis of new (E)-α-benzylsulfonyl chalcones is described. Oxidation of phenacyl benzylsulfides **4** with 30% hydrogen peroxide in the presence of glacial acetic acid gave phenacyl benzylsulfones **7**. Condensation of phenacyl

benzylsulfones **7** with aldehydes **5** in glacial acetic acid and ammonium acetate provided α-benzylsulfonyl chalcones **8**. Alternatively, these sulfonyl chalcones **8** were also prepared by the oxidation of benzylthio chalcones **6** with an excess of m-chloroperbenzoic acid (*m*-CPBA) in chloroform. Compound **6t,** α-benzylsulfoxide chalcone was prepared by the controlled oxidation of **6c** with 1 equivalent of *m*-CPBA in chloroform.



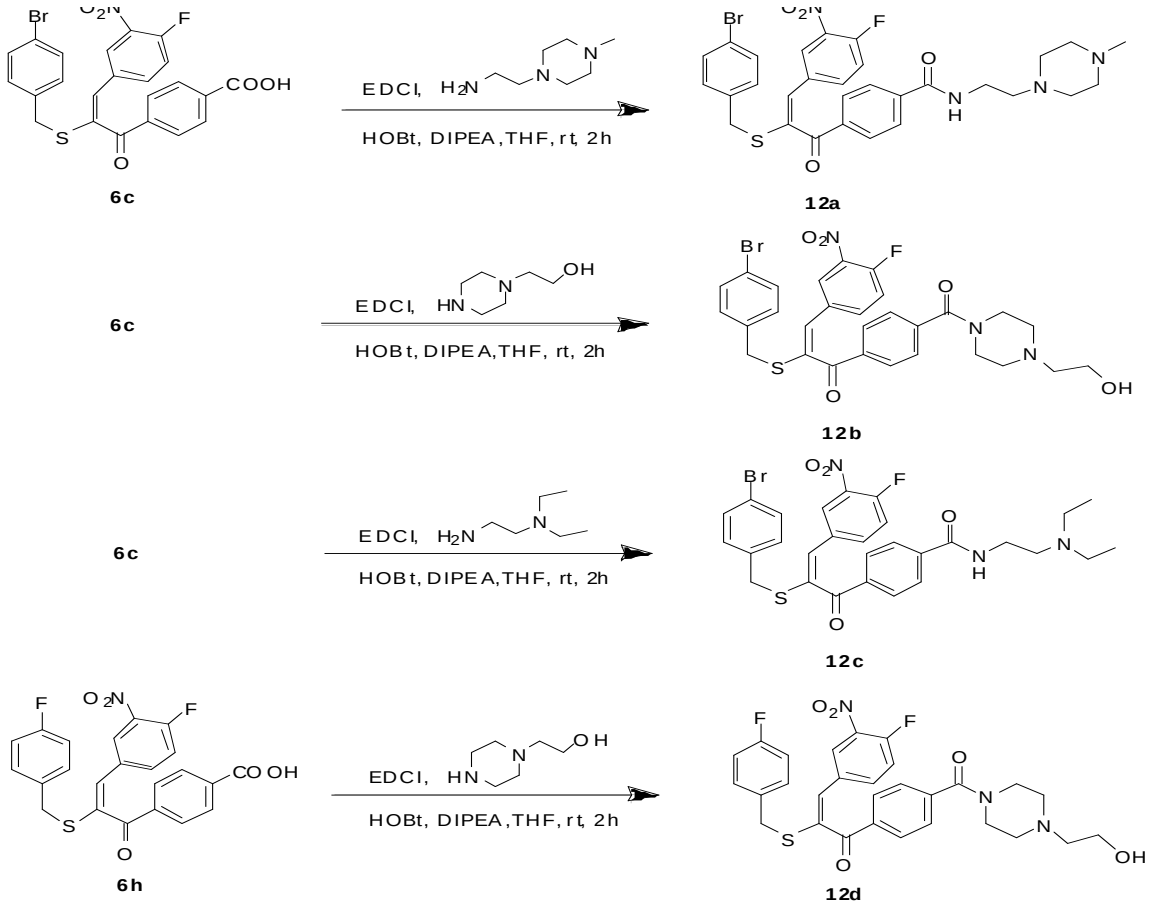
Scheme 2. Synthesis of (*E*)-α-benzyl sulfoxide/sulfonyl chalcones. Reagents and conditions: (a) 30% H_2O_2, AcOH, 70 ^{0}C, 2 h; (b) NH_4OAc, AcOH, reflux, 8 h; (c) *m*-CPBA (3 eq), $CHCl_3$, rt, 2 h; (d) *m*-CPBA (1 eq), $CHCl_3$, 0 ^{0}C - rt, 2h.

To delineate the role of benzylthio moiety in the biological activity of **6**, we synthesized a few molecules replacing the benzyl moiety with aryl group. In Scheme 3 we have outlined the synthesis of α-arylthio chalcones starting from **2**. Condensation of **2** with aryl mercaptans **9** in methanolic sodium hydroxide solution produced phenacyl arylsulfides **10**, which on Knoevenagel type condensation with aromatic aldehydes **5** in the presence of a base yielded (*E*)-α-arylthio chalcones **11.** [3]



Scheme 3. Method for the synthesis of (E)-α-arylthio chalcones. Reagents and conditions: (a) Br_2, AcOH, 70 ^{0}C, 3 h; (b) NaOH, MeOH, rt, 2 h; (c) NH_4OAc, AcOH, reflux, 8 h; (d) $PhCH_2NH_2$, AcOH, reflux, 8 h.

To enhance the solubility and bioavailability of the active (E)-α-benzylthio chalcones, we modified the carboxylic acid group located at the *para* position of the benzoyl aromatic ring of **6** as amide substituted piperazine groups. In Scheme 4 benzylthio chalcones, **6c** and **6h** were treated either with primary or cyclic secondary amines to produce amide substituted piperazines **12a -12d** (Scheme 4).



Scheme 4. Synthesis of compounds **12a – 12d.**

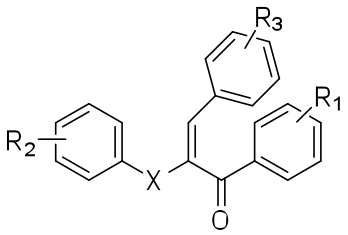
Structure-Activity Relationships (SAR)

After the synthesis of these compounds, their *in vitro* cytotoxicity was assessed using K562, a BCR-ABL positive leukemic cell line. As some of these compounds are highly potent against K562 cells, their ability to inhibit the phosphorylation of BCR-ABL was assessed by western blotting of the drug treated cell lysates with phospho anti BCR-ABL

antibodies. The results of this study are presented in Tables 1-3 and Figure 1. These studies show that the cytotoxicity of these compounds depends on i) the position of the thio group, ii) the oxidation state of the sulfur atom in the molecule and iii) the nature and site of the substituents in the aromatic rings. During our screening of about 600 α-benzylthio and sulfonyl chalcones, we found that some of these compounds showed a very good cytotoxicity (IC_{50} = 0.3-0.9 μM) in K562 cells (Table 1). To analyze the effect of the substituents on the aromatic rings of **6**, we synthesized a number of analogs containing methoxy, halo, methyl, nitro, carboxy and hydroxy groups on these rings. Cytotoxicity analyses of these analogues on K562 cells showed that the compounds with a fluoro atom at the fourth position and a nitro group at the third position on the styryl aromatic ring exhibited the best activity in these series. To further assess the significance of the 3-nitro and 4-fluoro groups on the styryl ring in **6**, we replaced these groups in **6c** with bromo at the site of nitro and hydroxyl at the position of fluoro atom (**6ab**). Both these replacements resulted in total loss of activity. Since double replacement of the 3-nitro and 4-fluoro substituents on styryl ring in **6c** caused a moderate loss of activity, we then made single changes either at 3-position keeping 4-position intact or vice versa and studied the effect of these changes in cytotoxic assay in tumor cells. Modifying the fluorine atom alone on the 4-position of styryl ring in **6c** with a hydroxyl group resulted in the loss of the activity (**6ac**). The activity of **6c** was found to be unaltered when fluorine at 4-position of styryl ring was replaced with chloro (**6a**) or bromo (**6b**) atoms. This confirms that a halogen atom at that position is essential for the activity of the molecule. Any modifications replacing fluorine with atoms or groups other than halogen atoms results in total loss of activity. To understand the role of the 3-position nitro group

on the cytotoxicity of the molecule, the nitro group of **6c** was replaced with a methoxy group (**6aa**). This substitution led to a drastic reduction in the cytotoxicity of the resulting molecule (**6aa**). Since both the nitro group and the fluorine atom on the ring seem to be critical for the cytotoxicity of the molecule, we tested whether their distribution on the ring other than at 3 and 4-positions has any effect on the tumor cell killing activity. To analyze the role of these two substituent locations on the ring, we synthesized **6ad**, **6ae**, **6af** and **6ag** where fluoro and nitro substituents are distributed at 2, 5 (**6ad**), 3, 4 (**6ae**), 4, 2 (**6af**) and 2, 4 (**6ag**) positions. Cytotoxicity data from K562 cells treated with these compounds showed that these alterations are drastic leading to the loss of cytotoxicity in the resulting molecules (Table 2). From this analysis it is evident that a nitro group at the third position and a fluorine atom at the fourth position on the styryl aromatic ring are critical for the anti-tumor activity of these molecules. It is also clear from Table 1 that the nature and position of the substituents on the benzoyl and benzylthio rings do not affect the cytotoxicity of the molecules as long as 3-nitro and 4-fluoro substituents are on the styryl ring. To determine the significance of sulfur oxidation state, we oxidized the sulfur atom in benzylthio group to sulfoxide and sulfone and then assayed the resulting molecules for their cytotoxicity. In both the cases, the sulfoxide (**6t**) or the sulfones (**8a** and **8b**) produced by the oxidation of **6c** were found to have significantly reduced cytotoxicity.

Table 1 *In vitro* cytotoxicity of α-aryl and benzyl thio chalcones.

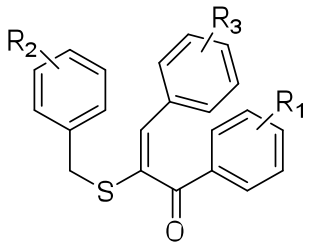


Compd.	R_2	X	R_1	R_3	IC_{50} (μM)	
					K562	pBCR/ABL
6a	4-Br	CH_2S	4-COOH	3-NO_2, 4-Cl	0.7	10
6b	4-Br	CH_2S	4-COOH	3-NO_2, 4-Br	0.9	10
6c	4-Br	CH_2S	4-COOH	3-NO_2, 4-F	0.3	0.5
6d	4-Cl	CH_2S	4-COOH	3-NO_2, 4-F	0.5	2.5
6e	4-Cl	CH_2S	4-COOH	3-NO_2, 4-Cl	0.5	10
6f	4-Cl	CH_2S	4-COOH	3-NO_2, 4-Br	0.6	2.5
6g	4-F	CH_2S	4-COOH	3-NO_2, 4-Cl	0.5	2.5
6h	4-F	CH_2S	4-COOH	3-NO_2, 4-F	0.4	2.5
6i	4-F	CH_2S	4-COOH	3-NO_2, 4-Br	0.4	15
6j	4-F	CH_2S	4-F	3-NO_2, 4-F	0.6	2.5
6k	2-F	CH_2S	4-COOH	3-NO_2, 4-Cl	0.8	10
6l	2-Cl	CH_2S	4-COOH	3-NO_2, 4-F	0.5	10
6m	2,4-Cl_2	CH_2S	4-COOH	3-NO_2, 4-Cl	0.6	2.5
6n	2,4-Cl_2	CH_2S	4-COOH	3-NO_2, 4-F	0.4	2.5
6o	2,4-Cl_2	CH_2S	4-COOH	3-NO_2, 4-Br	0.6	2.5

6p	4-CH_3	CH_2S	4-COOH	3-NO_2, 4-F	0.3	2.5
6q	4-CF_3	CH_2S	4-COOH	3-NO_2, 4-F	0.4	0.2
6r	4-Br	CH_2S	4-$COOCH_3$	3-NO_2, 4-F	0.3	0.75
6s	H	CH_2S	4-COOH	3-NO_2, 4-F	0.3	2.5
6t	4-Br	CH_2SO	4-COOH	3-NO_2, 4-F	75	>20
8a	4-Br	CH_2SO_2	4-COOH	3-NO_2, 4-F	5	>20
8b	4-Br	CH_2SO_2	4-COOH	3-NO_2, 4-Cl	25	>20
11	4-Br	S	4-COOH	3- NO_2, 4-F	1.5	20

These results show that these molecules exhibit much higher potency towards tumor cells when an un-oxidized sulfur atom is present in their structure. To understand the role of the benzyl methylene group in structure activity relationship, we replaced the benzylthio moiety with phenylthio group where the sulfur atom is directly connected to the aromatic ring instead of having a methylene bridge between the ring and the sulfur atom (**11**). This modification seems to have an adverse effect on the molecule resulting in a 5-fold reduction in cytotoxicity.

Table 2 *In vitro* cytotoxicity of α-benzyl thio chalcones



Compd.	**R_2**	**R_1**	**R_3**	**IC_{50} (μM)**	
				K562	**pBCR/ABL**
6aa	4-Br	4-COOH	3-OCH_3, 4-F	20	>20

6ab	4-Br	4-COOH	3-Br, 4-OH	35	>20
6ac	4-Br	4-COOH	3-NO_2, 4-OH	75	>20
6ad	4-Br	4-COOH	2-F, 5-NO_2	7.5	>20
6ae	4-Br	4-COOH	3-F, 4-NO_2	5.0	>20
6af	4-Br	4-COOH	2-NO_2, 4-F	20	>20
6ag	4-Br	4-COOH	2-F, 4-NO_2	7.5	>20

We next focused our attention on enhancing the aqueous solubility and bioavailability of the active molecules. As all the active molecules synthesized have very poor aqueous solubility (<1 mg/mL), these were dissolved in dimethyl sulfoxide (DMSO) to treat the cells or administer to the mice by intraperitoneal (IP) injection. Bioavailability and pharmacokinetic data from the mice injected with **6c** in DMSO showed very low levels of this compound in serum. To improve the solubility and bioavailability of these compounds, we converted the carboxylic acid on the benzoyl ring of **6c** and **6h** in to compounds **12a-12d** having secondary and tertiary amino groups. As shown in Table 3, secondary and tertiary amine salts of **6c** and **6h** were made and tested for their solubility and cytotoxicity. The amino hydrochloride salts (**12a-12d**) made from **6c** and **6h** displayed excellent aqueous solubility and retained their cytotoxicity in K562 cells. The bioavailability of these compounds is being tested.

Table 3 *In vitro* cytotoxicity of carboxamide derivatives of α-benzylthio chalcones

Compd.	**IC_{50} (μM)**		
	K562	**pBCR/ABL**	**Solubility**
12a	0.6	2.5	>10.0 mg/mL

12b	0.2	0.5	>10.0 mg/mL
12c	0.75	0.5	>10.0 mg/mL
12d	0.3	0.5	>10.0 mg/mL

Inhibition of BCR-ABL kinase activity

The cell based cytotoxicity assays reported above demonstrate that (E)-α-benzylthio chalcones exhibit nanomolar cytotoxicity against K562 cells. To assess whether these compounds directly inhibit the phosphorylation of BCR-ABL, we tested the cell lysates prepared from K562 cells treated with these compounds in western blot analysis with anti-phospho BCR-ABL antibody (Fig.1). These results showed that while all the compounds, in general, inhibited the phosphorylation of BCR-ABL, compounds **6c**, **6q**, **6r**, **12a**, **12b** and **12d** were most effective in this assay. Imatinib [5] was used as a positive control while DMSO was used as the negative control. The results of this study showed that some of the α-benzylthio chalcones (**6c**, **6q**, **6r**, **12b**, **12c** and **12d**) inhibited the phosphorylation of BCR-ABL with an IC_{50} (0.2 to 0.75 μM) comparable to that of imatinib (0.75 μM).

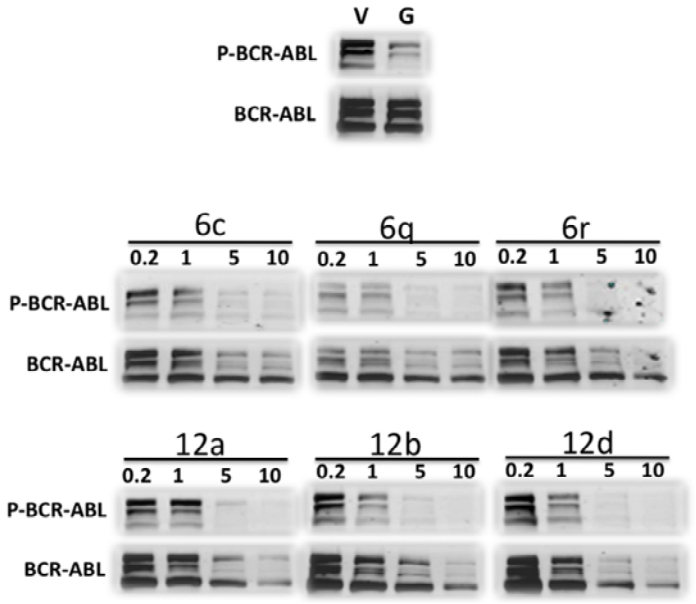


Figure 1. BCR-ABL kinase inhibition in human chronic myelogenous leukemic cells.

Our current study shows that (E)-α-benzylthio chalcones exert a cytotoxic effect by inhibiting BCR-ABL phosphorylation in leukemic cell lines that over express this protein, thereby causing growth arrest and cell death. We describe the development of novel small molecules that inhibit autophosphorylation of BCR-ABL at a concentration of 0.2–0.75 μM. While assessing the kinase inhibitory profile of **6c** against other kinases implicated in cancer, we found **6c** to be a specific inhibitor of BCR-ABL. **6c** exhibited 10-15 fold higher selectivity for BCR-ABL over other tyrosine kinases and more than 40-fold higher selectivity for BCR-ABL over serine/threonine kinases (Table 4). The optimization of **6c** to improve its bioavailability and pharmacokinetics is ongoing. The *in vivo* efficacy of the optimized version of **6c** needs to be tested in xenograft mouse models of leukemia and *ex vivo* on patient samples.

6c and its analogs do not resemble typical ATP mimetics in structure. Hence **6c** and its analogs are expected to target regions outside the ATP binding site of their target kinases and offer the potential to be unaffected by mutations in the kinase domain that make tumor cells resistant to ATP-competitive inhibitors.

Table 4 Kinase inhibition profile of **6c**

Kinase	**IC_{50} (μM)**
	6c
BCR-ABL	0.75
JAK-2	1.09
Lyn B	17.0
Src	13.2

ErbB2	>10.0
EGFR	>20.0
Plk-1	>50.0
Cdk-1	>50.0

We describe here, for the first time, the discovery and synthesis of a novel class of compounds, (E)-α-benzylthio chalcones, which possess potent kinase inhibitory activity and exhibit cytotoxicity against human tumor cells that express the oncogenic kinase BCR-ABL. While these compounds are comparable to imatinib in their *in vitro* efficacy they do not resemble typical ATP-mimetics. Hence, they offer the potential to be unaffected by mutations in the kinase domain that make tumor cells resistant to ATP-competitive inhibitors. These compounds possess a simple molecular structure and are easy to synthesize which makes them very attractive for further exploration as kinase inhibitors with application in cancer therapy.

References:

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2. Baliah V, Rangarajan T. *J. Chem. Soc.* **1960**, 4703.

3. Reddy, MVR. Reddy, S. *Acta. Chim. Hung.* **1985**, *120*, 275.

Key Research Accomplishments

1. Onconova chemists synthesized approximately 3000 compounds to screen for BCR-ABL inhibitors by Dr. Reddy's group.

2. Onconova scientists worked in collaboration with Dr. Reddy's group to develop the high thru put screening methods described in Dr. Reddy's report.

3. Onconova developed methods for large scale synthesis of ON044580, ON045000 and ON96030 and supplied gram quantities of these compounds to Drs. Reddy and Arlinghaus.

4. Onconova scientists completed studies to determine the oral bio-availability of ON043580 in mouse model system. The oral bio-availability studies in mice showed that ON044580 is not orally bioavailable and hence a decision was made to modify the back bone structure of this compound to enhance the oral bioavailability of this compound.

5. To accomplish this goal, Onconova chemists have designed and synthesized additional molecules and provided them to Dr. Reddy's group for further evaluation of their biochemical and biological properties.

6. Onconova developed methods for large scale synthesis of ON044580, ON045260 and ON044690 and supplied gram quantities of these compounds to Drs. Reddy and Arlinghaus.

7. Onconova scientists worked in collaboration with Dr. Reddy's group to develop the Caco cell assay and carried out HPLC analyses to determine encapsulation efficiency of ON044580 loaded transferrin nanoparticles.

Reportable Outcomes

1. Reddy MV, Pallela VR, Cosenza SC, Mallireddigari MR, Patti R, Bonagura M, Truongcao M, Akula B, Jatiani SS, Reddy EP. Design, synthesis and evaluation of (E)-alpha-benzylthio chalcones as novel inhibitors of BCR-ABL kinase. Bioorg Med Chem. 2010. **18**:2317-26.

Conclusions

We have developed methods for the large scale production of ON044580, ON045000 and ON96030 for pre-clinical and clinical development of these compounds.

Appendices

Manuscripts Published/in press

1. Reddy MV, Pallela VR, Cosenza SC, Mallireddigari MR, Patti R, Bonagura M, Truongcao M, Akula B, Jatiani SS, Reddy EP. Design, synthesis and evaluation of (E)-alpha-benzylthio chalcones as novel inhibitors of BCR-ABL kinase. Bioorg Med Chem. 2010. **18**:2317-26.

2. Jatiani SS, Cosenza SC, Reddy MVR, Ha JH, Baker SJ, Samanta AK, Olnes MJ, Pfannes L, Sloand EM, Arlinghaus RB, and Reddy EP. A Non–ATP-Competitive Dual Inhibitor of $JAK2^{V617F}$ and $BCR\text{-}ABL^{T315I}$ Kinases: Elucidation of a Novel Therapeutic Spectrum Based on Substrate Competitive Inhibition. Genes & Cancer. *In Press*.

3. Samanta AK, Chakraborty SN, Wang Y, Schlette E, Reddy ER, Arlinghaus RB. Destabilization of Bcr-Abl/Jak2 network by a Jak2/Abl kinase inhibitor ON044580 overcomes drug resistance in blast crisis chronic myelogenous leukemia (CML). Genes and Cancer *(*In Press 2010).

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Design, synthesis and evaluation of (*E*)-α-benzylthio chalcones as novel inhibitors of BCR-ABL kinase

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Chronic myelogenous leukemia

ABSTRACT

Novel (*E*)-α-benzylthio chalcones are reported with preliminary in vitro activity data indicating that several of them are potent inhibitors (comparable to imatinib, the reference compound) of BCR-ABL phosphorylation in leukemic K562 cells, known to express high levels of BCR-ABL. The ability of such compounds to significantly inhibit K562 cell proliferation suggests that this scaffold could be a promising lead for the development of anticancer agents that are able to block BCR-ABL phosphorylation in leukemic cells.

1. Introduction

The Philadelphia chromosome (Ph), discovered in 1960 by Nowell and Hungerford,[1] results from a reciprocal translocation between chromosomes 9 at band q34 and chromosome 22 at band q11.[2,3] This translocation fuses the breakpoint cluster region (BCR) and the ABL genes and creates the BCR-ABL oncogene.[4] Because the BCR-ABL protein is active in greater than 90% of chronic myelogenous leukemia (CML) cases, it has been possible to synthesize small molecules that inhibit BCR-ABL kinase activity in leukemic cells without adversely affecting the normal cell population. Imatinib (STI571, Gleevec®)[5] is a small molecule inhibitor that binds to the kinase domain of BCR-ABL when the protein is in its closed, inactive conformation,[5] thereby inhibiting its activity, and is now considered as a first-line therapy for the majority of CML cases due to its high efficacy and relatively mild side effects.[6] In spite of the fact that the majority of patients receiving imatinib respond to treatment at both the hematological and cytogenetic levels, relapse occurs in a large percentage of patients.[7] Several studies have attempted to address the mechanism(s) by which CML cells acquire imatinib resistance.[8–10] Results from these studies indicate that the mechanism that accounts for a majority of imatinib-resistant leukemias, in vivo, is mutation of the BCR-ABL gene itself. Mutation within the kinase domain is the most common and to date, more than 50 different clinically relevant point mutations within this domain have been identified. It is believed that certain amino acid substitutions interfere with the ability of imatinib to interact directly with the BCR-ABL kinase domain whereas others destroy or hinder the ability of the BCR-ABL kinase domain to adopt a conformation that is required for imatinib binding.[7,11]

The challenges of mutational relapse in CML patients undergoing imatinib therapy has paved the way for the development of second generation BCR-ABL inhibitors such as PD180970,[12] CGP76030,[13] BMS-354825,[14] AMN 107 or Nilotinib[15] and more recently AP24534 (Chart 1).[16] These new BCR-ABL inhibitors are all ATP-competitive agents and therefore will potentially encounter challenges similar to imatinib via accumulation of kinase domain mutations.

Because of the frequency of mutations within the ATP-binding site, efforts are now focused on the identification of novel inhibitors that inhibit the BCR-ABL signaling pathway by mechanisms other than competing with ATP. Different approaches have recently been described to overcome this resistance in at least some CML cases. Farnesyltransferase inhibitors, such as SCH66336, and the proteasome inhibitor Bortezomib have been shown to have growth inhibitory effects on certain imatinib-resistant leukemias.[17]

Abbreviations: SAR, structure–activity relationship; FBS, fetal bovine serum.

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It has also been argued that non-ATP-competitive kinase inhibitors might constitute better drug candidates.[18] Since there is a limited number of chemotypes that act as non-ATP competitive inhibitors, we undertook the synthesis and characterization of new chemotypes that are unrelated to ATP and other purine and pyrimidine nucleosides but still possess kinase inhibitory activity.

Here we report the discovery of (*E*)-α-benzylthio chalcones as a new class of inhibitors that potently inhibit the growth of cells harboring the activated BCR-ABL protein by inhibiting the phosphorylation of the kinase.

2. Chemistry

The general method for the synthesis of novel benzylthio chalcones is outlined in Scheme 1. For the synthesis of compounds **6a**–**6t** and **6aa**–**6ag**, phenacyl bromides **2** were synthesized from acetophenones **1** and then reacted with benzylmercaptans **3** to produce phenacyl benzyl sulfides **4**.[19] Knoevenagel condensation of **4** with aromatic aldehydes **5** in glacial acetic acid in the presence of ammonium acetate yielded α-benzylthio chalcones **6**.[20] Alternatively, the condensation between **4** and **5** was also carried out in glacial acetic acid in the presence of a catalytic amount of benzylamine to obtain **6**.[21]

In Scheme 2, a method for the synthesis of new (*E*)-α-benzylsulfonyl chalcones is described. Oxidation of phenacyl benzylsulfides **4** with 30% hydrogen peroxide in the presence of glacial acetic acid gave phenacyl benzylsulfones **7**. Condensation of phenacyl benzylsulfones **7** with aldehydes **5** in glacial acetic acid and ammonium acetate provided α-benzylsulfonyl chalcones **8**. Alternatively, these sulfonyl chalcones **8** were also prepared by the oxidation of benzylthio chalcones **6** with an excess of *m*-chloroperbenzoic acid (*m*-CPBA) in chloroform. Compound **6t**, α-benzylsulfoxide chalcone was prepared by the controlled oxidation of **6c** with 1 equiv of *m*-CPBA in chloroform.

To delineate the role of benzylthio moiety in the biological activity of **6**, we synthesized a few molecules replacing the benzyl moiety with aryl group. In Scheme 3 we have outlined the synthesis of α-arylthio chalcones starting from **2**. Condensation of **2** with aryl mercaptans **9** in methanolic sodium hydroxide solution produced phenacyl arylsulfides **10**, which on Knoevenagel type condensation with aromatic aldehydes **5** in the presence of a base yielded (*E*)-α-arylthio chalcones **11**.[21]

To enhance the solubility and bioavailability of the active (*E*)-α-benzylthio chalcones, we modified the carboxylic acid group located at the *para* position of the benzoyl aromatic ring of **6** as amide substituted piperazine groups. In Scheme 4 benzylthio

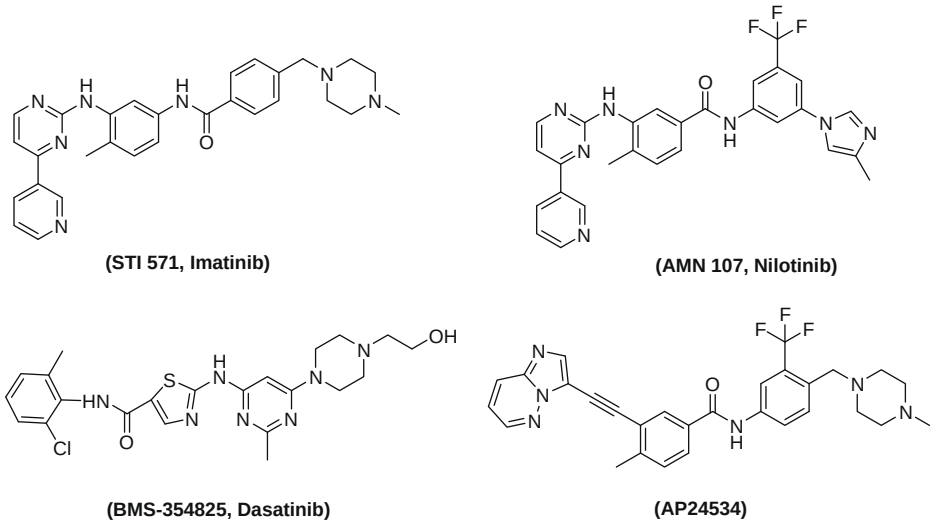
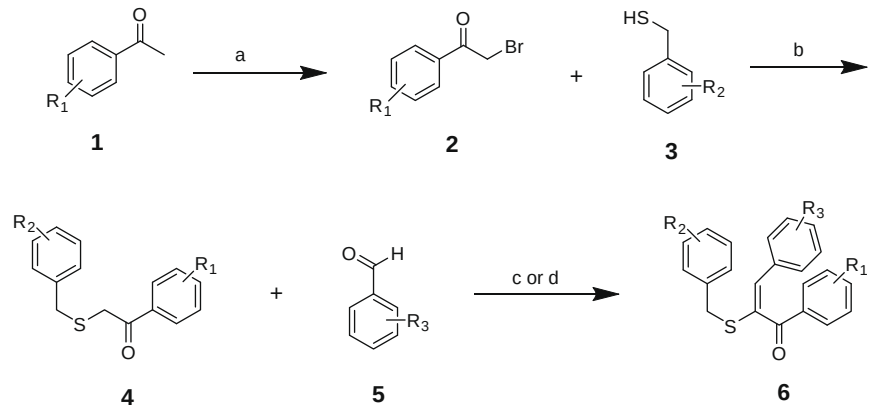
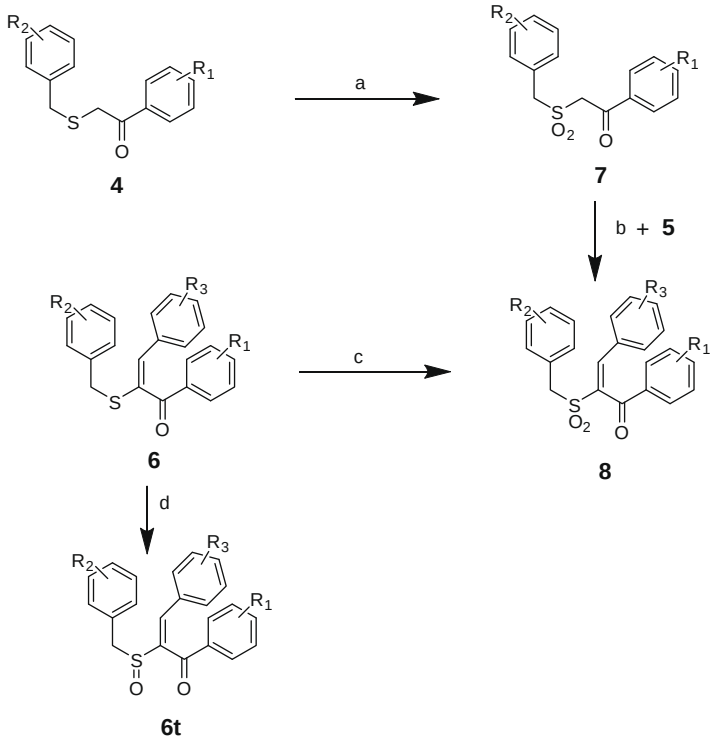


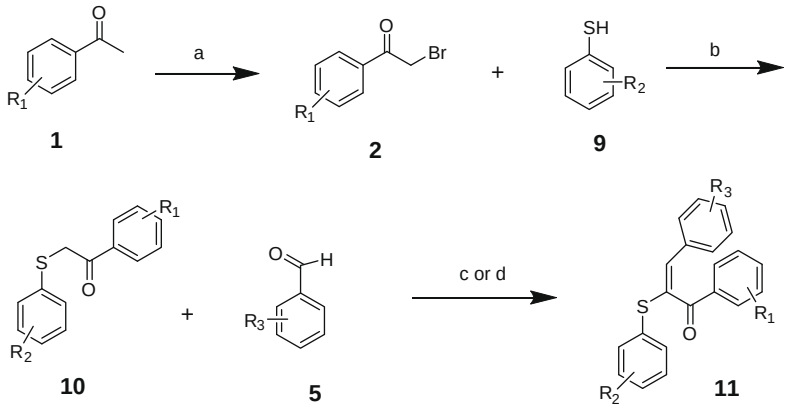
Chart 1. Structures of known BCR-ABL kinase inhibitors.



Scheme 1. General method for the synthesis of (*E*)-α-benzylthio chalcones. Reagents and conditions: (a) Br_2, AcOH, 70 °C, 3 h; (b) NaOH, MeOH, rt, 2 h; (c) NH_4OAc, AcOH, 70 °C, 3 h; (d) $PhCH_2NH_2$, AcOH, reflux, 8 h.



Scheme 2. Synthesis of (*E*)-α-benzyl sulfoxide/sulfonyl chalcones. Reagents and conditions: (a) 30% H_2O_2, AcOH, 70 °C, 2 h; (b) NH_4OAc, AcOH, reflux, 8 h; (c) *m*-CPBA (3 equiv), $CHCl_3$, rt, 2 h; (d) *m*-CPBA (1 equiv), $CHCl_3$, 0 °C to rt, 2 h.



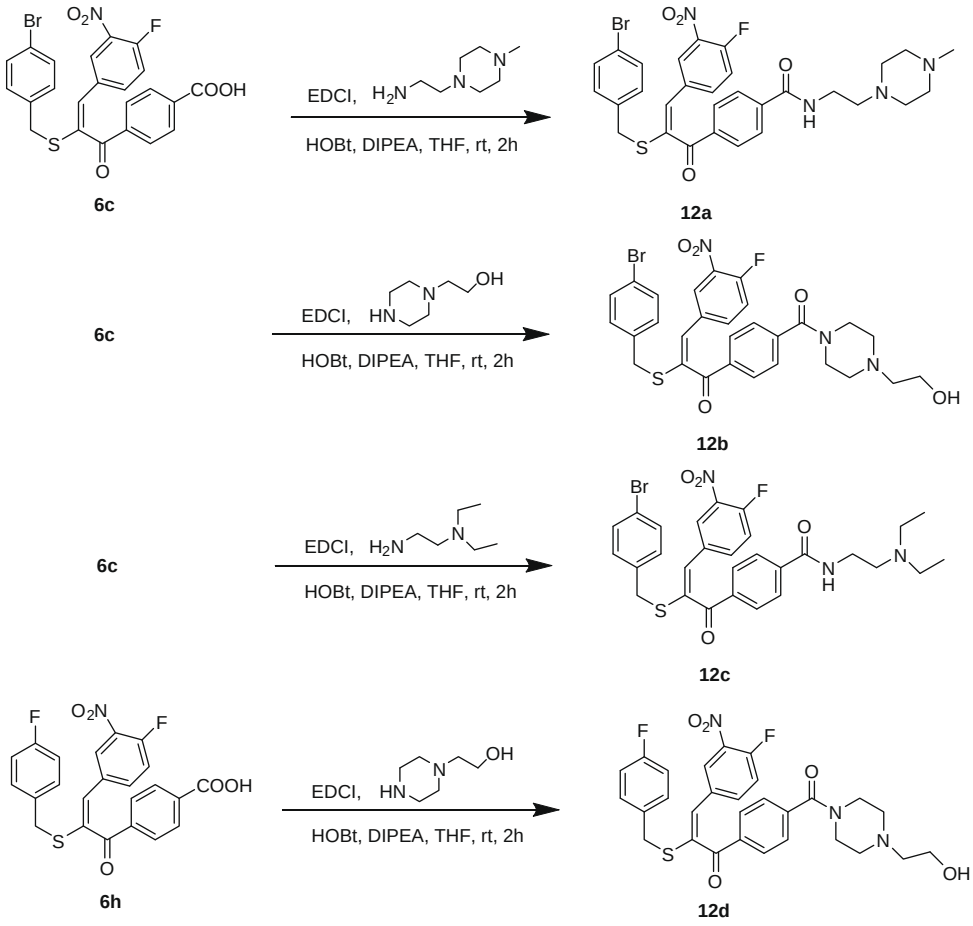
Scheme 3. Method for the synthesis of (*E*)-α-arylthio chalcones. Reagents and conditions: (a) Br_2, AcOH, 70 °C, 3 h; (b) NaOH, MeOH, rt, 2 h; (c) NH_4OAc, AcOH, reflux, 8 h; (d) $PhCH_2NH_2$, AcOH, reflux, 8 h.

chalcones, **6c** and **6h** were treated either with primary or cyclic secondary amines to produce amide substituted piperazines **12a**–**12d** (Scheme 4).

3. Results and discussion

3.1. Structure–activity relationships (SAR)

After the synthesis of these compounds, their in vitro cytotoxicity was assessed using K562, a BCR-ABL positive leukemic cell line. As some of these compounds are highly potent against K562 cells, their ability to inhibit the phosphorylation of BCR-ABL was assessed by western blotting of the drug treated cell lysates with phospho anti BCR-ABL antibodies. The results of this study are presented in Tables 1–3 and Figure 1. These studies show that the cytotoxicity of these compounds depends on (i) the position of the thio group, (ii) the oxidation state of the sulfur atom in the molecule and (iii) the nature and site of the substituents in the aromatic rings. During our screening of about 600 α-benzylthio and sulfonyl chalcones, we found that some of these compounds showed a very good cytotoxicity (IC_{50} = 0.3–0.9 μM) in K562 cells (Table 1). To analyze the effect of the substituents on the aromatic rings of **6**, we synthesized a number of analogs containing methoxy, halo, methyl, nitro, carboxy and hydroxy groups on these rings. Cytotoxicity analyses of these analogues on K562 cells showed that the compounds with a fluoro atom at the fourth position and a nitro group at the third position on the styryl aromatic ring exhibited the best activity in these series. To further assess the significance of the 3-nitro and 4-fluoro groups on the styryl ring in **6**, we replaced these groups in **6c** with bromo at the site of nitro and hydroxyl at the position of fluoro atom (**6ab**). Both these replacements resulted in total loss of activity. Since double



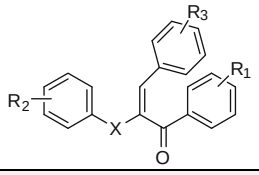
Scheme 4. Synthesis of compounds **12a–12d**.

replacement of the 3-nitro and 4-fluoro substituents on styryl ring in **6c** caused a moderate loss of activity, we then made single changes either at 3-position keeping 4-position intact or vice versa and studied the effect of these changes in cytotoxic assay in tumor cells. Modifying the fluorine atom alone on the 4-position of styryl ring in **6c** with a hydroxyl group resulted in the loss of the activity (**6ac**). The activity of **6c** was found to be unaltered when fluorine at 4-position of styryl ring was replaced with chloro (**6a**) or bromo (**6b**) atoms. This confirms that a halogen atom at that position is essential for the activity of the molecule. Any modifications replacing fluorine with atoms or groups other than halogen atoms results in total loss of activity. To understand the role of the 3-position nitro group on the cytotoxicity of the molecule, the nitro group of **6c** was replaced with a methoxy group (**6aa**). This substitution led to a drastic reduction in the cytotoxicity of the resulting molecule (**6aa**). Since both the nitro group and the fluorine atom on the ring seem to be critical for the cytotoxicity of the molecule, we tested whether their distribution on the ring other than at 3 and 4-positions has any effect on the tumor cell killing activity. To analyze the role of these two substituent locations on the ring, we synthesized **6ad**, **6ae**, **6af** and **6ag** where fluoro and nitro substituents are distributed at 2, 5 (**6ad**), 3, 4 (**6ae**), 4, 2 (**6af**) and 2, 4 (**6ag**) positions. Cytotoxicity data from K562 cells treated with these compounds showed that these alterations are drastic leading to the loss of cytotoxicity in the resulting molecules (Table 2). From this analysis it is evident that a nitro group at the third position and a fluorine atom at the fourth position on the styryl aromatic ring are critical for the anti-tumor activity of these molecules. It is also clear from Table 1 that the nature and position of the substituents on the benzoyl and benzylthio rings do not affect the cytotoxicity of the molecules as long as 3-nitro and 4-fluoro substituents are on the styryl ring. To determine the significance of sulfur oxidation state, we oxidized the sulfur atom in benzylthio group to sulfoxide and sulfone and then assayed the resulting molecules for their cytotoxicity. In both the cases, the sulfoxide (**6t**) or the sulfones (**8a** and **8b**) produced by the oxidation of **6c** were found to have significantly reduced cytotoxicity.

These results show that these molecules exhibit much higher potency towards tumor cells when an un-oxidized sulfur atom is present in their structure. To understand the role of the benzyl methylene group in structure–activity relationship, we replaced the benzylthio moiety with phenylthio group where the sulfur atom is directly connected to the aromatic ring instead of having a methylene bridge between the ring and the sulfur atom (**11**). This modification seems to have an adverse effect on the molecule resulting in a fivefold reduction in cytotoxicity.

We next focused our attention on enhancing the aqueous solubility and bioavailability of the active molecules. As all the active molecules synthesized have very poor aqueous solubility (<1 mg/mL), these were dissolved in dimethyl sulfoxide (DMSO) to treat the cells or administer to the mice by intraperitoneal (IP) injection. Bioavailability and pharmacokinetic data from the mice injected with **6c** in DMSO showed very low levels of this compound in serum. To improve the solubility and bioavailability of these compounds, we converted the carboxylic acid on the benzoyl ring of **6c** and **6h** in to compounds **12a**–**12d** having secondary and tertiary amino groups. As shown in Table 3, secondary and tertiary amine salts of **6c** and **6h** were made and tested for their solubility and cytotoxicity. The

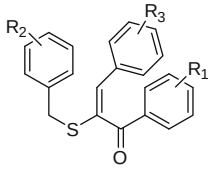
Table 1
In vitro cytotoxicity of α-aryl and benzyl thio chalcones



Compd	R_2	X	R_1	R_3	IC_{50} (μM)	
					K562	pBCR/ABL
6a[a]	4-Br	CH_2S	4-COOH	3-NO_2, 4-Cl	0.7	10
6b[a]	4-Br	CH_2S	4-COOH	3-NO_2, 4-Br	0.9	10
6c[a]	4-Br	CH_2S	4-COOH	3-NO_2, 4-F	0.3	0.5
6d[a]	4-Cl	CH_2S	4-COOH	3-NO_2, 4-F	0.5	2.5
6e[a]	4-Cl	CH_2S	4-COOH	3-NO_2, 4-Cl	0.5	10
6f[a]	4-Cl	CH_2S	4-COOH	3-NO_2, 4-Br	0.6	2.5
6g[a]	4-F	CH_2S	4-COOH	3-NO_2, 4-Cl	0.5	2.5
6h[a]	4-F	CH_2S	4-COOH	3-NO_2, 4-F	0.4	2.5
6i[a]	4-F	CH_2S	4-COOH	3-NO_2, 4-Br	0.4	15
6j[b]	4-F	CH_2S	4-F	3-NO_2, 4-F	0.6	2.5
6k[a]	2-F	CH_2S	4-COOH	3-NO_2, 4-Cl	0.8	10
6l[a]	2-Cl	CH_2S	4-COOH	3-NO_2, 4-F	0.5	10
6m[a]	2,4-Cl_2	CH_2S	4-COOH	3-NO_2, 4-Cl	0.6	2.5
6n[a]	2,4-Cl_2	CH_2S	4-COOH	3-NO_2, 4-F	0.4	2.5
6o[a]	2,4-Cl_2	CH_2S	4-COOH	3-NO_2, 4-Br	0.6	2.5
6p[a]	4-CH_3	CH_2S	4-COOH	3-NO_2, 4-F	0.3	2.5
6q[a]	4-CF_3	CH_2S	4-COOH	3-NO_2, 4-F	0.4	0.2
6r[b]	4-Br	CH_2S	4-$COOCH_3$	3-NO_2, 4-F	0.3	0.75
6s[a]	H	CH_2S	4-COOH	3-NO_2, 4-F	0.3	2.5
6t[a]	4-Br	CH_2SO	4-COOH	3-NO_2, 4-F	75	>20
8a	4-Br	CH_2SO_2	4-COOH	3-NO_2, 4-F	5	>20
8b	4-Br	CH_2SO_2	4-COOH	3-NO_2, 4-Cl	25	>20
11[c]	4-Br	S	4-COOH	3- NO_2, 4-F	1.5	20

[a] These compounds were synthesized by utilizing method A.
[b] These compounds were synthesized by utilizing method B.
[c] These compounds were synthesized by utilizing method A and method B.

Table 2
In vitro cytotoxicity of α-benzyl thio chalcones



Compd[a]	R_2	R_1	R_3	IC_{50} (μM)	
				K562	pBCR/ABL
6aa	4-Br	4-COOH	3-OCH_3, 4-F	20	>20
6ab	4-Br	4-COOH	3-Br, 4-OH	35	>20
6ac	4-Br	4-COOH	3-NO_2, 4-OH	75	>20
6ad	4-Br	4-COOH	2-F, 5-NO_2	7.5	>20
6ae	4-Br	4-COOH	3-F, 4-NO_2	5.0	>20
6af	4-Br	4-COOH	2-NO_2, 4-F	20	>20
6ag	4-Br	4-COOH	2-F, 4-NO_2	7.5	>20

[a] All the compounds were synthesized by utilizing method A.

amino hydrochloride salts (**12a**–**12d**) made from **6c** and **6h** displayed excellent aqueous solubility and retained their cytotoxicity in K562 cells. The bioavailability of these compounds is being tested.

3.2. Inhibition of BCR-ABL kinase activity

The cell based cytotoxicity assays reported above demonstrate that (*E*)-α-benzylthio chalcones exhibit nanomolar cytotoxicity against K562 cells. To assess whether these compounds directly inhibit the phosphorylation of BCR-ABL, we tested the cell lysates prepared from K562 cells treated with these compounds in western blot analysis with anti-phospho BCR-ABL antibody (Fig. 1). These results showed that while all the compounds, in general, inhibited the phosphorylation of BCR-ABL, compounds **6c**, **6q**, **6r**, **12a**, **12b** and **12d** were most effective in this assay. Imatinib[5] was used as a positive control while DMSO was used as the negative control. The results of this study showed that some of the α-benzylthio chalcones (**6c**, **6q**, **6r**, **12b**, **12c** and **12d**) inhibited the phosphorylation of BCR-ABL with an IC_{50} (0.2–0.75 μM) comparable to that of imatinib (0.75 μM).

Table 3
In vitro cytotoxicity of carboxamide derivatives of α-benzylthio chalcones

Compd	IC_{50} (μM)		Solubility (mg/mL)
	K562	pBCR/ABL	
12a	0.6	2.5	>10.0
12b	0.2	0.5	>10.0
12c	0.75	0.5	>10.0
12d	0.3	0.5	>10.0

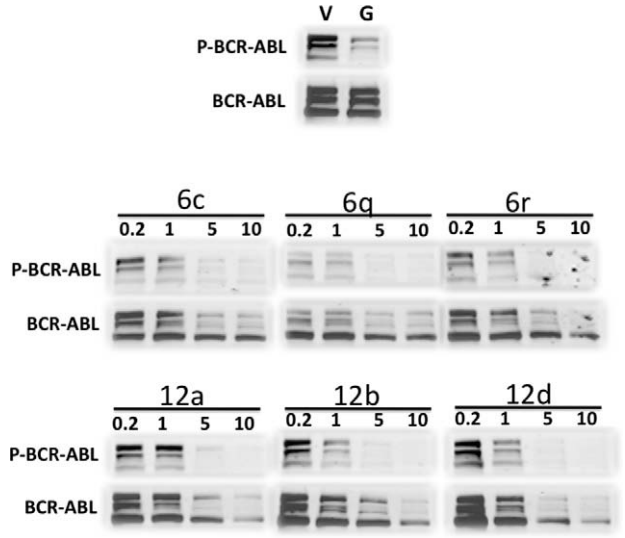


Figure 1. BCR-ABL kinase inhibition in human chronic myelogenous leukemic cells. K562 Cells expressing BCR-ABL p210, were treated with the indicated micromolar concentrations of each compound, 1 μM Gleevec(G) or Vehicle(DMSO) for 2 h. Total cellular proteins were harvested and resolved by 10%-SDS–PAGE. The gel was transferred and hybridized against antibodies specific for P-BCR-ABL or BCR-ABL. The western blot was treated with secondary antibodies conjugated with infrared dyes (LiCor) and scanned using Odyssey (LiCor) scanner. Percent inhibition was determined by quantifying each band using the software provided by LiCor, then normalizing the P-BCR-ABL signal to the parental BCR-ABL signal and determining% inhibition based on the vehicle control signal.

3.3. Discussion

Our current study shows that (*E*)-α-benzylthio chalcones exert a cytotoxic effect by inhibiting BCR-ABL phosphorylation in leukemic cell lines that over express this protein, thereby causing growth arrest and cell death. We describe the development of novel small molecules that inhibit autophosphorylation of BCR-ABL at a concentration of 0.2–0.75 μM. While assessing the kinase inhibitory profile of **6c** against other kinases implicated in cancer, we found **6c** to be a specific inhibitor of BCR-ABL. **6c** exhibited 10–15-fold higher selectivity for BCR-ABL over other tyrosine kinases and more than 40-fold higher selectivity for BCR-ABL over serine/threonine kinases (Table 4). The optimization of **6c** to improve its bioavailability and pharmacokinetics is ongoing. The in vivo efficacy of the optimized version of **6c** needs to be tested in xenograft mouse models of leukemia and ex vivo on patient samples.

Table 4
Kinase inhibition profile of **6c**

Kinase	IC_{50} (μM) **6c**
BCR-ABL	0.75
Lyn B	17.0
Src	13.2
ErbB2	>10.0
EGFR	>20.0
Plk-1	>50.0
Cdk-1	>50.0

Studies with kinase inhibitors have identified three general mechanisms for pharmacological inhibition of kinase activity: (1) direct binding in the ATP-binding site, (2) binding in the substrate-binding site, and (3) engagement of an allosteric site which results in the altered conformation of the kinase causing a block in proper substrate phosphorylation. The first kinase inhibitor to reach the market was Imatinib (Gleevec®),[5] an inhibitor of BCR-ABL tyrosine kinase that has been a remarkable success for the treatment of Philadelphia chromosome positive (Ph+) chronic myelogenous leukemia's (CMLs). This was followed by the development of a series of kinase inhibitors, which include gefitinib (Iressa),[22] erlotinib (OSI-1774; Tarceva),[23] lapatinib (GW-572016),[24] canertinib (CI 1033),[25] semaxinib (SU5416),[26] vatalanib (PTK787/ZK222584),[27] sorafenib (BAY 43-9006),[28] sutent (SU11248),[29] and leflunomide (SU101).[30] All of these compounds are ATP-competitive inhibitors and an understanding of the pharmacological properties and anticancer activities of these compounds have resulted in a rapid advance to our understanding of the advantages and limitations associated with this class of therapeutic agents. One of the important facts that has emerged in the past few years has been the realization that tumor cells often develop resistance to ATP-competitive kinase inhibitors as a result of accumulating mutations in the ATP-binding site of the kinase, which has been observed in patients undergoing treatment with imatinib[31] as well as gefitinib[32] and erlotinib.[33]

Compound **6c** and its analogs do not resemble typical ATP mimetics in structure. Hence **6c** and its analogs are expected to target regions outside the ATP-binding site of their target kinases and offer the potential to be unaffected by mutations in the kinase domain that make tumor cells resistant to ATP-competitive inhibitors.

4. Conclusion

In conclusion, we describe here, for the first time, the discovery and synthesis of a novel class of compounds, (*E*)-α-benzylthio chalcones, which possess potent kinase inhibitory activity and exhibit cytotoxicity against human tumor cells that express the oncogenic kinase BCR-ABL. While these compounds are comparable to imatinib in their in vitro efficacy they do not resemble typical ATP-mimetics. Hence, they offer the potential to be unaffected by mutations in the kinase domain that make tumor cells resistant to ATP-competitive inhibitors. These compounds possess a simple molecular structure and are easy to synthesize which makes them very attractive for further exploration as kinase inhibitors with application in cancer therapy.

5. Experimental

5.1. Chemistry

5.1.1. General information

All reagents and solvents were obtained from commercial suppliers and used without further purification unless otherwise stated. Solvents were dried using standard procedures and reactions requiring anhydrous conditions were performed under N_2 atmosphere. Reactions were monitored by Thin Layer Chromatography (TLC) on precoated Silica Gel F254 plates (Sigma–Aldrich) with a UV indicator. Column chromatography was performed with Merck 70–230 mesh Silica Gel 60 Å. Yields were of purified product and were not optimized. Melting points were determined using an Electro thermal Mel-Temp® 3.0 micro melting point apparatus and are uncorrected. ^{1}H NMR spectra were obtained with a Bruker AM 300 and 400 MHz spectrometer. The chemical shifts are reported in parts per million (δ) downfield using tetramethyl silane (Me_4Si) as internal standard and. Spin multiplicities are given as s (singlet), d (doublet), br s (broad singlet), m (multiplet), and q (quartet). Coupling constants (*J* values) were measured in hertz (Hz). The purity of the final compounds was determined by HPLC and is 95% or higher unless specified otherwise. Phenacylbromides were prepared according to the procedure reported in the literature.[34]

5.1.2. General procedure for the preparation of 2-(benzyl/aryl thio)-1-arylethanone (4 and 10)

To a solution of sodium hydroxide (20 mmol) in methanol (50 mL) benzylthiol **3** (20 mmol) or arylthiol **9** (20 mmol) was added and the contents were stirred at room temperature for 10 min. To this reaction mixture phenacylbromide **2** (20 mmol) was added and stirred for further 1–2 h. After completion of the reaction, the contents were cooled, poured on to ice-water and the solid obtained was collected by filtration. The crude product was recrystallized from 2-propanol to get pure **4** or **10**. The following 2-(benzyl/aryl thio)-1-arylethanones **4** and **10** were prepared using the above procedure.

5.1.2.1. 4-(2-(4-Bromobenzylthio)acetyl)benzoic acid (4a). Yield: 95%; white solid, mp 195–197 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.72 (s, 2H, CH_2), 3.95 (s, 2H, CH_2), 7.27–8.05 (m, 8H, Ar–H), 13.40 (br s, 1H, COOH). HRMS found *m*/*z* 364.78. Calcd for $C_{16}H_{13}BrO_3S$ $(M+H)^+$ *m*/*z* 364.98.

5.1.2.2. 4-(2-(4-Chlorobenzylthio)acetyl)benzoic acid (4b). Yield: 92%; white solid, mp 194–196 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.78 (s, 2H, CH_2), 4.05 (s, 2H, CH_2), 7.28–8.20 (m, 8H, Ar–H), 13.35 (br s, 1H, COOH). HRMS found *m*/*z* 321.08. Calcd for $C_{16}H_{13}ClO_3S$ $(M+H)^+$ *m*/*z* 321.04.

5.1.2.3. 4-(2-(4-Fluorobenzylthio)acetyl)benzoic acid (4c). Yield: 88%; white solid, mp 200–202 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.67 (s, 2H, CH_2), 3.92 (s, 2H, CH_2), 7.21–8.05 (m, 8H, Ar–H), 13.38 (br s, 1H, COOH). HRMS found *m*/*z* 305.10. Calcd for $C_{16}H_{13}FO_3S$ $(M+H)^+$ *m*/*z* 305.06.

5.1.2.4. 2-(4-Fluorobenzylthio)-1-(4-fluorophenyl)ethanone (4d). Yield: 84%; white solid, mp 68–70 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.60 (s, 2H, CH_2), 3.63 (s, 2H, CH_2), 7.18–7.85 (m, 8H, Ar–H). HRMS found *m*/*z* 279.06. Calcd for $C_{15}H_{12}F_2OS$ $(M+H)^+$ *m*/*z* 279.07.

5.1.2.5. 4-(2-(2-Fluorobenzylthio)acetyl)benzoic acid (4e). Yield: 72%; white solid, mp 189–190 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.82 (s, 2H, CH_2), 4.02 (s, 2H, CH_2), 7.15–8.14 (m, 8H, Ar–H), 13.35 (br s, 1H, COOH). HRMS found *m*/*z* 304.58. Calcd for $C_{16}H_{13}FO_3S$ $(M+H)^+$ *m*/*z* 304.58.

5.1.2.6. 4-(2-(2-Chlorobenzylthio)acetyl)benzoic acid (4f). Yield: 84%; white solid, mp 190–192 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.71 (s, 2H, CH_2), 3.93 (s, 2H, CH_2), 7.11–8.10 (m, 8H, Ar–H), 13.25 (br s, 1H,

COOH). HRMS found *m/z* 321.02. Calcd for $C_{16}H_{13}ClO_3S$ $(M+H)^+$ *m/z* 321.04.

5.1.2.7. 4-(2-(2,4-Dichlorobenzylthio)acetyl)benzoic acid (4g). Yield: 89%; white solid, mp 215–217 °C. ^1H NMR ($CDCl_3$, 300 MHz): δ 3.88 (s, 2H, CH_2), 4.12 (s, 2H, CH_2), 7.28–8.21 (m, 7H, Ar–H), 13.55 (br s, 1H, COOH). HRMS found *m/z* 354.97. Calcd for $C_{16}H_{12}Cl_2O_3S$ $(M+H)^+$ *m/z* 354.99.

5.1.2.8. 4-(2-(4-Methylbenzylthio)acetyl)benzoic acid (4h). Yield: 93%; white solid, mp 196–198 °C. ^1H NMR ($CDCl_3$, 300 MHz): δ 2.27 (s, 3H, CH_3), 3.68 (s, 2H, CH_2), 3.91 (s, 2H, CH_2), 7.10–8.04 (m, 8H, Ar–H), 13.25 (br s, 1H, COOH). HRMS found *m/z* 301.07. Calcd for $C_{17}H_{16}O_3S$ $(M+H)^+$ *m/z* 301.09.

5.1.2.9. 4-(2-(4-(Trifluoromethyl)benzylthio)acetyl)benzoic acid (4i). Yield: 78%; white solid, mp 162–164 °C. ^1H NMR ($CDCl_3$, 300 MHz): δ 3.68 (s, 2H, CH_2), 3.95 (s, 2H, CH_2), 7.11–8.10 (m, 8H, Ar–H), 13.35 (br s, 1H, COOH). HRMS found *m/z* 355.04. Calcd for $C_{17}H_{13}F_3O_3S$ $(M+H)^+$ *m/z* 355.06.

5.1.2.10. Methyl 4-(2-(4-bromobenzylthio)acetyl)benzoate (4j). Yield: 90%; white solid, mp 75–77 °C. ^1H NMR ($CDCl_3$, 300 MHz): δ 3.72 (s, 2H, CH_2), 3.92 (s, 3H, OCH_3), 4.05 (s, 2H, CH_2), 7.26–8.15 (m, 8H, Ar–H). HRMS found *m/z* 379.00. Calcd for $C_{17}H_{15}BrO_3S$ $(M+H)^+$ *m/z* 379.00.

5.1.2.11. 4-(2-(Benzylthio)acetyl)benzoic acid (4k). Yield: 93%; white solid, mp 182–184 °C. ^1H NMR ($CDCl_3$, 300 MHz): δ 3.88 (s, 2H, CH_2), 4.02 (s, 2H, CH_2), 6.98–8.04 (m, 9H, Ar–H), 13.50 (br s, 1H, COOH). HRMS found *m/z* 287.07. Calcd for $C_{16}H_{14}O_3S$ $(M+H)^+$ *m/z* 287.07.

5.1.3. 4-(2-(4-Bromobenzylsulfonyl)acetyl)benzoic acid (7)

To a mixture of **4a** (50 mmol) in glacial acetic acid (100 mL), 30% hydrogen peroxide (60 mL) was added and the contents were refluxed for 1 h. After completion of the reaction, the cooled reaction contents were poured on to ice-water and the separated solid was filtered and dried. The crude product was recrystallized from methanol to obtain pure sample of **7**.

Yield: 80%; white solid, mp 270–272 °C. ^1H NMR ($CDCl_3$, 300 MHz): δ 3.82 (s, 2H, CH_2), 4.05 (s, 2H, CH_2), 7.36–8.15 (m, 8H, Ar–H), 13.50 (br s, 1H, COOH). HRMS found *m/z* 396.98. Calcd for $C_{16}H_{13}BrO_5S$ $(M+H)^+$ *m/z* 396.98.

5.1.4. 4-(2-(4-Bromophenylthio)acetyl)benzoic acid (10a)

Yield: 94%; white solid, mp 188–190 °C. ^1H NMR ($CDCl_3$, 300 MHz): δ 3.85 (s, 2H, CH_2), 7.21–8.15 (m, 8H, Ar–H), 13.50 (br s, 1H, COOH). HRMS found *m/z* 350.98. Calcd for $C_{15}H_{11}BrO_3S$ $(M+H)^+$ *m/z* 350.97.

5.1.5. General procedure for the preparation of (*E*)-2-(benzylthio)-1,3-diphenylprop-2-en-1-one (6) and (*E*)-1,3-diphenyl-2-(phenylthio)prop-2-en-1-one (11)

Method A: A mixture of 2-(benzyl/aryl thio)-1-arylethanone (**4** or **10**) (10 mmol), araldehyde (**5**) (12.5 mmol) and ammonium acetate (25 mmol) in glacial acetic acid (10 mL) was refluxed for 5–8 h. After completion of the reaction, reaction mixture was cooled and separated product was filtered, washed with 2-propanol and petroleum ether and dried. If solid was not formed, the reaction mixture was poured on to crushed ice, extracted with ethyl acetate, washed with water, brine and dried over sodium sulfate. After concentration, crude product obtained was recrystallized in 2-propanol to yield analytically pure sample of **6** or **11**.

Method B: A mixture of 2-(benzyl/aryl thio)-1-arylethanone (**4** or **10**) (10 mmol), araldehyde (**5**) (10 mmol), glacial acetic acid (5 mL), and a catalytic amount (100 μL) of benzyl amine was refluxed for 5–8 h. After completion of the reaction (TLC monitoring, $CHCl_3$/MeOH on silica gel plate), the contents were cooled to room temperature, the precipitated product was filtered, washed with 2-propanol and petroleum ether and dried. If solid was not formed, the reaction mixture was poured on to ice-water and extracted with ethyl acetate. The organic layer was dried over sodium sulfate, filtered and concentrated under vacuum to obtain the crude **6** or **11**. The crude product was recrystallized form 2-propanol to yield an analytically pure sample of **6** or **11**.

5.1.5.1. (*E*)-4-(2-(4-Bromobenzylthio)-3-(4-chloro-3-nitrophenyl)acryloyl)benzoic acid (6a). Yield: 78%; light yellow solid, mp 148–150 °C. ^1H NMR ($CDCl_3$, 400 MHz): δ 3.92 (s, 2H, CH_2), 6.84–6.94 (m, 3H, Ar–H), 7.17–7.23 (m, 5H, Ar–H), 7.22 (s, 1H, CH), 7.55–7.57 (dd, 2H, Ar–H), 7.58–7.62 (m, 1H, Ar–H), 7.85–7.87 (d, 2H, *J* = 8.0 Hz, Ar–H), 8.04–8.15 (dd, 1H, Ar–H), 13.50 (br s, 1H, COOH). HRMS found *m/z* 533.98. Calcd for $C_{23}H_{15}BrClNO_5S$ $(M+H)^+$ *m/z* 533.96.

5.1.5.2. (*E*)-4-(2-(4-Bromobenzylthio)-3-(4-bromo-3-nitrophenyl)acryloyl)benzoic acid (6b). Yield: 80%; light yellow solid, mp 168–170 °C. ^1H NMR ($CDCl_3$, 400 MHz): δ 3.90 (s, 2H, CH_2), 6.85–6.92 (m, 3H, Ar–H), 7.22–7.28 (m, 5H, Ar–H), 7.25 (s, 1H, CH), 7.62–7.65 (dd, 2H, Ar–H), 7.60–7.62 (m, 1H, Ar–H), 7.82–7.84 (d, 2H, *J* = 8.0 Hz, Ar–H), 8.02–8.09 (dd, 1H, Ar–H), 13.40 (br s, 1H, COOH). HRMS found *m/z* 577.92. Calcd for $C_{23}H_{15}Br_2NO_5S$ $(M+H)^+$ *m/z* 577.91.

5.1.5.3. (*E*)-4-(2-(4-Bromobenzylthio)-3-(4-fluoro-3-nitrophenyl)acryloyl)benzoic acid (6c). Yield: 72%; light yellow solid, mp 170–172 °C. ^1H NMR ($CDCl_3$, 400 MHz): δ 3.72 (s, 2H, CH_2), 6.68–6.75 (m, 3H, Ar–H), 6.98–7.06 (m, 5H, Ar–H), 7.01 (s, 1H, CH), 7.30–7.37 (dd, 2H, Ar–H), 7.54–7.58 (m, 1H, Ar–H), 7.87–7.89 (d, 2H, *J* = 8.3 Hz, Ar–H), 8.13–8.16 (dd, 1H, Ar–H), 13.33 (br s, 1H, COOH). HRMS found *m/z* 518.10. Calcd for $C_{23}H_{15}BrFNO_5S$ $(M+H)^+$ *m/z* 517.99.

5.1.5.4. (*E*)-4-(2-(4-Chlorobenzylthio)-3-(4-fluoro-3-nitrophenyl)-acryloyl)benzoic acid (6d). Yield: 82%; light yellow solid, mp 165–168 °C. ^1H NMR ($CDCl_3$, 400 MHz): δ 3.75 (s, 2H, CH_2), 6.71–6.76 (m, 3H, Ar–H), 6.95–7.06 (m, 5H, Ar–H), 7.05 (s, 1H, CH), 7.28–7.35 (dd, 2H, Ar–H), 7.48–7.55 (m, 1H, Ar–H), 7.78–7.85 (d, 2H, *J* = 8.2 Hz, Ar–H), 8.10–8.14 (dd, 1H, Ar–H), 13.36 (br s, 1H, COOH). HRMS found *m/z* 472.05. Calcd for $C_{23}H_{15}ClFNO_5S$ $(M+H)^+$ *m/z* 472.04.

5.1.5.5. (*E*)-4-(2-(4-Chlorobenzylthio)-3-(4-chloro-3-nitrophenyl)acryloyl)benzoic acid (6e). Yield: 77%; light yellow solid, mp 170–162 °C. ^1H NMR ($CDCl_3$, 400 MHz):δ 3.82 (s, 2H, CH_2), 6.69–6.71 (m, 3H, Ar–H), 6.82–7.08 (m, 5H, Ar–H), 7.01 (s, 1H, CH), 7.18–7.24 (dd, 2H, Ar–H), 7.35–7.55 (m, 1H, Ar–H), 7.79–7.91 (d, 2H, *J* = 8.2 Hz, Ar–H), 8.04–8.10 (dd, 1H, Ar–H), 13.40 (br s, 1H, COOH). HRMS found 488.05. Calcd for $C_{23}H_{15}Cl_2NO_5S$ $(M+H)^+$ *m/z* 488.01.

5.1.5.6. (*E*)-4-(2-(4-Chlorobenzylthio)-3-(4-bromo-3-nitrophenyl)acryloyl)benzoic acid (6f). Yield: 85%; light yellow solid, mp 189–191 °C. ^1H NMR ($CDCl_3$, 400 MHz): δ 3.80 (s, 2H, CH_2), 6.68–6.75 (m, 3H, Ar–H), 6.82–7.08 (m, 5H, Ar–H), 7.11 (s, 1H, CH), 7.22–7.28 (dd, 2H, Ar–H), 7.41–7.56 (m, 1H, Ar–H), 7.81–7.91 (d, 2H, *J* = 8.0 Hz, Ar–H), 8.15–8.22 (dd, 1H, Ar–H), 13.50 (br s, 1H, COOH). HRMS found *m/z* 533.95. Calcd for $C_{23}H_{15}BrClNO_5S$ $(M+H)^+$ *m/z* 533.96.

5.1.5.7. (*E*)-4-(2-(4-Fluorobenzylthio)-3-(4-chloro-3-nitrophenyl)acryloyl)benzoic acid (6g). Yield: 68%; light yellow solid, mp 164–166 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.69 (s, 2H, CH_2), 6.65–6.75 (m, 3H, Ar–H), 6.80–7.09 (m, 5H, Ar–H), 7.08 (s, 1H, CH), 7.15–7.30 (dd, 2H, Ar–H), 7.35–7.48 (m, 1H, Ar–H), 7.78–7.89 (d, 2H, J = 8.1 Hz, Ar–H), 8.05–8.18 (dd, 1H, Ar–H), 13.50 (br s, 1H, COOH). HRMS found m/z 472.05. Calcd for $C_{23}H_{15}ClFNO_5S$ $(M+H)^+$ m/z 472.04.

5.1.5.8. (*E*)-4-(2-(4-Fluorobenzylthio)-3-(4-fluoro-3-nitrophenyl)acryloyl)benzoic acid (6h). Yield: 82%; light yellow solid, mp 160–162 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.97 (s, 2H, CH_2), 6.93–6.97 (m, 3H, Ar–H), 7.11–7.15 (m, 5H, Ar–H), 7.21 (s, 1H, CH), 7.67–7.69 (dd, 2H, Ar–H), 7.94–7.99 (m, 1H, Ar–H), 8.03–8.07 (d, 2H, J = 8.1 Hz, Ar–H), 8.48–8.50 (dd, 1H, Ar–H), 13.50 (br s, 1H, COOH). HRMS found m/z 456.05. Calcd for $C_{23}H_{15}F_2NO_5S$ $(M+H)^+$ m/z 456.07.

5.1.5.9. (*E*)-4-(2-(4-Fluorobenzylthio)-3-(4-bromo-3-nitrophenyl)acryloyl)benzoic acid (6i). Yield: 75%; light yellow solid, mp 185–187 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.93 (s, 2H, CH_2), 6.88–6.99 (m, 3H, Ar–H), 7.18–7.25 (m, 5H, Ar–H), 7.26 (s, 1H, CH), 7.55–7.68 (dd, 2H, Ar–H), 7.87–7.89 (m, 1H, Ar–H), 7.98–8.02 (d, 2H, J = 8.1 Hz, Ar–H), 8.35–8.40 (dd, 1H, Ar–H), 13.40 (br s, 1H, COOH). HRMS found m/z 517.98. Calcd for $C_{23}H_{15}FNO_5S$ $(M+H)^+$ m/z 517.99.

5.1.5.10. (*E*)-3-(4-Fluoro-3-nitrophenyl)-2-(4-fluorobenzylthio)-1-(4-fluorophenyl)prop-2-en-1-one (6j). Yield: 79%; light yellow solid, mp 88–90 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.02 (s, 2H, CH_2), 6.98–7.02 (m, 2H, Ar–H), 7.15–7.19 (m, 2H, Ar–H), 7.25 (s, 1H, CH), 7.62–7.67 (dd, 2H, Ar–H), 7.71–7.73 (d, 1H, J = 8.0 Hz, Ar–H), 7.98–8.03 (d, 2H, J = 8.1 Hz, Ar–H), 8.07–8.11 (dd, 1H, Ar–H), 8.52–8.54 (dd, 1H, Ar–H). HRMS found m/z 430.06. Calcd for $C_{22}H_{14}F_3NO_3S$ $(M+H)^+$ m/z 430.07.

5.1.5.11. (*E*)-4-(3-(4-Chloro-3-nitrophenyl)-2-(2-fluorobenzylthio)acryloyl)benzoic acid (6k). Yield: 66%; white solid, yield: 78%; mp 138–140 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.04 (s, 2H, CH_2), 6.95–7.02 (m, 2H, Ar–H), 7.09–7.18 (m, 2H, Ar–H), 7.20 (s, 1H, CH), 7.34–7.32 (dd, 2H, Ar–H), 7.73–7.80 (m, 2H, Ar–H), 7.95–8.06 (d, 2H, J = 8.1 Hz, Ar–H), 8.16–8.22 (dd, 1H, Ar–H), 8.44–8.59 (dd, 1H, Ar–H), 13.57 (br s, 1H, COOH). HRMS found m/z 472.01. Calcd for $C_{23}H_{15}Cl$ FNO_5S $(M+H)^+$ 472.03.

5.1.5.12. (*E*)-4-(2-(2-Chlorobenzylthio)-3-(4-fluoro-3-nitrophenyl)acryloyl)benzoic acid (6l). Yield: 76%; white solid, mp 198–200 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.15 (s, 2H, CH_2), 7.03–7.14 (m, 2H, Ar–H), 7.22–7.28 (m, 2H, Ar–H), 7.20 (s, 1H, CH), 7.58–7.62 (dd, 2H, Ar–H), 7.78–7.83 (d, 1H, J = 8.0 Hz, Ar–H), 7.98–8.03 (d, 2H, J = 8.0 Hz, Ar–H), 8.05–8.10 (dd, 1H, Ar–H), 8.43–8.54 (dd, 1H, Ar–H), 13.47 (br s, 1H, COOH). HRMS found 472.04. Calcd for $C_{23}H_{15}ClFNO_5S$ $(M+H)^+$ m/z 472.04.

5.1.5.13. (*E*)-4-(3-(4-Chloro-3-nitrophenyl)-2-(2,4-dichlorobenzylthio)acryloyl)benzoic acid (6m). Yield: 85%; white solid, mp 126–130 °C. ^{1}HNMR ($CDCl_3$, 400 MHz): δ 4.10 (s, 2H, CH_2), 6.93–7.12 (m, 2H, Ar–H), 7.24–7.29 (m, 2H, Ar–H), 7.26 (s, 1H, CH), 7.55–7.60 (dd, 2H, Ar–H), 7.82–7.89 (d, 1H, J = 8.1 Hz, Ar–H), 8.03–8.08 (d, 2H, J = 8.0 Hz, Ar–H), 8.11–8.18 (dd, 1H, Ar–H), 8.51–8.59 (dd, 1H, Ar–H), 13.50 (br s, 1H, COOH). HRMS found m/z 523.97. Calcd for $C_{23}H_{14}Cl_3NO_5S$ $(M+H)^+$ m/z 523.97.

5.1.5.14. (*E*)-4-(3-(4-Fluoro-3-nitrophenyl)-2-(2,4-dichlorobenzylthio)acryloyl)benzoic acid (6n). Yield: 80%; white solid, mp 124–128 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.92 (s, 2H, CH_2), 6.92–6.98 (m, 2H, Ar–H), 7.12–7.19 (m, 2H, Ar–H), 7.20 (s, 1H, CH), 7.25–7.33 (dd, 2H, Ar–H), 7.52–7.59 (d, 1H, J = 8.1 Hz, Ar–H), 7.68–7.73 (d, 2H, J = 8.0 Hz, Ar–H), 7.81–7.88 (dd, 1H, Ar–H), 8.10–8.16 (dd, 1H, Ar–H), 13.50 (br s, 1H, COOH). HRMS found m/z 506.00. Calcd for $C_{23}H_{14}Cl_2FNO_5S$ $(M+H)^+$ m/z 506.00.

5.1.5.15. (*E*)-4-(3-(4-Bromo-3-nitrophenyl)-2-(2,4-dichlorobenzylthio)acryloyl)benzoic acid (6o). Yield: 74%; white solid, mp 160–162 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.95 (s, 2H, CH_2), 6.90–6.98 (m, 2H, Ar–H), 7.15–7.21 (m, 2H, Ar–H), 7.25 (s, 1H, CH), 7.28–7.32 (dd, 2H, Ar–H), 7.49–7.53 (d, 1H, J = 8.1 Hz, Ar–H), 7.62–7.70 (d, 2H, J = 8.0 Hz, Ar–H), 7.78–7.84 (dd, 1H, Ar–H), 8.15–8.20 (dd, 1H, Ar–H), 13.48 (br s, 1H, COOH). HRMS found m/z 567.92. Calcd for $C_{23}H_{14}BrCl_2NO_5S$ $(M+H)^+$ m/z 567.92.

5.1.5.16. (*E*)-4-(3-(4-Fluoro-3-nitrophenyl)-2-(4-methylbenzylthio)acryloyl)benzoic acid (6p). Yield: 85%; white solid, mp 148–150 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.25 (s, 3H, CH_3), 3.90 (s, 2H, CH_2), 6.83–6.92 (m, 2H, Ar–H), 6.98–7.12 (m, 2H, Ar–H), 7.20 (s, 1H, CH), 7.25–7.30 (dd, 2H, Ar–H), 7.38–7.43 (d, 1H, J = 8.1 Hz, Ar–H), 7.67–7.70 (d, 2H, J = 8.0 Hz, Ar–H), 7.85–7.89 (dd, 1H, Ar–H), 8.22–8.33 (dd, 1H, Ar–H), 13.50 (br s, 1H, COOH). HRMS found m/z 452.11. Calcd for $C_{24}H_{18}FNO_5S$ $(M+H)^+$ m/z 452.10

5.1.5.17. (*E*)-4-(3-(4-Fluoro-3-nitrophenyl)-2-(4-(trifluoromethyl)benzylthio)acryloyl)benzoic acid (6q). Yield: 68%; white solid, mp 183–186 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.87 (s, 2H, CH_2), 6.73 (s, 1H, CH), 7.03–7.11 (m, 2H, Ar–H), 7.17–7.19 (d, 2H, Ar–H), 7.30–7.37 (d, 1H, J = 8.1 Hz, Ar–H), 7.59–7.65 (dd, 2H, J = 8.0 Hz, Ar–H), 7.88–7.98 (dd, 1H, Ar–H), 8.19–8.21 (dd, 1H, Ar–H), 13.50 (br s, 1H, COOH). HRMS found m/z 506.07. Calcd for $C_{24}H_{15}F_4NO_5S$ $(M+H)^+$ m/z 506.07.

5.1.5.18. (*E*)-Methyl4-(2-(4-bromobenzylthio)-3-(4-fluoro-3-nitrophenyl)acryloyl)benzoate (6r). Yield: 76%; white solid, mp 143–145 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.83 (s, 3H, OCH_3), 3.92 (s, 2H, CH_2), 6.83 (s, 1H, CH), 7.06–7.12 (m, 2H, Ar–H), 7.20–7.28 (d, 2H, Ar–H), 7.30–7.37 (d, 1H, J = 8.1 Hz, Ar–H), 7.55–7.65 (dd, 2H, J = 8.0 Hz, Ar–H), 7.78–7.88 (dd, 1H, Ar–H), 8.12–8.20 (dd, 1H, Ar–H), 13.50 (br s, 1H, COOH). HRMS found m/z 532.01. Calcd for $C_{24}H_{17}BrFNO_5S$ $(M+H)^+$ m/z 532.01.

5.1.5.19. (*E*)-4-(2-(Benzylthio)-3-(4-fluoro-3-nitrophenyl)acryloyl)benzoic acid (6s). Yield: 85%; white solid, mp 148–150 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.87 (s, 2H, CH_2), 6.76 (s, 1H, CH), 6.96–7.00 (m, 4H, Ar–H), 7.08–7.14 (m, 2H, Ar–H), 7.40–7.43 (d, 2H, J = 12 Hz, Ar–H), 7.67–7.71 (m, 1H, Ar–H), 7.93–7.95 (d, 2H, J = 8 Hz, Ar–H), 8.20–8.22 (dd, 1H, J = 8.0 Hz, Ar–H), 13.50 (br s, 1H, COOH). HRMS found m/z 438.08. Calcd for $C_{23}H_{16}FNO_5S$ $(M+H)^+$ m/z 438.08.

5.1.5.20. (*E*)-4-(2-(4-Bromobenzylsulfinyl)-3-(4-fluoro-3-nitrophenyl)acryloyl)benzoic acid (6t). To a mixture of **6c** (0.51 g, 10 mmol) in dry chloroform (10 mL), *m*-chloroperoxybenzoic acid (0.22 g, 10 mmol) in 5 mL dry chloroform was added drop wise at 0 °C. After the addition, the reaction was continued at this temperature for 1 h and at room temperature for an additional 1 h. The separated solid was filtered and recrystallized from chloroform to obtain a pure sample of **6t**. Yield: 78%; white solid, mp 213–215 °C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 4.47–4.63 (q, 2H, CH_2), 7.22–7.24 (d, 2H, Ar–H), 7.36–7.39 (d, 2H, Ar–H), 7.47–7.56 (m, 2H, Ar–H), 7.63–7.67 (m, 1H, CH), 7.81–7.98 (m, 1H, Ar–H), 8.02–8.05 (dd, 1H, Ar–H), 8.11–8.24 (m, 3H, Ar–H), 13.50 (br s, 1H, COOH). HRMS found m/z 531.90. Calcd for $C_{23}H_{15}BrFNO_6S$ $(M+H)^+$ m/z 531.99.

5.1.6. (*E*)-4-(2-(4-Bromobenzylsulfonyl)-3-(4-fluoro-3-nitrophenyl)acryloyl)benzoic acid (8a)

This compound was prepared from **7** and 3-nitro-4-fluorobenzaldehyde in 62% yield as described method A. Alternatively, this compound was also prepared by the oxidation of **6c** with excess (3 equiv) of *m*-chloroperoxybenzoic acid as described in **6t**. Yield: 62%; white solid, mp 178–180 °C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 4.82 (s, 2H, CH_2), 7.39–7.49 (m, 2H, Ar–H), 7.51–7.67 (m, 4H, Ar–H), 7.81 (s, 1H, CH), 7.91–7.99 (m, 3H, Ar–H), 8.10–8.14 (m, 1H, Ar–H), 13.50 (br s, 1H, COOH). HRMS found *m*/*z* 549.98. Calcd for $C_{23}H_{15}BrFNO_7S$ $(M+H)^+$ *m*/*z* 549.98.

5.1.6.1. (*E*)-4-(2-(4-Bromobenzylsulfonyl)-3-(4-chloro-3-nitrophenyl)acryloyl)benzoic acid (8b). Yield: 48%; white solid, mp 148–150 °C. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 4.53 (s, 2H, CH_2), 7.20–7.24 (m, 3H, Ar–H), 7.30–7.36 (m, 5H, Ar–H), 7.38 (s, 1H, CH), 7.43–7.55 (dd, 2H, Ar–H), 7.51–7.59 (m, 1H, Ar–H), 7.95–7.98 (d, 2H, *J* = 8.6 Hz, Ar–H), 8.09–8.21 (dd, 1H, Ar–H), 13.50 (br s, 1H, COOH). HRMS found *m*/*z* 565.92. Calcd for $C_{23}H_{15}BrClNO_7S$ $(M+H)^+$ *m*/*z* 565.95.

5.1.7. (*E*)-4-(2-(4-Bromophenylthio)-3-(4-fluoro-3-nitrophenyl)-acryloyl)benzoic acid (11)

Yield: 89%; white solid, mp 145–147 °C. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.12–7.25 (m, 2H, Ar–H), 7.28–7.31 (d, 2H, Ar–H), 7.65 (s, 1H, CH), 7.88–7.90 (d, 1H, *J* = 8.1 Hz, Ar–H), 8.00–8.03 (dd, 2H, *J* = 8.0 Hz, Ar–H), 8.24–8.27 (dd, 1H, Ar–H), 8.65–8.67 (dd, 1H, Ar–H), 13.50 (br s, 1H, COOH). HRMS found *m*/*z* 502.97. Calcd for $C_{22}H_{13}BrFNO_5S$ $(M+H)^+$ *m*/*z* 502.97.

5.1.7.1. (*E*)-4-(2-(4-Bromobenzylthio)-3-(4-fluoro-3-methoxyphenyl)acryloyl)benzoic acid (6aa). Yield: 65%; white solid, mp 72–74 °C. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 3.85 (s, 3H, OCH_3), 4.04 (s, 2H, CH_2), 6.84–6.94 (m, 2H, Ar–H), 7.18–7.27 (m, 5H, Ar–H), 7.29 (s, 1H, CH), 7.55–7.57 (dd, 2H, Ar–H), 7.58–7.62 (m, 1H, Ar–H), 7.85–7.87 (d, 2H, *J* = 8.0 Hz, Ar–H), 8.04–8.15 (dd, 1H, Ar–H), 13.50 (br s, 1H, COOH). HRMS found *m*/*z* 502.34. Calcd for $C_{24}H_{18}BrFO_4S$ $(M+H)^+$ *m*/*z* 502.36.

5.1.7.2. (*E*)-4-(2-(4-Bromobenzylthio)-3-(4-hydroxy-3-bromo) acryloyl)benzoic acid (6ab). Yield: 60%; yellow solid: mp 158–160 °C. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 3.45 (br s, 1H, OH), 3.98 (s, 2H, CH_2), 7.20–7.26 (m, 4H, Ar–H), 7.30–7.37 (m, 2H, Ar–H), 7.62–7.69 (m, 4H, Ar–H), 7.95–8.04 (dd, 1H, *J* = 12.0, 8.0 Hz, Ar–H), 8.52–8.61 (m, 1H, Ar–H), 13.48 (br s, 1H, COOH). HRMS found *m*/*z* 549.30. Calcd for $C_{23}H_{16}Br_2O_4S$ $(M+H)^+$ *m*/*z* 549.25.

5.1.7.3. (*E*)-4-(2-(4-Bromobenzylthio)-3-(4-hydroxy-3-nitrophenyl)acryloyl)benzoic acid (6ac). Yield: 78%; yellow solid: mp 200–202 °C. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 3.35 (br s, 1H, OH), 4.05 (s, 2H, CH_2), 7.00–7.04 (m, 2H, Ar–H), 7.11–7.21 (m, 4H, Ar–H), 7.63–7.65 (d, 2H, *J* = 8.0, Ar–H), 7.90–7.93 (dd, 1H, *J* = 12.0, 8.0 Hz, Ar–H), 8.00–8.02 (d, 2H, *J* = 8.0, Ar–H), 8.38–8.39 (d, 1H, Ar–H), 13.50 (br s, 1H, COOH). HRMS found *m*/*z* 515.32. Calcd for $C_{23}H_{16}BrNO_6S$ $(M+H)^+$ *m*/*z* 515.36.

5.1.7.4. (*E*)-4-(2-(4-Bromobenzylthio)-3-(2-fluoro-5-nitrophenyl)acryloyl)benzoic acid (6ad). Yield: 68%; yellow solid: mp 223–225 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.00 (s, 2H, CH_2), 6.95–7.02 (m, 2H, Ar–H), 7.12–7.19 (m, 2H, Ar–H), 7.29–7.35 (m, 2H, Ar–H), 7.38–7.43 (d, 1H, *J* = 8.1 Hz, Ar–H), 7.61–7.70 (d, 2H, *J* = 8.0 Hz, Ar–H), 7.92–7.98 (m, 2H, Ar–H), 8.28–8.33 (dd, 1H, Ar–H), 13.50 (br s, 1H, COOH). HRMS found *m*/*z* 517.32. calcd for $C_{23}H_{15}BrFNO_5S$ $(M+H)^+$ *m*/*z* 517.35.

5.1.7.5. (*E*)-4-(2-(4-Bromobenzylthio)-3-(3-fluoro-4-nitrophenyl)-acryloyl)benzoic acid (6ae). Yield: 90%; yellow solid, mp 198–200 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 4.01 (s, 2H, CH_2), 6.52 (s, 1H, CH), 6.92–7.09 (m, 2H, Ar–H), 7.16–7.27 (m, 4H, Ar–H), 7.38–7.46 (m, 1H, Ar–H), 7.62–7.67 (m, 2H, Ar–H), 8.11–8.36 (m, 2H, Ar–H), 13.50 (br s, 1H, COOH). HRMS found *m*/*z* 517.98. Calcd for $C_{23}H_{15}BrFNO_5S$ $(M+H)^+$ *m*/*z* 517.99.

5.1.7.6. (*E*)-4-(2-(4-Bromobenzylthio)-3-(4-fluoro-2-nitrophenyl)acryloyl)benzoic acid (6af). Yield: 86%; yellow solid: mp 194–196 °C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 3.89 (s, 2H, CH_2), 6.59 (s, 1H, CH), 6.91–7.06 (m, 2H, Ar–H), 7.16–7.27 (m, 4H, Ar–H), 7.40–7.49 (m, 1H, Ar–H), 7.68–7.74 (m, 2H, Ar–H), 8.16–8.36 (m, 2H, Ar–H), 13.49 (br s, 1H, COOH). HRMS found *m*/*z* 517.97. Calcd for $C_{23}H_{15}BrFNO_5S$ $(M+H)^+$ *m*/*z* 517.99.

5.1.7.7. (*E*)-4-(2-(4-Bromobenzylthio)-3-(2-fluoro-4-nitrophenyl)acryloyl)benzoic acid (6ag). Yield: 87%; yellow solid, mp 196–199 °C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 3.89 (s, 2H, CH_2), 6.52 (s, 1H, CH), 6.92–7.09 (m, 2H, Ar–H), 7.16–7.27 (m, 4H, Ar–H), 7.38–7.46 (m, 1H, Ar–H), 7.62–7.67 (m, 2H, Ar–H), 8.11–8.36 (m, 2H, Ar–H), 13.50 (br s, 1H, COOH). HRMS found *m*/*z* 517.98. Calcd for $C_{23}H_{15}BrFNO_5S$ $(M+H)^+$ *m*/*z* 517.99.

5.1.8. (*E*)-4-(2-(4-Bromobenzylthio)-3-(4-fluoro-3-nitrophenyl) acryloyl)-*N*-(2-(4-methylpiperazin-1-yl)ethyl)benzamide (12a)

A mixture of **6c** (0.155 g, 3 mmol), 1-hydroxybenzotriazole hydrate (0.040 g, 3 mmol), *N*,*N*-diisopropylethylamine (157 μL, 9 mmol), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (0.064 g, 3.3 mmol) in dry tetrahydrofuran (10 mL) were stirred at room temperature under nitrogen atmosphere for 30 min. To this reaction mixture, 2-(4-methylpiperazin-1-yl)ethanamine (0.039 g, 3 mmol) in THF (2 mL) was added drop wise and continued stirring for additional 2 h. The progress of the reaction was monitored by TLC (9:1 chloroform/methanol on silica gel plate). After completion of the reaction, THF was removed under reduced pressure, diluted with water and extracted with ethyl acetate. The ethyl acetate layer was washed with saturated sodium bicarbonate solution, water and brine and dried over anhydrous sodium sulfate. Removal of the solvent under reduced pressure afforded a crude product which was purified by silica gel column chromatography, eluting with chloroform/methanol (9:0.5), to yield compound **12a**. Yield: 75%; light orange solid, mp 60–62 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.19 (s, 3H, CH_3), 2.25–2.54 (m, 10H), 3.45–346, (d, 2H, NH–CH_2), 3.98 (s, 2H, CH_2), 6.80–6.95 (m, 2H, Ar–H), 7.12–7.25 (m, 3H, Ar–H), 7.39–7.49 (dd, 2H, Ar–H), 7.63–7.78 (m, 2H, Ar–H), 8.28–7.29 (d, 2H, *J* = 8.0 Hz, Ar–H), 8.58 (br s, 1H, NH). HRMS found *m*/*z* 643.12. Calcd for $C_{30}H_{30}BrFN_4O_4S$ $(M+H)^+$ *m*/*z* 643.12.

5.1.8.1. (*E*)-2-(4-Bromobenzylthio)-3-(4-fluoro-3-nitrophenyl)-1-(4-(4-(2-hydroxy-ethyl)piperazine-1-carbonyl)phenyl)prop-2-en-1-one (12b). Yield: 70%; white solid, mp 117–119 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.44–2.54 (m, 6H), 2.76 (br s, 1H, OH), 3.34–3.37 (d, 2H), 3.57–3.61 (m, 2H), 3.74–3.79 (m, 2H,), 3.89 (s, 2H, CH_2), 6.84–6.85 (s, 1H, Ar–H), 6.92–6.94 (d, 2H, Ar–H), 7.14–7.16 (d, 2H, Ar–H), 7.20–7.26 (m, 2H, Ar–H), 7.34–7.36 (d, 2H, Ar–H), 7.50–7.52 (d, 2H, Ar–H), 7.74–7.77 (m, 1H, Ar–H). HRMS found *m*/*z* 630.08. Calcd for $C_{29}H_{27}BrFN_3O_5S$ $(M+H)^+$ *m*/*z* 630.09.

5.1.8.2. (*E*)-4-(2-(4-Bromobenzylthio)-3-(4-fluoro-3-nitrophenyl)-acryloyl)-N-(2-(diethylamino)ethyl)benzamide (12c). Yield: 72%; white solid, mp 60–61 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.44–2.54 (m, 6H), 2.76 (br s, 1H, OH), 3.34–3.37 (d, 2H), 3.57–3.61 (m, 2H), 3.74–3.79 (m, 2H,), 3.89 (s, 2H, CH_2), 6.84–6.85 (s, 1H, Ar–H), 6.92–6.94 (d, 2H, Ar–H), 7.14–7.16 (d, 2H, Ar–H),

7.20–7.26 (m, 2H, Ar–H), 7.34–7.36 (d, 2H, Ar–H), 7.50–7.52 (d, 2H, Ar–H), 7.74–7.77 (m, 1H, Ar–H). HRMS found *m/z* 616.10. Calcd for $C_{29}H_{29}BrFN_3O_4S$ $(M+H)^+$ *m/z* 616.11.

5.1.8.3. (*E*)-3-(4-Fluoro-3-nitrophenyl)-2-(4-fluorobenzylthio)-1-(4-(4-(2-hydroxyethyl)piperazine-1-carbonyl)phenyl)prop-2-en-1-one (12d). Yield: 75%; light yellow solid, mp 68–70 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.21 (br s, 2H), 2.28–2.33 (m, 6H), 3.13 (br s, 1H, OH), 3.35–3.38 (t, 2H), 3.54 (br s, 2H,), 3.71 (s, 2H, CH_2), 6.51–6.55 (m, 2H, Ar–H), 6.65 (s, 1H, Ar–H), 6.80–6.86 (m, 2H, Ar–H), 6.98–7.03 (dd, 2H, Ar–H), 7.10–7.15 (m, 2H, Ar–H), 7.33–7.35 (d, 2H, Ar–H), 7.50–7.53 (m, 1H, Ar–H), 8.12–8.14 (dd, 1H, Ar–H). HRMS found *m/z* 568.15. Calcd for $C_{29}H_{27}F_2N_3O_5S$ $(M+H)^+$ *m/z* 568.16.

5.1.9. Biological assay methods, cells and culture conditions

5.1.9.1. Cell culture and growth inhibition assays. K562 cells were purchased from ATCC and maintained at 37 °C under 5% CO_2 in RPMI medium supplemented with 10% fetal bovine serum (Cell Generation, Co.) and penicillin–streptomycin. For growth inhibition studies, K562 cells were plated at 1×10^5 cells/mL and incubated with varying concentrations of each compound. DMSO was used as a negative control. After 96 h of treatment the cell viability was determined by Trypan blue exclusion and expressed as percent of DMSO control to determine GI_{50} values.

5.1.9.2. Western blotting. Exponentially growing K562 cells were treated for 2 h with increasing concentration of compound (dissolved in DMSO at 10 mM stock concentrations and diluted in DMSO to 1000× working stock solutions. Whole cell protein lysate was collected and 50 μg of the clarified lysate was resolved by 10%-SDS–PAGE and western blotted. The blots were sequentially probed against antibodies specific for phosphorylated BCR-ABL (Santa Cruz Biotechnologies sc-885) and BCR-ABL (Santa Cruz Biotechnologies N-20), respectively, using the protocol provided by Li-Cor, Inc, Co. The western was treated with secondary antibodies conjugated with infrared dyes (LiCor) and scanned using Odyssey (LiCor) scanner. Percent inhibition was determined by quantifying each band using the software provided by LiCor, then normalizing the P-BCR-ABL signal to the parental BCR-ABL signal and determining% inhibition based on the vehicle control signal.

5.1.9.3. Kinase assays and IC_{50} determination. For Src (Invitrogen P3044), LynB (Invitrogen P2907), Plk-1 (Invitrogen PV3501) and Cdk-1/cyclinB (Upstate 14–450) kinase assays 10 ng of recombinant kinase was used with 1 μg GST-Sam68 (aa 331–443; Santa Cruz Biotechnologies sc-4249), dephosphorylated α-Casein (Sigma C8032) or Histone H1 (Roche Diagnositics 223549) as protein substrate, respectively. The kinase reactions were initiated by the addition of 1 μg recombinant substrate, 20 μM ATP and 20 μCi γ-32P-ATP. The reactions were incubated at 30 °C for 20 min, terminated by the addition of 2× Laemmli sample buffer, boiled for 2 min, resolved by 12% acrylamide SDS–PAGE and subjected to autoradiography. The autoradiograms were scanned and the band corresponding to phosphorylation of substrate was quantitated using MacBas software. The densitometric values obtained were plotted as a function of log drug concentration using Prism 4 Graphpad software and IC_{50} values determined by plotting sigmoidal non-linear regression curves with variable slope. ErbB2 and EGFR assays were performed using cell based immunoprecipitation assays like those performed by us earlier.[35]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.01.051.

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A Non–ATP-Competitive Dual Inhibitor of JAK2^{V617F} and BCR-ABL^{T315I} Kinases: Elucidation of a Novel Therapeutic Spectrum Based on Substrate Competitive Inhibition

Shashidhar S. Jatiani[1,*,†], Stephen C. Cosenza[1,*,†], M.V. Ramana Reddy[1,†], Ji Hee Ha[1,‡], Stacey J. Baker[1,†], Ajoy K. Samanta[2], Matthew J. Olnes[3], Loretta Pfannes[3], Elaine M. Sloand[3], Ralph B. Arlinghaus[2], and E. Premkumar Reddy[1,†]

Abstract

Here we report the discovery of ON044580, an α-benzoyl styryl benzyl sulfide that possesses potent inhibitory activity against two unrelated kinases, JAK2 and BCR-ABL, and exhibits cytotoxicity to human tumor cells derived from chronic myelogenous leukemia (CML) and myelodysplasia (MDS) patients or cells harboring a mutant JAK2 kinase. This novel spectrum of activity is explained by the non–ATP-competitive inhibition of JAK2 and BCR-ABL kinases. ON044580 inhibits mutant JAK2 kinase and the proliferation of *JAK2*^{V617F}-positive leukemic cells and blocks the IL-3–mediated phosphorylation of JAK2 and STAT5. Interestingly, this compound also directly inhibits the kinase activity of both wild-type and imatinib-resistant (T315I) forms of the BCR-ABL kinase. Finally, ON044580 effectively induces apoptosis of imatinib-resistant CML patient cells. The apparently unrelated JAK2 and BCR-ABL kinases share a common substrate, STAT5, and such substrate competitive inhibitors represent an alternative therapeutic strategy for development of new inhibitors. The novel mechanism of kinase inhibition exhibited by ON044580 renders it effective against mutant forms of kinases such as the BCR-ABL^{T315I} and JAK2^{V617F}. Importantly, ON044580 selectively reduces the number of aneuploid cells in primary bone marrow samples from monosomy 7 MDS patients, suggesting another regulatory cascade amenable to this agent in these aberrant cells. Data presented suggest that this compound could have multiple therapeutic applications including monosomy 7 MDS, imatinib-resistant CML, and myeloproliferative neoplasms that develop resistance to ATP-competitive agents.

Keywords

JAK2, BCR-ABL, CMPD, MDS, CML

Introduction

The Janus Kinase (JAK) family of cytoplasmic protein tyrosine kinases are pivotal mediators of cytokine signaling pathways.[1,2] The JAK kinase family consists of 4 members: TYK2, JAK1, JAK2, and JAK3. Activation of JAK kinases[1-3] resulting from cytokine/receptor interaction leads to phosphorylation of corresponding interleukin receptors on multiple tyrosine residues, which in turn serve as docking sites for other signal-transducing proteins, the most important of which are the STAT family of transcription factors.[1-3] JAK2 is the primary tyrosine kinase activated by erythropoietin (Epo) and is essential for definitive erythropoiesis.[1]

The importance of JAKs in human cancer has been highlighted by the discovery of genetic alterations in this family of kinases, leading to hyperactivation of the pathways they regulate. These findings include translocations leading to the expression of various forms of JAK2 fusion protein such as TEL/ETV6-JAK2, PCM1-JAK2, BCR-JAK2, RPN1-JAK2, NFE2-JAK2, AML1-JAK2, SSBP2-JAK2, and PAX5-JAK2, which occur in lymphoid/myeloid leukemias and myelodysplasia (MDS).[4-15] In addition, amplification of the JAK2 locus has been shown to occur in Hodgkin's lymphomas,[16] and acquired activating mutations in the JAK2 gene have been found in chronic myeloproliferative

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disorders (CMPD),[17-19] acute lymphoblastic leukemias,[20-23] and myelogenous leukemias.[24-27]

A point mutation in the JAK2 kinase has been suggested as the causative molecular event in most patients with polycythemia vera (PV) as well as in half of the cases of essential thrombocythemia (ET) and chronic idiopathic myelofibrosis, all of which are classified as CMPD.[28-31] In addition, it has been reported that about half of refractory anemia ringed sideroblasts with thrombocytosis (RARS-T) patients, along with a subset of others with MDS and mixed MDS/CMPD, carry the JAK2 mutation.[18,32,33] Remarkably, every sample derived from such patients contained the same amino acid substitution (V617F). Based on the predicted JAK2 structure and atomic level simulations, this substitution is believed to disrupt an autoinhibitory interaction between the pseudokinase (JH2) and kinase (JH1) domains of the protein.[4,28,34] Studies using Epo receptor mutants have revealed the need for receptor-dependant dimerization of the mutant kinase for constitutive activation,[35] and a recent report provides biochemical evidence for a regulatory role of the FERM domain in hyperactivation of JAK2 with a V617F substitution.[36] This mutation has been found to confer Epo-independent growth of the mutant cells *in vitro* due to deregulation of signaling pathways downstream of JAK2.[28] Small interfering RNA-mediated knock-down of JAK2 has also been found to impair EEC formation from PV bone marrow.[29] Furthermore, PV patients who lacked the V617F point mutation were found to harbor other activating exon 12 mutations in JAK2,[37] making mutations of JAK2 the causative genetic lesion in all cases of this disease.

Activation of the JAK-STAT pathway has also been observed in diseases with signaling defects in proteins upstream of the Janus kinases. One such example is the constitutive activation of JAK2[38] and STAT1[39] in cells from monosomy 7 MDS patients, likely due to aberrant cytokine receptor signaling. Monosomy 7 is the second most frequently observed cytogenetic abnormality in MDS, with an incidence of 21%.[40] It is the most frequent karyotypic aberration occurring in bone marrow failure patients following immunosuppressive treatment, and it is associated with severe cytopenias and a high propensity for developing acute leukemia.[41,42] Patients who develop monosomy 7 AML are difficult to treat and often relapse quickly or die of infection.[43] Monosomy 7 is especially common in MDS secondary to exposure to alkylating drugs and in pediatric MDS. Monosomy 7 cells show increases in a differentiation-defective GCSFR isoform (IV) that fails to internalize following GCSF binding as normally occurs for the full-length receptor. It is also defective in facilitating phosphorylation of STAT-3, but its ability to signal phosphorylation of STAT-1 and -5 is unimpaired.[39,44] As a result, the cell's ability to differentiate is limited, whereas its ability to proliferate via JAK-2 remains intact.

These findings open new avenues for diagnosing and classifying patients with these disorders and identify JAK2 as a new molecular target for drug discovery. To date, a number of ATP-competitive JAK-2 inhibitors have been identified.[45-49] Here we report the discovery of a new JAK2 inhibitor that is non-ATP competitive and potently inhibits the kinase activity of both wild-type and mutant JAK2 kinase. It readily inhibits the proliferation of *JAK2*^{V617F}-positive leukemic cells and blocks the IL-3–mediated phosphorylation of JAK2 and STAT5, a known substrate of JAK2. Importantly, ON044580 selectively inhibits the proliferation of aneuploid cells in bone marrow samples from monosomy 7 MDS patients. Most interestingly, this compound also inhibits both wild-type and imatinib-resistant (T315I) forms of the BCR-ABL kinase and induces apoptosis of imatinib-resistant chronic myelogenous leukemia (CML) patient cells.

Results

The remarkable clinical success of imatinib,[50] an inhibitor of the BCR-ABL tyrosine kinase, was followed by the development of a series of kinase inhibitors that have found application in cancer therapy. In the past few years, there has been an increasing realization that tumor cells often develop resistance to ATP-competitive kinase inhibitors as a result of accumulating mutations in the ATP binding site of the kinase. This has been observed in patients undergoing treatment with imatinib.[51] Because selection of highly conserved mutable residues in the ATP binding site appears to be relatively common for many kinases, it has been argued that non–ATP-competitive inhibitors might constitute better drug candidates.[52] Because there are a limited number of chemotypes that act as non–ATP-competitive inhibitors, we undertook the synthesis and characterization of new chemotypes that are unrelated to ATP or other purine and pyrimidine nucleosides and yet possess kinase inhibitory activity. This effort led to the synthesis and identification of a new class of compounds, α-benzoyl styryl benzyl sulfides, that possess potent kinase inhibitory activity and exhibit cytotoxicity to human tumor cells that express oncogenic kinases.[53] Our preliminary screening of these compounds using a high-throughput cell-based assay in combination with kinase assays led to the identification of a compound, ON044580 (Fig. 1a) that is a potent inhibitor of JAK-2. *In vitro* studies using a recombinant JAK-2 protein produced in insect cells (that is commercially available) showed that this compound inhibits the kinase activity of recombinant JAK2 with an IC_{50} ranging between 0.9 and 1.2 μM (Figs. 1b and c). Under identical conditions, AG490 was able to inhibit JAK2 kinase activity with an IC_{50} of 36.4 μM, which is in agreement with published literature.[54] Following these observations, we examined the ability of

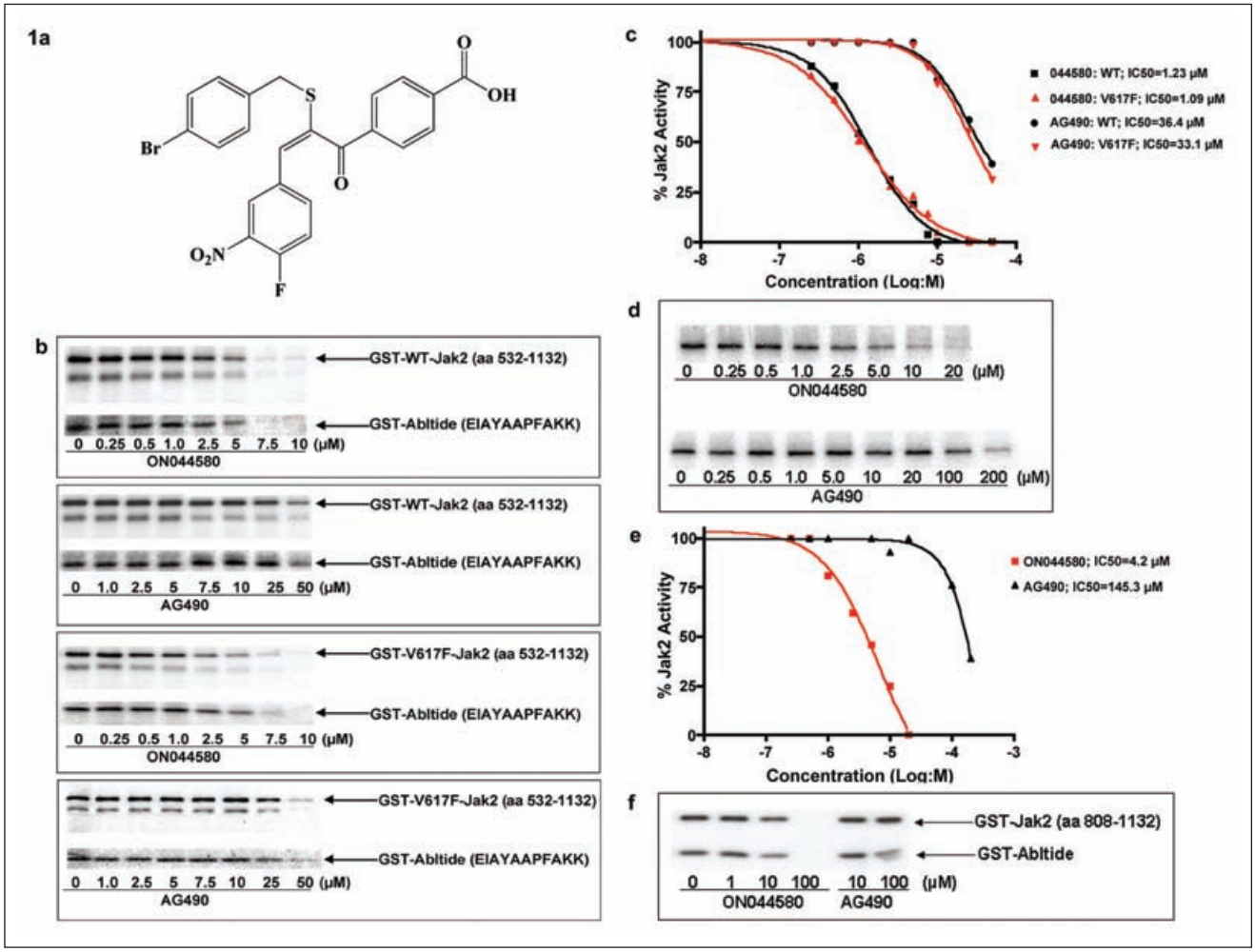

Figure 1. Identification of a novel JAK2 inhibitor. (**a**) Structure of the JAK2 inhibitor ON044580. (**b**) JAK2 inhibitory activity of ON044580. Recombinant wild-type or V617F mutant JAK2 (aa 532-1132) was mixed with increasing concentrations of ON044580 (0.25-10 μM) or AG490 (1-50 μM) and kinase assays performed as described in Methods using recombinant GST-Abltide as substrate. The reaction mixtures were subjected to SDS-PAGE and autoradiography. (**c**) Determination of IC_{50} values. From the autoradiograms described in **b**, the values of individual bands corresponding to autophosphorylation of JAK2 were analyzed using MacBas software and plotted as a function of drug concentration using Prism 4 Graphpad software. ON044580 exhibited a 30-fold greater inhibition of wild-type and mutated Jak2. (**d**) Inhibition of mammalian JAK2 activity by ON044580. Full-length JAK2 was immunoprecipitated from clarified lysates of BaF3:*JAK2*V617F cells stimulated with 5 ng/mL recombinant IL-3 for 1 hour, as described in the Methods section. The washed immunocomplexes were incubated for 30 minutes in the presence of increasing concentrations of ON044580 and processed for kinase assays as described in **b**. (**e**) The samples from **d** were quantitated, and IC_{50} curves were plotted as described in **c**. Although ON044580 showed inhibition with IC_{50} = 4.2 μM, AG490 had an IC_{50} value of 145.3 μM. (**f**) Involvement of the regulatory JH2 domain. Recombinant wild-type catalytic JH1 domain (aa 808-1132) of JAK2 was incubated with increasing concentrations of ON044580 and assayed for kinase activity. No inhibition of JAK2 activity was apparent at an ON044580 concentration of 10 μM in this truncated unregulated form of the kinase. The longer version of the kinase (aa 532-1132) encompassing both the catalytic and regulatory domains was inhibited at 1 μM (**1a** and **b**).

this compound to inhibit the activated V617F mutant form of JAK2 using commercially available recombinant protein produced in insect cells. The results of this study showed that ON044580 inhibited the kinase activity of mutant JAK2 with a similar IC_{50} (0.8 to 1.1 μM) as that seen with WT JAK2, whereas AG490 had an IC_{50} of 33.1 μM (Figs. 1b and c). Because the recombinant preparations of wild-type and mutant JAK2 proteins are truncated forms of the kinase, we examined the kinase inhibitory activity of these compounds using JAK2 kinase immunoprecipitated from the Ba/F3:*JAK2*V617F cell line that expresses the full-length wild-type and mutant forms of JAK2. These studies again showed that ON044580 inhibited the JAK2 kinase activity with an IC_{50} of approximately 4 μM. Under identical conditions, AG490 inhibited the kinase activity with an IC_{50} of 145 μM (Figs. 1d and e). The observation that

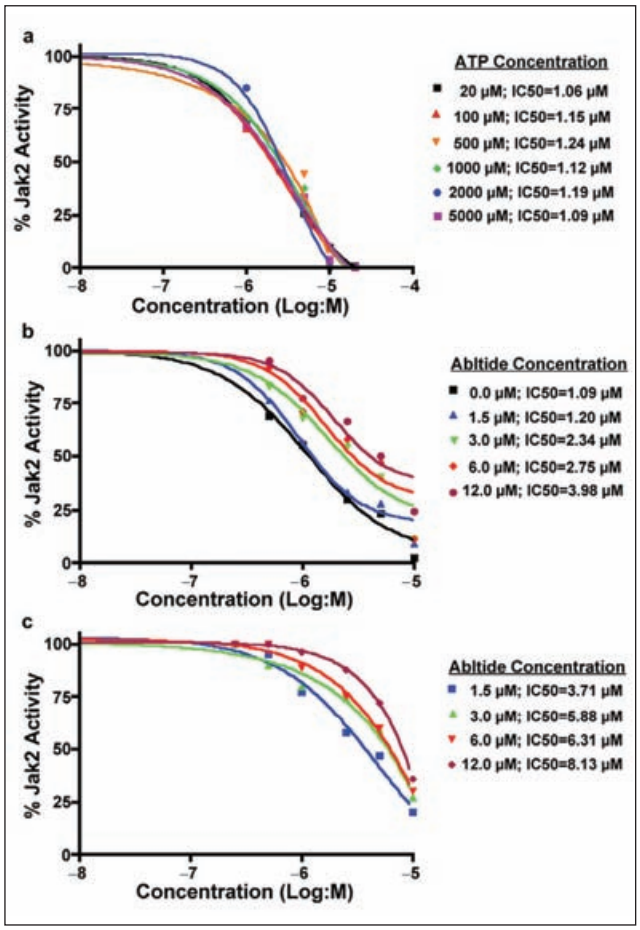

Figure 2. Steady-state kinetic analysis of JAK2 inhibition by ON044580 (**a**) ATP does not affect the JAK2-V617F inhibitory activity of ON044580. Recombinant JAK2-V617F (aa 532-1124) was mixed with the indicated concentrations of ON044580 and varying concentrations of ATP up to 5 mM. Kinase assays were performed as described in **Figure 1b**. The values from individual samples were analyzed and plotted as a function of log inhibitor concentration as described in **Figure 1c**. (**b, c**) Effect of substrate concentration on the inhibition of V617F-JAK2 autophosphorylation and GST-Abltide phosphorylation. IC_{50} curves for V617F-JAK2 kinase activity in the presence of varying concentrations of GST-Abltide substrate and ON044580 were generated as described in (**a**). The upper band, like that seen in **Figure 1b** was quantitated for autophosphorylation of JAK2-V617F (**b**) and the lower band for phosphorylation of GST-Abltide (**c**).

ON44580 inhibits recombinant JAK2 (aa 532-1132) led us to ask whether this compound binds to the catalytic kinase domain (JH1) or the regulatory pseudokinase domain (JH2) of JAK2. To test this, we made use of the commercially available recombinant form of JAK2 containing just the kinase domain (JH1 domain spanning amino acids 808-1132). Interestingly, ON044580 failed to inhibit JAK2 kinase domain (JH1) activity at a concentration of 10 µM, whereas complete inhibition was observed only at 100 µM (Fig. 1f). This significant increase in the inhibitory concentration suggests the ATP-binding kinase domain of JAK2 (JH1) is not the primary site of action of ON044580.

ON44580 is a non–ATP-competitive inhibitor. Our observation that the pseudokinase domain is required for the kinase inhibitory activity of ON044580 further led us to postulate that it is not an ATP-competitive JAK2 inhibitor. We directly tested this hypothesis by carrying out kinase inhibition assays either in the presence of increasing amounts of ATP or in the presence of increasing amounts of substrate. The results of this study, shown in Figure 2, demonstrate that increasing the ATP concentration in the kinase reaction mixture did not affect the inhibitory activity of ON044580 (Fig. 2a). On the other hand, increasing the substrate concentration in the reaction mixture resulted in a reduction of the kinase inhibitory activity of ON044580. This was seen to be true for both the autophosphorylation of JAK2 kinase itself (Fig. 2b) and the transphosphorylation of GST-Abltide substrate (Fig. 2c).

In vivo *inhibition of Jak2 autophosphorylation and STAT-5 phosphorylation by ON044580 in Ba/F3:JAK2V617F cells*. To test the *in vivo* kinase inhibitory activity of ON044580, we treated Ba/F3:*JAK2*V617F cells with increasing concentrations of the compound for 2 hours in the presence of recombinant IL-3 (which enhances the phosphorylation status of JAK2). At the end of the 2-hour incubation period, cells were washed and lysed in detergent containing buffer, and the clarified lysates were subjected to SDS-PAGE followed by Western blotting to detect the phosphorylation status of JAK2. The results of this study (Fig. 3a) showed that ON044580 was able to inhibit the phosphorylation of JAK2 in a concentration-dependent manner. AG490, under identical conditions, did not inhibit JAK2 phosphorylation, which could be due to the high IC_{50} values seen for full-length JAK2 kinase with this compound. As part of this study, we also examined the time course of inhibition in which we added 10 µM of ON044580 for periods of time ranging from 15 to 60 minutes and examined the phosphorylation status of JAK2 using Western blot analysis. The results of this study presented in Figure 3b showed that in as little as 15 to 30 minutes, the compound was able to inhibit IL-3–mediated JAK2 phosphorylation.

Using a similar approach, we also examined the phosphorylation status of STAT-5 (a natural substrate of JAK2) in Ba/F3:*JAK2*V617F cells treated with increasing concentrations of the compound. The results presented in Figures 3c and d show that ON044580 inhibited STAT-5 phosphorylation in a concentration-dependent and time-dependent manner. Because similar results were seen with JAK2 phosphorylation, these studies suggest that the 2 events are interrelated.

Cellular inhibition of constitutive JAK/STAT signaling by ON044580. The U266 multiple myeloma cell line expresses wild-type JAK2 but has constitutive activation of the IL-6 receptor/JAK2/STAT-3 pathway.[55] We treated U266 cells

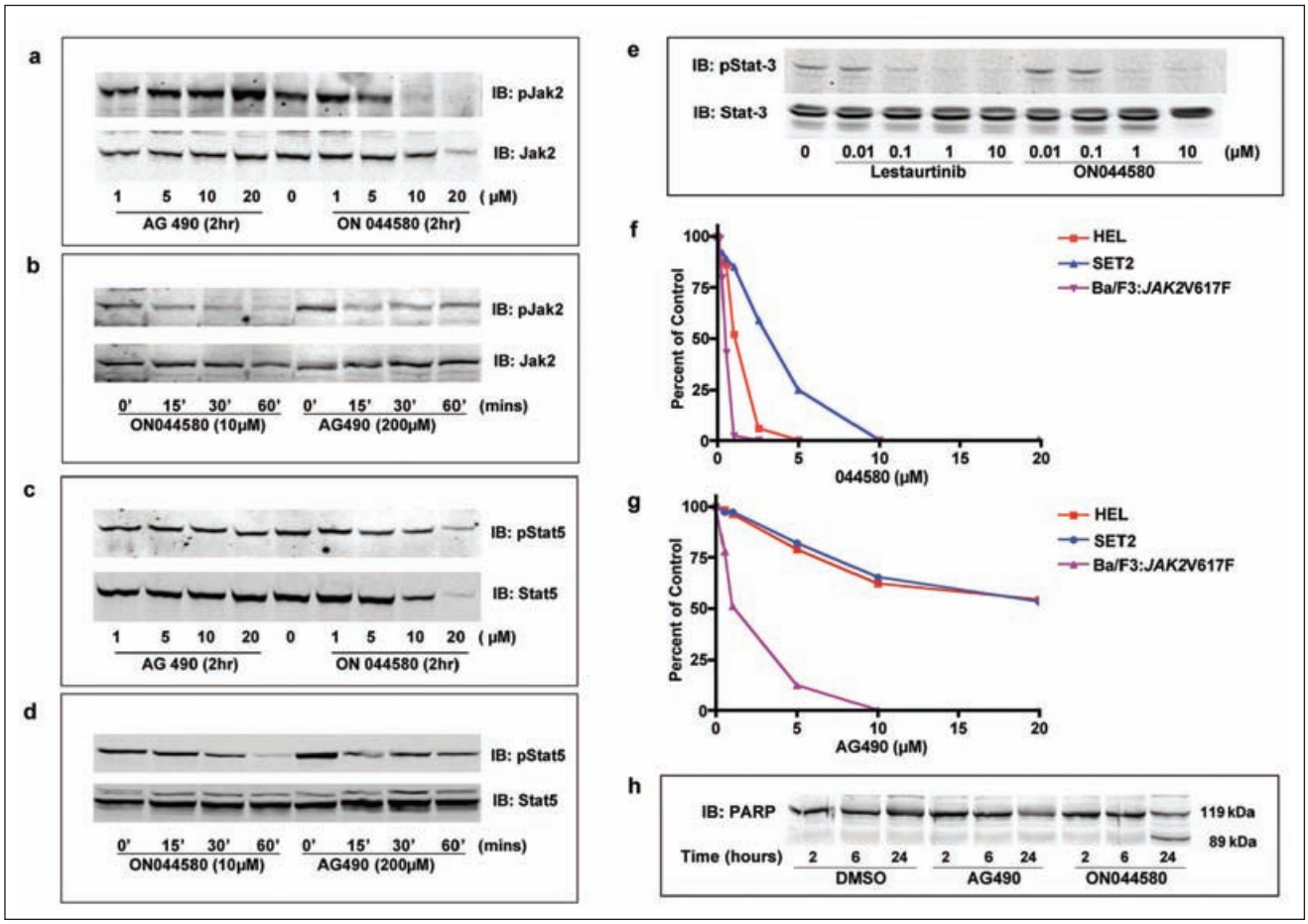

Figure 3. Effects of ON044580 on cellular JAK2-dependent cytokine signaling. (**a**) Inhibition of JAK2 autophosphorylation by ON044580 in IL-3–stimulated Ba/F3:*JAK2*V617F cells. Mid-log phase Ba/F3:*JAK2*V617F cells were treated for 2 hours with indicated concentrations of ON044580 and 1 hour with 5 ng/mL recombinant IL-3. Washed cells were lysed in detergent containing buffer, and the clarified lysates were subjected to SDS-PAGE followed by immunoblotting to detect phosphorylation status of JAK-2. (**b**) ON044580 inhibits JAK2 autophosphorylation in Ba/F3:*JAK2*V617F cells within 15 minutes. Mid-log phase Ba/F3:*JAK2*V617F cells were treated for the indicated times with 10 μM ON044580 or 200 μM AG490 and processed as above. For all time points, the total time for IL-3 stimulation was 60 minutes. (**c**) Inhibition of STAT-5 phosphorylation by ON044580 in IL-3–stimulated Ba/F3:*JAK2*V617F cells. Ba/F3:*JAK2*V617F cells were treated and processed as in (**a**). Immunoblotting was carried out to assess STAT-5 phosphorylation. **(d)** ON044580 inhibits STAT-5 phosphorylation in Ba/F3:*JAK2*V617F cells within 30 minutes. Ba/F3:*JAK2*V617F cell treatments were carried out as described in (**b**). Western blot analysis was performed to assay degree of STAT-5 phosphorylation. (**e**) ON044580 inhibits JAK2-dependent STAT-3 phosphorylation in U266 cells. Exponentially growing U266 cells were treated for 2 hours with increasing concentrations of ON044580. The ATP-competitive JAK2 inhibitor, lestaurtinib, was used as a control. Cells were washed and lysed, and clarified lysates were resolved by SDS-PAGE for immunoblot analysis to assess phosphorylated STAT-3 levels. (**f, g**) Growth inhibition of JAK2-V617F–expressing cells. Ba/F3:*JAK2*V617F cells (ectopic expression), HEL cells (homozygous *JAK2*V617F), and SET-2 cells (hemizygous *JAK2*V617F) were grown in the presence of varying concentrations of ON044580 (**e**) or AG490 (**f**) for 72 hours. Cell viability was measured by Trypan blue exclusion. GI_{50} values were calculated by plotting the percentage of viable cells as a function of drug concentration. (**h**) ON044580 induces apoptotic cell death in cells expressing JAK2-V617F. Ba/F3:*JAK2*V617F cells were treated with 0.5 μM ON044580 or 2 μM AG490 for 2, 6, and 24 hours. Thereafter, the cells were harvested, washed, lysed, and probed with anti-PARP antibody following Western blotting.

with increasing concentrations of ON044580 for 2 hours to test whether this compound could inhibit aberrant JAK/STAT signaling instigated by mechanisms other than genetic alterations in the JAK2 gene itself. The results of this study showed that such a treatment led to a dose-dependent inhibition of STAT-3 phosphorylation at concentrations comparable to a known ATP-competitive JAK2 inhibitor[45] (Fig. 3e).

Growth inhibition of JAK2-V617F–expressing cells. To determine whether ON044580 inhibits the proliferation of *JAK2*V617F-positive leukemic cells, we studied its effect on the growth and viability of 3 different cell lines that express the mutant form of JAK2. These included the Ba/F3:*JAK2*V617F cells that were transfected with an expression vector that encodes the mutant JAK2 and two human leukemic cell lines that were derived from leukemic patients

who naturally contained this mutation in their *JAK2* loci. One of them, HEL, is homozygous for V617F mutation, whereas the second cell line, SET2, is hemizygous for the V617F mutation. The results of this study, presented in Figure 3f, show that ON044580 readily inhibited the proliferation of all three cell lines at nanomolar or low micromolar concentrations. Thus, the GI_{50} for Ba/F3:*JAK2*V617F cells was approximately 250 nM, whereas the GI_{50} for HEL cells was approximately 900 nM. Interestingly, the SET2 cell line that was hemizygous for V617F mutation was more resistant to the cell-killing activity of the compound with a GI_{50} value of 3.0 μM. In a similar experiment using AG490, the GI_{50} value for Ba/F3:*JAK2*V617F cells was 1.0 μM, whereas that for HEL and SET-2 cells was greater than 20 μM (Fig. 3g).

ON044580 exerts its antiproliferative effect by inducing apoptosis in Ba/F3:JAK2V617F cells. After demonstrating effective inhibition of myeloproliferation by ON044580, we examined the mechanisms associated with this cytotoxic effect. Upon microscopic observation of cells treated with ON044580, we did not find evidence for autophagic vacuoles or mitotic arrest. To test if ON044580 activates the apoptotic pathways, we treated Ba/F3:*JAK2*V617F cells for 2, 6, and 24 hours with ON044580 and AG490 at concentrations twice the GI_{50} values obtained in the growth inhibition studies (shown in Fig. 3f). DMSO was used as a control. These cells were then harvested and probed for the status of poly-(ADP-ribose) polymerase (PARP), a marker for apoptosis induction.[56] The results of this experiment show that treatment with ON044580 did indeed lead to proteolytic cleavage of PARP in 24 hours (Fig. 3h). A similar though less pronounced effect was observed with AG490.

In vitro *inhibition of wild-type and T315 mutant forms of BCR-ABL kinase by ON044580*. The studies presented above suggest that ON044580 inhibits the JAK2 kinase activity either by binding to the STAT-5 binding domain of JAK2 or by binding to an allosteric site, which results in altered conformation and inhibition of the kinase activity of the protein.[57] It is now well established that STAT-5 is also a substrate of the BCR-ABL kinase, and if the two kinases contain a similar substrate-binding structure, it is possible that ON044580 could also inhibit the BCR-ABL kinase. Hence, we evaluated the effect of ON044580 on the *in vitro* kinase activity of mammalian BCR-ABL proteins immunoprecipitated from cultured cells. Lysates prepared from K562 cells expressing the wild-type BCR-ABL or 32Dcl3 cells expressing the T315I mutant form were incubated with antibodies directed against the BCR-ABL protein. Kinase assays were performed on the washed immunoprecipitates in the presence of different concentrations of ON044580 as described in the Materials and Methods section. Imatinib was used as a control in all of these assays. These studies show that imatinib readily inhibited the kinase activity of WT BCR-ABL but failed to do so with the T315I-BCR-ABL kinase. On the other hand, ON044580 inhibited both WT and T315I mutant forms of the BCR-ABL kinase (Figs. 4a and b), suggesting that mutations that affect the kinase inhibitory activity of imatinib do not affect the inhibitory activity of ON044580. It is interesting to note that ON044580 was more effective in inhibiting the T315I mutant form compared to the wild-type BCR-ABL kinase.

Cellular inhibition of the kinase activity of BCR-ABL. Having demonstrated direct biochemical inhibition of wild-type and imatinib-resistant BCR-ABL kinase activity, we proceeded to evaluate the *in vivo* inhibition of the BCR-ABL activity by ON044580. We examined the autophosphorylation status of BCR-ABL protein as well as the phosphorylation status of STAT-5 and CrkL in cells treated with increasing concentrations of this compound for 2 hours. Data presented in Figure 4c show that ON044580 inhibited the autophosphorylation of wild-type BCR-ABL protein expressed in K562 cells. This compound also inhibited the phosphorylation of STAT-5 but did not affect the phosphorylation status of CrkL. Imatinib (Gleevec®) was used as a positive control in all of these experiments. These results suggest that ON044580 is selective in its inhibitory activity of BCR-ABL substrates.

Following the establishment of its *in vivo* activity toward WT BCR-ABL kinase, we next examined the ability of ON044580 to inhibit the autophosphorylation of T315I-BCR-ABL kinase and transphosphorylation of STAT-5. For these studies, we used the 32D:*p210*T315I cell line that expresses high levels of the T315I-BCR-ABL kinase and is known to be resistant to imatinib. As was done with K562 cells, we treated 32D:*p210*T315I cells with increasing concentrations of ON044580 for 2 hours followed by Western blot analysis of cell lysates to determine the ability of this compound to inhibit the phosphorylation of BCR-ABL and STAT-5. These studies (Fig. 4d) show that ON044580 was very effective in inhibiting autophosphorylation of T315I-BCR-ABL and STAT-5 phosphorylation in 32D:*p210*T315I cells, whereas imatinib failed to do so. These results suggest the possibility that ON044580 does not bind to the ATP-binding domain of the BCR-ABL kinase but acts via binding to the substrate-binding domain (that is specific to STAT-5 but not to CrkL) or to an allosteric domain of the BCR-ABL kinase that results in the impairment of its ability to phosphorylate itself and STAT-5. Interestingly, the steady-state levels of BCR-ABL-T315I kinase itself were reduced upon treatment with ON044580, suggesting enhanced degradation of the protein in these murine cells.

In vitro *tumor cell–killing activity of ON044580*. We next examined the ability of ON044580 to inhibit the proliferation

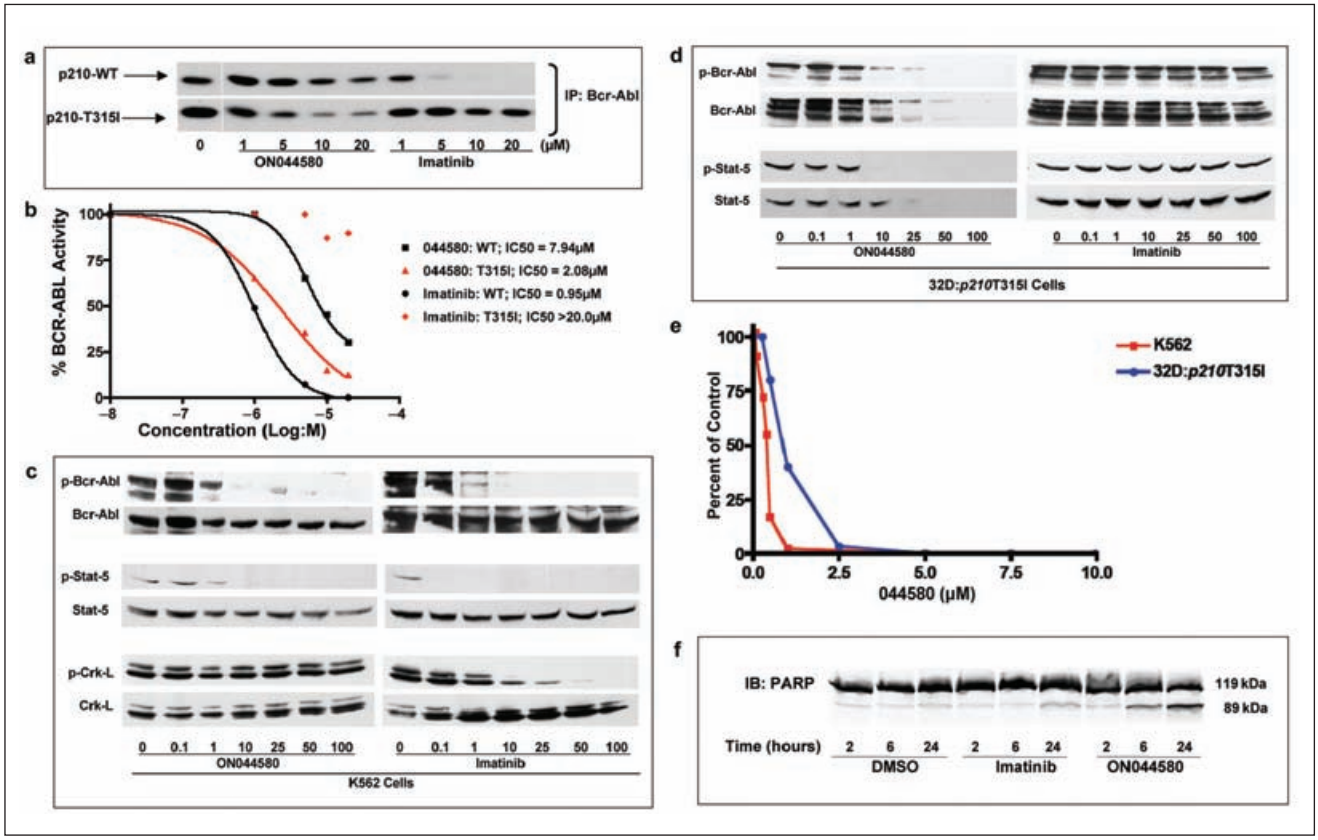

Figure 4. ON044580 is a dual JAK2/BCR-ABL kinase inhibitor. (**a**) ON044580 inhibits imatinib-sensitive and -resistant forms of BCR-ABL isolated from mammalian cells. BCR-ABL was immunoprecipitated from K562 and 32D:*p210*T315I cells. The immunocomplexes were incubated with inhibitor for 30 minutes, and radiometric kinase assays were performed. Imatinib was used as a control to inhibit wild-type BCR-ABL. (**b**) Determination of IC_{50} values. From the autoradiograms presented in (**a**), the values of individual bands corresponding to phosphorylatoin of GST-Abltide were analyzed using MacBas software and plotted as a function of drug concentration using Prism 4 Graphpad software. (**c**) Inhibition of BCR-ABL and STAT-5 phosphorylation by ON044580 in K562 cells. Exponentially growing K562 cells were treated for 2 hours with indicated concentrations of ON044580. Washed cells were lysed in detergent containing buffer, and the clarified lysates were subjected to SDS-PAGE followed by Western blotting and immunodetection with indicated antibodies. (**d**) Inhibition of BCR-ABL and STAT-5 phosphorylation by ON044580 in imatinib-resistant 32D:*p210*T315I cells. Exponentially growing 32D:*p210*T315I cells were treated for 2 hours with indicated concentrations of ON044580. Washed cells were lysed in detergent containing buffer, and the clarified lysates were subjected to SDS-PAGE followed by Western blotting. (**e**) ON044580 inhibits growth of cells expressing imatinib-sensitive and -resistant forms of BCR-ABL. K562 and 32D:*p210*T315I cells were grown in the presence of varying concentrations of ON044580 for 72 hours. Cell viability was measured by Trypan blue exclusion. (**f**) ON044580 induces apoptosis in chronic myelogenous leukemia cells. K562 cells were treated with 1 μM ON044580 or imatinib for 2, 6, and 24 hours. Thereafter, the cells were harvested, washed, lysed, and immunoblotted to ascertain PARP status.

of BCR-ABL–positive myeloid leukemias. For this study, we used K562 cells that express WT BCR-ABL kinase and 32D:*p210*T315I cells that express an imatinib-resistant form of BCR-ABL. The results presented in Figure 4e show that ON044580 was an effective inducer of myeloid tumor cell death with a GI_{50} of 300 to 400 nM in cells sensitive to imatinib. Imatinib, in the same assay system, showed a GI_{50} of 100 to 200 nM (data not shown). Significantly, ON044580 inhibited the proliferation of 32D:*p210*T315I cells with a GI_{50} of 500 to 900 nM, whereas imatinib showed a GI_{50} of 20 to 30 μM.

ON044580 induces apoptosis in K562 cells. After demonstrating effective growth inhibition of cells expressing imatinib-sensitive and imatinib-resistant forms of BCR-ABL by ON044580, we tested whether this effect was mediated by apoptotic cell death. K562 cells were treated with 1 μM ON044580 or imatinib for 2, 6, and 24 hours. DMSO was used as a control. We carried out Western blot analysis of these cell lysates to evaluate the status of PARP. Proteolytic cleavage of PARP upon ON044580 treatment was evident within 6 hours and became more pronounced in 24 hours (Fig. 4f). PARP cleavage was also observed to a lesser extent in K562 cells treated with imatinib for 24 hours.

Short-term ON044580 exposure causes growth inhibition of cells expressing mutated JAK2 and BCR-ABL kinases whereas normal bone marrow cells remain unaffected. Metabolic and/

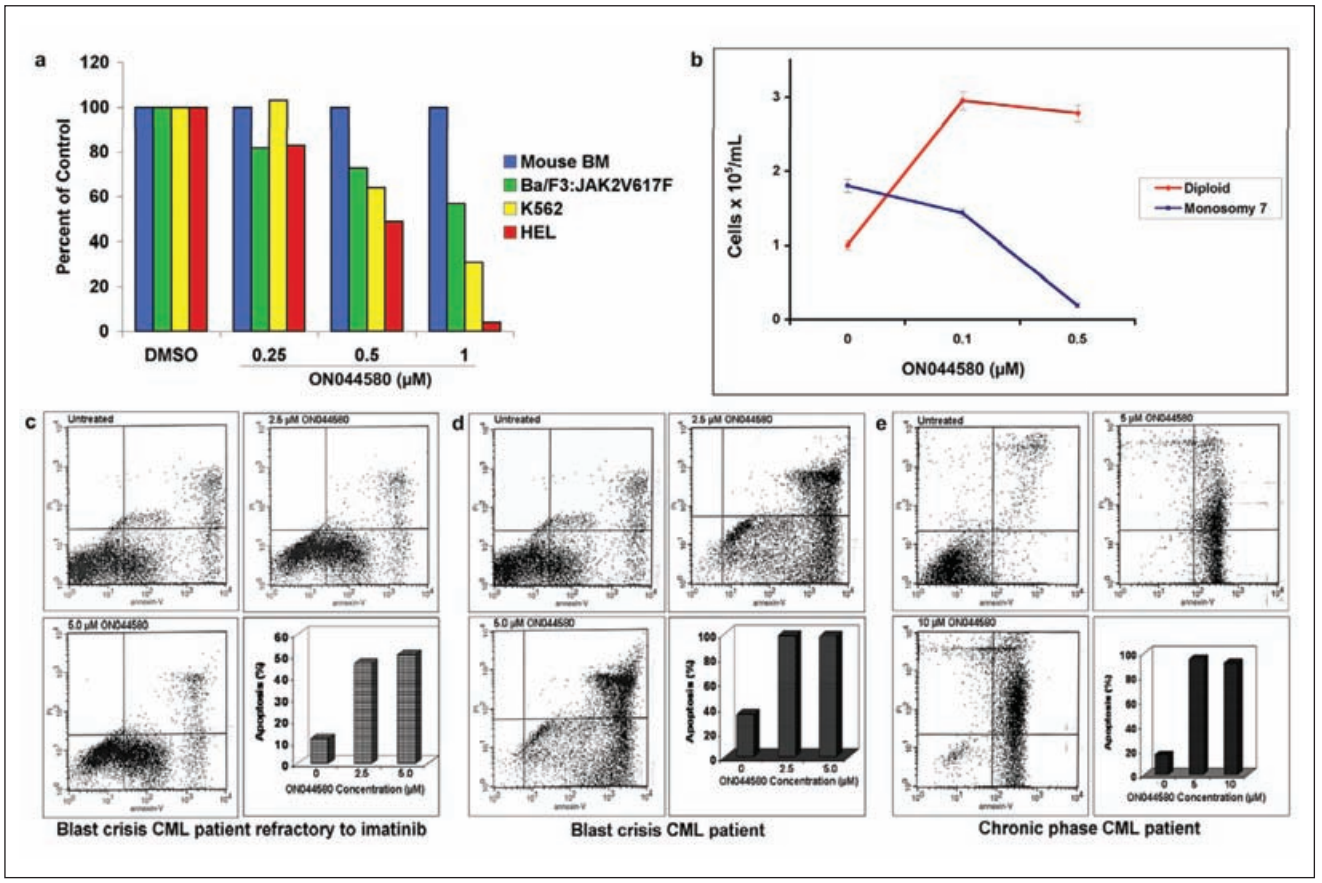

Figure 5. Effects of ON044580 on monosomy 7 MDS and chronic myelogenous leukemia (CML) patient samples. (**a**) Short-term exposure to ON044580 is cytotoxic to cells expressing oncogenic forms of JAK2 and ABL kinases but not to normal bone marrow cells. Ba/F3:*JAK2*-V617F cells, HEL cells, K562 cells, and mononuclear cells isolated from normal mouse bone marrow were treated with increasing concentrations of ON044580 for 2 hours. The cells were then washed and allowed to grow in complete medium in the absence of the compound. The data are plotted as percentage viability as compared to DMSO-treated controls. Concentration-dependent growth inhibition was observed for all three oncogenic cell lines, whereas mouse bone marrow cells subjected to identical conditions were unaffected. (**b**) *Ex vivo* treatment of monosomy 7 MDS patient bone marrow cells with ON044580 causes favorable cytogenetic changes. MDS patient bone marrow mononuclear cells were cultured in colony-supporting conditions and treated with 0.5 μM and 1.0 μM ON044580. This led to a dose-dependant decrease in the total number of monosomy 7 cells while maintaining/increasing the total number of diploid cells. (**c-e**) ON044580 induces apoptosis in refractory, chronic phase and blast crisis stages of CML patients. Purified blood samples from a refractory CML patient (**c**), a CML patient in blast crisis (**d**), and a chronic phase CML patient (**e**) were incubated with the indicated concentrations of ON044580, and one untreated sample was kept as a control. After 48 hours, the apoptosis was measured by Annexin-V/IP method. Cells accumulated in the lower right and upper right quadrants indicate the percentage of cells in the early and late stage of apoptosis, respectively.

or excretory elimination of drugs after administration is an important pharmacokinetic criterion. It is therefore pertinent that compounds be tested for their ability to effect changes in target cells within short times of exposure. To test these parameters, cells expressing mutated JAK2 (Ba/F3:*JAK2*V617F and HEL cells) and BCR-ABL (K562 cells) were treated with increasing concentrations of ON044580 for 2 hours, washed extensively, and returned to drug-free growth medium. Normal mouse bone marrow cells were used as a parallel control in all 3 sets of experiments. As can be seen in the results presented in Figure 5a, short-term exposure to ON044580 caused growth inhibition of Ba/F3:*JAK2*V617F cells in a concentration-dependent manner, whereas mouse bone marrow cells were unaffected. Similar results were obtained for HEL cells that are homogyzous for the *JAK2*V617F allele and K562 cells expressing the oncogenic BCR-ABL fusion protein. These results are corroborated by those presented in Figure 3b and 3d, where the inhibitory effects of ON044580 on JAK/STAT signaling were elicited within 30 minutes of treatment.

ON044580 effects favorable cytogenetic changes in primary bone marrow cells from monosomy 7 MDS patients. Monosomy 7 MDS bone marrow mononuclear cells preferentially express the truncated class IV G-CSF receptor, which leads to constitutive signaling through the JAK2 pathway.[39] Because ON044580 inhibited the activated IL-6 receptor/JAK2/STAT3 pathway in U266 cells (Fig. 3e) and did not show adverse effects on normal bone marrow cells (Fig. 5a), we were encouraged to test its effects on Monosomy 7 and

diploid hematopoietic colony formation from MDS marrow samples. To assess the effect of ON044580 on these cells with constitutive JAK2 activity, bone marrow aspirate mononuclear cells (BMMNCs) derived from patients with monosomy 7 MDS (confirmed by metaphase karyotyping and FISH) were grown in Mylocult media (Stem Cell Technologies, Vancouver, Canada) supplemented with 400 ng/mL G-CSF and growth factor cocktail as previously described.[58] Following treatment with ON044580 at 0.1 μM and 0.5 μM, cells harvested were examined for the number of aneuploid and diploid cells by FISH using centromeric probes specific for chromosomes 7 and 8. Results from this study showed that there was a reduction in the number of anueploid cells by 20% and 80%, respectively, compared to vehicle treatment control. The effect of ON044580 appeared to be limited to the aneuploid population as the total number of diploid cells in the treatment groups increased (Fig. 5b). These preliminary results indicate that ON044580 suppresses monosomy 7 bone marrow cell growth while stimulating growth of normal diploid cells, a finding that may translate into a novel targeted therapy for patients with monosomy 7 MDS.

ON044580 induces apoptosis in primary cells from CML patients refractory to imatinib. Because ON044580 was able to induce the apoptotic death of 32D:*p210*T315I cells, it was of interest to examine its effects on primary tumor cells derived from patients who were refractory to imatinib treatment. For this study, we used cells derived from three different patients: a blast crisis CML patient refractory to imatinib treatment, (Fig. 5c), a CML patient in blast crisis (Fig. 5d), and a CML patient in chronic phase (Fig. 5e). Cells were maintained for 48 hours in the absence of cytokines, to enhance the level of BCR-ABL+ cells in the blood cell population, and treated for an additional 48 hours with various doses of the drug. Our results show that the blast crisis and chronic phase cells were highly sensitive to apoptosis induction by ON044580, which suggests that ON044580 may be useful to treat the unusually resistant blast crisis patients.

Discussion

Chronic myeloproliferative disorders (CMPD) are clonal malignancies characterized by overproduction of one or more hematopoietic lineages with relatively normal differentiation. The molecular pathogenesis of several CMPD has been well characterized and is frequently attributable to mutations that result in constitutive activation of a protein tyrosine kinase. The classic CMPD are subdivided into chronic myeloid leukemia (Ph^+ CML) and the Ph-negative CMPD, that is, polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF).[59]

Although Ph+ CML is characterized at the molecular level by the expression of the BCR-ABL fusion protein with deregulated kinase activity, a novel gain of function mutation in the JAK2 tyrosine kinase (*JAK2*V617F) has been observed in about 95% of patients with PV and 50% of those with either ET or PMF. This mutation has also been found in patients with nonclassic CMPD such as RARS-T, chronic neutrophilic leukemia, atypical CML, and chronic myelomonocytic leukemia at 50%, 20%, 20%, and 3% incidence, respectively.[60] Both the BCR-ABL and JAK2-V617F proteins have been found to confer cytokine-independent growth of the mutant cells *in vitro* due to deregulation of signaling pathways downstream of JAK2. Expression of either of these mutant proteins were found to result in the constitutive activation of downstream effector proteins of the JAK signaling pathway, the STAT family of transcription factors. These findings have opened new avenues for diagnosing and classifying patients with these disorders and identify JAK2 as a new molecular target for the therapy of myeloproliferative disorders.

Selection of highly conserved mutable residues in the ATP binding site appears to be relatively common for many kinases. It has hence been argued that substrate-competitive inhibitors might constitute better drug candidates.[53] In addition, many of the kinase inhibitors that are ATP mimetics inhibit their enzyme targets at nanomolar concentrations while requiring micromolar concentration for tumor cell growth inhibition, and this discrepancy may be related to the need to overcome millimolar concentrations of ATP known to exist inside the cell.[53] Mutations occur at residues directly implicated in imatinib binding or, more commonly, at residues important for the ability of the kinase to adopt the specific closed (inactive) conformation to which imatinib binds.[61] Because of the frequency of mutations, efforts are now focused on the identification of novel inhibitors that are active against imatinib-resistant mutants of BCR-ABL.

In this report, we describe the development of a novel small-molecule inhibitor of JAK2 and BCR-ABL that appears to target regions outside the ATP-binding site of its target and offers the potential to be unaffected by mutations in the kinase domain that make tumor cells resistant to ATP-competitive inhibitors. The results presented in this study show that this novel pharmacophore,[53] which inhibits recombinant JAK2 kinase (both wild-type and mutant forms) at a concentration of 0.9 to 1.2 μM, is noncompetitive with ATP (Fig. 2a). However, the addition of increasing concentrations of the substrate peptide to the reaction mixture results in an increase in the IC_{50} of the compound, suggesting that this could be a substrate-competitive inhibitor (Figs. 2b and c). It is interesting to note that both autophosphorylation and substrate phosphorylation are affected by increasing concentrations of the substrate

peptide, suggesting that the binding of the compound to JAK2 might affect both these activities. Our studies also suggest that at least a portion of the regulatory JH2 domain is essential for the inhibitory activity of ON044580 (Fig. 1f).

ON044580 is also able to actively down regulate IL-3 signaling by inhibiting the phosphorylation of JAK2 and STAT5 proteins in cells harboring mutant JAK2 kinase within 30 minutes following the addition of the compound. This, in turn, appears to result in growth arrest and apoptosis of human and mouse leukemic cells expressing the *JAK2*V617F protein (Figs. 3f and h). Because both BCR-ABL and JAK2-V617F proteins confer cytokine-independent growth of mutant cells *in vitro* due to deregulation of signaling pathways downstream of JAK2 and share several of the substrates associated with cytokine signaling (such as STAT5), it was of interest to see if ON044580 exhibits any cross-reactivity with BCR-ABL kinase activity. Initially, we carried out experiments aimed at determining the ability of ON044580 to inhibit the kinase activity of recombinant BCR-ABL kinase produced in insect cells. Our results presented in Supplementary Figure S1 show that this compound failed to inhibit the kinase activity of recombinant BCR-ABL even at a concentration of 20 μM. It has been known that some of the non–ATP-competitive inhibitors of BCR-ABL do not exhibit *in vitro* kinase inhibitory activity with recombinant enzymes.[57] An explanation for the lack of *in vitro* activity against recombinant protein could be the absence of posttranslational modifications that might affect the conformation of the protein as well as the binding of the inhibitor to the protein.

When we carried out the *in vitro* kinase inhibition assays with BCR-ABL protein immunoprecipitated from K562 cells or 32D:*p210*T315I cells, we could detect direct inhibition of both the wild-type and mutant T315I forms of BCR-ABL kinase by ON044580 (Figs. 4a and b). Notably, ON044580 exhibited a better inhibitory activity against the mutant kinase (IC_{50} = 2.08 μM) as compared to the wild type (IC_{50} = 7.94 μM). It is known that the T315I mutation confers imatinib resistance partly by phosphorylation of endogenous BCR, suggesting aberrant substrate activation by BCR/ABL harboring the T315I mutation.[62] It is conceivable that ON044580, being a substrate competitive inhibitor, has an additional site to effect its activity against the T315I version of BCR-ABL as opposed to the wild-type protein, which does not display such aberrant substrate activation. Alternatively, it is possible that the conformation adapted by the mutant BCR-ABL protein allows a tighter binding of ON044580.

We also examined whether ON044580 inhibits the *in vivo* autophosphorylation and substrate (STAT5) phosphorylation of BCR-ABL kinase. These studies showed that incubation of K562 cells with ON044580 resulted in an inhibition of cellular phosphorylation of the BCR-ABL kinase in a dose-dependent manner. In 2 hours, autophosphorylation levels started to decrease at 0.3 μM and were undetectable at a concentration of 10 μM. In the same assay, imatinib exhibited similar inhibitory activity with respect to BCR-ABL autophosphorylation (Fig. 4c). Notably, such inhibition required lower concentrations of ON044580 compared to the *in vitro* inhibition of immunoprecipitated BCR-ABL (IC_{50} = 8 μM). This could be due to interference by the antibody used to immunoprecipitate the BCR-ABL protein or subtle changes that the protein undergoes during immunoprecipitation resulting in a poorer binding of the compound to the kinase.

Most interestingly, ON044580 could bring about a similar reduction in the autophosphorylation status of BCR-ABL-T315I kinase (Fig. 4d), suggesting that mutations in the kinase domain of BCR-ABL do not adversely affect the inhibitory activity of ON044580. As can be expected, in a similar assay, imatinib failed to affect the autophosphorylation status of BCR-ABL-T315I kinase even at a concentration of 100 μM. Interestingly, ON044580 could readily inhibit the phosphorylation status of STAT5 in cells expressing the WT or the T315I mutant forms of BCR-ABL but failed to inhibit CrkL phosphorylation. These results suggest that substrate-competitive inhibitors may not inhibit phosphorylation of all substrates and thus differ from ATP-competitive inhibitors such as imatinib. We also observed enhanced degradation of BCR-ABL-T315I kinase in ON044580-treated cells (Fig. 4d). A similar effect is seen on the levels of JAK2 and STAT5 in lysates from murine myeloid cells treated with 20 μM ON044580 (Figs. 3a and c). It has been previously shown that inhibition of JAK2 kinase activity in CML cells by AG490, its analogs, or JAK2-directed siRNA results in the destabilization of the large BCR-ABL-JAK2 multiprotein network leading to the degradation of BCR-ABL kinase.[63-66] Our preliminary studies show that ON044580 brings about similar destabilization of the large BCR-ABL-JAK2 multiprotein complex (which contains STAT5) and could account for its degradation.

To further substantiate the usefulness of this compound in CML therapy, we examined the effects of this compound on freshly isolated human leukemic cells from patients who were refractory to imatinib. In this study, we used cells derived from three different patients, a blast crisis CML patient refractory to imatinib treatment (Fig. 5c), a CML patient in blast crisis (Fig. 5d), and a CML patient in chronic phase (Fig. 5e), for treatment with various doses of the drug. Our results show that the blast crisis and chronic phase cells were highly sensitive to apoptosis induction by ON044580, which suggests that this compound may be useful to treat the unusually resistant blast crisis patients.

The observation that ON044580 inhibits the wild-type JAK2 (Fig. 1b and c) led us to test its effect in U266 multiple myeloma cells that have constitutively activated IL-6

recpetor/JAK2/STAT-3 pathway. As would be expected, we found ON044580 inhibited the phosphorylation of STAT-3 at nanomolar concentrations (Fig. 3e). We further tested the usefulness of ON044580 in shutting down aberrant JAK/STAT signaling in bone marrow samples from monosomy 7 MDS patients. Monosomy 7 MDS is characterized by constitutive activation of JAK2 and STAT1. Treatment with ON044580 specifically decreased the monosomy 7 population whereas normal diploid cells continued to proliferate (Fig. 5b). These results along with the induction of apoptosis in cells expressing the activated V617F mutant form of JAK2 (Figs. 3f and h) suggest that ON044580 also holds promise for use as a therapeutic against both myelodysplastic syndromes and myeloproliferative neoplasms where JAK2 is aberrantly activated.

In the past few years, several cases have been reported in which both the BCR-ABL translocation and JAK2-V617F mutation have been observed concomitantly in bone marrow samples from CMPD patients.[67-70] These studies have revealed that JAK2-V617F mutation-associated CMPD develops predominantly after selective treatment of Ph+ CML with imatinib. Furthermore, the emergence of the BCR-ABL translocation on the background of $JAK2^{V617F}$ CMPD seems to be unrelated to prior myelosuppressive treatment (standard treatment for CMPD). Finally, $JAK2^{V617F}$ mutation seems to precede the acquisition of the Philadelphia chromosome.[71] Importantly, there has been recent evidence showing that the kinase activity of JAK2 is required for the stability of BCR-ABL protein and thus maintenance of the oncogenic signal. In this context, JAK2 has been shown to be activated in Bcr-Abl+ hematopoietic cells and CML cell lines.[58,64,72,73] In these cell lines, activated JAK2 appears to be important for stimulating the PI-3-kinase/Akt pathway. Jak2 is also involved in elevating SET levels,[58] which inhibits the PP2A/Shp1 pathway leading to the maintenance of high levels of pTyr Bcr-Abl.[74]

It is significant that ON044580 is the first reported dual JAK2/BCR-ABL inhibitor and holds the unique clinical promise to eliminate all clones that have both or either of these 2 genetic lesions. In addition, ON044580 can potentially reduce the outgrowth of $JAK2^{V617F}$ clones from Ph+ cells and prevent the development of resistance.

In conclusion, we have identified ON044580, a substrate-competitive kinase inhibitor active against two oncogenic kinases, BCR-ABL and activated JAK2 that appear to play a central role in CMPD. This inhibitor is active against mutant versions of these kinases and causes apoptotic cell death of imatinib-resistant CML clones on one hand while selectively inhibiting the proliferation of monosomy 7 MDS clones on the other. Being a dual JAK2/BCR-ABL inhibitor, ON044580 could possibly become the first in a class of inhibitors effective in treating a variety of myeloproliferative disorders such as CML, Ph-negative CMPD, and MDS typified by aberrant JAK/STAT signaling.

Materials and Methods

Kinase assays and IC_{50} determination. Ten nanograms of GST-JAK2 (aa 808-1132; Invitrogen PV4210), 200 ng of GST-JAK2-WT (532-1132; Invitrogen PV4393), 200 ng of GST-JAK2-V617F (532-1132; Invitrogen PV4336), or 100 ng affinity purified GST-p210-WT kinase (baculovirally expressed in Sf21 cells; Invitrogen B821-01) was diluted into kinase buffer (20 mM Tris pH 7.5, 10 mM $MgCl_2$, 0.01% NP-40, 1 mM EGTA, 2 mM DTT) and incubated with the indicated concentration of inhibitor at room temperature for 30 minutes. The kinase reactions were initiated by the addition of 1 μg (1.5 μM) recombinant GST-Abltide (Upstate 12-525), 20 μM ATP, and 20 μCi γ-^{32}P-ATP. The reactions were incubated at 30°C for 20 minutes, terminated by the addition of 2x Laemmli sample buffer, boiled for 2 minutes, resolved by 12% acrylamide SDS-PAGE, and subjected to autoradiography. For Src (Invitrogen P3044), LynB (Invitrogen P2907), Plk-1 (Invitrogen PV3501), and Cdk-1/cyclinB (Upstate 14-450) kinase assays, 10 ng of recombinant kinase was used with 1 μg GST-Sam68 (aa 331-443; Santa Cruz Biotechnologies sc-4249), dephosphorylated α-Casein (Sigma C8032), or Histone H1 (Roche Diagnostics 223549) as protein substrate, respectively. Full-length BCR-ABL (WT and T315I) and JAK2-V617F were immunoprecipitated from cultured cells and the immune-complexes processed as above to assay kinase activity. The autoradiograms were scanned, and the band corresponding to autophosphorylation of JAK2 or phosphorylation of GST-Abltide was quantitated using MacBas software. The densitometric values obtained were plotted as a function of log drug concentration using Prism 4 Graphpad software and IC_{50} values determined by plotting sigmoidal nonlinear regression curves with variable slope.

Cell culture. Ba/F3:*JAK2*V617F cells were maintained in RPMI medium 1640 supplemented with 10% FBS, 1 U/mL penicillin-streptomycin, and 1% WEHI-3B conditioned medium as a source of IL-3. 32D:*p210*WT and 32D:*p210*T315I cells were maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS, 1 U/mL penicillin-streptomycin. K562 (DSMZ no. ACC 10) cells were maintained in RPMI medium 1640 with 10% FBS and 1 U/mL penicillin-streptomycin. U266 (DSMZ no. ACC 9), HEL (DSMZ no. ACC 11), and SET-2 (DSMZ no. ACC 608) cells were maintained in RPMI medium 1640 containing 10%, 20%, and 10% heat-inactivated FBS, respectively. Patients' samples were diluted with PBS in a 1:1 ratio and were separated by Histopaque-1077 (Sigma-Aldrich). After separation of the whole blood, the cells were

washed with PBS, suspended in RPMI-1640 supplemented with 10% FBS, and treated with various doses of ON044580 for 48 hours. Subsequently, the cells were assayed for apoptosis by Annexin-V/IP method involving flow cytometry.

Western blot analysis and immunoprecipitation. Mid-log phase Ba/F3:*JAK2*V617F cells were treated for 0 to 120 minutes with indicated concentrations of inhibitor and 1 hour with 5 ng/mL recombinant mouse IL-3 (R&D Systems 403-ML). Washed cells were lysed in detergent containing buffer, and the 50 µg of the clarified lysates were resolved by SDS-PAGE and analyzed by Western blotting using anti-phopho-STAT5A/B (Tyr694/699) clone 8-5-2 (Upstate 05-495) and anti-phosho-JAK2 (Tyr1007/1008; Upstate 07-606) antibodies and the corresponding HRP-conjugated secondary antibodies (GE Healthcare). Subsequently, the blots were stripped and reprobed with anti-STAT5A/B (Upstate 06-588) and anti-JAK2 (Upstate 06-255) antibodies. In apoptosis studies, cells were treated with compounds (or DMSO) for 2, 6, and 24 hours. The harvested cells were washed in PBS and lysed as above. Fifty micrograms of clarified lysates resolved by SDS-PAGE were analyzed by Western blotting using anti-PARP antibody (Cell Signaling Technology 9542). For immunoprecipitation, cells grown to mid-log phase were stimulated with 5 ng/mL recombinant IL-3 for 1 hour, washed with PBS, lysed in detergent containing buffer (25 mM HEPES pH 7.5, 0.1% Triton X-100, 150 mM NaCl, 1 mM DTT, 1 mM EGTA, 1.5 mM $MgCl_2$, 20 mM β-glycerophosphate, 0.2 mM Na_3VO_4, and 1x protease inhibitor mixture [Roche Diagnostics]), and 500 µg of the clarified lysate was incubated with 5 µL anti-JAK2 antibody and 25 µL (50% slurry) of Protein A Sepharose CL-4B beads (GE Healthcare 17-0780-01) for 2 hours at 4°C. The immune-complexes were washed 3 times in PBS and used for *in vitro* kinase assays or Western blot analysis. Exponentially growing K562 cells and 32D:*p210*T315I cells were treated for 2 hours with indicated concentrations of inhibitor. Cells were washed with PBS, and 50 µg of the clarified lysate was used for Western blot analysis. BCR-ABL was immunoprecipitated from 500 µg of the clarified lysates using 5 µL of anti-Bcr (N-20) antibody (Santa Cruz Biotechnologies sc-885) and processed as above. For kinase assays, untreated 32D:*p210*WT and 32D:*p210*T315I cells were used for immunoprecipitation. Western blot analysis was carried out with antiphosphotyrosine antibody (Santa Cruz Biotechnologies sc-885) to check the levels of p210 autophosphorylation. The blot was stripped and reprobed for levels of p210 protein with anti-Bcr (N-20) antibody. Similarly, anti-phospho-CrkL (Tyr207; Cell Signaling Technologies 3181S) and anti-CrkL (32H4; Cell Signaling Technology 182) antibodies were used to assay levels of CrkL phosphorylation.

Growth inhibition and GI_{50} determination. Ba/F3:*JAK2* V617F cells, HEL cells, SET-2 cells, K562 cells, and 32D:*p210*T315I cells were grown in the presence of varying concentrations of ON044580 for 72 hours. Cell viability was measured by Trypan blue exclusion. GI_{50} values were calculated by plotting percentage viable cells as a function of drug concentration using Prism 4 Graphpad software.

Short-term exposure wash-out experiments. Ba/F3:*JAK2* V617F cells, HEL cells, and K562 cells were treated with increasing concentrations of ON044580 or vehicle for 2 hours (in duplicate), washed 3 times in growth medium, and plated in 12-well dishes. The total number of viable cells was determined 96 hours later by Trypan blue exclusion. Bone marrow was harvested from femur and tibia of CD-1 mice and treated with increasing concentrations of ON044580 or vehicle (in duplicate) for 2 hours. The cells were washed 3 times in IMDM medium and cultured in methylcellulose medium supplemented with 50 ng/mL rmStem Cell Factor, 10 ng/mL rmIL-3, 10 ng/mL rhIL-6, 200 µg/mL human Transfeffin, and 3 U/mL rhErythropoietin (Stem Cell Technologies). Cultures were seeded in duplicates using 35-mm plastic petri dishes, and colony-forming units were determined after 1 week. Histograms of percentage viable cells/colony-forming units (compared with DMSO-treated controls) as a function of drug concentration were plotted using Excel software.

Treatment of primary cells from CML patients. Blood samples were received from 3 CML patients through our approved lab protocol. One was from a blast crisis CML patient refractory to imatinib treatment. The other was from a CML patient in blast crisis. The 3rd was a sample from a CML patient in chronic phase. White blood cells were maintained for 48 hours in the absence of cytokines, to enhance the level of BCR-ABL+ cells in the blood cell population. Cells were treated for an additional 48 hours with various doses of the inhibitor. Cells were assayed for apoptosis by the annexinV/PI method involving flow cytometry. Apoptotic cells accumulate in the lower right (quadrant 3) and upper right quadrants (quadrant 4).

Treatment of bone marrow samples from monosomy 7 MDS patients. BMMNCs were obtained from patients with monosomy 7 MDS confirmed by metaphase karyotyping and FISH after informed consent according to protocols approved by the Institutional Review Board of the National Heart, Lung, and Blood Institute. BMMNCs were aspirated from the posterior iliac crest into syringes containing RPMI media supplemented 1:10 with heparin (O'Neill and Feldman, St. Louis, MO) and prepared by density gradient centrifugation using lymphocyte separation medium (Organon, Durham, NC). Cryopreserved BMMNCs were grown in Mylocult media

(Stem Cell Technologies) supplemented with 400 ng/mL G-CSF and growth factor cocktail as previously described.[75]

FISH studies. FISH studies on cryopreserved BMMNCs were performed as described previously[75] using centromeric probes for chromosome 7 and 8 (Vysis, Downers Grove, IL). Percentage positive staining was based on counting of 400 cells. Three different observers, who were blinded with respect to sample identity, scored 3 different sets of slides. Scores were averaged, and the mean of the 3 was recorded. The total number of aneuploid and diploid cells was calculated by multiplying the percentage aneuploidy by the number of viable cells in each sample.

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Declaration of Conflicting Interests

Dr. E.P. Reddy is a stockholder, board member, and consultant for Onconova Therapeutics, Inc. Dr. M.V. Ramana Reddy is a stockholder and consultant for Onconova Therapeutics, Inc. Drs. Stephen C. Consenza and Stacey J. Baker are consultants for Onconova Therapeutics, Inc. Drs. Jatiani, Ha, Samanta, Olnes, Pfannes, Sloand, and Arlinghaus declare no potential conflicts of interest.

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Destabilization of Bcr-Abl/Jak2 Network by a Jak2/Abl Kinase Inhibitor ON044580 Overcomes Drug Resistance in Blast Crisis Chronic Myelogenous Leukemia (CML)

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Abstract

Abstract: Bcr-Abl is the predominant therapeutic target in chronic myeloid leukemia (CML), and tyrosine kinase inhibitors (TKIs) that inhibit Bcr-Abl have been successful in treating CML. With progression of CML disease especially in blast crisis stage, cells from CML patients become resistant to imatinib mesylate (IM) and other TKIs, resulting in relapse. Because Bcr-Abl is known to drive multiple signaling pathways, the study of the regulation of stability of Bcr-Abl in IM-resistant CML cells is a critical issue as a possible therapeutic strategy. Here, the authors report that a new dual-kinase chemical inhibitor, ON044580, induced apoptosis of Bcr-Abl+ IM-sensitive, IM-resistant cells, including the gatekeeper Bcr-Abl mutant, T315I, and also cells from blast crisis patients. In addition, IM-resistant K562-R cells, cells from blast crisis CML patients, and all IM-resistant cell lines tested had reduced ability to form colonies in soft agar in the presence of 0.5 μM ON044580. In *in vitro* kinase assays, ON044580 inhibited the recombinant Jak2 and Abl kinase activities when the respective Jak2 and Abl peptides were used as substrates. Incubation of the Bcr-Abl+ cells with ON044580 rapidly reduced the levels of the Bcr-Abl protein and also reduced the expression of HSP90 and its client protein levels. Lysates of Bcr-Abl+ cell lines were found to contain a large signaling network complex composed of Bcr-Abl, Jak2, HSP90, and its client proteins as detected by a gel filtration column chromatography, which was rapidly disrupted by ON044580. Therefore, targeting Jak2 and Bcr-Abl kinases is an effective way to destabilize Bcr-Abl and its network complex, which leads to the onset of apoptosis in IM-sensitive and IM-resistant Bcr-Abl+ cells. This inhibitory strategy has potential to manage all types of drug-resistant CML cells, especially at the terminal blast crisis stage of CML, where TKIs are not clinically useful.

Keywords

CML, Bcr-Abl, Jak2, drug resistance, apoptosis

Introduction

In chronic myelogenous leukemia (CML), Bcr-Abl, the fusion protein derived from Philadelphia chromosome, is the constitutively activated protein tyrosine kinase, which is largely unregulated.[1-3] It is widely known that Bcr-Abl drives several important signaling pathways—the Ras, PI-3 kinase, STAT5, STAT3, and Jak2 pathways that cause oncogenesis in CML.[4-10] Since these important pathways are derived from Bcr-Abl, it is considered the critical target molecule for CML therapy. Imatinib mesylate (IM) is an effective inhibitor of the Bcr-Abl tyrosine kinase and is the first-line treatment of CML since about 75% of early chronic phase CML patients favorably respond to IM treatment. During longer term treatment with IM, progression of the disease and drug resistance can develop in patients for several reasons.[11-20] Continuous targeting of Bcr-Abl can lead to blastic transformation[21] due to activation of other oncogenes and inactivation of tumor suppressor genes. The remission rate of the accelerated phase is 50%, and for the blast crisis phase, the remission rate is 20%.[17,22] Alterations of tumor suppressors such as PP2A, mutation of p53, inactivation of tyrosine phosphatases (Shp1), and overexpression of new proteins (e.g., SET) lead to the terminal blast crisis stage and ultimately death of the patients. More potent forms of IM (i.e., Nilotinib, NS-187) have been developed for the treatment of IM-resistant patients,[23] but they fail to kill cells from the blast crisis stage. The dual-kinase inhibitor dasatinib (Bcr-Abl and Lyn) is successful in the induction of apoptosis of several IM-resistant Bcr-Abl mutant cells in blast crisis patients,[24] but dasatinib fails to kill T315I Bcr-Abl mutant cells. Dasatinib-resistant CML has been reported, as 20 of 21 patients treated with dasatinib developed resistant CML cells containing the T315I

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mutation (S. Soverini *et al*., 2006, 2007[AQ: 1]).[25,26] Several other second-generation drugs were developed for CML therapy, but each drug has its own limitations.[27] Although overcoming IM resistance can be achieved for some forms of IM resistance caused by mutations in BCR-ABL, specific drugs for the T315I BCR-ABL IM-resistant mutant have not yet been developed, nor are drugs available to treat blast crisis CML. The untreated chronic phase may last for several years, the accelerated stage lasts for only 4 to 6 months, and the terminal blast crisis stage, characterized by rapid expansion of either myeloid or lymphoid differentiation-arrested blast cells (blast crisis), lasts for only a few months.[17,18] No successful therapeutic strategy of blast crisis exists at the present time. Allogeneic stem cell transplantation with high chemotherapy has been found to be successful in a small percentage (10%) of patients. New target molecules and specific inhibitor(s) need to be developed to treat advanced stages of CML, particularly in blast crisis patients.

Since Bcr-Abl is considered the primary therapeutic target molecule in CML, the stability and regulation of Bcr-Abl in CML cells is one of the critical issues for development of new therapeutic strategies required to overcome drug resistance. Neviani *et al*.[28] demonstrated that Bcr-Abl regulates its own stability by inhibiting PP2A-Shp1 phosphatases by inducing expression of tumor suppressor protein SET.[28,29] Our previous studies demonstrated that Jak2 is a major downstream signaling molecule in CML. It has been shown that Jak2 interacts with Bcr-Abl,[9] induces high-level c-Myc expression,[30] induces tyrosine phosphorylation of Gab2 on YxxM sequences needed for activation of PI-3 kinase,[31] is part of a Bcr-Abl network involving proteins such as Akt and GSK3β,[31] and regulates SET protein in Bcr-Abl+ cells.[32] Jak2 also maintains Lyn kinase in its functionally active form in Bcr-Abl+ cells through a Jak2-SET-PP2A-Shp1 signaling loop where PP2A-Shp1 remained inactive by Jak2-activated SET expression.[32] These results indicate that Jak2 is one of the important signaling molecules in Bcr-Abl+ cells.

HSP90, a major molecular chaperone, is known to interact with proteins involved in transcriptional regulation and signal transduction pathways for maintaining the stability and functional conformation of signaling proteins.[33-36] HSP90 acts as a biochemical buffer against genetic instability during cancer. HSP90 is responsible for the maturation and functional stability of a plethora of polypeptides called client proteins. HSP90 is overexpressed in leukemia and also in many other cancers, and it is assumed that in cancer, the requirement of HSP90 is critical since most of the client proteins of HSP90s are active participants in signal transduction pathways of cancer cells.[33,36-38] These qualities and functional aspects of HSP90 make it a potential target for anticancer drugs. Although several small molecules have been identified as anti-HSP90 candidates during past years, none of them has yet been successful in the clinic.[39,40] Gorre and colleagues[14] first showed that inhibition of HSP90 expression by 17-AAG caused reduction of wild-type and mutant Bcr-Abl proteins, leading to inhibition of growth. Later, Blagosklonny *et al*.[41] demonstrated that BCR-ABL+ cells were induced to undergo apoptosis upon treatment with 17-AAG. These qualities and functional aspects make HSP90 a potential target for the development of anticancer drugs.

In the current study, we have shown that ON044580 shows strong apoptotic activities in Bcr-Abl+ cells and overcomes drug resistance. These apoptotic events were initiated in part due to destabilization of the Bcr-Abl protein from where major signaling pathways originate. We have further demonstrated that ON044580 disrupted a high molecular weight Bcr-Abl/Jak2/HSP90 network structure. These results were obtained due to the unique Jak2 and Bcr-Abl kinase inhibitory properties of ON044580, which make it a novel and potentially useful compound for CML therapy.

Results

ON044580, α-benzoyl styryl benzyl sulfide, is a new compound synthesized by Dr. Reddy's group[42] that is not an adenosine triphosphate (ATP) competitor like many of the tyrosine kinase inhibitors such as IM but inhibits the catalytic activities of Abl (and Bcr-Abl) and Jak2. We present results on the role of ON044580 in modulating Bcr-Abl-driven cell signaling pathways and its effects on cell viability, apoptosis, and colony formation in soft agar.

Recombinant Abl and Jak2 kinase assays. To examine the effects of ON044580 on Abl and Jak2 kinases, we performed *in vitro* kinase assays with purified recombinant Abl (45-kDa Abl kinase) and Jak2 kinase (JH1-JH2) using Abl tide substrate for assays with Abl kinase and Jak2 peptide containing the Tyr 1007 activation site for the Jak2 kinase, respectively. IM inhibited the phosphorylation of Abl tide by recombinant Abl about 85%, whereas ON044580 at 5 μM and 10 μM reduced the Abl kinase activity by 50% and 75%, respectively (**Fig. 1a**). In the Jak2 kinase assay with JH1-JH2 domains, ON044580 strongly reduced Jak2 kinase activity in a dose-dependent-manner (**Fig. 1b**). As a positive control TG101209, an authentic Jak2 inhibitor[43] was used that strongly reduced phosphorylation of the Jak2 peptide. These studies indicate that both recombinant Abl kinase and Jak2 kinase are strongly inhibited by ON044580, suggesting that ON044580 is a dual-kinase inhibitor (**Figs. 1 a and b**).

ON044580 strongly inhibited Jak2 and Bcr-Abl tyrosine kinase activity in kinase assays performed with immune complexes from Bcr-Abl+ 32D cells. To further investigate the effects of ON044850 on the Jak2 kinase, we performed *in vitro* autophosphorylation assays of Jak2 using Bcr-Abl+

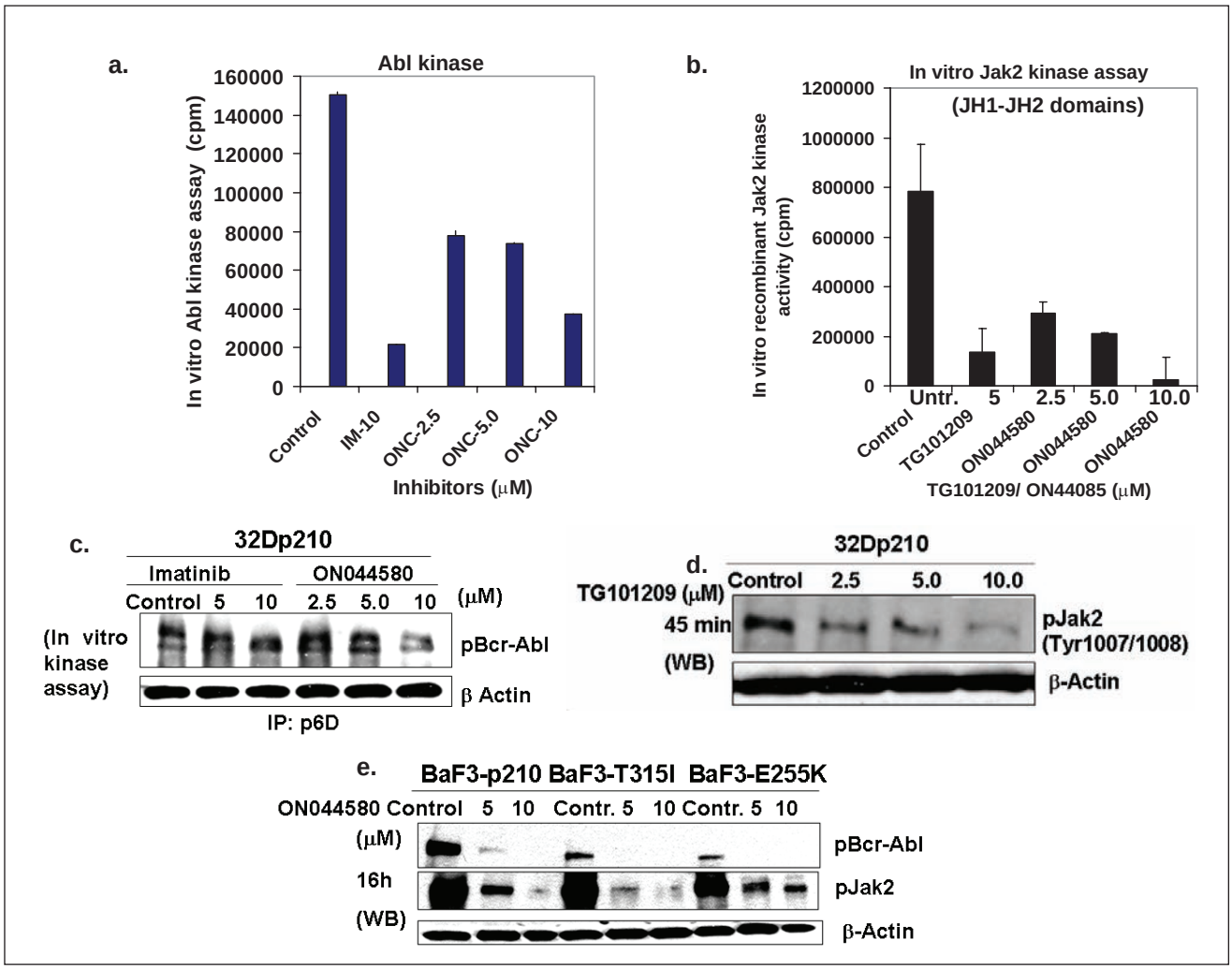

Figure 1. pJak2 and pBcr-Abl are inhibited by ON044580. (**a**) Inhibition of recombinant Abl kinase by ON044580 during *in vitro* kinase assay using the Abl tide peptide as substrate. Recombinant Abl (45 kD) was used in an *in vitro* kinase assay using Abl tide peptide as substrate and 32P gamma adenosine triphosphate (ATP) following the protocol of the manufacturer. Abl kinase inhibitor imatinib (IM) was used as a control; the effects of ON044580 on the Abl kinase were examined in a dose-dependent manner. (**b**) Inhibition of recombinant Jak2 kinase (JH1 and JH2 domains) by ON044580 during *in vitro* kinase assay using a Jak2 peptide as substrate. For *in vitro* Jak2 kinase assay, recombinant Jak2 kinase (JH1 and JH2 domains) was used to phosphorylate the Jak2 peptide containing tyrosine 1007/8 sequences. The Jak2 inhibitor TG101209 (TargeGen, San Diego, CA) was used as a positive control. (**c**) Inhibition of pBcr-Abl by ON044580 during *in vitro* kinase assay of Bcr-Abl. Detergent extracted Bcr-Abl+ cell lysates were immunoprecipitated with p6D anti-Abl antibody following the standard protocol. *In vitro* kinase assay for Bcr-Abl was carried out in the presence of different doses of imatinib (positive control) and ON044580 for 30 minutes. The supernatant of the kinase reaction was analyzed by Western blotting using 4G10 antibody. The lysates after immunoprecipitation were used for Western blotting for β actin levels. (**d**) Inhibition of Jak2 by ON044580 during *in vitro* kinase assay. Since Jak2 and Bcr-Abl are physically associated in Bcr-Abl+ cells, we immunoprecipitated Jak2 using p6D antibody, and the *in vitro* Jak2 kinase assay was carried out in the presence of a different amount of ON044580 following the standard protocol. After kinase reaction, the supernatant was used for Western blotting for the detection of pJak2 (Tyr1007/1008) signals. (**e**) pBcr-Abl and pJak2 inhibition in Bcr-Abl+ IM-sensitive and IM-resistant cells. Wild-type Bcr-Abl+ BaF3, BaF3 T315I, and BaF3 E255K cells were incubated with different amounts of ON044580 for 16 hours. The detergent lysates were analyzed by Western blotting with pTyr antibody (4G10) to detect pTyr Bcr-Abl and pTyr Jak2.

cell lysates. Our previous findings indicate that Jak2 is associated with the C-terminus of Bcr-Abl.[9] On the basis of that observation, for the Jak2 kinase assay, we immunoprecipitated Bcr-Abl from detergent-extracted Bcr-Abl+ 32D cell lysates with Abl-specific antibody (P6D). After repeated washing of the immunoprecipitates, the kinase assays were performed using the protocol described for Jak2 kinase.[9,44] The kinase supernatant was analyzed by Western blotting using anti-pTyr (4G10) to detect tyrosine-phosphorylated P210 BCR-ABL (**Fig. 1c**) and anti-pJak2 (Tyr1007/1008) to detect activated Jak2 (**Fig. 1d**). We observed that both Bcr-Abl kinase and Jak2 kinase activities were reduced in the presence of ON044580 (**Figs. 1 c and d**).

Treatment of IM-resistant cells with ON044580 reduced pTyr Bcr-Abl and pTyr Jak2. We incubated Bcr-Abl+ IM-sensitive (BaF3p210) and IM-resistant cells (BaF3p210 T315I and BaF3p210 E255K cells) with different doses of ON044580 for 16 hours. Cell lysates were prepared by detergent extraction, and the lysates were analyzed by Western blotting using anti-pTyr antibody (4G10). We observed that the levels of both pTyr Jak2 and pTyr Bcr-Abl were sharply reduced with 16-hour incubation (**Fig. 1e**). However, the Bcr-Abl protein was found to rapidly disappear from the lysate within 2 hours of 10 μM ON044580 treatment, whereas Jak2 protein levels were not affected during these 2-hour treatments. The dose needed to reduce the Bcr-Abl protein levels began at 2.5 μM and was complete at 10 μM (Suppl. Figs. S1 a and b). These studies indicate that treatment of Bcr-Abl+ cells with ON044580 may affect either the stability or solubility of Bcr-Abl.

Bcr-Abl, Jak2, and their downstream signaling molecules are reduced in amount by ON044580 in Bcr-Abl+ cells. We addressed the question of whether treatment of Bcr-Abl+ cells with ON044580 affected downstream signaling molecules of Bcr-Abl. To examine this possibility, we incubated Bcr-Abl+ 32D cells for 6 hours using 10 μM ON044580 and for 16 hours with increasing amounts (0-10 μM) of the inhibitor. The detergent-extracted lysates were analyzed by Western blotting using several antibodies. We observed that in addition to the reduction of Bcr-Abl, pTyr Jak2, STAT3, and Akt levels were also reduced during 6-hour incubation of Bcr-Abl+ cells with ON044580 (**Fig. 2a**). We further observed that a 16-hour incubation of Bcr-Abl+ cells with ON044580 reduced not only Jak2 and STAT3 levels but also pTyr705 and pSer727 STAT3 levels. Interestingly, Lyn was unaffected (**Fig. 2b**). It is known that Bcr-Abl, Jak2, and STAT3 are the client proteins of HSP90,[45-48] but Lyn has not been reported to be a client protein of HSP90. Thus, our results also suggest that Lyn is not a client protein of HSP90.

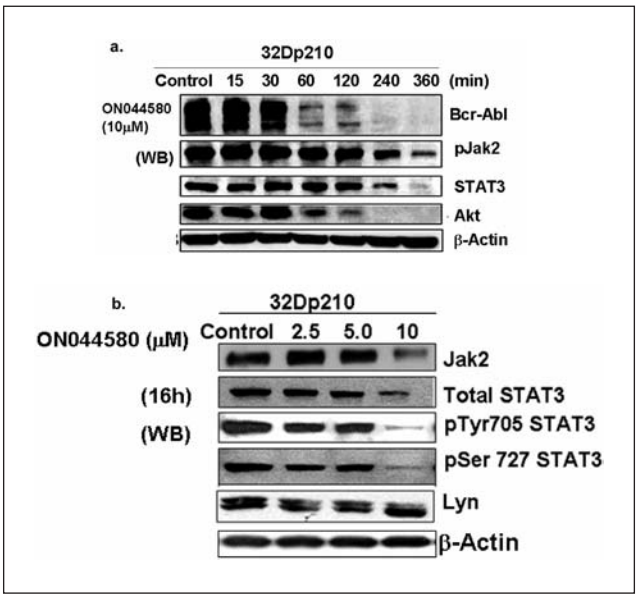

Figure 2. ON044580-mediated inhibition of Jak2 and Bcr-Abl kinases induced reduction of downstream targets of Bcr-Abl signaling molecules. (a) ON044580 reduces expression levels of Bcr-Abl downstream signaling molecules. Bcr-Abl+ 32D cells were incubated with 10 μM ON044580 for different time periods as marked. Actin was used as the loading control. The detergent cell lysates were analyzed by Western blotting using different antibodies—Bcr-Abl (8e9), pJak2 (pTyr 1007/8), STAT3, Akt, and β-actin. (b) ON044580 reduces the levels of Jak2 and STAT3 but not Lyn. The results show reduced expression of STAT3, pTyr 705 STAT3, and pSer 727 STAT3 by different doses of ON044580 for 16 hours.

ON044580 reduced binding of STAT3 to its consensus sequence in Bcr-Abl+ cells. It is known that tyrosine phosphorylation of STAT3 plays a key role in the dimerization of STAT3, nuclear translocation, and binding to specific DNA consensus sequence of STAT3, whereas serine phosphorylation of STAT3 is essential for maximum transcriptional activity.[49,50] Since Tyr 705 STAT3 phosphorylation was reduced by ON044580, it was expected that DNA binding of STAT3 to its consensus sequence would be interrupted. Therefore, we examined the binding of STAT3 to its consensus sequence by electrophoretic mobility shift assays (EMSA). STAT3, obtained from nuclear extracts of ON044580-treated Bcr-Abl+ 32D cells (16 hours), was allowed to interact with its radiolabeled consensus STAT3 oligonucleotide DNA sequence.[51] Bcr-Abl+ cells treated with ON044580 had strongly reduced the STAT3-specific DNA binding activity in a dose-dependent manner (**Fig. 3a**). The assay signal for STAT3 is specific because competition with nonradioactive consensus sequences strongly competed with the radioactive target oligonucleotides in a dose-dependent manner (**Fig. 3b**, right panel). Similarly, addition of STAT3 antibody to the nuclear lysate caused a mobility shift of the STAT3 complex (not shown), indicating that the signals for STAT3 in EMSA (**Fig. 3a**) are STAT3 specific.

ON044580 decreased the levels of HSP90 in Bcr-Abl+ cells. HSP90 is reported to be a chemotherapeutic target molecule for many cancers, including CML.[35,36,48,52] Some of the critical signaling molecules in Bcr-Abl+ cells are client proteins of HSP90.[14,47,3] We examined whether ON044580 regulated the expression of HSP90 at the transcriptional level. For this, we performed RT-PCR assays using HSP90 primers. We treated 32Dp210 cells with ON044580 for 16 hours. We note that the HSP90α promoter has a binding site

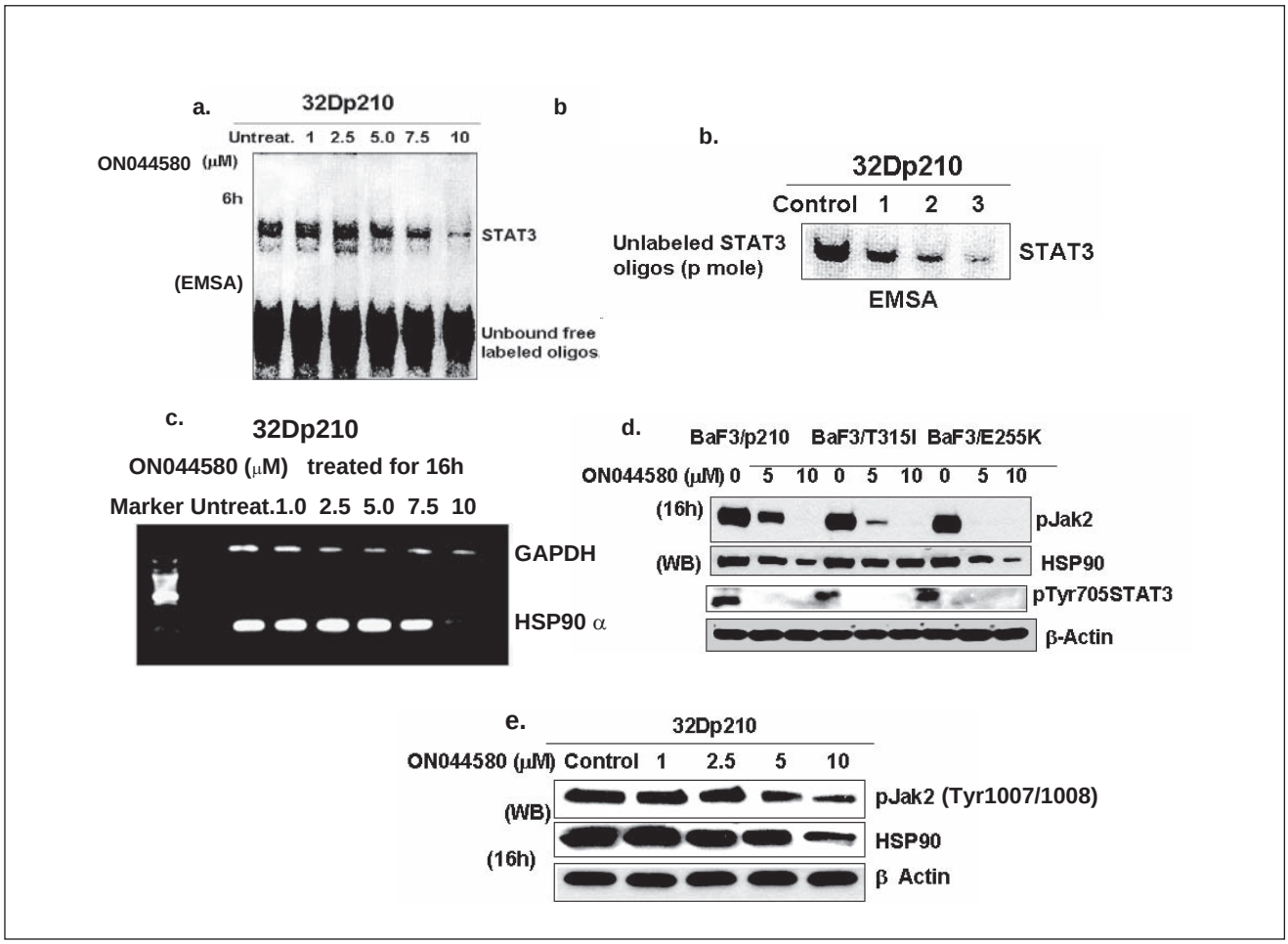

Figure 3. ON044580 reduced binding of STAT3 to its consensus sequence and also reduced expression of HSP90 at transcription and translational levels. (**a**) Binding of STAT3 (obtained from nuclear extract preparation of Bcr-Abl+ 32D cells) to its consensus oligonucleotide radiolabeled with 32P adenosine triphosphate (ATP) was reduced by ON044580 in a dose-dependent manner as elucidated by the electrophoretic mobility shift assay (EMSA). (**b**) The signals for STAT3 binding are specific as an increasing amount of unlabeled STAT3 oligos replaced the radiolabeled STAT3 oligos binding to the consensus STAT3 DNA sequence. (**c**) ON044580 reduced HSP90 expression at the transcriptional level. Bcr-Abl+ cells were incubated with ON044580 for 16 hours, and RNA was extracted from the treated cells. Reverse transcriptase–polymerase chain reaction (RT-PCR) was carried out for HSP90 using specific primers for HSP90 and GAPDH (loading control). (**d**) Exposure of ON044580 to the Bcr-Abl+ imatinib (IM)–sensitive and IM-resistant cells for 16 hours reduced HSP90 and pJak2 at protein levels. (**e**) ON044580 reduced the levels of pJak2, HSP90 in Bcr-Abl+ cells. After incubation of Bcr-Abl+ cells with ON044580 for 16 hours, the detergent-extracted cell lysates were analyzed by Western blotting, and the membrane was probed with pJak2 (Tyr1007/1008) and HSP90 antibodies.

for STAT3 (not shown). Of interest, ON044580 at 10 μM strongly reduced HSP90α transcripts at 16 hours of treatment (**Fig. 3c**), which coincides with the amount of ON044580 required to inhibit STAT3 binding to its consensus sequence (**Fig. 3a**). HSP90α protein levels in IM-sensitive and IM-resistant cells were also reduced by incubation of cells with 5 and 10 μM ON044580 for 16 hours. However, T315I cells were partially resistant to HSP90 reduction by ON044580 at 16 hours despite the high sensitivity to ON044580 to reduction of activated STAT3 (**Fig. 3d**). Nevertheless, these results suggest that Jak2 kinase may regulate expression of HSP90α through Jak2's ability to activate STAT3 in Bcr-Abl+ cells (**Figs. 3 a-e**).

Identification of a large network complex in Bcr-Abl+ cells and disruption of that complex in ON044580-treated cells. From our previous studies with various co-immunoprecipitation experiments, we showed that immunoprecipitation of one member of the Bcr-Abl signaling pathway co-precipitated other members of the pathway. Therefore, we predicted the presence of a large molecular network complex in Bcr-Abl+ CML cells.[31] To identify, characterize,

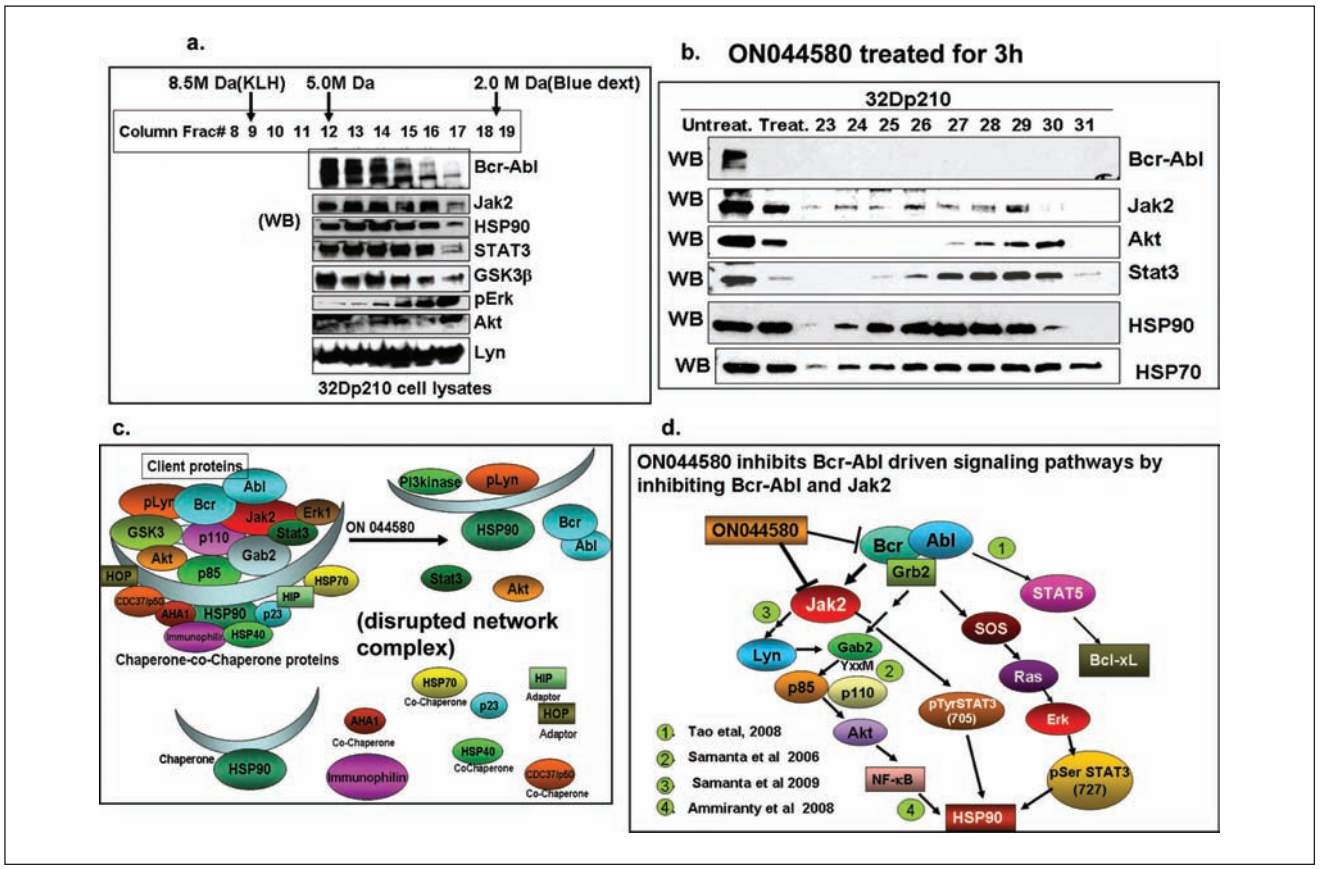

Figure 4. ON044580 induced disruption of the Jak2/Bcr-Abl/STAT3/HSP90 network complex. (**a**) Detection of a large molecular weight signaling network complex comprised Bcr-Abl, Jak2, and HSP90 and other proteins (e.g., STAT3, Akt, Erk, GSK3, and Lyn) by gel filtration column chromatography. Proteins from the detergent extracted lysate of Bcr-Abl+ 32D cells were eluted from the column by a detergent-containing buffer. From each eluant, a 25-μL aliquot was taken and analyzed by Western blotting, and the membrane was probed with different antibodies as indicated. (**b**) Treatment of Bcr-Abl+ 32D cells with 10 μM ON044580 for 3 hours disrupted the Bcr-Abl/Jak2/HSP90 network structure. The procedure for analysis was the same as used for (**a**). (**c**) Diagrammatic representation shows how ON044580 interrupted Bcr-Abl-driven signaling pathways affecting Jak2 and Bcr-Abl kinases and disrupted the Bcr-Abl/Jak2/HSP90 network complex. (**d**) Model showing how ON044580 inhibited Jak2 and Bcr-Abl and how the events of that inhibition affect downstream target molecules in Bcr-Abl+ cells.

and estimate the relative size of the Bcr-Abl/Jak2 network complex, we performed gel filtration column chromatography as a means to determine whether the Bcr-Abl/Jak2 network complex could be detected in a high molecular weight region of the column eluant. In collaboration with our Proteomics Core Facility, we optimized and calibrated the gel filtration column with different marker proteins ranging up to 8 million molecular weight (Suppl. Fig. S1e). Cell lysates of Bcr-Abl+ 32 D cells (32Dp210) were fractionated on the gel filtration column and eluted with a buffer containing NP-40 and glycerol. Fractions were analyzed by Western blotting with various antibodies so as to detect several proteins thought to be present in this network complex (**Fig. 4a**). We detected several signaling proteins, including HSP90, in the same fractions of the column eluant (e.g., fraction 12), suggesting the presence of high molecular weight protein complexes (**Fig. 4a**), which were estimated to be in the 4 to 6 million Da molecular size fraction. The Bcr-Abl/Jak2 network proteins included pTyrJak2 (1007/8), pLyn (Tyr 396), Lyn, Akt, STAT3, GSK3β, pErk, and HSP90; several column fractions contained these high molecular weight complexes (**Fig. 4a** and Suppl. Fig. S1e). Similar results were obtained with lysates of K562 cells (Suppl. Fig. S1d). The decrease in levels of Bcr-Abl and several other signaling proteins by treatment with ON044580 (**Fig. 2a**) suggested that this dual-kinase inhibitor might disrupt the network structure. To determine whether the elution pattern of the network would be affected by ON044580 treatment, we incubated 32Dp210 cells with 10 μM ON044580 for 3 hours and loaded the cell lysate into the column. We observed that the Bcr-Abl/Jak2/HSP90 network complex was disrupted, as Bcr-Abl protein was

severely reduced in amount, as were other members of the network. Importantly, HSP90 and other the client proteins eluted at a much lower molecular size (**Fig. 4b**). Although the levels of Jak2, STAT3, and Akt were reduced in the column fractions of ON044580-treated lysates, the levels of HSP90 remained almost unchanged but eluted at a much lower molecular size, as the position of the HSP90 protein shifted from elution at the higher molecular weight fractions (e.g., 12-15) to the lower size fractions (fractions 24-27), indicating that network had been disrupted. These results suggest the following: (1) that the Bcr-Abl/Jak2 network is bound to HSP90 and (2) that decrease in Bcr-Abl and inhibition of both Bcr-Abl and Jak2 kinases lead to disruption of the network structure by separation of Bcr-Abl and Jak2 from its signaling partners. We hypothesize that HSP90 client proteins such as Bcr-Abl are more susceptible to proteolytic degradation when the network structure is disrupted by treatment with ON044580. Under identical conditions, lysates of Bcr-Abl+ 32D cells treated with 10 μM imatinib for 6 hours did not show degradation/dissociation of signaling molecules (Suppl. Fig. S1c). A hypothetical model for disruption of the network by ON044580 is shown in **Figure 4c**.

ON044580 induces apoptosis in Bcr-Abl+ cells and overcomes drug resistance in Bcr-Abl+ leukemia cells. Our studies demonstrate that ON044580 strongly inhibits Jak2 and Abl kinase activities, and as a result, the levels of downstream signaling molecules are reduced, and the large Bcr-Abl/Jak2/HSP90 network complex is disrupted. We next examined how these inhibitory effects on the Bcr-Abl/Jak2/HSP90 network structure affected cell survival. For that purpose, we did cell viability/proliferation assays (MTT), apoptosis assays, and colony formation assays. We first assessed the effects of ON044580 on cell viability and proliferation by MTT assays. IM-sensitive Bcr-Abl+ cells (32Dp210) and IM-resistant cells (e.g., K562R) were inhibited by ON044580, as the viability was reduced in a dose-dependent manner (the IC_{50} of ON044580 for 32Dp210 and K562R cells was 3-5 μM; Suppl. Figs. S2 a and b). Apoptosis assays on several Bcr-Abl+ IM-sensitive and IM-resistant hematopoietic cell lines (**Figs. 5 a-c** and Suppl. Figs. S2 c and d, S3 a and b) were conducted by staining with annexin and propidium iodide followed by flow cytometric analysis. Results from this study showed that ON044580 was a potent inducer of apoptosis at concentrations of 1 to 5 μM. IM-sensitive Bcr-Abl+ cells (32Dp210) and BaF3-p210 cells were very sensitive to ON044580 to apoptosis induction, and 5 μM of ON044580 induced >80% apoptosis. IM-resistant cells such as T315I mutant cells and E255K and K562-R cells, although resistant to IM, were very sensitive to apoptosis induction by ON044580 (**Fig. 5b**). The T315I mutant is termed the gatekeeper mutation,[11] and all known kinase inhibitors that target the ATP binding domain of the Bcr-Abl tyrosine kinase fail to induce apoptosis in T315I cells (**Fig. 5b**). Therefore, it is quite significant that ON044580 induced apoptosis in T315I mutant cells. Similar results were obtained with the E255K IM-resistant mutant of Bcr-Abl (not shown).

ON044580 induces apoptosis in primary cells from CML patients. After examination in IM-sensitive and IM-resistant Bcr-Abl+ cell lines, we tested the ability of ON044580 to kill cells from blast crisis CML patients, which are largely resistant to many drugs. As can be seen in **Figures 5 d-f** and Supplement Figures S4 a-c, white blood cells from the peripheral blood of blast crisis CML patients are quite resistant to IM (5 and 10 μM) but are very sensitive to ON044580. Most interestingly, primary CML cells are very sensitive to low doses (1-2 and 0.5 μM) of ON044580. We observed that blast crisis patient cells, some of which are resistant to IM, are induced to undergo apoptosis by ON044580 with values ranging from 70% to 90% (**Figs. 5 d-f** and Suppl. Fig. S3 c-e).

ON044580 strongly inhibited colony formation at low doses in IM-sensitive and IM-resistant Bcr-Abl+ cells. Anchorage-independent growth is a cell culture surrogate for tumor behavior in mice. We assessed the ability of ON044580 to inhibit colony formation in soft agar cultures. Cells were seeded into soft agar culture medium at the single cell level. Cultures were allowed to incubate for two weeks in the presence of different doses of ON044580. Colonies were stained, photographed, and counted to assess the remaining colony number after the drug treatment. Cells that were both IM sensitive and IM resistant were tested (**Figs. 6 a-d**). In general, colony formation was completely inhibited at 0.5 μM ON044580. Importantly, IM-resistant forms of Bcr-Abl+ cells were also inhibited at similar concentrations (**Figs. 6 c and d**). The results showed that ON044580 severely inhibited colony formation at levels between 0.1 and 0.5 μM (right panels of **Figs. 6 a-d**). These results suggest that oncogenic ability of IM-sensitive and IM-resistant Bcr-Abl+ cells are inhibited by ON044580 at lower concentrations compared to the concentrations required for apoptosis and MTT assays.

Discussion. In this article, we investigated the mode of action and functional properties of a new non-ATP competitive kinase inhibitor, ON044580, in Bcr-Abl+ mouse hematopoietic cell lines, IM-resistant cell lines, and cells from blast crisis CML patients. Our studies (see **Fig. 1**) and those of Jatiani *et al*.[42] indicate that ON044580 is a dual-kinase inhibitor that inhibited both Bcr-Abl and Jak2 kinases.

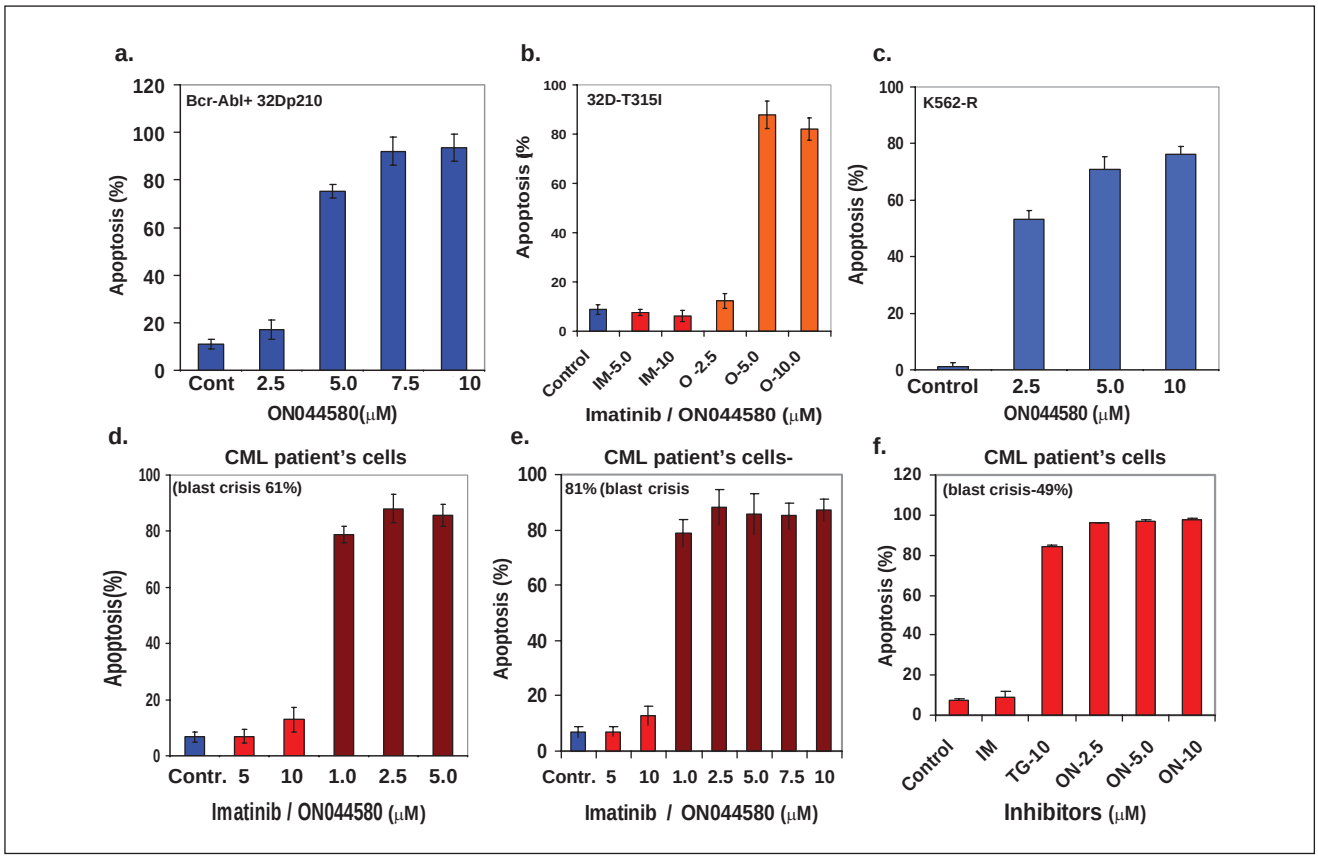

Figure 5. ON044580 induced apoptosis in Bcr-Abl+ imatinib (IM)–sensitive and IM-resistant cell lines and cells from blast crisis chronic myelogenous leukemia (CML) patients. ON044580 induced apoptosis in Bcr-Abl+ cell lines incubated with ON044580 for 48 hours as measured by annexin V/PI flow cytometry. (**a**) IM-sensitive Bcr-Abl+ 32D cells. (**b**) IM-resistant T315I Bcr-Abl+ 32D cells. (**c**) IM-resistant K562-R cells. (**d-f**) IM-resistant blast crisis CML cells.

Importantly, ON044580 induced apoptosis in IM-sensitive and IM-resistant cells and cells from the late stage of CML patients (**Figs. 5 a-e**). Our findings further showed that ON044580 induced rapid disappearance of Bcr-Abl protein from the detergent-soluble fraction of leukemic cells (**Fig. 2a** and Suppl. Fig. S1a), which affects downstream signaling of Bcr-Abl (**Figs. 2 a and b**) and disrupts the Bcr-Abl/Jak2/HSP90 network complex (**Figs. 4 a-c**). The rapid disappearance of Bcr-Abl from Bcr-Abl+ cells caused by ON044580 makes it a novel compound with potential for clinical application in CML. The possible mechanism of a rapid decrease of Bcr-Abl protein by ON044580 is not yet established, but preliminary experiments with a potent Jak2 inhibitor suggest that Jak2 inhibition only is sufficient for rapid disappearance of Bcr-Abl from the detergent-soluble fraction of cell. Since proteosomal inhibitors (MG132 and lactocyteine) failed to protect the rapid disappearance of Bcr-Abl from the detergent-soluble fraction within 2 to 4 hours (data not shown), we predict that upon inhibition with ON044580, Bcr-Abl and Jak2 dissociate from the network complex, and Bcr-Abl rapidly migrates to a detergent-insoluble compartment of the cell. How ON044580 treatment may accomplish this is under study in our laboratory. Nevertheless, upon dissociation of Bcr-Abl and Jak2 from the network complex, oncogenic signaling would be greatly reduced, and the leukemogenic properties of CML cells would similarly be greatly reduced.

The Bcr-Abl/Jak2 dual-kinase inhibitory effects of ON044580 are a critical aspect of this compound. Thus, unlike IM, where resistant mutations arise in BCR-ABL, ON044580 has the capacity to also inhibit the Jak2 kinase, which induces apoptosis in IM-resistant Bcr-Abl mutant cells, including those expressing the gatekeeper mutant T315I (**Fig. 5**). In addition, signals produced from both Bcr-Abl and Jak2 in IM-sensitive cells will be downregulated (**Figs. 2 a and b**) by treatment with ON044580.

In our previous study, we reported that Bcr-Abl is associated with several signaling proteins and forms a signaling network that includes Jak2, Gab2, Akt, and GSH3β.[31] In these studies, we showed in co-immunoprecipitation experiments that Bcr-Abl was associated with various members of its downstream signaling targets. For example, immunoprecipitation of Bcr-Abl+ cells with anti-Jak2 detected Jak2, Akt, GSK-3β, and Bcr-Abl. In addition, immunoprecipitation with antibody against GSK-3β co-precipitated Bcr-Abl, and immunoprecipitation with an Akt antibody

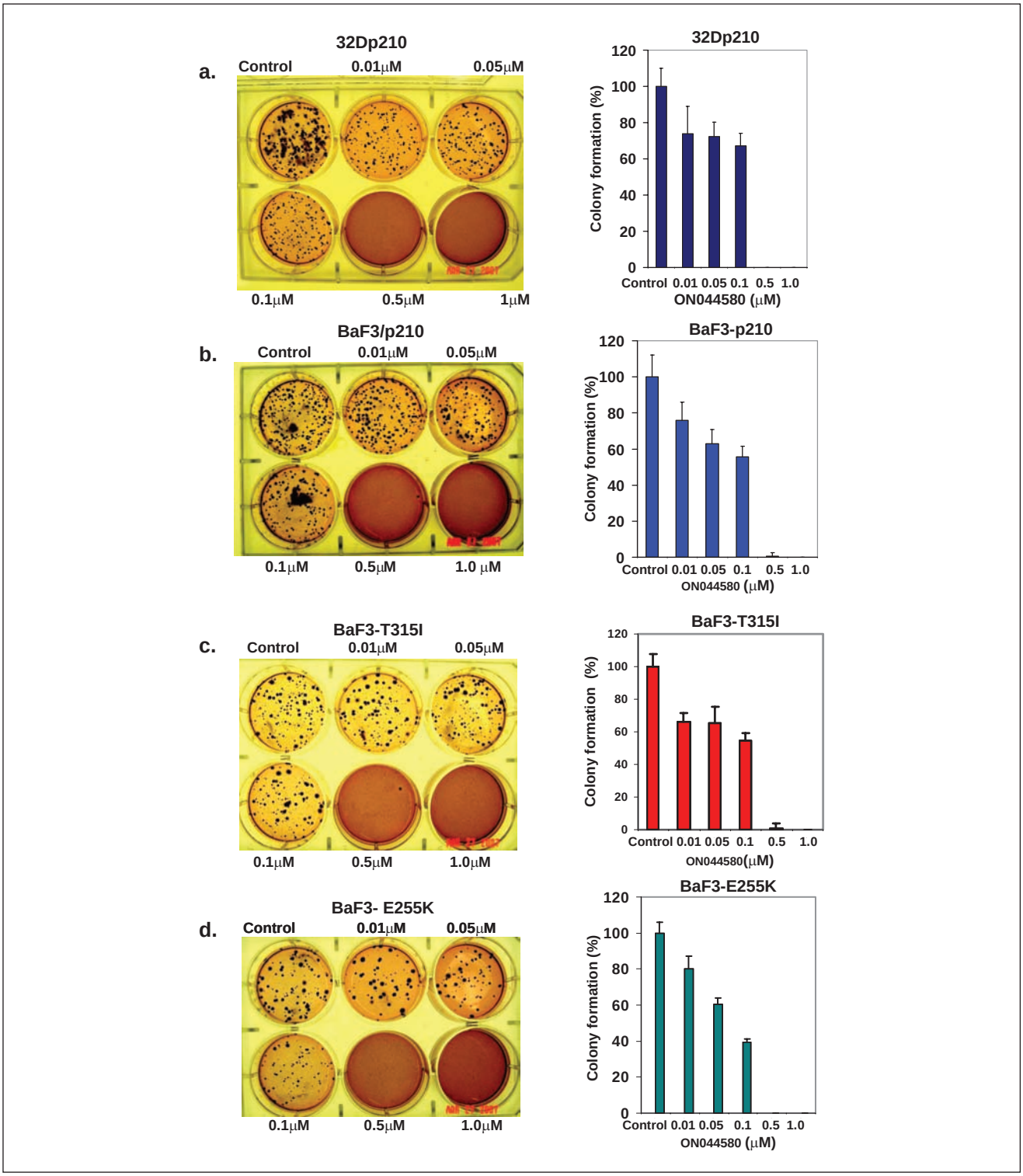

Figure 6. ON044580 reduced soft agar colony formation of imatinib (IM)–sensitive and IM-resistant Bcr-Abl+ cells in a dose-dependent manner. The experiments were carried out in duplicate plates, and the mean counts of colonies in percentages are graphically presented in the right panels. (**a**) and (**b**) represent the reduction of colony formation of IM-sensitive Bcr-Abl+ 32D and BaF3, respectively. (**c**) IM-resistant BaF3-T315I cells. (**d**) IM-resistant Bcr-Abl+ BaF3-E255K cells.

also co-precipitated Bcr-Abl. Normal serum controls established the specificity of these co-immunoprecipitation experiments. We concluded that Bcr-Abl and members of the signaling network described by Samanta *et al.*[29] [AQ: 2]were present in a network complex. The gel filtration experiments in **Figure 4** and Supplement Figure S1 support this conclusion and indicate that the network is quite large in terms of molecular size, possibly more than 6 million Da.

It is known that HSP90 is a therapeutic target molecule for solid tumor cancers and CML.[14,54] It is also reported that the critical signaling molecules, such as Bcr-Abl, Jak2, Akt, pErk, and STAT3, are physically associated with HSP90,[14,47,55,56] which plays a role in their conformational maturation and functional performance and also provides protection from proteases.[47,56,57] Any disturbance of HSP90 synthesis will eventually lead to proteolytic degradation of the client proteins.[58-60] Our studies in Bcr-Abl+ cells indicate that ON044580 treatment, because of its ability to inhibit both Jak2 and Bcr-Abl kinases (**Figs. 1 and 4d**), led to inhibition of STAT3, and since STAT3 appears to control HSP90α transcription, this eventually leads to a decrease in HSP90α transcription and decreased HSP90α protein levels (**Figs. 3 c-e**). Whether STAT3 is the direct cause of STAT3 transcription is not known at this point, but further experiments are planned to clarify the pathway from STAT3 to HSP90. Importantly, we noticed that the promoter of HSP90α contains binding sites for STAT3 (at -125 bases; our observation) and also for NF-κB.[61] Jak2 inhibition also leads to downregulation of NF-κB.[31] We note also that leukemia cells express predominantly the HSP90α form.[41,62] However, the reduction of HSP90α protein levels follows the initial inhibitory event of ON044580, which is the decrease in Bcr-Abl levels within 2 to 3 hours of ON044580 treatment. Our findings also showed that Bcr-Abl, Jak2, and HSP90α exist in a high molecular weight network structure (estimated to be about 6 million in size) (**Fig. 4a**) (a similar structure was seen in K562 cells; Suppl. Fig. S1d) that houses a number of other signaling proteins, including Akt, Erk, GSK3β, and STAT3 (**Fig. 4a**). Treatment of Bcr-Abl+ 32D cells with ON044580 for 3 hours caused destruction of this large network structure (**Fig. 4b**). It is of interest that IM treatment of Bcr-Abl+ 32D cells had little effect on the Bcr-Abl/Jak2/HSP90 network complex during a 6-hour treatment (Suppl. Fig. S1c). Thus, these results suggest that Jak2 inhibition by ON044580 is the critical inhibitory activity caused by ON044580 that leads to rapid destruction of the Bcr-Abl/Jak2/HSP90 network complex. However, ON044580, by also decreasing levels of Bcr-Abl, might contribute to the rapid destabilization of the network complex. It is likely that this destruction of the Bcr-Abl/Jak2/HSP90 network structure will induce apoptosis and death of the leukemia cell.

Our mechanistic studies (**Fig. 4c**) regarding ON044580 treatment of Bcr-Abl+ cells is likely to explain why it induces apoptosis in the IM-sensitive and IM-resistant Bcr-Abl+ cells such as Bcr-Abl mutant T315I and E255 cells, as well as the IM-resistant CML cell line (K562-R) and drug-resistant CML blast crisis patient cells (**Figs. 5 a-e**). We have proposed a model that describes the roles of Bcr-Abl and Jak2 in signaling pathways that operate in CML cells and the effects of ON044580 (**Fig. 4d**). In this model, our findings suggest that both Bcr-Abl and Jak2 have important roles in activating STAT3, and importantly, ON044580 treatment of leukemia cells will downregulate both Bcr-Abl and Jak2 kinase-induced effects. The reduction of STAT3 will lead to reduced transcripts of HSP90α, which in turn will reduce HSP90α protein levels. Although these inhibitory effects on HSP90 protein expression probably play an important role in the final apoptotic consequences of ON044580 treatment, we emphasize that the rapid reduction of Bcr-Abl protein levels and the inhibition of the Jak2 kinase are the primary events that initiate the destruction of the Bcr-Abl/Jak2/HSP90 signaling complex. On the basis of our findings, we propose that targeting Jak2/Bcr-Abl/HSP90 is an excellent strategy for inducing apoptosis in drug-resistant CML cells of all types, including advanced stages of CML-like blast crisis, and thus ON044580 may have potential for treatment of many forms of drug-resistant CML.

Materials and Methods. Cell lines and tissue culture. In all our major experiments, we used IM-sensitive Bcr-Abl transformed mouse cell lines 32Dp210 and BaF3p210 cell lines. For examination in IM resistance, we used Abl point mutant cell lines 32D/BaF3-T315I and BaF3-E255K and Lyn upregulated human CML line K562-R. All the cell lines were cultured in RPMI 1640 supplemented with penicillin and streptomycin and 10% fetal bovine serum. K562-R cells were cultured in 5 μM imatinib.

Methods for Western blot and immunoprecipitation. The cells were washed with cold phosphate-buffered saline (PBS) followed by washing with low salt buffer (pH 7.4), 25 mM NaCl, and 1 mM DTT. The cells were lysed in lysis buffer (1% NP-40 in 20 mM HEPES [pH 7.4]), 150 mM NaCl, 1 mM EDTA, and protease inhibitors 1 mM AEBSF/PMSF, 2 μg/mL aprotinin, 2 μg/mL leupeptin, 50 mM NaF, and 500 μg/mL benzamidine.

For immunoprecipitation, 1 μg primary antibody was added to 400 μg cell extract and mixed with 400 μL lysis buffer for 90 minutes at 4°C. After that time period, 35 μL 50% suspension of protein A/G agarose was added, and the rotation was continued for another 90 minutes. After that incubation, the agarose beads were washed twice with lysis buffer and twice with low salt buffer. Then the beads were

treated with 30 µ [AQ: 3]2x sample buffer and boiled for 5 minutes. For direct Western blot, 50 µg cell lysate was mixed with an equal amount of 2x sample buffer and boiled for 5 minutes, loaded into wells, and separated in 4% to 20% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gradient gel using protein markers. The proteins were transferred onto a PVDF membrane and blocked by either 3% bovine serum albumin (BSA; for detection of pTyr) or 5% nonfat dry milk diluted in TBST[AQ: 4]. Primary antibodies were diluted in TBST (at room temperature; monoclonal) or in milk (polyclonal) and incubated for 1 hour. After washing 3 times with TBST, the appropriate secondary antibodies conjugated to horseradish peroxidase were incubated at room temperature for 1 hour. After washing, the membrane was developed using ECL/ECL plus reagents (Amersham Biotech Company, Piscataway, NJ). β-Actin (Sigma, St. Louis, MO) was used as a loading control.

Electrophoretic mobility shift assay (EMSA). EMSA was carried out following the method of Samanta *et al*.[51] After treatment with the appropriate inhibitors, cytosolic and nuclear extracts were prepared. The nuclear extract was used either immediately after preparation or stored at –70°C. For each EMSA, 6 to 8 µg of nuclear extract protein was incubated with poly (dI:dC), 10% NP-40, and ^{32}P-labeled consensus oligonucleotide was annealed to make it double stranded. From ON044580-treated cells, nuclear extracts were prepared and incubated with gamma radiolabeled ^{32}P STAT3 consensus DNA binding site prepared as above at 37°C. Then the whole contents were separated in a 6.6% polyacrylamide gel.

(5′-AGCTTCATTTCCCGTAAATCCCTA-3′ consensus STAT3 binding site[AQ: 5]

5′-TAGGGATTTACGGGAAATGAAGCT-3′ complementary strand. Autoradiography of the dried gel provided the results of the experiments. Since there were inhibitory effects of ON044580 for this binding, the amount of STAT3 protein bound to the radioactive consensus sequence was reduced in a dose-dependent manner in ON044580-treated leukemia cells. DNA binding specificity was determined by competition with unlabeled wild-type or mutated oligonucleotides. The mixture was incubated at 37°C for 15 minutes, and the reaction was terminated by addition of 4 µL 2x DNA loading dye and analyzed in a 6.6% polyacrylamide gel. After autoradiography, STAT3-DNA complexes were detected using a PhosphoImager (Molecular Dynamics, Sunnyvale, CA).

Reverse transcriptase–polymerase chain reaction (RT-PCR). From Bcr-Abl+ CML cells, total cellular RNA was prepared by the TRIzol method following the manufacturer's protocol (GIBCO, Carlsbad, CA). RT was carried out using 500 ng total RNA in a first-strand cDNA synthesis reaction with superscript reverse transcriptase as recommended by the manufacturer (Invitrogen, Carlsbad, CA). The sequence for HSP90α is as follows: forward 5′-GCGGCAAAGACAAGAAAAAG-3′ and reverse 5′-CAAGTGGTCCTCCCAGTCAT-3′. GABDH was used as an internal control. The sequences for GABDH are as follows: forward 5′-CATGATGGCTTCCTTAGATGCCCAG-3′ and reverse 5′-CCGTGTGTCATGTAGTGAACCTTTAAG-3′, and an expected product size was 316 bp. PCR reaction was carried out by adding 1 µL RT product into a 25-µL volume reaction mixture containing 1x buffer and 200 µM of each dNTPs, oligonucleotide primer, and 0.2 U AmpliTaq polymerase. For amplification of DNA, cDNA was denatured at 94°C for 1 minute and subjected to primer annealing at 60°C for 1 minute, followed by DNA extension at 72°C for 1 minute for 30 cycles in a thermal cycler (Applied Biosystems, Foster City, CA). Amplified products were analyzed by DNA gel electrophoresis in 1% agarose and visualized by the Alpha Imager 3400 (Alpha Innotec, Santa Clara, CA).

Gel filtration column chromatography. The protein separation column selected for this purpose was 50 cm length × 0.7 cm diameter (Ecoho [AQ: 6]column, Bio-Rad, Hercules, CA), and the column material selected for this purpose was Superose 6 prep grade gel filtration (Amersham-Biosciences, part of GE Healthcare, Piscataway, NJ), which can achieve high-resolution separations across an exceptionally broad molecular weight range. The bed volume of the column was 17.5 mL, and the void volume was 6.0 mL. The composition of the elution buffer was 30 mM HEPES (pH 7.4) containing 150 mM NaCl, 10% glycerol, and 0.5% NP-40. Elution rate was 4.56 mL/h. The column was standardized with the mixture of protein markers containing keyhole limpet hemocyanin (KLH; MW 8.5 million Da), blue dextran (2 million Da), β-amylase (200 kDa), BSA (66 kDa), and cytochrome C (12.4 kDa) (Suppl. Fig. S1e). The fractions were collected in 500-µL microfuge tubes in a fraction collector. The elution of the markers detected in 280 nm was plotted against the log of the molecular weight of the standard proteins. From this standard elution pattern, the size of the Bcr-Abl protein network was estimated to be between 2 and 6 million. In a preequilibrated column, we loaded 150 µL (~3 mg) protein onto the column, and the proteins were separated into 40 tubes, each containing approximately 500 µL column eluant. All the column fractions were stored at –20°C. From each column fraction, 25 µL was taken for analysis by Western blotting with various antibodies. Elution analysis of fractions 8 to 24 were performed in 3 premade gradient SDS-PAGE gels (4%-20%). The proteins were transferred to PVDF membranes. The membranes were blocked with BSA for detection of pTyr, and for detection of Bcr-Abl and other proteins, blocking

was carried out with 5% milk, and Western blot was carried out as described earlier.

Preparation of cell free lysates. A detergent-extracted cell-free lysate was prepared from the Bcr-Abl-positive cell lines 32Dp210 or K562. Lysis buffer used for this cell-free extract was 1% NP-40 in 20 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EDTA, and protease inhibitors 1 mM PMSF, 2 µg/mL aprotinin, 2 µg/mL leupeptin, 50 mM NaF, 1 mM sodium venadate, and 500 µg/mL benzamidine. The cells were usually incubated with the lysis buffer for 30 minutes in ice with mild mixing with a vortex mixer. The cell lysate was centrifuged at 4°C for 10 minutes. The supernatant was removed and kept in a separate tube, and protein concentration was measured by a Bio-Rad reagent (Bio-Rad) using BSA as a standard (Pierce, Rockford, IL).

Kinase assays for Jak2 and Bcr-Abl. Jak2 kinase assay was carried out following the methods of Xie *et al*.[9] and Sandberg *et al*.[44] Cell-free lysate of 32Dp210 was prepared by treating the cells with lysis buffer containing 20 mM Tris-HCl, 100 mM NaCl, 1% NP-40, and protease inhibitors. Detergent-extracted lysate was aliquoted to each Eppendorf tube containing 500 µg protein/500 µL lysis buffer and pre-screened with protein G agarose conjugate. The supernatant was incubated with 50 µL Abl antibody (P6D) for 1 hour followed by 30 µL protein G agarose beads for another 1 hour for co-immunoprecipitation for Bcr-Abl/Jak2. After washing with lysis buffer followed by washing with kinase buffer (containing 50 mM HEPES [pH 7.6], 100 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$), the agarose beads were suspended in kinase buffer. Different amounts of ON044580 were added and incubated for 10 minutes, and the reaction was initiated by addition of 2.5 mM ATP. The reaction was continued for 30 minutes at room temperature, and the reaction was stopped by addition of 2x sample buffer. The signals for kinase reaction were detected in Western blotting with pJak2 Tyr1007/1008 antibody.

Autophosphorylation of Bcr-Abl kinase was performed following the method of Bartholomeusz *et al*.[63] by immunoprecipitating Bcr-Abl with P6D antibody. Immunoprecipitates were incubated with various amounts of ON044580. Kinase reactions were initiated with addition of cold ATP, Mg++, and 1 mM dithiothreitol (DTT) at 30°C for 30 minutes. Kinase activity was detected by Western blotting with anti-pTyr antibody (4G10).

In vitro kinase assay for Jak2 and Abl kinases with recombinant proteins. Recombinant Jak2 kinase and Abl kinase were assayed *in vitro* following modified methods.

Recombinant Jak2 kinase assay: Recombinant Jak2 (JH1 and JH2 domains) was preincubated for 10 minutes with different amounts of ON044580 in an incubation mixture as described above for the cold kinase assay. After 10 minutes, the reaction was initiated with cold ATP (5 µM), 10 µCi/assay 32P gamma ATP, and 5 µM Jak2 peptide substrate (994-Asp-Phen-Gly-Leu-Thr-Lys-Val-Leu-Pro-Glu-Lys-Glu-Tyr^{1007}-Tyr^{1008}-Lys-Val-Lys-Glu-Pro-Gly-Glu-Ser-Pro-Iso-Phen-1019) as originally described,[9] and the incubation was continued for 10 minutes at 30°C. The reaction tubes were kept in ice, 250 µg BSA was added, and finally an equal volume of trichloroacetic acid (TCA, 40%) was added and incubated for 30 minutes. After centrifugation, the pellet was washed with 20% TCA twice, and the pellet was used for counting 32P gamma ATP incorporated in the pellet in a gamma counter.

Recombinant Abl kinase assay: For Abl kinase assay, 20 ng recombinant Abl kinase was mixed with the same kinase buffer used for the Jak2 kinase assay. A different amount of ON044580 was added to the incubation mixture and preincubated for 10 minutes. The reaction was initiated by adding the substrate for Abl kinase (5 µM Abltide), 5 µM unlabeled ATP, and radiolabeled ATP (10 µCi). The reaction was stopped by addition 5 µL of 3% phosphoric acid from the mixture, and 10 µL of the mixture was dropped on Whatman filter paper (2.5-cm disk, grade p81) in triplicate. The disks were washed in 180 mM phosphoric acid 2 times with shaking and finally washed with 100% methanol. Then the count present in each disk was measured by a scintillation counter.

Apoptosis assay: Apoptosis measurement was carried out following the manufacturer's protocol using the Annexin V/PI method in a flow cytometer (BD Pharmingen, San Diego, CA). The cells were incubated with either IM or ON044580 in different doses for 48 hours. Then the cells were processed for measurement of apoptosis following the manufacturer's protocol (BD Pharmingen).

Colony formation assay. Colony formation assay was carried out following the method described.[28]

CML patient cells. Cells from CML donors were obtained under an approved institutional protocol. CML cells were separated by centrifugation through Histopaque 1077 (Sigma), and the cells were suspended in RPMI medium with 10% fetal bovine serum for 48 hours in the presence of 5 and 10 µM imatinib and 2.5 to 10 µM ON044580 and incubated for 48 hours. Cells were processed for flow cytometry with Annexin V and propidium iodide staining to measure late-stage apoptosis.

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Declaration of Conflicting Interests

Dr. E.P. Reddy is a stockholder, board member, and consultant for Onconova Therapeutics, Inc. Drs. Samanta, Chakraborty, Wang, Schlette, and Arlinghaus declare no conflicts of interest.

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