Oxidation of Protein Tyrosine Phosphatases as a Pharmaceutical Mechanism of Action: A Study Using 4-Hydroxy-3,3-dimethyl-2H-benzo[g]indole-2,5(3H)-dione

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ABSTRACT
Growth factor and insulin signal transduction comprise series of protein kinases and protein phosphatases whose combined activities serve to propagate the growth factor signal in a regulated fashion. It was shown previously that such signaling cascades generate hydrogen peroxide inside cells. Recent work has implied that one function of this might be to enhance the feed-forward signal through the reversible oxidation and inhibition of protein tyrosine phosphatases (PTPs). We identified compound 4-hydroxy-3,3-dimethyl-2H-benzo[g]indole-2,5(3H)-dione (BVT.948) as an agent that is able to inhibit PTP activity in vitro noncompetitively, a mechanism involving oxidation of the catalytic cysteine residue. We investigated the pharmaceutical utility of this compound by examining its effects in a series of in vitro cellular and in vivo assays. Results showed that BVT.948 was able to enhance insulin signaling in cells, although it did not increase tyrosine phosphorylation globally. Furthermore, the compound was active in vivo, enhancing insulin tolerance tests in ob/ob mice, therefore apparently enhancing insulin sensitivity. BVT.948 was able to inhibit several other PTPs tested and also was efficient at inhibiting several cytochrome P450 (P450) isoforms in vitro. The data suggest that inhibitors of PTPs that display noncompetitive kinetics must be viewed with caution because they may oxidize the enzyme irreversibly. Furthermore, although such compounds display interesting biological effects in vitro and in vivo, their general pharmaceutical utility may be limited due to undesired effects on P450 enzymes.

Growth factor signal transduction comprises a series of ordered reversible protein and lipid phosphorylation reactions that serve to transfer the signal inside the cell and evoke a cellular response (for example, see Avruch, 1998). The receptor-proximal stages of the signaling cascade often involve protein tyrosine phosphorylation and dephosphorylation reactions, and more distal steps use protein serine and threonine phosphorylation. The feed-forward kinase reactions are countered by dephosphorylation reactions catalyzed by protein phosphatases; as a result, the strength and duration of a signal is very finely regulated. Thus, signaling cascades can behave with robust adaptation, where the signal is restricted to a short increase in signaling cascade activity before the resting steady state is reestablished or with proportional adaptation, where a new higher steady state of signaling is established for prolonged periods (Bhalla and Iyengar, 1999; Lauffenburger, 2000; Bhalla et al., 2002; Bagowski et al., 2003).

Therefore, feedback regulation of signaling cascades is a crucial component through which appropriate biological activity is attained. Protein tyrosine kinase reactions are countered by dephosphorylation by PTPs. The human genome encodes a large family of diverse PTPs containing upwards of 100 members (Wang et al., 2003). These include enzymes that are largely cytosolic in nature and proteins that traverse the plasma membrane and are regulated by extracellular ligands (receptor PTPs). PTPs play a wide variety of roles and display significant biological specificity. The functions of individual PTPs are now being more and more delineated through knockout studies in which enzymes are ablated in animals. Examples include T-cell PTP, lack of which leads to compromised immune function and death (You-Ten et al., 1997); the closely related PTP1B, which causes enhanced

ABBREVIATIONS. PTP, protein tyrosine phosphatase; BVT.948, 4-hydroxy-3,3-dimethyl-2H-benzo[g]indole-2,5(3H)-dione; pNPP, para-nitrophenylphosphate; OD, optical density; DTT, dithiothreitol; BVT.2274, 2,4-dihydroxy-3,3-dimethyl-2,3-dihydro-5H-benzo[g]indole-5-one-1-oxide; DMSO, dimethyl sulfoxide; α-MEM, minimum essential medium-α; P-Tyr, phosphorylated tyrosines; IR, insulin receptor; PBS, phosphate-buffered saline; P450, cytochrome P450; LAR, leukocyte antigen-related PTP; SHP-2, 5H2-containing PTP; TCPTP, T-cell PTP.
insulin and leptin sensitivity (Elchelby et al., 1999; Klaman et al., 2000; Cheng et al., 2002; Zablotny et al., 2002); the receptor PTP CD45, which causes defects in thymocyte development and immune cell dysfunction (Byth et al., 1996); and the LAR PTP, which causes metabolic abnormalities (Ren et al., 1998).

It has been shown that growth factor signal transduction causes the generation of hydrogen peroxide in cells (Sundaresan et al., 1995; Lee et al., 1998). Recent work has demonstrated that a possible physiological function of this is to inhibit PTP activity, allowing protein tyrosine phosphorylation reactions to prevail transiently, thus increasing the transduction of the signal (Mahadev et al., 2001). Thus, peroxide leads to the reversible oxidation of the catalytic cysteine residue of PTPs, which inhibits their activity against phosphorylated tyrosine residues. Furthermore, two recent studies have shown that the oxidation state of inactive PTP1B involves the formation of a sulfynylamide intermediate, permitting the oxidation to be reversed and the enzyme to be reactivated when needed (Salmeen et al., 2003; van Montfort et al., 2003). An interesting question to pose stemming from this work is whether such an oxidation mechanism would be harnessable in a pharmaceutical compound, thus allowing new therapies to be developed against human disease involving PTPs. We identified compound 4-hydroxy-3,3-dimethyl-2H-benzo[g]indole-2,5(3H)-dione (BVT.948) (Fig. 1) as a compound able to inhibit PTP activity in vitro through an oxidation mechanism. Therefore, we used this to address the possibility to utilize the oxidation mechanism therapeutically, here using insulin-dependent functional studies as a model system. Our data show that promising biological effects can be seen but that side effects of such oxidizing pharmaceuticals may be profound.

**Materials and Methods**

**Materials.** Catalase, the alkaline phosphatase assay kit (N-2770), para-nitrophenyl phosphate (pNPP) were from Sigma-Aldrich (St. Louis, MO). Coomassie Plus protein assay reagent was from Pierce Chemical (Rockford, IL). 2-Deoxy-[3H]-glucose was from PerkinElmer Life and Analytical Sciences (Boston, MA). Trypsin was from Roche Diagnostics (Indianapolis, IN). Human insulin (Actrapid) was from Novo Nordisk (Malmo, Sweden). Cell culture reagents were from Invitrogen (Carlsbad, CA). All reagents for time resolved fluorescence were from PerkinElmer Wallac (Gaithersburg, MD). An antibody raised to the triply-phosphorylated activation loop of the insulin receptor (catalogue no. 44-804) was from BioSource International (Camarillo, CA). Human LAR and T cell PTP were purchased from New England Biolabs. Unless stated otherwise, all other reagents were from Sigma-Aldrich and were the highest grade available.

**Expression and Purification of Recombinant Human PTPs.** A plasmid encoding amino acids 1 to 298 of human PTP1B without an affinity tag was expressed in *Escherichia coli*, and cultures were grown to an OD600 of approximately 2. Cells were lysed in lysis buffer consisting of 50 mM Tris-HCl, pH 7.5, 10% glycerol, 1 mM EDTA, 1 mM DTT, to which lysozyme was added to a concentration of 25 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% (w/v) BSA, and 50 mM Heps, pH 7.2 (for TCPTP). Trypsin was from Roche Diagnostics (Indianapolis, IN). Human insulin (Actrapid) was from Novo Nordisk (Malmo, Sweden). Cell culture reagents were from Invitrogen (Carlsbad, CA). All reagents for time resolved fluorescence were from PerkinElmer Wallac (Gaithersburg, MD). An antibody raised to the triply-phosphorylated activation loop of the insulin receptor (catalogue no. 44-804) was from BioSource International (Camarillo, CA). Human LAR and T cell PTP were purchased from New England Biolabs. Unless stated otherwise, all other reagents were from Sigma-Aldrich and were the highest grade available.

**Measurement of PTP Activity.** Protein tyrosine phosphatase activity was measured using pNPP as the substrate. Assays were performed in 200-μl volumes in buffers consisting of 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% (w/v) BSA, and 50 mM Heps, pH 7.2 (for PTP1B, LAR, and SHP-2) or 25 mM Tris-HCl/0.01% (w/v) Brij 35 (for TCPTP). pNPP was used at concentrations corresponding to the *Km* values for the individual enzymes (0.6 mM for TCPTP, 1.25 mM for PTP1B, and 6.25 mM for LAR and SHP-2). Assays were performed for different times for different enzymes (45–90 min, ensuring linearity was maintained) and were terminated by addition of 100 μl of 0.1 N NaOH. The OD405 was measured after 10 min, and the extent of reaction was calculated using a molar extinction coefficient of 18,000 M/cm. The effects of BVT.948 and BVT.2274 on PTP activity were measured in assays as described above where the enzyme was added last to wells containing all assay components including the compound. Experiments examining the effects of catalase on PTP1B activity were performed as above except that catalase at a final concentration of 25 μg/ml was included from the beginning. To determine the reversibility of the inhibition of PTP activity by BVT.948, 50 ng of PTP1B was incubated in 100 μl of assay buffer (see above) with 20 μM BVT.948 for 10 min in a centric-10 concentra-
tion device. The sample was then centrifuged at 14,000 rpm at 4°C for 12 min. The concentrate was subsequently washed three times with 100 μl of assay buffer followed by centrifugation. After washing, 190 μl of assay buffer was added to the sample, increasing the volume to 200 μl. Twenty microliters was used in assays measuring enzyme activity remaining using pNPP as a substrate as described above. Controls included enzyme, which was treated with inhibitor but not washed, and enzyme, which was not treated with BVT.948 but was put through the incubation and washing procedures.

**Intraperitoneal Insulin Tolerance Test.** Male mice 12 to 14 weeks old (C57BL/6Jbom-Lepob, ob/ob; Bomholtgaard, Ry, Denmark) were used in this study. They were divided into equal groups (n = 9) based on blood glucose levels. At time 0, the mice were injected with vehicle (NaCl with 10% DMSO) or BVT.948 (0.3 and 3 μmol/kg) and 1 U/kg insulin intraperitoneally. Blood glucose was determined from tail vein sampling at 0, 30, 60, and 120 min using a glucometer (Accutrend Sensor Comfortii; Roche Diagnostics Scandinavia, Stockholm, Sweden).

**Determination of Insulin Receptor Phosphorylation.** L6 myocytes were maintained in minimum essential medium-alpha (αMEM) supplemented with 10% fetal bovine serum and 100 IU/ml penicillin-streptomycin at 37°C in 5% CO2. Cells were seeded into 24-well plates, and the medium was replaced with αMEM containing 2% fetal calf serum to induce differentiation into myotubes. The medium was changed every other day, and cytidine (0.24 mg/ml medium) was added to the cultures at days 7 to 9 to suspend cycling cells. The cells were used in experiments after overnight serum starvation at days 11 to 16. They were treated with or without 25 μM BVT.948 for 30 min followed by 5 min of insulin (25 nM) stimulation. After freezing with liquid N2, the cells were lysed with a Tris-HCl buffer, pH 7.4, containing 1% Nonidet-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, and complete protease inhibitor cocktail (Roche Diagnostics). The cell extracts were centrifuged at 14,000g for 10 min, and the supernatants were used in the Delfia assay.

A lanthanide-based fluorescent assay (Delfia) was used to detect phosphorylated tyrosines (P-Tyrs) on the insulin receptor (IR). IRs in the cell lysates were captured on a FluoroNunc 96-well plate pre-coated with goat antibody to rabbit IgG (Cappel Laboratories, Durham, NC) and coated with rabbit antibody to IR (sc-711; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). P-Tyr was detected with a biotinylated monoclonal antibody to P-Tyr (PT-99B; Santa Cruz Biotechnology, Inc.) followed by europium-labeled streptavidin (PerkinElmer Life and Analytical Sciences). By addition of enhancement solution, a fluorescent chelate was formed with a long excited half-life, which permitted time-resolved emission measurement after a delay to avoid background interference.

**Glucose Uptake Assay.** L6 muscle cells were cultured and differentiated as described above, and cells were starved overnight in α-MEM without serum. Cells were pretreated in the presence or absence of 100 nM wortmannin for 30 min as described for individual experiments and were stimulated with 17 nM insulin for 60 min in the presence or absence of BVT.948 and in the continued presence of wortmannin where appropriate. Cell monolayers were washed twice with warm PBS (37°C), and PBS containing 1μCi/ml radioactive 2-deoxy-glucose was added for a further 4 min. Cells were subsequently washed with ice-cold PBS, and the cell monolayer was solubilized in 250 μl of 0.5M NaOH for 3 hours at room temperature. Radioactivity was measured in 200 μl of lysate, and the protein concentration was determined in the remainder using the Coomassie Plus kit from Pierce Chemical.

**Cytochrome P450 Inhibition Assay.** Microsomes from baculovirus-infected insect cells (BTI-TN-5B1-4) overexpressing human cytochrome P450 isoforms were purchased from BD Gentest (Woburn, MA). BVT.948 was dissolved in DMSO and added directly into incubations. The final DMSO concentration was held at maximum 1%. DMSO was added to control incubations (without BVT.948). Inhibitor concentration was between 0.1 and 100 μM in a total incubation volume of 100 μl with 25 mM KPO4 buffer, pH 7.4 (CYP2C9 and CYP2C19) or 100 mM KPO4 buffer, pH 7.4 (CYP1A2, CYP2D6, CYP2E1, and CYP3A4). Incubations were performed in triplicate. Concentrations of marker substrates were held at respective Km values. Marker substrates were 3-cyano-7-ethoxycoumarin (CYP1A2 and CYP2C19), 14C-diclofenac (CYP2C9), 14C-dextromethorphan (CYP2D6), 14C-p-nitrophenol (CYP2E1), and testosterone (CYP3A4), respectively. Reactions were started by addition of NADPH to a concentration of 1 mM and terminated with 50 μl of acetonitrile or 60% acetonitrile/40% 0.1 M Tris, pH 9.0 (CYP1A2 and CYP2C19).

**Results**

**In Vitro Characteristics of PTP Inhibition.** Compound BVT.948 was identified in a high-throughput screen looking for inhibitors of the PTP PTP1B. Kinetic analysis of the inhibition showed that the compound behaved noncompetitively (Fig. 2) with a Ki of 1.2 μM against PTP1B. Because PTPs are relatively susceptible to oxidation by virtue of their catalytic mechanism (Denu and Tanner, 1998; Zhu et al., 2001), and because compound BVT.948 reduced Vmax without affecting Km, we investigated the reversibility of the inhibition. PTP1B was incubated in the presence and absence of...
BVT.948 prior to washing in a centricon 10 device. Measurements of PTP activity afterwards showed that although 50% of the initial activity could still be detected from enzyme incubated without BVT.948, almost no activity from compound-treated enzyme was recovered (Fig. 3A). The reduced control activity was presumably due to losses in the centricon device. These data indicated that the inhibition was irreversible. Therefore, we investigated the role of hydrogen peroxide in the inhibitory mechanism by incubating PTP1B in the presence and absence of BVT.948 and catalase and measured time courses of activity (Fig. 3B). The data showed that BVT.948 was ineffective in inhibiting PTP1B activity in the presence of catalase (Fig. 3B, closed squares) but that the compound inhibited PTP1B in a time-dependent fashion when catalase was omitted (Fig. 3B, open squares). Thus, BVT.948 inhibits PTP1B activity irreversibly in vitro through catalyzing the hydrogen peroxide (or HO₂⁻) dependent oxidation of the enzyme. Interestingly, a variant of BVT.948 that is not a redox reactive compound (BVT.2274) did not oxidize the enzyme. These data indicated that the inhibition was irreversible. Therefore, we investigated the role of hydrogen peroxide in the inhibitory mechanism by incubating PTP1B in the presence and absence of BVT.948 and catalase and measured time courses of activity (Fig. 3B). The data showed that BVT.948 was ineffective in inhibiting PTP1B activity in the presence of catalase (Fig. 3B, closed squares) but that the compound inhibited PTP1B in a time-dependent fashion when catalase was omitted (Fig. 3B, open squares). Thus, BVT.948 inhibits PTP1B activity irreversibly in vitro through catalyzing the hydrogen peroxide (or HO₂⁻)-dependent oxidation of the enzyme. Interestingly, a variant of BVT.948 that is not a redox reactive compound (BVT.2274) was ineffective as an inhibitor of PTP1B (Table 1).

Using matrix-assisted laser-desorption ionization-mass spectrometry, we have observed that only the catalytic cysteine residue in PTP1B is oxidized to the sulfonic (SO₃H) form in the presence of BVT.948 and that all other cysteines are not modified (A. Tjernberg, unpublished data). These data agree with those reported for the effects of hydrogen peroxide on PTP1B (Denu and Tanner, 1998) and emphasize the role of hydrogen peroxide in the inhibitory mechanism of BVT.948.

**Cellular Activity of BVT.948.** BVT.948 was found to be able to cross cell membranes using CaCo-2 cells in culture (data not shown; Garberg et al., 1999). Therefore, we investigated whether BVT.948 exerted biological effects in cells by virtue of inhibiting PTPs, using insulin-dependent readouts as a model system. Rat L6 myotubes were incubated in the presence and absence of BVT.948 with and without insulin and wortmannin, and uptake of radiolabeled glucose was measured. BVT.948 did not increase insulin-stimulated glucose transport (data not shown) due to the fact that insulin readily stimulates a maximal response that cannot be further increased. However, BVT.948 alone was able to stimulate a near-maximal increase in glucose uptake (Fig. 4). Furthermore, the increased uptake of glucose was dependent on the activity of phosphoinositide 3-kinase because wortmannin completely blocked increased uptake. Stimulation of cells with insulin alone was used as a control (Fig. 4) and showed that BVT.948 exerts similar effects on L6 myotubes to insulin.

To investigate the cellular effects of BVT.948 further, we examined the ability of the compound to alter tyrosine phosphorylation both specifically, on the insulin receptor and globally, on all soluble cytosolic proteins. Using a simple immuno-capture assay and time-resolved fluorescence detection, we measured the phosphorylation status of the insulin receptor from L6 myotubes. This assay has been validated by examining a variety of different cell lines. Figure 5A shows insulin dose curves in HepG2 hepatoma cells, Fao rat hepatoma cells, and L6 myotubes. The EC50 values for phosphorylation of the insulin receptor in each of these cell lines was 2.2 ± 0.9, 35 ± 5, and 127 ± 24 nM, respectively. Thus, the assay allows the sensitivity of different cell lines to insulin to be determined. Figure 5B shows in CHO cells overexpressing the human insulin receptor that approximately 80% of the signal can be competed away with an unlabelled antibody raised to the phosphorylated activation loop of the insulin receptor. In contrast, the basal phosphorylation signal seen in unstimulated cells is not affected by the presence of the competing antibody. Thus, the use of a labeled antiphosphotyrosine antibody in the fluorescence assay detects predominantly the phosphorylation of the activation loop of the receptor and, thus, gives a measure of the activation of the

**TABLE 1**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>IC₅₀ BVT.948</th>
<th>IC₅₀ BVT.2274</th>
</tr>
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<tbody>
<tr>
<td>PTTP1B</td>
<td>0.9 ± 0.3</td>
<td>&gt;100</td>
</tr>
<tr>
<td>TCPTP</td>
<td>1.7 ± 0.1</td>
<td>&gt;100</td>
</tr>
<tr>
<td>SHP-2</td>
<td>0.09 ± 0.01</td>
<td>ND</td>
</tr>
<tr>
<td>LAR</td>
<td>1.5 ± 0.4</td>
<td>ND</td>
</tr>
<tr>
<td>YopH</td>
<td>0.7 ± 0.05</td>
<td>ND</td>
</tr>
<tr>
<td>Phage λ Ppase</td>
<td>&gt;100</td>
<td>ND</td>
</tr>
</tbody>
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ND, not determined.
kinase activity of the receptor itself. Figure 5C shows the effect of preincubation of L6 myotubes with BVT.948 on the phosphorylation status of the insulin receptor. The compound alone did not alter the basal phosphorylation of the receptor, whereas it doubled the level of phosphorylation stimulated by suboptimal concentrations of insulin (Fig. 5C, left panel). When cells were incubated with BVT.2274 instead of BVT.948, insulin-stimulated insulin receptor phosphorylation was not significantly affected (Fig. 5C, right panel). These data are consistent with in vitro data that show that BVT.2274 is not able to inhibit PTP activity (Table 1) and suggest that the effects of BVT.948 in increasing insulin receptor phosphorylation with cells is related to its ability to inhibit PTP activity. Figure 5D shows that the effect of BVT.948 was to strengthen the insulin signal and had no effects on the duration of the signal. Thus, after 35 min of stimulation, the phosphorylation status of the receptor had returned to basal, irrespective of treatment with BVT.948 or not. To investigate the effects of BVT.948 on the phosphorylation of other proteins, crude cell lysates from cells treated with or without compound were resolved on SDS-PAGE and blotted with antiphosphotyrosine antibody. No alterations in the insulin-stimulated phosphorylation of other proteins could be observed with this method (not shown).

In Vivo Activity of BVT.948. To test the effects of BVT.948 in vivo, we elected to examine possible insulin-sensitizing effects in insulin tolerance tests in ob/ob mice. Mice were treated with BVT.948 and insulin simultaneously, and blood glucose concentrations were monitored over 3 hours. Results showed that 3 μmol/kg BVT.948 significantly enhanced glucose clearance from the blood stream in response to insulin compared with vehicle-treated controls (Fig. 6). Thus, the cellular effects of BVT.948 reported above translated into consistent in vivo effects, leading to reductions in hyperglycaemia in this mouse model of diabetes.

Specificity of Effects of BVT.948. PTPs all utilize the same nucleophilic cysteine mechanism in dephosphorylating their substrates. Therefore, we examined the effects of BVT.948 on a variety of PTPs, reasoning that all PTPs ought to be susceptible to inhibition through this oxidation mechanism (Table 1). BVT.948 was able to inhibit four other PTPs tested, although with different efficacies. Thus, the compound was most effective against SHP-2 and least effective against TCPTP. Interestingly, BVT.2274 was unable to inhibit either PTP1B or TCPTP, strengthening the evidence that hydrogen peroxide (or HO₂) is integral to the mechanism. In addition, BVT.948 was not effective against phase λ phosphatase, an enzyme that uses another catalytic mechanism.

Many xenobiotic substances are metabolized in the body in part through hydroxylation catalyzed by P450 enzymes. To catalyze this reaction, P450 proteins undergo cycles of reduction and oxidation, involving both NADH or NADPH cofactors and molecular oxygen. Due to the fact that BVT.948 appeared to be a redox active compound, as seen in the generation of hydrogen peroxide, we investigated the effects of BVT.948 on the activity of a selection of P450 isomers in vitro. P450 activity was measured as described under Materials and Methods in the presence of 1, 10, and 100 μM BVT.948. Results showed that BVT.948 was a very effective inhibitor of all P450 isoforms tested, with especially pronounced effects on CYP2C19 and CYP2D6 (Fig. 7). Therefore, BVT.948 appeared to be an effective inhibitor of both PTP activity and P450 activity.

Discussion

In this work, we report the discovery and characterization of a small molecule inhibitor of PTP activity that causes the oxidation of the enzyme itself. Therefore, the compound mimics a recently described physiological mechanism, whereby signal transduction-generated hydrogen peroxide inhibits the activity of cellular PTPs, preventing the rapid downregulation of the signaling event (Meng et al., 2002). Inhibition of PTP activity would be expected to enhance growth factor signaling in cells and animals, and we elected to analyze insulin-regulated events to test this.

BVT.948 was found to be an effective irreversible inhibitor of PTPs in vitro, through the generation of hydrogen peroxide, and led to the specific oxidation of the catalytic cysteine 215 residue (A. Tjernberg, unpublished data). None of the other six cysteine residues in the recombinant PTP1B protein was affected after treatment with BVT.948. These data are in agreement with the effects of hydrogen peroxide on PTP1B (Denu and Tanner, 1998). This effect is presumably due to the low pH, value of the catalytic cysteine residue compared with that of other cysteines in the proteins (Peters et al., 1998). Thus, the pH, value of the catalytic cysteine residue in PTP1B is around 5.5, compared with the usual range of 8 to 11 for cysteines in polypeptide structures. This renders the residue more susceptible to electrophilic oxidation, therefore leading to oxidation of cysteine 215 in PTP1B while leaving the others unmodified. The irreversibility of the inhibition induced by BVT.948 in contrast to hydrogen peroxide (Denu and Tanner, 1998) is possibly due to different chemical mechanisms behind the oxidation of the enzyme. Thus, as shown in Fig. 8, BVT.948 is able to produce hydrogen peroxide itself or hydrogen peroxy radicals. It has been reported that the latter is almost 10-fold more effective in inactivating PTP1B (Barrett et al., 1999) than hydrogen peroxide; therefore, the

![Fig. 4. BVT.948 stimulates glucose uptake into L6 myotubes in a PI 3-kinase-dependent fashion. L6 myotubes were starved of serum overnight and stimulated with insulin or treated with 25 μM BVT.948 as described under Materials and Methods after a 30-min preincubation with (dark gray bars) or without (light gray bars) 100 nM wortmannin. After washing, glucose uptake was measured. Data are from four different treatments.](image-url)
enzyme is oxidized further to the irreversible sulfinic and sulfonic forms more readily with BVT.948 than hydrogen peroxide. We have not measured if the inhibition of PTP activity by BVT.948 in cells is irreversible, and it is possible that the oxidation there is reversible due to the subsequent glutathionylation of PTP1B as has been reported previously (Denu and Tanner, 1998; Barrett et al., 1999).

In cell-based assays, BVT.948 doubled the tyrosine phosphorylation of the insulin receptor at suboptimal concentrations of insulin, consistent with the activity of inhibiting cellular PTPs. The compound did not prolong the phosphorylation of the insulin receptor in this muscle cell line but rather increased it, an effect that is consistent with what has been seen in PTP1B knockout animals in which insulin receptor phosphorylation was not prolonged in the muscle (as compared with the liver) but was strengthened. However, BVT.948 alone did not have significant measurable effects on the basal phosphorylation of the insulin receptor. In contrast, BVT.948 was able to increase basal glucose transport in cultured myotubes, which was dependent on cellular signal transduction as seen in the ability of wortmannin (which inhibits phosphoinositide 3-kinase activity) to block this effect completely. The molecular basis for this apparent disparity in the actions of BVT.948 is probably due to limits of detection in current assay methodologies. It is possible that BVT.948 is able to increase tyrosine phosphorylation of cellular proteins, including the insulin receptor but that this is beneath the level of detection for both the fluorescence assay we have employed and Western-blotting detection methods. The Delfia fluorescence assay we have used is more sensitive than Western-blotting detection using enhance chemiluminescence because we are able to measure insulin receptor phosphorylation in cell lines that express the insulin receptor only sparsely, which we are not able to do by Western blotting (P. Hydén and S. R. James, unpublished data). However, the EC50s for insulin-stimulated phosphorylation of the in-

Fig. 5. BVT.948 increases insulin-stimulated insulin receptor phosphorylation. A, time-resolved fluorescence immunocapture assay was validated in three different cell lines. L6 myotubes (open circles), Fao hepatoma (filled circles), and HepG2 hepatoma (open triangles) were stimulated with increasing concentrations of insulin for 10 min, and insulin receptor phosphorylation was measured in lysates as described under Materials and Methods. Data show the means ± S.D. of results from three to five experiments for each cell line, normalized to the maximum response for each cell line. Solid lines are fits of the data to a hyperbola from which EC50 values were derived. B, competition with an unlabeled antibody raised to the triply labeled activation loop of the insulin receptor shows that most of the signal from the Delfia assay is due to phosphotyrosine in the activation loop of the receptor. L6 myotubes were left unstimulated (light bars) or stimulated with insulin (dark bars) as described under Materials and Methods. Receptor phosphorylation was measured as described above except that the assay was performed in the presence of increasing concentrations of unlabeled competing antibody as indicated. Error bars where visible represent the S.D. of triplicate determinations. C, L6 myotubes were incubated in the absence (light bars) or presence (dark bars) of BVT.948 (left panel) or absence (light bar) and presence (patterned bar) of BVT.2274 (right panel) and stimulated with the indicated concentrations of insulin, as described under Materials and Methods. Insulin receptor phosphorylation was then determined as above. D, L6 myotubes were incubated with BVT.948 (dark bars) or without BVT.948 (light bars) and stimulated with 25 nM insulin for the indicated times prior to measurement of receptor phosphorylation. Data in C and D were performed in triplicate and are representative of at least two independent experiments.
at the same time point. Data are taken from three separate experiments performed on different groups of animals. *, p < 0.05 with respect to vehicle controls at the same time point.

![Graph](image)

**Fig. 6.** BVT.948 enhances insulin sensitivity in the ob/ob mouse, a model of obesity and diabetes. Male mice were divided into three groups (nine animals per group) and treated with vehicle (open circles), 0.3 μmol/kg BVT.948 (filled circles) or 3 μmol/kg BVT.948 (open squares) and 1 U/kg insulin simultaneously. Blood glucose was monitored at various time points. Data are the means ± S.D. of triplicate determinations.

![Graph](image)

**Fig. 7.** The effects of increasing concentrations of BVT.948 on P450 activity in vitro. The indicated recombinant P450 enzymes were incubated with 1 (light gray bars), 10 (dark gray bars), and 100 (black bars) μM BVT.948, and activities were measured as described under Materials and Methods. Data are the means ± S.D. of triplicate determinations.

We also have seen that BVT.948 catalyzes the consumption of oxygen using a Clarke electrode, in the presence of NADPH cytochrome P450 reductase and NADPH (B. Lundgren, unpublished data) and that BVT.948 also inhibits the reductase enzyme itself. We hypothesize that BVT.948 catalyzes the production of hydrogen peroxide in a two-step reaction from molecular oxygen, itself being oxidized in the process (Fig. 8, steps 1 and 3). Cytochrome P450 reductase subsequently catalyzes the reduction of BVT.948 to the parent compound (steps 2 and 4), which can then catalyze the production of further hydrogenperoxy radicals from oxygen. As such, this represents a cycle in which BVT.948 is the catalyst of the production of hydrogen peroxide radicals and hydrogen peroxide, and cytochrome P450 restores the compound to its initial state mediated by NADPH. Thus, the P450 enzymes appear to be inhibited by BVT.948 in in vitro assays as shown in Fig. 7 due to the fact that they are fully occupied with reducing BVT.948 to its initial state. Considering the importance of P450 enzymes to the elimination of xenobiotics in the body, these data suggest the effects of BVT.948 represent a severe limitation to its utility as a pharmaceutical from which to develop compounds to treat diseases through the inhibition of PTP activity.

![Chemical Diagram](image)

**Fig. 8.** The catalytic cycle proposed for the production of hydrogen peroxide by BVT.948. BVT.948 is oxidized by cytochrome P450 to yield hydrogen peroxide, which then attacks the catalytic site of cytochrome P450 reductase enzyme itself. We hypothesize that BVT.948 catalyzes the consumption of oxygen using a Clarke electrode, in the presence of NADPH cytochrome P450 reductase and NADPH (B. Lundgren, unpublished data) and that BVT.948 also inhibits the reductase enzyme itself. We hypothesize that BVT.948 catalyzes the production of hydrogen peroxide in a two-step reaction from molecular oxygen, itself being oxidized in the process (Fig. 8, steps 1 and 3). Cytochrome P450 reductase subsequently catalyzes the reduction of BVT.948 to the parent compound (steps 2 and 4), which can then catalyze the production of further hydrogenperoxy radicals from oxygen. As such, this represents a cycle in which BVT.948 is the catalyst of the production of hydrogenperoxy radicals and hydrogen peroxide, and cytochrome P450 restores the compound to its initial state mediated by NADPH. Thus, the P450 enzymes appear to be inhibited by BVT.948 in in vitro assays as shown in Fig. 7 due to the fact that they are fully occupied with reducing BVT.948 to its initial state. Considering the importance of P450 enzymes to the elimination of xenobiotics in the body, these data suggest the effects of BVT.948 represent a severe limitation to its utility as a pharmaceutical from which to develop compounds to treat diseases through the inhibition of PTP activity.

BVT.948 is the second compound series that we have identified that acts as a catalyst for the production of hydrogen peroxide that subsequently inhibits PTP activity in vitro (Liljebris et al., 2002; Tjernberg et al., 2004). The catalytic mechanisms used in vitro by BVT.948 and the pyridazine compound series we described earlier are similar, where the compounds oxidize chemical reducing agents (DTT or tricarboxyethyl phosphate) in the assay buffer to generate hydrogen peroxide (Tjernberg et al., 2004). However, the effects of the pyridazine compounds (Liljebris et al., 2002) are slightly more enhanced by the compound described here than by BVT.948 (Liljebris et al., 2002).
different from BVT.948 insofar as the inhibition of PTP activity seen in vitro was reversible, the inhibition of P450 activity against its marker substrates was less marked, and the compounds did not catalyze the consumption of oxygen to a degree detectable with a Clarke electrode (P. Baranczewski and B. Lundgren, unpublished data). Therefore, we conclude from work with BVT.948 and the pyridazine series of compounds that, although reversible oxidation of PTPs to allow the feed forward of biochemical signal transduction is a powerful and effective biological mechanism, the mechanisms by which these compounds generate hydrogen peroxide and act as electron shuttles make it unlikely that it will be possible to harness them into small molecule pharmaceuticals to treat human disease. Any future small molecule therapies seeking to inhibit PTP activity by oxidizing the active site cysteine and act as electron shuttles make it unlikely that it will be possible to harness them into small molecule pharmaceuticals to treat human disease.

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References
Ren JM, Li P-M, Zhang WR, Sweet LJ, Cline G, Shulman GI, Livingstone JN, and

Fig. 8. Hypothetical scheme by which BVT.948 generates hydrogen peroxide for the inhibition of PTP activity. BVT.948 reduces molecular oxygen dissolved in the buffer via HO2 to hydrogen peroxide in two steps (steps 1 and 3). In the intervening steps (steps 2 and 4), BVT.948 is reduced to its original form by the action of P450. A cycle of activity, thus, is established, in which oxygen dissolved in the buffer is consumed for the production of hydrogen peroxide by BVT.948 and P450 activity is, therefore, apparently inhibited toward its marker substrates as it catalyzes the reformation of the reduced form of BVT.948.


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