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Mechanism of action of pyridazine analogues on protein tyrosine phosphatase 1B (PTP1B)

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Abstract—The inhibitory effect on PTP1B caused by the addition of pyridazine analogues has been investigated. Biophysical techniques, that is, mass spectrometry (MS), nuclear magnetic resonance (NMR), and isothermal titration calorimetry (ITC) were used for the characterization. Pyridazine analogues cause catalytic oxidation of the reducing agent, generating hydrogen peroxide that oxidizes the active site cysteine on the enzyme, leading to enzyme inactivation. Two additional compound classes show the same effect. We found one common structural feature in these molecules that allows the reaction with triplet molecular oxygen to be less endothermic. A proposed mechanism for the catalytic redox cycle is described.

Protein tyrosine phosphatase 1B (PTP1B) is a well established drug target for non-insulin dependent diabetes mellitus (NIDDM) and obesity.[1] Protein tyrosine phosphatases (PTPs) serve to hydrolyze phosphotyrosine moieties in target proteins, using a common catalytic mechanism.[2,3] The active site cysteine residue on the enzyme receives the phosphate from the phosphotyrosine residue on the target protein to form a phosphocysteine intermediate. The enzyme is returned to the free thiol form by transfer of the phosphate group to an activated water molecule in the active site. The integrity of the reduced form of the catalytic cysteine residue is central and oxidation renders the enzyme inactive.

Several studies have implicated PTB1B in dephosphorylation of the insulin receptor, turning off insulin signalling.[4–6] PTP1B knock-out mice (lacking the PTP1B gene) display reduced glucose and insulin levels in blood plasma, and have lean phenotypes.[7,8] Inhibiting PTP1B in vivo is believed to enhance insulin signalling by prolonging phosphorylation of the insulin receptor. Recently a report from Biovitrum was published describing a novel class of pyridazine analogues as non-competitive and reversible inhibitors of PTP1B.[9] The apparent physicochemical properties of these compounds make them attractive starting points for developing orally bioavailable drugs. However, despite a large number of compounds being synthesized, only a single compound displayed selectivity towards related PTPs and it was difficult to establish a robust structure–activity relationship (SAR) for PTP1B inhibition. Furthermore, co-crystallization attempts were not successful, and precipitated protein in the presence of inhibitor was observed. For inhibitors belonging to other compound classes co-crystal structures have readily been obtained.[10,11] Upon additional studies using biophysical techniques, that is, mass spectrometry (MS), calorimetry and nuclear magnetic resonance (NMR), it was possible to establish that pyridazine analogues inhibit PTP1B via an indirect mechanism. The same inhibition mechanism could also be confirmed for two other compound classes, pyrimido[5,4-*e*][1,2,4]triazine-5,7(1*H*,6*H*)-diones and 4-hydroxy-2*H*-benzo[*g*]indole-2,5(3*H*)-diones.

Keywords: Mechanism of action; Irreversible oxidation; Enzyme inhibition; Reducing agent.
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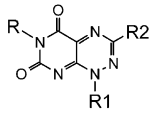
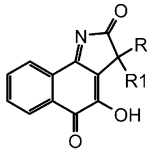


Pyrimido[5,4-e][1,2,4]triazine-5,7(1H,6H)-dione

In this article we describe experimental data obtained for the model compound **1** in the pyridazine analogue series, 3-amino-6,6-dimethyl-2-phenyl-2,8-dihydro-6*H*-pyrano[3,4-*c*]pyridazine-4-carbonitrile (Fig. 4).



4-hydroxy-2H-benzo[g]indole-2,5(3H)-dione

One dimensional ^{1}H NMR experiments with mixtures of PTP1B and compound **1** in the presence of reducing agent (DTT) showed a dramatic decrease of the protein signal intensities within ten min of the addition of the compound. This was interpreted as formation of large protein aggregates due to (at least partial) unfolding of PTP1B. Precipitate was not observed until after several hours. No effect on the protein signal intensities was observed when small molecule peptidomimetic PTP1B inhibitors were used.[11] Differential scanning calorimetry (DSC) experiments showed a well-characterized temperature induced unfolding behaviour of PTP1B in the absence of inhibitor, while in the presence of compound **1**, a temperature induced unfolding could not be detected. Both experiments were performed in the presence of DTT. Thus, the protein loses its tertiary structure in the presence of reducing agents and compound **1**.

Isothermal titration calorimetry (ITC) experiments were performed where compound **1** was added to the protein solution in the presence of reducing agents (TCEP and GSH) (Fig. 1). Even at a molar ratio of 0.1 between compound **1** and PTP1B the calorimetric response resulted in an exothermic heat burst. The maximum thermal power observed was more than 100 times higher than expected from a normal ligand binding experiment. Also the reaction lasted for more than 3 h. Apparently processes other than ligand binding occurred in the calorimetric vessel. The same experiment was performed in the absence of PTP1B. The calorimetric response was almost as high as in the previous experiment. However, the kinetics were faster and the heat evolved was larger in the presence of protein. ITC experiments on the same system without any reducing agent present showed no abnormalities. In fact, compound **1** and PTP1B formed a 1:1 complex.

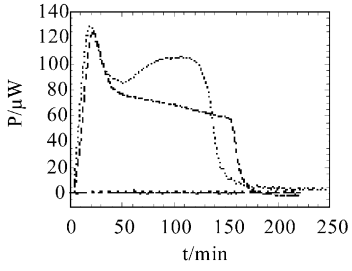


Figure 1. Three ITC experiments showing the heat evolved from adding compound **1** to different solutions. The dashed thermogram was obtained from consecutive additions of 6.5 μL 195 μM compound **1** in 3 mM TCEP, 50 mM sodium phosphate and 0.4 v/v% DMSO to 900 μL of the same buffer, the dotted thermogram is in the presence of 10 μM PTP1B. The solid line shows the titration of compound **1** to 10 μM PTP1B in 50 mM sodium phosphate and 0.4 v/v% DMSO pH 7.4. The time between the first and second injections was 30 min and the time between the consecutive 24 injections was 8 min. Experiments were performed at 25 °C using a TAM calorimeter (Thermometric AB, Järfälla, Sweden).

One-dimensional ^{1}H NMR experiments were used to study the effect of compound **1** on DTT. Fresh DTT (reduced) was mixed with compound **1** followed by immediate (within 10 min) NMR analysis. No NMR signals of reduced DTT could be observed. In the absence of compound **1** the NMR signals from reduced DTT were readily observed.

The initial heat burst in the ITC experiment and the rapid disappearance of the reduced form of the reducing agent in ^{1}H NMR experiments when compound **1** was present could be confirmed by oxygen measurements with a Clarke electrode. Compound **1** at 20 μM did not cause any significant consumption of O_2 in 50 mM sodium phosphate buffer. However, compound **1** at 1μM caused a rapid consumption of dissolved O_2 when the buffer contained 3mM DTT or 3mM TCEP at −25 mM/min and −30 mM/min, respectively.

An in vitro enzymatic assay was performed with and without reducing agent present (Fig. 2). The inhibitory effect of the compounds in the presence of catalase (a radical scavenger) was also assayed. Notably PTP1B did not lose any activity upon addition of the presumed

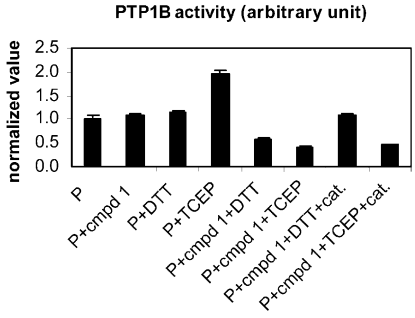


Figure 2. Enzymatic activity of PTP1B (P) in the presence of compound **1**. The PTP1B phosphatase activity was measured using *para*-nitrophenylphosphate (*p*NPP) as the substrate. The assay was performed in 200 μL volumes in buffers comprising 50 mM NaCl, 1 mM EDTA, 50 mM Hepes pH 7.2, and 1.25 mM pNPP. The PTP1B concentration was 50 ng/mL equivalent to approximately 1.5 nM. Compound **1** was added to a concentration of 10 μM, DTT, TCEP and catalase were added (when applicable) to final concentrations of 1 mM, 1 mM and 25 μg/mL, respectively. The enzymatic reaction was terminated after 60 min by addition of 100 μL NaOH. The optical density measured at 405 nm was taken as a measure of PTP1B activity. The graph shows the PTP1B activity in presence of inhibitor, with and without reducing agent (DTT or TCEP) and catalase. All values were normalized against the PTP1B activity in the absence of DTT or any additives (first bar).

inhibitor (compound **1**) in the absence of any reducing agents. In contrast, inhibition of PTP1B occurred only in the presence of both compound **1** and DTT or TCEP. TCEP by itself increased the PTP1B activity compared to the baseline activity. This observation suggests that a partial oxidation of the enzyme is reversed by TCEP but not by DTT. Furthermore, addition of catalase abolished the inhibitory effect observed in the presence of DTT. In the presence of TCEP on the other hand, catalase could not restore the PTP1B activity. Most likely the TCEP phosphine coordinates strongly to the heme group of the active site of the catalase and thus acts as an inhibitor.[12] It is evident that the compounds do not inhibit PTP1B via a direct mechanism.

In order to investigate the actual cause of the inhibitory effect, tryptic digestion followed by matrix assisted laser desorption ionisation time-of-flight (MALDI-TOF) MS analysis was performed. Compound **1** was added to PTP1B (1:1) in the presence of DTT or TCEP and tryptic digestion was performed overnight. MS data revealed that the molecular mass of the peak corresponding to the tryptic peptide containing the active site cysteine at M + H 2175 Da (ESGSLSPEHGPVVVHCSAGIGR) was to a large extent increased by 48 Da, resulting in an abundant peak at M + H 2223 Da (Fig. 3A). This effect was not observed in the absence of reducing agent, nor in the sample lacking the compound (Fig. 3B and C). The mass increase of 48 Da corresponded to an oxidation of the cysteine to a sulfonic acid. Electrospray ionization tandem MS (ESI MS/MS) analysis was performed for the additional confirmation of the oxidative modification using peptide sequencing. ESI MS/MS fragmentation of the peptide of interest at M + H 2223 Da confirmed the modification as addition of sulfonic acid at the active site cysteine (Fig. 3, inset). MS data recorded in the presence of TCEP displayed the same dependence on reducing agent for irreversible sulfonation (not shown).

Taken together our data show that treatment with compound **1**, together with millimolar quantities of reducing agent induce irreversible formation of the sulfonic derivative (SO_3H) of the active site cysteine. Interestingly, the sulfinic derivative (SO_2H) at M + H 2207 Da was sometimes also found in the absence of inhibitor whilst the sulfonic derivative only appeared in the presence of both compound **1** and reducing agent. No covalent interaction of compound **1** with PTP1B was observed.

PTPs are sensitive to oxidation. Inhibitory mechanisms dependent on oxidation of the active site cysteine have previously been described.[13–18] Treatment of PTPs with hydrogen peroxide or compounds such as Alendronate (4-amino-1-hydroxybutylidene 1,1-bisphosponate) or 2-phenyl-isoxazolidine-3,5-dione which generate reactive oxygen species, resulted in inhibition of the phosphatase activity through irreversible or reversible oxidation of the catalytic cysteine residue A plausible in vivo mechanism is proposed for the signal transduction pathway involving superoxide radical anion-mediated inactivation and glutathionylation of PTP1B.[17,18] They showed that the initial oxidative product, the sulfenic derivative (SOH), can easily be oxidized further to its irreversible sulfinic and sulfonic derivatives. This step

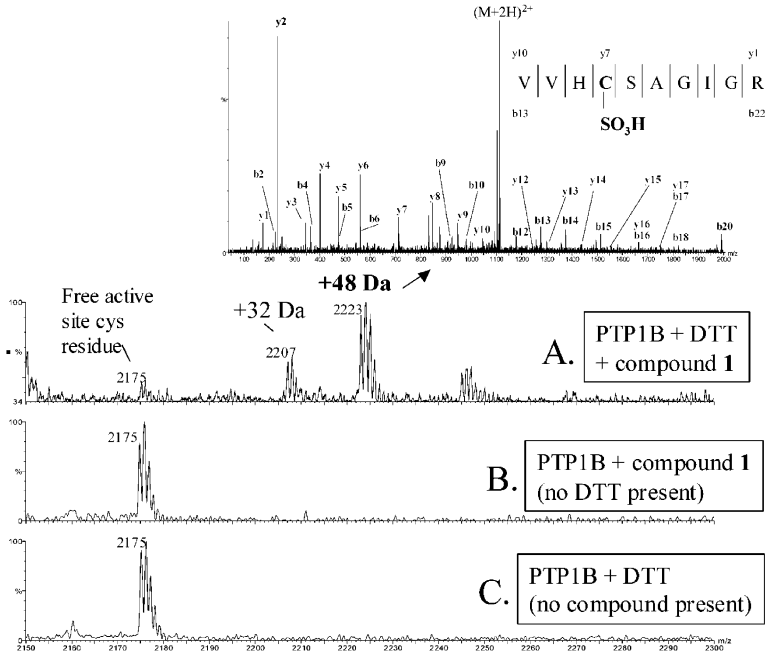


Figure 3. MALDI-TOF MS spectra of trypsin cleaved PTP1B. The enzyme was incubated in the presence and absence of inhibitor and/or DTT. Compound **1** was added to PTP1B (30 μM), in a solution consisting of 20 mM sodium phosphate, 10 mM NaCl, 1 mM DTT, and 0.3% (v/v) DMSO at pH 7.5. A molar ratio protein:ligand of 1:1 was normally used. For the proteolytic digestion and subsequent MALDI preparation the samples (2 μL each) were prepared following standard methods. A TofSpec SE (Micromass Inc., Manchester, UK) MALDI-TOF MS operated in reflectron mode was used. ESI MS/MS analysis (inset) of the doubly charged tryptic peptide at M + H 2223 Da was performed on a Q-Tof-1 (Micromass Inc., Manchester, UK). Of each sample, 3 μL was applied to a nanoESI spray needle. Fragmentation data were recorded in positive ion mode. Argon was used as collision gas and the collision energy was 30 V.

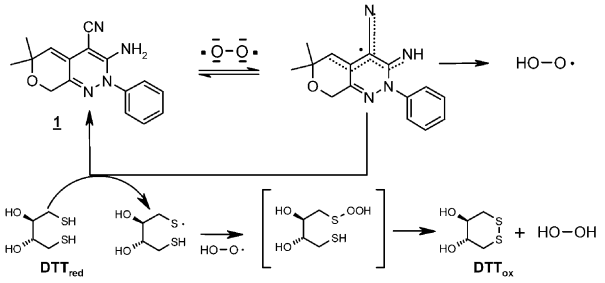


Figure 4. Formation of HO_2• and H_2O_2 as a result of the catalytic redox cycle of compound **1** and molecular oxygen in the presence of DTT as reducing agent. The initial reaction of O_2 and compound **1** appears left shifted when no reducing agent is added. DTT_{red} by itself is slowly (several days) oxidized to DTT_{ox} under aerobic conditions. Under the same conditions, with added compound **1** (cat), DTT_{ox} is quantitatively formed within min together with H_2O_2. The property of the participating reducing agent appears not to be critical for the three classes of compounds investigated, with compound **1** as illustrative example. Besides DTT, reducing agents such as TCEP and GSH are also effective. The combination of a reducing agent and compound **1** (or a compound of any of the two compound classes mentioned in the text) considerably accelerates the reduction of molecular oxygen to H_2O_2.

was prevented in vivo by glutathionylation of the sulfenic derivative to form a *S*-glutathionylated PTP1B, which could be reactivated by DTT or thioltransferase. A sulphenyl-amide derivative was found in addition to the previously known derivatives.[15,16]

For most organic compounds the reaction with triplet molecular oxygen is endothermic and occurs to a negligible extent at ordinary temperatures. We have found three classes of organic compounds that react rapidly at room temperature with triplet oxygen. One common structural feature of these molecules allows the reaction to be less endothermic. In our systems the radical product R• is part of a conjugated system and is highly stabilized by resonance delocalization. This resonance stabilization in the radical product decreases the heteroatom–proton bond energy sufficiently that the reaction can and does occur at physiological temperatures. It is a reactive product of the reaction with O_2 (Fig. 4) that attacks the enzyme. It appears that in the absence of any reducing agent the initial reaction of the compound with O_2 is shifted to the left (no evidence of oxidation of the active site cysteine in the absence of reducing agent). As soon as a reducing agent is added, the radical product R• is reduced, depleting the right hand side of the reaction Scheme. The oxidation–reduction cycle is repeated until all reducing agent has been consumed allowing rapid formation of reactive species H_2O_2 (or HO_2•) which oxidizes the cysteine-SH to the observed sulfonic derivative.

The previous report[9] described the pyridazine analogues as noncompetitive inhibitors of PTP1B and thus not binding within the active site of the enzyme. Our study, utilizing biophysical techniques, reveals that the pyridazine analogues do not inhibit PTP1B by binding to the enzyme. The inhibitory effect is solely due to the fact that pyridazine analogues cause catalytic oxidation of the reducing agent generating hydrogen peroxide that efficiently oxidizes the enzyme. Inhibition (oxidation) of PTP1B by hydrogen peroxide is reversible at moderate peroxide concentrations.[14] The speed of formation, and amount of peroxide formed, in the presence of pyridazine analogues and reducing agent will depend on their respective starting concentrations. This explains why under certain conditions PTP inhibition is reversible,[9] whereas in other cases (when the peroxide concentration reaches high enough concentration to drive the oxidation of the active site cysteine to the SO_3H state) it is irreversible.

Furthermore the apparent selectivity over T-Cell PTP observed for a few compounds in the series[9] may reflect a differential sensitivity to oxidation of the active site cysteine of the two proteins.

In conclusion, our investigations have identified three compound classes catalysing the reduction of molecular oxygen to H_2O_2 in the presence of a reducing agent. The common structural feature of these compound classes is a conjugated keto- or imino-quinoid or semiquinoid system that stabilizes an intermediate radical via resonance delocalization. In combination with a reducing agent, compounds possessing such a quinoid system may catalytically act as an efficient electron shuttle in redox systems. Under aerobic conditions HO_2• and H_2O_2 are likely to be formed, which may cause adverse and unpredicted reactions.

When working with enzyme systems sensitive to oxidation, it is always desirable to investigate whether oxidation of the active site cysteine has occurred after addition of new types of inhibitors. The described method using tryptic digestion followed by MALDI-TOF MS can rapidly answer this question.

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