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The design and preliminary structure–activity relationship studies of benzotriazines as potent inhibitors of Abl and Abl-T315I enzymes

Jianguo Cao,[a] Richard Fine,[b] Colleen Gritzen,[a] John Hood,[a] Xinshan Kang,[b] Boris Klebansky,[b] Dan Lohse,[a] Chi Ching Mak,[a] Andrew McPherson,[a] Glenn Noronha,[a,*] Moorthy S. S. Palanki,[a] Ved P. Pathak,[a] Joel Renick,[a] Richard Soll,[a] Binqi Zeng[a] and Hong Zhu[a]

[a]*TargeGen, Inc., 9380 Judicial Drive, San Diego, CA 92121, USA*
[b]*BioPredict, Inc., 660 Kinderkamack Road, Oradell, NJ 07649, USA*

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Abstract—We describe the design, synthesis and structure–activity relationship studies in optimizing a series of benzotriazine compounds as potent inhibitors of both Abl and Abl-T315I enzymes. The design includes targeting of an acid functional residue on the αC-helix that is available only upon kinase activation. This designed interaction provides an advantage in overcoming the challenges arising from the T315I mutation of Abl and transforms poor (ca. 10 μM) inhibitors into those with low nM potency.

Abelson tyrosine kinase (Abl) is a non-receptor tyrosine kinase that is usually under tight control, present in a wide range of cells, and involved in cell growth and proliferation. About 95% of patients with chronic myeloid leukemia (CML) have the c-Abl gene from chromosome 9 fused with the breakpoint cluster (Bcr) gene from chromosome 22 resulting in the Philadelphia chromosome.[1] The formation of the Philadelphia chromosome results in the production of constitutively active Bcr-Abl, which has all the catalytic activity of Abl and phosphorylates a broad range of substrates. This Bcr-Abl protein transforms and proliferates cells and makes them growth factor independent.[2] Bcr-Abl is causative for both the onset and uncontrolled proliferation of myeloid cells as well as the inhibition of apoptosis in CML.

Gleevec® (Imatinib mesylate), a Bcr-Abl inhibitor, was approved by the FDA for the treatment of CML.[3] Although impressive success has been achieved in treating CML, a high percentage of clinical relapse has been reported from long-term treatment due to resistance to Gleevec.[4] The majority of the resistance in these patients is through the selection and propagation of hematopoietic stem cells containing point mutations at the ATP binding pocket of the Abl kinase domain of Bcr-Abl. Sprycel® (dasatinib) was recently approved by the FDA for the treatment of CML patients who are resistant to Gleevec.[5] Sprycel potently inhibits Bcr-Abl and fourteen of its point mutations in the nanomolar range.[6] Tasigna® (nilotinib) was also recently approved for Gleevec resistant CML.[7]

Among the several mutations of Bcr-Abl, the T315I mutation is resistant to all approved Bcr-Abl inhibitors and other compounds that are in the developmental stage. Only Bosutinib, a dual inhibitor of both Src and Abl, weakly inhibits the Abl-T315I mutant. The Abl-T315I mutation, the gatekeeper residue Thr315 to Ile315, is one of the most prevalent mutations among Imatinib resistant patients (~12%).[8] Thus, developing new compounds that inhibit Bcr-Abl-T315I remains the largest unmet need in treating CML patients today.

In the course of developing inhibitors for Src that would have potential utility in oncology therapy, we have identified a series of benzotriazine based inhibitors as a new class of Src targeting molecules.[9,10] Since most ATP competitive inhibitors of Src are also inhibitors of Abl,[11] we will describe a few key molecules in terms of

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* Corresponding author. Tel.: +1 858 678 0760; fax: +1 858 678 0762; e-mail: noronha@targegen.com

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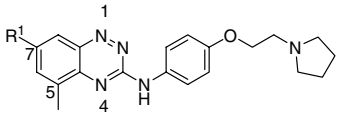
Table 1. In vitro evaluation of compounds with R^1 modifications on the 3-aminobenzo-[1,2,4]-triazine



Compound	R^1	K_i (nM)		
		Src	Abl	Abl-T315I
1	2,6-Me_2phenyl	7	11	4240
2	2,6-Cl_2phenyl	11	20	10,300
3	2-Clphenyl	35	42	2340
4	2-Cl-5-OHphenyl	1.3	1.3	55

their Src binding in order to indicate how we initiated our efforts aimed at Abl-T315I.

Both the dimethyl benzotriazine analog **1** and the dichloro analog **2** are potent inhibitors of Src and inhibit the enzyme in the low nanomolar range (Table 1). The monosubstituted phenyl ring **3** was also a good Src inhibitor showing potency comparable to **2**. Based on a homology model of fully activated Src complexed with **3** (Fig. 1), we observed that the carboxylic acid of Glu310 located on the αC-helix in the N-terminal lobe is oriented toward the hydrophobic pocket in a close proximity to the 7-phenyl group. In fact, crystal structures (Fig. 2a) of both inactive Src (gold) and active Src (blue) indicate that the αC-helix of activated Src moves as much as 10 Å towards the hydrophobic pocket proximal to the ATP binding site; positioning the carboxylic group in close proximity to the 7-phenyl ring.[12]

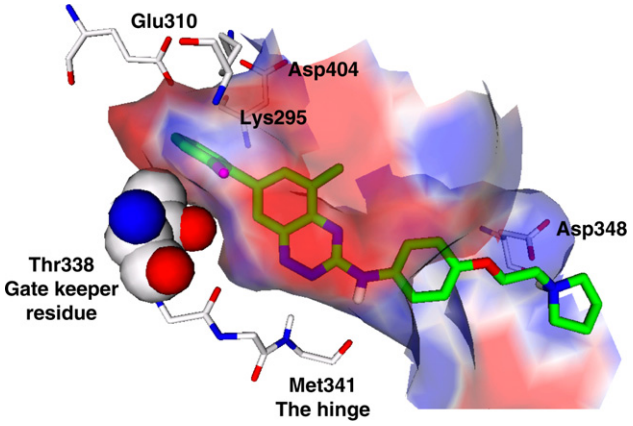


Figure 1. The binding of compound **3** at the ATP pocket of Src.

At the time, we knew of no reports involving systematic design of potent inhibitors targeting the active kinase via a group presenting only upon kinase activation.[13] We envisioned that we could incorporate an appropriately positioned donor group for targeting this glutamic acid residue. Molecular modeling (Fig. 1) suggested that the 5-position of the 7-phenyl ring of **3** might be optimal for making such an interaction.

Figure 2b depicts a minimized binding mode of a benzotriazine inhibitor (**11**) in the ATP pocket of the activated Src model. The donor group (hydroxyl) could favorably interact with the carboxylate (Glu310) and facilitate its potency. The introduction of a *meta* donor hydroxyl group on the C-7 phenyl resulted in compound **4** with a 20-fold improved potency against Src (**4** vs **3**). The concept of utilizing a carboxylic group buried deep with in a hydrophobic region to improve the potency worked very well with Src, and resulted in potent compounds.[14]

Because several biochemical, crystallographic, and mutagenesis studies revealed that the biochemical regulation and catalytically active state of Abl are very similar to that of Src,[15–17] we started to examine whether we could develop our benzotriazine Src inhibitors to target Abl and more importantly the Abl-T315I mutant by incorporating an appropriately positioned donor interaction in the back pocket. Unsurprisingly the inhibitors of Src showed good potency against Abl as seen with compound **1** and **2**. This led us to continue to investigate whether one could take Src inhibitors and optimize them against the Abl-T315I.[18] Modeling of these benzotriazines in Abl revealed that these inhibitors would bind to ATP pocket of Abl (Fig. 3a) in a similar fashion to

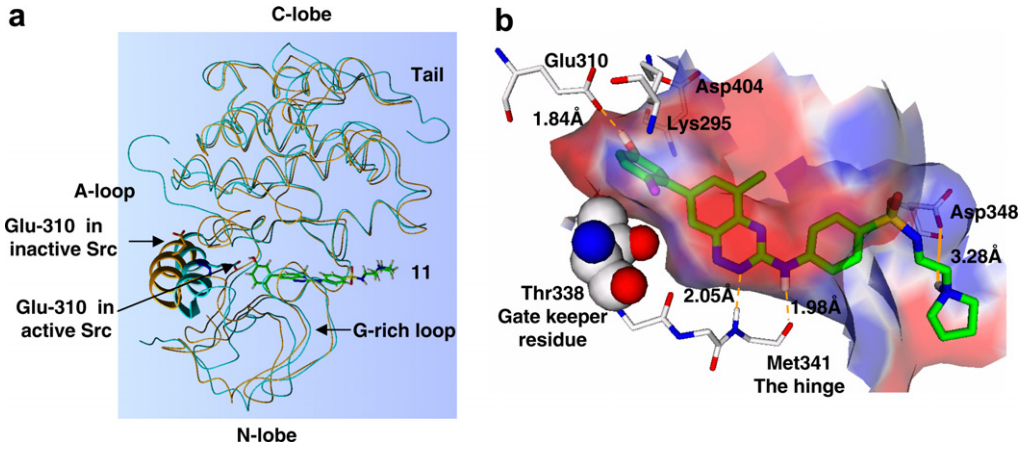


Figure 2. (a) Ribbon diagram of Src superposing Src in the inactive (gold) and active (blue) states. Note the difference in the position of the αC-helix and Glu310 indicating movement from the inactive to the active state. Compound **11** is docked at the ATP pocket. (b) The binding of compound **11** at the ATP pocket of active Src indicating key interactions between **11** and the active Src. Note the hydrogen-bond with Glu310 from the αC-helix.

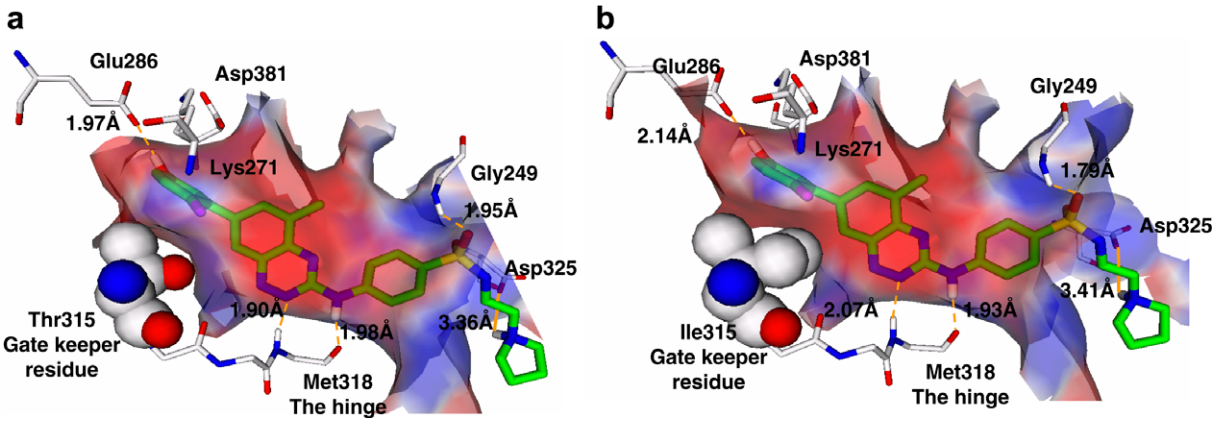


Figure 3. (a) The binding of compound **11** at the ATP pocket of active Abl. The hydroxyl group of the 7-phenyl ring interacts with Glu286 from the αC-helix. (b) The binding of compound **11** at the ATP pocket of Abl-T315I mutant. The hydroxyl group of phenyl interacts with Glu286 from the αC-helix. The gatekeeper residue Ile-315 forms hydrophobic interactions with the aromatic ring of the benzotriazine.

the binding of these inhibitors in Src. More importantly, we observed that these compounds also inhibit the Abl-T315I mutant. We also noticed that one of the fused aromatic rings is in close proximity to the isoleucine (Fig. 3b), thus providing an opportunity for optimization against the more hydrophobic mutated gatekeeper residue 315I. When the analog **3** without a suitably positioned donor group was tested in Abl-T315I, the compound showed poor potency, indicating that hydrophobic contact is not sufficient for obtaining low nM potency against Abl-T315I.

On addition of the appropriately positioned donor group to make contact with the Glu residue in the back of the hydrophobic pocket (**4**), a 30-fold improvement in potency is seen in Abl and a 40-fold improvement in potency in Abl-T315I (Table 1). The donor (hydroxyl group) on the 7-phenyl ring forms a hydrogen-bond with Glu286 on the αC-helix. While the hydroxyl group in Src and Abl provides enhanced potency, the donor interaction in Abl-T315I appears necessary for obtaining low nM potency. Encouraged by these results, we continued lead optimization of this series against the Abl-T315I mutant.[19,20]

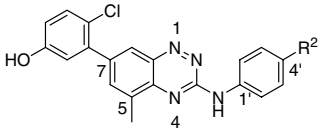
The substitution of the 3-pyrrolidinylpropyl group, as in **5**, in place of the 2-pyrrolidinylethoxy group resulted in a 20-fold improvement in Abl activity but only a 2-fold improvement in Abl-T315I activity. Other changes to this portion of the inhibitor, **6**–**8**, had little to no effect on the potency against Abl, and only a minor effect on Abl-T315I as in the case of **7**. In fact, the substitution of 1,1-dioxodothiomorpholinyl analog **9** and sulfonamide analog **10** did not result in any change in potency versus Abl, but showed a dramatic drop in potency in Abl-T315I.

The introduction of a sulfonamide group in the place of an oxygen atom in **4**, resulted in compound **11** with dramatic improvement in Abl-T315I potency. Modeling studies revealed a strong interaction between the two sulfonamide oxygens towards Gly249 and Asn322 in the solvent accessible channel. The pyrrolidine nitrogen is also interacting with Asp325 near the lip area of solvent accessible region. The combination of these interactions boosted the potency of **11** in Abl-T315I. The methylation of the NH of the sulfonamide improved the potency 10-fold in Abl and 3-fold in Abl-T315I. While the introduction of ethyl (**13**) and propyl (**14**) groups did not change the potency in Abl, but dramatically reduced the potency in Abl-T315I.

A few additional sulfonyl groups (**15**–**19**) were explored, and all showed comparable potency to **11** in Abl, but dramatically reduced potency in Abl-T315I except for compound **18**. These studies demonstrated that the hydrophobic channel in Abl is tolerant to a wide range of substitutions. However, the hydrophobic channel in Abl-T315I has specific requirements and not as tolerant to various groups.

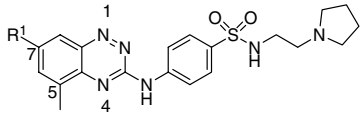
It is evident from the above structure–activity relationship studies that the (2-pyrrolidin-1-ylethyl)sulfonamide substituted analog is one of the most potent compounds. Therefore, we continued our lead optimization efforts while keeping this (2-pyrrolidin-1-ylethyl)sulfonamido group constant and explored various substituted aryl groups at the 7-position (Table 3). While adding or changing the substituents on the aryl ring led to compounds with improved potency against Abl, **21** and **22**, the potency against Abl-T315I remained unchanged or was severely reduced, **20**–**26**, especially when trying to replace the 5-hydroxy with an indole donor group (**24**). Interestingly, the use of an amino group (**25**) in the place of the hydroxyl group in **11**, resulted in a compound with a 5-fold drop in potency in Abl and more than a 700-fold drop in Abl-T315I activity. Moving the hydroxyl group in **11** from the 5-position to 6-position as in **26** did not affect the potency towards Abl, but dramatically reduced the potency towards Abl-T315I mutant. While Abl tolerated several of these compounds at the binding site, the Abl-T315I mutant was more sensitive to changes in structures. The interaction between the 5-hydroxy group and Glu286 appears to be more critical in the Abl-T315I mutant than in Abl. The pK_a and angle of the H-bond donor to the Glu286 are crucial

Table 2. In vitro evaluation of compounds with R^2 modifications on the 3-aminobenzo-[1,2,4]-triazine



Compound	R^2	K_i (nM)	
		Abl	Abl-T315I
4	(2-Pyrrolidin-1-ylethoxy)	1.3	55
5	(3-Pyrrolidin-1-ylpropyl)	0.062	26
6	(2-Pyrrolidin-1-ylethylaminocarbonyl)	2.6	48
7	(Piperazin-1-ylcarbonyl)	1.7	15
8	[(4-Methylpiperazin-1-yl)carbonyl]	0.5	21
9	[(1,1-Dioxodothiomorpholin-4-yl)carbonyl]	4.2	820
10	Sulfonamide	7.7	687
11	[(2-Pyrrolidin-1-ylethyl)sulfonamido]	3	3.4
12	[*N*-Methyl-*N*-(2-pyrrolidin-1-ylethyl)sulfonamido]	0.3	1.2
13	[*N*-Ethyl-*N*-(2-pyrrolidin-1-ylethyl)sulfonamido]	0.4	66
14	[*N*-*i*-Propyl-*N*-(2-pyrrolidin-1-ylethyl)sulfonamido]	2.9	956
15	[*N*-Methyl-*N*-(2-morpholino-4-ylethyl)sulfonamido]	5.1	150
16	(Piperazin-1-ylsulfonyl)	4.4	140
17	[(3-Pyrrolidin-1-ylpropyl)sulfonyl]	1.7	16
18	(Piperidin-4-ylsulfonyl)	3.5	4.7
19	({1-[2-(Diethylamino)ethyl]piperdin-4-yl}sulfonyl)	3.5	163

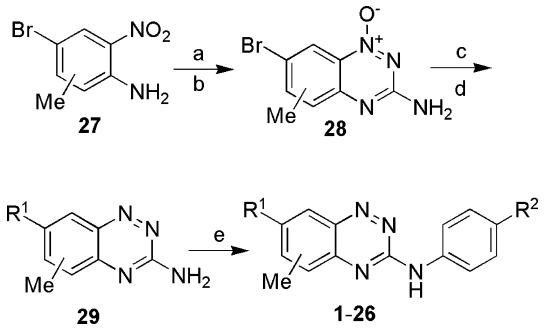
Table 3. In vitro evaluation of compounds with R^1 modifications on the 3-aminobenzo-[1,2,4]-triazine



Compound	R^1	K_i (nM)	
		Abl	Abl-T315I
20	2,6-Cl_2-5-OHphenyl	27	50
21	2-Cl-6-F-5-OHphenyl	1.3	570
22	2,6-F_2-5-OHphenyl	3.4	51
23	2-Br-5-OHphenyl	1	76
24	1H-Indol-4-yl	24	1770
25	5-NH_2-2-Clphenyl	16	>2500
26	2-Cl-6-OHphenyl	5	1620

to obtain Abl-T315I potency. Optimization of other portions of the molecule is required to further tune the potency of these Abl-T315I inhibitors. The threonine to isoleucine mutation results in an enzyme that has very restricted structural requirements for an inhibitor. This partly explains the challenges involved in developing a potent inhibitor targeting the Abl-T315I mutant, and why so many inhibitors of other mutated Abl enzymes are inactive or far less potent against the Abl-T315I mutation.

The synthesis of the compounds mentioned in Tables 1–3 (**1**–**26**) is outlined in Scheme 1. 5- or 6-Methyl substituted 4-bromo-2-nitroanilines were treated with cyanamide in 37% aqueous hydrochloric acid at reflux temperature to give intermediate guanidines. These guanidines without further purification were cyclized to 1-oxobenzotriazines (**28**) using 30% aqueous sodium hydroxide at reflux temperature in a one pot process.[21,22] 1-Oxobenzotriazines were reduced using Raney nickel in ethanol to yield aminobenzotriazines, which were converted to substituted benzotriazines (**29**) by treatment with various aryl boronic acids under Suzuki



Scheme 1. Reagents and conditions: (a) NH_2CN, 37% aq HCl, 110 °C, 1.5 h; (b) 30% aq NaOH, 110 °C, 0.5 h, 65% over two steps; (c) 10% Raney Ni, H_2, EtOH, rt, 4 h, 80%; (d) $R^1B(OH)_2$, $Pd(PPh_3)_4$, K_2CO_3, DME/EtOH/H_2O (4:1:1), Δ, 3 h, 80%; (e) 4-R^2PhBr, $Pd_2(dba)_3$, Xantphos, Cs_2CO_3, dioxane, Δ, 12 h, 70%.

conditions.[23] The final compounds **1**–**26** were prepared from **29** by treatment with various aryl bromides using Buchwald–Hartwig cross-coupling reactions utilizing palladium and Xantphos.[24]

Compound **11** was further evaluated in a cell-based assay for its ability to block both Bcr-Abl and Bcr-Abl-T315I mutant enzymes from phosphorylating downstream substrates (Fig. 4).[25] Both STAT5 and Crkl are phosphorylated by Bcr-Abl as well as by Bcr-Abl-T315I. In both Bcr-Abl and Bcr-Abl-T315I transfected Ba/F3 cells, compound **11** inhibited the phosphorylation of STAT5 and Crkl. The data presented here confirms that Bcr-Abl-T315I functions in a fashion similar to Bcr-Abl in hematopoietic stem cells and thus, inhibition of Bcr-Abl-T315I by **11** is sufficient to induce apoptosis. Further characterization of **11** in both in vitro and in vivo assays is beyond the scope of this manuscript and will be presented separately.

It is established that Bcr-Abl kinase activity is not only essential for proliferation but also required for cell survival since inhibition of Bcr-Abl kinase induces apoptosis in hematopoietic stem cells. Induction of apoptosis in Bcr-Abl cells would help in minimizing the relapse of CML following the termination of administration of Bcr-Abl inhibitors. Thus, the apoptosis-inducing activity of **11** was determined in Bcr-Abl and Bcr-Abl-T315I cells by two apoptotic assays: caspase3 and DNA laddering assays.

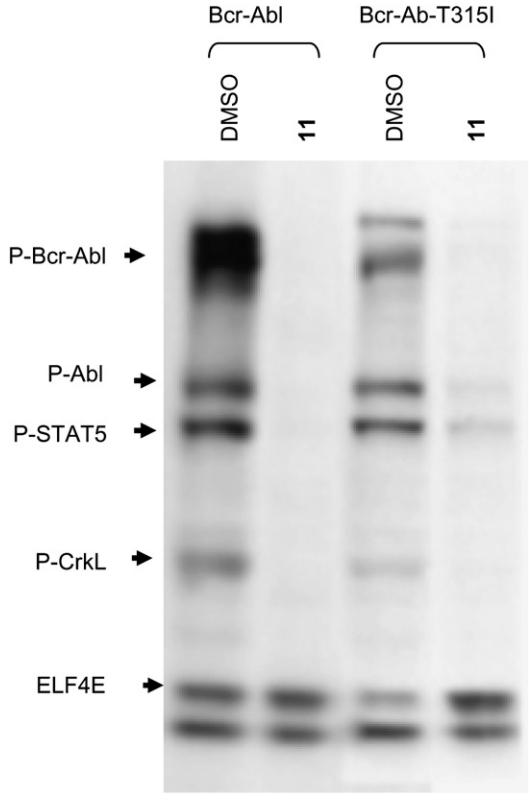


Figure 4. Compound **11** was tested in cell-based assay to study its inhibitory activity. Phosphorylation of both STAT5 and Crkl were blocked in two different Ba/F3 cells containing Bcr-Abl and Bcr-Abl-T315I mutant enzymes.

Caspase3 is the major apoptosis executioner in all cell types tested and identified as a convergent point of multiple apoptosis signaling pathways. In a caspase3 assay,[26] **11** was able to activate caspase3 in both Bcr-Abl and Bcr-Abl-T315I cells. Encouragingly, the pro-apoptotic activity of **11** was not observed in Ba/F3 cells, suggesting that **11**-induced apoptosis is dependent on the Bcr-Abl kinase.

To further confirm that **11**-induced apoptosis by specifically inhibiting Bcr-Abl or Bcr-Abl-T315I, a genomic DNA laddering assay was conducted to detect genomic DNA fragmentation, which represents a point of no return in apoptosis.[27] Compound **11**-induced DNA fragmentation in both Bcr-Abl and Bcr-Abl-T315I cells. The genomic DNA isolated from **11**-treated Ba/F3 cells was intact.

In summary, we have presented a novel strategy to design inhibitors of Abl-T315I. The design took advantage of a hydrogen-bond interaction to a Glu residue buried deep within the hydrophobic pocket and available only after kinase activation. In Abl-T315I, we have found that introducing this interaction provides dramatically improved potency. We have further elucidated the key structural requirements for optimizing this series against the mutant Abl-T315I enzyme. Further, we have utilized the design strategy outlined here to develop two related series with more favorable drug properties and details of these advances will be presented shortly.

Constitutively activated kinases are found in certain malignancies as in the case of CML where abnormal Bcr-Abl protein results in a constitutively active kinase that is the hallmark of the disease. In this particular letter we have described our approach in targeting and developing novel benzotriazine inhibitors of the constitutively activated Bcr-Abl-T315I, a particularly challenging and unresolved problem in Gleevec resistant patients.

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18. K_i values for compounds were determined against Abl (Invitrogen) and the T315I mutant form of Abl (Upstate Cell Signaling Solutions). In white, flat-bottom, 96-well plates (Nunc) assays were run at room temperature at a final volume of 50 μL. Each well contained 40 μL of buffer consisting of 75 mM Tris buffer, pH 7.2 containing 95 mM $MgCl_2$, 1.5 mM EGTA, 0.35 mM Triton X-100, 10 μM β-mercaptoethanol and an appropriate amount of either Abl or T315I-Abl such that the assay was linear over 60 min. Varying amounts (1–100 μM) of Abltide substrate (Upstate Cell Signaling Solutions) in water were added in the presence of a fixed varied amount of compound. The fixed varied concentrations of compounds were generated by adding the appropriate amount in 2.5 μL of DMSO; the DMSO present in each assay was constant at 5%. The reaction was initiated by the addition of ATP to a final concentration of 0.5 mM. After the reaction was allowed to proceed for 60 min, 50 μL of Kinase-Glo reagent (Promega) was added to terminate the reaction. The solution was then allowed to proceed for an additional 10 min to maximize the luminescence reaction. Values were then measured using an Ultra 384 instrument (Tecan, Charlotte, NC, USA) set for luminosity measurements. A control reaction containing no peptide substrate was used for a zero point. Enzyme reaction rates were derived by calculating the difference between kinase catalyzed and uncatalyzed reactions at a specific compound concentration. K_i values were derived from rate data using the non-competitive enzyme kinetics curve fitting capabilities of Prism (Version 4; GraphPad Software, San Diego, CA, USA).
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25. Full-length human Bcr and Abl cDNA were purchased from OriGene (Rockville, MD) and ligated to mimic Philadelphia chromosome formation. The Bcr-Abl cDNA was subcloned into a retroviral vector, pFN-Neo (Strategene, San Diego, CA). Point mutations were introduced into Abl kinase domain by the QuickChange site-directed mutagenesis kit (Strategene, San Diego, CA). The recombinant retroviral vectors were transfected into 293 cells (ATCC, Manassas, VA) to produce recombinant retroviral particles. The retroviral particles were transduced into a mouse pro-B cell line, Ba/F3, (DSMZ, Germany) and selected for by 1 mg/mL G418 in RPMI + 10% FBS + 10 ng/mL IL3 (R&D Systems, Minneapolis, MN). IL3 was omitted from the growth medium for the G418 resistant cells. 2×106 cells were seeded in each 10-cm dish. Compounds were added to the cells following 1 h incubation in CO_2 incubator. Cells were treated with compound for 4 h before being harvested by centrifugation (890 RCF, 4 °C, 5 min). Each cell pellet was lysed with 0.5 mL RIPA buffer (Boston BioProducts, Worcester, MA). Total protein concentrations were obtained by BCA method (Pierce, Rockford, IL). Fifty micrograms of each sample were resolved by 4–12% gradient SDS–PAGE. Following transferring proteins from the gels to nitrocellulose membranes, the membranes were blocked with 5% non-fat dry milk (VONS, San Diego, CA) in TBST. Primary antibodies were incubated with the membranes in 1% non-fat dry milk in TBST followed by incubation with secondary antibodies in 5% non-fat dry milk in TBST. Target proteins were visualized by chemoluminescent reaction (Pierce, Rockford, IL).

26. To determine whether **11** activates capase3, Ba/F3, Bcr-Abl and Bcr-Abl-T315I cells cultured in 96-well plates were treated with **11** at eight final concentrations ranging from 10 to 0.4 μM with 3-fold dilution for 4 h. A fluorogenic caspase3 substrate (Image-iT) was added to the cells which covalently bound to the active caspase3 in apoptotic cells. The fluorescent intensity of stained cells in each well was collectively quantified by a fluorescence plate reader (TECAN).
27. Ba/F3, Bcr-Abl and Bcr-Abl-T315I cells cultured in 10-cm dishes were treated with 2 μM **11** for 24 h, followed by isolation of genomic DNA. Genomic DNA fragmentation was visualized by electrophoresis on agarose gels.