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Inhibitors of the Abl kinase directed at either the ATP- or myristate-binding site

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ABSTRACT

The ATP-competitive inhibitors dasatinib and nilotinib, which bind to catalytically different conformations of the Abl kinase domain, have recently been approved for the treatment of imatinib-resistant CML. These two new drugs, albeit very efficient against most of the imatinib-resistant mutants of Bcr–Abl, fail to effectively suppress the Bcr–Abl activity of the T315I (or gatekeeper) mutation. Generating new ATP site-binding drugs that target the T315I in Abl has been hampered, amongst others, by target selectivity, which is frequently an issue when developing ATP-competitive inhibitors. Recently, using an unbiased cellular screening approach, GNF-2, a non-ATP-competitive inhibitor, has been identified that demonstrates cellular activity against Bcr–Abl transformed cells. The exquisite selectivity of GNF-2 is due to the finding that it targets the myristate binding site located near the C-terminus of the Abl kinase domain, as demonstrated by genetic approaches, solution NMR and X-ray crystallography. GNF-2, like myristate, is able to induce and/or stabilize the clamped inactive conformation of Abl analogous to the SH2-Y527 interaction of Src. The molecular mechanism for allosteric inhibition by the GNF-2 inhibitor class, and the combined effects with ATP-competitive inhibitors such as nilotinib and imatinib on wild-type Abl and imatinib-resistant mutants, in particular the T315I gatekeeper mutant, are reviewed.

1. Introduction

About one-third of the protein targets under investigation by the pharmaceutical industries are either protein kinases or lipid kinases. To date, several small molecular weight kinase inhibitors have been launched [1–6]. In addition, more than 60 kinase drugs targeted to a handful of protein and lipid kinases are in clinical development, with many more in various stages of pre-clinical development [1–7]. Given the roles played by various protein and lipid kinases in cell proliferation and apoptosis, it is not surprising that the majority of investigational kinase inhibitors are being developed to treat human malignancies. This first wave of ATP-site directed kinase inhibitors may be viewed as first generation molecules. Although we have a good knowledge of the structural determinants for the ATP binding site with respect to kinase inhibitors [4,7], selectivity, as well as a limited set of chemotypes targeting the ATP binding site – a highly crowded area – have become major issues in protein and lipid kinase drug discovery.

Imatinib been has shown to target dominant oncogenes including Abl, Kit, and PDGFR that are constitutively activated in various forms of human malignancies [8–10]. Although responses to imatinib treatment are durable, patients with advanced disease frequently become resistant to imatinib treatment, due to mutations in the tyrosine kinase domain of the target kinases Bcr–Abl, Kit, DDR and/or PDGFR that impair imatinib binding [2,10–12,22]. To date, two novel ATP-competitive inhibitors, nilotinib and dasatinib, have been registered for the treatment of imatinib-resistant CML. These drugs show different selectivity profiles because they bind to catalytically different conformations of the Abl kinase domain [13–16,33].

ATP binds in a cleft between a small N-terminal lobe and a larger C-terminal lobe of the protein kinase domain via two hydrogen bonds to the connector of the two lobes also referred to as the hinge while the adenine group is surrounded by two hydrophobic pockets, the entrance of one of which is regulated by the so-called gatekeeper residue [2,10]. The ATP cleft is lined by structural elements responsible for the catalytic activity of the kinase including the activation loop (A-loop), which represents the platform for the binding of the protein substrate. Both nilotinib and imatinib which have one hydrogen bond contact to the hinge are known to stabilize a particular inactive conformation of the

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Abl kinase also referred to as the DFG-out [2,10,41]. The DFG motif, which is located at the N-terminus of the so-called A-loop, can adopt different conformations ranging from the fully active (DFG-in) to the fully inactive (DFG-out) [2,10,41]. In contrast, dasatinib targets the active conformation (DFG-in) of the Abl kinase as demonstrated by X-ray and solution NMR [2,15,36], which may be one of the reasons why nilotinib and imatinib have a more restricted in vitro selectivity profile compared to dasatinib [35].

Although dasatinib and nilotinib are very efficient against most of the imatinib-resistant mutants of Bcr–Abl, neither drug effectively inhibits the Bcr–Abl activity of the T315I mutation, also known as the gatekeeper mutation [13–15,33]. This single amino-acid substitution causes a disruption of the inactive conformation of the Abl kinase domain [10,22,43,44] achieved by stabilization of the so-called hydrophobic spine – a network of hydrophobic interactions in the kinase domain – that promotes the assembly of the active kinase conformation [42]. A recent elegant study reported that the gatekeeper mutation is activating in various tyrosine kinases [43]. One potential approach to inhibit the T315I gatekeeper mutation of Bcr–Abl would be to target the destabilized hydrophobic spine by ATP-site directed compounds [43]. Although many attempts have been undertaken to target the ATP binding in order to inhibit the gatekeeper mutation of Bcr–Abl [2,16,33,43], with one exception none of these compounds have entered clinical trials.

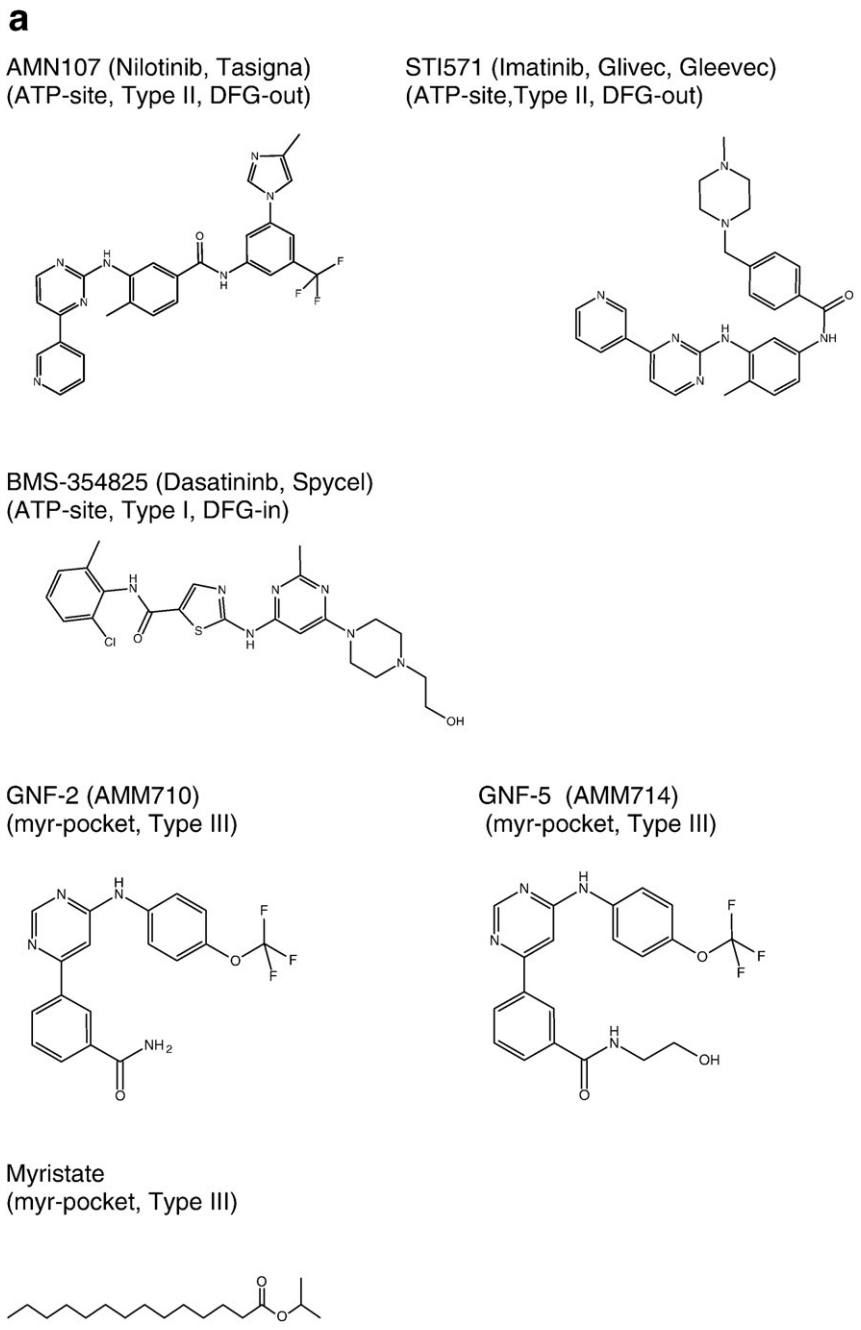

Fig. 1. Structures of myr-pocket and ATP-site binders and structure of the Abl kinase domain liganded to imatinib and GNF-2. a) The structures of nilotinib, imatinib, dasatinib, myristate, GNF-2 and GN5 are shown. b) 2 representations of Abl kinase domain (Abl229–515) with GNF-2 in the C-terminal lobe of the Abl kinase domain and imatinib occupying the ATP binding cleft between the N-terminal and C-terminal lobes of the Abl kinase domain. Iα = I helix; Cα = C helix. In the left representation the ATP and the protein subsrate binding site are indicated.

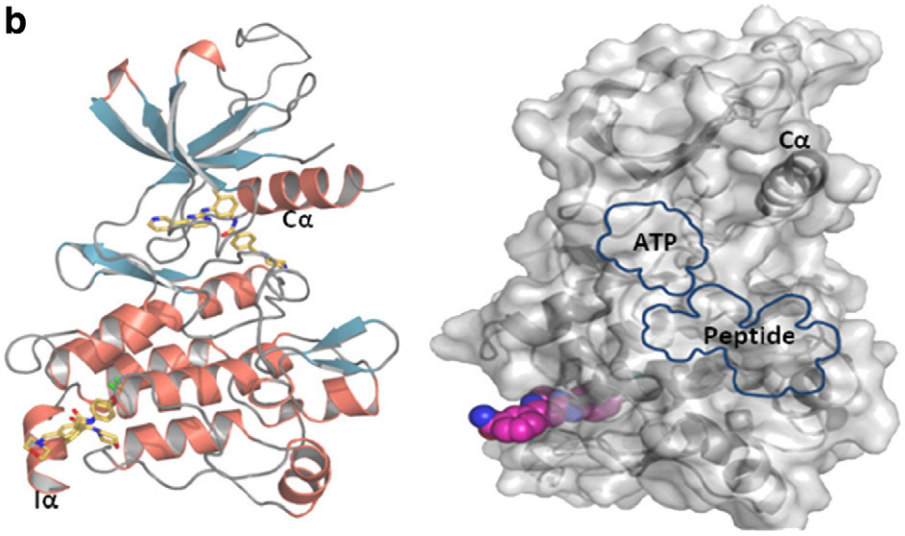

Fig. 1 (*continued*).

Recently AP24534, a potent, orally available ATP-competitive multi-targeted purine-based inhibitor active against the T315I and other Bcr–Abl mutants has entered Phase I clinical trials [37,38]. According to preliminary analysis this promiscuous inhibitor, a characteristic of most compounds targeting the gatekeeper mutation, appears to show evidence of clinical antitumor activity in patients with resistance to the T315I mutation of Bcr–Abl [38].

Another possibility to override the T315I gatekeeper mutation is to target the Abl kinase outside of the ATP-binding pocket. In this respect, GNF-2, a 4-6-di-substituted pyrimidine, has been indentified, which demonstrates an exquisite selectivity towards the Abl kinase and Bcr–Abl transformed cells without inhibiting the kinase domain of Abl, represents an interesting starting point [17,18]. Recent data indicate the presence of a myristate binding pocket (myr-pocket) in the C-terminal lobe of the kinase domain of Abl to which GNF-2-type compounds are able to bind resulting in the stabilization of the clamped inactive conformation of Abl [17,18]. The molecular mechanism of the allosteric inhibition by the myr-pocket binders GNF-2 and the combined effects with ATP-competitive inhibitors such as nilotinib, imatinib and dasatinib on the Abl and Bcr–Abl are reviewed in this report.

2. Experimental procedures

2.1. Expression and purification of protein kinase

Expression and purification of human Abl was performed using standard expression purification procedures. The following Abl proteins were generated and used for *in vitro* kinase assays: Abl64–515 (1a amino acid numbering), also referred to as SH3SH2SH1-Abl), and the respective point mutants T315I–Abl64–515 and E505K–Abl64–515, as well as different lengths of the catalytic domains of Abl, namely Abl229–515, Abl229–580, Abl229–515, Abl218–500, Abl229–500 and the gatekeeper mutant T315I–Abl229–515. The recombinant kinase domains of Abl were purified as described earlier [10,17] while the recombinant human SH3SH2H1-Abl proteins were produced by a modifications of published procedures [18–20,34]. The latter proteins were generated by a co-expression vector carrying the DNA fragments for Abl (1a isoform, as well as mutants encompassing residues 64–515, with an N-terminal His6-tag followed by a PreScission protease cleavage site) and the human protein tyrosine phosphatase-1B (residues 1–283, untagged), using the dual expression vector pCDF Duet-1 (Novagen). The His-Abl was expressed in *E. coli* BL21 (DE3) and the Abl proteins were isolated by Ni-affinity on a Ni-NTA column (Qiagen). The His-tag was removed by PreScission protease (GE Healthcare) and the non-phosphorylated Abl further purified on a Mono Q HR 10/10 (GE Healthcare, mono-phosphorylated Abl is about 10–20% of total Abl protein) and HiLoad 16/60 Superdex 200 size exclusion column (GE Healthcare) [10,17,34]. Non-phosphorylated Abl64–515 proteins were analyzed by Mass Spec analysis and flash-frozen in aliquots and stored at −80 °C. Src (amino acids 83–535 or Src83–535) was expressed and purified as previously described [21].

2.2. Radiometric filter binding assays

For determination of Abl kinase activity, the radiometric filter binding assay was used [10,13,17,21]. The assay was performed by mixing 10 µL of the compound pre-diluted with 10 µL of ATP (20 µM ATP with 0.1 µCi [γ-33P]-ATP) with the phospho-acceptor peptide poly[Ala6Glu2LysHBr5Tyr1] = poly-AEKY) in 20 mM Tris/HCl pH 7.5, 1 mM DTT, 10 mM $MgCl_2$, 0.01 mM Na_3VO_4, 50 mM NaCl as described elsewhere [10,13,17,21]. 10 µL of enzyme (ranging between 5 nM to 20 nM) was added to initiate the reaction. Pre-incubation of enzyme with compounds (when stated) was performed by exposing the enzyme to compounds prior to addition of the substrate mixture (ATP and/or peptide substrate). After 15 min at room temperature, the reaction was stopped by the addition of 50 µL 125 mM EDTA, and the peptide-bound 33P separated on filter-plates (PVDF or MAIP; Millipore, Volketswil, Switzerland) prepared according to the manufacturer's instructions. Filter-plates were washed 3× with 0.5% H_3PO_4, followed by addition of 30 µL scintillation cocktail (Microscint, Perkin Elmer) per well and then analyzed in a TopCount NXT scintillation counter (Perkin Elmer). Results were expressed as IC_{50} values as earlier described [10,17,21].

The K_m values for ATP were determined by assaying the Abl kinase with increasing concentrations of ATP and keeping the exogenous acceptor protein substrate (poly-AEKY) at a constant concentration (at about 2-fold its K_m) and *vice versa*. K_m and V_{max} were calculated according to Eadie–Hofstee as described previously [10]. The data were plotted as *V versus V/S*, where *V* is the velocity of the reaction at a given substrate (*S*) concentration, and fitted to a straight line using linear regression analysis, where the slope of the line corresponds to $-K_m$ and the *Y*-intercept represents the V_{max}.

2.3. Cellular auto-phosphorylation of Bcr–Abl (pELISA) and viability assay

The phosphorylation status of the cellular targets in lysates from cells was determined using a capture ELISA as described previously

[13,22]. Cells grown in 96-well wells were treated with serial compound dilutions followed by removal of culture supernatants after 1 hour. Cells were then lysed as described [13,22] and 50 µL of the lysates were transferred to black ELISA plates (NUNC-Maxisorp) coated with the anti-Abl SH3 domain specific polyclonal Ab (50 ng/well; Upstate Biotechnologies). Following incubation and washing, the phosphorylation status of Bcr–Abl was detected using a commercial anti-phospho-Tyr-Ab, labeled with alkaline phosphatase (PY20-AP, Zymed). Detection was done using the chemi-luminescent AP substrate (CDPStar RTU with Emerald II, Applied Biosystems), and luminescence quantified by measuring counts per second (cps) with a Packard Top Count Microplate Scintillation Counter. IC_{50} values were calculated by graphical extrapolation of the dose–response curves as described [13,22].

Cell viability was determined by luminescent ATP detection (ATPLite™, Perkin Elmer Life Sciences) [13,22], which is based upon the production of light (luminescence) caused by the reaction of ATP with added luciferase and D-luciferin. Untreated cells were used as control, and medium without cells was used to determine the assay background signal. After 70 h incubation with compounds at 37 °C in 5% CO_2, the cells were lysed and luciferase and D-luciferin were added. After 5 min shaking and 10 min dark-adaptation of the plates, light emission was measured with a Packard TopCount. IC_{50} values were determined from the dose–response curves by graphical extrapolation as described [13,22].

2.4. Combination experiments

To determine the nature of the drug interaction (synergism, additivity or antagonism) with respect to in vitro kinase inhibition, the combination index method based on the median dose effect principle developed by Chou and Talalay [29,39,40] was used. The Combination Index (CI) is calculated by the isobologram equation: $CI = (D)1/(Dx)1 + (D)2/(Dx)2]$ where $(D)1$ and $(D)2$ are the doses of drug 1 and drug 2 in combination that cause *x*% kinase inhibition and $(Dx)1$ and $(Dx)2$ are the doses of drug 1 and drug 2 alone, respectively, that cause *x*% kinase inhibition. $CI<1$ or $CI>1$ indicates greater than additive effects. For synergism the smaller the CI value is the greater the degree of synergy and in the case of antagonism the greater the value the greater the antagonism. Additivity, antagonism or synergism were examined by isobologram where the *X* and *Y* intercepts indicate the concentrations of either compound alone resulting in a 50% kinase inhibition (IC_{50}). The data point that falls between the axes indicates the concentration of the drug combination that inhibits the kinase activity. Data point above or below the straight line joining the intercepts (IC_{50}) indicate antagonistic or synergistic the effect, respectively, while data points that fall on or close to the line joining the intercepts are indicate additive effects. It should be noted that significant synergism or antagonism is obtained when $CI<0.5$ and $CI<2.0$, respectively.

3. Results

3.1. The myristate binding pocket in Abl

Recent structural evidence indicates the presence of a myr-pocket in the C-terminal lobe of the kinase domain of Abl [16–18,47]. This pocket has recently been targeted by compounds which include the 4,6-di-substituted pyrimidines also known as GNF-2 and GNF-5 (the hydroxy-ethylamide analog of GNF-2) [17,18] (Fig. 1a). Solution phase NMR and X-ray crystallography, unambiguously demonstrate that GNF-2 binds to this recently identified myr-pocket [16–18]. These results also confirm earlier findings demonstrating that the N-myristoylated peptide of Abl is able to displace Bcr–Abl or Abl from a GNF-2 affinity matrix [17]. Therefore, these compounds are referred to as myr-pocket binders to differentiate them from the ATP-pocket binders like nilotinib, imatinib or dasatinib (BMS354825) (Fig. 1a).

GNF-2, GNF-5, myristate and the N-terminal myr-Abl peptide (N-myristoylated peptide corresponding amino acids 2–16 of Abl) are able to bind to the myr-pocket of Abl229–515, but not to the shorter version of the Abl kinase domain (Abl229–500) as demonstrated by solution NMR (Table 1 and [17,18]). The kinase domain of Abl lacking the 15 amino acids at the C-terminus (Abl229–500) is unable to bind myr-pocket binders because it cannot form the helix-I (Iα) which is an important structural feature for the binding of the myristate moiety [18] (Fig. 1).

Fig. 1b shows the overall crystal structure of Abl kinase domain (Abl229–515) with GNF-2 liganded to the myr-pocket and imatinib bound to the ATP-binding site. It should be emphasized, that only those Abl kinase domain structures that contain imatinib bound to the ATP-binding pocket have been able to be solved with the myr-pocket binders. The requirement for ATP ligands in the form of ATP-site-directed inhibitors is essential to obtain stable of the Abl kinase domain for X-ray crystallography [10,21,44]. There is very little difference in the ATP-binding site as well as between the relative orientations the N- and terminal C-lobe of the Abl kinase domain when comparing the Abl–imatinib complex with the Abl–imatinib–GNF-2 or Abl–imatinib–myristate complexes [18,23,24,47]. A detailed description of the residues lining the myr-pocket and involved in binding GNF-2 has been recently reported [18]. GNF-2 binds in an extended conformation into the myr-pocket, the majority of the interactions being hydrophobic where the trifluor-methoxy

Table 1
Effects of ATP-site and myr-pocket binders on the kinase activity of Abl in enzymatic assays and cells.

	Enzyme kinase activity (IC50 µM)				Kd by NMR (µM)		Cellular pELISA (IC50 µM)		
	SH1	SH3SH2SH1	SH3SH2SH1	SH3SH2SH1	SH1	SH1	p210	p210	p210
	wt	wt	T315I	K505E	229–515	229–500	wt	T315I	K505E
AMN107	0.028	0.025	1.8	0.054	na	na	0.028	>10	0.012
STI571	0.033	0.012	>10	0.038	na	na	0.543	>10	0.242
BCV841	0.002	0.003	6.002	0.006	na		0.003	>10	0.005
GNF2	>10	0.009	>10	>10	0.2	>10	0.212	>10	>10
GNF5	>10	0.017	>10	>10	na	na	0.121	>10	>10
Abl-myrpep	>10	1.002	>10	>10	na	na	na	na	na
Src-myrpep	>10	0.982	>10	>10	na	na	na	na	na
myr	>10	2.782	>10	>10	20	na	na	na	na
CPD-X	>10	0.009	>10	>10	0.02	na	na	na	na

The kinase domain of Abl (SH1: Abl229–515 and Abl229–500), Abl64–515 (SH3SH2SH1) and the respective T315I and E505K mutants were prepared and the kinase assay was carried out as described in Experimental procedures. Kd by NMR was performed exactly as described in [18]. Capture ELISA (pELISA) of autophosphorylated Bcr-Abl-p210 (wt, T315 and E505K mutants) expressed in BaF3 cells was carried out as described in Experimental procedures. The E505K data are from reference [18]. na = not done. Results are means of at least three independent experiments without SD (omitted for space reasons).

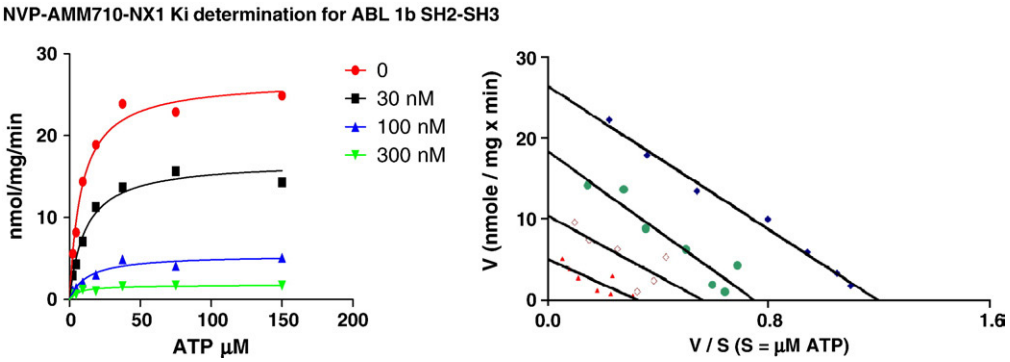

Fig. 2. Effects of increasing concentrations of AMM710 (GNF-2) on Abl64–515. Enzyme kinetics of GNF-2. The ATP concentrations were varied between 1.25 and 100 µM while GNF-2 concentrations varied between 30 and 300 nM. Left panel shows raw data and right panel shows an Eadie–Hofstee representation (plotted as V versus V/S as described in Experimental procedures).

group plays an important role [18]. Except for the positions of a few residues, the overall structure of the Abl kinase domain bound with GNF-2 is very similar to that of the myristate-bound form.

3.2. Effects of binding of GNF-2 and derivatives to the myr-pocket of Abl

In contrast to the ATP-site directed inhibitors dasatinib, nilotinib or imatinib, the protein kinase activity of the Abl kinase domain (Abl229–515 or Abl229–500) was not affected by the presence of myr-pocket binders (myristate, GNF-2, GNF-5 and myr-peptide) (Table 1). These data confirm previous findings [17,18] and indicate that the binding to the myr-pocket has no functional consequences on the kinase activity of Abl. In contrast, there was a dose-dependent inhibition of the protein kinase activity of the Abl kinase carrying the SH3 and SH2 domains (Abl64–515), in the presence of increasing concentrations of the myr-pocket binders (Table 1). Both ABL1 and ABL2 also known as Abl and Arg, respectively, which comprise the Abl family of non-receptor tyrosine kinases, have an isoform that is myristoylated at the N-terminus and the other that is deficient in N-myristoylation due to an alternative splicing of the first exon [8]. The N-terminal myristoyl group together with the SH3 and SH2 modules that are located N-terminal to the kinase domain induce and stabilize the assembled inactive state as predicted from the 3-dimensional Abl kinase structure [16,18,23,47]. The assembly of the N-myristoyl deficient Abl carrying the SH3 and SH2 domains (Abl64–515) into the clamped catalytically inactive state can be mimicked by binding of myristate or other myr-pocket binders resulting in the inhibition of the kinase activity (Table 1). The Abl myr-pocket appears to function also in the oncogenic form of Bcr–Abl as major anchor point for the assembly of the inactive state as demonstrated by the finding that Bcr–Abl auto-phosphorylation in cells is potently inhibited by the myr-pocket binders GNF-2 and GNF-5 (Table 3) [17,18].

Enzyme kinetics with Abl64–515 revealed that GNF-2 is non-competitive with respect to ATP (Fig. 2, change in V_{max} with no change in K_m). Similar ATP-non-competitive kinetics was observed with all of the other myr-pocket binders like GNF-5, CPD-X and the N-terminal myristoylated peptides. Increasing the concentration of GNF-2 in combination with GNF-5 resulted in additive effects with respect to inhibition of the Abl64–515 kinase activity (Fig. 3) indicating that these two compounds act in a similar way to inhibit the protein kinase activity of Abl64–515. Taken together, all of these data strongly indicate that the myr-pocket binder act in an ATP-non-competitive manner and achieve inhibition of Abl kinase activity by stabilizing the assembled inactive conformation of Abl which is stabilized by docking of the SH3 and SH2 domains onto the Abl kinase domain [16,18,23,47].

3.3. The myristate binding sites in Src

A myristate binding site similar to that found in Abl was recently described in the C-terminal lobe of the kinase domain of Src which displays an overall kinase architecture similar to Abl [21]. No effects on the Src kinase activity were observed when Src containing the SH3 and SH2 domains (Src83–535) was incubated with the N-terminal myristoylated peptide derived for either Src or Abl (Table 2). Consequently no effects of myristate or GNF-2 were observed on the kinase activity of Src. In contrast, both N-terminal myristoylated peptides derived from either Src or Abl encompassing amino acids 2–16 of the respective kinase were very effective in inhibiting the kinase activity of Abl64–515 (Table 2). In agreement with previous findings the only ATP-site-binders capable of inhibiting the activity of Src was dasatinib [11,15,21]. These data indicate that myr-pockets when present in protein kinases may serve different purposes. In Src, the myr-pocket appears not to contribute to the assembly of the clamped inactive state while myristoylation of the N-terminus of Abl, which occurs in only in one of the two Abl splice variants [8], is proposed to induce a closed and assembled inactive conformation of Abl. In Src the assembled inactive conformation occurs mainly via binding of the SH2 to the C-terminally phosphorylated Y527 [25,26].

N-myristoylation and N-palmitoylation have also been shown to serve as a mechanism for targeting proteins to cellular membranes [25,27]. Recent results suggest that GNF-2 inhibits the kinase activity of non-myristoylated Abl (or Arg) as potently as that of the myristoylated Abl (or Arg) leading to differential localization of the

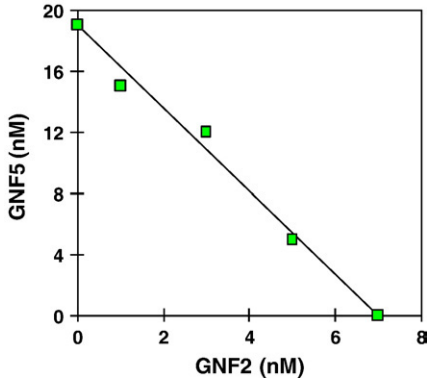

Fig. 3. Additive effects of increasing concentrations of GNF-2 and GNF-5 on the kinase activity Abl64–515. Isobologram analysis showing simultaneous exposure of Abl64–515 to increasing concentrations of GNF-2 and GNF-5. Starting concentrations were 10 µM for each compound with 3 step dilutions down to 0.003 µM. Isobologram analysis was performed as described in Experimental procedures.

Table 2
Effects of ATP-site and myr-pocket binders on the kinase activity of Src.

	Src kinase activity (IC50 μM)
BCV841	0.003 ± 0.001
AMN107	>10
STI571	>10
GNF2	>10
GNF5	>10
Abl-myrpep	>10
Src-myrpep	>10
myr	>10

The Src kinase containing SH2 and SH3 was prepared and kinase assay was carried out as described in Experimental procedures. Results are mean of at least three independent experiments.

myristoylated Abl compared to its non-myristoylated counterpart [28]. In addition to cellular relocation, the myr-pocket of Abl may also be used for the recruitment of celullar N-myristoylated proteins or protein kinases to the site of action of Abl, in particular for the splice forms of Abl and Arg that are deficient in N-myristoylation. Moreover, the myr-pocket in Src or Abl may serve as a home base for its own myristoylated N-terminus which depending on the activation state of Src or Abl can be used as anchor to locate and tether Src or Abl following its activation in cells to membranes or to other proteins that have similar myr-pockets [27]. Alternatively, the myr-pocket of Src or Abl may be used to recruit other N-myristoylated proteins or protein kinases to the Src or Abl kinases. Alignment of the primary sequences of Src and Abl encompassing the myr-pocket did not reveal any evidence for similarity suggesting that the presence of a myr-pocket in protein kinases (and other proteins) may become only evident from the 3-dimensional structure.

3.4. Effects of myr-pocket binders on imatinib-resistant mutations of Abl or of Bcr–Abl

GNF-2 and GNF-5 showed a higher potency in the biochemical kinase assay as compared to the IC50 obtained using the auto-phosphorylation of Bcr–Abl in BaF3 cells, indicating that the assembly of the inactive state of the p210-Bcr–Abl may be more difficult to achieve compared to Abl64–515 in the biochemical assay (compare Tables 1 and 3). Point mutations in and around the ATP-binding sites of Bcr–Abl usually result in a loss of inhibitory potency of the ATP-site binders in particular imatinib, nilotinib and dasatinib as determined by reduced auto-phosphorylation of Bcr–Abl in cell-based assays or substrate phosphorylation in biochemical assay using the kinase Abl domain [22,45]. Many of these mutations have been shown to be responsible for the clinical resistance of Bcr–Abl to these drugs [9–12,30,33]. Therefore, various combinations of site directed mutagenesis and cellular read outs following exposure of cells to increasing concentrations of drugs have been used *in vitro* to obtain and predict resistance to Bcr–Abl drugs targeting the ATP-binding site [18,30,31]. Two independent mutagenesis approaches resulted in GNF-2 resistant Bcr–Abl mutants which were found to cluster mainly around the myr-pocket, the SH2 and SH3 domains [18]. In particular, one mutation, the E505K, which is located in the myristate binding site of Bcr–Abl abolished the inhibitory activities of the myr-pocket binders in vitro (Tables 1 and 3). According to the crystal structure, the E505K mutation which is located in the second shell of residues forming the myrsitate binding site is likely to have unfavorable steric effects with respect to the GNF-2 binding [18]. When the E505K mutation was transferred to the Abl64–515 (E505K–Abl64–515) the protein kinase activity was shown to be completely insensitive to all of the myr-pocket binders, but still as sensitive to inhibition by the ATP-site-binders as the non mutated Abl64–515 version (Table 1). Most importantly, the T315I gatekeeper mutation which completely abrogates the inhibition of the ATP-site-binders dasatinib, nilotinib or imatinib was also totally insensitive to the myr-pocket binders, not only in the biochemical assay but also in cells (Tables 1 and 3). Point mutations in the ATP-binding pocket of Abl or Bcr–Abl, other than the T315I gatekeeper are also known to increase resistance to imatinib [9–12,30,31,33]. As shown in Table 3, some of the other imatinib-resistant mutations [10] were found to have increased resistance against the myr-pocket binders as well as ATP-site-binders. In particular the mutations in amino acids 250, 255, 351 and 317 which are known to destabilize the inactive conformation of the Abl and Bcr–Abl kinase [10,44] also showed a significant reduction in the ability of the myr-pocket binders to assemble the inactive clamped conformation of Abl and Bcr–Abl (Table 3). However, none of these mutations was as effective as T315I in abrogating the inhibitory activity of ATP-site- and myr-pocket binders (Table 3). While the E505K resistance can be explained with the available structural information of the GNF-2 bound to the myr-pocket of Abl kinase domain, it remains an enigma why myr-pocket binders are unable to assemble the inactive conformation of the gatekeeper mutation of Abl64–515 or Bcr–Abl. The T315I substitution has been shown to results in a disruption of the inactive conformation of the Abl kinase domain by stabilization of the so-called hydrohobic spine in the kinase domain that assembles the active kinase conformation [10,22,42–44]. Thus, the gatekeeper mutation that leads to the resistance of ATP-site and myr-pocket binders is an activating mutation which apparently locks the Abl kinase in a permanently activated state. Attempts to purify the T315I Abl kinase for X-ray crystallography either with or without the SH3–SH2 domains in the absence of compounds have been hampered by the fact that the T315I mutation of the Abl protein is rather unstable. This is in stark contrast to the wt Abl which can be purified with good yields. It appears as if the gatekeeper mutation is able to lock Abl into the active conformation resulting in an unstable protein.

Table 3
Effects of ATP-site and myr-pocket binders on the auto-phosphorylation and proliferation of BafF3 cells expressing p210Bcr–Abl and p210Bcr–Abl mutants.

	Cell-based (IC50 μM)														
	Growth	pELISA	Growth	pELISA	Growth	pELISA	Growth	pELISA	Growth	pELISA	Growth	pELISA	Growth	pELISA	Growth
	BaF3	p210	p210	p210	p210	p210	p210	p210	p210	p210	p210	p210	p210	p210	p210
	wt	wt	wt	T315I	T315I	K505E	K505E	E255K	E255K	G250E	G250E	M351T	M351T	F317L	F317L
Imatinib	>10	0.612	0.494	>10	>10	0.242	0.321	2.345	5.781	4.825	3.374	0.696	2.076	0.968	2.254
GNF2	>10	0.182	0.576	>10	>10	>10	>10	1.0812	7.045	5.321	3.642	0.418	5.098	0.946	>10
GNF5	>10	0.121	0.145	>10	>10	>10	>10	0.338	1.664	2.991	1.094	0.097	1.176	0.432	5.237
CPD-X	>10	0.009	0.025	0.517	>10	>10	>10	0.069	0.368	0.196	0.397	0.022	0.351	0.078	1.73

Proliferation (Growth) and capture ELISA (pELISA) of autophosophorylated p210 (wt, T315, E505K, G250E, E255K, F317L and M351T p210-Bcr–Abl) expressed in BaF3 cells were carried out as described in Experimental procedures. Results are means of at least three independent experiments without SD (omitted for space reasons). The E505K data are from reference 18. BaF3 wt denotes BaF3 cells that do not express any oncogene and which grow in an IL-3-dependent manner.

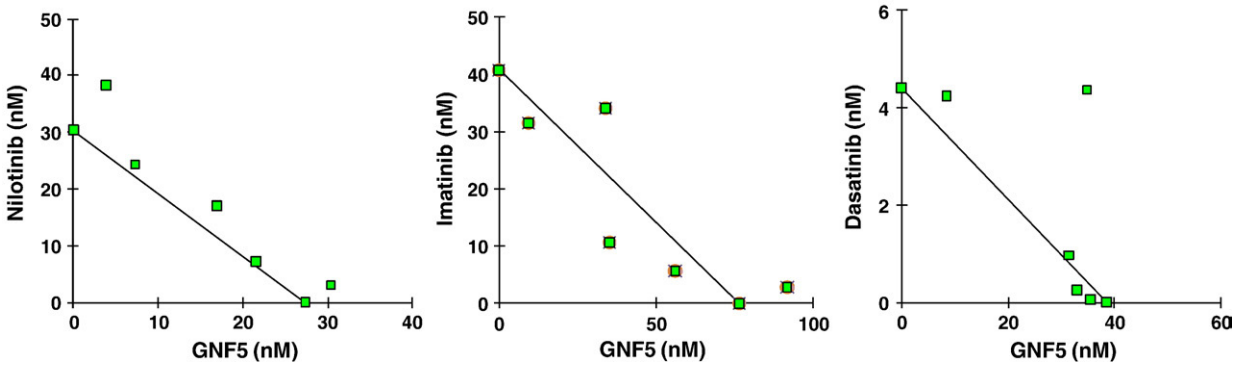

Fig. 4. Additive effects of increasing concentrations of GNF-5 and Imatinib on the kinase activity Abl64–515. Isobologram analysis showing simultaneous exposure of Abl64–515 to increasing concentrations of Nilotinib (A), Imatinib (B), Dasatinib (C) with GNF-5. Starting concentrations were 10 μM for each compound with 3 step dilutions. Isobologram analysis was performed as described in Experimental procedures [29].

3.5. Effects of combination of myr-pocket binders and ATP-site binders on imatinib-resistant mutations of Abl or of Bcr–Abl

One strategy to address the T315I mutation would be a more potent myr-pocket binder capable of restoring the assembled inactive conformation. However, the possibility cannot be ruled out that the T315I is completely incompatible with the assembled state of the Abl molecule. An alternative strategy would be small molecular weight inhibitors targeting the ATP binding site and showing complementarity to the dismantled hydrophobic spine such that they inhibit the T315I gatekeeper mutation of Abl [37,38]. A third possibility to override the T315I mutation would be to use the myr-pocket in combination with the ATP-site binders.

According to the isobologram analysis, the combinations of myr-pocket and ATP-site binders were shown to be additive with respect to inhibition of the protein kinase activity of Abl carrying the SH3 and SH2 domains (Abl64–515) in biochemical assays (Fig. 4). The sequence of incubation with either of the myr-pocket or ATP-site-binders as well as length of incubation did not change the shape of the isobologram suggesting additivity between myr-pocket and ATP-site binder in inhibiting the protein kinase activity of Abl64–515 (Fig. 4). There was no evidence for a significant difference in additivity between dasatinib, nilotinib or imatinib which are known to target different conformations of the Abl kinase [2,10,15,36]. Nilotinib and imatinib are known to target the inactive (DFG-out), while dasatinib binds the active (DFG-in) conformation of Abl. The assembled inactive clamped conformation of the Abl64–515 is compatible with binding of ATP-pocket binder irrespective of their binding mode [23,24,45,46]. Similar additive effects of myr-pocket binders and ATP-site inhibitors with respect to the inhibition of both auto-phosphorylation and proliferation were noted in BaF3 expressing wt p210-Bcr–Abl. Whether there is a more subtle cross-talk between the ATP-binding pocket and the myr-pocket as has been recently postulated by using hydrogen exchange mass spectrometry which allows the dynamics of a protein to be investigated by measuring the exchange of backbone amide hydrogen with the bulk solvent [18,32], remains to be studied more in detail.

GNF-2 and GNF-5 were developed as single-agent inhibitors of Bcr–Abl and there may be the potential that another class of myristate ligands could be discovered that exhibit greater synergy for inhibition of Bcr–Abl in combination with ATP-site binders. Additivity between the myr-pocket and ATP-site binder was observed against the T315I mutant in cells or with recombinant T315I–Abl64–515 using concentrations well above 10 μM of either of type of compound. Additivity between myr-pocket and the ATP-site binders against the T315I mutant has been previously noted *in vitro* as well as *in vivo* animal studies [17,18]. Although these reported experiments appear promising the degree of additivity between myr-pocket binder (GNF-5 or GNF-2) and ATP-site binders (nilotinib or imatinib) was seen only at supra-pharmacological concentrations *in vitro* [17,18]. Therefore, further chemical optimization will likely be required before these concepts can be explored in more details.

Using a structure based approach we have generated more potent myr-pocket binders (Table 3, Fig. 5). The structure activity relationship obtained between the inhibition of Abl64–515 kinase activity and the inhibition of the p210-Bcr–Abl auto-phosphorylation in BaF3 cells showed an acceptable correlation (Fig. 5). It should be noted, that the kinase assay with Abl64–515 was at least one order of magnitude more sensitive than the auto-phosphorylation of p210-Bcr–Abl in cells (Fig. 5). One of the most potent compounds found by this approach, termed CPD-X, inhibited the kinase activity of the T315I–Abl64–515 as well as the auto-phosphorylation of the p210-Bcr–Abl–T315I expressed in BaF3 cells with an IC50 of around 0.5 μM (Tables 1 and 3). However, inhibition of the auto-phosphorylation of the gatekeeper mutant of p210-Bcr–Abl–T315I in BaF3 cells did not translate into the expected anti-proliferative effect. Like the other two myr-pocket binders GNF-2 and GNF-5, CPD-X was not generally cytotoxic as it neither inhibited the IL-3 dependent BaF3 cells as well as their T315–p210 Bcr–Abl expressing counterparts (Table 3). Combination of CPD-X with ATP-site binders like nilotinib showed that it was more potent in inhibiting the proliferation of BaF3 cells expressing the T315–p210-Bcr–Abl than the combination of the ATP-site binder nilotinib and the myr-pocket binder GNF-5. Nevertheless, also the combination of CPD-X and nilotinib still required

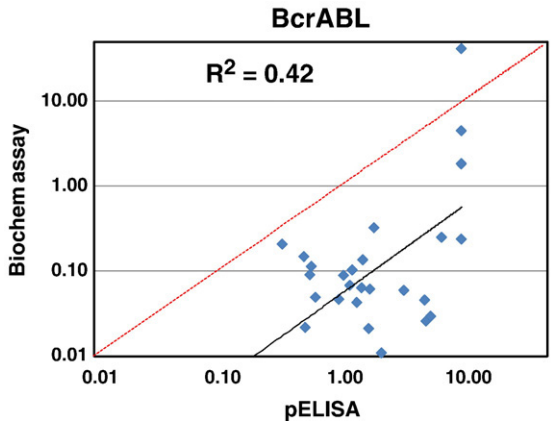

Fig. 5. Biochemical and cellular SAR of myr-pocket binder. Correlation of myr-pocket binder between cell-based and biochemical assays. IC50 of myr-pocket binder were generated in parallel using the Abl64–515 (biochemical assay) and in cell-based auto-phosphorylation assay (pELISA) using p210-Bcr–Abl expressing BaF3 cells as described in Experimental procedures. The red dotted line represents the ideal correlation; the black line is the calculated trend line.

concentrations well above 3 μM in order to obtain a combination effects which could be quoted as synergistic.

Taken together, these data would suggest that more potent myr-pocket antagonists in combination with a potent ATP-site directed inhibitor may be useful to override the T315I gatekeeper mutation.

4. Concluding remarks

Although clinical remission is achieved in early-stage CML with the ATP-site targeting drug imatinib (STI571), nilotinib (AMN107) and dasatinib (BMS-354825) advanced-stage patients usually relapse mainly due to the emergence of the gatekeeper T315I mutation which is located in the ATP binding site of the kinase domain of Bcr–Abl. The T315I mutation has remained elusive, thus far, and only AP24534 a multi-kinase inhibitors has been tested in patients.

Using an unbiased differential cytotoxic approach, myr-pocket binders were identified capable of inhibiting the kinase activity of Abl or Bcr–Abl and shown to be efficacious in Bcr–Abl-dependent myeloproliferative disease models in mice. While these myr-pocket binders displayed *in vitro* and *in vivo* efficacy in combination with ATP-site binder against the T315I mutant it is also apparent that micromolar concentrations are required to obtain combination effects *in vitro*.

In designing more potent myr-pocket binders therapeutically relevant inhibition of the gatekeeper mutation of p210-Bcr–Abl activity can be achieved in combination with ATP-site binders. Further studies will be required to investigate the potential of combinations of ATP and myr-site binders to suppress the initial emergence of resistance which would represent another potential clinical application. Thus the combination of inhibitors that bind to the myr-pocket, and to the ATP-site inhibitors may become clinically useful in overcoming the resistance of the major imatinib-resistant mutation, the T315I.

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