A novel strategy for the development of selective active-site inhibitors of the protein tyrosine phosphatase-like proteins islet-cell antigen 512 (IA-2) and phogrin (IA-2 β)

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Islet-cell antigen 512 (IA-2) and phogrin (IA-2 β) are atypical members of the receptor protein tyrosine phosphatase (PTP) family that are characterized by a lack of activity against conventional PTP substrates. The physiological role(s) of these proteins remain poorly defined, although recent studies indicate that IA-2 may be involved in granule trafficking and exocytosis. To further understand their function, we have embarked upon developing low-molecular-mass inhibitors of IA-2 and IA-2 β . Previously, we have shown that a general PTP inhibitor, 2-(oxalylamino)benzoic acid (OBA), can be developed into highly selective and potent inhibitors of PTP1B. However, since wild-type IA-2 and IA-2 β lack conventional PTP activity, a novel strategy was designed whereby catalytically active species were generated by ‘back-mutating’ key non-consensus catalytic region residues to those of PTP1B. These mutants were then used as tools with which to test the potency and selectivity of OBA and a variety of its derivatives. Catalytically competent IA-2 and IA-2 β species were generated by ‘back-mutation’ of only three key residues (equivalent to Tyr⁴⁶, Asp¹⁸¹ and Ala²¹⁷ using the human PTP1B numbering) to

those of PTP1B. Importantly, enzyme kinetic analyses indicated that the overall fold of both mutant and wild-type IA-2 and IA-2 β was similar to that of classic PTPs. In particular, one derivative of OBA, namely 7-(1,1-dioxo-1*H*-benzo[*d*]isothiazol-3-yloxy-methyl)-2-(oxalylamino)-4,7-dihydro-5*H*-thieno[2,3-*c*]pyran-3-carboxylic acid (‘Compound 6’ shown in the main paper), which inhibited IA-2 β _(S762Y/Y898P/D933A) (IA-2 β in which Ser⁷⁶² has been mutated to tyrosine, Tyr⁸⁹⁸ to proline, and Asp⁹³³ to alanine) with a K_i value of $\approx 8 \mu\text{M}$, appeared ideal for future lead optimization. Thus molecular modelling of this classical, competitive inhibitor in the catalytic site of wild-type IA-2 β identified two residues (Ser⁷⁶² and Asp⁹³³) that offer the possibility for unique interaction with an appropriately modified ‘Compound 6’. Such a compound has the potential to be a highly selective and potent active-site inhibitor of wild-type IA-2 β .

Key words: autoantigen, back-mutation, diabetes, enzyme kinetics, molecular modelling, phosphatase inhibitor.

INTRODUCTION

IA-2 (islet-cell antigen 512) and IA-2 β (phogrin) are integral membrane glycoproteins that are predominantly expressed in dense-core secretory granules of a variety of neuroendocrine tissues, including pituitary cells, chromaffin cells of the adrenal medulla and α - and β -cells of the pancreatic islets of Langerhans (reviewed in [1]). Both proteins are major autoantigens in Type 1 diabetes with $\approx 70\%$ of newly diagnosed patients presenting autoantibodies to IA-2, and, to a lesser extent, IA-2 β [1]. At present the physiological roles of IA-2 and IA-2 β are incompletely defined, although recent studies indicate that IA-2 may be involved in granule trafficking and exocytosis [2,3]. Such a role is supported by the observation of reduced glucose-stimulated insulin secretion in IA-2^{-/-} mice [4]. Owing to an increasing incidence of Type 1 diabetes [5], there is considerable interest in further elucidating the physiological function(s) of IA-2 and IA-2 β .

Both IA-2 and IA-2 β possess a luminal ectodomain, a short transmembrane segment and an intracellular domain that shares high identity with the ≈ 280 -amino-acid catalytic domain of

protein tyrosine phosphatases (PTPs). IA-2 and IA-2 β share approx. 74% identity in their intracellular domains and possess the hallmark amino acid sequence, the so-called P-loop (Cys-Xaa-Arg, where Xaa is ‘any’ amino acid), which characterizes this enzyme family [6]. To further unravel the physiological role(s) of IA-2 and IA-2 β , it would be advantageous to develop selective, small-molecule inhibitors that could be used to probe their function at both a cellular and an *in vivo* level. Since no inhibitors are currently available, the present study was conducted as a first step towards gaining the structure–activity information necessary for the design of such compounds.

Since, in marked contrast with most other PTPs, recombinant IA-2 and IA-2 β do not display enzyme activity towards conventional PTP substrates, a novel strategy was required to develop such inhibitors. The lack of IA-2 and IA-2 β activity is due, at least in part, to substitutions of key, highly conserved residues that are considered essential for catalysis. Indeed, early studies have shown that limited IA-2 and IA-2 β enzyme activity can be generated by ‘back-mutating’ a number of these variant residues [7–9] to the PTP consensus. By superimposing the X-ray structures of seven different PTP catalytic domains, we previously

Abbreviations used: GST, glutathione S-transferase; PTP, protein tyrosine phosphatase; IA-2, islet-cell antigen 512; IA-2 β , phogrin [islet-cell-antigen-related PTP (IAR), PTP-NP, ICAAR, NE-6]; IA-2 β _(S762Y/Y898P/D933A), IA-2 β in which Ser⁷⁶² has been mutated to tyrosine, Tyr⁸⁹⁸ to proline, and Asp⁹³³ to alanine; OBA, 2-(oxalylamino)benzoic acid; pNPP, *p*-nitrophenyl phosphate; r.m.s.d., root-mean-square displacement; WPD loop, Trp-Pro-Asp loop; DTT, 1,4-dithio-*D*-L-threitol.

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Figure 1 Chemical structures of OBA [2-(oxalylamino)benzoic acid] and derivatives

Compound **1**, OBA; Compound **2**, 3-(oxalylamino)naphthalene-2-carboxylic acid; Compound **3**, 2-(oxalylamino)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylic acid; Compound **4**, 2-(oxalylamino)-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3-carboxylic acid; Compound **5**, 2-(oxalylamino)-4,7-dihydro-5H-thieno[2,3-c]pyran-3-carboxylic acid; Compound **6**, 7-(1,1-dioxo-1H-benzo[d]isothiazol-3-ylloxymethyl)-2-(oxalylamino)-4,7-dihydro-5H-thieno[2,3-c]pyran-3-carboxylic acid.

demonstrated that classic PTPs have a highly conserved fold and consistent C α -backbone trace [10]. This striking conservation at the structural level allowed us, using a combined bioinformatics and predictive structure-based design approach, to generate highly selective PTP1B inhibitors [11–13]. We reasoned that, if a PTP1B-like enzyme could be obtained by back-mutating only a few amino acid residues of IA-2 and IA-2 β , this would demonstrate that the fold of these proteins was also very similar to those of conventional PTPs. Furthermore, this would support the notion that the lack of IA-2 and IA-2 β activity against conventional PTP substrates was exclusively due to a few critical side-chain substitutions and not to differences in three-dimensional structure. Accordingly, if compounds could be found that bound with sufficient affinity to the mutated forms of IA-2 and IA-2 β , structural predictions were also likely to apply to the wild-type proteins.

In the following study, primary sequence-alignment analyses were used to identify additional non-consensus residues in IA-2 and IA-2 β that could potentially contribute to their observed lack of enzyme activity. As a result, mutant forms of IA-2 and IA-2 β were generated that displayed similar catalytic activity against the widely used PTP substrate *p*-nitrophenyl phosphate (*p*NPP) to that of the archetypal PTP, PTP1B. An enzyme kinetic assay was then used to screen a small library of low-molecular-mass non-peptide compounds for their ability to inhibit IA-2- and IA-2 β -catalysed *p*NPP dephosphorylation. Using this technique, a relatively potent inhibitor of IA-2 β _(S762Y/Y898P/D933A) (mouse IA-2 β in which Ser⁷⁶² has been mutated to tyrosine, Tyr⁸⁹⁸ to proline, and Asp⁹³³ to alanine) was identified. We anticipate that the present study provides significant information necessary for the future development of selective inhibitors of the wild-type IA-2 and IA-2 β enzymes.

EXPERIMENTAL

Materials

BSA, *p*NPP, 1,4-dithio-DL-threitol (DTT), GSH and GSTrapTM GSH-Sepharose columns were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Synthetic oligonucleotides were purchased from DNA Technology (Århus, Denmark) and TAG Copenhagen (Copenhagen, Denmark). NuPAGE[®] pre-cast BisTris/10% (w/v) polyacrylamide gels and electrophoresis reagents were obtained from Invitrogen (Groningen, The Netherlands). Protein assay reagent was purchased from Bio-Rad Laboratories (Hercules, CA, U.S.A.). Compounds **1–6** (see Figure 1) were synthesized as described previously [11–13]. Most other reagents were of analytical grade and from Merck (Darmstadt, Germany).

Site-directed mutagenesis, cloning, expression and purification of recombinant proteins

Cloning and expression of wild-type and mutant forms of human PTP1B^{1–321} (GenBankTM accession no. M31724), mouse IA-2^{621–979} (Q60673) and mouse IA-2 β ^{626–1000} (U57345) glutathione *S*-transferase (GST)-fusion-protein constructs was performed as described previously [11,14,15]. The following mutants (see Figure 2) were made by overlap extension PCR amplification [16] using appropriate restriction sites for cloning purposes:

IA-2: (i) A877D, (ii) D911A, (iii), A877D and D911A, (iv) H740Y, A877D and D911A

IA-2 β : (i) Y898P, (ii) D933A, (iii) Y898P and D933A, (iv) S762Y, Y898P and D933A

PTP1B: (i) Y46H, (ii) Y46S, (iii) P180Y (iv) D181A, (v) A217D

Figure 2 GST-fusion protein constructs prepared

Schematic depiction of IA-2^{621–979} (dark-grey bars), IA-2^{626–1000} (mid-grey bars) and PTP1B^{1–321} (white bars) GST-fusion mutants prepared showing the location of the point mutation within catalytic domain (based on the crystal structure of human PTP1B [32]). WPD loop, Trp-Pro-Asp loop.

Figure 3 SDS/PAGE of GST-IA-2

Following elution from two GSH-Sepharose (GSTRapTM) columns connected in tandem, approx. 0.5 μ g GST-IA-2 protein (from the peak protein fraction) was subjected to 10% SDS/PAGE using a NuPAGE[®] precast BisTris 10%-(w/v)-polyacrylamide gel, followed by Coomassie Brilliant Blue staining. The molecular mass of GST-IA-2 was about 66 kDa. The photograph is representative of the GST-proteins purified in the present study.

All constructs were inserted into pGEX expression vectors (Amersham Pharmacia Biotech) and mutations confirmed by DNA sequencing. Following protein expression [12], GST-fusion proteins were purified using two 1ml GSTRapTM GSH-Sepharose columns connected in tandem according to the manufacturer's (Amersham Pharmacia Biotech) protocol. Proteins were found to be $\approx$ 95% pure as assessed by SDS/PAGE and Coomassie Blue staining of the gels (a representative SDS/PAGE gel is shown in Figure 3). Protein concentrations were determined using Bio-Rad Laboratories protein assay reagent with BSA as standard, according to the manufacturer's protocol. Note that all experimental data and amino acid numbering presented in the

Table 1 Kinetic constants of wild-type and mutant PTP1B, IA-2 and IA-2 β GST-fusion proteins using *p*NPP as a substrate at pH 6.5 and 25 °C

Results are means $\pm$ S.E.M. of three or four determinations.

Enzyme	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ · M ⁻¹)
IA-2	No activity	No activity	
IA-2 _(A877D)	No activity	No activity	
IA-2 _(D911A)	Negligible activity	Negligible activity	
IA-2 _(A877D/D911A)	37.4 $\pm$ 3.6	5.2 $\pm$ 0.1	140 $\pm$ 10
IA-2 _(H740Y/A877D/D911A)	3.4 $\pm$ 0.1	7.1 $\pm$ 3.6	2120 $\pm$ 314
IA-2 β	No activity	No activity	
IA-2 β _(Y898P)	No activity	No activity	
IA-2 β _(D933A)	46.5 $\pm$ 4.4	6.5 $\pm$ 0.3	140 $\pm$ 20
IA-2 β _(Y898P/D933A)	69.0 $\pm$ 13.2	12.5 $\pm$ 2.0	190 $\pm$ 10
IA-2 β _(S762Y/Y898P/D933A)	1.6 $\pm$ 0.1	11.8 $\pm$ 0.3	7440 $\pm$ 370
PTP1B	2.3 $\pm$ 0.1	23.8 $\pm$ 0.7	10280 $\pm$ 310
PTP1B _(A217D)	No activity	No activity	
PTP1B _(D181A)	No activity	No activity	
PTP1B _(Y46H)	6.3 $\pm$ 0.1	11.3 $\pm$ 0.8	1810 $\pm$ 110
PTP1B _(Y46S)	16.8 $\pm$ 0.5	1.1 $\pm$ 0.1	70 $\pm$ 0.5
PTP1B _(P180Y)	0.7 $\pm$ 0.1	12.1 $\pm$ 0.1	18550 $\pm$ 610

present study refers to human PTP1B and mouse forms of IA-2 and IA-2 β .

Assay of PTP activity using *p*NPP as substrate

Enzyme reactions were carried out in microtitre plates using a range of *p*NPP concentrations (0.22–14.2 mM final assay concn.), essentially as described previously [12], except that the assay buffer used consisted of 50 mM 3',3'-dimethylglutaric acid, 50 mM NaCl, 0.1% (w/v) BSA and 5 mM DTT, pH 6.5 (total volume 100 μ l). Reactions were started by the addition of enzyme and carried out at 25 °C for 60 min. Reactions were terminated by the addition of 20 μ l of a 1:1 mixture of 0.5 M NaOH and 50% (v/v) ethanol. Enzyme activity was determined by measurement of the A_{405} , with appropriate corrections for absorbance of the compounds and *p*NPP. Presented values for K_m , k_{cat} and k_{cat}/K_m (Table 1) are the means $\pm$ S.E.M. of at least three independent determinations.

Determination of inhibitor constants

Enzymic reactions were carried out using a microtitre plate assay as described previously [12]. Briefly, appropriately diluted inhibitors (four different concentrations: diluted 1-, 3-, 9- and 27-fold respectively) were added to reaction mixtures containing seven different concentrations of the substrate, *p*NPP (range 0.22–14.2 mM final assay concn.). The same assay buffer (total volume 100 μ l) was used as that described above. Reactions (25 °C for 60 min) were started by the addition of enzyme and terminated as described above. Data were analysed using non-linear regression hyperbolic fit to classical Michaelis–Menten enzyme kinetic models. Inhibitor constants, K_i (expressed in μ M), were determined by replottting the apparent K_m values as a function of the inhibitor concentration [17]. For each compound, the inhibitor constant was given as a mean of the K_i values calculated for the different inhibitor concentrations. Only K_i values which were in the range of 1/10 and two times the corresponding inhibitor concentration were used for the calculation of the mean

value. Experiments were repeated if all K_i values were outside the range or if the correlation coefficient, R^2 , of the hyperbolic fit was less than 0.98. Values represent the means of at least two independent determinations.

Construction of wild-type and mutant IA-2 β models

The X-ray-crystallographic structure of human PTP1B, solved in complex with a hexapeptide, DADEpYL-NH₂ (one-letter amino acid code; pY is phosphotyrosine; the NH₂ indicates that the N-terminus is amidated) [18] (Protein Data Bank [19], entry code: 1ptu), was used as a template with which to construct models of wild-type IA-2 β and IA-2 $\beta_{(S762Y/Y898P/D933A)}$. Three-dimensional models were generated using the MODELLER package [20,21] a package based on an algorithm using spatial restraints extracted from an alignment of the target sequence with the template structure [22].

The quality of model structures was assessed by: (1) an analysis of how PTP structural motifs and single conserved residues were maintained in the model structures and (2) the application of PROCHECK [23], PROVE [24] and WHATIF [25]. Minor unfavourable side-chain contacts were relaxed by minimizing for a brief period. This involved 75 steps of both steepest decent and conjugated gradient minimization. The root-mean-square displacement (r.m.s.d.) for C α atoms between PTP1B and IA-2 β models ranged from 4.78 to 5.66 Å (1 Å = 0.1 nm). The lower r.m.s.d. value (4.78 Å) was obtained by omitting three loop regions (namely amino acids 775–785, 852–858 and 945–947) from IA-2 β that showed significant displacement compared with PTP1B. These regions were distant from the active-site region and therefore were not expected to have any influence on the binding of small molecules such as *p*NPP. Analysis using PROCHECK (main-chain-dihedral-angles check), PROVE (atomic-volume check) and WHATIF (structural-quality check) indicated that the model structures were very similar to the analysis performed for PTP1B.

RESULTS AND DISCUSSION

Generation and kinetic characterization of IA-2 and IA-2 β mutants

In agreement with previous studies [7–9], no detectable activity towards *p*NPP was observed for the wild-type forms of IA-2 and IA-2 β (Table 1). We therefore took a stepwise approach to find the minimal changes required to generate IA-2 and IA-2 β mutants with a catalytic activity similar to that of conventional PTPs. A number of GST-fusion proteins, consisting of the intracellular domains of mouse IA-2 or IA-2 β , were prepared (Figure 2) whereby variant residues were back-mutated, either individually or in combination, to those of the extensively characterized and catalytically active PTP human PTP1B. A kinetic analysis of these mutants was performed using *p*NPP as substrate which, to our knowledge, represents the first direct kinetic comparison of catalytically active IA-2 and IA-2 β mutants.

First, we studied the effect of changing a key residue in the P-loop. In classic PTPs, residue 217 (PTP1B numbering) is alanine, whereas the corresponding residue in IA-2 or IA-2 β is aspartic acid. Mutation of Ala²¹⁷ of PTP1B to an aspartate residue resulted in an absence of activity (Table 1), illustrating the importance of this residue for catalysis of *p*NPP [8,9]. While no activity could be measured by back-mutating this residue in IA-2, the equivalent IA-2 β mutant displayed appreciable activity (Table 1) with a value for k_{cat} of $\approx 6.5 \text{ s}^{-1}$ (i.e. only about 4-fold less than that observed for wild-type PTP1B). However, K_m was 20-fold higher than that

for PTP1B. As a consequence, k_{cat}/K_m was only $140 \text{ s}^{-1} \cdot \text{M}^{-1}$. Therefore, additional mutations had to be prepared for both IA-2 and IA-2 β .

Numerous X-ray-crystallographic and enzyme-kinetic studies have shown the importance of the WPD (Trp-Pro-Asp) loop for catalysis [6,26]. Since both IA-2 and IA-2 β differ from classic PTPs in this region, we next explored whether back-mutation within the WPD loop alone, to the equivalent residues in PTP1B, could promote IA-2 or IA-2 β activity. However, as shown in Table 1, neither IA-2_(A877D) nor IA-2 $\beta_{(Y898P)}$ displayed any detectable activity toward *p*NPP. Mutating PTP1B to the equivalent residue in IA-2 within this region of the catalytic domain [namely PTP1B_(D181A)] resulted in an enzyme with no measurable activity (Table 1). Such an observation is in accordance with previous work that has identified this as a mutant that, by attenuating cleavage of the scissile P–O bond of the substrate, acts as a substrate ‘trap’ [27].

These observations indicated that any possible effects of the IA-2 and IA-2 β WPD-loop mutations described above were masked by the presence of an aspartic acid residue in the P-loop. Therefore, combined P-loop and WPD-loop mutations were required. In agreement with previous work [7], a pronounced increase in IA-2_(D911A) catalytic efficiency was observed for the IA-2_(A877D/D911A) double mutant. A similar, but less pronounced, increase in k_{cat} and k_{cat}/K_m was also observed for IA-2 $\beta_{(Y898P/D933A)}$ [cf. IA-2 $\beta_{(D933A)}$]. The effect on k_{cat}/K_m was offset by a slight increase in K_m for this mutant.

Although we had now generated active enzymes, IA-2_(A877D/D911A) and IA-2 $\beta_{(Y898P/D933A)}$ displayed catalytic-efficiency (k_{cat}/K_m) values that were approx. 60-fold lower than that observed for wild-type PTP1B (Table 1). To allow for more direct comparisons of inhibitor potency, our strategy was to generate IA-2 and IA-2 β mutants with catalytic efficiencies similar to that of wild-type PTP1B (with respect to *p*NPP dephosphorylation). We therefore sought to address an additional variant IA-2 and IA-2 β residue, which, via back-mutation to the PTP consensus, could further improve the activity of the double-mutant enzymes. Through primary sequence alignment (Figure 4), an additional striking difference between IA-2 and IA-2 β and the PTP consensus was seen with the residue corresponding to Tyr⁴⁶ of PTP1B. Tyr⁴⁶ resides in the so-called ‘phosphotyrosine recognition’ region of PTP1B. This key and highly conserved residue helps define the depth of the active-site pocket and hence selectivity for phosphotyrosine, and, through its aromatic ring, is considered important for both recognition of phosphotyrosine and optimal substrate orientation [28]. Both IA-2 and IA-2 β show a substitution at this key position (see Figure 4), with IA-2 possessing a histidine residue (His⁷⁴⁰), whereas IA-2 β has a serine residue (Ser⁷⁶²). We therefore next examined how back-mutation of this additional residue influenced the enzyme activity of IA-2_(A877D/D911A) and IA-2 $\beta_{(Y898P/D933A)}$. Incorporating a H740Y point mutation on to IA-2_(A877D/D911A) to create IA-2_(H740Y/A877D/D911A) resulted in an 11-fold decrease in K_m for *p*NPP to 3.4 mM and an approx. 15-fold increase in k_{cat}/K_m (Table 1). Even more pronounced was the effect of the equivalent substitution for IA-2 $\beta_{(Y898P/D933A)}$. Here, substitution of Ser⁷⁶² by the consensus tyrosine residue led to an approx. 43-fold decrease in the K_m value for *p*NPP and an approx. 40-fold increase in catalytic efficiency that, compared with IA-2_(H740Y/A877D/D911A), more closely approached values observed for wild-type PTP1B (Table 1). The importance of this residue in classic PTPs is further illustrated by the finding that substituting Tyr⁴⁶ of PTP1B with the equivalent residue in IA-2 [namely PTP1B_(Y46H)] led to a 5-fold decrease in k_{cat}/K_m (Table 1). Introduction of a serine residue in position 46, the corresponding residue in IA-2 β , resulted

Figure 4 Primary amino-acid-sequence alignment of the intracellular domains of mouse IA-2 and IA-2β with the PTP catalytic domain of human PTP1B

IA-2 (U11812), IA-2β (U57345) and PTP1B (M31724) were aligned using the PILEUP program from GCG. The letters preceding the PTP 'name' represent the following species: h, human; m, mouse. Identical residues are indicated by solid black boxes. Grey boxes show residues that are conserved between IA-2 and IA-2β. Regions enclosed in boxes represent the 'phosphotyrosine recognition' motif (1), the WPD loop (2) and the PTP signature motif (P-loop) (3). IA-2 and IA-2β residues highlighted in **bold**/numbered within these regions are addressed in this study and indicate a major variance from the general PTP catalytic domain consensus (highlighted in **bold** and underlined).

in a marked 7-fold increase in the K_m value for p NPP and a pronounced approx. 150-fold decrease in k_{cat}/K_m for this mutant [PTP1B_(Y46S)] (Table 1). The more pronounced effect of a serine-versus-histidine residue in this position is readily explained by the fact that the aromatic nature of a histidine residue may to some extent serve the same function as a tyrosine residue.

Inhibition of active IA-2 and IA-2β mutants by OBA [2-(oxalylamino)benzoic acid] and associated compounds

Having generated IA-2 and IA-2β triple mutants with catalytic efficiencies similar to that of PTP1B (in addition to partially active IA-2 and IA-2β double mutants), we were now equipped with the necessary tools to evaluate potential inhibitors. Previous work by our laboratory established OBA to be a general competitive inhibitor of a number of PTPs [11]. We therefore investigated whether the mutant forms IA-2 or IA-2β could be inhibited by either OBA or a number of its derivatives (Compounds 1–6; Figure 1).

Compound 1

As shown in Table 2, OBA (Compound 1) was unable to inhibit either IA-2_(A877D/D911A) or IA-2_(H740Y/A877D/D911A) activity. Likewise, OBA was ineffective at inhibiting IA-2β_(Y898P/D933A). However, exchanging Ser⁷⁶² with a tyrosine residue [namely IA-2β_(S762Y/Y898P/D933A)] resulted in weak ($K_i > 500 \mu\text{M}$) inhibition of this mutant. In agreement with previous studies [11], OBA inhibited PTP1B with a K_i of approx. 230 μM . Substituting Tyr⁴⁶ of PTP1B with a histidine or serine residue, the equivalent residues in IA-2 and IA-2β respectively, led to a marked decrease in the inhibitory potency of this compound. This is likely to be due to a loss of aromatic–aromatic interaction between the enzyme and the inhibitor.

Compound 2

Since our previous studies showed that the incorporation of a second phenyl ring into the OBA structure allowed for additional

Table 2 Inhibition of wild-type and mutant IA-2-, IA-2β- and PTP1B-catalysed p NPP hydrolysis by Compounds 1–6 at pH 6.5 and 25 °C

Results are means of two or three determinations. Abbreviation: N/I, no inhibition at 1 mM.

Enzyme	Compound ...	K_i (μM)					
		1	2	3	4	5	6
IA-2 _(A877D/D911A)		N/I	N/I	> 1000	N/I	N/I	N/I
IA-2 _(H740Y/A877D/D911A)		N/I	> 1000	> 500	> 1000	N/I	310
IA-2β _(Y898P/D933A)		N/I	N/I	467	N/I	> 500	> 500
IA-2β _(S762Y/Y898P/D933A)		> 500	198	24	297	20	8
PTP1B		233	82	29	8	62	< 1
PTP1B _(Y46H)		> 500	403	62	72	317	10
PTP1B _(Y46S)		N/I	N/I	> 500	N/I	N/I	224
PTP1B _(P180Y)		208	52	30	2	76	< 1

hydrophobic interactions between the inhibitor and PTP [11,29,30], we next tested whether one such compound (Compound 2) showed improved potency towards our IA-2 and IA-2β mutants. This was indeed the case, with Compound 2 inhibiting IA-2β_(S762Y/Y898P/D933A) with a K_i value of 198 μM (Table 2). A weak but reproducible ($K_i > 1000 \mu\text{M}$) inhibition of IA-2_(H740Y/A877D/D911A) was also elicited by this compound. However, in both cases enzyme inhibition was only observed when a tyrosine residue was in position 46. Compound 2 inhibited PTP1B with an approx. 3-fold greater potency than Compound 1, an observation comparable with the 2.3-fold increase in inhibition measured in our previous study at pH 5.5 [11]. It is notable that Compound 2 was also able to inhibit PTP1B_(Y46H) activity, albeit at an approx. 5-fold lower potency than for the wild-type enzyme, indicating that, with respect to inhibitor binding, histidine in position 46 could partially substitute for a tyrosine residue. The failure of Compound 2 to inhibit IA-2_(A877D/D911A), where a histidine residue was located at position 46, is therefore likely to be due to factors in addition to the lower efficacy of this inhibitor towards a histidine group. The lack of an inhibition of either PTP1B_(Y46S)

or IA-2 β _(Y898P/D933A) by Compound 2 indicates that, as noted for Compound 1, a serine residue in position 46 of the target PTP is extremely unfavourable for this inhibitor.

Compound 3

To improve enzyme–inhibitor interaction together with inhibitor potency and selectivity, previous studies indicated that it was advantageous to introduce additional groups on to the second ring structure of Compound 2 while maintaining its hydrophobic characteristics [11]. We therefore next tested Compound 3 [12], an inhibitor that contains a saturated ring (Figure 1). For IA-2 β _(S762Y/Y898P/D933A), Compound 3 was approx. eight times more potent an inhibitor than Compound 2, inhibiting this mutant with a K_i value of 24 μ M (Table 2). In contrast with Compound 2, Compound 3 was also able to inhibit, albeit weakly, IA-2 β _(Y898P/D933A) (K_i 467 μ M) and, to a lesser extent, both IA-2_(H740Y/A877D/D911A) (K_i > 500 μ M) and IA-2_(A877D/D911A) (K_i > 1000 μ M). This is likely to be due to the greater flexibility of the cyclohexane ring structure of Compound 3 compared with the naphthyl ring of Compound 2, leading to improved hydrophobic interactions of Compound 3 with IA-2 and IA-2 β . Compound 3 inhibited PTP1B with similar potency (K_i 29 μ M) to that observed for IA-2 β _(S762Y/Y898P/D933A). However, it is notable that the approx. 3-fold improvement in potency of Compound 3 for PTP1B (cf. Compound 2) was substantially less than the 8-fold increase in efficacy measured for IA-2 β _(S762Y/Y898P/D933A). This disparity is indicative of subtle differences in the architecture of the active-site pockets of IA-2 β and PTP1B, which may be utilized in future optimization studies. Substitution of Tyr⁴⁶ by a histidine residue [namely PTP1B_(Y46H)] led to an approx. 3-fold decrease in the affinity of this inhibitor for PTP1B. Again, this decrease in inhibitor potency was much more marked when a serine residue replaced Tyr⁴⁶ in PTP1B [namely PTP1B_(Y46S)], indicating the importance, for inhibitor efficacy, of a ring structure in position 46 of the active site.

Compound 4

Using structure-based design we recently demonstrated that a highly selective inhibitor of PTP1B could be obtained by incorporating a basic nitrogen atom into Compound 3, affording Compound 4 [12]. The presence and position of this positive charge in the saturated ring structure of Compound 4 (Figure 1) allowed for the formation of a salt bridge with Asp⁴⁸ of PTP1B and led to a corresponding increase in inhibitory potency [12]. By contrast, PTPs containing an uncharged asparagine residue in this position were less efficiently inhibited, owing to repulsive forces between the positive charge on Compound 4 and the asparagine side chain [12]. Since IA-2, like PTP1B, contains an aspartic acid residue in position 48 (Figure 4), we hypothesized that Compound 4 would be a more potent inhibitor of the active mutants of this protein (cf. IA-2 β mutants which contain an alanine residue in this position). Unexpectedly, however, Compound 4 failed to inhibit IA-2_(A877D/D911A) and was only a very weak inhibitor of IA-2_(H740Y/A877D/D911A) activity with a K_i value (K_i > 1000 μ M) greater than that observed for Compound 3 (Table 2). We speculate that the lack of activity of Compound 4 against IA-2_(H740Y/A877D/D911A) is due to a rotamer conformation of aspartate that is different to that observed for PTP1B in complex with Compound 4 [12].

Compared with Compound 3, Compound 4 was also a weaker inhibitor of the IA-2 β mutants tested, the affinity for IA-2 β _(S762Y/Y898P/D933A) being $\approx$ 10-fold lower than that observed with Compound 3. This decrease in potency may be explained, at least in part, by the charged tetrahydropyridine ring of

Compound 4 being able to form less favourable interactions with the hydrophobic Ala⁷⁶⁴ residue of IA-2 β (compared with the hydrophobic cyclohexane ring structure of Compound 3).

Compound 5

Of Compounds 1–4, Compound 3 was the most potent inhibitor of both IA-2_(H740Y/A877D/D911A) (K_i > 500 μ M) and IA-2 β _(S762Y/Y898P/D933A) (K_i 24 μ M), but was also a strong inhibitor of PTP1B (K_i 29 μ M). In order to improve inhibitor selectivity and potency towards IA-2 and IA-2 β , we wished to introduce side groups on to this compound that would allow unique interactions with these two proteins. Although interesting from an enzyme-kinetic perspective, Compound 3 was found to be an unsuitable template from which to synthesize further compounds. We therefore tested whether a similar, but pyran-based, compound (Compound 5), on which it was easier to incorporate side groups, could be used as an alternative template. As shown in Table 2, Compound 5 inhibited IA-2 β _(S762Y/Y898P/D933A) with a similar K_i value (K_i 20 μ M) to that observed with Compound 3 (K_i 24 μ M), confirming its suitability for further studies. By contrast, Compound 5 inhibited PTP1B with a 3-fold lower potency (K_i 62 μ M).

Compound 6

Recent work from our laboratory has shown that Gly²⁵⁹ in PTP1B forms the base of a so-called ‘258/259 gateway’ that, due to its lack of side-chain atoms, allows easy access to the active site for a broad range of substrates, whereas bulky residues in the equivalent position of other PTPs cause steric hindrance and reduced substrate-recognition capacity [31]. These observations have formed the basis of recent work that has led to the development of substantially more potent and selective inhibitors of PTP1B [13]. By introducing a group, designed to address the region of the PTP surrounding residues 258 and 259, into the fused ring system of the inhibitor, improved selectivity was obtained for PTP1B due to steric hindrance imposed by bulky position-259 residues in other PTPs [13]. Since IA-2 and IA-2 β also possess a glycine residue in the equivalent position to Gly²⁵⁹ of PTP1B (Figure 4), a similar approach was adopted in the present study. Compound 6 (Figure 1), a derivative of Compound 5, whereby a saccharin moiety was introduced in the 7-position of the fused ring system, was now tested. Compound 6 inhibited IA-2 β _(S762Y/Y898P/D933A) with a K_i value of 8 μ M, a 2-fold improvement over the potency of Compound 5. Compound 6 was a classical competitive inhibitor of IA-2 β _(S762Y/Y898P/D933A), as demonstrated by Figure 5. Compound 6 was an even more potent inhibitor of PTP1B, inhibiting this enzyme with a K_i value of < 1 μ M. Thus the significant increase in potency with Compound 6 was obtained at the expense of the greater selectivity of the parent, pyran-based compound (Compound 5) towards IA-2 β _(S762Y/Y898P/D933A).

IA-2 β _(Y898P/D933A), containing a serine residue in the equivalent position to Tyr⁴⁶ of PTP1B, was considerably less potently inhibited by Compound 6 (K_i > 500 μ M). Likewise, substituting Tyr⁴⁶ of PTP1B with a serine residue resulted in substantially weaker inhibition by Compound 6 (K_i 224 μ M).

In marked contrast with the other inhibitors tested, Compound 6 also promoted substantial inhibition of IA-2_(H740Y/A877D/D911A) activity (K_i 310 μ M). However, Compound 6 was without effect on IA-2_(A877D/D911A), where a histidine residue resides in position 46. By contrast, although inhibitory potency was diminished, Compound 6 remained a very potent inhibitor of PTP1B_(Y46H) (K_i 10 μ M).

Figure 5 Compound 6 is a competitive inhibitor of IA-2 β (S762Y/Y898P/D933A)

Effect of Compound 6 on IA-2 β (S762Y/Y898P/D933A)-catalysed hydrolysis of pNPP at pH 6.5. (A) Lineweaver-Burk plot; (B) apparent K_m values [K_m (app)] versus inhibitor concentration; (C) apparent V_{max} values [V_{max} (app)] versus inhibitor concentration. $\diamond$, No addition of Compound 6; $\bullet$, addition of 37.4 μ M Compound 6; $\blacktriangle$, addition of 111.1 μ M Compound 6; $\blacksquare$, addition of 333.3 μ M Compound 6.

Molecular modelling of Compound 6 in the catalytic site of IA-2 β

On the basis of enzyme kinetic measurements, Compound 6 was identified to be the most potent inhibitor of IA-2 β (S762Y/Y898P/D933A) activity (K_i 8 μ M) in the present study. Since previous studies indicated this compound to be a substantially weaker inhibitor (K_i = 91–> 500 μ M) of a variety of other PTPs, including SHP-1 (Src-homology 2-containing protein tyrosine phosphatase-1), PTPH1, PTP α , PTP ϵ , PTP-LAR (leucocyte-antigen-related PTP) and GLEPP-1 (glomerular epithelial protein-1) [13], we considered Compound 6 to be a promising starting point from which to design a more selective inhibitor of the wild type IA-2 β enzyme. Given that Compound 6 and the other compounds (Compounds 1–5) tested were substantially less potent inhibitors of mutant IA-2 activity, we chose to only focus on IA-2 β for the molecular-modelling component of this study. As noted above, Compound 6 was also a potent inhibitor of PTP1B (K_i 1 μ M). To assist future lead optimization attempts, we explored the possibility of modifying Compound 6 in such a way as to create a

more potent inhibitor of IA-2 β while reducing affinity towards PTP1B. To aid this process, we next performed molecular-modelling studies of Compound 6 in the active-site cleft of IA-2 β (Figure 6). Since the tertiary structure of IA-2 β remains to be elucidated, a model structure of the active site of IA-2 β was constructed using the crystal structure of PTP1B as template [18]. Active-site models were created for both IA-2 β (S762Y/Y898P/D933A) and the wild-type IA-2 β enzyme. Compound 6 was now docked into the active sites of each of these model structures by superimposition with the calculated low-energy conformation of PTP1B co-crystallized with Compound 6 [13].

As can be seen from Figure 6(A), a number of potential sites of IA-2 β (S762Y/Y898P/D933A)–Compound 6 interaction were identified. As indicated by enzyme-kinetic data (see above), it appears that Tyr⁷⁶² (equivalent to Tyr⁴⁶; PTP1B numbering) plays a key role in the greater affinity of Compound 6 for IA-2 β (S762Y/Y898P/D933A) compared with IA-2 β (Y898P/D933A). The potential for Tyr⁷⁶² of IA-2 β (S762Y/Y898P/D933A) to form hydrogen bonds with the carboxy group of five-membered ring of Compound 6, together with possible hydrophobic interactions between the six-membered ring of Compound 6 and phenyl group of Tyr⁷⁶² (Figure 6A) contrast markedly with IA-2 β (Y898P/D933A). Here, as also shown by the wild-type IA-2 β model (Figure 6B), a serine residue is in position 762, hydrophobic interactions with the six-membered ring of Compound 6 are no longer possible, and, owing to a greater distance between the hydroxy group of Ser⁷⁶² and the carboxy group of the five-membered ring of the inhibitor, hydrogen bonding is impossible. Since the ultimate goal of future studies is to develop a potent and selective inhibitor against the wild-type IA-2 β molecule, we propose to modify Compound 6 so that it binds with greater affinity when a serine residue is in the 762 position. Such a rationale has the potential to offer high selectivity towards IA-2 β , since a serine residue in this position is unique to this PTP; the majority of PTPs have a consensus tyrosine residue (Tyr⁴⁶; PTP1B numbering) in this position. One possible strategy would be to introduce a side group on to the six-membered ring of Compound 6 so that it can now function as a potential hydrogen acceptor (hence form hydrogen bonds with the hydroxy group of Ser⁷⁶²), but will simultaneously reduce the potential for hydrophobic interactions with the inhibitor when a consensus tyrosine residue is in this position (see Area 1 of Figure 6B). A second potential unique point of interaction between IA-2 β and Compound 6 could utilize Asp⁹³³ at the base of the P-loop of the enzyme (see Area 2 of Figure 6B). We propose replacing the sulphur group of the five-membered ring of Compound 6 with a hydrogen donor. Since the majority of PTPs have a consensus alanine residue in position 933, only PTPs such as IA-2 and IA-2 β , which have an aspartate residue in this position, would be able to form hydrogen bonds with such a modified compound 6. It is recognized, however, that the longer Asp⁹³³ molecule (cf. the consensus alanine residue) may interfere with the orientation by which Compound 6 lies in the active-site pocket of IA-2 β . Nevertheless, when Asp⁹³³ is in an orientation where it swings away from the active site P-loop, enough space is available to accommodate such a modified Compound 6, with distances between Asp⁹³³ and an NH group on the five-membered ring of this modified compound, allowing the possibility for hydrogen bonding.

In summary, we have combined enzyme-kinetic analysis of active mutants of IA-2 and IA-2 β with molecular modelling to design a potentially unique active-site inhibitor of wild-type IA-2 β . Future studies will be conducted to synthesize and test the selectivity and potency of this inhibitor, with a similar approach applied in the design of a specific inhibitor of IA-2.

Figure 6 Model structures of the catalytic domains of (A) IA-2 β (S762Y/Y898P/D933A) and (B) wild-type IA-2 β docked with Compound 6

White lines indicate distances in Å (1 Å = 0.1 nm); The P-loop (located at the base of the catalytic pocket) and WPD loop (located above the catalytic pocket) are coloured red; residues denoted with yellow lettering indicate point mutations; Areas (1 and 2) encircled by broken yellow lines indicate potential unique sites of interaction between wild-type mIA-2 β and a modified Compound 6 (see the text for details).

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