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# 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

Charlotta Liljebris, Pawel Baranczewski, Eva Björkstrand, Styrbjörn Byström, Bo Lundgren, Agneta Tjernberg, Malin Warolén, Stephen R. James *

Biovitrum AB, SE 112 76 Stockholm, Sweden

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* Author for Correspondence: Department of Biology, Biovitrum AB, SE 112 76 Stockholm, Sweden. Telephone: +46 8 6972824. Fax. +46 8 6185882. E-mail:

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Abbreviations IR, insulin receptor; P-tyr, phospho-tyrosine; PI3K, phosphoinositide 3-kinase; PTP, protein tyrosine phosphatase; CYP, cytochrome P450; *p*NPP, *para*-nitro phenylphosphate;

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**Abstract**

Growth factor and insulin signal transduction comprises series of protein kinases and protein phosphatases whose combined activities serve to propagate the growth factor signal in a regulated fashion. It was previously shown that such signalling 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 (PTP). We identified compound BVT.948 as an agent that is able to inhibit PTP activity in vitro non-competitively, 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 signalling 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 was also efficient at inhibiting several cytochrome P450 (CYP) isoforms in vitro. The data suggest that inhibitors of PTPs which display non-competitive kinetics must be viewed with caution as they may oxidise the enzyme irreversibly. Furthermore, whilst such compounds display interesting biological effects in vitro and in vivo, their general pharmaceutical utility may be limited due to undesired effects on CYP 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 (see for example (Avruch, 1998)). The receptor-proximal stages of the signalling 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 catalysed by protein phosphatases and as a result, the strength and duration of a signal is very finely regulated. Signalling cascades can thus behave with robust adaptation, where the signal is restricted to a short increase in signalling cascade activity before the resting steady state is re-established, or with proportional adaptation, where a new higher steady state of signalling is established for prolonged periods (Bhalla et al., 2002), (Bhalla and Iyengar, 1999), (Bagowski et al., 2003), (Lauffenburger, 2000).

Feedback regulation of signalling cascades is therefore 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 which are largely cytosolic in nature, and proteins which traverse the plasma membrane and are regulated by extracellular ligands (receptor PTPs, RPTP). 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 insulin and leptin

sensitivity [(Klaman et al., 2000), (Elchelby et al., 1999), (Cheng et al., 2002), (Zablotny et al., 2002), the RPTP 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 sulfenyl-amide intermediate, permitting the oxidation to be reversed and the enzyme to be re-activated when needed (van Montfort et al., 2003), (Salmeen 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 BVT.948 (4-hydroxy-3,3-dimethyl-2H-benzo[g]indole-2,5(3H)-dione, Figure 1), as a compound able to inhibit PTP activity in vitro through an oxidation mechanism. We used this therefore to address the possibility to utilise 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 oxidising pharmaceuticals may be profound.

## Methods

### Materials

Catalase, the alkaline phosphatase assay kit (N-2770) and *para*-nitrophenyl phosphate (*p*NPP) were from Sigma. Coomassie Plus protein assay reagent was from Pierce. 2-Deoxy-[3H]-glucose was from NEN Life Science Products. Trypsin was from Boehringer Mannheim (Indianapolis, IN, USA). Human insulin (Actrapid) was from Novo Nordisk, Denmark. Cell culture reagents were from Life Technologies. All reagents for time resolved fluorescence were from Wallac. An antibody raised to the triply-phosphorylated activation loop of the insulin receptor (catalogue number 44-804) was from Biosource International. Human LAR and T cell PTP were purchased from New England Biolabs. Unless stated otherwise, all other reagents were from Sigma and were the highest grade available.

### Expression and purification of recombinant human PTPs

A plasmid encoding amino acids 1-298 of human PTP1B without an affinity tag was expressed in *E. coli* and cultures were grown to an $OD^{600}$ of approximately 2. Cells were lysed in lysis buffer comprising 50mM Tris-HCl pH 7.5, 10% glycerol. 1mM EDTA, 3mM DTT to which was added lysozyme to a concentration of 0.2mg/ml, 1mg/ml DNAseI and 3mM $MgCl_2$. After dilution and filtration, the extract was resolved by serial chromatography on ion-exchange chromatography, hydrophobic chromatography (after exchanging to a high salt buffer) and gel filtration. All chromatographic steps were performed using an ÄKTA 2 system (Amersham Pharmacia Biotech). Recombinant PTP1B was >95% pure and yields were approximately 6mg per gram of *E. coli* paste. Enzyme was stored in a buffer comprising 25mM Tris-HCl pH 7.5, 300mM NaCl, 1mM EDTA and 3mM DTT at

-70$^{o}$C. A GST fusion protein containing the active site of SHP-2 was expressed and purified as described previously (Löthgren et al., 2001).

**Measurement of PTP activity**

Protein tyrosine phosphatase activity was measured using *p*NPP as the substrate. Assays were performed in 200µl volumes in buffers comprising 50mM NaCl, 1mM EDTA, 1mM DTT, 0.1% (w/v) BSA and 50mM Hepes pH 7.2 (for PTP1B, LAR and SHP-2) or 25mM Tris-HCl/ 0.01% (v/v) Brij 35 (for TCPTP). *p*NPP was used at concentrations corresponding to the Km values for the individual enzymes (0.6mM for TCPTP, 1.25mM for PTP1B and 6.25mM for LAR and SHP-2). Assays were performed for different times for different enzymes (45-90 minutes, ensuring linearity was maintained) and were terminated by addition of 100µl 0.1N NaOH. The $OD^{405}$ was measured after 10 minutes and the extent of reaction was calculated using a molar extinction coefficient of $18000M^{-1}$ $cm^{-1}$. 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 25µg/ml was included from the beginning.

To determine the reversibility of the inhibition of PTP activity by BVT.948, 50ng PTP1B was incubated in 100µl assay buffer (see above) with 20µM BVT.948 for 10 minutes in a centricon-10 concentration device. The sample was then centrifuged at 14000rpm at 4$^{o}$C for 12 minutes. The concentrate was subsequently washed three times with 100µl assay buffer followed by centrifugation. After washing, 190µl assay

buffer was added to the sample increasing the volume to 200µl. 20µl was used in assays measuring enzyme activity remaining using *p*NPP 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 (ITT)**

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

**Determination of insulin receptor phosphorylation**

L6 myocytes were maintained in minimum essential medium-alpha (α-MEM) supplemented with 10% foetal bovine serum (FBS) and 100 IU/ml penicillin-streptomycin at 37$^{o}$C in 5% $CO_2$. Cells were seeded into 24-well plates and the medium was replaced with αMEM containing 2% FCS 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 day 7-9 to suspend cycling cells. The cells were used in experiments after over night serum starvation at day 11-16. They were treated with or without 25 µM BVT.948 for 30 min followed by 5 min insulin (25 nM) stimulation. After freezing with liquid $N_2$ the cells were lysed with a Tris-HCl buffer, pH7.4, containing 1% Nonidet-40, 0.25% sodium deoxycholate, 150mM NaCl, 1mM

EDTA, 1mM EGTA, 1mM sodium orthovanadate, 10mM β-glycerophosphate, 5mM sodium pyrophosphate and complete protease inhibitor cocktail from Boehringer. 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-tyr) on the insulin receptor (IR). IR's in the cell lysates were captured on a FluoroNunc 96-well plate pre-coated with goat antibody to rabbit IgG (from Cappel) and coated with rabbit antibody to IR (sc-711 from Santa Cruz Biotechnology). P-tyr was detected with a biotinylated monoclonal antibody to P-tyr (PY-99B from Santa Cruz Biotechnology) followed by europium-labelled streptavidin (from Perkin Elmer). By addition of enhancement solution, a fluorescent chelate was formed with a long excited halftime, 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 αMEM without serum. Cells were pre-treated in the presence or absence of 100nM wortmannin for 30 minutes as described for individual experiments and were stimulated with 17nM insulin for 60 minutes 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$^{o}$C) and PBS containing 1μCi/ml radioactive 2-deoxy-glucose was added for a further 4 minutes. Cells were subsequently washed with ice-cold PBS and the cell monolayer was solubilised in 250μl 0.5M NaOH for three hours at room temperature. Radioactivity

was measured in 200µl lysate and the protein concentration was determined in the remainder using the Coomassie Plus kit from Pierce.

**Cytochrome P450 Inhibition Assay**

Microsomes from baculovirus infected insect cells (BTI-TN-5B1-4) over expressing human cytochrome P450 isozymes were purchased from Gentest Corp., MA, USA. 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 0.1-100µM in a total incubation volume of 100µl with 25mM $KPO_4$ buffer, pH 7.4 (CYP2C9 and CYP2C19) or 100mM $KPO_4$ buffer, pH 7.4 (CYP1A2, CYP2D6, CYP2E1 and CYP3A4). Incubations were performed in triplicates. Concentrations of marker substrates were held at respective Km values. Marker substrates were 3-cyano-7-ethoxycoumarin (CYP1A2 and CYP2C19), $^{14}$C-diclofenac (CYP2C9), $^{14}$C-dextromethorpan (CYP2D6) $^{14}$C-p-nitrophenol (CYP2E1) and testosterone (CYP3A4), respectively. Reactions were started by addition of NADPH to a concentration of 1mM and terminated with 50µl acetonitrile or 60% acetonitrile/40% 0.1M 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 non-competitively (Figure 2) with a Ki of 1.2μM 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 whilst 50% of the initial activity could still be detected from enzyme incubated without BVT.948, almost no activity from compound-treated enzyme was recovered (Figure 3A). The reduced control activity was presumably due to losses in the centricon device. These data indicated that the inhibition was irreversible. We therefore 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 (Figure 3B). The data showed that BVT.948 was ineffective in inhibiting PTP1B activity in the presence of catalase (closed squares in Figure 3B) but that the compound inhibited PTP1B in a time-dependent fashion when catalase was omitted (open squares, Figure 3B). Thus, BVT.948 inhibits PTP1B activity irreversibly in vitro through catalysing the hydrogen peroxide (or $HO_2$•) -dependent oxidation of the enzyme. Interestingly, a variant of BVT.948 which is not a redox reactive compound (BVT.2274, Figure 1) was ineffective as an inhibitor of PTP1B (Table 1). Using MALDI-MS, we have observed that only the catalytic cysteine residue in PTP1B is oxidised to the sulphonic ($SO_3H$) form in the presence of

BVT.948 and that all other cysteines are not modified (Agneta Tjernberg, unpublished). These data agree with those reported for the effects of hydrogen peroxide on PTP1B (Denu and Tanner, 1998) and emphasise 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)). We therefore 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 radio-labelled 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 which cannot be further increased. However, BVT.948 alone was able to stimulate a near-maximal increase in glucose uptake (Figure 4). Furthermore, the increased uptake of glucose was dependent on the activity of phosphoinositide 3-kinase (PI3K) as wortmannin completely blocked increased uptake. Stimulation of cells with insulin alone was used as a control (Figure 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±0.9nM, 35±5nM and 127±24nM, respectively. The assay thus allows the sensitivity of different cell lines to insulin to be determined. Figure 5B shows in CHO cells over-expressing 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. By contrast, the basal phosphorylation signal seen in unstimulated cells is not affected by the presence of the competing antibody. Thus, the use of a labelled anti-phosphotyrosine 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 kinase activity of the receptor itself. Figure 5C shows the effect of pre-incubation 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 sub-optimal concentrations of insulin (Figure 5C left panel). When cells were incubated with BVT.2274 instead of BVT.948, insulin-stimulated insulin receptor phosphorylation was not significantly affected (Figure 5C right panel). These data are consistent with in vitro data which 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 minutes 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 anti-phosphotyrosine 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-sensitising 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 three hours. Results showed that 3μmol/kg BVT.948 significantly enhanced glucose clearance from the blood stream in response to insulin compared to vehicle-treated controls (Figure 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 utilise the same nucleophilic cysteine mechanism in dephosphorylating their substrates. We therefore 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 $HO_2$•) is integral to the mechanism. In addition, BVT.948 was not effective against phase λ phosphatase, an enzyme which uses another catalytic mechanism.

Many xenobiotic substances are metabolised in the body in part through hydroxylation catalysed by CYP enzymes. To catalyse this reaction, CYP 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 CYP isoforms in vitro. CYP activity was measured as described in Methods in the presence of 1, 10 and 100μM BVT.948. Results showed that BVT.948 was a very effective inhibitor of all CYP isoforms tested, with especially pronounced effects on CYP2C19 and CYP2D6 (Figure 7). BVT.948 therefore appeared to be an effective inhibitor of both PTP activity and CYP activity.

## Discussion

In this work we report the discovery and characterisation of a small molecule inhibitor of PTP activity that causes the oxidation of the enzyme itself. The compound therefore mimics a recently described physiological mechanism, whereby signal-transduction-generated hydrogen peroxide inhibits the activity of cellular PTPs, preventing the rapid down-regulation of the signalling event (Meng et al., 2002). Inhibition of PTP activity would be expected to enhance growth factor signalling in cells and animals and we elected to analyse 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 (Agneta Tjernberg, unpublished). No other of the 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 pKa value of the catalytic cysteine residue compared to that of other cysteines in the proteins (Peters et al., 1998). Thus, the pKa value of the catalytic cysteine residue in PTP1B is around 5.5, compared to the usual range of 8-11 for cysteines in polypeptide structures. This renders the residue more susceptible to electrophilic oxidation, therefore leading to oxidation of cysteine 215 in PTP1B whilst 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 Figure 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 and the enzyme is therefore oxidised 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 (Barrett et al., 1999), (Denu and Tanner, 1998).

In cell-based assays, BVT.948 doubled the tyrosine phosphorylation of the insulin receptor at sub-optimal 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 to the liver) but was strengthened. However, BVT.948 alone did not have significant measurable effects on the basal phosphorylation of the insulin receptor. By 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 PI3K 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 chemoluminescence, as we are able to measure insulin receptor phosphorylation in

cell lines which express the insulin receptor only sparsely, which we are not able to do by western blotting (Hydén & James, unpublished). However, the EC50s for insulin-stimulated phosphorylation of the insulin receptor we report here for three different cell lines represent supra-physiological concentrations of insulin, indicating that the assay is not able to measure changes in phosphorylation that would be evoked by physiological concentrations of insulin (approximately 100pM). Thus, we suspect that BVT.948 stimulates glucose uptake in L6 cells in an insulin receptor-dependent fashion, but the phosphorylation of the receptor is beneath the levels of detection in our assays.

The data from these in vitro studies indicated that BVT.948 is able to enhance insulin-mediated signal transduction (as seen in the phosphorylation assay) and is able also to act as an insulin mimetic, stimulating the uptake of glucose itself. We reasoned therefore that the compound may also enhance the effects of insulin in vivo, such as uptake of glucose from the blood stream and inhibition of glucose production from the liver. Using insulin tolerance tests, we tested the former hypothesis, measuring the rate of clearance of glucose from the blood of ob/ob mice in insulin tolerance tests in which the animals were treated with insulin and BVT.948 simultaneously. The data showed that glucose was cleared with greater efficiency in animals treated with BVT.948 compared to vehicle. Thus, the inhibition of PTPs by BVT.948 was apparently translated into a physiological effect in response to an insulin challenge.

BVT.948 was able to inhibit several different PTPs that utilise a catalytic mechanism in which a nucleophilic cysteine residue attacks the phosphate moiety of

phosphotyrosine in protein substrates. The compound therefore was a non-selective inhibitor of PTPs although it displayed apparently greater potency against SHP-2, which may be due to the relative susceptibility of SHP-2 to oxidation by hydrogen peroxide or due to the properties of the molecule such that it fits better near the active site of this enzyme. The compound could therefore in principle have been viewed as a potential lead molecule for the development of more selective compounds for specific PTPs that employ oxidation via hydrogen peroxide as the molecular mechanism of action.

However, proteins other than PTPs may be susceptible to the actions of hydrogen peroxide generated in biological systems, thus limiting the utility of BVT.948 and related molecules. Furthermore, we measured the effects of BVT.948 on cytochrome P450 activity in vitro since the activity of this class of enzymes may be particularly susceptible to compounds which themselves catalyse the reduction of molecular oxygen. We found that BVT.948 was effective at inhibiting all CYP 450 isoforms tested, in particular CYP2C19 and CYP2D6. We have also seen that BVT.948 catalyses the consumption of oxygen using a Clarke electrode, in the presence of NADPH cytochrome P450 reductase and NADPH (Bo Lundgren, unpublished) and that BVT.948 also inhibits the reductase enzyme itself. We hypothesise that BVT.948 catalyses the production of hydrogen peroxide in a two-step reaction from molecular oxygen, itself being oxidised in the process (steps 1 and 3 in Figure 8). Cytochrome P450 reductase subsequently catalyses the reduction of BVT.948 to the parent compound (steps 2 and 4) which can then catalyse 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. The CYP enzymes thus appear to be inhibited by BVT.948 in in vitro assays as shown in Figure 7, due to the fact that they are fully occupied with reducing BVT.948 to its initial state. Considering the importance of CYP 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, in the press). The catalytic mechanisms used in vitro by BVT.948 and the pyridazine compound series we described earlier are similar, where the compounds oxidise chemical reducing agents (DTT or TCEP) in the assay buffer to generate hydrogen peroxide (Tjernberg et al, in the press). The effects of the pyridazine compounds (Liljebris et al., 2002) are however slightly different from BVT.948 insofar as the inhibition of PTP activity seen in vitro was reversible, the inhibition of CYP activity against its marker substrates was less marked and the compounds did not catalyse the consumption of oxygen to a degree detectable with a Clarke electrode (Baranczewski and Lundgren, unpublished). We conclude therefore from work with BVT.948 and the pyridazine series of compounds that, whilst 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 oxidising the active site cysteine residue will need to employ other mechanisms such as mechanism-based oxidation in which the compound in the active site causes cysteine oxidation at some stage in the catalytic cycle of the enzyme. Furthermore, as reversible inhibition is to be preferred, a molecule which induces only the first step of thiol oxidation of the cysteine residue (to the sulphenic form) and preserves the ability of this form of the enzyme to form the recently-reported sulphenyl-amide form (Salmeen et al., 2003; van Montfort et al., 2003) will be the most viable way forward in the production of compounds that harness the biological mechanism of enzyme oxidation to inhibit PTP activity.

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## Legends for Figures

### Figure 1

Chemical structures of BVT.948 (4-hydroxy-3,3-dimethyl-2H-benzo[g]indole-2,5(3H)-dione) and the control non-redox xompound BVT.2274 (2,4-dihydroxy-3,3-dimethyl-2,3-dihydro-5H-benzo[g]indole-5-one-1-oxide).

### Figure 2

Kinetic analysis of inhibition of PTP1B by BVT.948. Upper panel: Velocity curves performed with 20ng PTP1B in the presence of increasing concentrations of BVT.948 (0.1-2μM). Data are representative of several experiments, each point the average of quadruplicates. Solid lines are fits of the data to the modified version of the Henri Michaelis Menten equation $v=(V_{\max}.[S].\frac{1}{1+[I]/K_i})/K_m+[S]$, assuming non-competitive inhibition. Lower panel: Lineweaver-Burke analysis of the data in the upper panel showing the profile typical for non-competitive inhibitors.

### Figure 3

BVT.948 inhibits PTP1B irreversibly using hydrogen peroxide. A) 50ng PTP1B was treated with 20μM BVT.948 for 10 minutes and washed as described in the Methods section. Enzyme activity remaining was measured and compared to enzyme activity where incubation with BVT.948 was omitted or washing was omitted, as indicated. Data are from one experiment which has been repeated twice at two concentrations of BVT.948 with similar results. B) PTP1B activity was determined in the absence (circles) and presence (squares) of BVT.948 and in the absence (open symbols) and

presence (closed symbols) of 25μg/ml catalase, as described in the Methods section. Error bars where visible represent the standard deviation of triplicate determinations.

**Figure 4**

BVT.948 stimulates glucose uptake into L6 myotubes in a PI 3-kinase dependent fashion. L6 myotubes were starved of serum overnight and were stimulated with insulin or treated with 25μM BVT.948 as described in the Methods section after a 30 minute pre-incubation with (dark grey bars) or without (light grey bars) 100nM wortmannin. After washing, glucose uptake was measured. Data are from four different experiments and normalised to no-insulin control responses. *$p<0.05$, **$p<0.005$ with respect to the corresponding no-wortmannin treatments.

**Figure 5**

BVT.948 increases insulin-stimulated insulin receptor phosphorylation. A) The 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 minutes and insulin receptor phosphorylation was measured in lysates as described in the Methods section. Data show the means ± SD of results from 3-5 experiments for each cell line, normalised to the maximum response for each cell line. Solid lines are fits of the data to a hyperbola from which $EC_{50}$ values were derived. B) Competition with an un-labelled antibody raised to the triply-labelled activation loop of the insulin receptor shows that most of the signal from the Delfia assay is due to phospho-tyrosine in the activation loop of the receptor. L6 myotubes were left unstimulated (light bars) or stimulated with insulin (dark bars) as described

in the Methods section. Receptor phosphorylation was measured as described above except that the assay was performed in the presence of increasing concentrations of unlabelled competing antibody as indicated. Error bars where visible represent the standard deviation 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 in the Methods section. 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 25nM 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.

**Figure 6**

BVT.948 enhances insulin sensitivity in the ob/ob mouse, a model of obesity and diabetes. Male mice were divided into 3 groups (9 animals per group) and treated with vehicle (open circles), 0.3µmol per kg BVT.948 (filled circles) or 3µmol per kg BVT.948 (open squares) and 1U/kg insulin simultaneously. Blood glucose was monitored at various time points. 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.

**Figure 7**

The effects of increasing concentrations of BVT.948 on CYP activity in vitro. The indicated recombinant CYP enzymes were incubated with 1µM (light grey bars),

10μM (dark grey bars) and 100μM (black bars) BVT.948 and activities measured as described in the Methods section. Data are the means ± SD of triplicate determinations.

**Figure 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 $HO_2$• 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 CYP. A cycle of activity is thus established, in which oxygen dissolved in the buffer is consumed for the production of hydrogen peroxide by BVT.948 and CYP activity is therefore apparently inhibited towards its marker substrates as it catalyses the re-formation of the reduced form of BVT.948.

**Table 1**

**Inhibition Constants for Inhibition of Various PTPs by BVT.948 and BVT.2274**

| **Enzyme** | **$IC_{50}$ BVT.948** | **$IC_{50}$ BVT.2274** |
|---|---|---|
| PTP1B | 0.9±0.3μM | >100μM |
| TCPTP | 1.7±0.1μM | >100μM |
| SHP-2 | 0.09±0.01μM | ND |
| LAR | 1.5±0.4μM | ND |
| YopH | 0.7± 0.05 | ND |
| Phage λ Ppase | >100μM | ND |

**ND**, not determined

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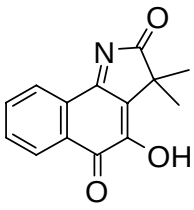
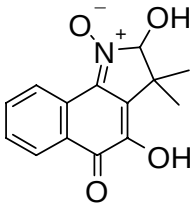
**Figure 1**




**BVT.948**




**BVT.2274**

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**Figure 2**

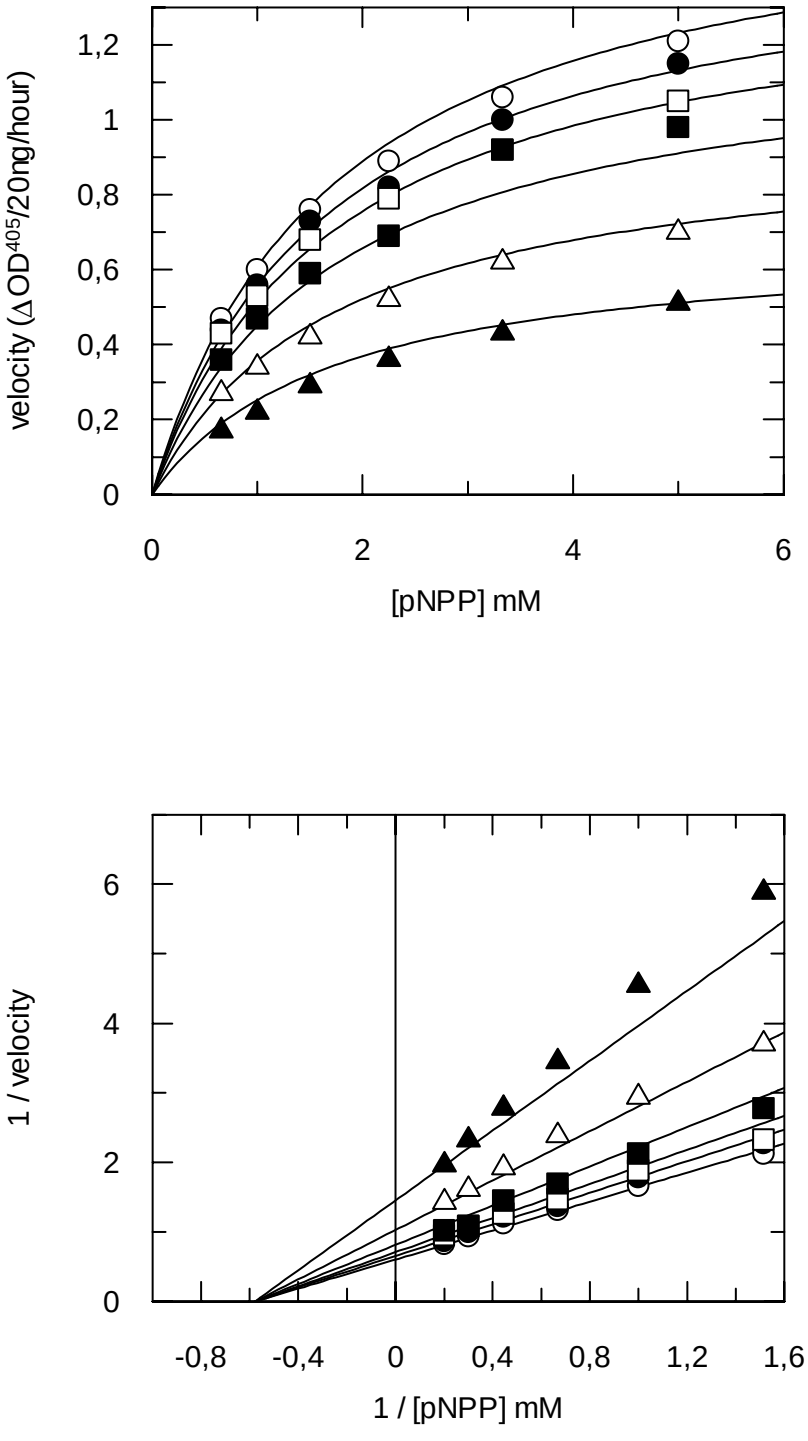

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**Figure 3A**

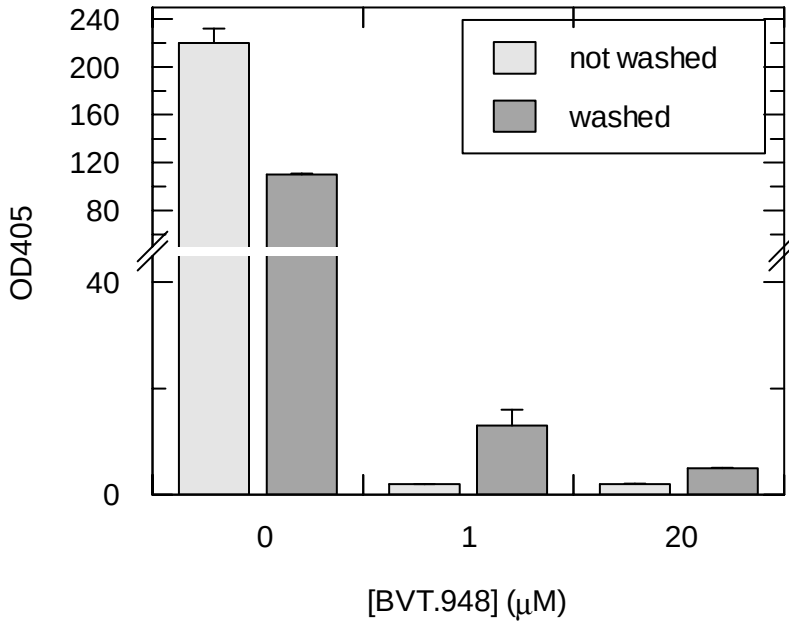

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**Figure 3B**

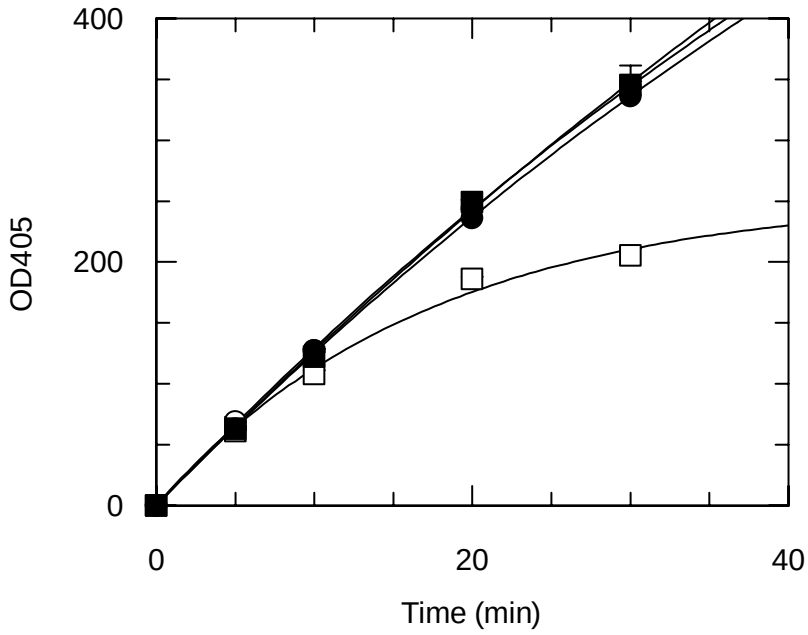

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**Figure 4**

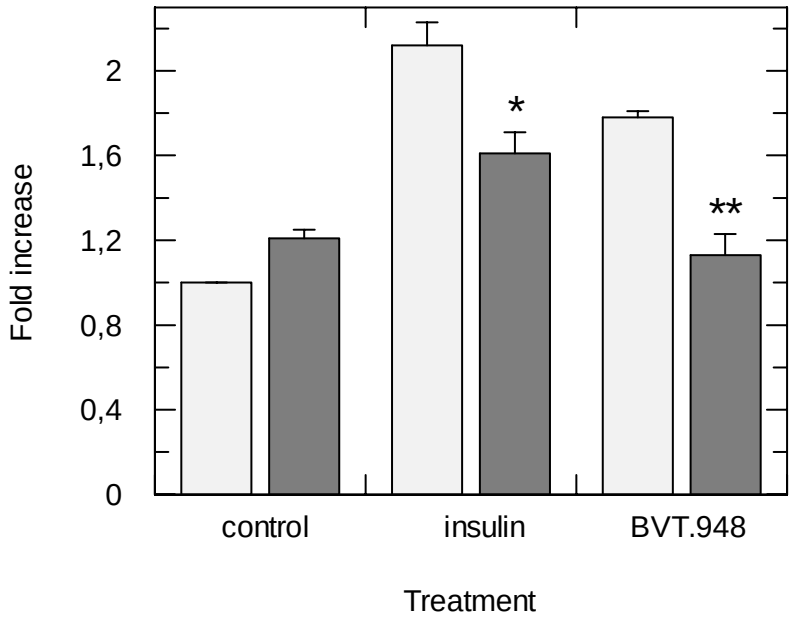

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**Figure 5A**

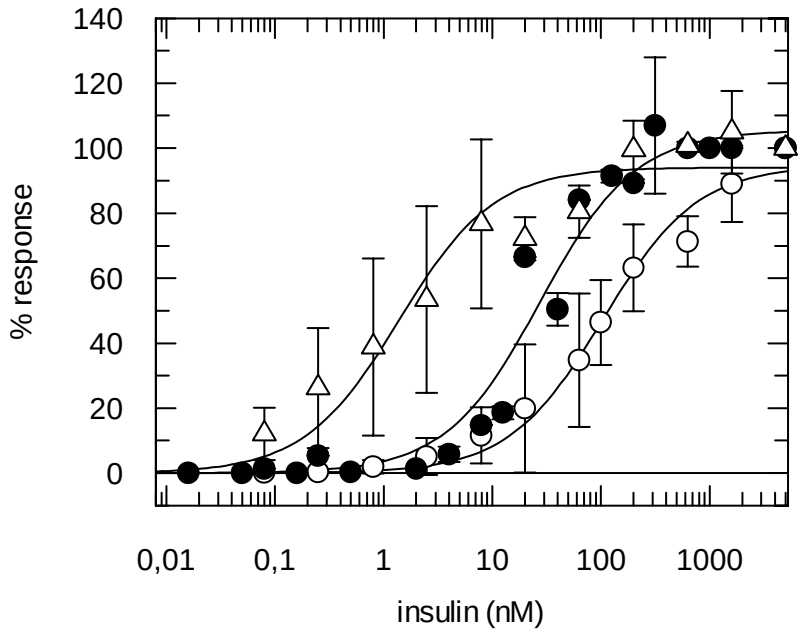

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**Figure 5B**

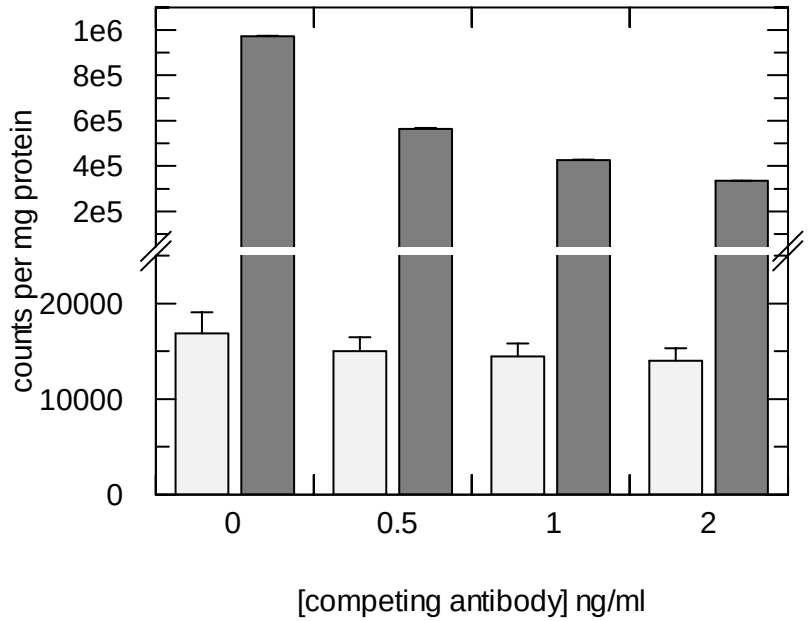

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**Figure 5C**

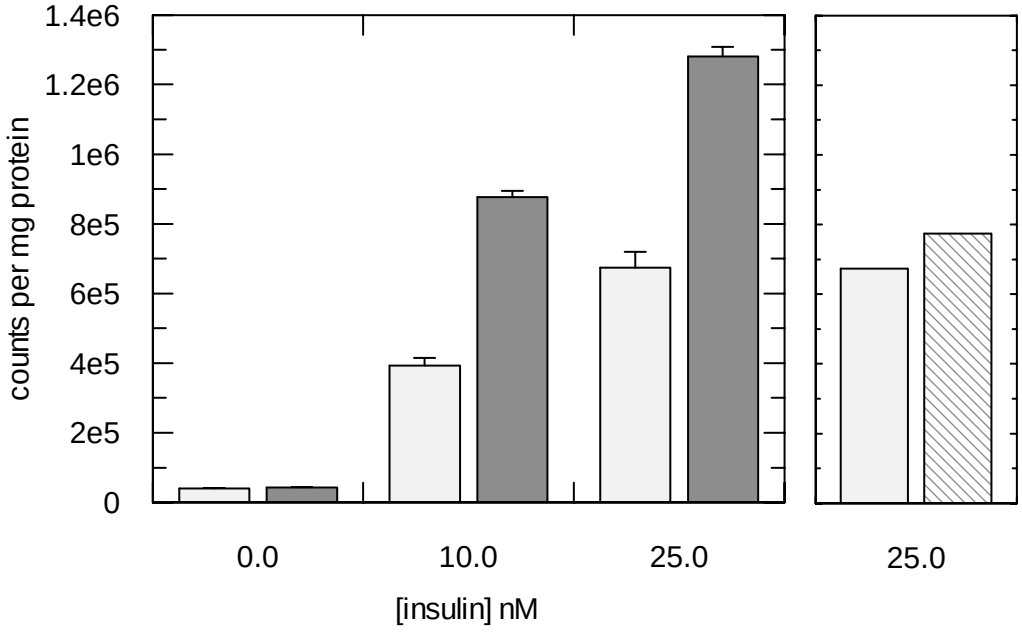

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**Figure 5D**

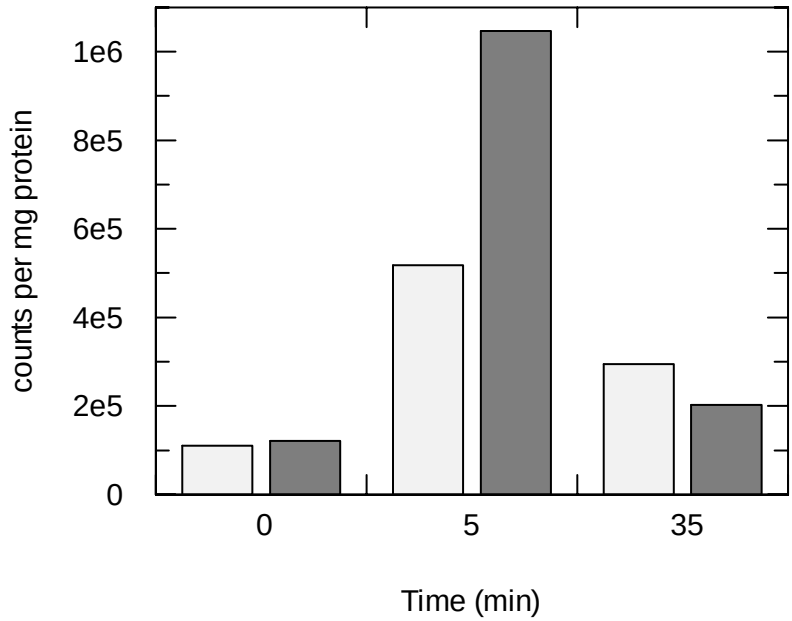

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**Figure 6**

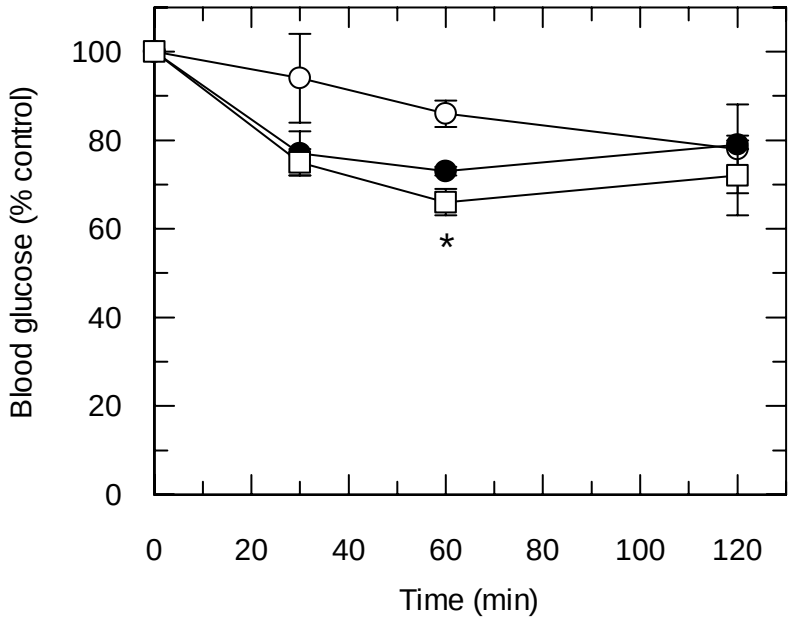

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**Figure 7**

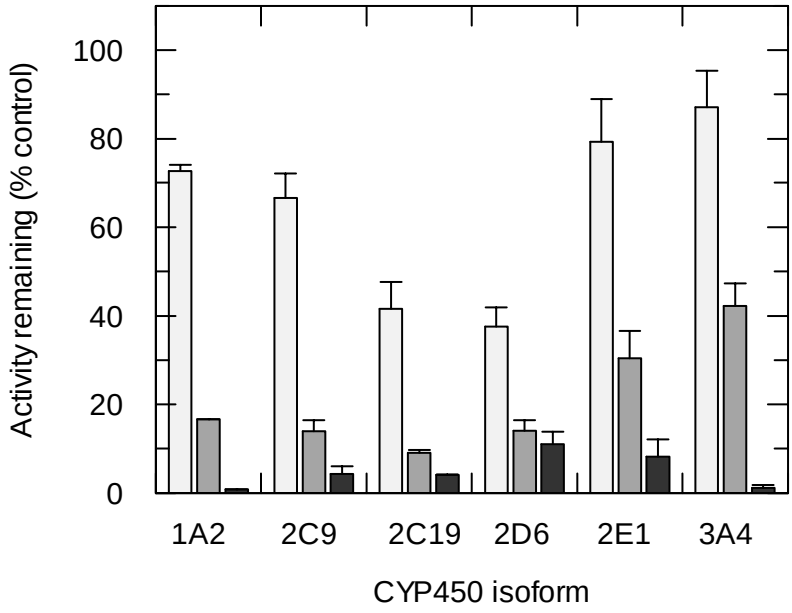

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**Figure 8**