Screening of Marine Microbial Extracts for Tyrosine Phosphatase 1B Inhibitors

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Abstract Protein tyrosine phosphatase 1B (PTP1B) acts as a negative regulator of insulin signaling, and selective inhibition of PTP1B has served as a potential drug target for the treatment of type 2 diabetes. As part of our searching for PTP1B inhibitors from natural products, the extracts of marine microorganisms were screened for the inhibitory effects on the activity of protein tyrosine phosphatase 1B (PTP1B). Among the tested 304 extracts, 29 extracts exhibited inhibition rate ranging 40.1 - 83.6 % against PTP1B at the concentration level of 30 μg/mL.

Key words : Marine microorganisms, protein tyrosine phosphatase 1B (PTP1B) inhibitor, diabetes mellitus

Introduction

Several protein tyrosine phosphatases (PTPases) play a critical role in the regulations of a variety of cellular processes, such as growth, proliferation and differentiation, metabolism, immune response, cell-cell adhesion, and cell-matrix contacts [3,5]. Particularly, protein tyrosine phosphatase1B (PTP1B) is a major non-transmembrane phosphotyrosine phosphatase in human tissues and a negative regulator of the insulin-stimulated signal transduction pathway by dephosphorylating the insulin receptor (IR) as well as its substrate, insulin receptor substrate-1 (IRS-1) and insulin receptor substrate-2 (IRS-2)[2,7,12]. The protein levels are increased in insulin-resistant diabetes patients and the deletion of PTP1B in mice has been shown to increase insulin sensitivity [1,6,15]. Thus, inhibiting PTP1B action by using antisense oligonucleotides or small molecule inhibitors represents one of the novel therapeutic approaches for the treatment of insulin resistance [4,13]. Although there have been a number of reports on the designing and development of synthetic PTP1B inhibitors little has been studied for PTP1B inhibitors derived from natural resources such as plant or microbial resources [4,6]. Et-3,4-dephostatin is one of few PTP1B inhibitors derived from microbial source. As a stable analogue
of dephostatin isolated from *Streptomyces*, Et-3,4-dephostatin has been shown to strongly inhibit PTP1B activity *in vitro*, and to potentiate insulin-related signal transduction in cultured mouse adipocytes [13]. Recently, a dimeric depside-type fungal metabolite KS-506a was isolated as a competitive PTP1B inhibitor with the Ki value of 2.7 μM by bioassay guided fractionation from the EtOAc extract of *M. ramannianus* var. *angulisporus* [10].

Considering the track record of success in the development of a number of useful therapeutics, it seems reasonable to search for PTP1B inhibitors from natural resources. In our continuing search for PTP1B inhibitors from natural resources, a number of bacterial and fungal isolates were screened for their ability to produce PTP1B inhibitory secondary metabolites.

Materials and Methods

Collection of samples

Samples of sediments and sea water were collected by hand in several geographically different areas in south coast of Korea. Six samples were obtained form Masan Bay area in January 2006. Forty-five samples were ob-

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tained form Geojedo area in August 2006. Eight samples were obtained form Geojedo area in April 2007. Six samples were obtained form Dadaepo area in April 2006.

Culture medium and agar plates

Modified ZoBell 2216e agar medium was used for isolation of bacteria, which contained (per liter) 5 g of Bacto Peptone (Difco), 1 g of yeast extract (Difco), 10 mg of $FePO_4$, 15 g of agar, and 750 g of seawater. The medium was adjusted to a pH of 7.2 and a volume of 1 L with distilled water. Potato dextrose agar (PDA, Difco) medium was used for isolation of fungi.

Bacterial/fungal isolation

The samples were suspended in 10 ml of sterile seawater and then serially diluted with seawater from 10^{-1} to 10^{-5}. Isolation of bacteria and fungi strains was achieved by spreading 0.5 ml of the diluted samples on Emerson agar plates. The plates were maintained at 28°C for 14 days to permit bacteria and fungi growth. The gross cell morphology of the resulting colonies were examined microscopically, sub-cultures were set up by transferring bacterial and fungal colonies to new plates until pure culture were isolated on the basis of distinct colony morphology. The purified strains were stored at -70°C in the presence of glycerol.

Culture and extraction

Isolated strains were incubated at 25 - 30°C for 4 - 5 days in a medium of bacteria (culture medium description) or in a medium of fungi (culture medium description). After the cultivation, the agar plate was extracted with MeOH, and the extract was concentrated *in vacuo* to dryness. All dried extracts were dissolved in DMSO and were used for the assay. The medium materials used for the cultivation was examined as background.

Table 1. Inhibition of PTP1B activity by the crude extracts of marine-derived bacterial strains

Strain Number	Collection site	% Inhibition
1005	Masan Bay (January 2006)	48.1
1017	Masan Bay (January 2006)	45.5
1050	Masan Bay (January 2006)	40.6
1095	Geojedo (August 2006)	43.1
1127	Geojedo (August 2006)	56.9
1139	Geojedo (August 2006)	83.6
1154	Geojedo (August 2006)	65.9
1157	Geojedo (August 2006)	66.5
1164	Geojedo (August 2006)	55.0
1165	Geojedo (August 2006)	50.5
1171	Geojedo (August 2006)	42.4
1174	Geojedo (August 2006))	47.8
1178	Geojedo (August 2006)	44.3
1185	Geojedo (August 2006)	43.9
1234	Geojedo (April 2007)	49.7
1245	Geojedo (April 2007)	40.1
1249	Geojedo (April 2007)	41.0
1272	Geojedo (April 2007)	48.8

Table 2. Inhibition of PTP1B activity by the crude extracts of marine-derived fungal strains

Strain Number	Collection site	% Inhibition
5060	Geojedo (April 2007)	69.6
5062	Gojedo (April 2007)	57.6
5064	Gojedo (April 2007)	45.2
5067	Gojedo (April 2007)	53.3
5073	Gojedo (April 2007)	62.0
5090	Gojedo (April 2007)	74.4
5095	Gojedo (April 2007)	71.2
5097	Gojedo (April 2007)	75.2
5099	Gojedo (April 2007)	65.0
5114	Gojedo (April 2007)	65.8
5116	Gojedo (April 2007)	55.1

PTP1B enzyme assay

PTP1B (human, recombinant) was purchased from BIOMOL Research Laboratories, Inc., and the enzyme activity was measured using *p*-nitrophenyl phosphate (pNPP) as substrate. For inhibition assay, inhibitors were added to the reaction mixture (final volume 100 μL) containing PTP1B (0.05 - 0.1 μL) and 2 mM pNPP in a buffer solution [50 mM citrate (pH 6.0), 0.1 M NaCl, 1mM EDTA and 1mM dithiothreitol (DTT)]. The reaction mixture was placed in a 30°C incubator for 30 min, and the reaction was terminated by addition of 1 M NaOH solution (10 μL). The amount of produced *p*-nitrophenol was estimated by measuring the absorbance at 405 nm. The non-enzymatic hydrolysis of 2 mM pNPP was corrected by measuring the absorbance at 405 nm in the absence of PTP1B enzyme.

Results and Discussion

The prevalence of diabetes is rapidly increasing in industrialized countries, and type 2 diabetes particularly accounts for more than 90% of cases. In type 2 diabetes, insulin-resistance is one of the characteristic pathogenesis, and several drugs to increase the insulin sensitivity are currently being used in clinic. However, currently available drugs for type 2 diabetes have a number

of limitations, such as adverse effects and high rates of secondary failure [8]. Among the various potential drug targets for improving insulin sensitivity, protein tyrosine phosphatase1B (PTP1B) has recently emerged as a promising one in the effective treatment of type 2 diabetes [11]. This is solidly supported by a number of accumulated evidences suggesting PTP1B as a major negative regulator of insulin receptor signaling [6,11].

Natural products have long proved to be valuable sources bioactive agents as well as of various enzyme inhibitors. By comparison to synthetic studies for the development of PTP1B inhibitors, reports on the screening for PTP1B inhibitors from natural products are relatively rare. However, it is conceivable that the screening of crude biological extracts for PTP1B inhibitor could provide certain benefit since each extract likely contains countless primary and secondary metabolites, many of which will not have been previously characterized, and this will allow us to dramatically increase the chemical space being sampled [14].

To identify the crude extract(s) with PTP1B inhibitory effects from marine-derived microbial sources, the crude extracts from the bacterial and fungal isolates, which were DMSO solutions that contained 1 mg/mL of dried methanolic extracts, were tested at a dilution of 3 μL in a final assay volume of 100 μL (30 μg/mL final extract concentration). The enzymatic activity of PTP1B was found to be completely unaffected by the addition of this amount of DMSO (3%). A known PTP1B inhibitor, ursolic acid (IC_{50} = 3.5 μM), was employed as a positive control [15,16]. Among the total of 276 extracts from bacterial strains isolated, 18 extracts exhibited moderate inhibition rate ranging 40.1 - 83.6 % against PTP1B. Eleven extracts from the total the total of 128 extracts from fungal strains isolated were also identified to have inhibitory effects on PTP1B activity with inhibition rate ranging 45.2 - 75.2 %. Overall 6.5% of bacterial extracts and 8.5% of fungal extracts were considered as hits in the screening for PTP1B inhibitory extracts, and further investigation on these strains to identify PTP1B inhibitors is on process.

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