NOVEL SMALL MOLECULE INHIBITORS OF PTP1B

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ABSTRACT

Protein tyrosine phosphatase is a classical non receptor tyrosine phosphotase enzyme and is an important regulator in several signaling pathways including insulin and leptin. PTP1B is a negative regulator of insulin receptor (IR) which dephosphorylates tyrosine residues of Insulin Receptor tyrosine Kinase (IRK) domain and induces resistance to insulin sensitivity and obesity upon over expression of PTP1B. Thus PTP1B is a vital therapeutic target for Type II diabetes and developing a selective PTP1B inhibitor is an essential breakthrough in controlling the disease which has been afflicting 1/3 of world population. We have identified few small molecule PTP1B inhibitors which have shown micro molar range inhibition by in vitro pNPP assay method.
KEY WORDS

IR: Insulin Receptor, IRK: Insulin Receptor Kinase, pNPP: Para Nitro Phenol Pyrophosphate

INTRODUCTION

Co-ordinate action of Phosphorylation and dephosphorylation of cellular proteins by protein kinases (PTKs) and protein phosphatases (PTPases) mediates wide variety of cellular functions like growth, division and cell-cell interactions etc. Defective or improper regulation of these enzymes can lead aberrant protein tyrosine phosphorylation which contributes to the development of many human diseases including cancer and diabetes.\(^1\,^2\) Protein kinases are broadly divided into tyrosine kinases or serine/threonine kinases where as protein phosphotases are divided into protein tyrosine phosphotase (PTP) and protein serine/threonine (PSP) and dual specificity phosphotases based on the structure and substrate specificity.\(^3\) Protein tyrosine phosphatases are signal transduction enzymes play key roles in variety of important physiological processes including cell growth, metabolism, differentiation and cell-cell communication etc. \(^4\,^5\) PTP1B is the first enzyme which was isolated and characterized well of all PTPases.\(^6\) PTP1B is ubiquitously expressed non-receptor protein tyrosine phosphatase and has been emerged as a potential target for the treatment of type II diabetes and obesity.\(^7\,^8\) We have identified three low molecular weight inhibitors which have shown inhibition to PTP1B by in vitro assay.

MATERIALS AND METHODS

CLONING

The catalytic domain of PTP1B (1-321) gene was amplified from commercial clone obtained from open Biosystems (Cat. No: MHS1011-76533) by Oligo nucleotide primers using PCR. The amplified gene was cloned into Pet28a Novagen vector (Cat No: 69864-3) between SalI and HindIII. The recombinant plasmid was confirmed by restriction digestion and sequencing analysis.

PROTEIN EXPRESSION & PURIFICATION:

Catalytic domain of human PTP1B (1-321) was
expressed in E.coli as described previously\textsuperscript{10}. According to the Novagen protocol recombinant plasmid was transformed with BL21 (DE3) competent cells (Novagen). A single colony was inoculated from transformation plate containing kanamycin at 50µg/ml in to 10ml LB medium and allowed to grown at 37°C for 3 Hrs at 180rpm. The starter culture was used for inoculating 1.0L of LB broth for large scale protein expression. When the A600 reached 1.0, induced the cells with 0.2 Mm IPTG (Isopropyl-β-D-Thiogalactopyranoside) for 18Hrs of shaking at 160 rpm at18°C. The culture was pelleted at 4000rpm and immediately processed.

The cell pellet was resuspended in Lysis buffer A(50mMTris pH:8.0, 200mM NaCl,0.5Mm EDTA ,10% Glycerol and 1mM DTT including protease inhibitor cocktail. The cell suspension was lysed by sonication(12cycles-on/40cycles-off/15 cycles at10µ amplitude) using sonicator from Sonodyne. The cell lysate was centrifuged at12000rpm for 45mins at 4°C and the supernatant containing soluble recombinant PTP1B(1-321 with N-ter. His tag) was mixed with Ni-NTA metal affinity resin at a ratio of 10(protein):1(resin). The protein-resin mix then transferred to a column and washed with buffer A for 10 column volumes till the A280 ≤ 0.1. The protein was eluted from the resin by washing buffer A containing increasing imidazole concentration by batch mode, gel picture of protein purified by Ni-NTA chromatography can seen in fig.1 The protein eluted from Ni-NTA was applied on to Superdex-75(AmershamBioscences)Gel filtration chromatography,using Buffer B(3mM EPESpH:7.5,150mM NaCl, 0.5mM EDTA and 0.1%β-Me) and the protein eluted at 12ml volume was loaded on SDS PAGE and was 95% pure. The eluted protein from gel filtration column was checked on SDS PAGE (Fig: 2a & 2b) and was flash frozen using liquid nitrogen and stored at -80°C.

![Figure 1](image_url)

**Figure 1**

PTP1B protein purification by Ni-NTA Chromatography and eluted samples on SDS PAGE.
BIOCHEMICAL ASSAY:
The enzymatic activities of PTP1B catalytic domain were determined by hydrolysis of (Para Nitro Phenol Pyrophosphate)pNPP at room temperature. Para-nitrophenyl phosphate is a chromogenic substrate for most of the phosphatases including tyrosine phosphatases. The reaction yields pNP (para-nitrophenol) which becomes an intense yellow soluble product under alkaline conditions. The absorbance can be measured at 405 nm on a spectrophotometer. The Km value of pNPP for PTP1B catalytic domain was determined using assay buffer (25mM Tris pH: 7.5, 75mM NaCl, 0.1% BSA, 0.1mM DTT) and was found to be 800µM. Based on the docking analysis using Gold suite protein-ligand software, shortlisted 35 compounds from the 2500 Aurigene chemical depository (data not shown) out of which only 3 compounds i.e. AU-0008, AU-0247 & AU-2525 (Fig:3) had shown inhibition to PTP1B protein. All the compound’s IC50 values were determined in a typical 100µl reaction and were carried out in 96 well flat-bottom plate at room temperature for 1 Hr. The Reaction was stopped by adding 1N NaOH and measured the OD405 using spectromax 190 micro plate reader. The compound’s IC50 values were determined by fitting initial rates of p-nitrophenol production to a sigmoidal dose response equation using prism3.0 (Graph pad software).

RESULTS AND DISCUSSIONS
The catalytic domain of PTP1B (1-321) protein was purified by two step purification. The initial step was by Ni-NTA affinity chromatography and further protein was separated to monomer by Gel filtration chromatography using Amershams Superdex -75 column. Virtual screening of Compounds from Aurigene library by using Gold suite docking software was done and identified 35 compounds. All the 35 compounds from the depository were taken for in vitro pNPP assay to check the activity of the compounds. Of all the 35 compounds only 3 compounds i.e. AU-0008, AU-0247 and AU-2525 have shown inhibition. The compounds were further for the IC50 determination. The IC50 values were compiled in table 1 and the results were interpreted in Fig.4a, 4b and 4c. Since these compounds were competitive binding inhibitors (data not shown) and are low molecular weight compounds which can be viewed as initial start-up low affinity inhibitors of PTP1B. The catalytic active site of PTP1B is surrounded by pTyr loop (44-49), WPD loop (179-185) and Q-loop (262-269).
CONCLUSION

Since the active site of PTP1B is highly flexible and can accommodate wide variety of compounds from different scaffolds and more over there are three different sites which were identified in PTP1B protein can influence the compound affinity and specificity. These sites are namely Site A/ Catalytic site (Cys215-Arg221), Site B/Second phosphate binding site (R24, R254)\textsuperscript{11} and Site C/ Allosteric site (Lys41, Arg 47).\textsuperscript{12} Since the three compounds identified in this study were low molecular weight

<table>
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<tr>
<th>Compound name</th>
<th>IC\textsubscript{50} VALUES (µM)</th>
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<tbody>
<tr>
<td>AU-0008</td>
<td>86</td>
</tr>
<tr>
<td>AU-0247</td>
<td>219</td>
</tr>
<tr>
<td>AU2525</td>
<td>27</td>
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Table 1
Compilation of IC\textsubscript{50} values of the compounds

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competitive inhibitors which could occupy only Site A. The affinity of these compounds might be further enhanced by increasing molecular weight by reaching Site B or Site C.

REFERENCES