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Title Page

Title: A Glutathione S-Transferase π Activated Pro-drug Causes Kinase Activation Concurrent with S-glutathionylation of Proteins

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S-glutathiolation with a NO releasing prodrug.

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Abbreviations footnote: PABA/NO, O^2-{2,4-dinitro-5-[4-(*N*-methylamino) benzoyloxy]phenyl} 1-(*N*,*N*-dimethylamino)diazen-1-ium-1,2-diolate; PDI, protein disulfide isomerase; PTP1B, protein tyrosine phosphatase 1B; ROS, reactive oxygen species; RNS, reactive nitrogen species; NO, nitric oxide, GSTπ, glutathione S-transferase.

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Abstract

Nitric oxide (NO) is an endogenous, diffusible, trans-cellular messenger shown to impact regulatory and signaling pathways with impact on cell survival. Exposure to NO can impart direct post-translational modifications on target proteins such as nitration and/or nitrosylation. Alternatively, after interaction with oxygen, superoxide, glutathione or certain metals NO can lead to S-glutathionylation, a post-translational modification potentially critical to signaling pathways. A novel GSTπ activated pro-drug, O^2-{2,4-dinitro-5-[4-(*N*-methylamino)benzoyloxy]phenyl} 1-(*N*,*N*-dimethylamino)diazen-1-ium-1,2-diolate or PABA/NO liberates NO and elicits toxicity *in vitro* and *in vivo*. We now show that PABA/NO induces nitrosative stress resulting in undetectable nitrosylation, limited nitration and high levels of S-glutathionylation. After a single pharmacologically relevant dose of PABA/NO, S-glutathionylation occurs rapidly (<5 minutes) and is sustained for ~ 7h, implying a half-life for the deglutathionylation process of approximately 3 hours. 2D SDS-PAGE and immunoblotting with a monoclonal antibody to S-glutathionylated residues indicated that numerous proteins were S-glutathionylated. Subsequent MALDI-TOF analysis identified 10 proteins, including β-lactate dehydrogenase, Rho GDP dissociation inhibitor β, ATP synthase, elongation factor 2, protein disulfide isomerase, nucleophosmin-1, chaperonin, actin, PTP1B and glucosidase II. In addition, we showed that sustained S-glutathionylation was temporally concurrent with drug-induced activation of the stress kinases, known to be linked with cell death pathways. This is consistent with the fact that PABA/NO induces S-glutathionylation and inactivation of PTP1B, one phosphatase that can participate in deactivation of kinases. These effects were consistent with the presence of intracellular PABA/NO or metabolites, since MRP1 over-expressing cells were less sensitive to the drug and had reduced levels of S-glutathionylated proteins.

Introduction.

The human genome is comprised of <30,000 genes and yet complexity of protein structure/function seems distinctly more layered. In the proteomics era, it becomes clear that the dogma of genetic determinism is distinctly influenced by a number of processes including, polymorphic variation, gene splicing events, exon shuffling, protein domain rearrangements and a number of post-translational modifications that contribute to alterations in tertiary and quaternary protein structure. Amongst these, phosphorylation, glycosylation, methylation and acetylation can account for a large proportion of modifications. Cellular homeostasis is an intricate balance of survival and death signals the control of which can occur through post-translational modification of target proteins. Protein phosphorylation is regulated by kinase/phosphatase interplay, frequently allowing an adaptive response to a changing environment. In addition to the more studied post-translational modifications, cysteine residues localized in a basic environment can have low pKa values making them targets for the addition of glutathione, i.e. can be S-glutathionylated. This modification can regulate cellular response to stresses such as reactive oxygen (ROS) and nitrogen (RNS) species (Shelton et al., 2005). Figure 1 shows how ROS and RNS can interact with cysteine residues resulting in the formation of S-glutathionylated proteins. This reaction can serve to protect the protein from further oxidative or nitrosative damage, or can effect a change in conformation and/or charge that may alter protein function. Critical in ascribing any regulatory function (i.e. equivalence to phosphorylation/dephosphorylation) to this process is the reversibility of S-glutathionylation by small molecule cysteine rich proteins such as glutaredoxin and/or sulfiredoxin (Shelton et al., 2005), (Beer et al., 2004), (Findlay et al, 2005).

It seems plausible that both endogenous and exogenous ROS/RNS can cause S-glutathionylation. For example, endogenous ROS can be by-products of lipid peroxidation and the electron transport chain, whereas exogenous sources include X- and UV irradiation. Elevated levels of nitric oxide (NO) provide the primary source of RNS. NO is an endogenous diffusible trans-cellular messenger shown to participate in survival and death pathways (Moncada et al., 1997) and can alter protein function directly through post-translational modifications (nitration or nitrosylation) or indirectly through

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interactions with oxygen, superoxide, thiols, and heavy metals, the products of which can ultimately lead to S-glutathionylation, Figure 1 (for review see (Chung et al., 2001)).

Dysregulation of endogenous NO can lead to the release of RNS and ROS, each of which have been implicated in a number of human pathologies, including neurodegenerative disorders, cystic fibrosis, aging and cancer (Ilic et al., 1999; Tieu et al., 2003; Townsend et al., 2003; van der Vliet et al., 1997). This observation has led to investigations of the capacity of NO to induce cytotoxicity, with particular reference to anti-tumor activities (Cui et al., 1994). The coalescence of NO and GST biology led to the design and synthesis of a novel anticancer pro-drug, O^2-{2,4-dinitro-5-[4-(*N*-methylamino)benzoyloxy]phenyl} 1-(*N*,*N*-dimethylamino)diazen-1-ium-1,2-diolate or PABA/NO (Saavedra et al, 2005). The GSTπ isozyme is expressed at elevated levels in a variety of human tumors and is linked with the development of resistance to a number of anti-cancer agents (O'Brien et al., 2000) (Townsend and Tew, 2003). Such characteristics have been instrumental in the development of a GSTπ activated phosphorodiamidate pro-drug, Telcyta that has undergone pre-clinical evaluation and has just entered Phase III clinical trial (Rosario et al., 2000). Catalytic activation of PABA/NO by GSTπ releases NO that elicits anti-tumor activity both *in vitro* and *in vivo* . The release of NO also causes an activation of stress response pathways that involve p38 and c-jun-NH_2-terminal kinase (JNK), eventually leading to apoptosis . In the present study, the capacity of PABA/NO to induce post-translational modifications such as nitration, nitrosylation and S-glutathionylation was viewed in the context of the cytotoxic properties and mechanism of action of the drug.

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Materials and Methods

Reagents and Cell Culture. PABA/NO was prepared as previously described (see also structure revision in Saavedra et al., submitted). The following reagents were commercially available: peroxynitrite and purified PTP1B (Upstate Biotechnologies, Lake Placid, NY), hydrogen peroxide, oxidized glutathione, and the MTT reagent (Sigma, St. Louis, MO), S-glutathionylated HeLa cell lysate (ViroGen Corp, Watertown, MA), and the Bradford reagent (Bio-Rad Laboratories, Hercules, CA). Cell lines were maintained in Dulbecco's modified Eagle's medium (NIH3T3 and NIH3T3-MRP) or RMPI (HL60 and HL60-ADR) supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, 100 U/ml penicillin, and 2 mM L-glutamine at 5% CO_2 and 37^{o}C.

Cytotoxicity Assays. 10,000 cells were seeded in 96 well plates in 50 μl medium. Increasing drug concentrations were added to a final volume of 100 μl and maintained in drug for 72 h. Following drug exposure, cell viability was assayed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) conversion assay. Each drug concentration was represented in quadruplicate and the experiments were repeated in 3 independent experiments. Mean values and S.E. were computed for each group.

Protein Preparation. 500,000 cells per treatment group were collected and washed with 1X phosphate-buffered saline (PBS). Cell pellets were suspended in lysis buffer containing 20 mM Tris-HCl, pH 7.5, 15 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% triton-X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate with freshly added phosphatase inhibitors (5 mM NaF and 1 mM Na_3VO_4) and a protease inhibitor cocktail (Sigma). Cells were incubated for 30 minutes on ice, sonicated 3 times for 10 sec and centrifuged for 30 minutes at 10,000 x g at 4^0 C. Protein concentrations in the supernatant were determined with the Bradford method using IgG as a standard (Bradford, 1976).

2-D SDS-PAGE. For the first dimension, 100 μg of whole cell protein was resuspended in 8 M urea with 0.5% carrier ampholytes and run on immobilized pH gradients (IPG) covering exponentially the pH range 3 to 10. For the second dimension, the IPG strips were separated on 10% polyacrylamide gels. Non-reducing conditions were used for electrophoresis (Righetti et al., 1991). Protein spots of interest were excised and subject to trypsin digestion. Peptide mass was analyzed by MALDI-TOF mass spectrometry at

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the Proteomics Core Facility of the Medical University of South Carolina. Protein identification was performed using software from the National Center for Biotechnology Information (NCBI) protein database.

Immunoblot Analysis. For analysis of S-glutathionylation and nitration, equivalent amounts of protein were separated under non-denaturing conditions on 10% SDS-polyacrylamide gels; unmodified proteins were separated under denaturing conditions. Gels were transferred overnight onto nitrocellulose membranes (Bio-Rad). Non-specific binding was reduced by incubating the membrane in 10% blocking buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% bovine serum albumin, 0.1% Tween 20, 1x protease inhibitors, 5mM NaF, and 1mM Na_3VO_4 for 1 hour. Membranes were incubated with specific antibodies (5% blocking buffer) overnight at 4^0C, washed 3x with PBS for 15 minutes and incubated with the appropriate secondary antibody conjugated to horseradish peroxidase for 1 hour. The membranes were washed 3 times and developed with ECL detection reagents (Bio-Rad). The relative abundance of proteins were evaluated using Quantity-One-4.5.2 software and plotted as arbitrary units in relation to actin.

Antibodies. Antibodies were purchased from the following sources: anti-active-c-Jun N-terminal kinase (JNK) and p38 (Promega, Madison, WI), anti-p38, anti-ERK and phospho-specific ERK (Santa-Cruz Biotechnology, Santa Cruz, CA), anti-JNK2 (BD Biosciences PharMingen, San Diego, CA), anti-nitrotyrosine (Upstate Biotechnology), anti-actin and anti-his (In Vitrogen), anti-SSG (ViroGen Corp.).

***In vitro* S-glutathionylation of PTP1B.** 1 μg His-tag purified PTP1B (Upstate Biotechnology) was incubated at 37^0 C for 0 to 30 minutes with 100 μM PABA/NO, 10 mM glutathione, 50 mM KPO_4- buffer. The samples were separated by SDS-PAGE under non-reducing conditions. The gels were transferred overnight to nitrocellulose membranes and immunoblotted with anti-S-glutathionylation and anti-His antibodies.

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Results

Detection of S-glutathionylation. Because drug treatment can lead to multiple post-translational changes, the specificity of the monoclonal antibodies used to detect S-glutathionylation was first tested. HL60 cells were treated for 30 minutes with peroxynitrite (a known inducer of nitration) and PABA/NO. We utilized S-glutathionylated HeLa cell extracts provided by Virogen Corp as a positive control for the anti-S-glutathionylation antibody and a negative control for the anti-nitrotyrosine antibody. Figure 2 shows that neither S-glutathionylation (panel A) nor nitration (panel B) were detected in untreated cell lysates. Nitration of proteins was observed as a consequence of peroxynitrite treatment. A positive reaction for S-glutathionylation was observed in the control HeLa cell extract and in the PABA/NO treated cells. These data were consistent with specific antibody reactivity and validate the sensitivity and specificity comparisons for detection of S-glutathionylated proteins.

PABA/NO induces S-glutathionylation of proteins in a dose and time dependent manner. Toxicity of PABA/NO and three potential hydrolysis products of PABA/NO (JS-42-25, JS-43-19 and 4-methylamino benzoic acid) was measured in HL60 cells along with an analog of PABA/NO incapable of releasing NO (JS-39-93) (Table 1). The IC_{50} value of PABA/NO was 33 $\pm$ 2.9 μM whereas the metabolites and NO-free control showed a three-fold increase in the IC_{50} value. HL60 cells treated with 0-250 uM of the metabolites and NO-free control failed to S-glutathionylate proteins (data not shown). These data suggest that intact PABA/NO is essential to elicit the cytotoxic effects and post-translational modification. The approximate IC_{50} concentration (30 uM) was used to examine the temporal modification of proteins. SDS-PAGE and immunoblot analysis showed that PABA/NO treatment induced S-glutathionylation of numerous proteins within 5 minutes (Figure 3) and that in the presence of drug, this was sustained > 6h (Figure 5), implying that deglutathionylation occurs in the absence of drug with a half-life of approximately 3h.

In previous studies we showed the toxicity of PABA/NO to be significantly diminished in NIH/3T3 cells over-expressing MRP1, with an IC_{50} value of 50 μM vs 11 μM in wild-type (Findlay, 2004). We utilized these cells to address whether PABA/NO induced S-glutathionylation is independent of toxicity, Figure 4. The data show that a 30

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μM PABA/NO treatment in NIH3T3 wild-type cells results in rapid S-glutathionylation of a variety of proteins within 15 min. However, S-glutathionylation in 3T3-MRP cells was not observed until ~ 1 hour. A dose- and time- dependent delay of S-glutathionylation in resistant cells was concurrent with a decrease in cytotoxicity. These data are consistent with our hypothesis that a glutathione-containing metabolite of PABA/NO elicits the cytotoxic effects and acts as both a substrate for MRP1 efflux and a donor for S-glutathionylation.

Activation of the MAP kinase pathway. PABA/NO-induced cytotoxicity could be mediated through downstream effects on kinase cascades (Findlay et al., 2004). To assess whether S-glutathionylation was linked to this effect, HL60 cells were treated with 30 μM PABA/NO for 1 hour, drug removed and cells were analyzed for temporal S-glutathionylation and MAP kinase pathway activation during the recovery phase (Figure 5). Maximal S-glutathionylation was observed at the time point when drug was removed (1 hour). A diminishment over 6 hours followed and led to undetectable levels at 6 hours (Figure 5A). Activation of kinases linked to stress response and cell death pathways was evaluated by measuring phosphorylated p-38, JNK, and c-jun. Following immunoblot with phospho-antibodies, the membranes were stripped and probed for the unmodified protein. While no changes in total protein were observed, phosphorylation of p38 and JNK was maximal 1 hour after PABA/NO treatment. The relative abundance of active p38 and JNK were comparable to untreated lysates at 2 and 3 hours, respectively. Phosphorylation of c-jun increased following drug exposure, reaching a maximum at 2 hours and declined to basal levels at 6 hours.

Protein Nitration. Nitration of proteins was evaluated with the anti-nitrotyrosine antibody (Figure 5B). Three proteins were detected. The immunoreactive spots were excised from a 2D SDS-PAGE gel, trypsin digested, and their identity analyzed by MALDI-TOF mass spectrometry. The proteins were identified as actin (41 kDa), ATP synthase (51 kDa) and protein disulfide isomerase, PDI, (57 kDa). Nitration did not decrease throughout the experiment, consistent with irreversible nature of this post-translational modification**.** The data shown in Figure 5 are representative of >5 experiments, all of which showed a temporal relationship between S-glutathionylation and activation of these kinases.

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Identification of S-glutathionylated proteins. PABA/NO treatment leads to rapid S-glutathionylation of numerous proteins in HL60 cells. In order to gain insight into the biological importance, 100 μg of protein equivalents of whole cell lysates from HL60 cells were separated by 2D-SDS-PAGE. Coomassie stained gels of treated and control cells confirmed equivalent loading (Figure 6A and C). In untreated cells, S-glutathionylated proteins were not detected, whereas PABA/NO treatment produced>20 modified proteins (Figure 6D). Ten immunoreactive spots were excised from the gel, trypsin digested, and identified by MALDI-TOF mass spectrometry (Table 2). S-glutathionylated proteins are listed according to their subcellular localization: (cytoplasm) β lactate dehyrogenase, Rho GDP dissociation inhibitor β, elongation factor 2, PTP1B, and actin; (nucleus) nucleophosmin; (endoplasmic reticulum) protein disulfide isomerase and glucosidase II; (mitochondria) ATP synthase β subunit and chaperonin.

***In vitro* S-glutathionylation of PTP1B**. PABA/NO treatment caused S-glutathionylation of PTP1B *in vivo* (Table 2) and *in vitro* (Figure 7). We have shown that PABA/NO treatment reduces PTP1B activity from 13 $\pm$7 to 3.7$\pm$1.2 nmoles/min/mg (Findlay et al., 2005). These data are in agreement with other studies showing reversible S-glutathionylation of the phosphatase PTP1B has been shown to inhibit activity and thereby impact kinase signaling (Barrett et al., 1999).

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Discussion

PABA/NO is a diazeniumdiolate where the N-methyl-para-aminobenzoic acid substituent is bound to the dinitrobenzene ring through the carboxyl oxygen (Saavedra et al., 2005). GST mediated activation via Meisenheimer complex intermediate results in a number of metabolites and the release of NO, the species considered to be critical to pharmacological activity . Our data suggest that PABA/NO produces detectable nitration of three proteins. While these modifications may be pertinent to the cytotoxic efficacy of the drug, they are quantitatively quite limited in extent when compared to cells treated with peroxynitrite. Nitration is an irreversible modification that could decrease the half-life and therefore lower the detection of target proteins, which might further be compromised by the lack of sensitivity of the commercially available antibody. However, our data show categorically that PABA/NO is a potent inducer of S-glutathionylation whereas peroxynitrite is not. We propose the lack of nitrated proteins is a reflection of the fact that hydrolysis products of PABA/NO cause higher levels of cysteine modifications. PABA/NO induced S-glutathionylation of numerous proteins occurred within minutes of drug treatment and was sustained in the absence of the drug for ~6 hours. The reduced and delayed S-glutathionylation in NIH3T3-MRP1 provides support for the conclusion that active metabolite of PABA/NO is responsible for causing S-glutathionylation of target proteins. In this and prior studies we demonstrate that despite equivalent GSTπ expression, S-glutathionylation and cytotoxicity are diminished in MRP1 over-expressing cells compared to the parental line. Collectively these data would imply that a glutathione conjugate of PABA/NO can act both as a substrate for MRP1 efflux and as a donor for S-glutathionylation. Whether the metabolite is the *proximal* donor for the S-glutathionylation reaction has yet to be determined.

S-glutathionylation preceded activation of the MAP kinases and followed a decay rate that was similar to kinase dephosphorylation. PTP1B is an important cellular tyrosine phosphatase that is a negative regulator of the insulin-stimulated kinase pathway. However, several studies have shown that PTP1B participates in various signal transduction pathways (for review see (Zhang et al., 2002)). Other laboratories have shown that when PTP1B is S-glutathionylated on Cys 215 it is rendered catalytically

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inactive (Barrett et al., 1999; Li and Whorton, 2003). Other studies in our laboratory have shown that deglutathionylation of PTP1B by sulfiredoxin can reinstate enzyme activity (Findlay et al., 2005). At least one model of drug action consistent with our present data is where PTP1B S-glutathionylation following PABA/NO allows the activation of the kinase pathway that leads to apoptosis. The deglutathionylation of PTP1B (and other targeted proteins) after 6 hours leads to phosphatase activation and subsequent dephosphorylation of the kinase cascade, consistent with Figure 6.

The decay of S-glutathionylation following removal of PABA/NO is not accompanied by any diminution of protein stain intensity in individual spots on gels. This suggests that S-glutathionylated proteins are not targeted for degradation. Our data infer a half-life for S-glutathionylation of approximately 3 hours. It seems plausible that this modification may serve to protect important cysteine residues while preventing degradation. When thiol groups are exposed to ROS, the flexible valency of the sulfur can allow the formation of sulfenic, sulfinic or sulfonic acids. Only the latter of these appears irreversible with resultant proteolysis (Paget and Buttner, 2003). With PABA/NO the proximal donor of the S-glutathionylation could be one of a number of drug metabolites. As shown in Fig 1, nitrosoglutathione (GSNO), oxidized glutathione (GSSG), glutathione sulfenate (GSOH), glutathione disulfide monoxide and dioxide have all been characterized as plausible donors of -SG (Tao and English, 2004). GSNO is a likely substrate for MRP1 and its efflux would be consistent with the reduced levels of S-glutathionylation in MRP1 over-expressing cells. GSOH, glutathione disulfide monoxide and dioxide would be plausible down-stream products of PABA/NO metabolism. Obviously, the rapid onset of S-glutathionylation predicts that PABA/NO has a short decomposition half-life, at least with respect to liberation of NO. Indeed, this is commensurate with the measured half-life of the drug with respect to NO release .

Alterations in redox balance by exposure to RNS or ROS cause dose-dependent changes in glutathione:GSSG ratios that can potentially influence a number of target proteins by causing oxidation and disulfide exchange reactions at specific cysteine residues. The importance of modifying cysteine residues is not necessarily restricted to redox regulation, but now seems to be a plausible event that can lead to changes in signaling processes. By adding the glutathione tripeptide to a target protein, an additional

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negative charge is introduced (as a consequence of the glu residue) and a change in protein conformation is made likely. A recent report (Manevich et al., 2004) showed that GSTπ is capable of acting in a catalytic manner to S-glutathionylate peroxiredoxin, a non-selenium dependent lipid peroxidase that converts lipid hydroperoxides to corresponding alcohols. The catalytically important cysteine residue on peroxiredoxin is sterically inaccessible within the globular dimeric complex and GSTπ facilitates glutathione transfer to this site. The resultant enzyme activation serves a cellular regulatory role, particularly with respect to response to oxidative stress. Actin is a prevalent structural protein, the very abundance of which makes it a primary target for S-glutathionylation. Indeed, hydrogen peroxide induced ROS seemed to result almost exclusively in S-glutathionylation of actin. S-glutathionylation of actin has been shown to be physiologically relevant. Under normal conditions a proportion of G-actin is S-glutathionylated at Cys374, and since deglutathionylation increases the rate of polymerization, by extrapolation, this modification limits polymerization into F-actin (Wang et al., 2001). Inhibition of deglutathionylation suppresses growth factor mediated actin polymerization (Wang et al., 2003). As elegantly discussed by Shelton et al (Shelton et al., 2005), redox modification of actin fulfils many of the criteria important in defining a regulatory role for reversible S-glutathionylation.

Using a variety of methods, other investigators have identified a number of distinct S-thiolated proteins that may be involved in cell regulation (Eaton et al., 2002), (Fratelli et al., 2002), (Baty et al., 2002). Using the monoclonal antibody technique, our data showed that ~20 distinct proteins were modified following PABA/NO treatment. This is the first evidence that the cytoxicity of an antitumor agent may elicit concomitant S-glutathionylation of proteins. This is a reasonably small proportion of the total proteome and suggests that there would be value in identifying those members of this group that might be involved in regulation/signaling, particularly as related to NO cytotoxicity and the mechanism of action of PABA/NO. To this end, we identified 10 S-glutathionylated proteins (c.f. Table 2). Of particular significance to PABA/NO- induced apoptosis, is ATP synthase β subunit. This enzyme is located in the inner mitochondrial membrane and catalyzes ATP formation through the energy of proton flux during oxidative phosphorylation. Nitration of numerous proteins including ATP synthase, was

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proposed to contribute to the pathogenesis of familial amyotrophic lateral sclerosis (Casoni et al., 2005). In a separate study, inhibition of beta-oxidative respiration with trinitazidine resulted in a dose- dependent decrease in ATP levels and an induction of apoptosis (Andela et al., 2005). These authors postulated that inhibition of β-oxidative respiration is a therapeutic window associated with the anti-cancer activity of PPAR gamma agonists. The present data suggest that ATP synthase is an indirect target of the RNS generated by GST mediated activation of PABA/NO and may contribute to the antitumor activity of the drug.

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Footnotes:

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Legends for Figures

Figure 1. Nitrosative stress leads to the formation of S-nitrosation and S-glutathionylation modifications. Both posttranslational modifications alter protein function.

Figure 2. Anti-S-glutathionylation antibodies do not cross react with nitration modifications. Cells in logarithmic growth were treated for 30 minutes with inducers of oxidative and nitrosative stress. 30 μg protein lysate were separated on a non-reducing SDS-PAGE and analyzed by immunoblot using: (A) anti-S-glutathionylation antibody and (B) anti-nitrotyrosine antibody. Lanes: 1) RPN800 molecular markers 2) untreated cells 3) 5mM glutathione 4) 195 μM peroxynitrite 5) 195 μM peroxynitrite + 5mM glutathione 6) 30 μM PABA/NO 7) 30 μM PABA/NO + 5mM glutathione 8) S-glutathionylated HeLa cell lysate

Figure 3. PABA/NO induces S-glutathionylation in a dose and time dependent manner. (A) HL60 cells were treated with varying concentrations of PABA/NO. Cell viability was measured at 72 hours with the MTT assay. (B) 1.5 x 10^6 HL60 cells were treated with varying concentrations of PABA/NO for 1 hour. (C) 1.5 x 10^6 HL60 cells were treated with 30μM PABA/NO for 0-120 minutes. Following treatment, 20 μg lysate were separated by SDS-PAGE and analyzed for S-glutathionylation by immunoblot.

Figure 4. S-glutathionylation is delayed in NIH3T3 cells over-expressing MRP1. NIH3T3 (A) and NIH3T3/MRP1 cells (B) were treated with 30 μM PABA/NO for 0-120 minutes.

Figure 5. Temporal S-glutathionylation and activation of kinases following PABA/NO treatment. HL60 cells were treated for 1 hour with 30 μM PABA/NO, resuspended in

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complete media and aliquots removed hourly for 6 hours. Each time point was analyzed by immunoblot (top) and the corresponding relative abundance of proteins were plotted as the ratio to actin (bottom) for (A) S-glutathionylation, the relative abundance of two bands, 37kDa (■) and 18 kDa (●) are plotted in arbitrary units relative to actin; (B) Nitration, (C) Phosphorylated and unmodified kinases and actin as a loading control. Phosporylated p38 (●), JNK, (■), and jun (▲) are plotted in arbitrary units relative to actin.

Figure 6. Two-dimension analysis of HL60 cells (B) untreated and (D) treated with 30 μM PABA/NO. 100 μg protein lysate were separated under nondenaturing conditions and immunoblotted with the anti-S-glutathionylation antibody. Coomassie stained gels of (A) untreated and (B) treated represent equivalent loading.

Figure 7. 1μg PTP1B was treated for varying times with 100 μM PABA/NO in the presence of 10 mM glutathione. Immunoblot analysis with anti-S-glutathionylation and anti-His antibodies confirm *in vitro* modification of PTP1B.

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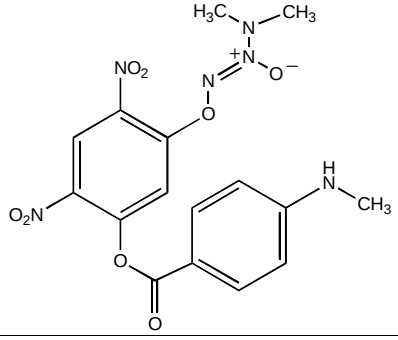
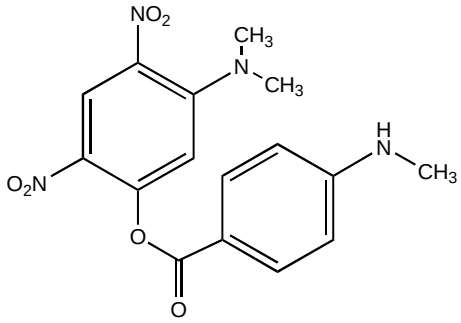
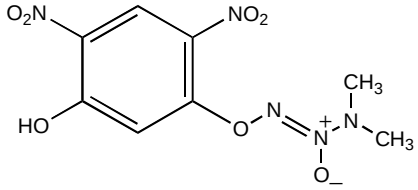
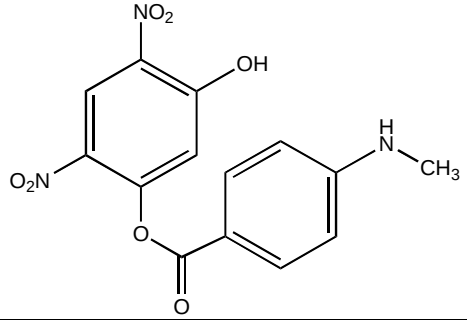
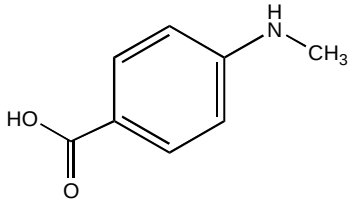
Table 1. Cytotoxicity and S-glutathionylation properties of PABA/NO and related compounds.

Drug	Structure	MW	IC_{50} (μM)	Glutathionylation
PABA/NO		420	33.16 ±2.9	yes
JS-39-93		360	84.2 ±0.2	no
JS-42-25		287	93.6 ±0.3	no
JS-43-19		333	100.1 ±2.6	no
4-Methylamino Benzoic acid		151	88.6 ± 3.5	no

***** HL60 cells were treated with varying concentrations of PABA/NO and its metabolites. Cell viability was measured after 72 hours with the MTT assay. The IC_{50} mean value of 3 independent experiments ± SD is shown. Immunoblot analysis of lysates treated with 0 – 250 μM of PABA/NO or its metabolites for 2 hours show that S-glutathionylation is specific to intact PABA/NO.

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Table 2. MALDI-TOF identification of S-glutathionylated proteins in HL60 cells treated with PABA/NO

Protein	NCBI accession number	MW (Da)	pI	Protein Confidence Interval
β lactate dehydrogenase	49259212	36516	6.2	100%
Rho GDP dissociation inhibitor β	56676393	22973	5.1	99.6%
Nucleophosmin-1	40353734	29446	4.64	100%
Protein disulfide isomerase	2098329	57080	5.9	100%
ATP synthase β subunit	1374715	51170	4.9	100%
Chaperonin	41399285	61016	5.5	100%
Glucosidase II	2274968	106832	5.74	100%
Elongation factor 2	4503483	95277	6.42	100%
Actin	15277503	41736	5.29	99.9%
PTP1B	18031	49966	5.8	99.9%

Fig. 1

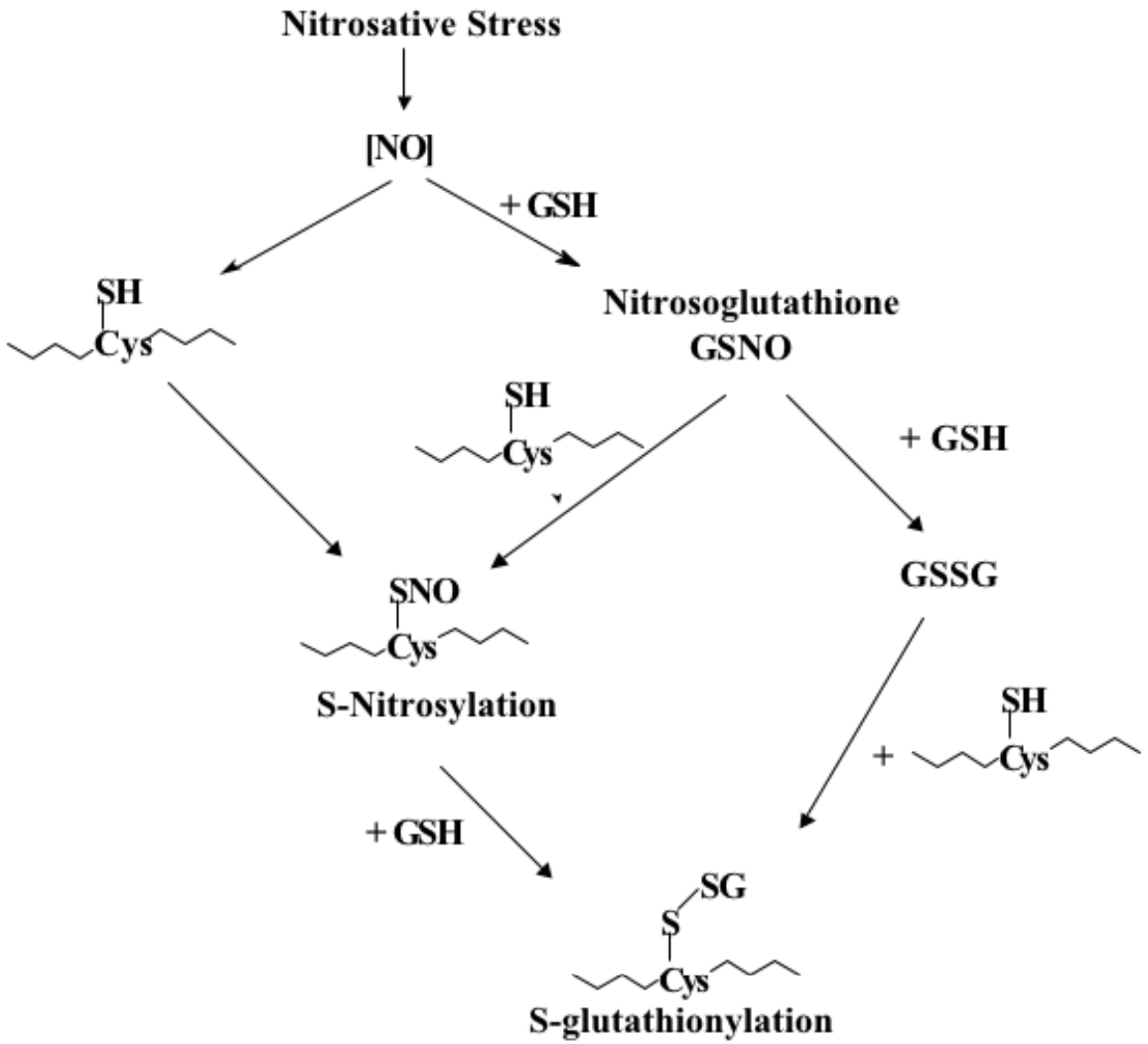


Fig 2

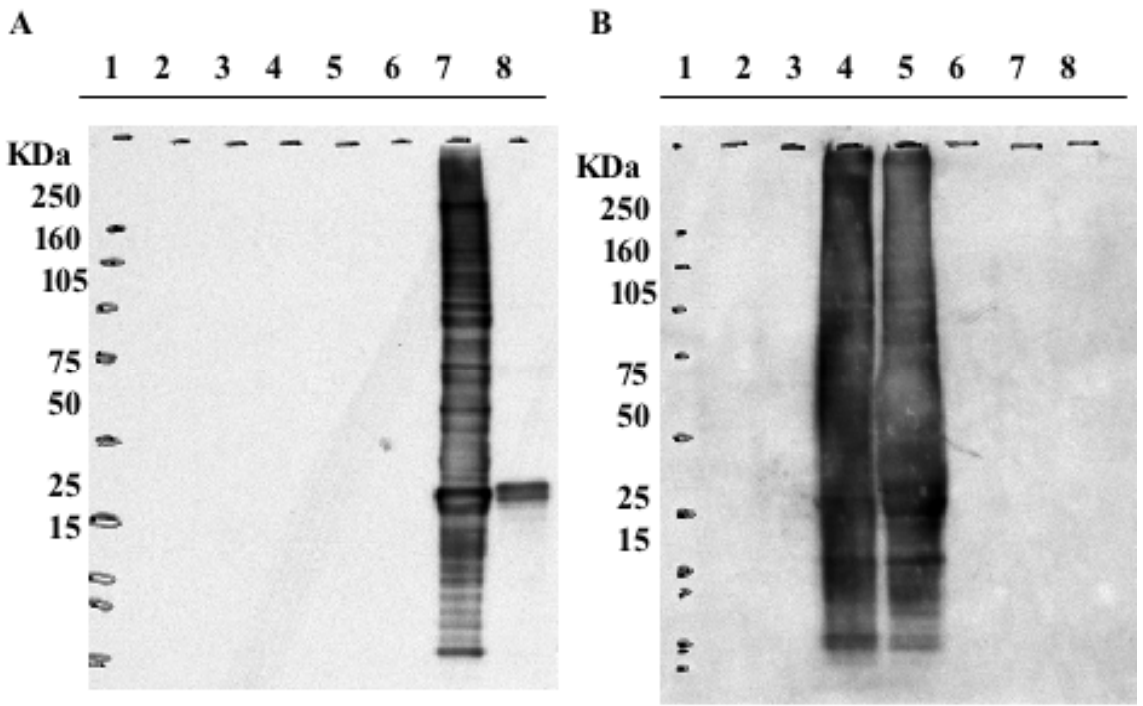
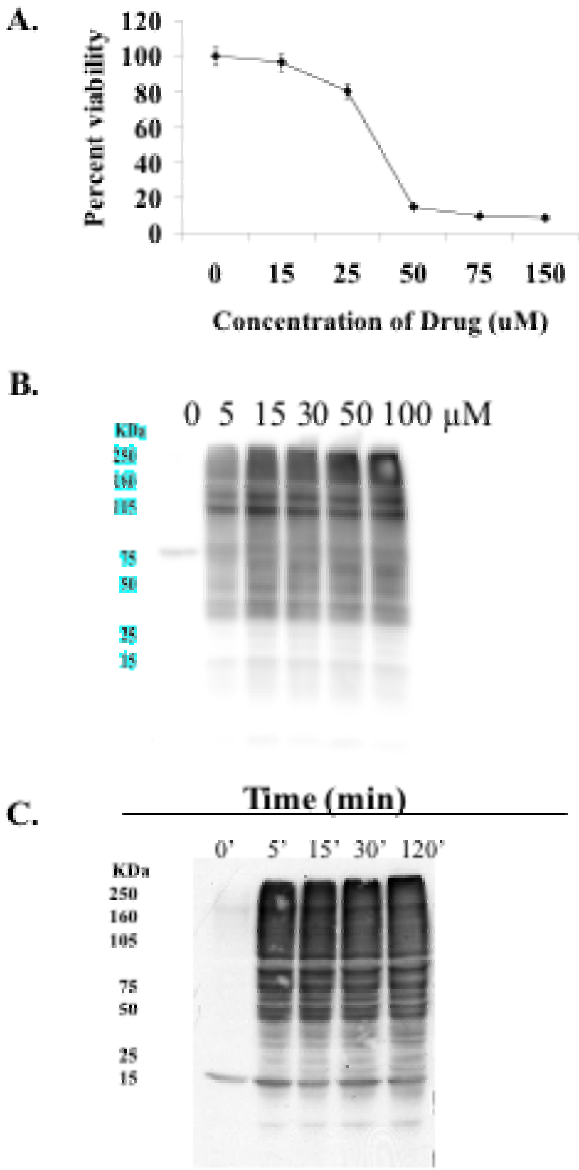


Fig 3



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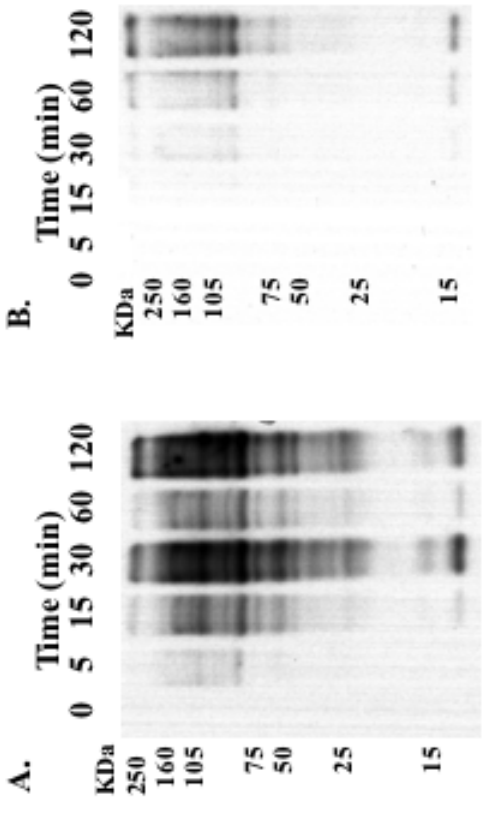

Fig 4

Fig 5

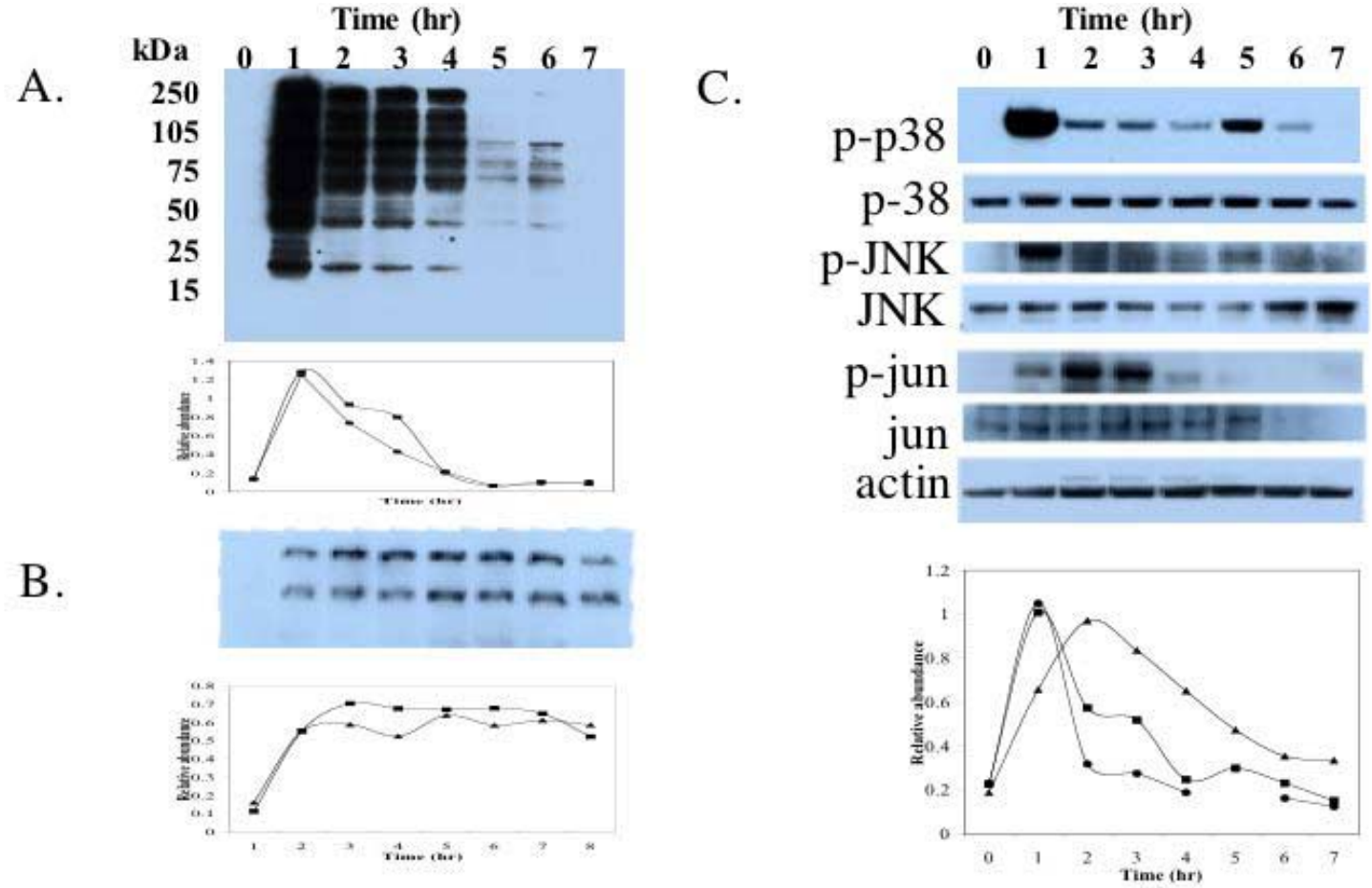

Fig 6

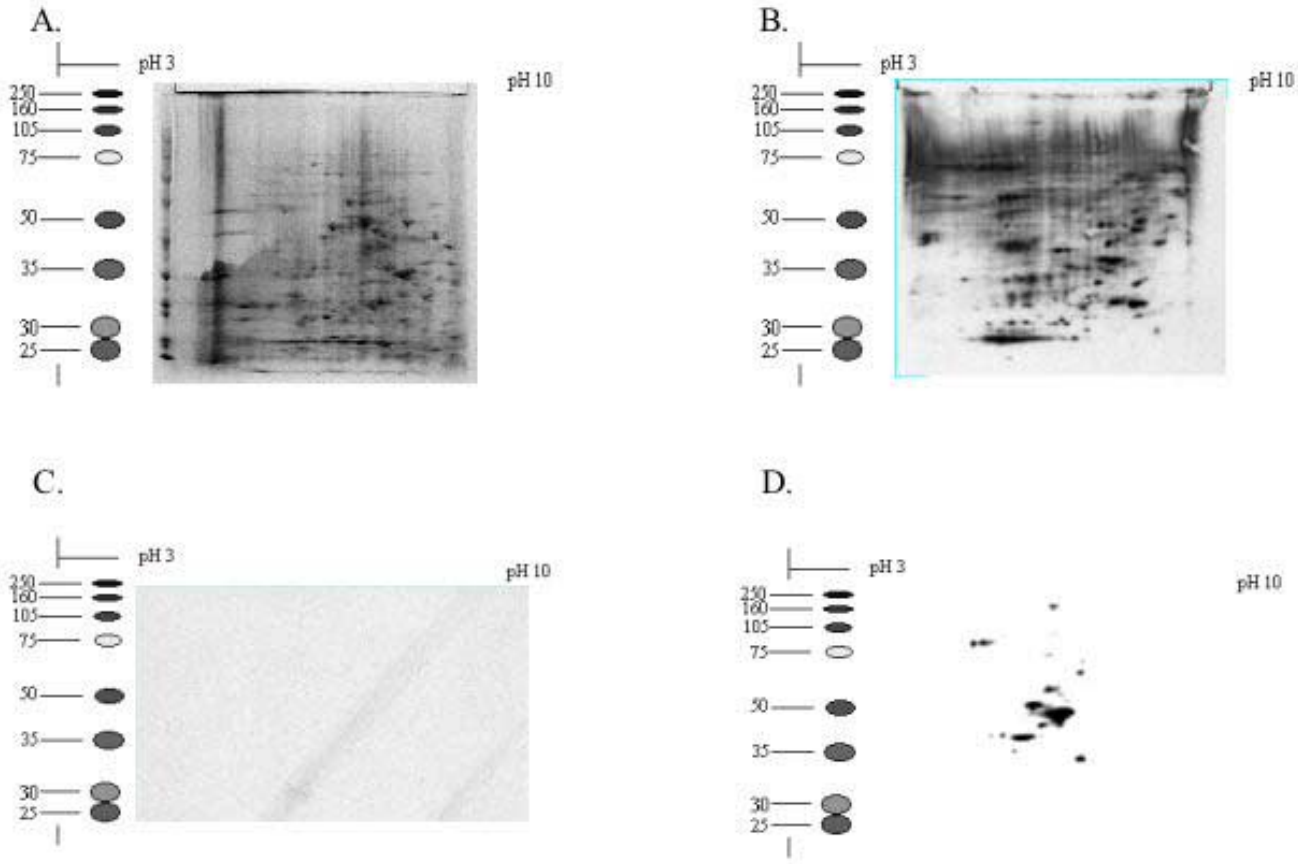

Fig 7