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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 up on 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 1/3rd 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.Protein tyrosine phosphotases are signal transduction enzymes play key roles in variety of important physiological processes including cell growth, metabolism, differentiation and cell-cell communication etc. PTP1B is the first enzyme which was isolated and characterized well of all PTPases.PTP1B is ubiquitously expressed non-receptor phosphotase enzyme,contains N-terminal catalytic domain and C-terminal hydrophobic domain which targets the enzyme to the cytoplasmic face of the endoplasmic reticulum. A series of biochemical, cellular and knock down mouse studies have revealed that PTP1B functions as a negative regulator of both insulin and leptin signaling receptor pathways.[3-6] There is substantial evidence suggesting that PTP1B is mainly responsible for the dephosphorylation of insulin receptor and hence acts to negatively regulate insulin signaling.[7] All the members of PTP family consist of signature motif HCXXGXXR which contains cysteinal residue (Cys 215), is essential for catalysis. The signature motif which forms the base of the catalytic cleft which is surrounded by loops namely WPD loop, Q loop and pTyr loops.PTP1B dephosphorylates the tyrosine residue of the substrate in two steps mechanism. The first step of the tyrosine dephosphorylation of substrate tyrosine is initiated by nucleophilic attack by sulfur atom of the Cys215 on the substrate phosphate coupled with protonation of leaving substrate by side chain of conserved Asp181residue acting as general acid, which forms cysteinyl-phosphate intermediate. The second step,the Gln262 coordinates a water molecule and Asp181 which functions in this step as general base which hydrolyzes the catalytic intermediate which releases phosphate.

Protein tyrosine phosphatase (PTP1B) is a non-receptor protein tyrosine phosphatase and has been emerged as a potential target for the treatment of type II diabetes and obesity.[8-9] we have identified three low molecular weight inhibitors which have shown inhibition to PTP1B by in vitro assay.

MATERIALS AND METHODS

CLONING

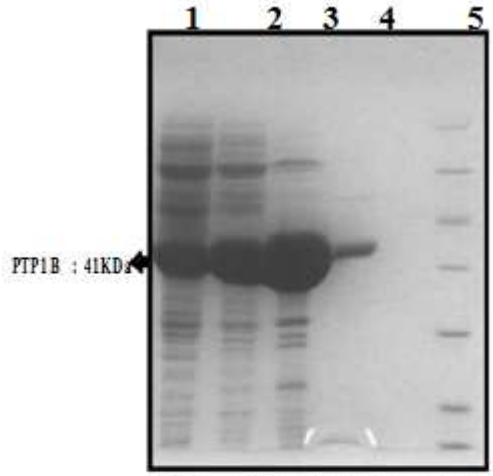
The catalytic domain of PTP1B (1-321) gene was amplified from commercial clone obtained from open Biosystems (Cat. No: MHS1011-76533) by Oligo nucleotide primers using PCR. The amplified gene was cloned into Pet28a Novagen vector (Cat No: 69864-3) between SalI and HindIII. The recombinant plasmid was 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[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 SDSPAGE and was 95% pure. The elutedprotein from gel filtration column was checked on SDS PAGE (Fig: 2a & 2b) and was flash frozenusing liquid nitrogen and stored at -80°C.



Lane1: 25mM Imidazole elute.
Lane2: 50mMImidazole elute.
Lane3: 150mM Imidazole elute.
Lane4: 250mM Imidazole elute.
Lane5: Protein Molecular weight Marker

Figure 1

PTP1B protein purification by Ni-NTA Chromatography and eluted samples on SDS PAGE.

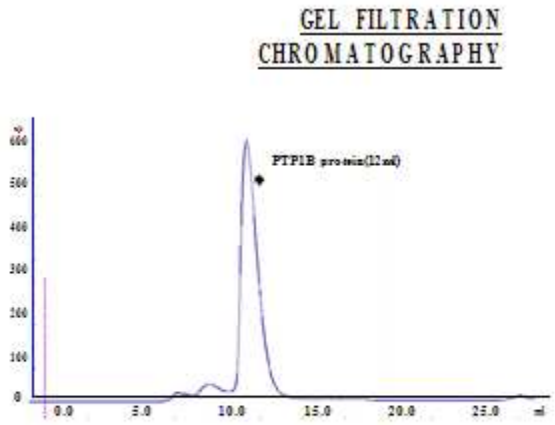


Figure 2a
PTP1B protein Gel filtration chromatogram

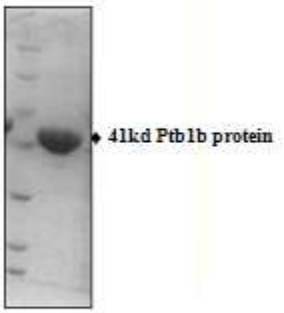


Figure 2b
PTP1B protein eluted from Superdex-75 column on SDSPAGE

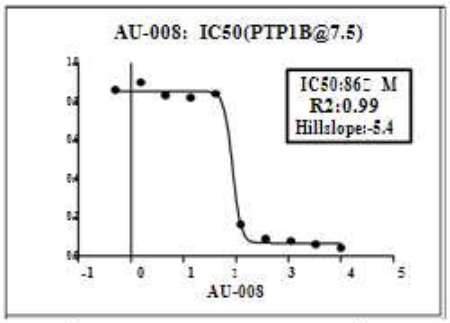
BIOCHEMICAL ASSAY:

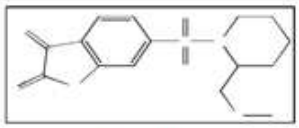
The enzymatic activities of PTP1B catalytic domain were determined by hydrolysis of (Para Nitro Phenol Pyrophosphate)pNPP at room temperature.Para-nitrophenyl phosphate is a chromogenic substrate for most of the phosphatases including tyrosine phosphatases. The reaction yields pNP (para-nitophenol) which 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 (25mMTrispH:7.5, 75mM, NaCl,0.1%BSA,0.1mM DTT) and was found to be 800µM. Based on the docking analysis using Gold suit 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 IC 50 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 IC 50 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 Amersham Superdex -75 column.

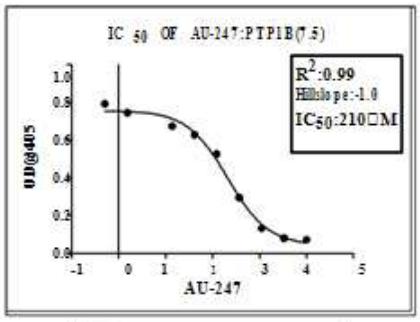
Virtual screening of Compounds from Aurigene library by using Gold suite docking software was done and identified 35 compounds. All the 35 compounds from the depository were taken for in vitro pNPP assay to check the activity of the compounds. Of all the 35 compounds only 3 compounds i.e.AU-0008, AU-0247 and AU-2525 have shown inhibition. The compounds were taken further for the IC_{50} determination. The IC_{50} 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).

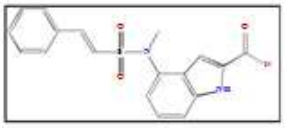




AU-0008

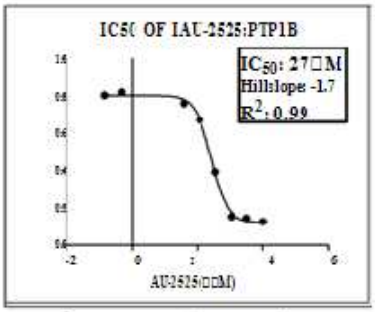
Figure 4a: IC50 value and structure of AU-0008

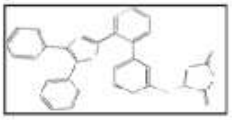




AU-0247

Figure 4b: IC50 value and structure of AU-0247





AU-2525

Figure 4c: IC50 value and structure of AU-2525

Table 1
Compilation of IC 50 values of the compounds

Compound name	IC 50 VALUES(µM)
AU-0008	86
AU-0247	219
AU2525	27

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)[11] and Site C/ Allosteric site (Lys41, Arg 47).[12] Since the three compounds identified in this study were low molecular weight

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.

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