Radio Frequency Tag Encoded Combinatorial Library Method for the Discovery of Tripeptide-Substituted Cinnamic Acid Inhibitors of the Protein Tyrosine Phosphatase PTP1B

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The introduction of chemical tagging methods in combinatorial library split synthesis has enabled the preparation of a number of small molecule libraries.[1] However, tagging strategies may be incompatible with certain synthetic methods used to generate such libraries. An alternative to chemical tagging is a coding system that uses radiofrequency (RF) encodable microchips. Similar RF encoded systems[2] are widely used to tag and identify laboratory mice *via* subcutaneous injection of a transponder: a glass-encased microchip that is pretuned to emit a unique binary code when pulsed with electromagnetic radiation. Binary codes are of sufficient size to allow a virtually unlimited number of unique identifier tags. For more complex applications, encodable transponders allow downloading of digital information onto the microchip. Later, uploading this data from the microchip provides a history of the transponders' manipulation throughout any experiment.

We have developed a combinatorial library method which couples an RF encodable microchip with a polypropylene capsule of derivatized polystyrene resin such that each unique synthesis site can be tagged with a unique identifier code. The inert nature of the RF transponder construction renders this tagging strategy compatible with virtually all synthetic methods. Additionally, the noninvasive transmission or retrieval of information from any capsule is unambiguous and instantaneous, avoiding the possibility of long reaction and/or analysis times associated with chemical tags. This strategy has been successfully applied to the discovery of novel inhibitors of the protein tyrosine phosphatase PTP1B.[3]

Protein tyrosine phosphatases[4] (PTPases) play a crucial role in regulating levels of phosphorylation during many signal transduction events. For example, CD45 is essential for the activation of $p56^{lck}$ in T-cells.[5] Mitogenic signaling may also involve positive effector roles for PTPases, such as SH-PTP2 in the growth factor-stimulated signal transduction in fibroblasts[6] and PTP1B in breast and ovarian cancers.[7] Therefore, the inhibition of these PTPases may hold promise in the treatment of a variety of diseases.

In our laboratory, para-substituted cinnamates have exhibited moderate inhibition of PTP1B.[8] A library was designed to assess the structural requirements for more potent inhibition of PTP1B by this compound class. A tripeptide amide **1** acylated at the *N*-terminus with *p*-carboxycinnamic acid was chosen as the library substrate (Figure 1). Rink resin[9] was used, and amino acid building blocks were chosen such that various chemical functionalities would be represented in the library. Thus, leucine for lipophilicity, tyrosine for aromatic structure, glutamate for anionic charge, lysine for cationic character, and glycine for conformational flexibility were included. Three synthetic steps with five amino acid building blocks per step required a series of 5^3 or 125 RF transponder containing resin capsules for the experiment. We utilized a modified split synthesis strategy[10] wherein each capsule was scanned and distributed between synthesis rounds to ensure that all possible sequences in the library would be synthesized. The capsules, each loaded with 0.025 mmol of resin, were first treated with 25% piperidine in DMF to remove the 9-fluorenylmethyloxycarbonyl (Fmoc) group from the resin and then divided into five pools of 25 capsules each. The pool location of each capsule was recorded *via* scanning of its RF transponder code. The codes were uploaded to a PC database program,[11] and the first round of synthesis was performed with a single amino acid in each pool using standard coupling protocols.[12] The capsules were scanned and redistributed according to a flow chart provided by the program. For example, after the first round of synthesis the 25 capsules from the glycine pool were distributed equally into five new pools for coupling with a single amino acid in the second round. This provided five copies of all dimer combinations with glycine in the first position. Again scanning and redistributing according to the program flow chart, each one of these 25 capsules was appropriately distributed in the third round to ensure the synthesis of all 25 possible trimers with glycine in the first position. A histogram of the synthesis was developed in the PC database by following this determinate distribution procedure[13] for all capsules. After the third round of amino acid couplings, all capsules were combined, Fmoc-deprotected, and acylated with *tert*-butyl 4-carboxycinnamate.[14] Transponders were scanned and distributed into individual wells of a

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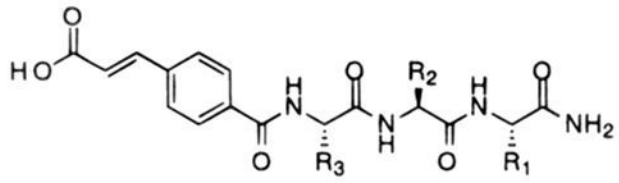

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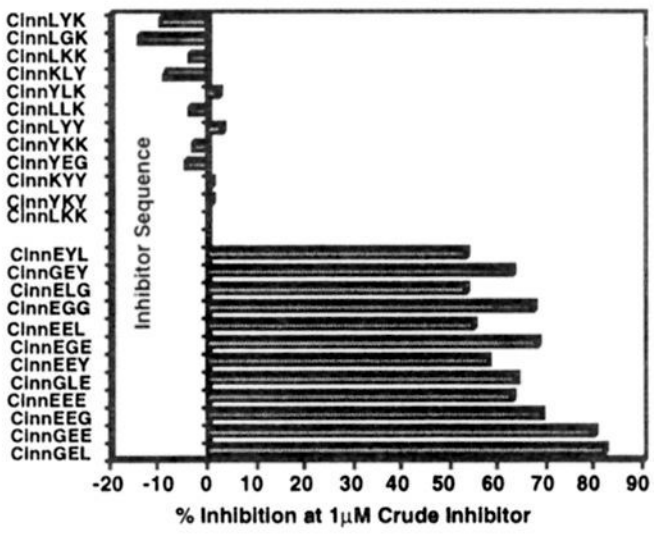
Figure 1. General structure of the tripeptide-substituted cinnamic acid: $CinnX_3X_2X_1$.

Chart 1. Lowest and Highest Ranking Inhibitors of PTP1B



96-well solid phase reaction block system.[15] Thus the cinnamate-capped peptides were cleaved directly into 96-well plates for screening.

A molecular weight prediction program was written to complement the database code program in our analysis.[11] The 96-well format electrospray MS data was matched with the predicted masses for each compound in the array. Sequences were sorted by the program such that the presence of at least one lysine identified those suitable for positive ion mode detection and all others for negative ion mode detection. *A success rate of 100%* was achieved in the synthesis as all 125 predicted masses were confirmed by MS.

The results from screening the crude library at an estimated ligand concentration of 1 μM for PTP1B inhibition are shown in Chart 1.[16] A striking presence of glutamate residues is observed in the most potent inhibitors. Less apparent is a position preference for this residue. Eight out of the top 12 compounds have glutamates in the third position, adjacent to the cinnamate cap. However, the two most potent inhibitors, CinnGEL (**2**) and CinnGEE (**3**), are lacking this position preference (Figure 2). With the notable exception of lysine, amino acids other than glutamate in the first and second positions do not lessen the inhibitory potency of the compounds. Lysine is not present in the sequences of any of the most potent compounds in this library. In fact, the inclusion of lysine appears to impede inhibition as its presence is abundant in the poorest inhibitors of PTP1B. Compound charge is a critical feature in determining an inhibitor's relative affinity for PTP1B. This observation is consistent with the PTP1B structure data.[17] Electron density maps of the protein surface surrounding the putative catalytic site show a plethora of positively charged residues. Perhaps electrostatic interactions occur between the glutamic acid carboxylate of the most potent inhibitors and these neighboring surface residues of the protein.

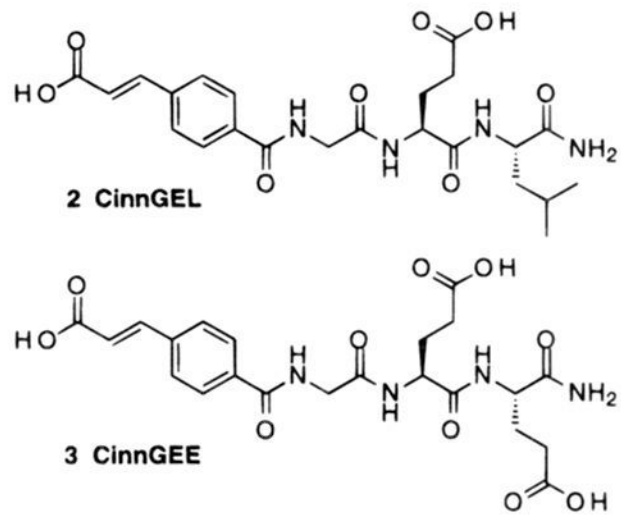


Figure 2.

After the initial screen, the inhibitors **2** and **3** as well as the two least potent compounds, CinnLYK and CinnLGK, were purified by reverse-phase HPLC. The IC_{50} data for CinnGEL and CinnGEE are 1.3 μM and 44 nM, respectively. Further analysis revealed that the sequence CinnGEL has a K_i of 490 nM and the sequence CinnGEE has a K_i of 79 nM. Both inhibitors are competitive with *p*-nitrophenyl phosphate substrate. In contrast, IC_{50} values of 49 and 46 μM were determined for CinnLYK and CinnLGK, respectively. Mixed noncompetitive behavior was observed for these sequences. Thus, there appears to be about a 1000-fold range of inhibitory activity within the library. Similar ranges in activity have been observed for compounds within this library when assayed against other tyrosine phosphatases. The selectivity of these compounds toward the inhibition of other phosphatases will be reported elsewhere.

The inert nature of the RF tag allows broader application of this encoded combinatorial library method to cover a vast array of chemistries. Organic synthesis, microchip technology, and robotics can be integrated in a powerful method for the generation of new chemical libraries.

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Supporting Information Available: ^{1}H NMR, ^{13}C NMR, electrospray MS, and HRMS (FAB) data of the four selected compounds, the experimental protocol for synthesis of the library, cloning, expression, and isolation of PTP1B, the inhibition assay protocol, and a description of the instrumentation used (6 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(13) With the Furka split synthesis strategy (ref 10) a stochastic distribution of multiple copies of individual synthesis sites (resin beads) ensures the successful synthesis of all possible chemical combinations. In the method employed here the determinate distribution of synthesis sites (capsules) ensures the successful synthesis of all possible chemical combinations. Of course, determinate distribution of capsules between synthetic steps is possible only if the code on an individual capsule can be retrieved during the synthesis.

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(15) An automated system for the solid phase synthesis of small organic molecules in a 96-well format has been developed in our labs. Chemistry takes place on resin in a 96-well reaction block. After cleavage, product is released directly into standard 96-well plates. The mating of these reaction blocks with automated reagent delivery systems, i.e., Tecan Model 5032, and other special purpose equipment allows for unattended multistep synthesis and cleavage of product off the solid support. This same system can be used for the rapid processing of individual RF tagged capsules. The advantages of having compounds cleaved in a spatially dispersed manner can be achieved in practice for chemical library sizes of approximately 2×10^4 or less. Thus, in this approach, individual compounds and not mixtures are available for screening.

(16) A 50% yield per compound was assumed in the library synthesis. The crude, neat compounds were dissolved in 1.25 mL of DMSO to give 10 mM stock solutions. PTP1B activity was assayed in 96-well microtiter plates, using 10 μL of enzyme at 2 μg/mL in assay buffer (100 mM sodium acetate pH 6.0, 1 mM EDTA, 0.1% TritonX-100 and 15 mM βme), 10 μL of 9 mM *p*-nitrophenyl phosphate, and 70 μL of assay buffer; 10 μL of inhibitor stock solution was added to yield a final assay volume of 100 μL. The assay was incubated at 37 °C for 65 min, 10 μL of 0.5 M NaOH−50% EtOH was added, and activity was determined by reading absorbance at 405 nm. Inhibition is relative to 500 μM sodium pervanadate (100%) and buffer blank (0%).

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