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Screening a Hydroxystilbene Library for Selective Inhibition of the B Cell Antigen Receptor Kinase Cascade

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Abstract: Protein tyrosine phosphorylation is a key post-translational modification used by eukaryotic cells in receptor mediated signal transduction. Selective inhibition of cellular phosphorylation would aid efforts to elucidate the individual events in a signaling pathway. A combinatorial library of putative kinase inhibitors has been screened using an anti-phosphotyrosine blotting assay that can detect inhibition of individual phosphorylation events in whole cells. One member of the library, 3-hydroxy-4-methoxy-4'-nitro-*trans*-stilbene (2B), has been found to selectively disrupt the phosphorylation of several proteins in the B cell receptor mediated cascade while not affecting other cellular phosphorylation events. The kinase specificity of stilbene 2B is compared to known natural and synthetic kinase inhibitors.

INTRODUCTION

Protein kinases have emerged as leading drug design targets because of their involvement in disease associated cellular control pathways[1-5]. Constitutive activation of growth factor receptor tyrosine kinases causes oncogenic transformation of cells[6]. T cell activation begins with a cascade of cytoplasmic protein tyrosine kinases resulting in lymphokine gene transcription and other immune effector functions including organ transplant rejection (Figure 1)[7]. B cell lymphomas are constitutively activated antibody producing cells which have high levels of cellular protein tyrosine kinase activity (Figure 1)[8]. Stimulation of platelets with platelet derived growth factor (PDGF) involves several waves of tyrosine phosphorylation leading to secretion of intracellular granules and platelet aggregation (Figure 1)[9]. The protein tyrosine kinase family of enzymes controls the earliest detectable cellular signal transduction events following cell surface receptor engagement, making tyrosine kinases appealing targets for therapeutic strategies seeking to "rewire" disease causing cellular control pathways. Moreover, since the direct substrates of individual tyrosine kinases in most cascades are not known, inhibitors of specific kinases may aid in the identification of the substrates of protein kinases[10, 11].

The major obstacle in the design and discovery of inhibitors which uniquely target one kinase is the stunning size and high degree of homology within the protein kinase family. There are estimated to be 2000 protein kinases in the human genome (2% of all proteins) which are highly homologous to one another in

the core catalytic domain[10]. In addition to the catalytic domain, the tyrosine kinases also contain non-catalytic domains which are critical for cellular localization of the protein tyrosine kinase as well as assembly of multiprotein complexes with other components of the signal transduction machinery [12, 13]. Since these non-catalytic domains (src-homology regions 2[14] and 3[15]–in the case of src family tyrosine kinases) contain more sequence variation than the catalytic domains, recent attempts to "rewire" intracellular signal transduction events have targeted the non-catalytic domains for inhibitor design[2, 16]. Our approach, however, focuses a panel of stilbenes against B cell cytoplasmic tyrosine kinases to probe for unique inhibitors of receptor mediated phosphorylation. By the use of new, more diverse collections of putative inhibitors created by combinatorial methods[17], it may be possible to identify highly selective and highly potent inhibitors for any target kinase[18-21]. The challenge is to rapidly screen combinatorial libraries against as many kinase targets as possible in a physiologically relevant context in order to identify useful pharmacological agents to dissect these pathways[19].

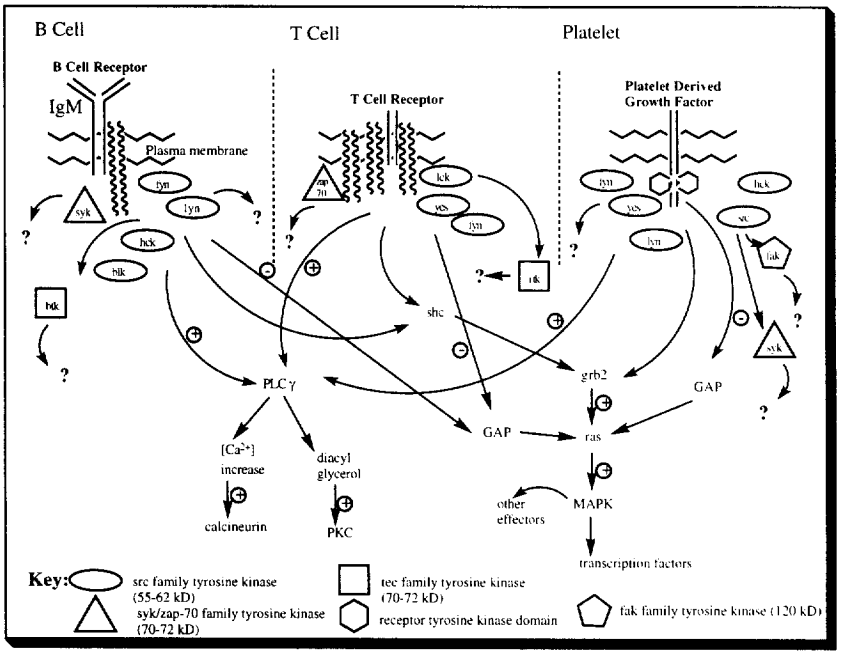


Figure 1: Tyrosine kinases are early elements of many signal transduction cascades.

To date, three general methods of screening libraries have been used for the identification of potent signal transduction inhibitors. 1.) Candidate inhibitors are screened *in vitro* against an enzyme which is thought to be important in a pathway[20]. 2.) Candidate inhibitors are screened in a whole cell using a reporter gene. Reporter gene assays (for example: β–galactosidase or chloramphenicol acetyltransferase) measure the end-point of a given signal transduction pathway. 3.) Candidate inhibitors can be screened for their ability to inhibit cell proliferation[22]. While these screening approaches have identified compounds that

inhibit gene expression, none of them provide immediate information about inhibition of specific upstream signaling events.

Here we describe the screening of a combinatorial library of putative kinase inhibitors for selective disruption of specific tyrosine phosphorylation events during B cell activation (Figure 3). The screening method that we have chosen to exploit allows for the activity of many tyrosine kinases to be assayed simultaneously in a physiologically relevant and time dependent context. The assay of tyrosine kinase activation following B cell stimulation is based on the use of an anti-phosphotyrosine antibody (4G10) which binds phosphotyrosine containing proteins regardless of the particular protein context in which the phosphotyrosine moiety occurs. Therefore, this antibody can recognize almost any phosphotyrosine containing protein in a cell. Anti-phosphotyrosine protein blotting assays have been powerful tools for dissecting the path of signal transduction by protein tyrosine kinases in many cell types, including B cells [22-24](Figure 2). Using this technique, the extent of phosphorylation of many different cellular tyrosine kinase substrates can be determined in a single assay. Furthermore, this assay provides a real test of a given inhibitor's action in the context of a dynamic, highly cooperative, and tightly regulated kinase cascade[25]. By combining new chemical methods for inhibitor synthesis with an extremely sensitive immunological whole cell assay we hoped to identify new highly selective pharmacological agents.

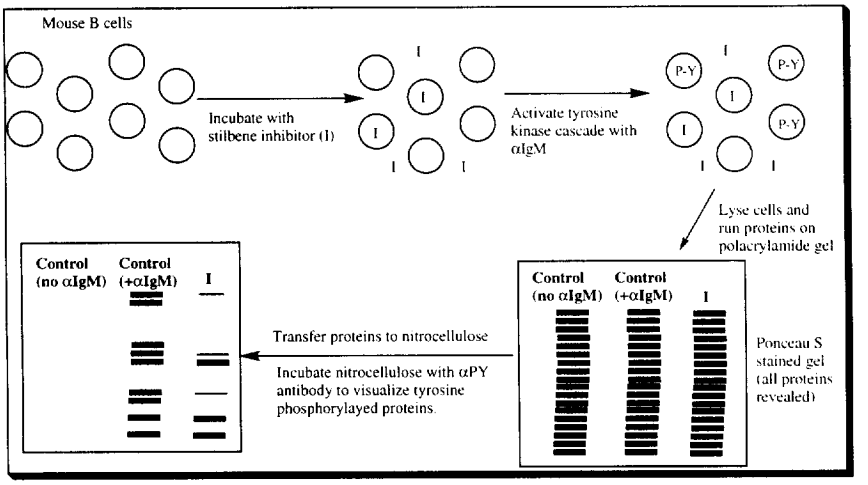


Figure 2: Whole cell anti-phosphotyrosine protein blotting assay for kinase inhibitors.

EXPERIMENTAL

B Cell Stimulation and Phosphotyrosine Assay

Splenic lymphocytes were isolated from 6-20 week old Balb/c or C57/B6 mice. The cells were washed out of the spleen into RPMI media and the red blood cells were lysed by incubation in 17 mM tris-ammonium chloride, pH 7.2. Cell density was adjusted to $2x10^7$ cells/mL. $4x10^6$ isolated cells were incubated at 37°C for 30 minutes with 250 μM of each individual inhibitor. B cell stimulation was initiated

by the addition of 2μg of goat anti-mouse IgM (Jackson Immuno Research, cat# 115-005-075) and subsequent incubation for 5 minutes at 37°C. The cells were isolated by centrifugation (13,000 rpm, 1 min) and lysed (lysis buffer: 1% Triton X-100, 50 mM tris pH 7.4, 1 mM EDTA, 150 mM NaCl, 100 μM phenylmethylsulphonyl fluoride, 1 mM sodium orthovanadate, 10 μg/mL leupeptin, 10 μg/mL apoprotin). The cellular debris was then pelleted at 13,000 rpm for 15 min.

Cellular proteins were separated by 10% polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane by Western blotting (3 hours, 60 V)[26]. The nitrocellulose membrane was incubated in a 1:5000 dilution in phosphate buffered saline of the anti-phosphotyrosine antibody (Upstate Biotechnology, Inc., cat# 05-321) for 1 hour followed by a 1 hour incubation with a horseradish peroxidase linked secondary antibody (VWR, cat# 7101332). Visualization of the phosphotyrosine containing proteins was achieved by incubation of the nitrocellulose membrane with a chemiluminescent substrate kit (Pierce, cat# 1856135, 1856136). Film was then exposed to the chemiluminescent membrane, causing the phosphoproteins to appear as positive bands. Phosphoprotein sizes were determined by staining molecular weight markers with Ponceau S following protein blotting. Gel data is representative of ≥2 experiments for each library member.

RESULTS AND DISCUSSION

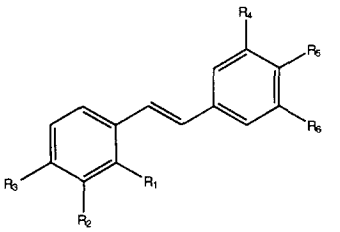
Selection of combinatorial library of kinase inhibitors.

The chemical library we chose to assay for inhibition of B cell signaling is based on the natural product piceatannol (3,4,3',5'-tetrahydroxy-*trans*-stilbene) isolated from seeds of *Euphorbia lagascae*[27]. Piceatannol was shown to be an inhibitor of the protein tyrosine kinases p40 and lck but not the protein ser/thr kinase cAMP dependent protein kinase[28]. Subsequent studies have led to discovery of tyrosine kinase inhibitors based on the hydroxy-*trans*-stilbene core structure, suggesting that a library with piceatannol as the parent structure might be a good starting point for development of selective kinase inhibitors [29]. We chose to assay a small library of hydroxy-*trans*-stilbenes that had originally been synthesized for another purpose, identification of compounds with estrogenic activity[30]. It had already been shown that these compounds displayed the proper physical and biological activities necessary to be good pharmacological agents. The stilbenes are cell permeable, stable, water soluble, and are non-cytotoxic. Furthermore, the stilbenes were appealing because they were prepared on adequate scale (>1 mmole) to allow for detailed assessment of their activity in our assay without repeating the synthesis[30].

Stimulation of B cell antigen receptor signal transduction

One of the most well characterized signal transduction cascades involving cytoplasmic tyrosine kinases is initiated upon crosslinking of the B cell antigen receptor [31] (IgM, see Figure 1, left panel). The tyrosine kinase cascade resulting from antigen receptor engagement has been characterized through the use of anti-phosphotyrosine protein blots following receptor engagement[24], gene knock-out experiments[32], *in*

vitro kinase reactions following selective kinase isolation by immunoprecipitation[33], and other methods[34, 35]. Pharmacological agents which disrupt this pathway typically do so in an all or nothing fashion because most known tyrosine kinase inhibitors inhibit multiple kinases within the same subfamily and, thus, result in broad inhibition of many phosphorylation processes in a cell (see Figure 6 for a representative example).



Compound	R_1	R_2	R_3	R_4	R_5	R_6
1A	H	OH	H	H	H	H
1B	H	OH	H	H	NO_2	H
1C	H	OH	H	H	Br	H
1D	H	OH	H	OCH_3	H	OCH_3
1E	H	OH	H	H	F	H
1F	H	OH	H	F	H	H
2A	H	OH	OCH_3	H	H	H
2B	H	OH	OCH_3	H	NO_2	H
2C	H	OH	OCH3	H	Br	H
2D	H	OH	OCH_3	OCH_3	H	OCH_3
2F	H	OH	OCH_3	F	H	H
3A	H	NO_2	OH	H	H	H
3B	H	NO_2	OH	H	NO_2	H
3C	H	NO_2	OH	H	Br	H
3D	H	NO_2	OH	OCH_3	H	OCH_3
3E	H	NO_2	OH	H	F	H
3F	H	NO_2	OH	F	H	H
4A	Cl	H	OH	H	H	H
4B	Cl	H	OH	H	NO_2	H
4C	Cl	H	OH	H	Br	H
4E	Cl	H	OH	H	F	H
4F	Cl	H	OH	F	H	H

Figure 3: Library of 22 substituted *trans*-stilbenes.

Inhibition of B cell Antigen Receptor Signal Transduction by Substituted Stilbenes

When assayed for their effect on the B cell antigen receptor initiated signaling cascade, the 22 stilbene analogs displayed one of four general effects. Treatment of cells with 250 μM of 1B, 1D, 1E, 1F, 2D, 2F, 4A, 4B, and 4F caused almost complete ablation of the phosphotyrosine signal (see Figure 4, lane 8 for a representative example). These compounds, however, are not broad spectrum tyrosine kinase inhibitors. After detailed investigation they were shown to globally disrupt proper cell pelleting (at this concentration), giving rise to the artificial absence of phosphotyrosine containing proteins. As a result, this group of stilbenes was not studied further.

Of the remaining 13 compounds, eight (1A, 1C, 2A, 3B, 3C, 3D, 4C, 4E) showed almost no inhibition of tyrosine phosphorylation upon IgM crosslinking (see Figure 4, compare lanes 4 and 7 with lane 2). A third category included compounds 2C, 3A, 3E, and 3F. This set of stilbenes showed modest inhibition of antigen receptor induced kinase activity when B cells were treated at 250 μM. This inhibition

appeared to be general as all protein phosphotyrosine levels were reduced by similar amounts with respect to the untreated cascade (see Figure 4, compare lanes 3, 5, and 6 with lane 2).

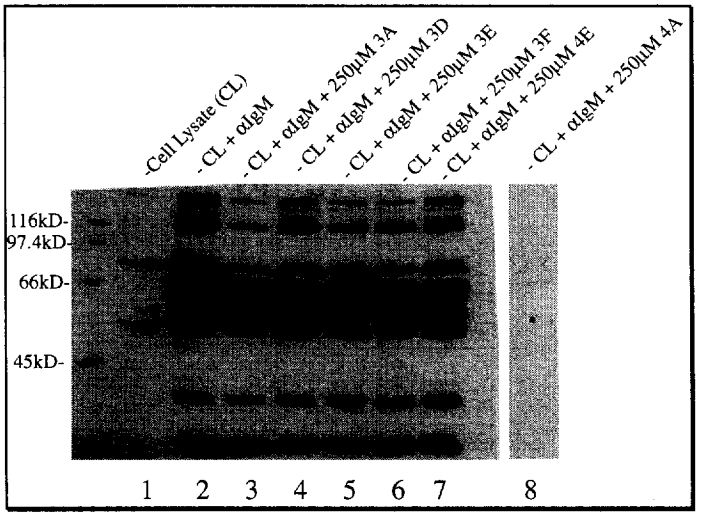


Figure 4: Anti-phosphotyrosine immunoblots for 3A, 3D, 3E, 3F, 4E, and 4A treated B cells.

Stilbene 2B proved to be the most interesting tyrosine kinase inhibitor identified in the library. At 250 μM, 2B was a potent inhibitor of the phosphorylation of four of the proteins (>116 kD, 72 kD, 55 kD, 38 kD) that are significantly phosphorylated upon receptor engagement (see Figure 5, compare lane 4 with lane 2). Three bands, corresponding to molecular weights of approximately 64 kD, 58 kD, and 35 kD are relatively unaffected by treatment with compound 2B. This suggests that 2B is not a completely general tyrosine kinase inhibitor but does display some modest level of selectivity for different protein kinases.

To verify that none of the stilbenes enhanced cellular tyrosine phosphorylation by phosphatase inhibition or kinase activation, we also screened the library in unstimulated B cells (no anti-IgM). None of the library members showed any detectable increase in phosphotyrosine signal at 250 μM (data not shown).

The many studies of the B cell receptor tyrosine kinase cascade using monoclonal kinase-specific antibodies allow for the identification of each phosphoprotein based on its molecular weight. The subset of phosphoproteins in the 55-62 kD range consists of src family tyrosine kinases (blk, lyn, fyn, hck)[35]. These proteins have been shown to be abundantly phosphorylated upon antigen receptor crosslinking. The 72 kD phosphoprotein which does not appear to be phosphorylated in the 2B treated cells is likely to be the dual SH2 domain containing kinase, syk. Syk is highly expressed in B cells and is known to become phosphorylated upon antigen receptor crosslinking[36]. The zap-70 family of tyrosine kinases, to which syk belongs, is particularly important in tyrosine kinase cascades because mutations in the *zap-70* gene cause an autosomal recessive form of severe combined immunodeficiency (SCID)[37]. Likewise, syk deficient B cells fail to trigger phospholipase Cγ-2 phosphorylation, leading to ablation of intracellular Ca^{2+} mobilization and inositol 1,4,5 trisphosphate production[32]. If stilbene 2B acts by either inhibiting the kinase which

phosphorylates syk or by activation of the phosphotyrosine phosphatase which dephosphorylates syk, it may be an important tool for understanding the cellular role of the syk/zap-70 kinase family in immunodeficiency.

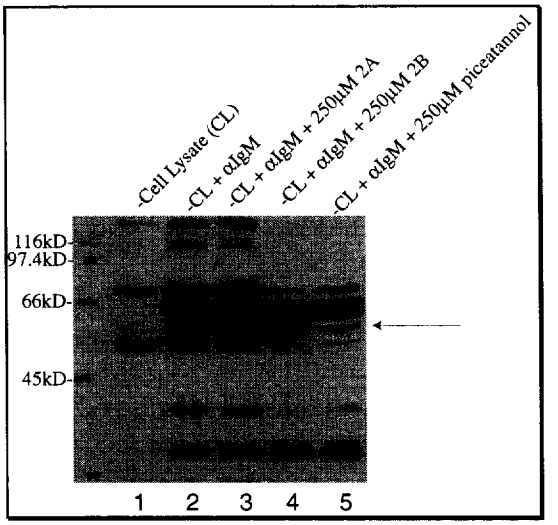


Figure 5: Anti-phosphotyrosine immunoblots for 2A, 2B, and piceatannol treated B cells.

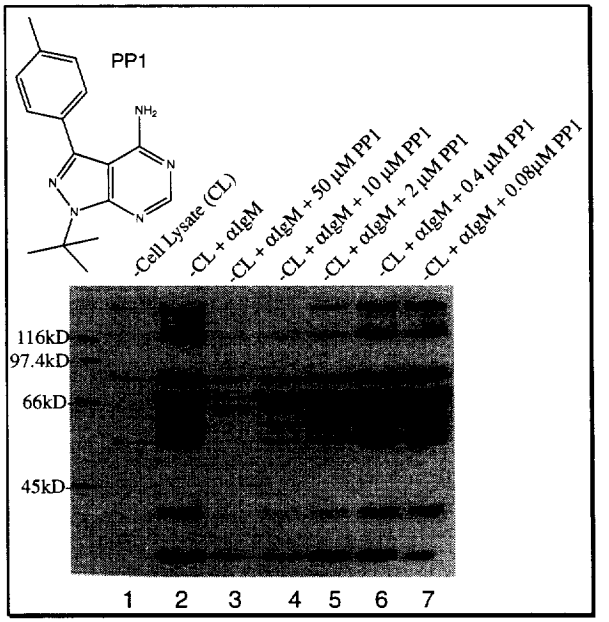


Figure 6: Anti-phosphotyrosine immunoblots treated with indicated concentrations of PP1.

One of our goals in the screening of the stilbene library was the identification of important aromatic substitution patterns that give selective inhibition of B cell phosphorylation events to guide synthesis of more targeted libraries. A library approach allows for the investigation of the effects of small structural perturbations to inhibitor structure with minimum effort, especially if a pre-existing library is used. To date we have not identified any general structure/function tyrosine kinase inhibition trends within the stilbene library. We have, however, demonstrated the wide diversity of cellular activities present within even the smallest of combinatorial pools. Likewise, we have identified a stilbene substitution pattern , 3-hydroxy-4-methoxy-4'nitro, that gives noticeable selectivity and can be exploited in second generation libraries. It is interesting to note that compound 2B showed no estrogenic activity in the original screen of these compounds[30]. This result demonstrates the value of "remining" synthetic libraries for inhibitors of distinct biological targets.

To determine if the aromatic substitution pattern in 2B altered the kinase selectivity with respect to the natural product, piceatannol, we compared the phosphoprotein pattern after treatment with the two inhibitors (see Figure 5, compare lanes 4 and 5 with lane 2). Piceatannol shows significant inhibition of several phosphoproteins, some of which are also inhibited by 2B. However, the two compounds give a distinctly different pattern of inhibition of phosphoproteins in the 50-72 kD region of the gel (especially compare the band at 58 kD (arrow)). We conclude that the synthetic stilbene 2B with the 4-methoxy and 4'-nitro groups, as opposed to the hydroxylated piceatannol, is selective for a different spectrum of tyrosine kinases involved in B cell antigen receptor signal transduction.

Next we compared our most selective stilbene, 2B, with one of the most highly selective tyrosine kinase inhibitors known [38]. The pyrazolopyrimidine, PP1 (Figure 6, insert), was developed by Hanke and coworkers as a highly selective inhibitor of src family kinases which have been shown to be critical components of B cell signal transduction[31]. We reasoned that such a selective inhibitor might show disruption of individual tyrosine phosphorylation events distinct from those inhibited by 2B. In this way we could determine if 2B was targeting the same set or a different set of tyrosine kinases. We synthesized PP1[39] and verified its potency against the fyn tyrosine kinase (IC_{50}= 1 μM) using a peptide phosphorylation assay (not shown). Shown in Figure 6 is the anti-phosphotyrosine protein blot of PP1 treated cells following B cell receptor engagement. Interestingly, the phosphotyrosine levels of each protein appear to be decreased by a similar amount upon PP1 treatment, even over a wide concentration range. The apparent general inhibition of all phosphoproteins by a highly selective src-family kinase inhibitor might simply be a reflection of the importance of src family kinases in initiating the entire kinase cascade. Importantly, however, the different pattern of inhibition by 2B relative to PP1 suggests that different kinases are targeted by 2B and that these kinases may operate downstream of the src family tyrosine kinases. Further efforts to confirm this target selectivity will require *in vitro* inhibition studies with purified kinases.

CONCLUSIONS

Despite only screening a small library of putative kinase inhibitors, we have identified an inhibitor of specific cellular phosphorylation events, demonstrating the potential of focusing combinatorial panels of small molecules at complicated signal transduction cascades. Even with very limited structural diversity, we observed widely varying effects on B cell receptor associated tyrosine phosphorylation. Although no general structure/function relationships were apparent , the 3-hydroxy-4-methoxy-4'-nitro stilbene substitution pattern of 2B was found to be important for selective inhibition of the phosphorylation of one or more src family kinases, as well as the zap-70 kinase family member, syk. From a relatively simple screen, compound 2B has been identified as a possible parent compound for a second generation library of stilbenes. The use of whole cell assay methods which simultaneously track the activity of multiple kinases should facilitate the discovery of highly selective inhibitors from combinatorial libraries of small organic molecules.

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