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PRINCIPAL INVESTIGATOR: E. Premkumar Reddy

CONTRACTING ORGANIZATION: Temple University
Philadelphia PA 19140

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E. Premkumar Reddy

E-Mail: reddy@temple.edu

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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 chemical modification of the compound to facilitate the oral bio-availability of the compound. This resulted in the derivation of two compounds, ONO45260 and ON044690 which retained the BCR-ABL inhibitory activity of the parent compound. They also retained the ability to inhibit kinase activities 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.

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BCR-ABL, Imatinib Resistance

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Introduction

Clinical studies conducted during the past few years have shown that a majority of CML patients harboring the Philadelphia chromosome which encodes the BCR-ABL oncogene respond well to imatinib, an inhibitor of the BCR-ABL tyrosine kinase (Deininger et al, 2005). However, a significant proportion of these patients chronically treated with imatinib develop resistance due to acquisition of mutations in the kinase domain of BCR-ABL (Deininger et al, 2005). 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 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, (Shah et al, 2004) and AMN107 or Nilotinib (Weisberg et al, 2005) 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 Deininger et al, 2005), there is an urgency to develop alternative compounds that are capable of inhibiting this particular (as well as other) amino acid substitution.

BODY

Work Accomplished by Dr. Reddy's Group at the Fels Institute

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. In the

previous year's progress report, we had described the identification of three 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. All of the three compounds were found to be non-ATP competitive and readily induced the down-regulation of BCR-ABL auto-phosphorylation and STAT-5 phosphorylation. Of these, ON044580 was found to be the most active and hence we proceeded to further characterize the pharmacological properties of this compound and for further clinical development.

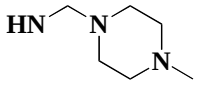
Oral Bioavailability Studies.

In order to determine the oral bioavailability of ON044580.Na (sodium salt of ON044580), we treated mice with ON044580.Na by oral gavage and tested plasma to determine the amount of ON044580.Na at various time points. Specifically, ON 04580.Na was formulated in sterile dd$H_2$0 at 1 mg/ml and administered to the mice by taking up the appropriate volume of solution using a syringe equipped with a bulb tipped gavage feeding needle. At various times after injection (30, 80, 120, 240 minutes), mice (N=2) were euthanized and whole blood was collected and placed into heparin containing tubes. The tubes were centrifuged for 10 minutes and the plasma was removed and frozen at -80ºC. Compound levels were determined by HPLC/MS/MS procedures. Briefly, to each 50 ul of sample solution, an internal standard was added, the samples were vortexed followed by sonication for 30 minutes. The samples were treated with acetonitrile and analyzed by injection into an Agilent 1100 and Sciex API 3000 LC/MS/MS machine. Analysis of the samples showed that there were no detectable levels of ON 044580.Na in the plasma at any of the time points analyzed. This data showed that ON044580 is not orally bio-available and chemical modification of ON044580 is required in order to obtain an orally bioavailable compound.

Chemical Modification of ON044580 for Oral Bioavailability

Since 0N044580 is poorly bioavailable in plasma after orally administering the compound, we decided to incorporate several side chains in the molecules without

affecting the BCR-ABL inhibitory activity of the compound. To accomplish this goal, we decided to convert the carboxylic acid group present in the *para*-position of the benzoyl ring. Literature survey revealed that the addition of chemical groups such as piperazine ethanol or N-ethylaminomorpholine or pyridinopiperazine have a remarkable effect on the oral bioavaiability of many compounds. One of the interesting properties of these prodrugs is the observation that following the uptake of the compounds by tumor cells, the ether or ester or amide linkages used to attach these side chains are cleaved by the cellular enzymes resulting in the release of the original drug into the cytoplasm of the cell. Some of the orally bioavailable kinase inhibitors that are currently used in cancer therapy have the following groups, which have been found to facilitate profoundly their oral bioavailability.



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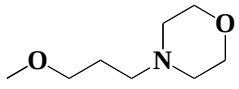

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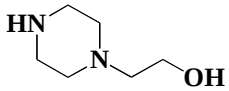

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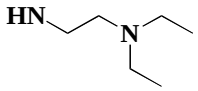
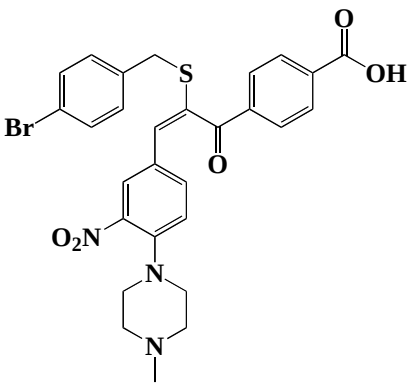


Sunitinib

Our structure activity relationship (SAR) analysis of ON044580 family of compounds showed that the critical substituent is the 3-NO_2 group on the phenyl ring. Modification of either the position or the group resulted in the loss of activity. On the other hand, we found that the replacement or modification of carboxylic acid group in the *para* position of the benzoyl ring did not affect the biological activity of the molecule. Hence, we selected this position for modification to enhance bioavailability and water solubility of the compound. Taking this into consideration, we have designed and synthesized the following molecules and evaluated them for their biological activity. The synthesis of these compounds was carried out by Onconova Therapeutics Inc who is one of the

participants in this grant. The structures of the compounds synthesized along with those of ON044580 and ON045000 is shown below.



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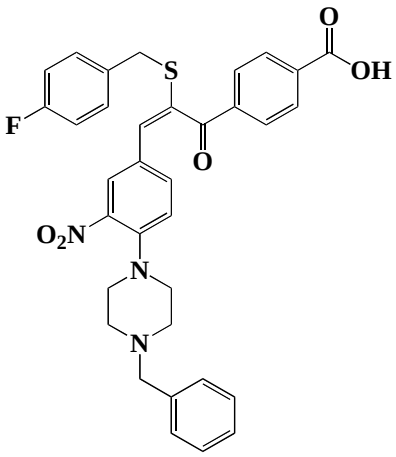

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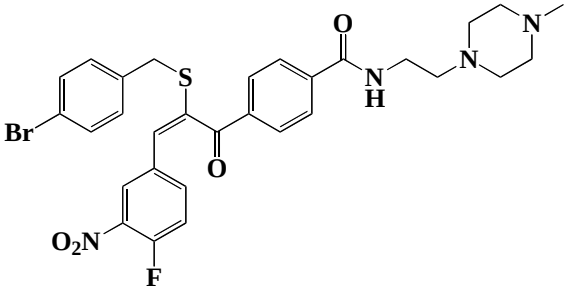

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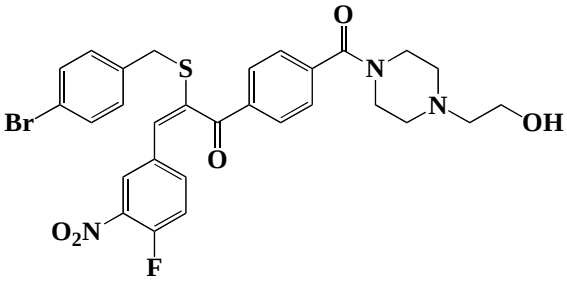

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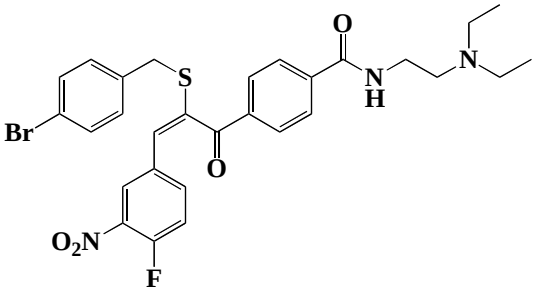

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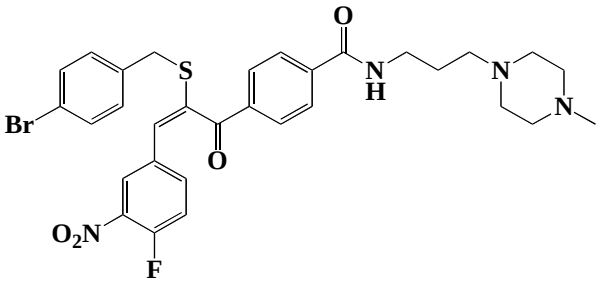

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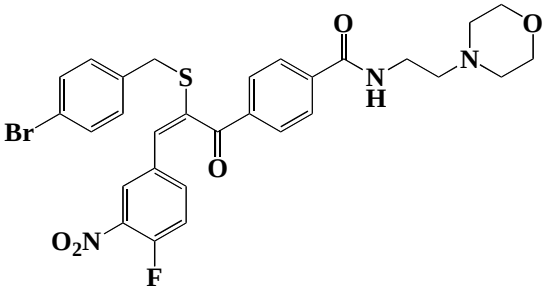

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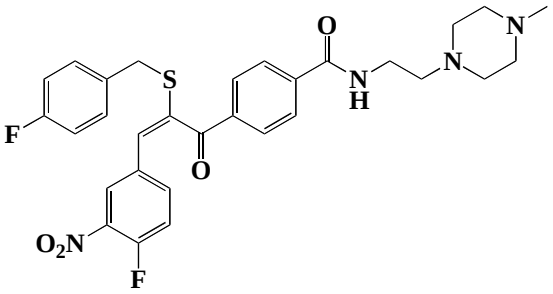

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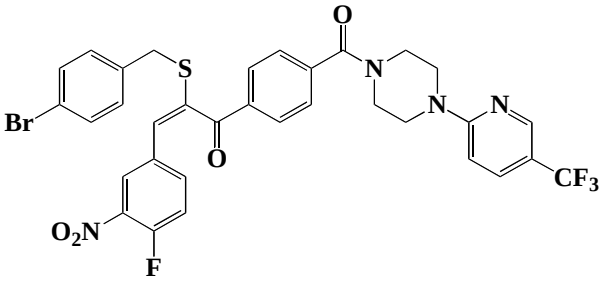

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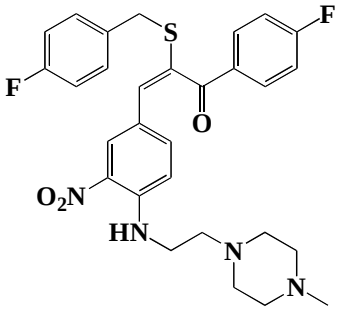

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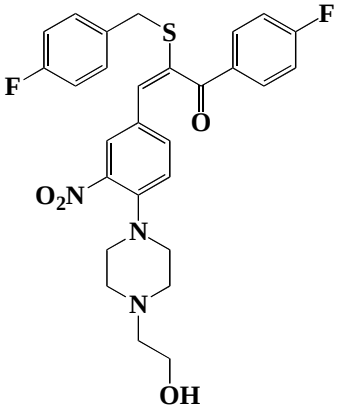

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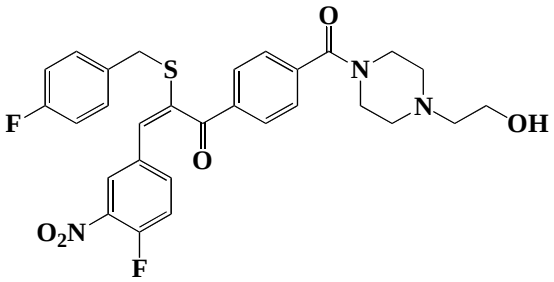

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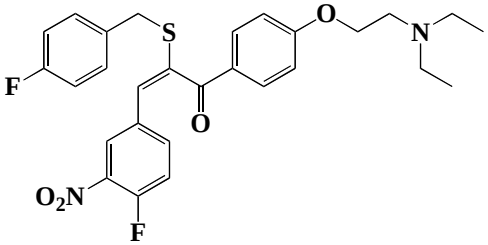

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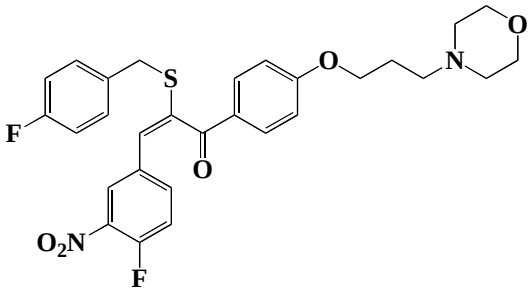

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Inhibition of *in vivo* kinase activity of BCR-ABL

To evaluate the *in vivo* inhibition of BCR-ABL activity by the compounds under study, we examined the autophosphorylation status of BCR-ABL protein as well as the phosphorylation status of STAT-5 and CrkL in cells treated with these 13 new α-benzyl styryl benzyl sulfides. For these studies, we used K562 cells (Ph+ human cell line from CML patient) that express the BCR-ABL protein. We treated these cells with increasing concentrations of the compounds for 2 hrs followed by western blot analysis of cell lysates to determine the ability of these compounds to inhibit the phosphorylation status of BCR-ABL, STAT-5 and CrkL in comparison to ON044580 and ON045000 (two of the three compounds we reported last year). Primary screening revealed that of the 13 new compounds, both ON044690 and ON045260 showed BCR-ABL inhibition activity comparable to ON044580 and ON045000. Data presented in Figure 1 shows that ON044690 inhibits the autophosphorylation of BCR-ABL protein expressed in K562 cells. Data presented in this figure also shows that ON044690 inhibits the phosphorylation of STAT-5 in these cells. The data for ON045260 is presented in Figure 2. However, just like ON044580 and ON045000, both ON044690 and ON045260 fail to inhibit CrkL phosphorylation in K562 cells. Imatinib (Gleevec) was used as a positive control in all these experiments.

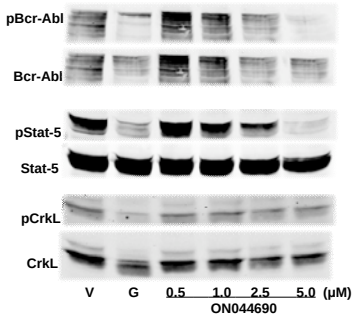

Figure 1. ON 044690 inhibition of Bcr-Abl pathway in K562 Cells. K562 cells were treated with the indicated concentrations of ON 044690, Vehicle (DMSO; V) or Gleevec (G; 10 μM) for 2 hours. The cells were harvested and total cellular lysates were prepared. Equal amounts of protein was resolved by 10% SDS-PAGE and analyzed for indicated proteins by infrared labeled secondary antibodies and scanning with Odyssey scanner (LiCor Technology).

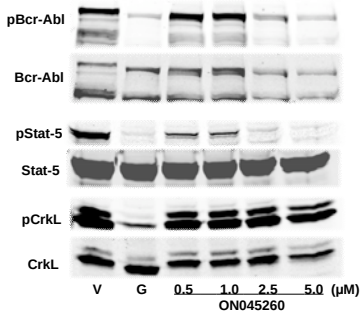

Figure 2. ON 045260 inhibition of Bcr-Abl pathway in K562 Cells. K562 cells were treated with the indicated concentrations of ON 045260, Vehicle (DMSO; V) or Gleevec (G; 10 μM) for 2 hours. The cells were harvested and total cellular lysates were prepared. Equal amounts of protein was resolved by 10% SDS-PAGE and analyzed for indicated proteins by infrared labeled secondary antibodies and scanning with Odyssey scanner (LiCor Technology).

Following the establishment of the *in vivo* activity of ON044690 and ON045260 towards WT BCR-ABL kinase, we next examined its ability to inhibit autophosphorylation and STAT-5 phosphorylation of T315I-BCR-ABL kinase. For these studies we used the 32D/T315I-BCR-ABL cell line which expresses high levels of the T315I-BCR-ABL kinase and was found to be resistant to imatinib. As was done with K562 cells, we

treated 32D/T315I-BCR-ABL cells with increasing concentrations of the compounds for 2 hours followed by western blot analysis of cell lysates to determine the ability of these compounds to inhibit the phosphorylation of BCR-ABL and STAT-5. The results of these studies are shown in Figure 3. These studies show that both ON044690 and ON045260 are very effective in inhibiting autophosphorylation and STAT-5 phsophorylation in the 32D/T315I-BCR-ABL cells while imatinib fails to do so.

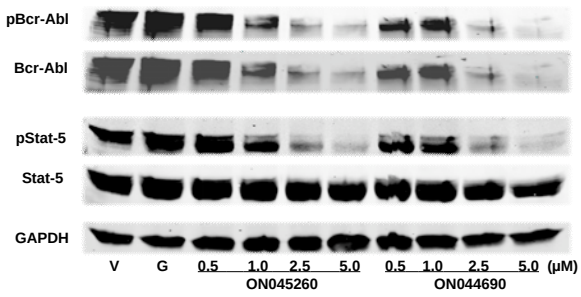

Figure 3. Inhibition of Bcr-Abl pathway by ON045260 and ON044690 in 32D-T315I Cells. 32D-Cl3 cells transformed with Bcr-Abl having the T315I mutation were treated with the indicated concentrations of ON 045260 or ON 044690, Vehicle (V; DMSO) or Gleevec (G; 10 μM) for 2 hours. The cells were harvested and total cellular lysates were prepared. Equal amounts of protein was resolved by 10% SDS-PAGE and analyzed for indicated proteins by infrared labeled secondary antibodies and scanning with Odyssey scanner (LiCor Technology).

These studies suggest that ON044690 and ON045260 do not bind to the ATP-binding domain of BCR-ABL kinase, but act via binding to the substrate-binding domain (which is specific to STAT-5 but not to Crk-L) or to an allosteric domain of the BCR-ABL kinase that results in the impairment of its ability to phosphorylate itself and STAT-5.

In vitro BCR-ABL kinase inhibitory activity of ON compounds

Having determined that ON044690 and ON045260 inhibit wild type (WT) and T315I BCR-ABL activity *in vivo*, we next examined whether these compounds inhibit their *in vitro* kinase activities. We assayed the inhibitory activity of these compounds on mammalian BCR-ABL proteins immunoprecipitated from mammalian cells. For these studies, cell lysates were prepared from K562 cells expressing the Wild Type BCR-ABL or 32D cells expressing the T315I mutant form of BCR-ABL. The BCR-ABL protein was immunoprecipitated with antibodies directed against the BCR-ABL protein. The immunoprecipitates were washed, re-suspended in kinase buffer and used for kinase assays using the GST-Abltide as a substrate. Immunoprecipitates derived from 200 μg of total protein were used for a single assay reaction and were mixed with different concentrations of the inhibitor. The kinase assays were performed as described previously by us (Gumireddy et al, 2005). Imatinib was used as a control in all of these assays.

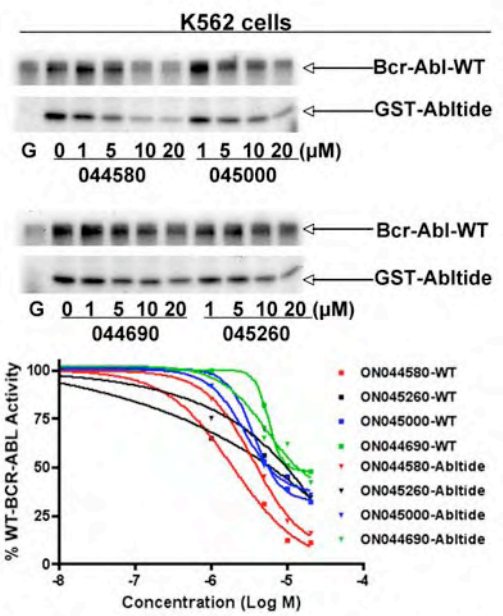

Figure 4. ON044580, ON045000, ON044690 and ON045260 inhibit the kinase activity of wild type (WT) BCR-ABL immunoprecipitated from K562 cells. Cell lysates from K562 cells were immunoprecipitated with antibodies directed against the BCR-ABL protein. Immunoprecipitates derived from 200 ug of the total cell protein were mixed with different concentrations of the indicated inhibitor and kinase assays performed using GST-Abltide as a substrate to measure substrate phosphorylation G: Gleevec (Imatinib).

Imatinib readily inhibits the kinase activity of WT BCR-ABL but fails to do so with the T315I-BCR-ABL kinase when GST-Abltide is used as a substrate. As shown in Figure 4, ON044580 potently inhibited the autophosphorylation of WT BCR-ABL and phosphorylation of GST-Abltide with an IC_{50} value of 1.9 μM. The inhibition of WT kinase autophosphorylation achieved by ON04500, ON044690 and ON045260 was modest in comparison at 5.5 μM, 10.0 μM and 6.0 μM. The value for inhibition of GST-Abltide phosphorylation by WT kinase for ON044580 was 4.0 μM and that for ON04500, ON044690 and ON045260 was also lower at 5.5 μM, 12.8 μM and 9.3 μM, respectively.

We next compared the inhibitory activity of these compounds against the imatinib resistant T315I mutant form of BCR-ABL. Figure 5 shows that when the kinase activity of the mutant kinase was assayed, ON044580 inhibited its autophosphorylation very potently with an IC_{50} value of 0.37 μM as compared to 0.98 μM for ON044690 and 1.1 μM for ON045260. All three compounds were 5-10 fold more effective in inhibiting the imatinib resistant mutant as compared to WT kinase autophosphorylation. At 5.6 μM ON045000 was found not to be selective in inhibiting the autophosphorylation of the mutant kinase. However, the phosphorylation of GST-

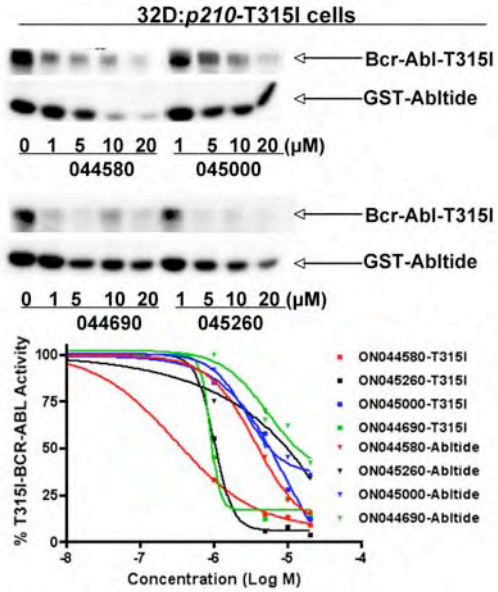

Figure 5. ON044580, ON045000, ON044690 and ON045260 inhibit the kinase activity of T315I BCR-ABL protein immunoprecipitated from 32D/T315I-BCR-ABL cells. Cell lysates from 32D/T315I-BCR-ABL cells were treated as described for K562 cells in Figure 5. The immunoprecipitates were incubated with indicated concentrations of the different inhibitors and assays for kinase activity using GST-Abltide as protein substrate in the reaction.

Abltide by T315I BCR-ABL was equally well inhibited by all four compounds with IC_{50} values in the 3.9 - 4.7 µM range.

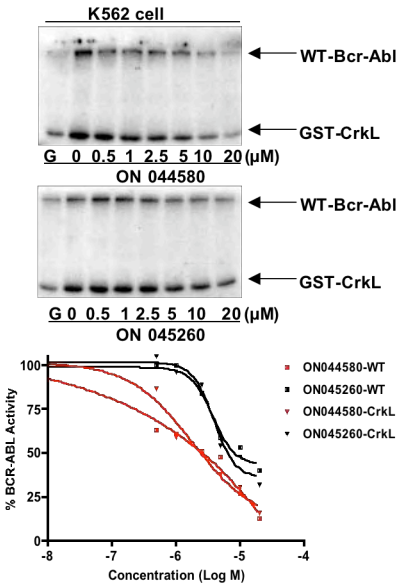

Figure 6a. ON044580 and ON045260 inhibit the phosphorylation of GST-CrkL by wild type (WT) BCR-ABL immunoprecipitated from mammalian cells. Cell lysates from K562 cells were immunoprecipitated with antibodies directed against the BCR-ABL protein. Immunoprecipitates derived from 200 µg of the total cell protein were mixed with different concentrations of the indicated inhibitor and kinase assays performed using GST-CrkL as substrate. G: Gleevec (Imatinib).

We further studied this difference in the inhibition of substrate phosphorylation by switching over to GST-CrkL instead of GST-Abltide as the protein substrate in the reaction and comparing ON044580 with ON045260.

As shown in Figure 6a ON044580 inhibited autophosphorylation of WT-BCR-ABL and phosphorylation of GST-CrkL by the WT kinase with IC_{50} values of 2.5 and 2.4 µM, respectively. ON045260 showed relatively lower inhibitory activity with IC_{50} values of 6.5 and 6.9 µM. This difference matches well with the results of Figure 4.

However, as can be seen in Figure 6b, the inhibition of T315I-BCR-ABL authophosphorylation by these compounds was not as dramatic when CrkL was used as the protein substrate. ON044580 inhibited the autophosphorylation the mutant kinase and the phosphorylation of GST-CrkL with IC_{50} values of 2.5 µM and 2.4 µM (identical to its activity against the WT kinase). Similarly the respective IC_{50} values for ON045260 were 7.9 µM and 6.3 µM were no different from those obtained for WT kinase. This could be related to the fact, that ON compounds

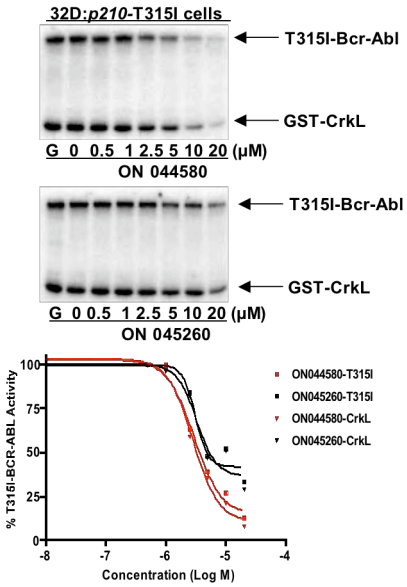

Figure 6b. ON044580 and ON045260 inhibit the phosphorylation of GST-CrkL by T315I BCR-ABL immunoprecipitated from mammalian cells. Cell lysates from 32D/T315I-BCR-ABL cells were immunoprecipitated with antibodies directed against the BCR-ABL protein. Immunoprecipitates derived from 200 µg of the total cell protein were mixed with different concentrations of the indicated inhibitor and kinase assays performed using GST-CrkL as a substrate. G: Gleevec (Imatinib).

seem to exhibit varying BCR-ABL inhibitory activities depending on the downstream substrate and hence the downstream pathway being addressed (as reported by us last year).

Imatinib inhibits only the WT form of BCR-ABL. In contrast our compounds inhibit both WT and T315I mutant forms of BCR-ABL kinase, suggesting that mutations that affect the kinase inhibitory activity of imatinib do not affect the inhibitory activity of ON044580, ON045000, ON044690 and ON045260.

In vitro tumor cell killing activity of ON compounds

We next examined the ability of our compounds to inhibit the proliferation of BCR-ABL positive myeloid leukemias. Initially, we studied the ability of these compounds to inhibit the growth of myeloid 32D cells overexpressing WT and T315I imatinib resistant forms of BCR-ABL. The results presented in Figure 7 show that both ON044580 and ON045260 were effective in killing 32D:p210 cells that express the WT form of BCR-ABL and that ON044580 was slightly more active with GI_{50} value of 700 nM while ON045260 showed a GI_{50} of 1 μM. In the case of the imatinib resistant T315I form of BCR-ABL, ON044580 showed a GI_{50} value of 800-900 nM while that for ON045260 was 2 μM.

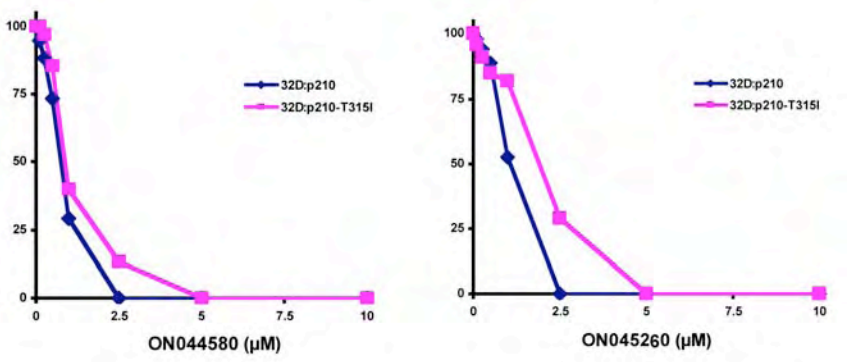

Figure 7. ON044580 and ON045260 inhibit the growth of cells expressing wild type and imatinib resistant forms of BCR-ABL. 32D:p210 and 32D:p210-T315I cells were plated at 2.5 x10^4 cells/ml/well. The cells were treated with increasing concentrations of each compound and the total number of viable cells was determined following trypan blue staining and counting using a haemocytometer 96 hours later. The date is plotted as the percent total viable cells compared to DMSO treated controls.

Next we assayed the in vitro tumor cell killing activities of ON044580 and ON045260. For this study, we used imatinib sensitive K562 cells which express WT BCR-ABL

kinase, imatinib resistant K562-R cells which overexpress Lyn and the human prostrate cancer cell line DU145. The results presented in Figure 8 show that both ON044580 and ON045260 were effective inducers of myeloid tumor cell death exhibiting GI_{50} values of 350 nM and 200 nM for K562 cells, respectively. Imatinib, in the same assay system showed a GI_{50} of 100-200 nM (data not shown). Further, both compounds were active against the imatinib resistant K562-R cell line with GI_{50} values of 150-200 nM. Imatinib in the same assay system showed a GI_{50} value greater than 25 μM. As a comparison, when we tested our compounds against human prostate cancer DU145 cells and found that ON044580 and ON045260 displayed much higher GI_{50} values of 2-2.5 μM.

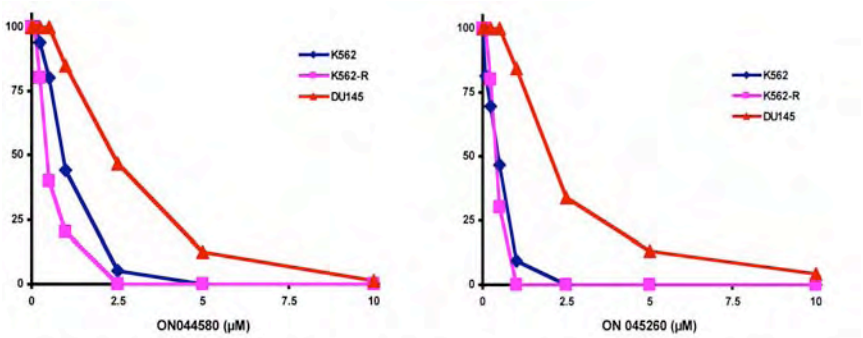

Figure 8. *In vitro* tumor killing activity of ON044580 and ON045260. Imatinib sensitive human erythroleukemia K562 (CML) cells, the Lyn-overexpressing imatinib resistant human erythroleukemia K562-R cells, and human prostate cancer DU145 cells were plated at 2.5 x10^4 cells/ml/well. The cells were treated with increasing concentrations of each compound and the total number of viable cells was determined following trypan blue staining and counting using a haemocytometer 96 hours later. The date is plotted as the percent total viable cells compared to DMSO treated controls.

JAK2 inhibitory activity of ON compounds

Last year we reported the development of ON44580 as a dual inhibitor of BCR-ABL and JAK-2 kinases. This year we report our studies with derivatives of ON044580 belonging to the α-benzyl styryl benzyl sulfide chemotype that we tested against BCR-ABL (detailed above) as equally potent inhibitors of JAK-2 kinase. We find that of the 13 new compounds tested ON045260 shows JAK-2 kinase inhibitory activity comparable to the parent molecule (ON044580) and the results for this compound are presented below.

To test the JAK2 inhibitory activity of ON compounds, we used the recombinant constitutively activated V617F mutant form of this protein protein including the catalytic (JH1) and regulatory (JH2) domains produced in insect cells (which is commercially available).

We have also improved our *in vitro* kinase assay system. In our previous system we used GST-Abltide as a substrate for JAK-2 mediated phosphorylation. The signal obtained from thus phosphorylated 330 ng of GST-Abltide is very weak and much less intense than the autophosphorylation of the 200 ng of insect cell expressed JH1-JH2-V617F-JAK2 kinase. Abltide has the amino acid sequence: EIAYAAPFAKK. A recent report (Li et al) demonstrated that a peptide fragment with a sequence of PQDKEYYKVKE derived from the autophosphorylation sites of human JAK2 when expressed as a GST fusion protein serves as a highly specific substrate for JAK2V617F. This agrees well with work from Dr. Carter-Su's group who showed that the JAK2 target is YxxL/I/V and not YxxP that is in Abltide. Therefore we made our own construct for expression of GST-PQDKEYYKVKE through pGEX-2T plasmid and used it for bacterial expression and affinity purification of the substrate. We named this peptide GST-*JAK*-S (for GST-tagged JAK substrate).

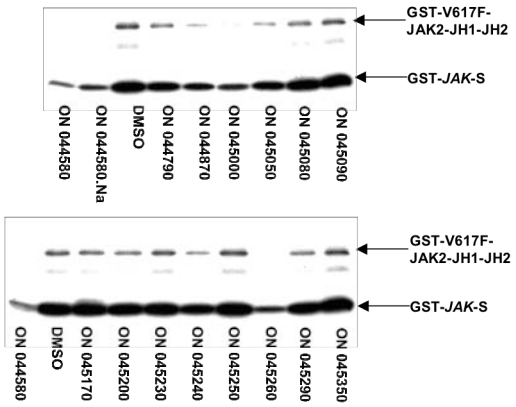

Figure 9. ***In vitro*** **inhibition of JAK2-V617F kinase activity:** 200 ng of recombinant JAK-2 was incubated with 20 μM of inhibitor for 30 minutes at room temperature. The kinase reaction was initiated by addition of substrate mix (330 ng GST-*JAK*-S, 20 μM ATP and 10 μCi γ^{32}P-ATP) and incubated for 20 minutes at 30ºC followed by SDS-PAGE and autoradiography.

In a primary screen using 20 μM of inhibitor we found that ON044580.Na (sodium salt of ON044580), ON045000 and ON045260 could inhibit JAK2 autophosphorylation and GST-*JAK*-S phosphorylation comparably to ON044580. This data is shown in Figure 9. Dose response experiments gave an IC_{50} value of 3.0 μM for ON044580 for the

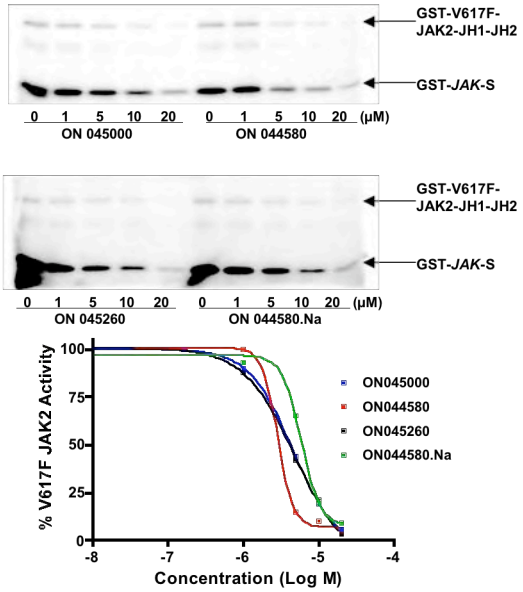

Figure 10. Dose-dependent inhibition of V617F-JAK2 ***in vitro*** **kinase activity.** 200ng of recombinant V617F-JAK2-JH1-JH2 was incubated with indicated inhibitor concentration for 30 minutes at room temperature. The kinase reaction was initiated by addition of substrate mix and incubated for 20 minutes at 30ºC followed by SDS-PAGE and autoradiography. Quantitation was performed by using MAC-Bas software and sigmoidal regression plots were obtained using GraphPad Prism software for estimation of IC_{50} values.

inhibition of GST-*JAK*-S phosphorylation by V617FJAK2. Of the three new compounds, ON045260 gave an IC_{50} value of 3.9 μM, followed by 4.2 μM for ON045000 and 6.0 μM for ON044580.Na. Figure 10 shows the sigmoidal regression plots for the inhibition of GST-JAK-S phosphorylation by these compounds.

The recombinant preparation of mutant JAK2 protein is a truncated form of the kinase (aa 532-1132), and we are in the process of examining the kinase inhibitory activity of these compounds using JAK2 kinase immunoprecipitated from the BaF3:V617F-JAK2 and HEL cell lines which expresses the full length form of mutant JAK2.

***In vivo* inhibition of STAT-5 phosphorylation by ON compounds in Ba/F3:V617F-JAK2 cells.**

To test the *in vivo* kinase inhibitory activity of ON044580.Na, ON045000 and ON045260 we treated Ba/F3:V617F-Jak2 cells with increasing concentrations of the compounds for 2 hrs in the presence of recombinant IL-3 (which enhances the phosphorylation status STAT-5 in response to JAK2 activation). 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 STAT-5. The results of this study, presented in Figure 11, showed that all three compounds were able to inhibit the phosphorylation of STAT-5 in a concentration dependent manner. Importantly, ON045260 exhibited the most potent *in vivo* JAK2 kinase inhibitory activity with an IC_{50} of ~1 μM that was slightly better than the parent compound ON044580. ON044580.Na, the sodium salt of the parent compound, showed the lowest inhibitory activity possibly owing to its poorer solubility when added to cell culture medium. We also observed significant protein degradation

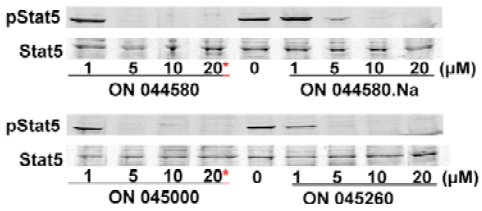

Figure 11. In vivo JAK-2 kinase inhibitory activity of ON compounds in Ba/F3:V617F-JAK2 cells. Mid log phase cells growing in the presence of recombinant IL-3 were treated for 2 hours with indicated inhibitor concentrations.Washed cells were lysed in detergent containing buffer and equal amounts of protein from the clarified lysates was resolved by 10% SDS-PAGE and analyzed for pSTAT-5 and STAT-5 by infrared labeled secondary antibodies and scanning with Odyssey scanner (LiCor Technology).*These samples showed >40% protein degradation

(total protein levels dropping by >40% at 20 μM in the 2 hour period) with ON044580 and ON045000. However, no such degradation was observed for ON045260.

Growth inhibition of V617F-Jak2 expressing cells.

To determine whether ON045260 inhibits the proliferation of V617F-Jak2-positive leukemic cells as well as ON044580, 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:V617F-Jak2 cells which 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, was homozygous for V617F mutation while the second cell line, SET2 was hemizygous for the V617F mutation. The results of this study, presented in Figure 12 show that both ON045260 and ON044580 could readily inhibit the proliferation and induce apoptosis of all the three cell lines at nanomolar or low micromolar concentrations. The GI_{50} of ON044260 for Ba/F3:V617F-Jak2 cells was approximately 300 nM which matches the GI_{50} value of 350 nM for ON044580. Interestingly, there were differences in the cell killing activity of these two compounds against cell lines derived from patients. While ON044580 was twice as effective in killing HEL cells with a GI_{50} of approximately 1 μM as compared to 2 μM of ON045260 while the converse was true for SET2 cells where ON045260 proved to be the better compound with a GI_{50} value ~ 1 μM.

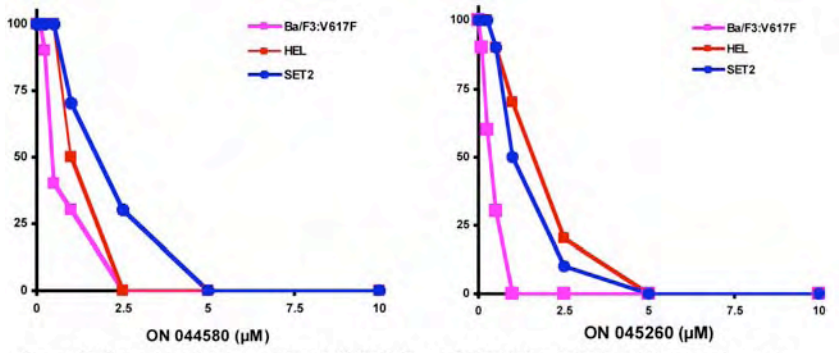

Figure 12. Growth inhibition activity of ON044580 and ON045260 in V617F-JAK2 expressing cells. BaF3:V617F-JAK2 cells (V617F-JAK2 overexpression), human erythroleukemia HEL cells (homozygous for V61F-JAK2) and human essential thrombocythemia SET2 cells (hemizygous for V617F-JAK2) were grown in the presence of varying concentrations of ON044580 and ON045260 for 72 hours. The total number of viable cells was determined following trypan blue staining and counting using a haemocytometer. Data is plotted as percent total viable cells as compared to DMSO controls.

Key Research Accomplishments.

1. 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. All of these compounds were found to be non-ATP competitive and readily induced the down-regulation of BCR-ABL auto-phosphorylation and STAT-5 phosphorylation.

2. Using ON044580 as the lead compound, we have carried out chemical modification of the compound to facilitate the oral bio-availability of the compound. This resulted in the synthesis of 13 different analogues which have been further tested for their BCR-ABL inhibitory activity. This resulted in the selection of 2 compounds, ONO45260 and ON044690 which retained the BCR-ABL inhibitory activity of the parent compound.

3. ON045260 and ON044690 also retained the ability to inhibit kinase activities of WT and V617F mutant forms of JAK2 and inhibit the proliferation and induce apoptosis of leukemic cell lines that express the V617F mutant form of JAK2.

Reportable Outcomes

None

Conclusions

1. Non-ATP competitive inhibitors of BCR-ABL are effective inhibitors of imatinib-resistant forms of BCR-ABL including the T315I-BCR-ABL.

2. Because of their substrate-competitive nature, some of these inhibitors also inhibit JAK2 kinase activity.

3. These compounds are useful therapeutic agents for CML as well as MPDs arising due to mutations in JAK2

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

INTRODUCTION:

Our previous findings have shown that ON044580 reduces the level of HSP90, a chaperone protein, a critically essential protein required for stabilization and conformational maturation of client proteins involved in signaling of cancer cells and leukemia [1,2]. It is known that HSP90 is a target for cancer therapy [3]. We have explored the mechanism of action of ON044580. Dr Reddy's lab has shown that ON044580 inhibits both Jak2 and Bcr-Abl kinases. Our findings indicate that both Bcr-Abl and Jak2 inhibition together will impact levels of HSP90 in BCR-ABL+ leukemia cells through inhibition of expression of STAT3. This stems from our findings that both pTyr 705 of STAT3 and pSer 727 of STAT3 are controlled by the Jak2 and Bcr-Abl, respectively. STAT3 is well known to control HSP90 transcription and to associate with HSP90. We hypothesize that HSP90 and/or one of the associated HSP90 members (e.g. HSP70 and HSP27) help maintain the macromolecular complex that assembled the Bcr-Abl/Jak2 signaling Network. Our published papers [4-7] show that Bcr-Abl activates Jak2. Jak2 activation leads to phosphorylation of Gab2 on the YxxM sequence required to activate the PI-3 kinase [8]. Our more recent findings have discovered that Lyn kinase is the tyrosine kinase that phosphorylates Gab2 on YxxM sequences, and that Lyn kinase is down-stream of Jak2. These studies also indicate that activation of SET expression is involved in maintaining Lyn kinase in an active state. SET expression was previously shown to be activated by Bcr-Abl [9,10]. Our findings show that activation of Jak2 is required to activate SET expression in Bcr-Abl+ cells (Samanta et al., revised submitted). SET expression inhibits PP2A, a Ser/thr phosphatase required to activate Shp1, a tyrosine phosphatase that when activated, dephosphorylates Bcr-Abl (leading to its degradation) and inactivates Lyn kinase. Thus, Jak2 activation by Bcr-Abl is required to maintain Bcr-Abl itself in an activated state and also maintain Lyn tyrosine kinase in an activated state. It is likely that Jak2 also activates Lyn kinase directly but further studies are required on this point.

Thus, our findings suggest the following sequence of events: Bcr-Abl activates Jak2 leading to activation of Lyn kinase that phosphorylates Gab2 on YxxM sequences leading to activation of the PI-3 kinase/Akt pathway. Jak2 activation by Bcr-Abl also allows Jak2 to stimulate expression of SET which in turn blocks the activity of PP2A which maintains Shp1 tyrosine phosphatase in an inactive state, thereby maintaining Bcr-Abl in an active state and Lyn kinase in an active state. Our studies indicate that the Bcr-Abl/Jak2 signaling pathway is part of a large multiprotein signaling Network. This Network is composed of Bcr-Abl, Jak2, Lyn, Gab2, SET, PI-3 kinase, Akt and GSK3 beta. Our recent findings suggest that the HSP90/HSP70/HSP27 chaperone proteins are also part of this Network, and may play a role in maintaining the Bcr-Abl signaling Network in a functional state. This pathway drives the activation of the NF-kB transcription factor leading to enhanced levels of c-MYC transcripts[7]. Through the inactivation of GSK3 beta, the level of c-Myc protein is also enhanced by preventing the degradation induced by active GSK3 beta [7].

The level of pTyr 705 STAT3 is also believed to be controlled by the Bcr-Abl/ Jak2 pathway [11]. However, the Jak kinase inhibitor used in these earlier studies was AG490, which is known to inhibit both Jak2 and Jak3, which we have confirmed in our studies (Samanta et al., submitted). We have used a new Jak kinase inhibitor hexabromocyclohexane (HBC), which we find inhibits only Jak2 and not Jak1 and Jak3 (Samanta et al, revised submitted). We will determine whether HBC inhibits pTyr 705 phosphorylation in BCR-ABL+ 32 D cells.

Bcr-Abl controls the phosphorylation of Ser 727 of STAT3 through the Ras pathway [11]. In this pathway Bcr-Abl autophosphorylates Bcr sequences causing the phosphorylation of tyrosine 177 [11,12]. This in turn causes binding of Grb2 which binds SOS that activates Ras. Ras activation activates Raf which in turn activates MEK and Erk kinases. These kinases phosphorylate STAT3 on Ser 727, which is the final activation step needed to fully activate STAT3 [11,13-15]. Thus, inhibition of both Bcr-Abl and Jak2 kinases by Onconova 044580 (see the Reddy data from last year) is a powerful strategy to induce apoptosis in BCR-ABL+ leukemia cells, as both the PP2A/Shp1 pathway will be activated leading to inactivation of both Lyn tyrosine kinase and the Bcr-

Abl kinase. Levels of c-Myc and levels of active NF-kB will be down-regulated and the expression of STAT3 transcription factor will be inhibited leading to reduction of the HSP90 chaperone complexes and the dismantling of the Bcr-Abl/Jak2 Network. As a result, BCR-ABL+ cells will lose their anti-apoptotic ability caused by STAT5 activation of Bcl-XL, Akt inhibition of pro-apoptotic Bad, and the anti-apoptotic effects of NF-kB inactivation.

BODY:

We have summarized below our findings obtained in the second year of this grant.

Last year's summary. We showed that ON044580 reduced the viability of a variety of Bcr-Abl+ cell lines both imatinib–sensitive and–resistant cells. Similarly ON 044580 induced late stage apoptosis in these same kinds of cells, using Annexin V/PI staining and flow cytometry. Similar findings were shown for cells from early and late stage CML patients. ON 044580 also was a potent inhibitor of anchorage-dependent growth of these Bcr-Abl + cell lines, both imatinib-sensitive and –resistant cell lines. We also showed that ON 044580 reduced expression of some members of the Bcr-Abl/Jak2 Network. HSP90 expression was also reduced by 5-10 uM ON 044580.

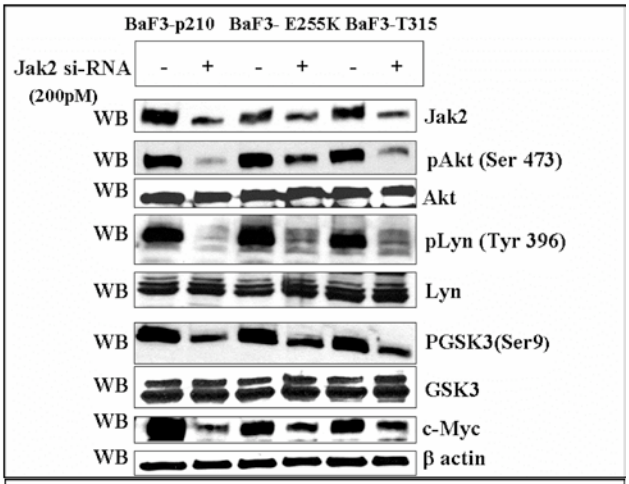


Fig. 1. IM-resistant BCR-ABL+ BaF3 cells were transfected with Jak2-specific siRNA, and the extract was processed for Western blotting with various antibodies.

ON044580 inhibits expression of STAT3. It is known that STAT3 controls expression of HSP90. Our *hypothesis* is that ON044580 inhibits the expression of active STAT3, leading to reduction of the HSP90 complex of proteins that maintains the Bcr-Abl/Jak2 signaling Network. The second part of this *hypothesis* is that reduction of STAT3 will down-regulate the HSP90 complex of proteins, leading to disruption of the Bcr-Abl/Jak2 Network. By way of introduction, we explored the effects of Jak2 knockdown on the various members of the Bcr-Abl/Jak2

Network (Fig. 1). From last year's studies, Jak2 knockdown did not effect HSP90 expression to any great extent. As Fig. 2 shows, Jak kinase inhibitor AG490 (we know it inhibits Jak3 and Jak2) had some effects on Bcr-Abl expression (Fig. 2), as it reduced levels of Bcr-Abl, Akt and Jak2 proteins. We now know this is due to inhibition of SET expression, which leads to activation of PP2A and tyrosine phosphatase Shp1 (Samanta et al, submitted). Shp1 activation will lead to dephosphorylation of Bcr-Abl which in turn leads to degradation of Bcr-Abl [10]. How Akt levels decrease somewhat by AG490 treatment is unknown at this point. Of interest, AG490 treatment had little effect on levels of HSP90 (Fig. 2).

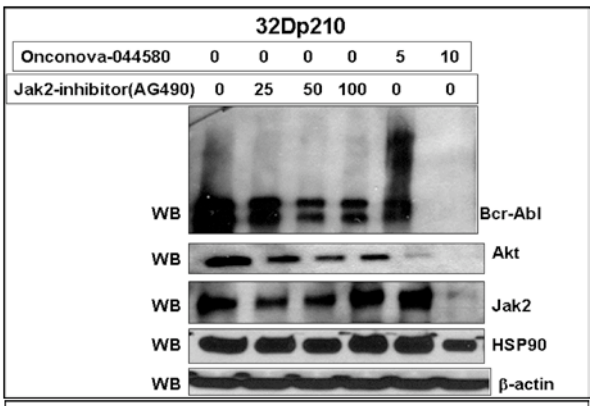


Fig. 2 Bcr-Abl+ cells were incubated with different doses of Jak2-inhibitor and ON 044580 for 16h and the cells were processed for Western blotting.

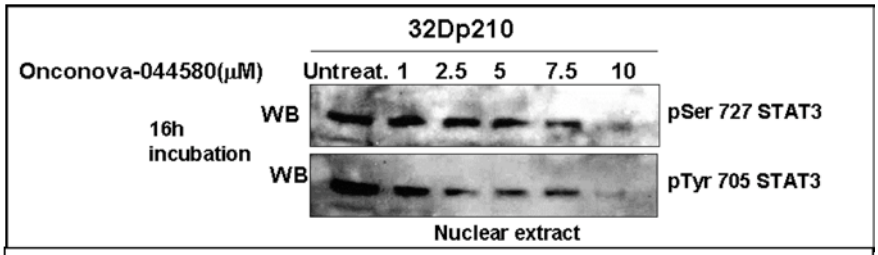


Fig. 3. ON044580 treated cells were used for preparation of nuclear extract. The extract was processed for Western blotting

ON044580 at low doses caused a dramatic reduction in levels of functional STAT3, as both pTyr 705 STAT3 and p727 STAT3 were severely decreased by ON 044580 (Fig. 3). Fig. 4 shows that nuclear lysates of ON 044850 treated cells had strongly reduced levels of active STAT3, and that transcripts of HSP90 were also reduced in cells treated with ON 044850 (Fig. 5).

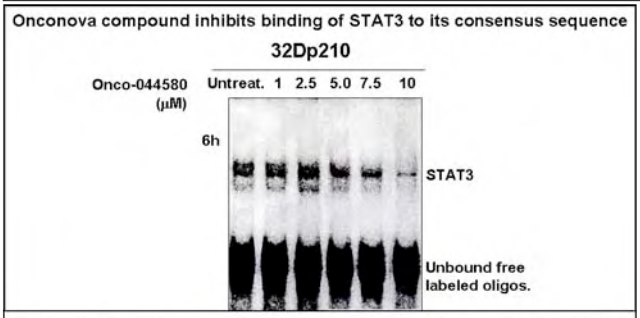


Fig. 4. Nuclear extract was prepared from ON 044580 treated 32Dp210 cells and then electrophoretic mobility shift assay (EMSA) was carried out. The dried gel was processed for autoradiography and the signals were detected by a phosphoimmager.

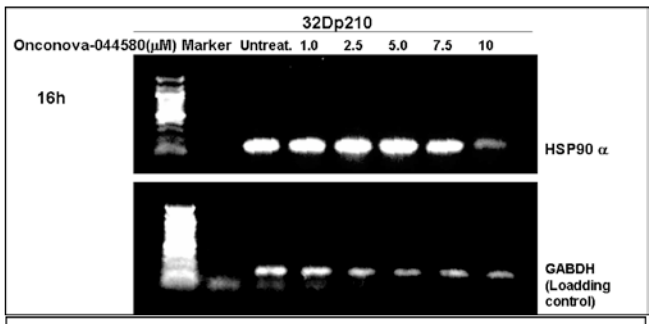


Fig. 5. RT-PCR was carried out using ON 044580 treated Bcr-Abl+ cells (32Dp210) and GAPDH was used as a loading control.

We examined the effects of ON044580 on STAT3 and HSP90 expression in Bcr-Abl+ cells. A 16 h treatment with ON 044850 decreased Jak2, STAT3 pTyr 705 STAT3, p727 STAT3, and HSP90 expression (Fig. 6). These effects are also observed within six h following ON 044850 treatment in a time-dependent manner (Fig. 7).

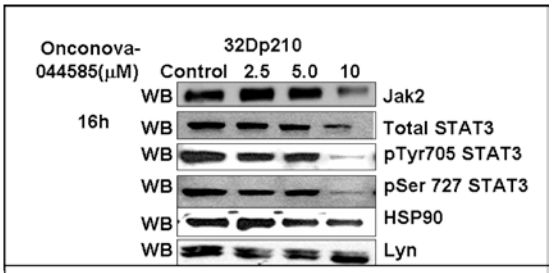


Fig. 6. 32Dp210 cells were treated with different doses of ON 044580. The cells were processed for Western blotting and the blot was treated with different antibodies to detect the respective signals.

Of interest, imatinib treatment of Bcr-Abl+ cells also reduced levels of HSP90 (Fig. 8). Of interest, IM had only partial inhibitory effects on pTyr 705 STAT3 but severely inhibited pSer 727 STAT3.

ON 044850 disrupts the Bcr-Abl/Jak2 Network. Our published studies suggest that the Bcr-Abl/Jak2 signaling pathway is part of a large protein Network complex [7]. We developed methods to fractionate large protein complexes by gel filtration chromatography (Fig. 9). Detergent lysates of 32D cells expressing Bcr-Abl migrated at a size of less than 5 million daltons but larger than 2 million daltons. Jak2, Lyn kinase and GSK3 beta co-migrated with Bcr-Abl. (Fig. 10 and 11). HSP90 and STAT3, Akt, and pErk also co-migrated with Bcr-Abl. We note that the Network migrated at a higher molecular size in detergent lysates from K562 cells (Fig. 12). This change was quite reproducible,

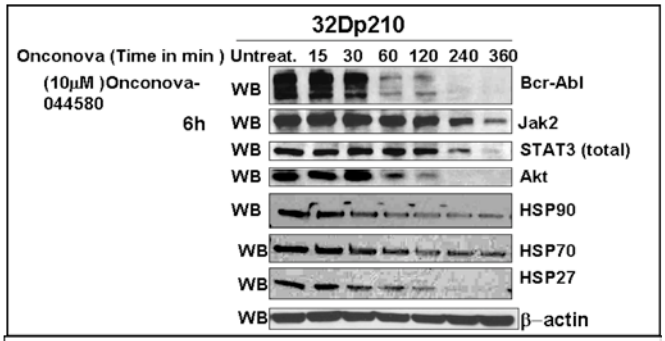


Fig.7. 32D cells were treated with 10 μM ON 044580 for 6h. Then the cells were processed for Western blotting as in Fig.6.

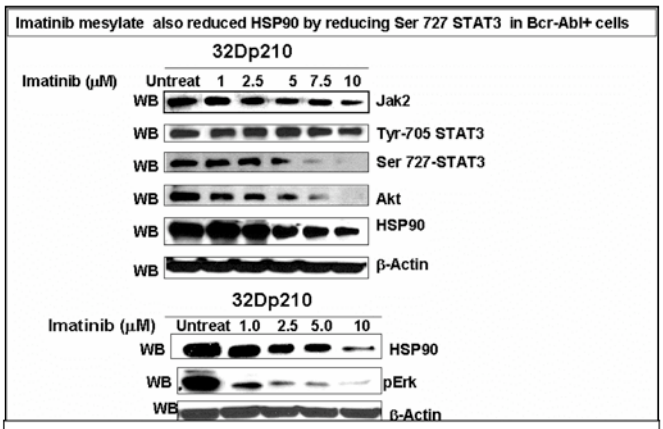


Fig. 8. Bcr-Abl+ cells were treated with different doses of imatinib for 16h. The cells were processed for Western blotting and the signals were detected by various antibodies.

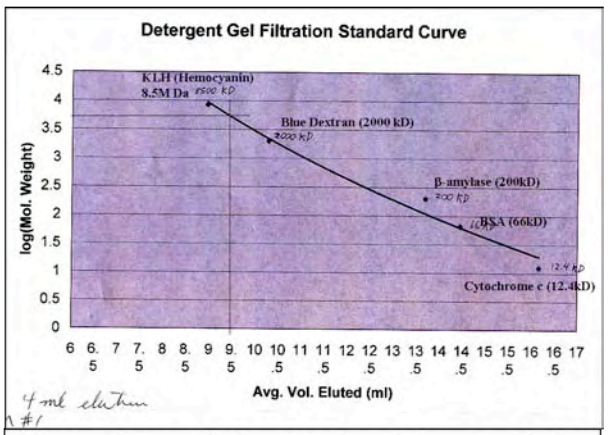


Fig. 9. The standard curve plotted from the elution profile of standard protein markers to determine the size of Bcr-Abl Network protein complex associated with HSP90

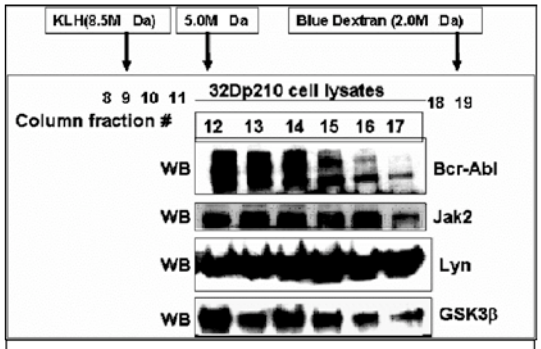


Fig. 10. Determination of molecular size of the Bcr-Abl/ Jak2 Network complex from the Bcr-Abl+ cell lysates.

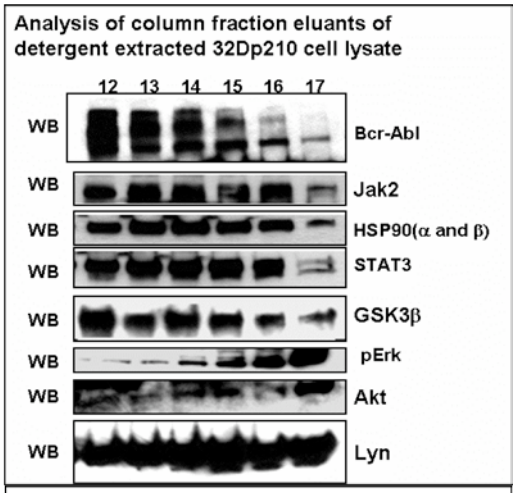


Fig. 11. Detection of HSP90 associated proteins by Western blotting from the fractions of gel filtration column loaded with Bcr-Abl+ cell lysate.

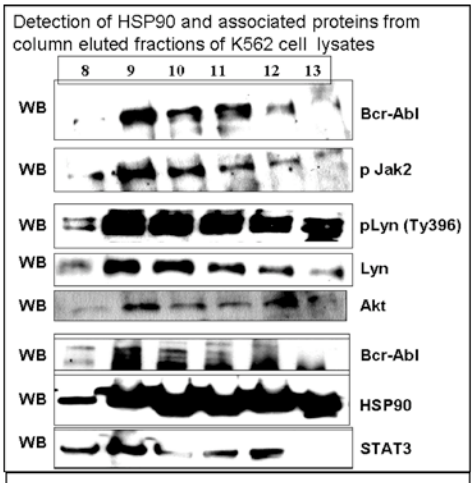


Fig. 12. Detection of Bcr-Abl and its signaling proteins in the same fraction of HSP90 as a high molecular weight complex from K562 cell lysates using our standard gel filtration column.

ON044580 treated for 3h

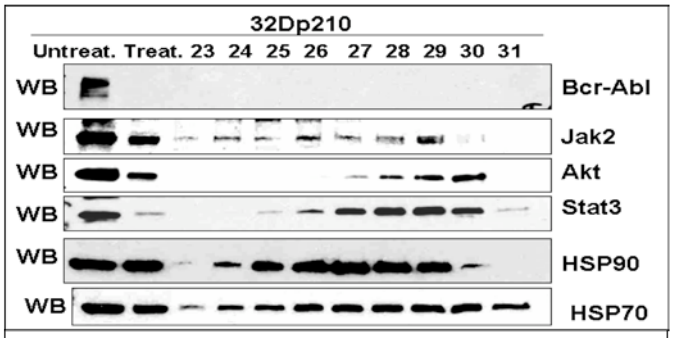


Fig. 13. 32Dp210 cells were treated with ON 044580 for 3h and the detergent extracted lysate was fractionated by gel filtration column chromatography and the fractions were processed for detection of HSP90 associated proteins.

suggesting that either the Network structure is more intact in the CML cell line than that from mouse 32D cells having forced expression of Bcr-Abl or that the structure is larger in K562 cells than Bcr-Abl+ 32D cells. Treatment of 32D cells expressing Bcr-Abl for 3 h at 10 μM ON 044850 drastically changed the elution pattern (Fig. 13), suggesting that ON 044850 treatment dismantles the Bcr-Abl/Jak2 Network, possibly due to decreased levels of the HSP90 complex of chaperone proteins. Of interest, STAT3 was still present but removed from the 5 million size structure as was HSP90, Akt and Jak2. We suggest that ON044850 dismantles the structure by either reducing the expression of the HSP90 protein or ON 044850 strongly inhibits Jak2, and that dismantles the Bcr-Abl/Jak2 Network. Further studies are needed to determine the actual cause of Network destruction.

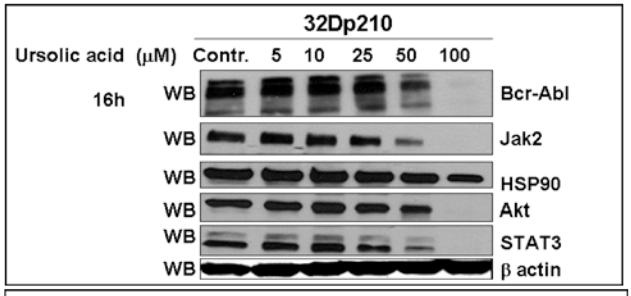


Fig. 14. Treatment of Bcr-Abl+ cells with Ursolic acid, a STAT3 inhibitor, reduced HSP90 and its client proteins.

STAT3 inhibitor Ursolic acid [16] reduced STAT3 and HSP90 levels in Bcr-Abl+ 32D cells. Our working *hypothesis* is that ON 044850 targets Bcr-Abl and Jak2 , causing severely decreased levels of active STAT3, leading to reduction of the HSP90 complex of proteins that maintains the Bcr-Abl/Jak2 signaling Network. To confirm the role of STAT3 in maintaining the Bcr-Abl/Jak2 Network, we examined the effects of two STAT3 inhibitors (Ursolic acid[16] and Capsiacin[17]) on HSP90 levels and whether these STAT3 inhibitors also

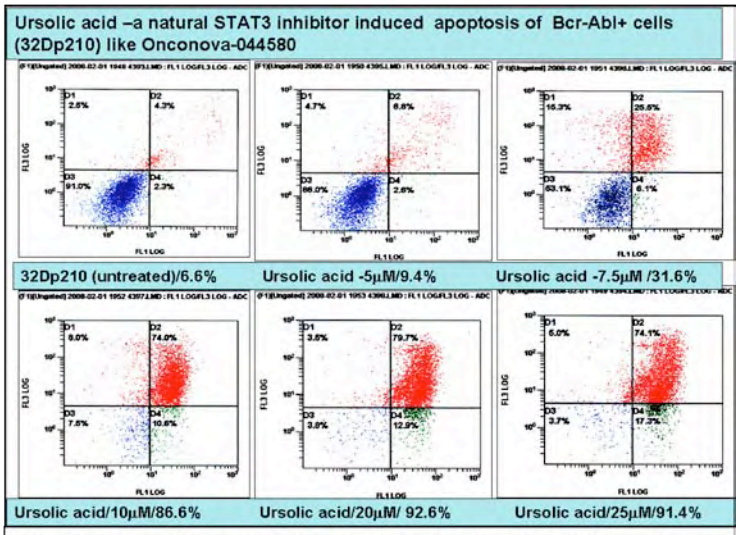


Fig. 15. Ursolic acid induced apoptosis in Bcr-Abl+ cells as measured by Annexin V/PI flow cytometry.

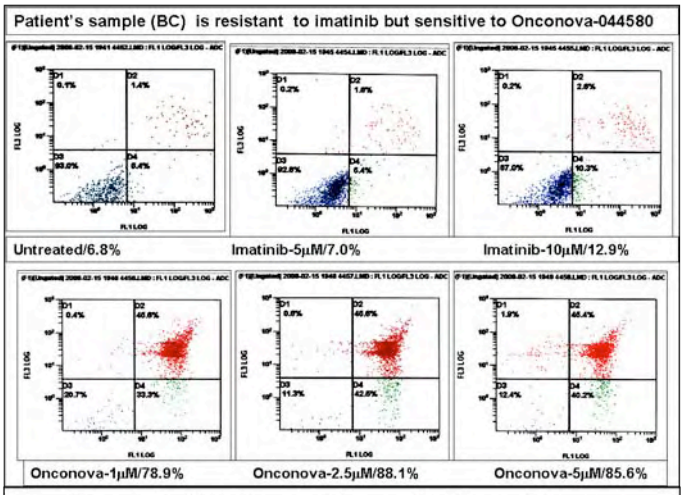


Fig. 16A. Cells from CML patients with blast crisis are resistant to imatinib but sensitive to ON 044580 for late stage apoptosis induction as measured by Annexin V/PI flow cytometry.

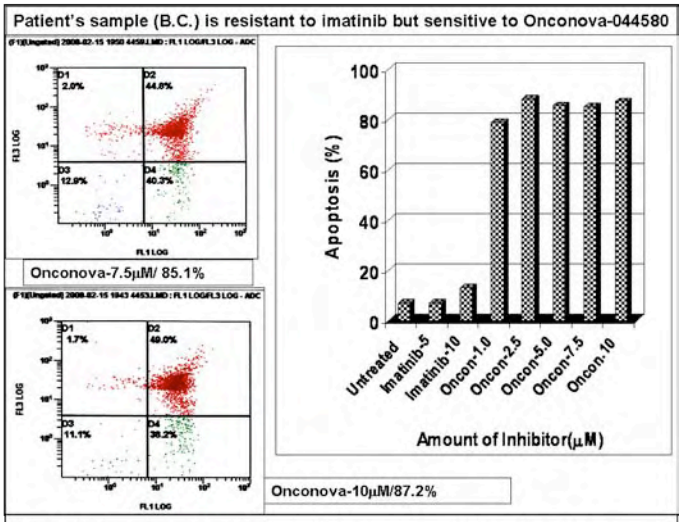


Fig. 16B. A graphical plot of apoptosis of the Bcr-Abl+ blast crisis cells treated with either Imatinib or ON 044580 using Annexin V/PI flow cytometry.

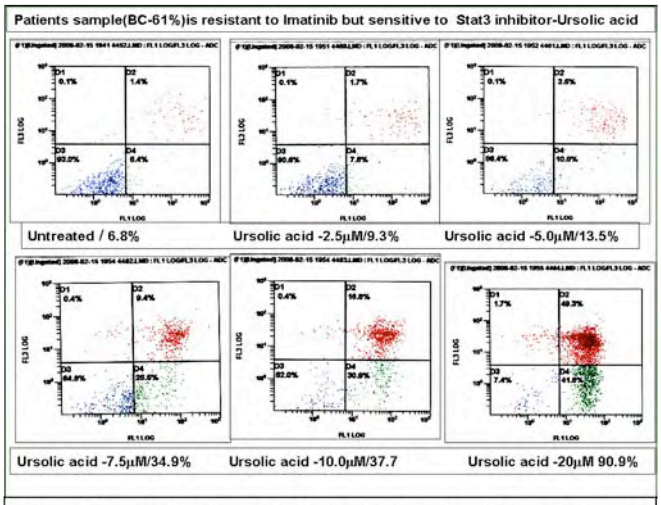


Fig. 17A. Cells from CML patients with blast crisis are resistant to imatinib but sensitive to ON 044580 as measured by Annexin v/PI flow cytometry.

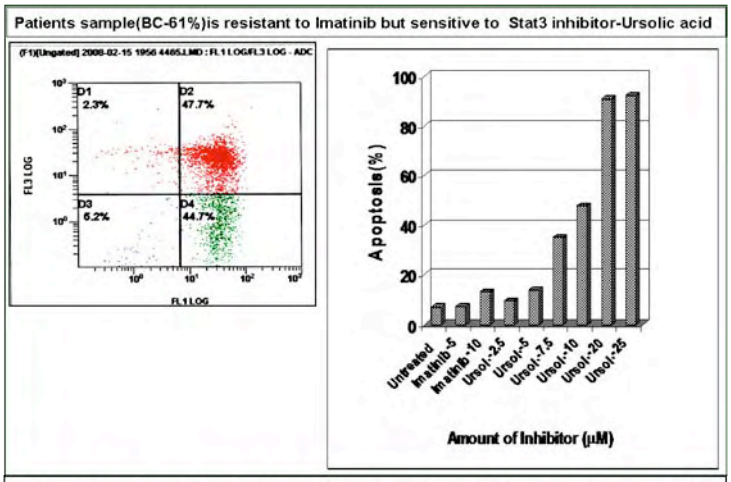


Fig. 17B. Graphical plot of apoptosis of the Bcr-Abl+ cells treated with Imatinib and ON 044580, as above.

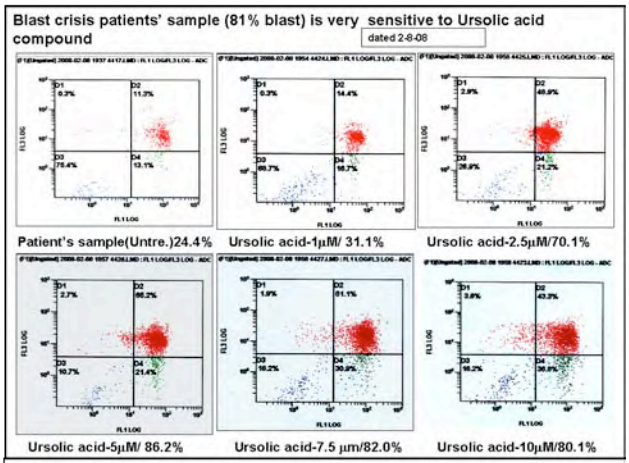
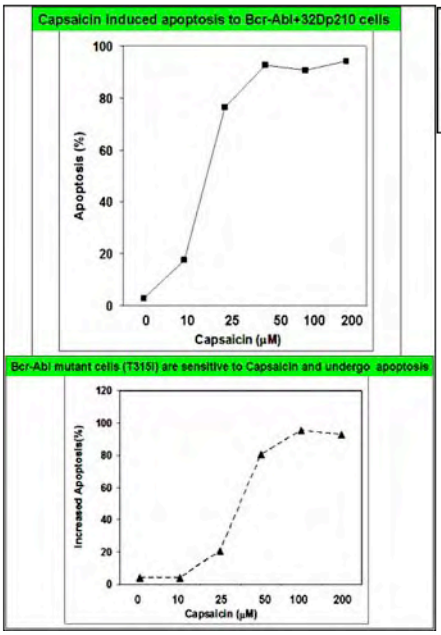
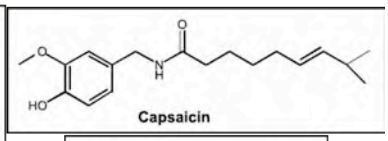


Fig. 18. Cells from CML patients with blast crisis are sensitive to apoptosis induction by Ursolic acid using Annexin V/Pi staining as above.





Structure of capsaicin

Fig. 19. Bcr-Abl+ and IM-resistant cells are sensitive to STAT3-inhibitor Capsaicin as measured by Annexin V/PI staining methods as above.

cause the dismantling of the Bcr-Abl/Jak2 signaling Network. Fig. 14 shows that levels of HSP90, Jak2, Akt and STAT3 were decreased in a dose-dependent manner by Ursolic acid. The disappearance of Bcr-Abl and Jak2 argues that the Network was disrupted as a result of a severe decrease in activated STAT3. Our findings indicate that ON044850 is reducing levels of STAT3 but not inhibiting STAT3. That Ursolic acid also induces extensive apoptosis induction, as we showed that it induces more than 90% of the Bcr-Abl cells to undergo late state apoptosis within 48 h (Fig. 15). Similar results were obtained in cells from blast crisis stage CML following treatment with ON 044850 , Ursolic acid and Capsiacin (Fig. 16 -19).

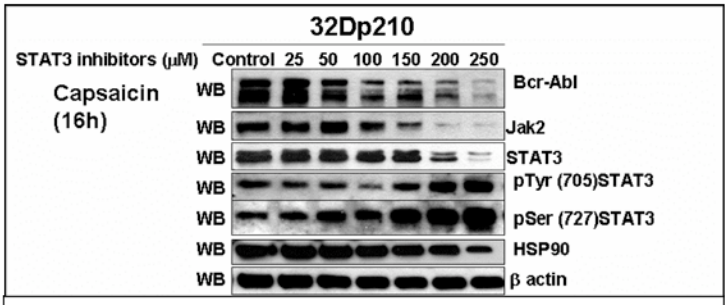


Fig. 20. Capsiacin increased the level of pTyr 705 STAT3 and pSerSTAT3 727 but reduced HSP90 and its client proteins in Bcr-Abl+ 32Dp210 cells.

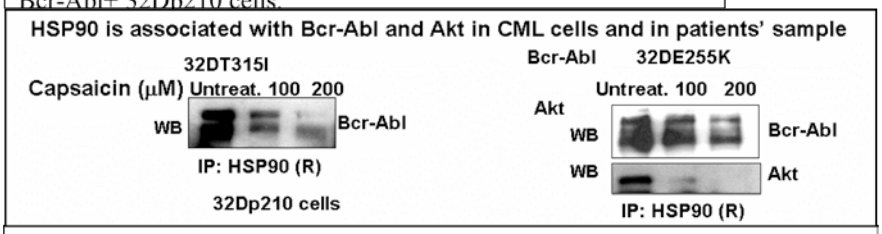


Fig. 21. Immunoprecipitation study (with anti-HSP90) to support that HSP90 is associated with Bcr-Abl and Akt. Capsaicin reduced the amount of client proteins Bcr-Abl and Akt associated with HSP90. IP-immunoprecipitation; WB, Western blotting.

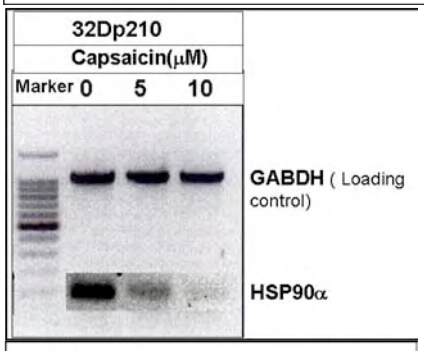


Fig.22. STAT3 inhibitor Capsaicin reduced the transcripts of HSP90 in Bcr-Abl+ 32Dp210 cell

STAT3 inhibitor Capsiacin also reduces HSP90 levels and disrupts the Bcr-Abl/Jak2 signaling Network. Fig. 20 and 21 shows that Capsiacin treatment reduced levels of HSP90 and members of the Bcr-Abl/Jak2 Network in a dose-dependent manner. As an example, we showed that Akt (one of the client proteins of HSP90) was associated with HSP90 in Bcr-Abl+ cells (Fig. 21). Importantly, Capsiacin also caused the accumulation of pTyr 705 STAT3 and pSer 727 STAt3, which we think are abortive forms of inactive STAT3. Fig. 22 shows that Capsiacin[18] treatment strongly reduced the levels of HSP90 alpha transcripts.

Capsiacin induces high levels of apoptosis in Bcr-Abl+ cells and IM-resistant T315I cells. Fig. 19 shows the results of apoptosis induction by 0N 044850. Over a 48 hr treatment period, apoptosis levels reach the 90% levels in both IM-sensitive Bcr-Abl+ cells and the IM-resistant T315I Bcr-Abl cells.

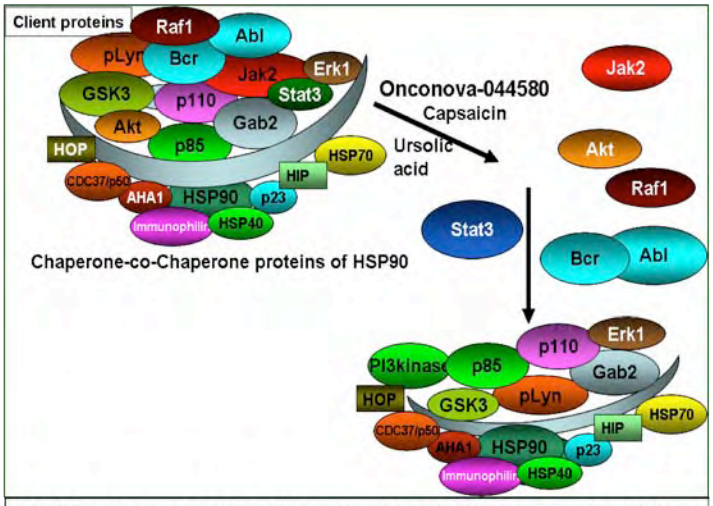


Fig.23. Treatment of Bcr-Abl+ cells with ON 044580/STAT3-inhibitors (Capsaicin and Ursolic acid) resulted in collapse of large Bcr-Abl/Jak2 Network complex and dissociated client proteins of Bcr-Abl signal transduction pathway from HSP90 chaperone complex.

Model for the mechanism of action of ON 044850. Fig. 23 and 24 show models depicting how ON 044850 affects Bcr-Abl signaling, leading to the induction of apoptosis. Briefly, ON 044850 destroys the Bcr-Abl/Jak2 Network, which is a signaling network that drives expression of anti-apoptotic pathways and other crucial pathways that prevent induction of apoptosis and allow the leukemic cell to be unregulated by normal control mechanisms. ON 044850 treatment accomplishes this by reducing the levels of STAT3 by inhibiting both

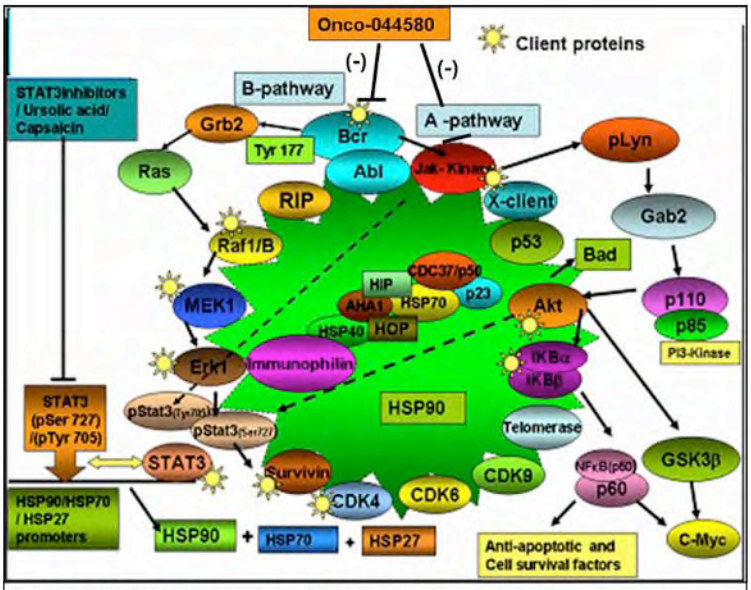


Fig.24. Model for effects of ON 044580 and STAT3 inhibitors on pathways involved in CML. Pathway A activates Jak2 and PI-3 kinase/Akt pathway that activates pTyr 705 STAT3; pathway B activates pSer STAT3, which controls HSP90 expression. Phosphorylation of STAT3 on serine 727 is believed to be the final step needed for STAT3 activation (see ref. 15)

pathways that are required to generate functional STAT3, that is, pTyr 705 STAT3 and pSer 727 STAT3. This leads to reduction of expression of HSP90 chaperone complexes, and then dismantling of the Bcr-Abl/Jak2 signaling Network.

KEY RESEARCH ACCOMPLISHMENTS:

1. We showed that ON 044850 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.
2. ON044850 causes reduction of STAT3 levels leading to reduced expression of HSP90.
3. ON 044850 induces rapid late stage apoptosis in imatinib mesylate (IM)-resistant CML cells and in cells from advanced stages of CML including the terminal blast crisis stage of the disease.

REPORTABLE OUTCOMES:

None at this time

CONCLUSIONS:

CML is a leukemia that is successfully treated in the early stage of the disease by IM treatment. At this point of the disease, the Bcr-Abl oncoprotein is the only oncogenic driver of the malignancy, and because IM inhibits the activity of the Bcr-Abl oncoprotein, the disease is effectively controlled. As time goes on, CML patients develop additional oncogenic events that along with Bcr-Abl also drive the leukemic disease. Our studies with ON 044850 suggest that it may be useful to treat those patients that fail IM treatment and those patients who do not respond to IM treatment with ON044850. More importantly, because ON044850 severely reduces the production of active forms of STAT3, and thus reduces HSP90 destroying the BCR-ABL/Jak2 Network, even advanced states of CML will be inhibited by ON 044850.

Of importance, we have elucidated the mechanism of action of ON 044850. Its primary target appears to be Jak2 and Bcr-Abl. Inhibition of the Jak2 and Bcr-Abl

pathways inhibits the expression of STAT3, which in turn caused reduction of HSP90 and other STAT3 transcriptional targets. This leads to destruction of the Bcr-Abl/Jak2 Network and a complete stoppage of anti-apoptotic signaling.

Future Plans.

1. We will complete the mechanism studies on ON 044850 necessary for manuscript completion.
2. Perform mouse leukemia model experiments with ON 044850 using various doses of ON 044850 by intraperitoneal injection.
3. We will characterize the mouse tumor cells and attempt to detect the Bcr-Abl/Jak2 signaling Network by gel filtration column chromatography and its absence and/or reduction in mice treated with ON 044850.
4. Characterize the pro-drug form of ON 044850, being synthesized, by several experiments including reduction of HSP90 and STAT3 expression and disruption of the Bcr-Abl Network in leukemia cell lines.

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Work Accomplished by Onconova Therapeutics

Body

1. 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.
2. To accomplish the goal, Onconova chemists have designed and synthesized the following molecules and provided them to Dr. Reddy's group for further evaluation of their biochemical and biological properties. The structures of the compounds synthesized are shown in Dr. Reddy's report.

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

Key Research Accomplishments

Please see Dr. Reddy's and Dr. Arlinghaus' reports.

Reportable Outcomes

None

Conclusions

We have developed methods for the large scale production of ON044580, ON045260 and on044690 for pre-clinical and clinical development of these compounds**.**