Molecular Basis of Protein Tyrosine Phosphatase Inhibition by Biologically Important Small Molecules with Relevance to Cell Signaling Pathways

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Approval Page

The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled:

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ABSTRACT

Protein tyrosine phosphatase 1B (PTP1B) is an abundant mammalian enzyme present ubiquitously in the cell. Initially it has been characterized as a central player in the insulin and leptin signaling pathways. Recent study supports the increasing importance and involvement of PTPs in other biological functions such as regulator of various transcription factor activation pathways, mediation in tumorgenesis, cancer, angiogenesis, apoptosis and the redox status of the cell.

Peracetic acid is a strong oxidant molecule produced by mammalian decarboxylase and other hydrolase enzymes. Peracetic acid uroporphyrin compound is isolated from the urine sample of the patient suffering from erythropoietic porphyria. Here we presented the evidence that peracetic acid can reversibly inactivate the PTPs function in nanomolar concentration and the inactivation is potent in presence of cellular reducing agent, glutathione. The result shows that peracetic acid can survive under reducing condition like H_2O_2 and other cell signaling messengers. The condition how peracetic acid is produced inside the cell is not yet known.

Our study shows that peracetic acid has the potential to regulate the PTPase function and might play a significant role during cell signaling pathways. Lipid peroxide (13S)-hydroperoxyoctadecanoic acid (13S-HPODE), a dietary metabolite of linoleic acid is known to prolong the phosphorylation of endothelial growth factor (EGF). Our *in vitro* study demonstrates that 13S-HPODE can inhibit the PTPase function in an identical manner as H_2O_2 and possibly lead to the increased EGF phosphorylation.

Oltipraz is a cancer preventive agent which is currently undergoing clinical phase trial II. Oltipraz potentiates its chemo preventive action by inducing the cell protecting enzymes by interacting with Keap1-Nrf2 pathways. We are the first to establish that oltipraz and its metabolite inhibits PTPase function like other cys-dependent proteins by reversible covalent modification. Our study also raises the possibility that oltipraz mediated PTPs inhibition might have the potential to trigger the NF-κB activation.

Hydrogen sulfide signaling has started getting attention in various aspects of cellular disease and therapeutics. Mechanism for many of the H_2S mediated signaling pathways is not yet known. Our preliminary study shows that H_2S metabolite sulfate (SO_3^{2-}) can individually and by combination with other reactive oxygen species inactivates the PTPase. Our work implicates that the metabolites of endogenous H_2S has potential to regulate the PTPase function during cell signaling.

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CHAPTER 2. Redox regulation of PTP1B function by organic peroxides

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Chapter 1: Introduction to the function and biological importance of protein tyrosine phosphatase 1B

1.1. Type 2 diabetes as a global concern.

Discovery of the insulin hormone in early 1920's by Banting is one of the great discoveries in medical science.[1] Insulin resistance has been accepted as the key cause of the type 2 diabetes.[2] Since the long journey to its discovery, study of its mechanism of action has been started over the last couple of years with the help of modern techniques like creation of knockout mice, genomic approaches, antisense oligonucleotides and specific antibodies. A statistical analysis shows that diabetes could turn out to be a global epidemic that affect approximately 250 million (almost 5 % of world population) by the year 2030.[3] A goal is to achieve the target specific therapeutic agent to ameliorate the disease. Metformin and thiazolidinediones (TZD) are the available drugs in the market for the treatment of diabetes (**Figure 1**).[4-8] With the aid of biotechnology and molecular cell biology, the mechanism of insulin resistance and lower glucose uptake in adipose tissue and skeletal muscle has been started for investigation. These will guide us to understand the molecular action of insulin as well as to comprehend the complicated biology of type 2 diabetes inside cell. Also, this will help us to design a more specific drug for the treatment of this existing medical threat.
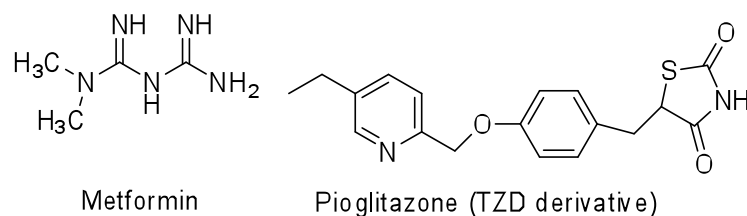
Figure 1: Structure of the available drug for the treatment type 2 of diabetes.[5,7,8]

1.2. Introduction to PTPs:

Protein phosphorylation and dephosphorylation are very important post translational modifications of macromolecules and play a crucial role in several biological pathways.[9] Protein kinases and phosphatases regulate the cellular phosphorylation and dephosphorylation events of the cell respectively.[10,11] Protein kinase installs the phosphate group to the amino acid residue to give phosphoamino acid residue (serine, threonine, and tyrosine) which functions as a biochemical switch for the amplification of the cellular signal.[12,13] Conversely, protein tyrosine phosphatase (PTPs) dephosphorylates the phosphoryl group from the phosphoamino acid residue (serine, threonine and tyrosine). Protein tyrosine phosphatase is a superfamily; almost more than 100 PTPs have been found and characterized in the human genome and many of them have been isolated and studied extensively.[14]

```
                    Protein Tyrosine Phosphatase family
                                     |
        ┌───────────────────┬────────┴─────────────┬──────────────────┐
        |                   |                       |                  |
  Classical 'pTyr'    Dual Specific PTPs         Cdc25          Low molecular
  specific PTPs       eg: Cdc14, PTEN, MKP,                     weight PTPs
        |                  VHR
        |                                      eg: Cdc25A,
  ┌─────┴─────┐                                Cdc25B, Cdc25C
  |           |
Receptor type   Intracellular
eg: LAR, CD45   eg: PTP1B,TCPTP
                Yop PTP, SHP1
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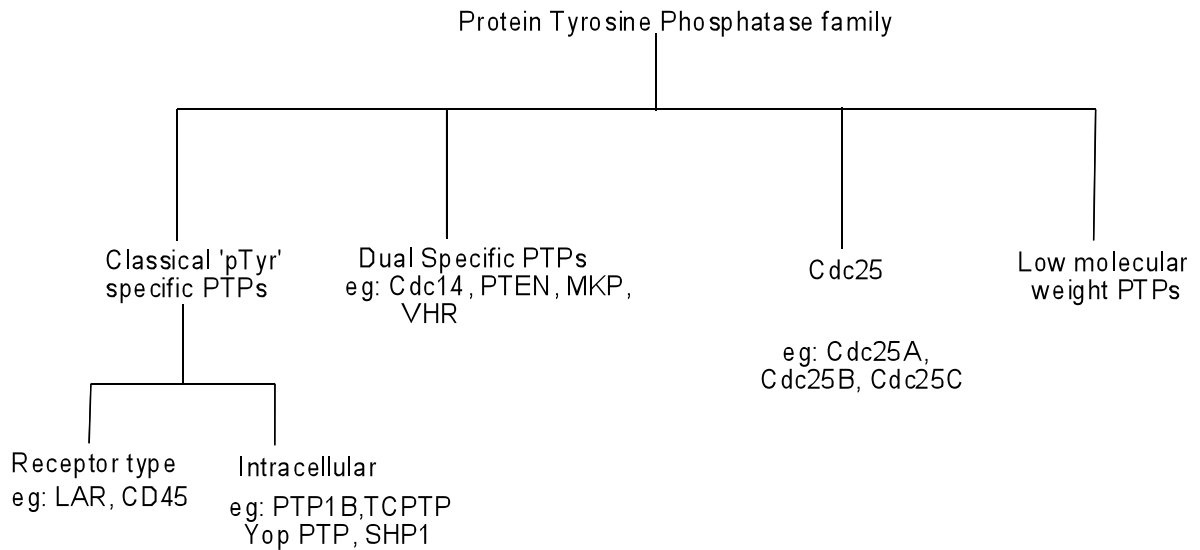
Figure 2: The different classes of PTP super family.[14]

There are different classes of phosphatase (PTPs) enzyme based on their structure, function and localization inside the cell. PTPs share a common structural motif and catalytic mechanism at the active site. They are protein tyrosine phosphatase (dephosphorylate p-tyrosine residue), dual specific phosphatase (dephosphorylate both tyrosine and serine phosphate group), Cdc25 (cell cycle regulatory phosphatase) and low molecular weight phosphatase (**Figure 2**). [14]

Among protein tyrosine phosphatase, there are two major subtypes, one is the intracellular PTPs (non-receptor type) and the other is receptor type PTPs. Protein tyrosine phosphatase 1B (PTP1B) is an abundant mammalian intracellular PTPs that is indentified as a major PTPs in the insulin signaling pathways.[13]

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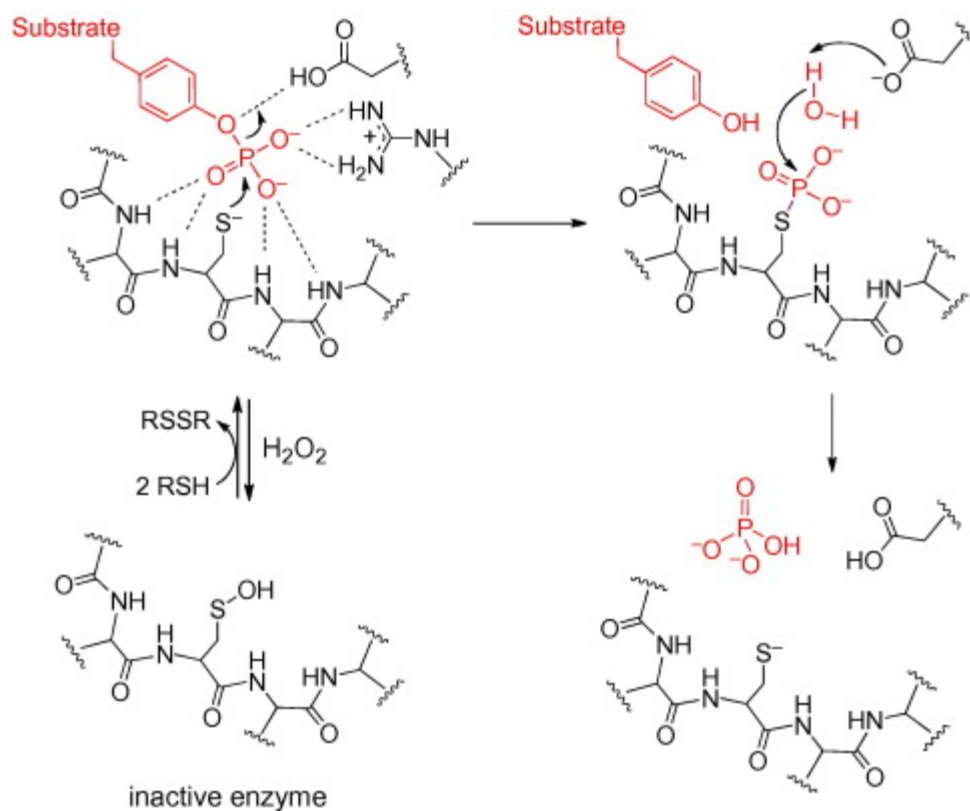
Scheme 1: General scheme of catalysis at the active site of PTPs [15]

The active site of PTP1B contains Cys-(Xaa)$_5$-Arg 11 amino acids as a signature motif and highly reactive cysteine thiolate (cys215) residue, buried in the deep catalytic core.[14] Unusual reactivity of Cys215 arises from its low pK_a of the thiol residue (pK_a 5.5) and stabilization of the thiolate by the neighboring amino acid residue (Ser216 and Ser222).[16] Catalytic reaction involves the removal of phosphate group from p-tyr substrate and produces a transient phosphocysteine intermediate,[17,18] followed by nucleophilic attack by a water molecule to generate the active enzyme.[19]

Binding of the p-tyr substrate involves the formation of electrostatic interaction of Arg221 with the negatively charged oxygen atom of the phosphate group and whereas the hydrophobic interaction with the Tyr 46 in Q loop and the Phe182 in WPD loop stabilizes the phenyl ring of the substrate at the active site (**Scheme 1**).[20,21] The substrate binding causes the WPD loop to move almost 10 Å to make a closed conformation over the substrate and then a nucleophilic attack by Cys215 on the substrate generates an unstable phosphocysteine intermediate, assisted by a proton transfer to the leaving group from Asp181 which acts as a general acid catalyst to stabilize the transition state.[13,20,21] This phosphocysteine intermediate is hydrolyzed by a water molecule activated by Asp181 and Gln262 at the active site to get the enzyme back for another round of catalytic cycle.[22]

1.3. Role of PTP1B during Insulin signaling:

PTP1B dephosphorylates the phosphotyrosine residue of insulin receptor (IR) and insulin receptor substrate (IRS1) and thus negatively regulates the insulin signaling pathways inside the cell (**Figure 3**). In brief, insulin causes phosphorylation of the insulin receptor which in turn phosphorylate the insulin receptor substrate (IRS-1).[23] The IRS-1 protein acts as a transmitter for the signal to several downstream signaling pathways. Elchebly reported that PTP1B knockout mice showed hypersensitivity towards insulin and lowered the blood glucose levels (**Figure 3**).[13,24]
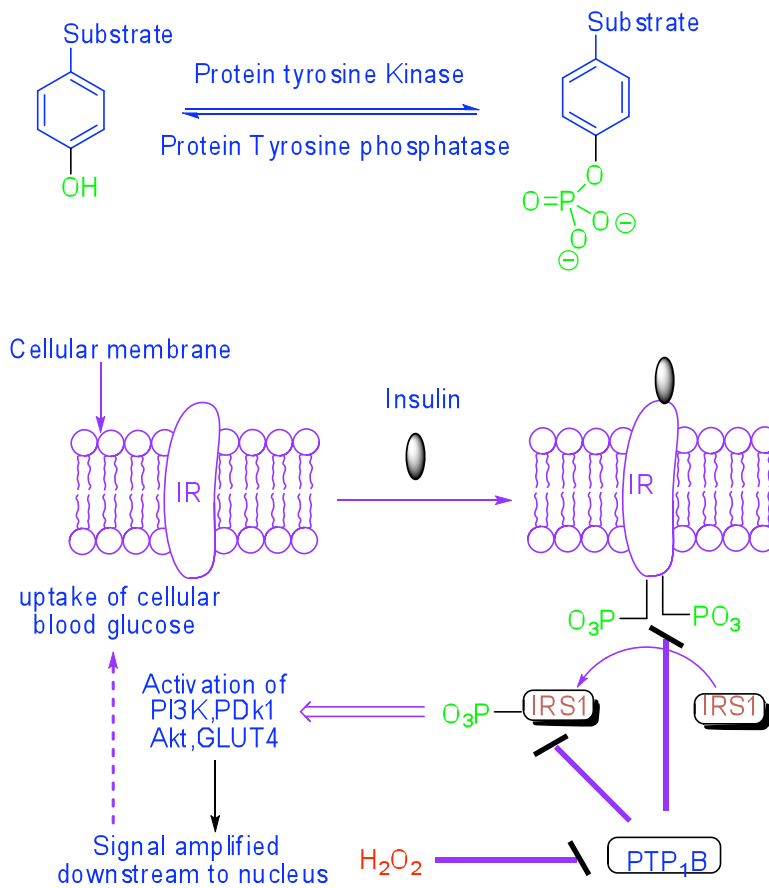
Figure 3: Scheme of insulin signal transduction pathways. Insulin binds with its receptor protein insulin receptor kinase (IR) and undergoes autophosphorylation. IR further transfers its phosphoryl group to IRS-1 (insulin receptor substrate 1) which activates phosphatidylinositol 3-kinse (PI3K) phosphorylation through binding with catalytic subunit. PI3K induces the activation of its downstream partner Akt (protein kinase B) and causes the transduction of insulin signal to translocation the of glucose transporter protein (GLUT4) and blood glucose uptake as biological response.[2,13]

Then IRS recruits PI-3 kinase as a substrate for its activation and which can further phosphorylate the Akt kinase and the signal is finally amplified downstream to express the glucose transporter protein (GLUT4) for the cellular uptake of blood glucose.[23]

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Insulin sensitivity of the PTP1B knockout mice has generated the urge in the design of selective PTP1B inhibitor molecules that inhibit PTP1B functions, may yield a hypoglycemic agent for diabetes. This finding made PTP1B an attractive target for the development of therapeutics for type II diabetes and obesity.[13]

1.4. Regulation of PTPs functions by ROS:

Reactive oxygen species (ROS) are well known to trigger various pathogenic processes inside the cell.[2,25-27] Roles of hydrogen peroxide and superoxide during insulin and other cytokine mediated signal transduction are known and efforts to identify their signaling targets have become increasingly prominent. Insulin signaling is associated with the "burst" of ROS and it was evidenced in 3T3-L1 adipocytes from the fluorescent signal of a redox sensitive dye 2,7-dichlorodihydrofluorescein (DCF).[28,29] Hydrogen peroxide is the oxidant generated during insulin stimulation of the cell and this was verified by the diminishing fluorescent signal of a dye in presence of catalase enzyme. Insulin-triggerd stimulation of cell causes the reduction of PTPase activity in HepG2 cell lysate and activity could be restored upon treating the samples with DTT.[29,30]

This result implicates the reversible oxidation of the PTPase on insulin treatment of the cell. Catalase also prevented the loss of PTPase activity in the insulin stimulated 3T3-L1 adipocyte lysate which was comparable to control samples.[25]

Addition of catalase also decreased the insulin stimulated autophosphorylation of insulin receptor (IR) and IRS, that indicates the oxidant produced during insulin signaling can target PTPs, negative regulator of IRS.[29]

Denu and Bunchok have shown from their *in vitro* study that hydrogen peroxide and superoxide radical anion can reversibly inactivate PTP1B and modulate its function.[31, 32] Later Salmeen and coworkers showed by protein crystallography that redox regulation of PTP1B function causes the generation of sulphenyl enzyme intermediate on treatment with H_2O_2 and then subsequent recovery of the active enzyme upon treatment with reducing agent thiol DTT.[33] Recent study shows that over activation of Nox4 enzyme has connection between the insulin mediated ROS generation and modulation of insulin signaling by regulation of PTP1B function.[25] Cells expressing Nox4 deletion constructs significantly decreased (56 %) the insulin receptor phosphorylation as well as the production of H_2O_2 by insulin stimulation as determined by DCF fluorescence.[34] These results all together suggest that ROS produced by Nox4 during insulin signaling can target the PTPase and effectively promote the insulin signaling pathways.

1.5. Reversible modification of oxidatively inactivated PTP1B:

Cellular assay shows that insulin mediated signal generates the ROS species (H_2O_2, O_2^-) can reversibly modulate the PTPase function.[25] In vitro experiments also support that hydrogen peroxide and superoxide can inactivate the PTP1B with a bimolecular rate constant of $10.0 \ M^{-1}s^{-1}$ and $334.5 \ M^{-1}s^{-1}$.[31,32]
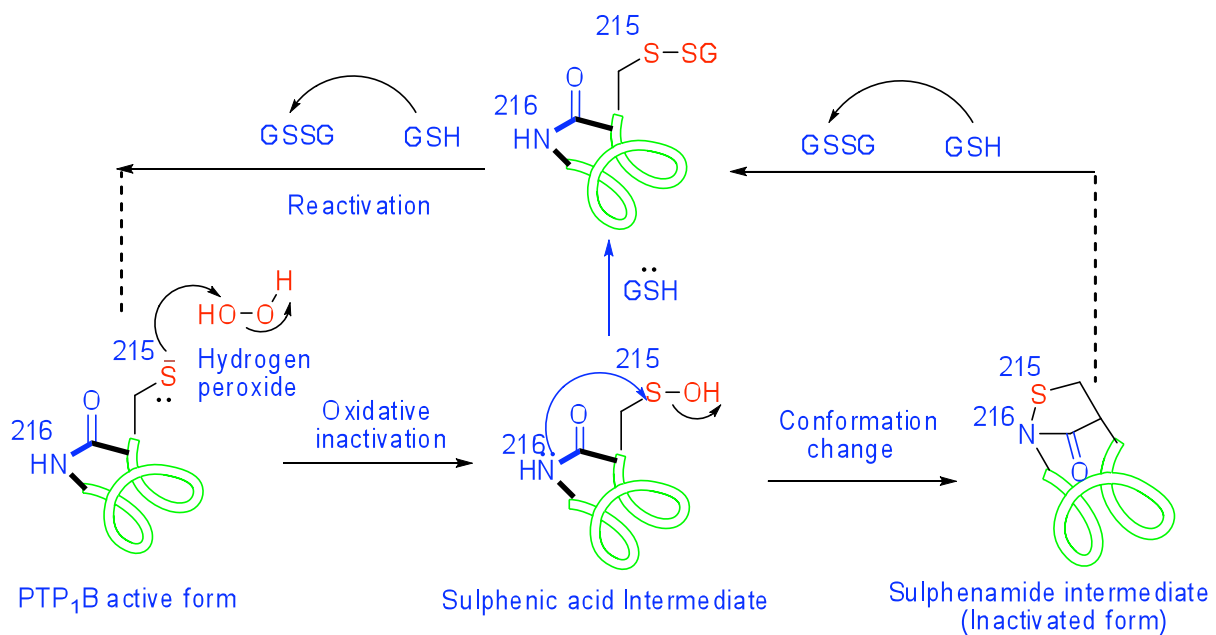
Scheme 2: Proposed mechanism for redox regulation of PTP1B function.[33,35-37]

It was hypothesized from the initial experimental evidence that redox regulation of PTP1B involves the formation of sulfenic acid intermediate (S-OH).[31,37] Further oxidation of the active site leads to the irreversible oxidation of PTP1B to give sulphinic acid (Cys-SO$_2$H) and sulfonic acid (Cys-SO$_3$H) form.[37] Reversible modification of PTP1B involves the formation of a novel five member sulphenyl amide intermediate and that result was supported by the X ray crystallographic data (**Scheme 2**).[33]

It was hypothesized that this intermediate was formed by the nucleophilic attack of proximal nitrogen atom of Ser216 on the sulfur atom of the Cys215 of the transient sulfenic acid (S-OH).[33]

This sulphenyl amide intermediate causes enzyme to undergo a conformational change of 7 Å in the P loop between Cys215 and Ser222.[33] The sulphenyl amide intermediate was formed even in presence of thiols and hence may be produced in the reducing environment of the cell.[33,37] Reversibility of this intermediate to the native enzyme by the treatment with thiol implicate that sulphenyl amide may serve as protective intermediate from its further oxidation to sulphinic acid (Cys-SO$_2$H) and sulfonic acid (Cys-SO$_3$H) form. Barrett has established that PTP1B can undergo glutathionylation of enzyme in presence of GSSG.[38] This modification is reversible not only by the treatment of chemical thiol (DTT) or biological thiol (GSH). The thioltransferase enzyme can also reactivate the glutathionylated enzyme.[38] This event suggests that besides sulphenyl amide formation, glutathionylation of active enzyme may also lead to the redox regulation of the PTPase function inside the cell.

Other PTPase also have been reported to undergo reversible modification of their function.[25] Redox regulation of Cdc25 functions is known to generate protein disulfide formation by a cysteine thiol attack (located at backdoor) on the sulfur atom of the active site sulfenic acid form.[39]

PTEN and map kinase phosphatase (MKP-1) contains redox active cysteine residue and modulate the downstream signaling of insulin and other signaling pathways.[40] ROS causes inactivation of the both the phosphatase function and more specifically causes disulfide formation with catalytic cysteine. The inactivated enzyme can be reactivated inside the cell by the thioredoxin.[41]

1.6. PTPase are emerging as a future therapeutics:

PTPase plays a role in several types of human disease and pathophysiological condition.[14,42] Studies are ongoing to understand how PTPase structure and function are related to normal and abnormal regulation inside the cell. So there is a need for the development of specific PTPase inhibitor for clinical purposes. The highly conserved and positively charged nature of active side PTP loop poses a challenge in the design of selective inhibitor. PTP1B null mice study has evolved a greater interest to take a combinatorial screening approach to identify the potential blocker of the PTPase function. Perturbing the function of PTPs could help us to understand its molecular role in the various cellular processes like cell signal transduction and could be useful towards the design of a disease specific drug. Here we will discuss several approaches that have been taken to design potent and selective PTP1B inhibitors.[42]

One approach involves the testing of natural product resources and their synthetic analogues as PTPase inhibitor. 4-Isoavenaciolide molecule **(Figure 4)** isolated from fungus showed strong inhibitory activity against VHR with an IC_{50} of 1.2 µM and similar inhibition against PTP1B, Cdc25B.[43]

4-Isoavenaciolide

Dancin
R= CN, OH

Dancin analogue

Dephostatin

Roseophilin

prodigiosin
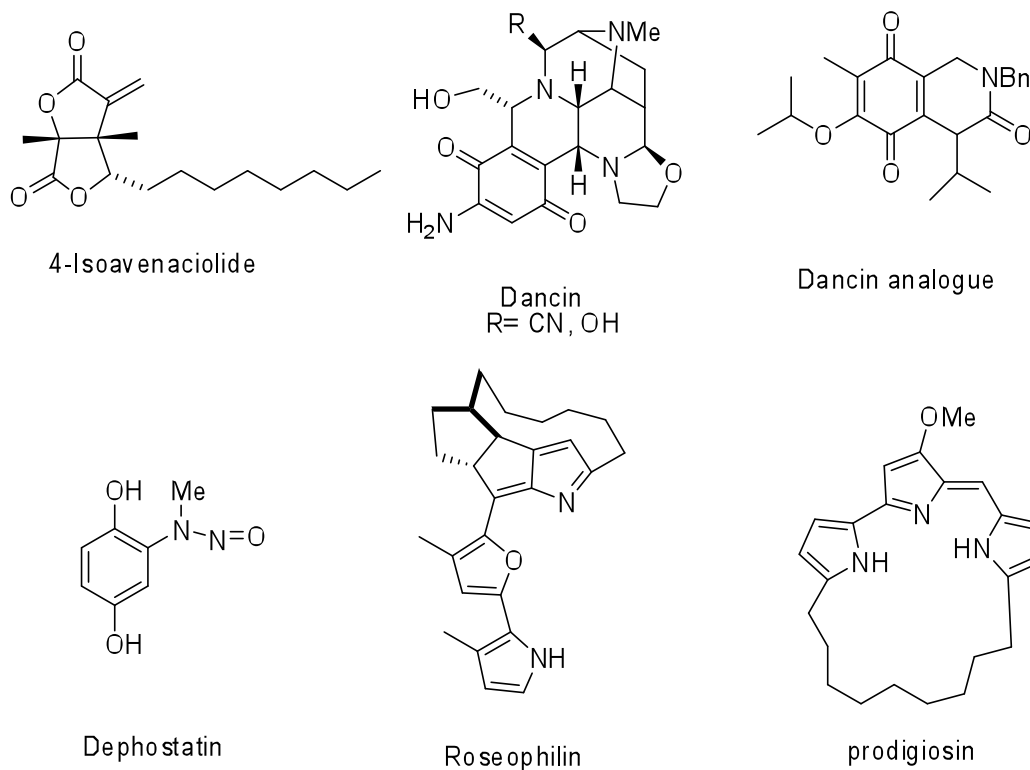
Figure 4: Natural product and its analogue as PTPase inhibitor.[42]

Mechanism study reveals that the covalent attachment of the active site cysteine and another surface cysteine residue with the Isoavenaciolide molecule and hydrophobic interaction near the active side pocket are essential also for binding.[42,44]

A natural product analogue of antitumor antibiotics Dancin A1 (**Figure 4**) showed inhibition of PTP1B, VHR, Cdc25A & B.[44, 45] The Compound showed selectivity for Cdc25 and PTP1B over VHR.

Natural product dephostatin (isolated from *Streptomyces*) and its analogue (**Figure 4**) showed competitive inhibition to PTP1B (IC_{50}= 0.94 μg mL^{-1}) and oral anti-diabetic activity without showing significant carcinogenic activity of nitrosamine group.[46, 47,48] Synthetic analogues of roseophilin and the prodigiosins also inhibit Cdc25A, PTP1B and VHR (**Figure 4**). [49,50,51]

Another approach was the design of PTPase inhibitor from the analysis of molecular modeling and the existing X-ray crystal structure of the phosphatase enzyme. Inhibitors with non-hydrolysable phosphotyrosine mimetic of enzyme were synthesized and screened against PTPase activity (**Figure 5**). Difluoromethylene phosphonate indeed increased the binding affinity of the substrate upon replacement of hydrogen atom of methylene with two fluorine atom.[14,52]

To build a specific PTP1B inhibitor, a secondary aryl phosphate binding sub-pockets were identified close to the active site of PTP1B.[14] This dual binding inhibitor compound **2** (**Figure 5**) is a very strong and highly selective inhibitor of PTP1B (K_i 2.4 nM). The binding interaction of dual binding inhibitor to the secondary pocket is found to be markedly different in various PTPase which help us to understand the molecular basis for their selectivity.[14]

But the problem associated with this compound was their poor bioavailability due to double negative charge. Subsequently oxalylaryl aminobenzoic acid, 2-(oxalyl amino) thiophene carboxylic acid and tripeptide cinnamic aldehydes were designed as phosphotyrosine mimetic.[53,54,55]
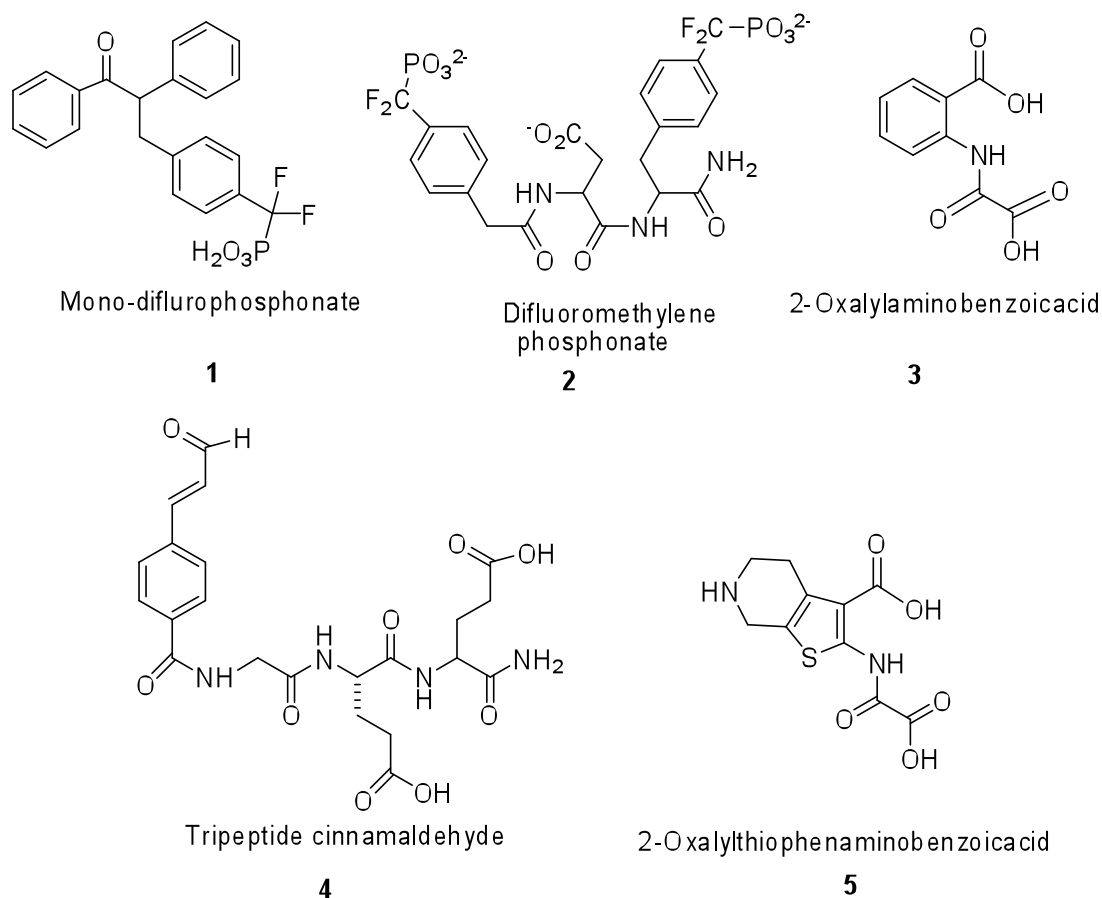
Figure 5: Phosphotyrosine and peptidomimetics as PTP1B inhibitor.[42]

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Other classes of inhibitors such as compound **5** (**Figure 5**) showed low micromolar K_i values for PTP1B and improved glucose uptake upon insulin treatment in cell. Biological or clinical data for many of these compounds are not available as they are still under investigation.

1.7. Conclusion:

We have seen the significant role of PTPase relevant to several cellular functions and physiological process. As PTPs possess a reactive thiolate residue, they are one of the key targets for the ROS mediated insulin signal transduction pathway. Insulin signal is propagated inside the cell via inhibition of PTPs function and lead to several biological responses, like glucose uptake inside the cell. Several PTPase inhibitors are known to exhibit promising biological activities *in vitro* and in cellular experiments. The selectivity of the compound among PTPase is a real concern and poses a great challenge to develop selective inhibitor for individual PTPs till date. Efforts are ongoing to achieve a potent but selective inhibitor or drug like molecule to use for clinical purposes. With the present structural information and mechanism known for PTPase, new drug for the treatment of disease associated with the regulation of PTPase is highly desired in the future.

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Chapter 2: Redox regulation of PTP1B functions by Organic Peroxides.

2.1. Introduction to biological importance of organic peroxides.

Peroxide compounds are well characterized and studied through the decades for their different chemical properties.[1] They are mainly used as oxidants in several organic reactions in laboratory to oxidize various functional groups like thiol, amine, double bond and other different functional groups.[2-5] Peroxides are used in water purification as disinfectants, as bleach in industry and also in explosives. But their usage is not only restricted as reagents in organic chemistry. Peroxide natural products are also produced by microorganism as metabolites and some of them are used in clinic as drug. Artesimin, a natural product isolated from bacteria is one of the leading antimalarial drugs used in clinic (**Figure 1**). [6]

Artemisinin   8,12-guaianolide hydroperoxide   Neoconcinndiol hydroperoxide

Figure 1: Peroxide natural products isolated from bacteria and marine organism.[6]

Besides artesimin, there are many other peroxide natural products known to exhibit biological properties.[6] Based on the structure, these peroxides are classified into two types; one is endoperoxides and other is exoperoxides. Usually peroxides are unstable compounds and reactive violently under normal reaction condition.[5] Many of them are very stable also.

Besides their use in organic and analytical chemistry, peroxide compounds are also generated inside biological systems. They are produced as a byproduct of several biochemical reactions. Example such as lipid peroxidation and protein peroxidation, is a very well known event inside cell (**Scheme 1**).[7]
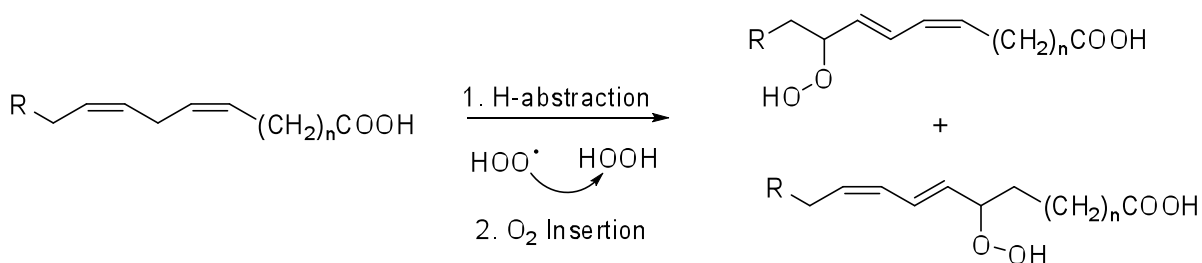
Scheme 1: General mechanism of lipid peroxidation.[7]

It is well known that reactive oxygen species (H_2O_2, $O_2^{\cdot-}$, $OH^{\cdot}$, $HNO_2^{\cdot-}$) are generated inside the cell as a consequence of cell stimulation by cytokine, cell signaling, pathogenic invasion.[7,8]

This reactive species can hit the protein and lipid component of the cell and produce protein peroxide and lipid peroxide respectively, as a reaction product. [7,8] Lipid peroxides can be further decomposed to give α,β-unsaturated aldehyde products that inactivates PTP1B.[9] Some peroxide compounds can be generated by enzymatic perhydrolysis of fatty acid ester (**Scheme 2**).[10] Although the concentration of H_2O_2 in this reaction is quite higher (in millimolar range) than physiologically concentration of H_2O_2. Perhydrolase, lipase and esterase can also produce peroxy acid as a side product of their enzymatic reaction (**Scheme 2**).[11-13]
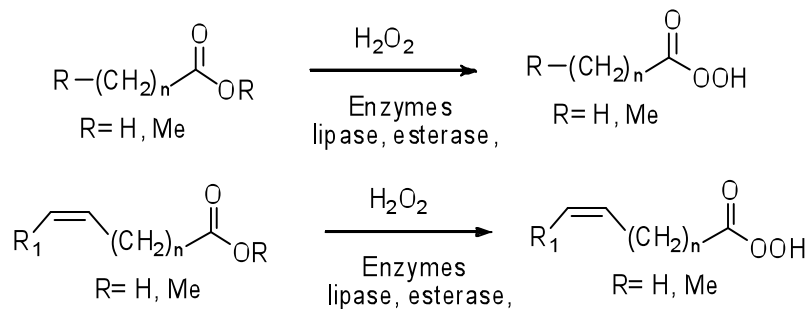
Scheme 2: Enzymatic perhydrolysis of fatty acid ester.[12,13]

Higher reactivity of acyl peroxide (peracid) allows them to modify biological macromolecules. Lipid peroxidation is well known as a biomarker for several diseases and pathophysiological status of the cell.[14]

Oxidative stress generates many of these organic peroxides and major of them have been identified to play crucial role in several cellular diseases like metabolic disorder (diabetics and obesity), neurodegeneration (Alzheimer's, Parkinson disease).[15,16]

Both lipid peroxide and protein hydro peroxides are known to cause DNA damage, strand cleavage and alters antioxidant status of cell.[17] Protein and amino acid hydroperoxides (His, Tyr, Trp, Val) are relatively stable (unlike H_2O_2) and have longer half-life in cell.[17-21] Thus, they are removed slowly and can play an important role in many biochemical event of the cell, like in cell signaling, modification of macromolecules. So, identification of their target protein and intracellular compartment that is attacked by this peroxidation product is very crucial. Amino acid, peptide and protein peroxide can cause the inhibition of thiol-containing enzymes (like caspases, cathepsin, etc) as they contain reactive cys residues for having low pK_a at their active side.[22-24] Davies has reported in a recent study that protein and amino acid hydroperoxides cause time-dependent inhibition of PTP1B activity.[25] Protein peroxides showed much more potency than amino acid peroxides. Only 1 μM RNAase peroxide was efficient to significantly inhibit the PTPase activity in cell lysate whereas similar inhibition was observed by H_2O_2 at 40 μM concentration.[25] This study shows the possible potential of protein hydroperoxide and amino acid hydroperoxide *in vitro and ex vivo* to regulate the PTPase function.

Protein hydroperoxide could play significant role during PTK/PTP based cell signaling and may contribute in many physiological and pathophysiological process.

2.2. Hypothesis:

Structural features of the amino acid hydroperoxide and protein peroxide were not elucidated in Davies's study. The mechanism, reversibility and kinetics of the inhibitions were not determined. It was hypothesized that sulfenic acid intermediate might be involved during the inhibition. In earlier study Denu showed that organic peroxides other than H_2O_2 did not inactivate PTPase.[26] PTPase inactivation by H_2O_2 is biologically relevant, but from the chemical reactivity of the peroxides, why other organic peroxide except H_2O_2 should not modify the PTPase was not clear to us. Based on the biological importance of many organic peroxides (lipid peroxides and protein peroxides) systematic study of the kinetics and mechanism of PTPase inhibition by hydroperoxide is relevant. That led us to investigate the role of structurally defined diverse organic peroxides to see their potential to regulate the PTP1B function like H_2O_2 (**Scheme 3**).
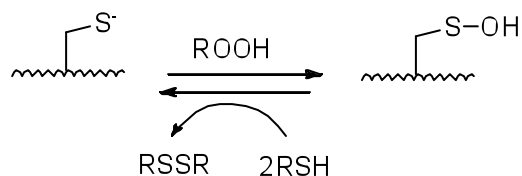
Scheme 3: Study of organic peroxides that can redox regulate the PTPase function.

2.3. Inactivation of PTP1B by organic peroxides:

We have tested the series of organic peroxides (**Figure 2**) to carry out thiol reversible and oxidative inactivation of PTP1B. PTP1B is often depicted as a representative member of the PTPase family of enzymes. Compound **1** (peracetic acid) showed rapid time dependent inactivation of the enzyme (**Figure 3**). [27]
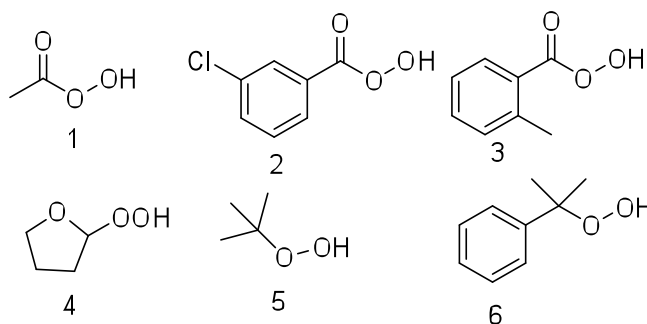
Figure 2: Structure of organic peroxides tested against PTP1B.[27]

Time dependent inactivation is consistent with process involving chemical modification. The inactivation data was fit to equation for 1^{st} order reaction (described in details in Experimental Section). The replot of apparent rate versus inactivator concentration yields a line that passes through origin. This result indicates there is no equilibrium association of **1** with the enzyme prior to covalent modification (**Figure 3A**).[28] The slope of the line (**Figure 3B**) reveals the bimolecular rate constant for the reaction of **1** with PTP1B is $2.1 \pm 0.2 \times 10^4$ M^{-1} s^{-1}. Peracetic acid is 2000 time more potent than hydrogen peroxide which inactivates the enzyme with a rate constant of 10 $M^{-1}s^{-1}$.
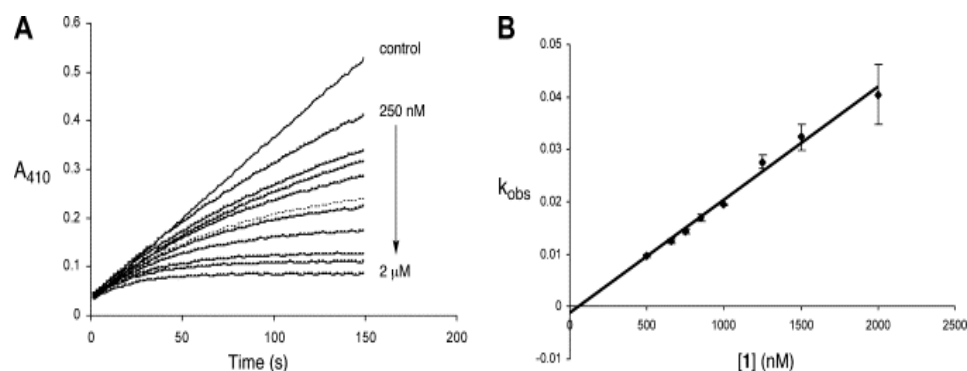
Figure 3: Rate of inactivation of PTP1B by compound **1**. (A) Progress curves for the inactivation of PTP1B by **1**. PTP1B was isolated as described previously and thiol-free samples of the enzyme were prepared by gel filtration of the protein through G25 Sephadex immediately prior to use according to reported procedures.[29] Thiol-free PTP1B (25 nM final) was added to a solution of **1** (250 nM–2 μM) in 3,3-dimethyl glutarate buffer (50 mM, pH 7.0) containing the substrate *p*-nitro phenyl phosphate (*p*-NPP, 20 mM) at 23 °C. The apparent rate constant (k_{obs}) for each concentration was calculated by the method of Voet and co-workers.[30] (B) Determination of the second-order rate constant for the inactivation of PTP1B by **1**; The apparent (pseudo-first-order) rate constants for inactivation of PTP1B by **1** were plotted versus concentration and the second-order rate constant for inactivation of PTP1B by **1** was extracted from the slope. The data was generated in triplicate (n=3) and Standard deviation (SD) was calculated and plotted in figure 3B. Inactivator concentrations >10-times the enzyme concentration were used in the plot. The concentration of **1** (and other peroxides) in stock solutions was determined by titration.[27,31]

The PTP1B inactivation by peracetic acid is not reversed by gel filtration of the inactivated enzyme (see experimental section). The inactivation was slowed down significantly in the presence of phosphate ion, a known active site inhibitor of PTP1B (K_i= 17 mM) (**Figure 4**).

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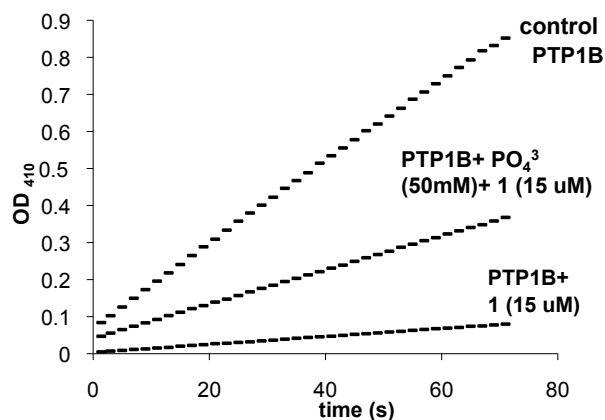
Figure 4: Inactivation of PTP1B by **1** was slowed by the addition of active site directed inhibitor (PO_4^{3-}). Thiol-free PTP1B (10 μM) was incubated with **1** (15 μM) in 3,3-dimethyl glutarate buffer (50 mM, pH 7.0) for 15 sec and then an aliquot of 15 μL was taken from the reaction mixture and added to a 1.0 mL of a solution containing the substrate *p*-nitrophenyl phosphate (*p*-NPP, 20 mM) at 23 °C and enzyme activity was measured by monitoring the enzyme-catalyzed release of *p*-nitrophenolate ion from the substrate *p*-NPP at 410 nm (The experiment was conducted in a quartz cuvette). Another assay was carried out similarly containing 1 (15 μM) and 50 mM (PO_4^{3-}) and after 15 sec the enzyme activity was measured exactly same way as mentioned above. A control reaction was carried out in identical manner except having no phosphate or 1 and all the remaining enzyme activity was measured with respect to the activity of the control.

Incubation of PTP1B with **1**(15 μM) for 15 sec did result in 91% inactivation of the enzyme with respect to a control (no inactivator), whereas in the presence of phosphate ion (50 mM) only 43% enzyme inactivation was observed. Together this result suggests that **1** inactivates the PTP1B by covalent modification of the active site.

2.4. Reversibility of the inactivation:

Inactivation of the PTP1B by peracetic acid is reversible by the treatment of the inactivated enzyme with thiol (**Figure 5**).

Specially the inactivation of PTP1B (750 nM) with low concentration of **1** (800 nM, 10 min incubation, 23 ^{0}C), followed by the treatment of the completely inactivated enzyme with dithiothreitol (DTT, 100 mM final concentration), led to substantial return of enzyme activity (73 % of the original activity). A similar control was performed under identical condition (except no inactivator) shows the full activity is retained under this condition. This suggests that the reaction of PTP1B with **1** predominantly converted the active site cysteine residue to sulfenic acid oxidation state. When the enzyme was treated with higher concentration of **1** (5 µM, 10 min incubation, 23 ^{0}C), only 10 % of the original activity returned upon treatment with DTT.
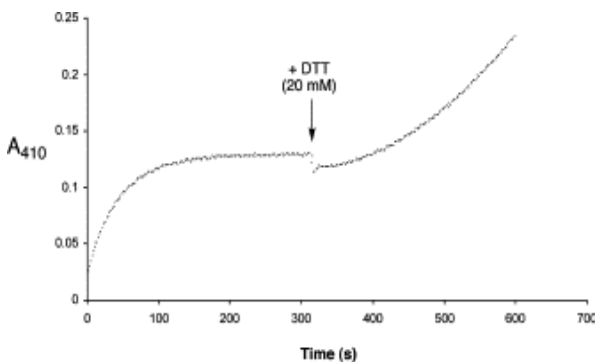
Figure 5. Inactivation of PTP1B by **1** can be reversed by addition of dithiothreitol (DTT). Thiol-free PTP1B (25 nM) was added to a solution of **1** (1 µM) in 3,3-dimethyl glutarate buffer (50 mM, pH 7.0) containing the substrate *p*-nitrophenyl phosphate (*p*-NPP, 20 mM) at 23 °C. When inactivation of the enzyme was nearly complete (300 s), a concentrated stock solution of DTT in water was added to give a final concentration of 20 mM. The experiment was conducted in a quartz cuvette and enzyme activity was measured by monitoring the enzyme-catalyzed release of *p*-nitrophenolate ion from the substrate *p*-NPP at 410 nm [27]

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High concentration of **1** presumably overoxidized the active site cysteine residue to sulfinic acid (RSO_2H) or sulfonic acid oxidation state (RSO_3H) that is not readily reduced back to active enzyme by DTT. It is known that cysteine residue in redox dependent enzymes can be over oxidized to sulfinic and sulfonic acids by hydrogen peroxide.[32,33]

2.5. Mechanism of Inactivation of PTP1B by peracetic acid:

Two possible pathways can be considered for the inactivation of PTP1B by **1** (**Scheme 3**). The first involves direct involvement of the active site cysteine residue with **1** to generate the sulfenic acid intermediate of the enzyme.[34-36] The other possibility involves metal mediated Fenton type reaction to produce diffusible oxygen radicals which could oxidize the active site cysteine thiolate.[37,38] Two lines of experimentation argue against a Fenton-type process in the inactivation of PTP1B by **1**. First, we find that addition of the classical oxygen radical scavenging agent mannitol, has little effect on the inactivation of PTP1B by **1**. Second, addition of $FeSO_4$ (5 μM) inhibits, rather facilitates, the inactivation process, presumably due to the fact that Fe (II) mediates destruction of the peracids. With the Fenton-type mechanism excluded, it is reasonable to suspect that the inactivation of PTP1B by **1** proceeds via the mechanism shown in (**Scheme 4**). Peracids shows more heterolytic cleavage over homolytic bond breakage because pK_a of the conjugate base that serves as the leaving group facilitates the process.[27]

A $\quad$ —S⁻ $\quad$ + $\quad$ HO—OR $\quad$ ⟶ $\quad$ —S—OH $\quad$ + $\quad$ ⁻OR

B $\quad$ HO—OR $\quad$ + $\quad$ Fe(II) $\quad$ ⟶ $\quad$ HO⁻ $\quad$ + $\quad$ •OR $\quad$ + $\quad$ Fe(III)
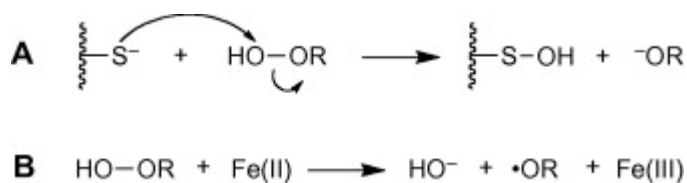
Scheme 4: Potential mechanisms for PTPase inactivation by hydroperoxides. [27]

2.6. Comparative study of the relative inactivation ability of the organic peroxide:

We find that aromatic peracids **2** and **3** inactivate PTP1B even more effectively than **1** (**Figure 6**).[39] We could not determine the bimolecular rate constant for the reaction of PTP1B with mCPBA (m-chloroperbenzoic acid) and MMPP (magnesium mono peroxyphthalate) under pseudo first order reaction condition due to extremely high reactivity of the compounds. For example, **2** and **3**, at 150 nM concentrations, completely inactivate the enzyme within milliseconds. In contrast, when the enzyme is treated with the same concentration of **1**, a significant amount of enzyme activity is remained at 1 min (**Figure 6**). The superior reactivity of peracid is due to their intrinsic higher reactivity compare to hydrogen peroxide and possibly associated with active site.[34] For another point of comparison, 150 nM H_2O_2 has no measurable effect on enzyme activity under these conditions. Indeed concentrations of H_2O_2 approaching 100 μM are required to yield inactivation rates comparable to 150 nM **1** (**Figure 6**).
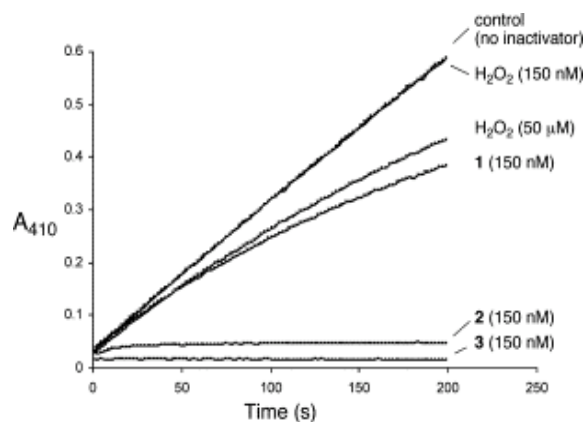
Figure 6. Comparison of the inactivation of PTP1B by compounds **1**, **2**, **3**, and hydrogen peroxide. Thiol-free PTP1B (25 nM) was added to a cuvette containing 3,3-dimethyl glutarate buffer (50 mM, pH 7.0), *p*-NPP (10 mM), and the hydroperoxide of interest (**1**, **2**, **3**, or H_2O_2) at 24 °C. Enzyme inactivation progress curves showing the amount of PTP1B activity remaining as a function of time were obtained by monitoring the enzyme-catalyzed release of *p*-nitrophenolate ion from the substrate *p*-NPP at 410 nm. Note: curves for control 'no inactivator' and 150 nM H_2O_2 are overlapping.[27]

In addition, we examined the ability of 2-hydroperoxytetrahydrofuran (**4**) to inactivate PTP1B. This reagent was synthesized by the method of Gold and co-workers.[40] We find that **4** inactivates PTP1B with a rate constant of $20.3 \pm 0.8 \ M^{-1} \ s^{-1}$. This is comparable to the literature reported value for hydrogen peroxide.[26,41]

Analogous to the inactivation of PTP1B by **1** and hydrogen peroxide, inactivation by **4** (50 μM, 1 h) is reversed upon reaction of the enzyme with DTT (54% return of activity, following treatment with 100 mM DTT for 1 h, see **Experimental Section**).

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Finally, it is noteworthy that **1**–**3** can inactivate the enzyme in the presence of the biological thiol, glutathione.[42] For example, treatment of PTP1B with **1** (5 μM, 15 s) causes a 29% loss of enzyme activity even in the presence of 1 mM glutathione (**Figure 7**).
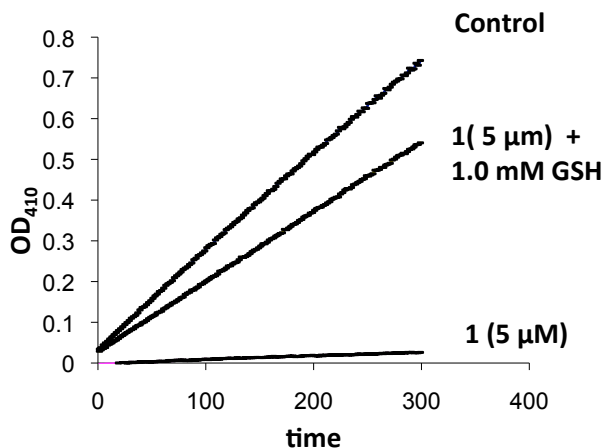
Figure 7: Inactivation of PTP1B by **1** was not completely abrogated by the presence of 1 mM glutathione (GSH). Thiol-free PTP1B (10 μM) was incubated with **1** (5 μM) in 3,3-dimethyl glutarate buffer (50 mM, pH 7.0) for 15 sec and then an aliquot of 15 μL was taken from the reaction mixture and added to a 1.0 mL of a solution containing the substrate *p*-nitrophenyl phosphate (*p*-NPP, 20 mM) at 23 °C and enzyme activity was measured by monitoring the enzyme-catalyzed release of *p*-nitrophenolate ion from the substrate *p*-NPP at 410 nm. Similarly another assay was carried out containing 1 (5 μM) and 1 mM (GSH) and after 15 sec the enzyme activity was measured exactly same way as mentioned above. A control reaction was carried out in identical manner except having no GSH or **1** and all the remaining enzyme activity was measured with respect to the activity of the control.

Tanner and Denu previously reported that *tert*-butyl hydroperoxide (**5**) and cumene hydroperoxide (**6**) do not inactivate PTPs.[26] As part of this work, we confirmed their findings.

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These hydroperoxides react with small-molecule thiols at rates comparable to hydrogen peroxide; therefore, it seems likely that their failure to inactivate PTPs stems, not from an inherent lack of reactivity, but from the fact that the substituents on these tertiary hydroperoxides form an 'umbrella' of steric bulk that prevents the peroxyl residue from penetrating the deep active site cavity of PTP1B to reach the catalytic cysteine residue. Indeed, as part of this study, we showed that less hindered hydroperoxide **4** inactivates PTP1B with potency comparable to hydrogen peroxide. Evidently, the planar peracids **1–3** clearly are not sterically precluded from entering the enzyme's active site.[27]

2.7. Potential of peracetic acid as putative endogenous regulator of PTPs:

Growing evidence suggest that protein acetylation is one of the very important post-translational modifications in biology.[43,44] Acetylation and deacetylation of protein play an important role in the perspective of cellular epigenetic.[43-45] Histone deacetylase (HDAC) has generated tremendous interest in many research groups for the design of inhibitor as therapeutics for cancer.[45] Apart from the post-translational modification, we will see the biological importance of protein acetylation in the light of cellular signal transduction and reactive oxygen species.

In vitro hydrogen peroxide is a very poor inactivator of PTPs with a low bimolecular rate constant of 10 $M^{-1}s^{-1}$. Still hydrogen peroxide can inactivate the PTPase inside the cell upon stimulation by insulin.[16]

It is known that insulin potentiates its action by generation of ROS which inhibits PTPase.[16] It remains unclear how H_2O_2 can inactivates PTPase inside cell, in presence of excess GSH while this transient ROS species are generated in low micro molar concentration. This differential reactivity of H_2O_2 towards PTPase *in vitro and in vivo*, led us to rationalize that there must be some secondary mechanism which plays important role during ROS mediated insulin signaling pathway. In a biochemically process H_2O_2 might get converted to a secondary oxidant which is potent enough in low concentration to modulate the insulin signaling by targeting the PTPase.

A recent study by Labutti shows that peroxymonophosphate ($=O_3POOH$) produced by the reaction of pyrophosphate and hydrogen peroxide and by the reaction of phosphoryl transferring enzyme, can reversibly regulate the PTP1B function.[29]
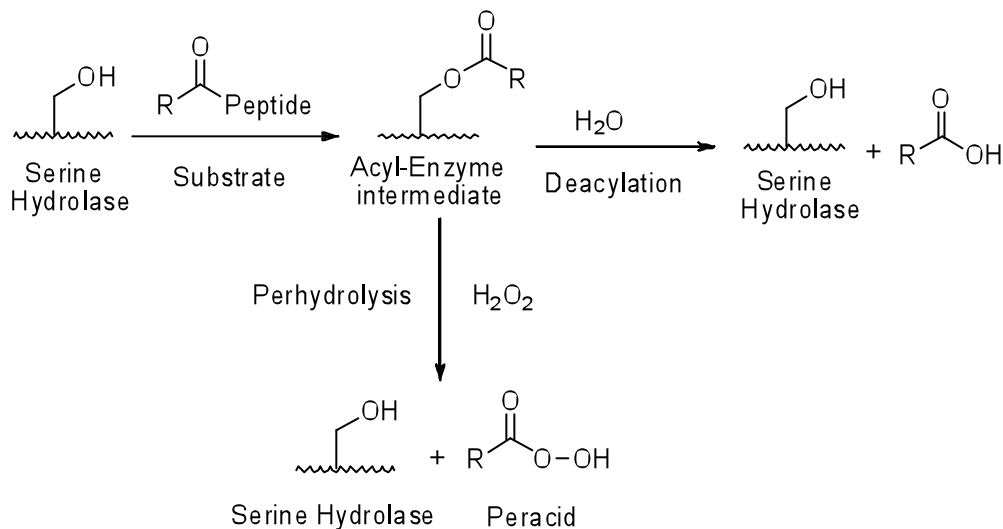
Scheme 5: General scheme of perhydrolysis side reaction of the hydrolase.[46]

This result inspired us to find whether peracids have the similar potential to be generated biochemically. A wealth of literature evidence shows that peracetic acids can be generated by various enzymes, as a catalyzed side reaction.[47] Various bacteria, higher plant enzymes, acetolactate synthase, pyruvate decarboxylase, esterase, lipase, hydrolase, haloperoxidase can lead to the formation of the peracetic acid as a side reaction (**Scheme 5**).[48-50] These results have been elucidated by structure, kinetics and mechanistic investigation.

It is known that hydrolase activity and perhydrolase activity are two completely distinguishable chemical reactions. Perhydrolase have similar catalytic triad (like Ser-His-Asp) like serine hydrolase. It was still unclear that which features guide them to catalyze a specific and distinct reaction. After rigorous structural alignment and analysis of six hydrolase and six perhydrolase amino acid sequence, Peter has made six mutants of an aryl esterase (from *pseudomonas fluorescens*) proteins to examine the possible role of the amino acid responsible for perhydrolase activity.[46] It was observed that only one amino acid mutation Leu29pro altered the behavior of hydrolase towards perhydrolase activity (28 fold increases, 0.24 to 6.8 U mg-1).[46]
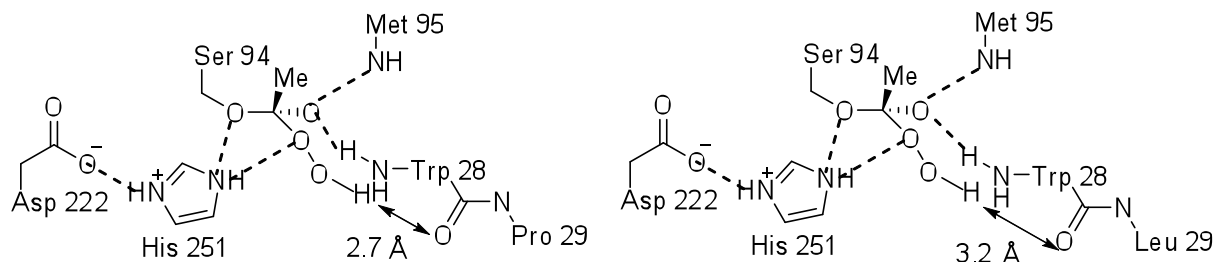
Figure 8: A proposed model for the preferential perhydrolase activity over hydrolase.[46]

A molecular modeling and proposed mechanism explain the result that how nucleophilic attack by hydrogen peroxide over water is facilitated by an additional hydrogen bond with the carbonyl carbon of proline29 and hydrogen atom of peroxide (**Figure 8**).[46] The increase perhydrolase activity is in agreement with the calculated energy (1.6 kcal/mol[-1]) for a new hydrogen bond.[46] The proposed model also shows that the electronic environment of the active site in the mutant diminished the attack by the water for deacetylation as that type of hydrogen bonding stabilization is absent over there.[46] This explanation shows us a molecular basis for hydrolase enzyme to behave as a perhydrolase. Beside perhydrolase, haloperoxidase can also use H_2O_2 as substrate. Hofman has reported the X-ray crystal structure of the cofactor free chloroperoxidase that use organic acid (benzoic acid) as its substrate for the catalytic reaction and peroxyacid formation as a reaction intermediate was proposed based on the electron density calculation.[51]

It will be worth exploring whether isoenzymes of mammalian haloperoxidase, hydrolase, esterase, lipase and can catalyze the formation of peracetic acid and fatty acyl peracid and thereby shed light upon their endogenous production in eukaryotes and important regulator during ROS mediated signaling.

Apart from the perhydrolase enzyme, decarboxylase enzyme has been observed to exhibit the property of catalyzing percatalytic reaction as a side reaction in a hydrogen peroxide independent pathways.[48] Study by Bunik shows that mitochondrial metabolic enzyme α-ketoglutarate dehydrogenase (KGDHC) can catalyze the formation of peracetic acid in relevant to neurodegenerative disease.[15]

KGDHC is a thiamine diphosphate (THDP) dependent enzyme, known to produce ROS in mitochondria.[52] Percatalytic reaction involves the oxidative decarboxylation of carbanionic intermediate and the consumption of oxygen as electrophile (**Scheme 6**).[15]
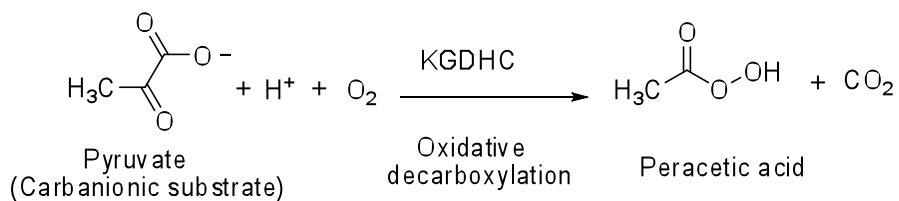
Scheme 6: Percatalytic reaction by α-ketoglutarate dehydrogenase enzyme.[15]

Isoenzyme of mammalian decarboxylase is also known to catalyze similar percatalytic reaction.[53-55] We have reported that nanomolar concentration of peracid is enough to abrogate the PTPase activity. Peracetic acid is stronger oxidant than H_2O_2 and thereby posses the potential to behave as a signaling molecule like H_2O_2 and might play a detrimental role during cell signaling.

Recently peracetic acid uroporphyrin compound has been isolated from the urine sample of the patient with erythropoietic porphyria and characterized by HPLC-MS (**Figure 9**) .[55] This result reinforces the endogenous formation of peracetic acid under pathophysiological condition. Under what circumstances and by which pathways it is produced inside the mammalian cell, needs to be determined.
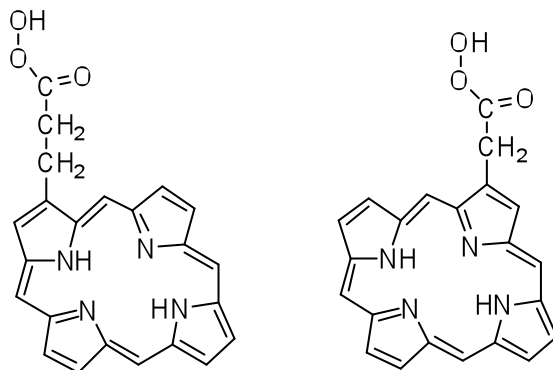
Figure 9: Uroporphyrin derivatives of peroxy propionic acid and peroxy acetic acid found in the urine sample of patient having erythropoietic porphyria.[55]

Accumulating evidence shows the relevance of serine protease behind the initial event of transmembrane signal transduction.[56] Animal study shows that insulin signaling pathways activates brush-border membrane hydrolase (BBM) and some of those proteins are the direct substrate of the IR.[58] But efforts are ongoing to determine the exact role of insulin signal for inducing BBM enzyme and see whether they can behave as perhydrolase.

2.8. Potential of lipid peroxides to modulate the PTPase function:

We have already seen how insulin signal propagates by ROS that inhibit the PTPase function and finally signal gets amplified downstream to a biological response of cellular glucose uptake. ROS can also modify the lipid to their peroxy metabolites (**Scheme 2**) which can cause damage to macromolecules.

A study on the effect EGF (epidermal growth factor) phosphorylation by linoleic acid metabolite (13S)-hydroperoxyoctadecanoic acid (13S)-HPODE led us to investigate this molecule relevant to the regulation of PTPase activity. Linoleic acid is an essential fatty acid in our nutrition and also found in lipids of the cell membrane. Metabolite of linoleic acid, 13S-HPODE (**Figure 10**) has found to modulate the tyrosine phosphorylation of epidermal growth factor (EGFR) and proteins involved in the signal transduction of mitogenic pathways.[59] Glasgow reported that treatment with 13S-HPODE augment the autophosphorylation of EGFR and several other GTPase binding protein in SHE cell lines.[59,60]

13S-HPODE did not alter the kinase activity of the EGF, rather prolonged the tyrosine phosphorylation intensely.[59] This result indicates that this metabolite can up regulate the EGF signaling by inhibiting the dephosphorylation of the EGFR.[59]

13(S)-hydroperoxyoctadecadienoic acid
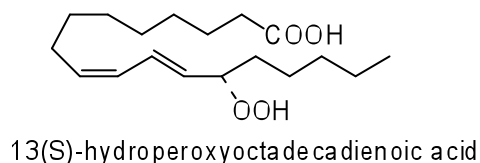
Figure 10: Structure of 13(S)- hydroperoxyoctadecanoic acid.[59]

The specific but undesignated cellular target of the 13S- HPODE is yet to be determined. Also the mechanism how it reduces the dephosphorylation of EGF in the cell is also not known yet.[59] It was hypothesized that 13S-HPODE could be regulating the EGFR phosphorylation by playing a balance between kinase and phosphatase activity in this signaling cascade.[59,61] The phosphatase specific for EGFR is not well known. Growing evidence shows that PTP1B is phosphorylated upon interaction with internalized EFGR receptor and could dephosphorylate the EGFR tyrosine kinase after internalization of the RTKs.[62,63] This finding led us to hypothesize that 13S-HPODE could modulate PTPase activity and thereby prolong the EGFR phosphorylation.

Also, the attenuation of the EGFR dephosphorylation could be due to the reversible modification of the PTP1B by 13S-HPODE in an identical manner to cellular hydrogen peroxide. We set out to test 13S-HPODE against PTPase activity by in vitro study.

Our initial study shows that lipid peroxide can inactivate the PTP1B and SHP-2 function and the inactivation is reversed by treating the inactivated enzyme with 50 mM DTT for 1 hr (**Figure 11B**).

11A.   **11B.**
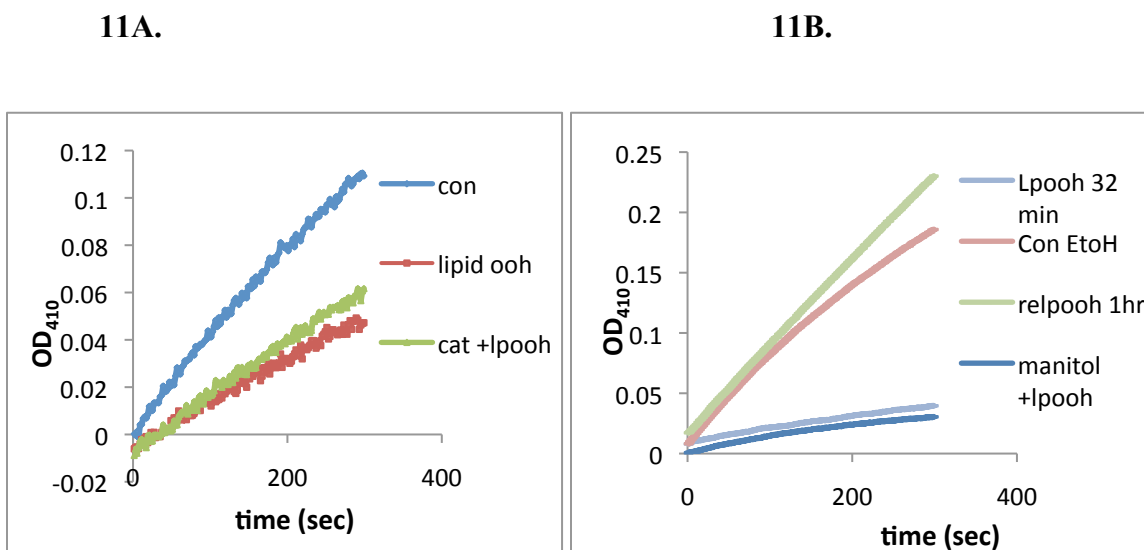
Figure 11: **(11 A)** PTP1B (0.25 µM) was incubated with 40 µM 13S-HPODE for 30 minutes in presence and absence of enzyme catalase, in 50 mM tris, 50 mM bis-tris, 100 mM NaOAc 10 mM DETAAC buffer (pH 7.2). An aliquot of 15 µL was taken from the reaction mixture and added to a 1 mL solution, containing 20 mM pNPP in the same assay buffer and the reaction progress was monitored by UV-vis for the generation of p-nitrophenolate at 410 nm to measure the % remaining enzyme activity. **(11 B)** SHP-2 (0.5 µM) was incubated with 40 µM 13S-HPODE for 30 minutes and the enzyme activity was assayed as mentioned above. A concentration of 100 mM DTT was added to the reaction mixture to reactivate the enzyme for 1 hr and after an hour, an aliquot of 15 µL was taken from the reaction mixture and assayed for enzyme activity. Enzyme activity was completely recovered. An identical incubation reaction was carried out in presence of 1 mM mannitol and enzyme activity was assayed similarly, found not to affect the inactivation. Abbreviation; con:control, lpooh: 13S-HPODE, cat: catalase, relpooh: reactivation of 13S-HPODE inactivated enzyme by DTT.

The addition of catalase and mannitol has no effect upon the PTPase mediated inactivation. This observation rules out the possibility of ROS involvement during 13S-HPODE mediated PTPase inactivation (**Figure 11A & 11 B**).This result shows that 13S-HPODE mediated PTPase inactivation involves redox regulation like H_2O_2, possibly involving sulfenic acid intermediate. The inactivation does not involve generation of any ROS intermediate. Future study is required to quantify the kinetics of the inactivation.

2.9. Conclusion:

Our work shows the evidence that organic peroxides can reversibly regulate the PTPase function involving sulfenic acid intermediate. Lipid peroxides and lipid metabolite (13S-HPODE) have the potential to modulate the PTPase function and therefore can upregulate the various receptor signaling pathways relevant to physiological and pathophysiological condition of cell. Peracetic acid is efficient to inactivate the PTP1B function in nano molar concentration and inactivation still occurs in presence of cellular reducing agent glutathione. Literature evidence shows that peracetic acid can be produced by several enzymes both in a ROS dependent and inpendent pathway. Peracetic acid derivatives are found in the urine sample of the patients suffering from erythropoietic porphyria. Therefore our study raises the possibility that peracetic acid could behave as a messenger molecule like H_2O_2 during ROS mediated cell signaling.

Future investigation is required to detect whether peracetic acid is generated during insulin signaling and other cytokine driven cell signaling pathways.

Experimental section:

 Materials and methods:

  Reagents: All the other chemicals and reagents of highest grade were purchased from Sigma, Fischer Chemicals Company and the dilute stock of inhibitors were prepared freshly before the assay from the concentrated stock. Dithiothreotol (DTT), 2-mercaptoehtanol (BME), substrate p-nitrophenolphosphate (pNPP), Glutathione (GSH) and m-chloroperoxybenzoic acid (70% mCPBA), Peracetic acid (19% CH_3CO_3H), magnesium peroxypthalate (MMPP), 3,3-dimethyl glutarate, sodium dihydrogen phosphate, sodium thiosulfate, sodium iodide, starch, nonionic detergent NP-40, 2,3 dihydrofuran were purchased from Sigma-Aldrich Company. Lipid peroxide (13S-hydroperoxyoctadecadienoic acid) was purchased from Caymon Chemical Company and 30 % H_2O_2, methyelene chloride, sodium bicarbonate, ammonium chloride, H_2SO_4 were purchased from Fischer Scientific Chemicals. Hewlett Packard 8953 model of UV machine was used to measure the UV-vis assay or the kinetics. Nuclear magnetic resonance spectra were recorded on Bruker 250 MHz spectrometer and in $CDCl_3$ unless, otherwise mentioned.

  Enzyme: Protein tyrosine phosphatase 1B (PTP1B) & SHP-2 were purified according to reported procedure.[29] The bacterial expression plasmid for human PTP1B was a kind gift from Prof. Nicholas Tonk, Cold Spring Harbor Laboratory, New York.

Thiols were removed from the enzyme stock during enzyme dilution before the enzyme assay by using G-25 sephadex bead (purchased from Sigma) spin column and Zeba^{TM} desalt spin column from Pierce.

Inactivation Assay of Protein Tyrosine Phosphatase 1B by Peracetic Acid (CH$_3$CO$_3$H):

Inactivation of PTP1B by peracetic acid was measured by the decrease of phosphatase activity towards phosphatase substrate p-nitrophenyl phosphate (*p*NPP). PTP1B inactivation assays were monitored by the generation of product p-nitrophenolate at 405 nm in a UV-vis spectrophotometer. Peracids are highly reactive and oxidizing agent and they can react with buffer component like hepes, tris and chelating agent like EDTA, DETAPAC. Very inert buffer component such as phosphate and 3,3-dimethyl glutarate buffer was used, in highly pure Millipore water to eliminate the presence of trace amount of free metal ion to avoid the decomposition of peroxides. Typically, all assay reactions contained (1.0 mL) 50 mM 3,3-dimethyl glutarate (pH 7.2), 1.0 % EtOH, 20 mM pNPP substrate, different concentration of peracetic acid (0- 2 µM) and (0.01- 0.25) µM PTP1B. PTP1B stock (20 µM) was allowed to pass through a G-25 sephadex spin column to remove the thiol, in order to require an absence of reducing agents during inactivation. Thiol free enzyme (0.01 µM final concentrations) was added as a last component to the reaction mixture to initiate the reaction and the reaction was monitored continuously by a UV-vis spectrophotometer at 405 nm.

The initial reaction rate or the apparent rate of inactivation was measured from the early regions of the reaction progress curve by fitting the data to prism software analysis for non-linear regression. All the assays were performed at room temperature in absence of any reducing agents and all the inhibitor stocks of peracetic acid were prepared fresh in Ethanol. Kinetics of PTP1B inactivation were measured according to the method described by Voet and co-worker.[30] The absorbance (A) vs time (t) data were fitted against equation

$$A_{410} = C - \exp(\ln v_0 - k't)/k'$$

using software Prism, the nonlinear regression was analyzed where v_0 is the initial reaction rate in absence of inactivator and k' is the observed inactivation constant. This method is based on a system which considers enzyme, inhibitor as well as substrate. Substrate concentration is kept constant throughout the assay and inhibitor concentration is varied. A secondary plot of k_{app} versus. I yielded a straight line passing through origin, giving the slope as a rate constant of bimolecular reaction (**Figure 3B**).

Reactivation of Peracetic acid Inactivated PTP1B Using DTT as Reducing Agents:

Inactivation of PTP1B medicated by peracetic acid (**1**) can be reversed with the treatment of thiol (DTT) like hydrogen peroxide,[26] by adding thiol as reducing agents. Thiol free PTP1B (0.5 µM) was incubated with 750 nM peracetic acid, in a 100 µL assay reaction for 15 min , containing 50 mM 3,3-dimethyl glutarate buffer (pH 7.2), 5.0 % EtOH, in order to make sure the enzyme was almost completely inactivated. An aliquot of 15 µL was taken from the reaction mixture and was added to a cuvette p-nitrophenolate phosphate (pNPP) as a substrate 50 mM 3,3-dimethyl glutarate buffer (pH 7.2) at 410 nm in Uv-vis spectrophotometer. The enzyme was almost completely inactivated (2.5 %). Then the completely inactivated enzyme was treated with 100 mM DTT and incubated for another 1 h in order to completely reactivate the enzyme and see whether inactivation involves redox regulation of the active site or not. After an hour 15 µL of reaction mixture was taken and diluted into 1 mL solution of glutarate buffer (pH 7.2) having 20 mM *p*NPP as substrate and enzyme activity was monitored continuously at 410 nm like before and enzyme activity was found to almost completely recovered (73%) by the treatment of DTT (**Figure 2**) and that result implicate the reversible modification of PTP1B function by peracetic acid. A control was done similarly only in absence of peracetic acid and treated with DTT as the inactivation was carried out. All the enzyme activity was measured from the slope of the reaction progress and calculated with respect to the control. A positive control was done with H_2O_2.

Gel filtration of peracetic acid inactivated PTP1B:

PTP1B (0.5 µM) was incubated with 1 µM **1** for 2 minutes in glutarate buffer (pH 7.2) (final reaction volume of 100 µL). An aliquot of 15 µL was taken from the reaction mixture after 2 minute and assayed for phosphatase activity. PTP1B was found to be completely inactivated. Remaining solution (85 µL) was gel filtered through G-25 spin column in order to free the residual peracetic acid. The exchange buffer contained 50 mM 3,3-dimethyl glutarate (pH 7.2), freshly added NP-40 as non ionic detergent. After 1 h of spin column, a 15 µL aliquot from the filtrate was taken to measure the PTP1B as described above.
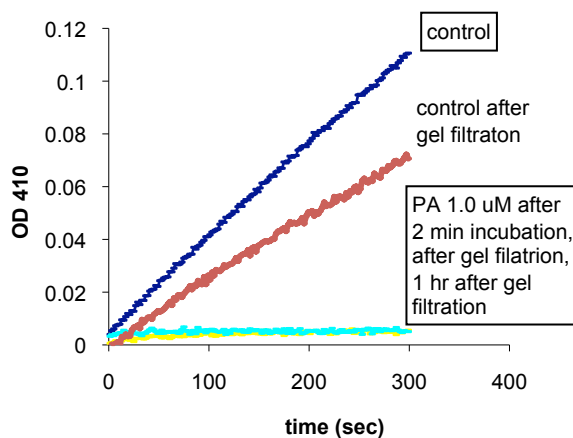
Figure 12: Gel filtration exp.

No significant return of PTP1B activity was observed compare to a control done in identical manner without addition of any peracetic acid (**Figure 12**). The control after gel filtration showed a reduction of activity before spin column, as some residual enzyme activity is lost during spinning through G-25 sephadex.

Does inactivation of PTP1B by peracid involves ROS:

PTP1B was incubated with 1 µM peracetic acid in presence of mannitol (1.0 mM) and metal ion (5 µM Fe^{+2}) and incubated for 10 minute and the enzyme activity was measured as mentioned before. Addition of mannitol and metal ion has no effect upon peracetic acid mediated inactivation of PTP1B (**Figure 13**). Individual control was done with Fe^{+2} and mannitol and it has no effect upon PTPase activity.
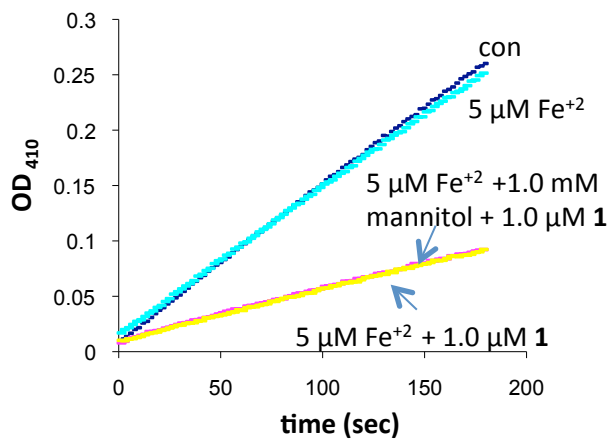
Figure 13: PTP1B inactivation by **1** (CH_3CO_3H) doesn't involve ay ROS.

Determination of Peracid concentration by Iodometric titration:

$$H_2O_2 + 2\ KI + H_2SO_4 ----> I_2 + K_2SO_4 + 2\ H_2O$$

$$I_2 + 2\ Na_2S_2O_3 ----> Na_2S_4O_6 + 2\ NaI$$
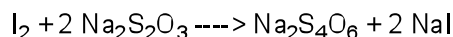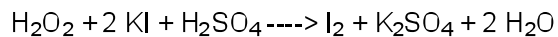
Hydrogen peroxide was titrated according to the procedure literature protocol and used as a positive control, before titrating peracids to determine its exact concentration.[31]

50 uL of H_2O_2 was taken from 30% hydrogen peroxide stock (500 mL).

It was titrated 3 times.

The titer value was 1. 11.2 mL. 2.11.0 mL 3. 11.2 mL

I took the average as 11.2 mL.

From the balanced equation it is quite clear that for 1 equivalent of H_2O_2, 2 equivalent of sodium thiosulfate is required. Even in terms of mole, for 1 moles of H_2O_2, 2 moles of thiosulfate is required.

The volume of thiosulfate required to titrate 50 uL of H_2O_2 is 11.2. The strength of the thiosulfate as written is 0.1 N.

Then, for 1 moles of H_2O_2, 2 moles of thiosulfate is required.

Then the mole of the $S_2O_3^{2-}$ required for titration = 11.2 x 0.1/ 1000

[Molarity and the normality of $Na_2S_2O_3.5H_2O$ is same]

So, the mole of H_2O_2 present in 50 μL sample = 1/2 * (11.2 x 0.1/ 1000)

The gm of the H_2O_2 present in 50 μL sample =1/2 * (11.2 x 0.1 / 1000) * 34

$$= 0.01904 \text{ gm}$$

So, the 500 mL bottle has = 190.4 gm

So, % of H_2O_2 in the bottle is = 38.08

Strength of the 500 mL bottle is = 190.4/34* 1000/500

$$= 11.2 \text{ M}$$

Peracetic acid was titrated according to the same procedure like above mentioned for the H_2O_2 titration.

Titer value of sodium thiosulfate for peracids: 1. 2.0 mL 2. 2.1 mL. 3. 2.0 mL.

The gm-equivalent of $S_2O_3^{2-}$ required for 100 µL of PA is = 2.01 x 0.1/ 1000 gm-equiv

So, the gm-equivalent of PA = 1/2* (2.01 x 0.1/1000) gm-equivalent

[Mol.wt of PA = 76.05 and the Equivalent wt of PA= 76.05].

So the gm of the PA present in 100 µL is = 1/2 * (2.01 x 0.1/ 1000) * 76

$$= 0.007638 \ gm$$

So, the amount of PA present in the 100 mL bottle = 7.643 gm

The % of PA in the bottle is 7.643 %.

So, the strength of the bottle is = 7.643/76.05* 1000/100

$$= 1.0049 \ M.$$

Other peracid like mCPBA and MMPP stock concentration was determined similarly.

Synthesis of 2-hydroperoxytetrahydrofuran: 2,3 dihydrofuran (5 g, 5.4 mL) was added drop wise for a period of 45 min to a 30 % H_2O_2 solution (11 g, 0.0964 mL) in presence of 0.02 mL conc. H_2SO_4 placed in a ice-bath (**Scheme7**). The stirring was continued for another 45 minutes at cold condition after the addition and made sure the temperature didn't rise above 10 ^{0}C.
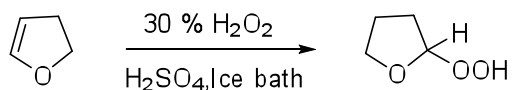
Scheme 7: Synthesis of 2-hydroperoxytetrahydrofuran.[40]

Then the reaction was quenched with NH_4Cl (solid) and extracted with CH_2Cl_2. After the organic layer was combined, it was extracted with 20 % NaOH and then combined organic layer was taken and the aqueous layer was adjusted to pH 7.2 (at cold) and then extracted with CH_2Cl_2 and then all the combined organic layer was washed with 10% $NaHCO_3$ (ice-cold) & then all the organic layer was combined and dried over Na_2SO_4 and rotary evaporated and dried at high vacuo to give oily compound; ^1H NMR (CDCl$_3$) δ 1.89 (m, 2, CH$_2$), 1.98 (m, 2, CH$_2$), 3.89 (m, 2, O-CH$_2$), 5.50 (m, 1, O-CH-OOH) and 9.85 (s,1, OOH); ^{13}C NMR (CDCl$_3$) δ 23.79 and 29.01 (CH$_2$'s), 67.57 (O-CH$_2$), 107.7 (O-CH-OOH) matches with literature. [40]

PTP1B inactivation by 2-hydroperoxytetrahydrofuran (THFOOH):
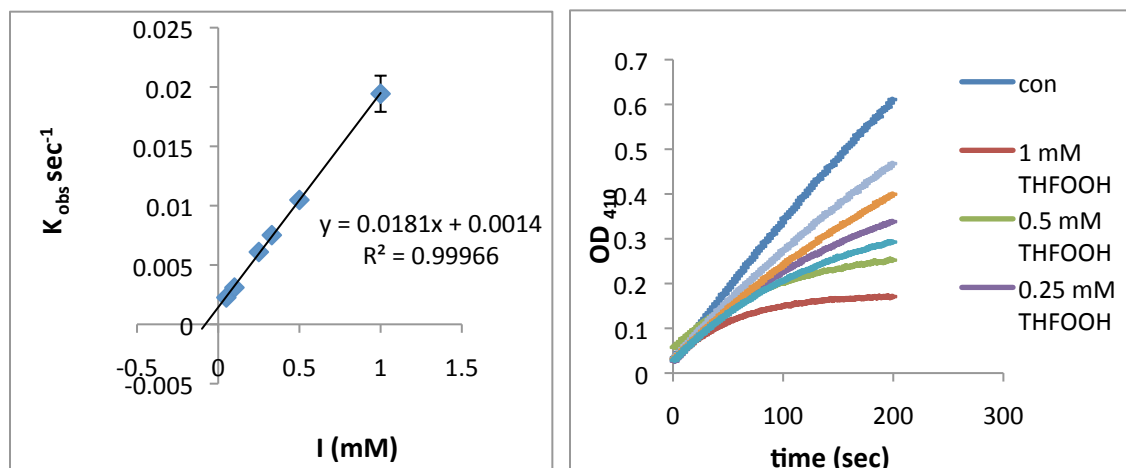
Figure 14: Rate of inactivation of PTP1B by compound **4**. Progress curves for the inactivation of PTP1B by **4**. Thiol-free samples of the enzyme were prepared by using through G25-Sephadex immediately prior to use according to reported procedures. The kinetic data was worked up as mentioned for peracetic acid.

Reactivation of 2-hydroperoxytetrahydrofuran (THFOOH) Inactivated PTP1b Using DTT as Reducing Agents:

The inactivation of PTP1B by THFOOH (50 μM) was carried out in exact similar way as described for reactivation of peracetic acid mediated inactivation. The 54 % of enzyme activity was recovered under identical experimental condition (**Figure 15**). The percentage activity was calculated from the slope of the line.
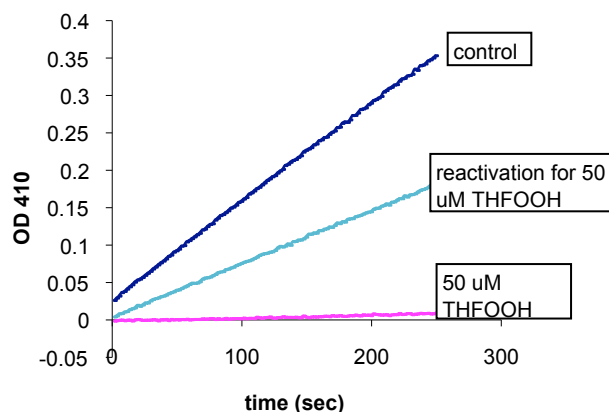
Figure 15: Thiol free PTP1b (0.5 µM) was incubated with 50 µM THFOOH, in a 100 µL assay reaction for 1 h, containing 50 mM 3,3-dimethyl glutarate buffer (pH 7.2), 5.0 % EtOH, in order to make sure the enzyme was almost completely inactivated. An aliquot of 15 µL was taken from the reaction mixture and was added to a cuvette p-nitrophenolate phosphate (pNPP) as a substrate 50 mM 3,3-dimethyl glutarate buffer (pH 7.2) at 410 nm in Uv-vis spectrophotometer. The enzyme was almost completely inactivated. Then the completely inactivated enzyme was treated with 100 mM DTT and incubated for another 1 hr in order to completely reactivate the enzyme. After an hour 15 µL of reaction mixture was taken and diluted into 1 mL solution of glutarate buffer (pH 7.2) having 20 mM *p*NPP as substrate and enzyme activity was monitored continuously at 410 nm like before and substantial enzyme activity was recovered (54%) by the treatment of DTT. A control was done similarly only in absence of THFOOH and treated with DTT as the inactivation was carried out. All the enzyme activity was measured from the slope of the reaction progress and calculated with respect to the control.

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THFOOH

1.716
1.718
1.726
1.729
1.743
1.745
1.750
1.754
1.759
1.761
1.763
1.768
1.770
1.773
1.775
1.787
1.792
1.855
1.857
1.866
1.872
1.875
1.883
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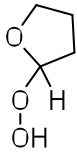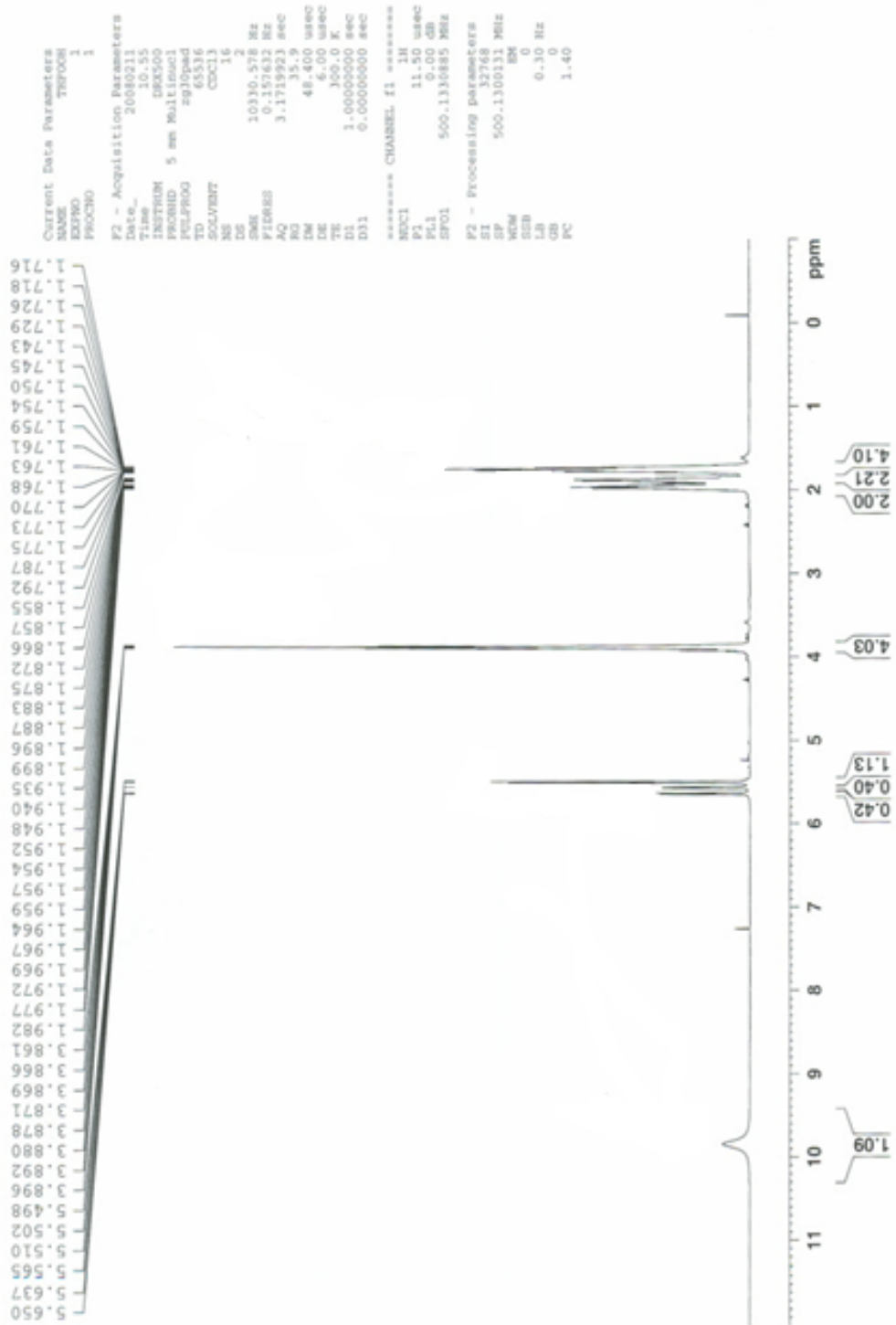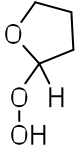
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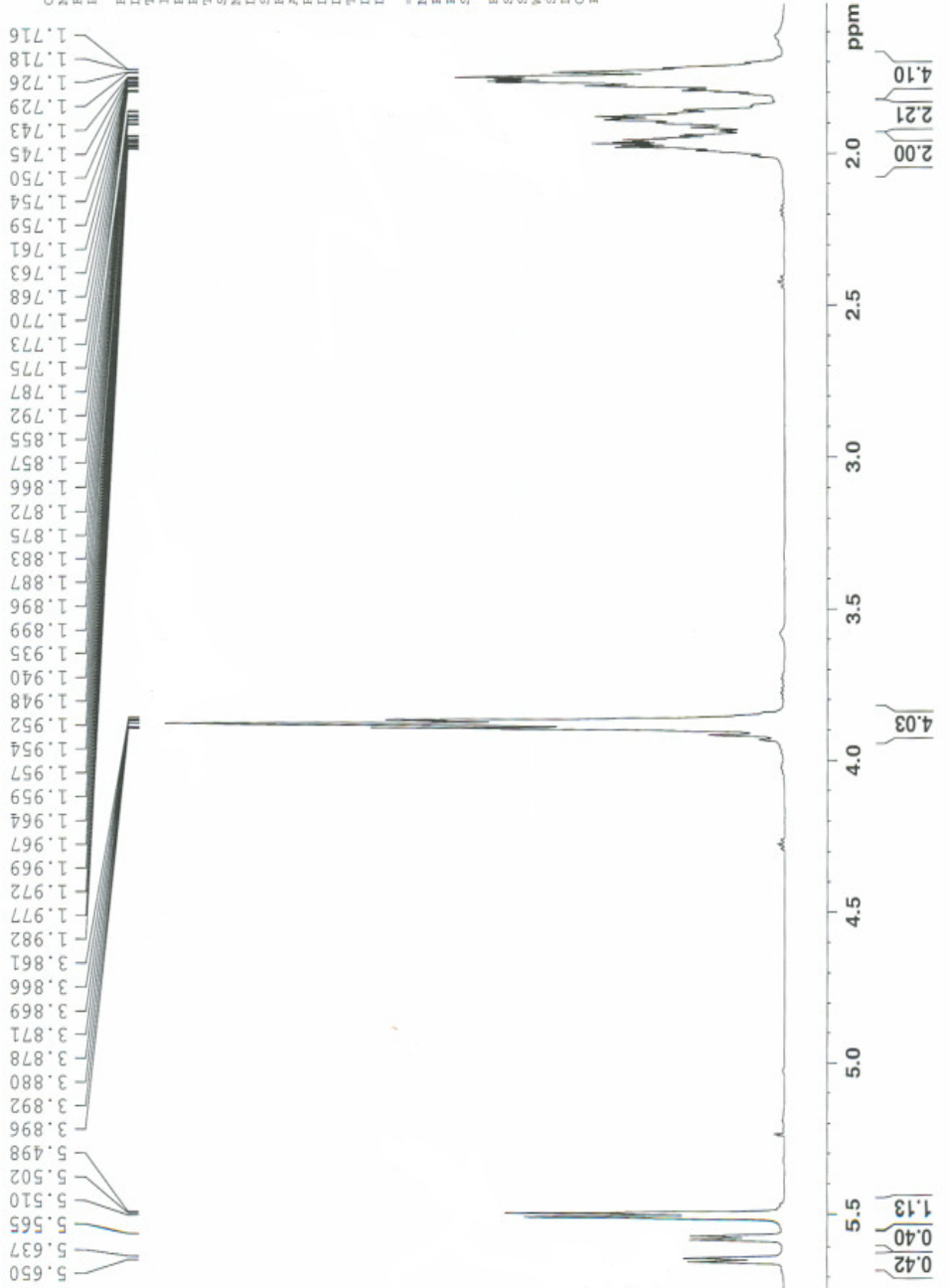
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23.79
29.01
29.29
29.32

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76.84
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77.35

106.14
107.42
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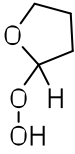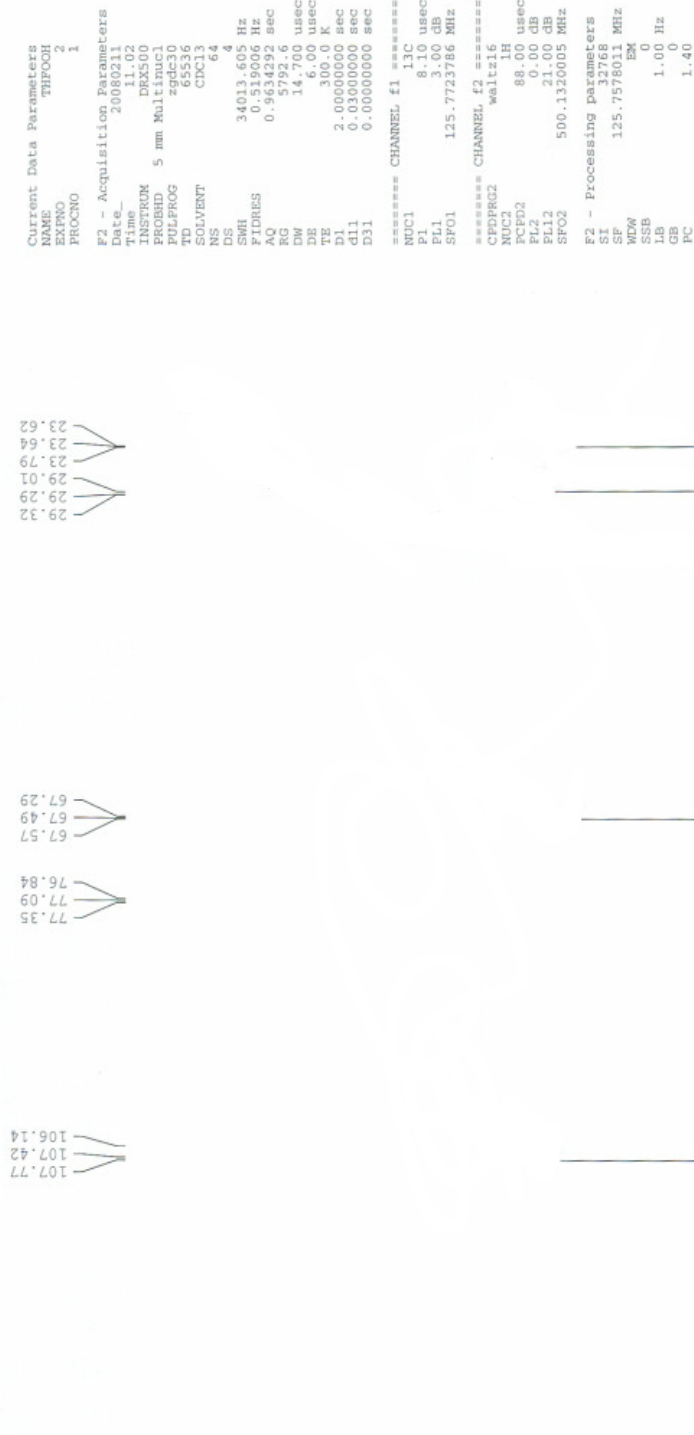
Chapter 3: Molecular basis of PTPs inhibition by oltipraz, a 3H-1,2-dithiol-3-thione compound and potent anticarcinogen: relevant to the activation of different biological pathways.

3.1. Introduction: Biological importance of 1,2-dithiol-3-thione compounds.

Phenyl isothiocyanate, sulphoraphane, retinoid and dithiolethiones exhibit the cancer preventive properties and are undergoing clinical trials to test their anticarcinogenic properties. [1, 2] Some of these compounds are found as a dietary component in cruciferous vegetables like broccoli and cauliflower. [3-5] Most of these anticarcinogenic compounds owe their activities to their inherently electrophilic character. [6-8]

4-methyl-5-(2-pyrazinyl)-
1,2-dithiol-3-thione (Oltipraz)   1,2-dithiol-3-thione   anithole dithione
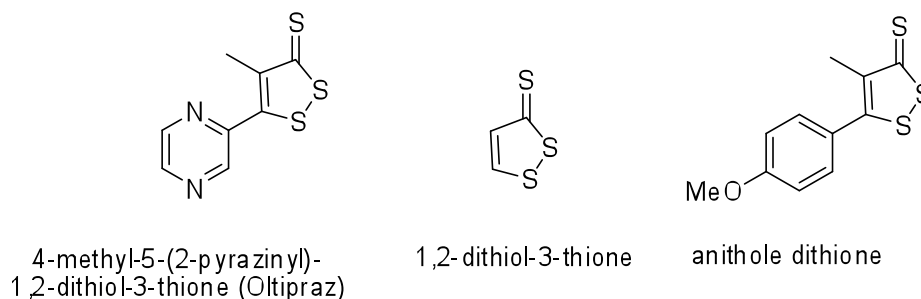
Figure 1: Structure of various dithiolethione compounds.

Oltipraz, (4-methyl-5-pyrazinyl-3H-1,2-dithion-3-thione) a synthetic analogue of 3H-1,2-dithion-3-thione is well documented for possessing cancer preventive properties and recently underwent clinical trial phase II (**Figure 1**),.[1, 9, 10] Several mechanisms have been proposed for the induction of the cellular protective enzyme by oltipraz.

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Oltipraz can rescue the cell from damage and detoxification of xenobiotics.[11] The protective enzymes induces by oltipraz includes NAD(P)H: (quinine acceptor) oxidoreductase (NQO1), epoxide hydrolase, glutathione S-transferase, UPD-glucuronosyl transferase, thioredoxin, peroxiredoxin etc.[11] The mechanism of oltipraz mediated cancer prevention is known to be triggered by the activation of Nre2-Keap1 transcription factor.[11] Keap1-Nrf2 pathways trigger the induction of house-keeping enzymes and cellular detoxifying enzyme.[11] Gene knock out experiments show that mice lacking Nrf2 genes are susceptible to carcinogenesis. Cytoprotective action of oltipraz was abrogated in Nrf2 silence mice and showed acute acetaminophen toxicity and higher amount of DNA adduct with carcinogen alfatoxin B1, benzo[a]pyrene.[11-17] Oltipraz treated mice caused augmentation of almost 292 gene levels by 24 h in wild type containing Nrf2 genes and most of them are expressed in mouse liver.[11] This is not seen in Nrf2 knockout mice and Nrf2-Keap1 double knockout mice.[11] Most of these cytoprotective enzyme, antioxidants, chaperone, immunity protein, cytokine, transport protein and detoxifying proteins are collectively known as phase 2 enzymes. Oltipraz ameliorated the toxicity of chemically induced carcinogenesis and that action was abrogated in Nrf2 knockout mice.[11]

It is proposed that Keap1 acts as a cytosolic repressor of the Nrf2 transcription factor which is sensitive to redox status of cell and cellular electrophilic and xenobiotic assaults.[14, 18, 19]

It is proposed that cysteine residues of the Keap1 interacts with oltipraz and causes translocation of Nrf2 to nucleus to trigger the expression of the mRNA and simultaneously the production of the antioxidant and cytoprotective enzymes (**Scheme 1**).[14, 18-21]
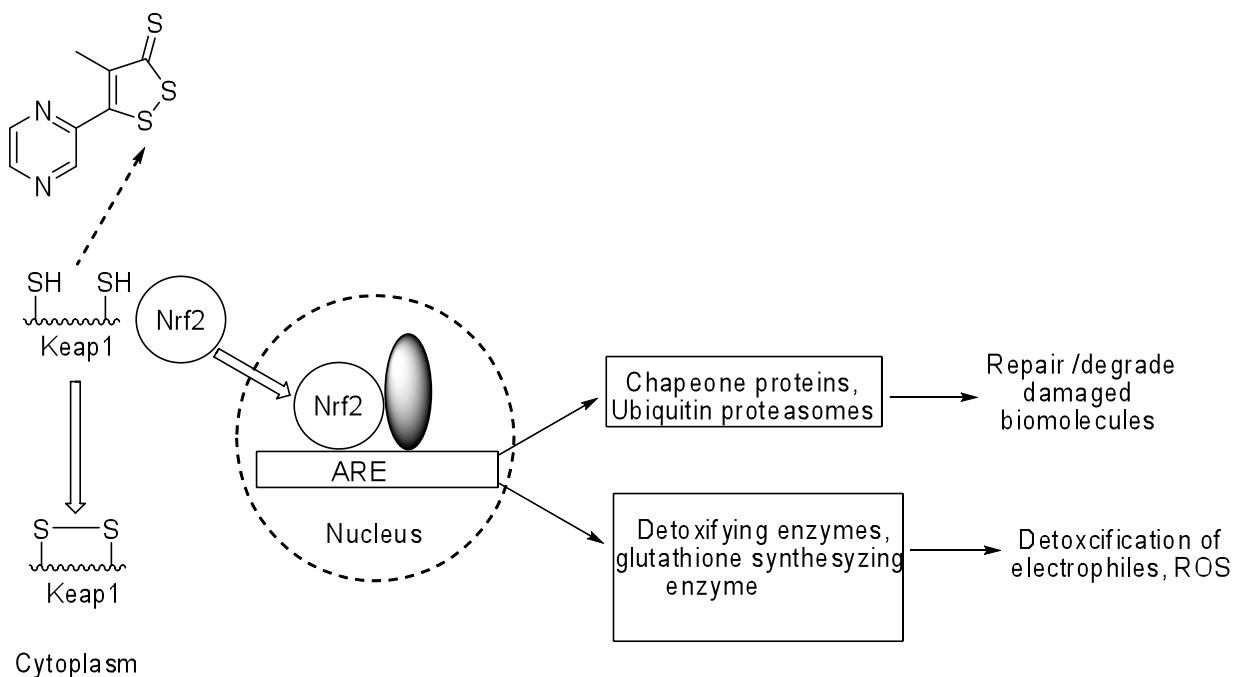
Scheme 1: Mechanism for induction of cellular detoxifying enzyme by nrf2-keap1pathways.[11] Keap1 protein act as a cytosolic suppressor of transcription factor nrf2. Oltipraz activate nrf2 by binding with keap-1 and subsequent translocation of nf2 into nucleus where it transcribe the gene for phase 2 enzyme.

This study was supported with the Keap1 and Keap1-Nrf2 double knockout mice. Elevated expression of these genes in wild type mice induced by oltipraz was not increased in double knockout mice.[11]

The knockout mice study clearly shows that the primary action of oltipraz mediated cancer prevention is Nrf2-Keap1 pathways and therefore a very important contributor of cellular longevity. [11]

The NF-κB (nuclear factor kappa B) is an important transcription factor, involved in pro-inflammatory and anti-apoptotic response for cell survival.[22] Nho reported the effect of oltipraz mediated expression of QR (quinine reductase) also involves the activation of NF-κB.[23-25] Oltipraz treatment of colon cancer cell HCT116 and HT29 reveals the induction of QR genes via NF-κB transcription pathways by activation of p65, IκB kinase (IKKα), IKKβ. [26, 27] The oltipraz treatment (100 µM) caused the tyrosine phosphorylation of inhibitory IKKβ of NF-κB transcription factor.[23] NF-κB exist as dimer (p50 and p65) and remains in inactive form in the cytoplasm by its repressor molecules IκBα, IκBβ.[28] Regulation of NF-κB signaling (upstream) by oltipraz treatment was induced through the tyrosine phosphorylation of IκBα, IκBβ of IKKα/β/γ complex and without proteasomal degradation of IκB.[29] A dominant mutant dnNIK (NIK is the suppressor of NF-κB) was used in HCT116 cell by transfection and significantly reduced the activation of NF-κB by oltipraz in a dose dependent manner.[23] The activation of QR promoter and mRNA transcription was strongly inhibited when an inactive NF-κB mutant was used.[23] This result clearly indicates the role of NF-κB for the expression of QR gene upon oltipraz treatment (100 µM) with colon cancer cells.

Manandhar has also reported the involvement of protein kinase pathways and phosphorylation level behind D3T mediated Nrf2 activation and regulation of the phase 2 enzyme production.[30,31-34] Extracellular receptor kinase (ERK1/2) have been found to be involved in the dithiolethione (D3T) stimulated production of NQO1in murine keratinocyte PE cells.[30] The ERK1/2 plays important role as an anti-apoptotic element under cellular stress condition and thus stress driven kinase could be involved in Nrf2 activation.[35] Use of ERK1/2 inhibitor decreased the expression of NQO1 triggered by D3T and severely attenuated the translocation of Nrf2 into nucleus.[30] Xu have shown that direct phosphorylation of Nrf2 by ERK1/2 helps the translocation of Nrf2 into nucleus.[36] This study establishes the function of ERK1/2 cascade in D3T treated murine keratinocytes and Nrf2 activation for cytoprotection against stress response and xenobiotic metabolism.

Besides the cancer preventive properties of oltipraz, other beneficial activities have been reported by Bae and co-workers.[37] The study shows that oltipraz can repair the insulin resistance caused by the hyperosmotic stress and restore the insulin signaling pathways via inhibition of S6K1 activation in HepG2 cell lines.[37] This result was evidenced by the use of dominant mutant of S6K1 which abrogated the impairing activity of the oltipraz in HepG2 transfected cell lines.[37] Serine phosphorylation of IRS-1 is a biomarker of hyperosmotic stress driven insulin resistance.[38, 39]

Oltipraz treatment eliminated the serine phosphorylation caused by hyperosmolarity and repaired the insulin signaling via tyrosine phosphorylation of IRS1.[37]

There is ample biological background showing oltipraz as cytoprotective agent in cellular context. Further research is required for identification of the specific upstream and downstream pathways involved in the oltipraz mediated cell signaling and protective action against cancer.

3.2. Metabolism of oltipraz and generation of ROS by oltipraz and its metabolite:

As oltipraz is undergoing clinical trial phase II, its metabolism has been studied extensively both in vitro and in vivo to better understand its molecular action.
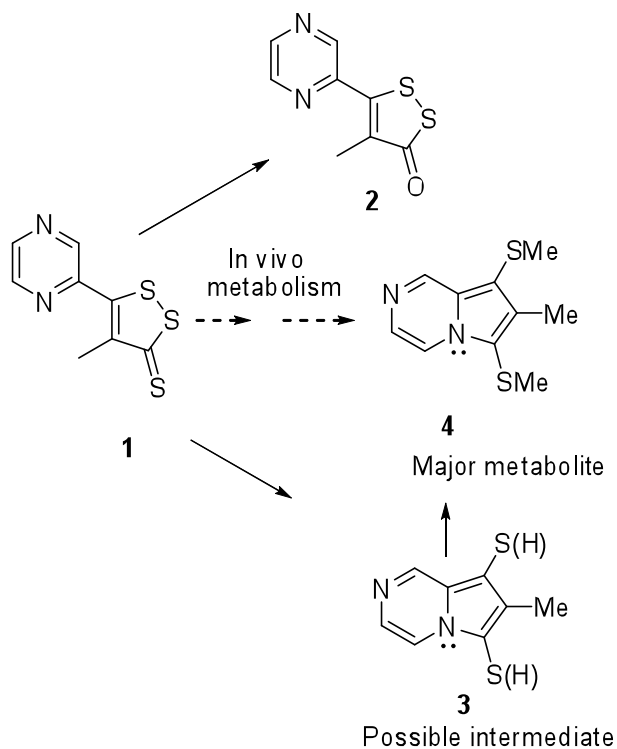
Scheme 2: *In vivo* metabolism of oltpraz.[40]

It is essential to know whether oltipraz itself or its metabolite can act as the reactive chemical probe and target the protein thiol and cause subsequent activation of transcription factor.
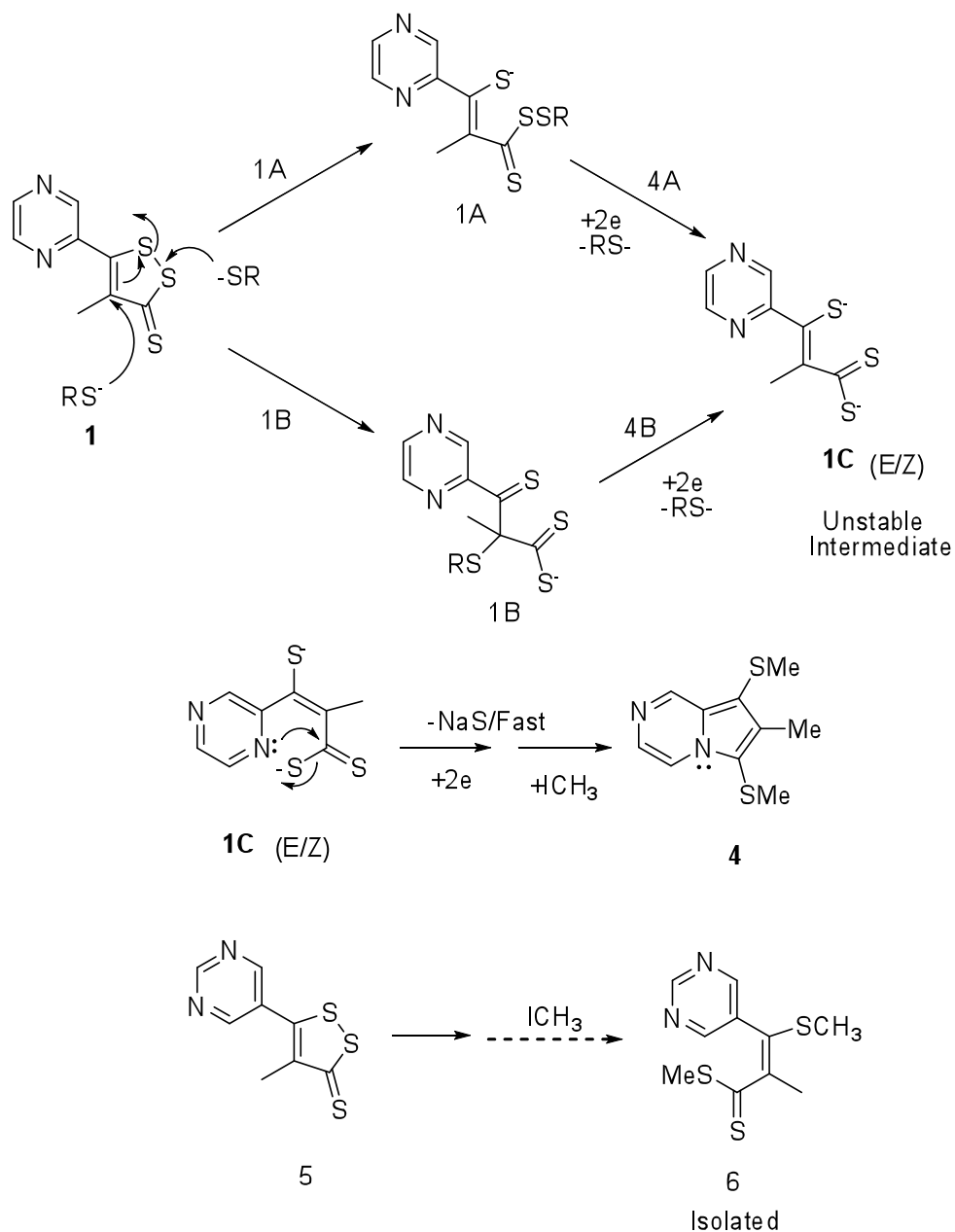
Scheme 3: *In vitro* metabolism of oltipraz.[41]

Metabolism study of oltipraz in humans reveal that the predominant product is compound **4** (pyrazine derivatives) and minor product (1 %) is its oxo analogue **2** (**Scheme 2**).[42, 43] When the pyrazine moiety of oltipraz was substituted with a phenyl of thiophene ring, the pharmacological activity of the compound was entirely lost.[41] Formation of compound **4** is hypothesized to be the outcome of biological methylation of **3**.[41, 44, 45] Fleury studied the *in vitro* metabolism of the oltipraz by GSH, cysteine, ethanethiol and alkoxide as nucleophiles.[41]
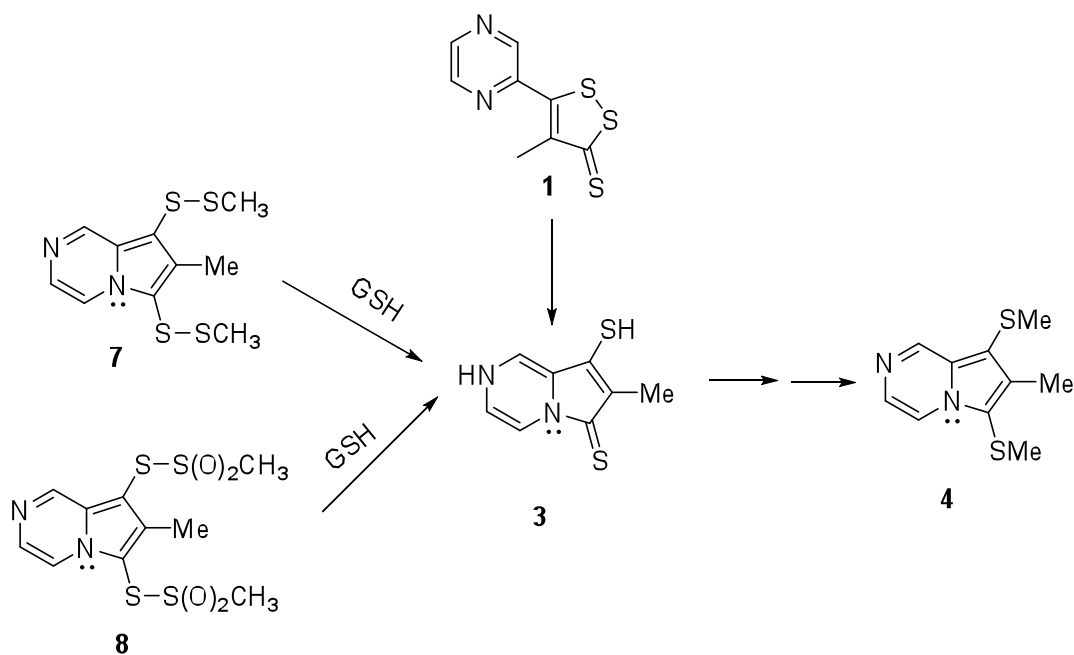
Scheme 4: Identification of a possible intermediate of oltipraz metabolite from a synthetic precursor.[44]

Their study shows that the dithiolethione undergoes nucleophilic attack by thiol ether at S-2 or C-4 position to yield a thione intermediate **1C** either by **1A** or **1B** pathways and **1C** could not be isolated due to its fast ring closure to compound **4** (**Scheme 3**). [41] To identify the intermediate, a similar reaction was carried out with an analogue of oltipraz, **5** (4-methyl-5-(5'-pyrimidinyl)-1,2-dithiol-3-thione). Under identical reaction condition, followed by treatment with methyl iodide, led to the formation of **6**, reinforcing the nucleophilic attack of thiol at C-4 or S-2 position (**Scheme 3**). It was proposed that the intramolecular ring closure yielded a transient cationic intermediate via Na_2S as leaving group and then subsequent reduction by excess thiol in the presence of methyl iodide produced the compound **4**.[41,42] Compound **4** did not cause phase II enzyme induction like oltipraz and its intermediate **3** does (**Scheme 3**). So the metabolite of oltipraz is also a biologically active compound.[44]

Fishbein has synthesized compound **7** (7-methyl-6,8-bis(methyl disulfanyl) pyrrolo[1,2-a]-pyrazine) and **8** (methane thiosulfonic acid S-((6-(methane sulfonyl)-7-methyl) pyrrlo [1,2-a]-pyrazin-8-yl) ester to study whether these compounds can act as possible precursors of oltipraz metabolized intermediate **3** to act as a phase 2 enzyme modulator.[40] They found that compounds **7** and **8** undergo facile reaction with cellular thiol glutathione at physiological condition to produce compound **3** which can be methylated to yield compound **3** which is a major metabolite of oltipraz (**Scheme 4**).

Recent study by Petzer clarify that intermediate **3** has comparable efficiency with oltipraz to trigger the phase 2 enzyme induction. [40, 46]

Kim reoprted that oltipraz and other 1,2-dithiol-3-thione compound causes DNA cleavage in presence of thiol and molecular oxygen by generating reactive oxygen radical.[47] Addition of metal chelating agent DETAPAC, hydroxyl radical scavenging agent mannitol and H_2O_2 destroying agent catalase considerably inhibited the DNA cleavage. This result clearly indicates the involvement of peroxy species as well as the trace metal catalyzed formation of reactive oxygen radical (by Fenton chemistry) cause DNA cleavage. DNA cleavage by oltipraz is also thiol dependent and requires the presence of oxygen. DNA cleavage is significantly reduced under anaerobic condition (degassed reaction mixture). This study suggests that oltipraz mediated DNA damage occurs through the generation of reactive oxygen species which is also known to induce gene expression inside the cell.[48-50]
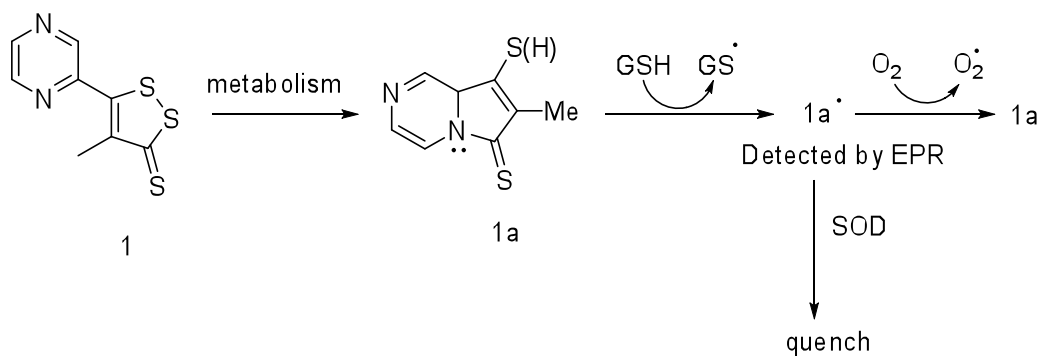
Scheme 5: ROS generation by the oltipraz metabolite.[51]

The relevance of ROS is very important in the light of PTPase as ROS can modulate the phosphatase function.[52] Murugesan reported that not only oltipraz but a metabolite of oltipraz is also capable of producing free radicals and thus could be very important in the light of oltipraz mediated chemoprevention.[51] Using the spin trap and DMPO they have shown that intermediate **3** from oltipraz metabolism can produce superoxide radical in presence of cellular thiol GSH at physiological pH and the addition of SOD quenched the formation DMPO-OH as evidenced by EPR.[51] The study by Murugesan et al shows that oltipraz and its metabolite could have the potential to activate the redox sensitive transcription factor and cytoprotective gene expression by generating ROS.[51]

3.3. Relevance of PTPs in the context cancer prevention:

Involvement of PTPase has been implicated in several cancer studies and increasing evidence strengthens the importance of PTPase as one of the key regulators in the cancer cell besides their classical role in insulin and leptin signaling. PTP1B has been isolated and characterized *in vitro* and *in vivo* as the major PTPs involved in c-Src activated cancer cell lines.[53] c-Src kinase is known to be hyperactive in several cancer cells and its activation is regulated by dephosphorylation of a specific tyrosine residue.[53-56] PTP1B have been found to activate c-Src by dephosphorylating the c-Src in breast cancer cell.[57-59]

Genetic mice models showed that PTP1B knockout mice or selective PTP1B inhibitor slows down the tumor-genesis.[60] PTP1B inhibition or PTP1B-/- mice lowered Akt phosphorylation which is highly active in several cancer cells and accelerated earlier apoptosis as evidenced by an increasing amount of caspase activation. [60] This result indicate that PTP1B possesses an oncogenic properties and thus it's inhibition in specific cancer cells can have the benefit as a novel therapeutic target for tumor.

Mannell showed that SHP-2 (src homologous phosphatase) inhibition triggers the anti-angiogenesis of mammalian endothelial cell lines (HUVEC) both *in vivo and in vitro*.[61] SHP-2 silencing triggered the decrease of blood vessel growth by augmenting endothelial apoptosis. FGF-2 (fibroblast growth factor) mediated angiogenesis occurs through SHP-2 regulated activation of ERK and deletion or inhibition of SHP-2 abrogated the FGF-2 dependent endothelial proliferation.[61] This indicates the central role of SHP-2 in FGF induced endothelial cell proliferation and could be an attractive target for the development of the therapeutics where blood vessel formation is undesirable such as tumor growth. Overall, inhibition of PTPase has the potential for multiple impacts in several diseases.

3.4. Hypothesis:

Various Biological events in the complex milieu of the cell need biochemical dissection in order to identify the exact molecular mechanism and pathways. This is true for dithiolethione and other chemopreventive agents in order to know the exact molecular basis of their cancer preventive properties. We have already seen that oltipraz activates the Nrf2 pathway by interacting with the active site cysteine residue of Keap1. We propose here that oltipraz can cause PTPase inhibition identical to other cys-dependent enzymes that could turn on various biological pathways. Oltipraz can activate the NF-κB by phosphorylating the tyrosine residue of the suppressor protein kinase IκB (IKK).[23, 29, 62] Imbert reported that PTPase inhibitor pervanadate led the activation of NF-κB by tyrosine phosphorylation of IκB-α.[62] Pervanadate caused the tyrosine phosphorylation of IκB-α, subsequent dissociation of the IκB-α/ NF-κB signalosome and activation of NF-κB.[22, 62, 63] Insulin and ROS can also activate the NF-κB transcription by tyrosine phosphorylation of IκB-α.[28]

This result makes a very clear connection. We know that insulin action is potentiated by ROS mediated PTP inhibition. This led us to hypothesize that oltipraz mediated PTPs inhibition might cause the tyrosine phosphorylation of IκB-α and thus subsequent activation of NF-κB (**Scheme 6**).
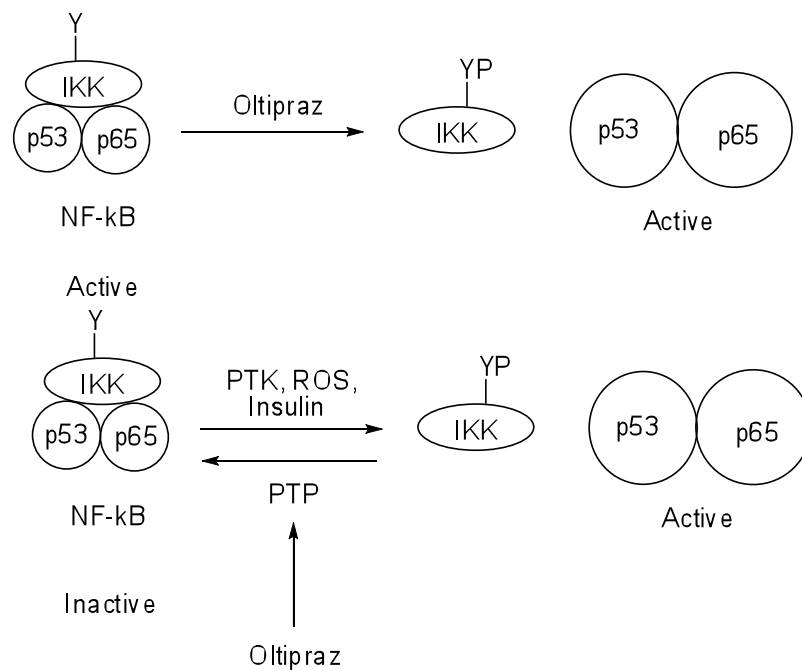
Scheme 6: Proposed mechanism of the Oltipraz mediated NF-κB activation pathways.[28] NF-κB remain as dimer in the cytosol, with two subunit p53 and p65 and IkB (IKK) kinase as cytosolic suppressor of NF-κB, undergoes activation via Tyr phosphorylation of IKK kinase subunit by endogenous insulin and ROS. Pervanadate mediated PTPase inhibition also causes the NF-κB activation.

Blockage of angiogenesis plays regulatory role in the control of tumor growth.[64-66] Oltipraz triggered anti-angiogenesis by inhibiting the fibroblast growth factor mediated endothelial cell proliferation and tumor progression in human and other animal models of the disease.[67] Administration of (0.4-100 μM) oltipraz completely prevented the capillary tube formation in human HUVEC cell lines.[67]

FGF-2

SHP-2

Substrate

Inhibition/
knockout

In tumor cell

Antiangiogenesis

Acitvation of MAKP pathway

Endothelial cell growth
and vessel formation
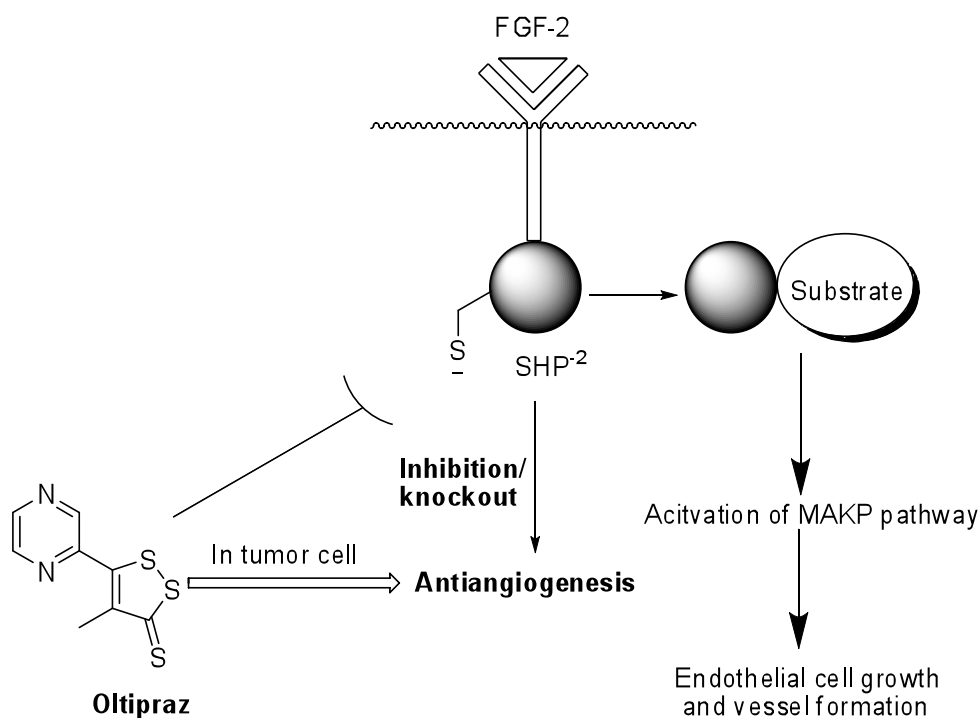
Oltipraz

Scheme 7: Proposed mechanism of oltipraz mediated anti-angiogenesis in tumor cell by SHP-2 inhibition.[61] Fibroblast growth factor (FGF-2) mediated SHP-2 (src homologous PTPs) dependent signaling in endothelial cell growth and vessel formation. SHP-2 activated by FGF-2 bind with PI3K and then subsequent binding with MAPKs led activation of Raf-ERK pathway to ultimately cause new vessel formation as biological response.

We have already discussed that SHP-2 inhibition or knockout promotes anti-angiogenesis and apoptosis of the endothelial cell. This event led us to hypothesize that possible mechanism of oltipraz mediated anti-angiogenesis might involve the SHP-2 inhibition by oltipraz (**Scheme 7**). Cell based assays are warranted to further confirm whether oltipraz can trigger anti-angiogenesis by reducing the PTPase activity or SHP-2 activity.

3.5. PTPs inactivation by 1,2-dithiol-3-thione compound oltipraz:

We have screened few 1,2-dithiol-3-thione compound (**Figure 1**) against protein tyrosine phosphatase to test our hypothesis and unveil the possible chemical basis of oltipraz mediated chemoprevention. We used PTP1B and SHP-2 tyrosine phosphatase for our study as they are referred as hierarchical members of their corresponding PTPase family. Oltipraz exhibited a time dependent inactivation of PTP1B in low micromolar concentration (**Figure 2**). The inactivation progress was in accordance with a process suggesting covalent modification of the enzyme. The inactivation data was fit to the equation for a first order reaction using Prism software to analyze the result (details described in **Experimental Section**).[68, 69]

The replot of the apparent inactivation rate versus oltipraz concentration yields a good linear fit, indicating the inactivation reaction is a second order process. The slope of the line gives the bimolecular rate constant for inactivation (k_{inact}=219.04 $\pm$ 18.75 M^{-1}s^{-1}).
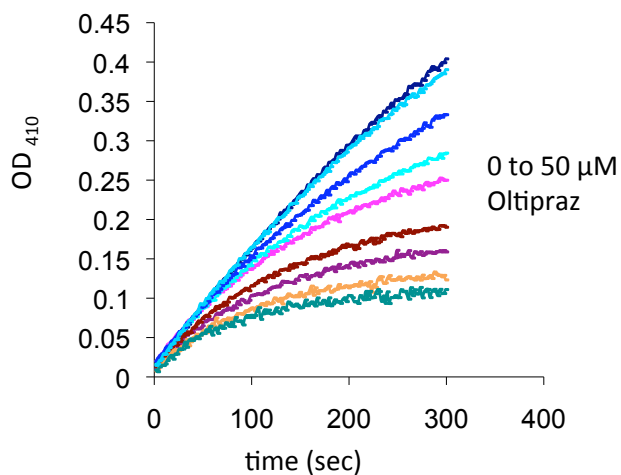
Figure 2: Progress curves for the inactivation of PTP1B by oltipraz. PTP1B was isolated as described previously and thiol-free samples of the enzyme were prepared by gel filtration of the protein through G-25 Sephadex, immediately prior to use. Thiol-free PTP1B (12.5 nM final) was added to a solution of oltipraz (0–50 µM) in 50 mM tris (pH 7.2), 50 mM bis-tris, 100 mM NaOAc 10 mM DTPAC buffer, 1.0 % DMF, containing the substrate (pNPP, 20 mM) at 23 ^{0}C. The reaction was monitored from the generation of p-nitrophenolate at 410 nm by UV-vis spectrophotometer.

Activity of the inactivated enzyme was not recovered upon gel filtration of the oltipraz-inactivated PTP1B. This result indicates a covalent binding between oltipraz and PTP1B. This inactivation was decreased substantially (26.5 % ± 4.9) at the presence phosphate (PO_4^{3-}), a known active site directed inhibitor of PTP1B (**Figure 3**). Incubation of PTP1B with oltipraz (50 µM) for 15 minute caused (80.3 ± 7.5) % inactivation of the enzyme compare to a control (only co-solvent DMF, no oltipraz) whereas in presence of PO_4^{3-} (50 mM) only (53.5 ± 4.9) % inactivation was noticed.

active site directed
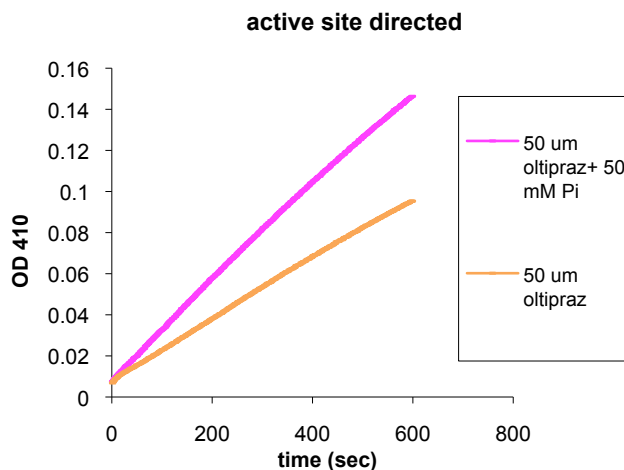
Figure 3: Thiol free PTP1B (0.5 µm) was incubated with 50 µM oltipraz for 15 min in 100 µL reaction volume, containing 50 mM tris, 50 mM bis-tris, 100 mM NaOAc and 10 mM DETPAC (buffer pH 7.2) at room temperature. Then 15 µL aliquot was taken from the reaction mixture and assayed to measure the phosphatase activity of the enzyme from the catalysis of pNPP at 405 nm as described earlier. The above reaction was also carried out in presence of 50 mM phosphate and after 15 min 15 µL aliquot was taken from the reaction and assayed from the remaining enzyme activity. A control was done having no oltipraz or phosphate, only co-solvent (5 % DMF) and enzyme activity was assayed in presence of 20 mM *p*NPP substrate in three component buffer. Oltipraz caused the loss of **75 %** enzyme activity whereas under identical experimental condition, **50 %** PTP1B activity loss was observed in presence of phosphate (50 mM). All the % remaining activity was calculated from the slope of the line with respect to the activity of the control. Abbreviation; P_i: phosphate.

This result clearly demonstrates that phosphate protects the active site of PTP1B from oltipraz and that oltipraz mediated inactivation is active site directed.

3.6. Thiol reversibility of the oltipraz mediated PTP1B modification:

We were interested to see whether modification of PTP1B by oltipraz is permanent or chemically reversible. Inactivation of PTP1B by oltipraz is chemically reversible by the treatment of DTT (100 mM) with inactivated enzyme for 1 h (**Figure 3A**).

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Reactivation was not observed by 1 mM GSH under identical condition (**Figure 3B)**. This result together with the active site directed experiment clearly suggests that oltipraz can target the active site of the PTP1B. That result led to us to study the chemical mechanism of the oltipraz mediated PTP1B inactivation.

6A.   **6B.**
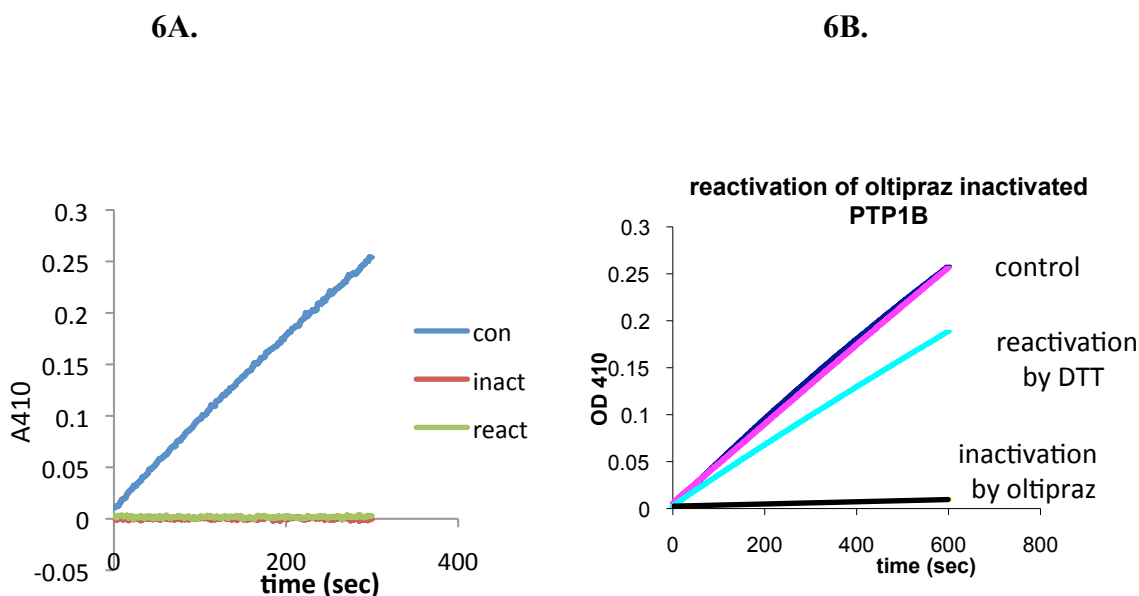
Figure 4: Reactivation of oltipraz Inactivated PTP1b Using DTT as Reducing Agents; **(6A)**Thiol free PTP1b (0.5 µM) was incubated with 50 µM oltipraz, in a 100 µL assay reaction for 1 hr, containing 50 mM tris (pH 7.2), 50 mM bis-tris, 100 mM NaOAc, 10 mM DTPAC, 5.0 % DMF. An aliquot of 15 µL was taken from the reaction mixture and was assayed with respect to 20 mM pNPP substrate in three component buffer (pH 7.2) at 410 nm by using Uv-vis. The enzyme was almost completely inactivated **(2.5 %)**. Then the completely inactivated enzyme was treated with 100 mM DTT and incubated for another 1 h, in order to completely reactivate the enzyme. After an hour 15 µL of reaction mixture was taken and diluted into 1 mL solution of 3-componenent buffer (pH 7.2) having 20 mM pNPP and enzyme activity was monitored continuously at 410 nm as mentioned before. Enzyme activity was found to be significantly recovered **(75%)** by the treatment of DTT. That result implicates the reversible modification of PTP1B function by oltipraz. A control was done similarly, in absence of oltipraz and treated with DTT as the inactivation was carried out. All the enzyme activity was measured from the slope of the reaction progress and calculated with respect to the control. **(6B)** Similar treatment of oltipraz mediated inactive PTP1B with 1.0 mM GSH could not return the enzyme activity. Abbreviation used; con: control, inact: inactivation, react: reactivation.

3.7. Mechanism of inactivation of PTP1B by oltipraz:

We have already seen that oltipraz and its metabolite have the potential to catalyze the formation of ROS. We have investigated whether oltipraz mediated PTP1B inactivation is ROS driven or that PTP1B undergoes chemical modification by oltipraz directly. We observed that addition of radical scavenging agent mannitol (1.0 mM) and catalase has no effect during PTP1B inactivation by oltipraz. This observation excludes the possibility of involvement of a Fenton-type process during inactivation. Thus, with the elimination of ROS contribution, the rationale for the direct covalent modification of PTP1B by oltipraz is reinforced (**Scheme 8**).

We were also interested to isolate and characterize the inactivated enzyme in order to precisely define the mechanism. From the proposed mechanism (**Scheme 8**), we can see that it is hard to identify the exact intermediate by using mass spectrometry as they both have identical mass. Thus our future goal is to explore the protein crystallography to determine the crystal structure of the inactivated PTP1B to reveal the exact attachment of oltipraz to PTP1B and thereby establish the mechanism of inactivation.
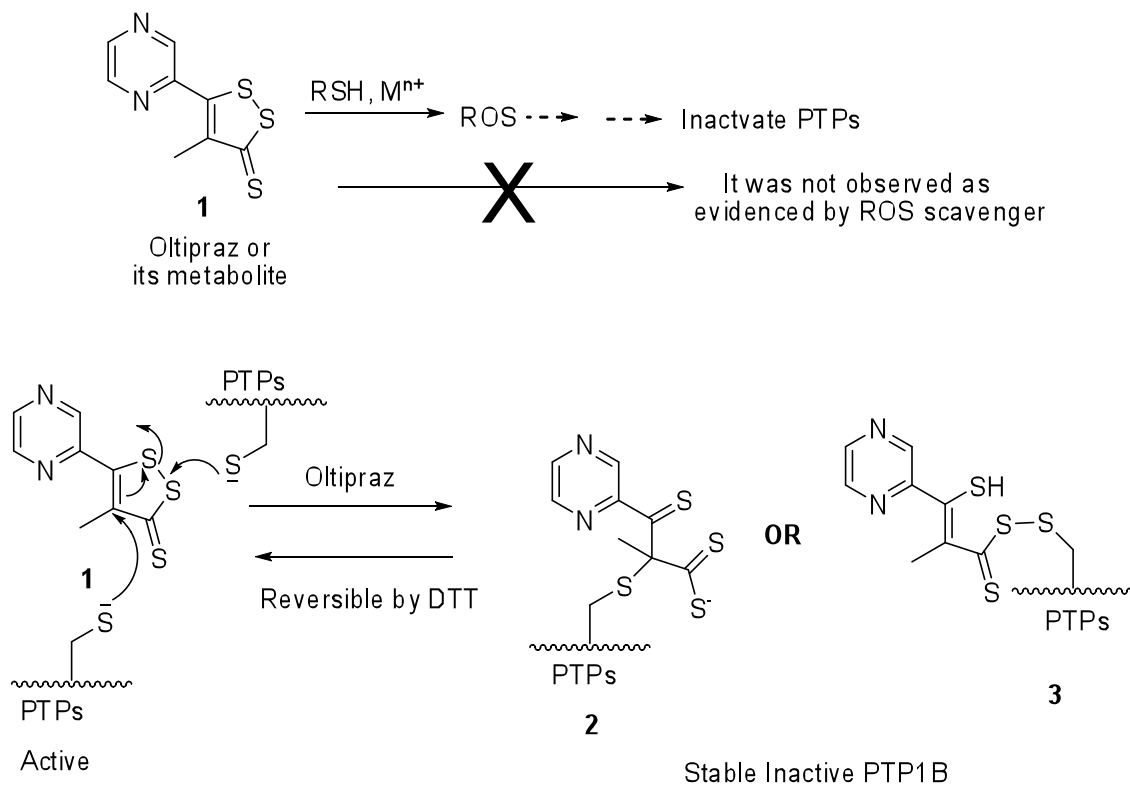
Scheme 8: Proposed mechanism of the PTPs inactivation by oltipraz.

3.8. Relative ability of PTP1B inactivation by various dithiolethione (D3T) compounds:

We have tested the relative ability of D3T to inactivate the enzyme. We have found that simple 1,2-dithiol-3-thione reacts strongly with PTP1B at rates comparable to oltipraz and anithole dithiolethione (**Figure 5A**).

7 A.
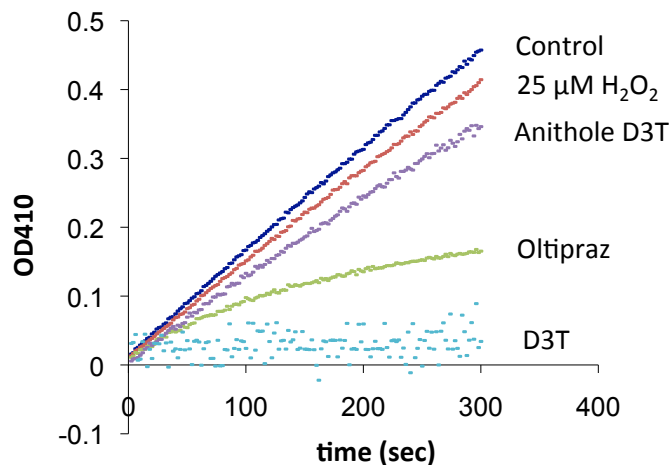
7 B.
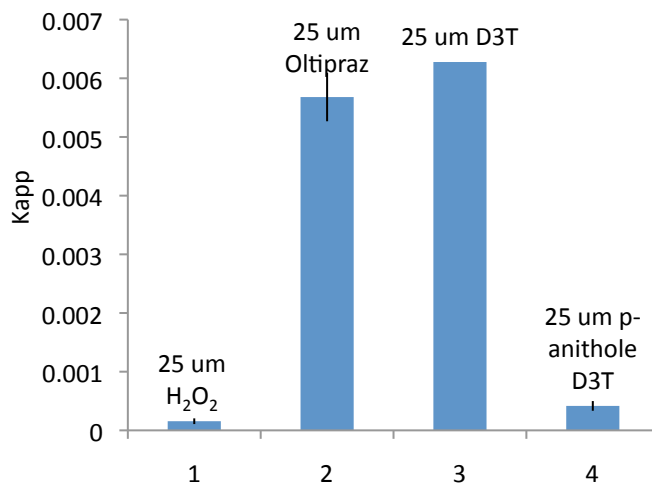
Figure 5: Relative inactivating ability of dithiolethione compounds towards PTP1B: **(5A)** Apparent rate of inactivation of PTP1B by compounds oltipraz, anithole, 2, 3 and H_2O_2 (figure 7). Thiol free PTP1B (12.5 nM final concentration) was added to a cuvette containing 20 mM pNPP substrate in 50 mM tris, 50 mM bis-tris, 100 mM NaOAc and 10 mM DTPA (buffer pH 7.2) and dithiolethione compound of interest at 23 °C. The reaction progress curve obtained as a function of time due to the release of p-nitrophenolate as a product of enzyme catalyzed reaction at 410 nm. The apparent rate of inactivation was obtained by non-linear fit. A control reaction was done without any in activator, having 1.0 % DMF as co-solvent.**(5B)** The k_{app} of inactivation for various dithiolethione was tabulated in a bar graph, representing the relative ability of dithiolethione for inactivation. The data were generated in triplicate (n=3). Abbreviation used;con: control, olt: oltipraz, D3T:3H-1,2-dithiol-3-thione.

Dithiolethione (25 µM) completely inactivated the enzyme possibly in microseconds and oltipraz (25 µM) almost significantly inactivated the enzyme towards complete loss of activity. In case of anithole dithiolethione (25 µM) considerable amount of enzyme activity was still observed. The result shows that oltipraz is a much more potent inactivator of PTP1B compare to H_2O_2 (positive control). Another set of continuous assay revealed that high concentration of H_2O_2 (1 mM) is required to almost completely inactivate the enzyme whereas only 50 µM oltipraz was sufficient to achieve identical apparent inactivation (**Figure 6**).
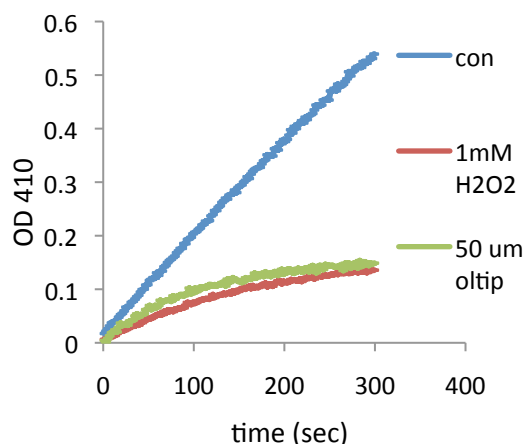
Figure 6: Comparative potential of oltipraz for PTP1B inactivation compare to positive control H_2O_2. Thiol free enzyme(12.5 nM final concentration) was added to a cuvette containing 20 mM *p*NPP substrate in 50 mM tris, 50 mM bis-tris, 100 mM NaOAc and 10 mM DTPA (buffer pH 7.2) and oltirpraz at 23 °C. The reaction progress curve obtained as a function of time due to the release of p-nitrophenolate as a product by enzyme catalyzed reaction at 410 nm. A control reaction was done similarly without any inactivator, having 1.0 % DMF as co-solvent. The data were generated in triplicate (n=3). Abbreviation; con: control, olt:oltipraz.

3.9. Does oltipraz inactivate other PTPase:

We know that most of the tyrosine phosphatase inhibitors are non-specific to their other family member due to their structural and functional similarity. We have screened oltipraz against SHP-2 (another member of PTPase family) and we have found that oltipraz shows time dependent inactivation of SHP-2 (**Figure 7**) and apparent rate of inactivation is comparable to PTP1B. We have discussed that SHP-2 plays central role in angiogenesis and oltipraz prevent tumor growth by causing anti-angiogenesis. So our work raises the possibility that oltipraz mediated SHP-2 inactivation might be involved as a possible mechanism for anti-angiogenic property of oltipraz in tumor cell (**Scheme 8**).
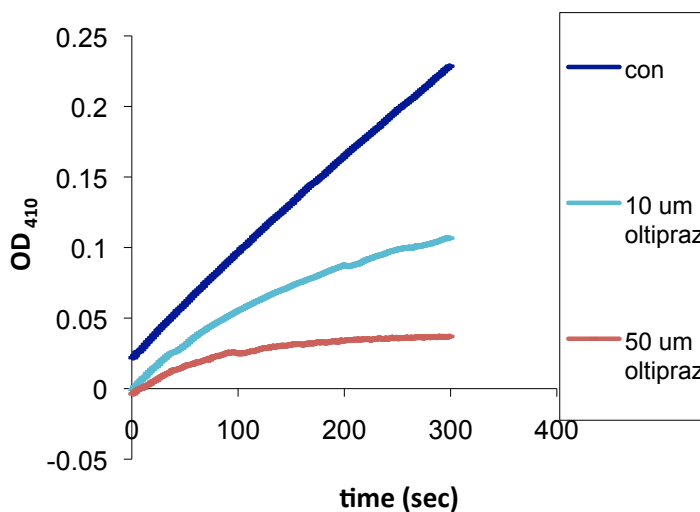
Figure 7: Progress curves for the inactivation of SHP-2 by oltipraz. Thiol-free SHP-2 (5 nM final) was added to a solution of oltipraz (0–50 μM) in 50 mM tris (pH 7.2), 50 mM bis-tris, 100 mM NaOAc, 10 mM DTPAC, 1.0 % DMF, containing the substrate (20 mM p-NPP) at 23 ^{0}C. The reaction was monitored by the formation of p-nitrophenolate at 410 nm by enzyme catalysis using UV-vis. Abbreviation; con: control, olt:oltipraz.

We have also observed that SHP-2 inactivation by oltipraz is completely reversible by the treatment of thiol (100 mM) DTT (**Figure 8**). After 30 min incubation of SHP-2 (0.5 μM) with 50 μM oltipraz, followed by the treatment of the completely inactivated SHP-2 with DTT (100 mM final concentration) for 1h, led to complete recovery of the enzyme activity.
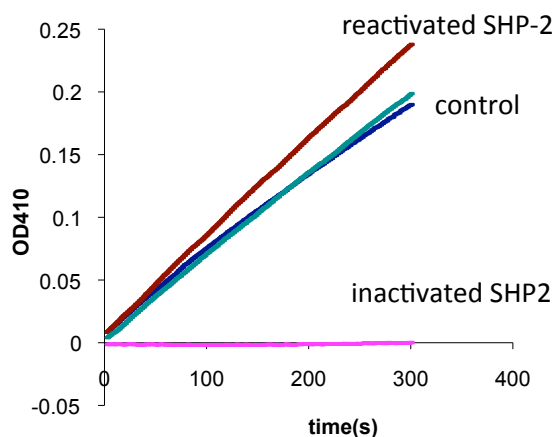
Figure 8: Reactivation of SHP-2 inactivated by oltipraz, Using DTT as reactivating Agents; Thiol free SHP2 (0.5 μM) was incubated with 50 μM oltipraz, in a 100 μL assay reaction containing 50 mM tris (pH 7.2), 50 mM bis-tris, 100 mM NaOAc, 10 mM DTPAC, 5.0 % DMF, for 1 h in order to make sure the enzyme was almost completely inactivated. An aliquot of 15 μL was taken from the reaction mixture and was assayed with respect to *p*-nitrophenol phosphate (20 mM pNPP) as a substrate in three component buffer (pH 7.2) at 410 nm by Uv-vis spectrophotometer. The enzyme was completely inactivated. Then the completely inactivated enzyme was treated with 100 mM DTT and incubated for another 1 h in order to completely reactivate the enzyme. After an hour of 15 μL of reaction mixture was taken and diluted into 1 mL solution of 3-componenent buffer (pH 7.2) having 20 mM *p*NPP as substrate and enzyme activity was monitored continuously at 410 nm like before and enzyme activity was found to almost completely recoverable by the treatment of DTT. A control was done similarly in absence of oltipraz and treated with DTT as the inactivation was carried out. All the enzyme activity was measured from the slope of the reaction progress and calculated with respect to the control.

Next we investigated whether oltipraz target the active site of SHP-2. We have used vanadate (VO_4^{3-}) as a known active site directed inhibitor to test whether vanadate can mask the active site of SHP-2 to protect from the oltipraz mediated inactivation. Incubation of SHP-2 (0.5 µM) with 10 µM oltipraz for 5 min caused 43 % inactivation of enzyme compare to a control (only DMF as co-solvent), whereas the presence of vanadate (7 mM) ion effectively shielded the inactivation of SHP-2, causing only 29 % loss of the SHP-2 activity (**Figure 9**). The above two line of experimental evidence implicate that oltipraz targets the active site of SHP-2 for covalent modification.
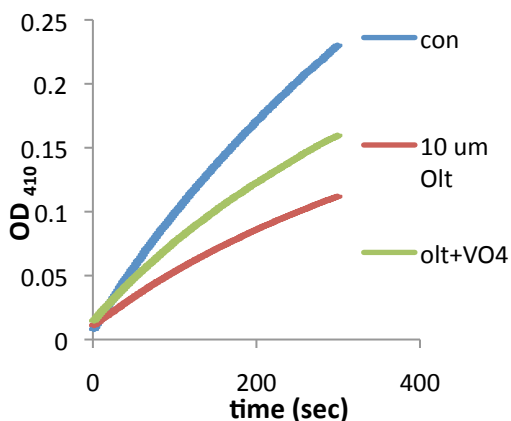
Figure 9: Thiol free SHP2 (0.5 µm) was incubated with 10 µM oltipraz for 5 min in 100 µL reaction volume, containing 50 mM tris, 50 mM bis-tris, 100 mM NaOAc and 10 mM DETPAC (buffer pH 7.2) at room temperature. Then 15 µL aliquot was taken from the reaction mixture after 5 min and assayed to measure the phosphatase activity of the enzyme from the catalysis of pNPP at 405 nm. Similarly another assay was carried out in presence of 7 mM vanadate and after 5 min 15 µL aliquot was withdrawn from the reaction and assayed for the remaining enzyme activity. A control was done having no oltipraz or vanadate, only co-solvent (5 % DMF). Oltipraz caused the **57 %** loss of enzyme activity whereas the presence of vanadate (7 mM) caused only **28 %** loss of SHP-2 activity. All the % remaining activity was calculated from the slope of the line with respect to the activity of the control. Abbreviation; con: control,olt: oltipraz, VO4: vanadate.

3.10. Potential of oltipraz metabolites to regulate the PTPase function:

We wanted to investigate whether metabolites of oltipraz also have the potential to modulate the PTPase activity. Because oltipraz metabolite also have been reported to be an inducer of the phase 2 protective enzyme. To test the hypothesis, we incubated PTP1B (0.5 µM) and SHP-2 (0.5 µM) with 100 µM and 50 µM oltipraz respectively, in presence of 1:1 GSH:GSSG (1 mM GSH and GSSG each) in 50 mM tris, 50 mM bis-tris, 100 mM NaOAc and 10 mM DETPAC (pH 7.2) buffer. After 30 min, an aliquot of 15 µL was withdrawn from the reaction mixture and added to a cuvette containing 20 mM substrate in three components buffer (pH 7.2) to measure the phosphatase activity. We found that oltipraz mediated PTP1B inactivation is abrogated in presence of GSH, whereas inactivation is potent in case of SHP-2 (**Figure 10A for PTP1B and 10B for SHP-2**). We used 1:1 mixture of GSH: GSSG in the assay in order to mimic the physiological condition.

We also pre-incubated oltipraz (50 µM) with 1 mM (GSH: GSSG) for 1 day, in order to give sufficient time to generate the active metabolite of oltipraz. We have found that oltipraz metabolite generated by GSH and GSSH mixture, cause significant inactivation of PTP1B (**Figure 11A**) and SHP-2 (**Figure 11B**). The survival of oltipraz in presence of GSH during SHP-2 inactivation is not quite surprising to us.

10 A:
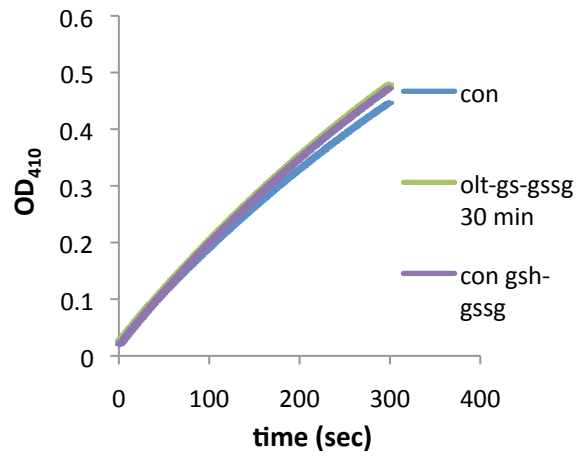
10B:
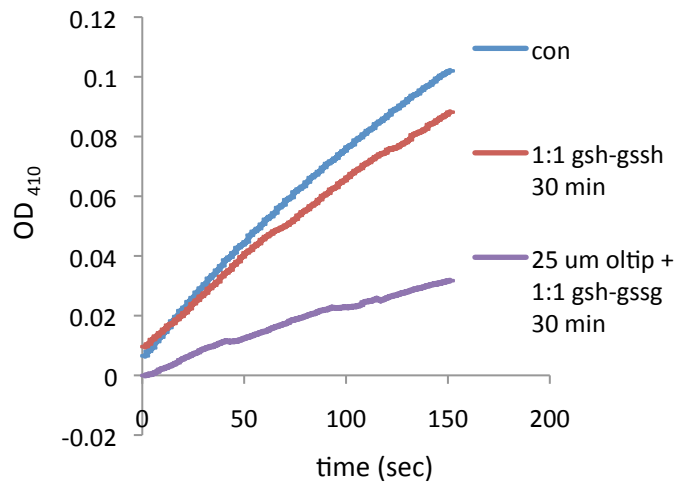
Figure 10 : PTP1B (0.5 μM) and SHP-2 (0.5 μM) was incubated with 1:1 GSH-GSSG (1 mM each) in presence of 100 μM and 50 μM oltipraz respectively. After certain 30 min, an aliquot of 15 μL was taken from the reaction mixture and added to the cuvette containing 20 mM PNPP in 3 component buffer (pH 7.2) with 10 mM DETAPAC and phophatase activity was measured as described before. Identical control was done in resence of 1:1 GSH:GSSG and with DMF and % remaining enzyme acivity was measuerd from the slope of the line compare to control. Oltipraz mediated inactivation of PTP1B was completely abrogated in presence of GSH-GSSG mixture. But under similar assay condition, SHP-2 showed potent inactivation of oltipraz in presence GSH-GSSG mixture. Abreviation; con:control, olt, oltipraz.

11A.
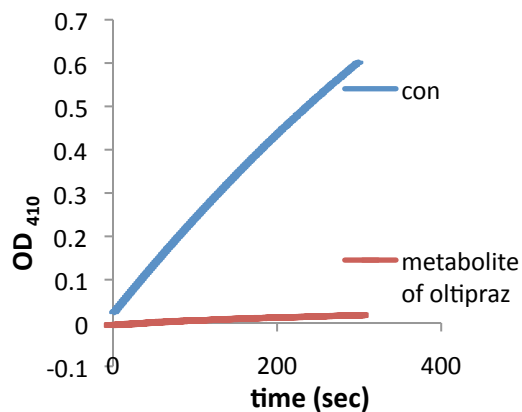
11B.
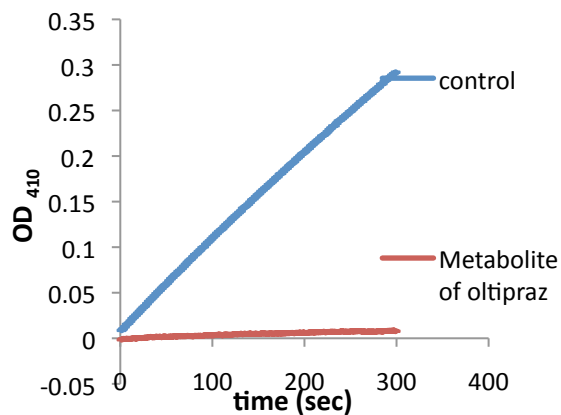
Figure 11: Oltipraz (100 µM) was pre-incubated with 1:1 GSH-GSSG (1 mM each) in presence of 100 µM oltipraz for 1 day. After 1 day, an aliquot of 30 µL was taken from the reaction mixture and added to 30 µL of PTP1B (5 µM final concetration) and SHP-2 (1.5 µM concentration). After 20 inute incubation, an aliquot of 15 µL was withdrawn from the reaction mixture and added to a cuvette containing 20 mM pNPP in 3 component buffer, with 10 mM DTPAC (pH 7.2) and phophatase activity was measured as described before. A control was done with DMF only and % remaining enzyme acivity was measuerd from the slope of the line compare to control. Abreviation; con:control, olt:olipraz.

Even SHP-2 is a member of PTPase family like PTP1B, it differs in many ways to PTP1B due to its unique structural alignment of the cysteine thiol in its active site.[70] Studies by Chen and co-workers reveal that H_2O_2 mediated modification of SHP-2 ultimately forms a disulfide link without affecting the active site thiol.[70] This result shows that SHP-2 behaves a bit differently than PTP1B.This result clearly explain the strutal differences and uniquenes of SHP-2 compared to PTP1B as well as the survival of oltipraz inside the cell and reenforce the potent biological activtiy of oltipraz in presence cellulalr GSH. Our work suggests that oltipraz metabolites irreversibly inactivate SHP-2 in the presence of GSH.

3.11. Conclusion:

Our *in vitro* study confirms that oltipraz can potentially modulate the PTPase function in low micromolar concentration. Oltipraz mediated enzyme modification is chemically reversible and active site directed in nature. Oltipraz metabolite also has the potential to regulate the PTPase function. Our mechanism study shows the biochemical basis of oltipraz mediated activation of NF-κB by PTPase inhibition. Cell based assays could confirm our *in vitro* studies and check whether oltipraz can reduce the PTPase activity and enhance tyrosine phosphorylation inside the cell during NF-κB activation. Our mechanism study also indicates that oltipraz mediated SHP-2 inhibition might be involved during anti-angiogenesis and endothelial apoptosis.

Our work can explain the possible molecular mechanism for oltipraz mediated anti-angiogenesis in tumor cell via SHP-2 inhibition. Biological assay is warranted to examine whether antiangiogenic effect of dithiolethiones correlate with decrease cellular activity of SHP-2.

Experimental Section:

Materials and methods: All the other chemicals and reagents of highest grade were purchased from Sigma, Fischer Chemicals Company and the dilute stock of inhibitors were prepared freshly before the assay from the concentrated stock. Dithiothreitol (DTT), 2-mercaptoehtanol (βME), glutathione disulfide (GSSG), sodium vanadate, mannitol, G-25 sephadex bid, dimethyl formamide (DMF), diethylenetriamine pentaacetic acid (DETAPAC), 2-(bis(2-hydroxyethyl) amino)-2-(hydroxymethyl) propane-1,3-diol (bis-tris), sodium acetate, tris(hydroxymethyl) aminomethane (tris), substrate 4-nitrophenylphosphate (pNPP) and glutathione (GSH) were purchased from Sigma-Aldrich Company and oltipraz, 1,2-dithiol-3-thione compound were synthesized as described.[47]

Inactivation Assay of Protein Tyrosine Phosphatase1B by Oltipraz: Inactivation of PTP1B by oltipraz was measured by the decrease of phosphatase activity against the phosphatase substrate p-nitrophenyl phosphate (*p*NPP). PTP1B inactivation assays were monitored from the generation of product p-nitrophenolate at 405 nm in a UV-vis spectrophotometer. Typically, all the assay reactions (1.0 mL) had 50 mM tris (pH 7.2), 50 mM bis-tris, 100 mM NaOAc 10 mM DTPAC, 1.0 % DMF, 20 mM pNPP substrate, different concentration of oltipraz (0- 50 μM) and (0.01-0.05) μM PTP1B.

PTP1B stock was (16 µM) allowed to pass through a G-25 sephadex spin column to remove the βME thiol agents during inactivation assay.

Thiol free enzyme (0.01 µM final concentrations) was added as a last component to the reaction mixture to initiate the reaction and the reaction was monitored continuously by a UV-Vis spectrophotometer at 405 nm. The apparent rate of inactivation was measured from the early regions of the reaction progress curve by fitting the data to Prism for non-linear regression analysis. All the assays were performed at room temperature in absence of any reducing agents and all the inhibitor stocks of oltipraz were prepared in DMF. Kinetics of PTP1b inactivation was measured according to the method described by Pei and co-workers).[68, 69] The absorbance (A_{410}) vs. time (t) data were fitted against equation

$$A_{410} = v_0(1-e^{-kt})/k$$

using software Prism, a nonlinear regression where v_0 is the initial reaction rate and k is the apparent inactivation constant. This method is based on a system which considers enzyme, inhibitor as well as substrate. Substrate concentration is kept constant throughout the assay and inhibitor concentration is varied. A secondary plot of k_{app} vs. I was fit against the following equation to give a line with slope of $k_{inact}/(1+[S]/K_m)$

$$K_{obs} = k_{inact} [I]_0 /(1+[S]/K_m).$$
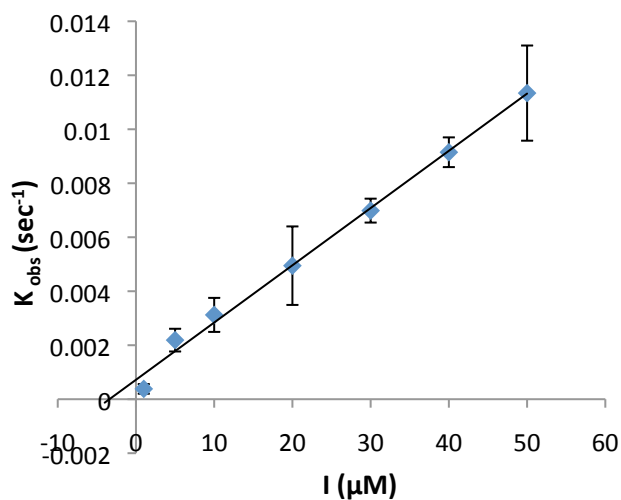
Figure 12: The apparent rate of inactivation (k_{obs}) for each concentration was calculated by the method as mentioned above. The apparent rate of PTP1B inactivation by oltipraz was plotted with inhibitory concentration of oltipraz (μM) and the second-order rate constant for inactivation was extracted from slope of the line ($k_{inact}= 219.04 \pm 18.75 \ M^{-1}s^{-1}$). The data were generated in triplicate (n=3) and standard deviation (SD) were calculated and plotted in excel.

Is oltipraz mediated inactivation ROS driven?

To check whether ROS could be involved during oltipraz mediated inactivation of PTP1B, we have used ROS destroying agents during inactivation assay. PTP1B inactivation was carried out in presence catalase and oxygen radical scavenger mannitol (1.0 mM) and monitored in a continuous fashion (**Figure 13**). Addition of catalase and mannitol has no effect upon the oltipraz mediated PTP1B inactivation.

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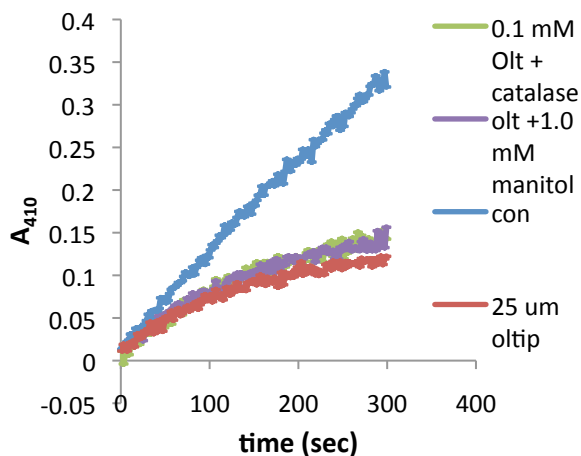
Figure 13: Thiol free enzyme (12.5 nM final concentration) was added to a cuvette containing 20 mM pNPP substrate in 50 mM tris, 50 mM bis-tris, 100 mM NaOAc and 10 mM DTPA (buffer pH 7.2) and 25 µM of oltipraz at 23 ^{0}C. The reaction progress curve obtained as a function of time due to the release of p-nitrophenolate by enzyme catalyzed reaction at 410 nm. Similar inactivation assay was carried out in presence of radical scavenger mannitol (1.0 mM), H_2O_2 destroying agent catalase. A control reaction was done similarly without any inactivator, having 1.0 % DMF as co-solvent. Abbreviation; con: control, olt: oltipraz,

That rules out the involvement of ROS during PTP1B inactivation by oltipraz. Oltipraz is also known to catalyze the formation of ROS in presence of thiol. Next we attempted to see whether addition of thiol has any impact during PTP1B inactivation by oltipraz. PTP1B was incubated with oltipraz in four different sets of reaction, containing (0.5 µM) enzyme, oltipraz (100 µM) and GSH (1.0 mM), catalase, 1.0 mM mannitol, in 50 mM 3,3-dimethyl glutarate buffer (pH 7.2).

An aliquot of 15 µL was drawn from the reaction mixture after 15 min and added solution containing 20 mM pNPP in 50 mM 3,3-dimethyl glutarate buffer (pH 7.2) to monitor the residual enzyme activity. The residual enzyme activities were compared in each case, with respect to a control which clearly shows that ROS has no participation during oltipraz mediated modification of PTP1B activity (**Figure 14**).
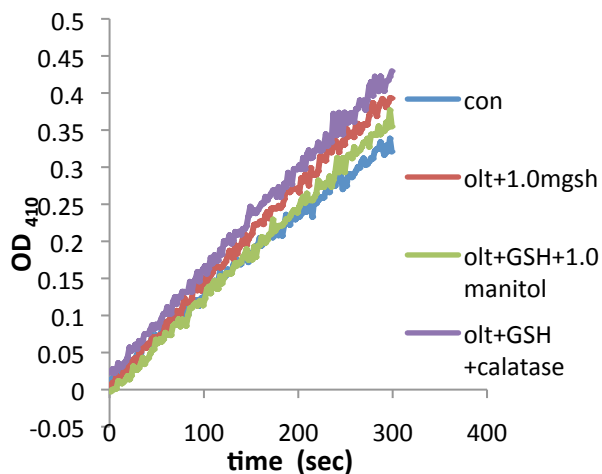
Figure 14: PTP1B (0.5 µM) was incubated with oltipraz (100 µM) and 1.0 m GSH for 15 min, in presence of catalase, radical scavenging agent mannitol (1.0 mM) in 50 mM 3,3-dimethyl glutarate buffer (pH 7.0), in separate reaction. After 15 min, an aliquot of 15 µL reaction mixture was taken and added to a cuvette of 1.0 mL reaction volume containing 20 mM pNPP substrate in of 50 mM 3,3-dimethyl glutarate assay buffer (pH 7.0) at 23 ^{0}C. The result shows that addition of catalase and mannitol (1.0 mM) in presence of exogenous thiol (1.0 mM GSH) has no effect on oltipraz mediated inactivation of PTP1B. Abbreviation; con: control,olt: oltipraz, GSH: glutathione.

Gel Filtration of the oltipraz inactivated PTP1B:
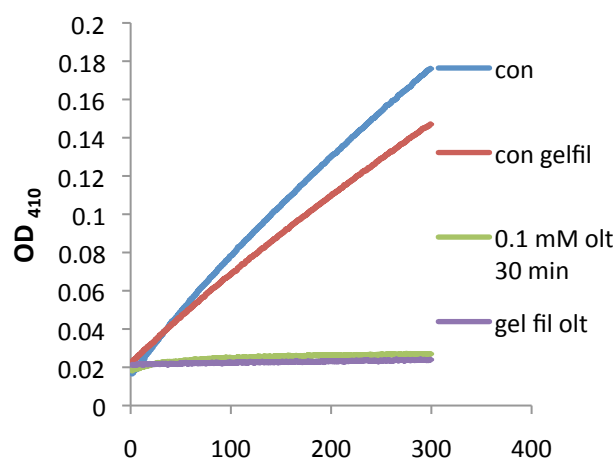
Figure 15: PTP1B (0.5 µM) was incubated with 100 µM oltipraz for 30 minutes in three component buffer (pH 7.2) and 10 mM DTPA (final reaction volume 100 µL). An aliquot of 15 µL was taken from the reaction mixture after 30 minute and assayed for the measurement of phosphatase activity. PTP1B was found to be completely inactivated. Remaining solution (85 µL) was gel filtered through G-25 spin column in order to free the residual amount oltipraz. The spin column was exchanged with 3 component buffers containing 10 mM DTPA (pH 7.2), freshly added NP-40 as non-ionic detergent. After spin column, a 15 µL aliquot from the filtrate was withdrawn and added to a 1 mL solution of three component buffer, containing 20 mM pNPP and PTPase activity was measured at 405 nm as mentioned before. No significant return of PTP1B activity was observed compare to a control done in an identical manner without addition of any oltipraz. Abbreviation; con:control, olt: oltipraz, gel: gel filtration.

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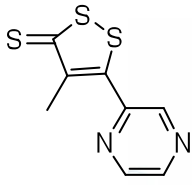
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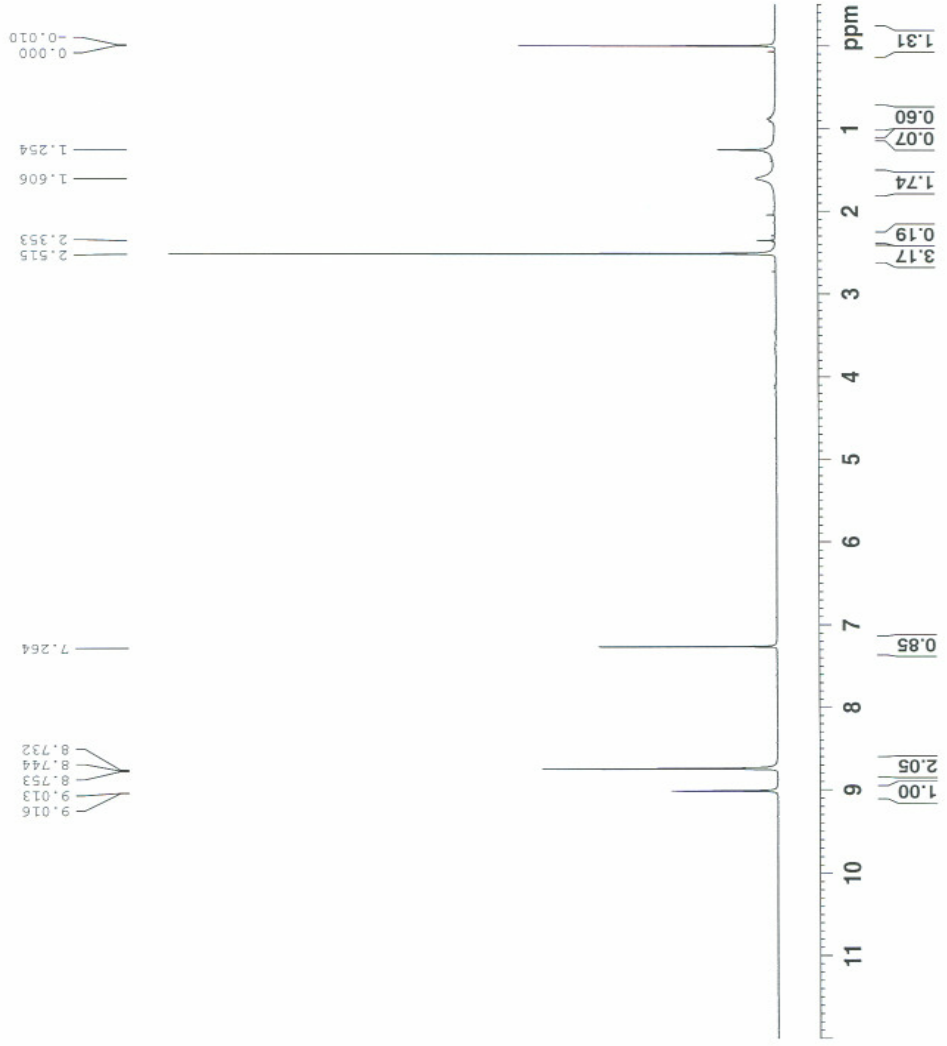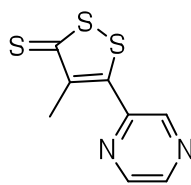
108

1,2-dithiol-3-thione

3H-1,2-dithilinone-3-thione pure stnadard

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Integral

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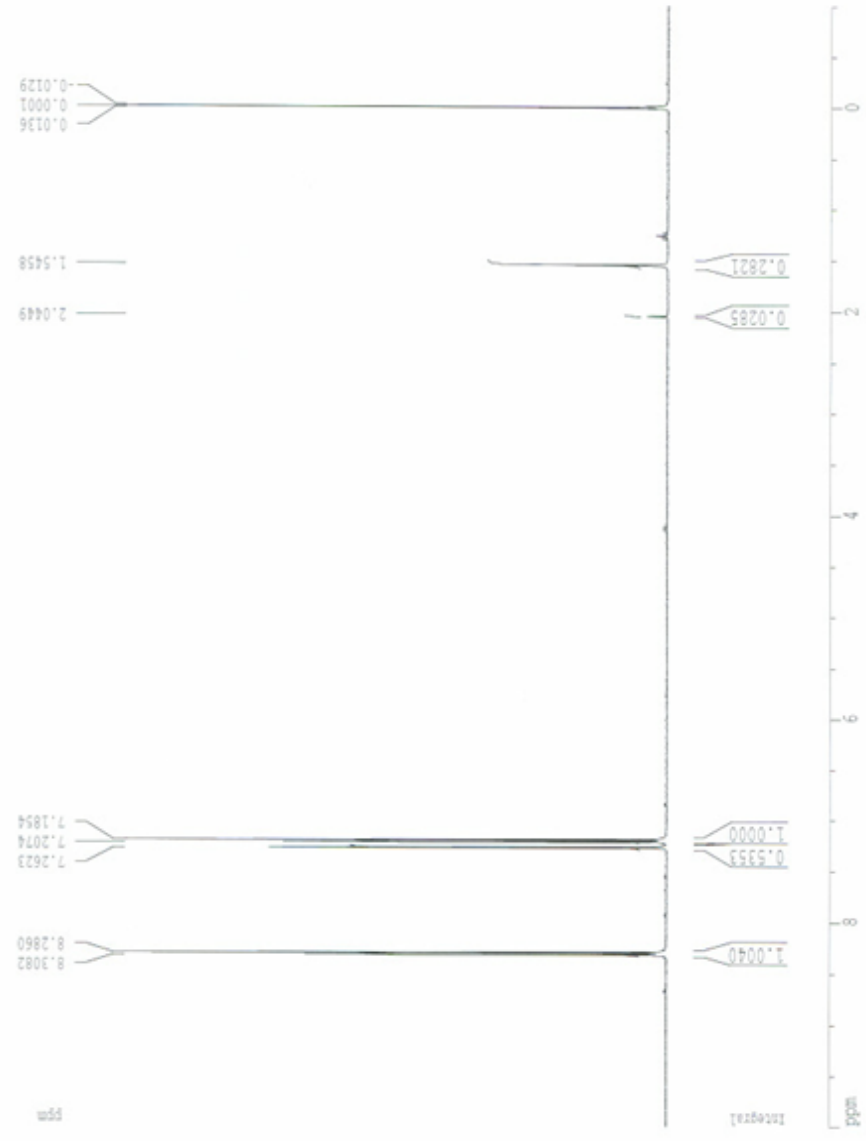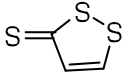
1,2-dithiol-3-thione

3H-1,2-dithilinone-3-thione pure standard

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Chapter 4: Impact of Hydrogen sulfide signaling and its metabolism upon PTPase function, a Preliminary study.

4.1. Introduction: Different source of Hydrogen sulfide.

Hydrogen sulfide is long known for more than 300 years as a foul smelling gas like rotten egg which was discovered by Scheele in 1777.[1] Volcanic eruption and spring water contain a significant amount of hydrogen sulfide.[2,3] H_2S is also found in sea water, pond, muddy and rudimentary area of the lake.[2,4] HS^- is susceptible to chemical oxidation and neutral or alkaline conditions facilitate the generation of thiosulfate ($S_2O_3^{2-}$), sulfate (SO_4^{2-}) and sulfur (S_0).[5] The SO_4^{2-} and S_2^- concentration in seawater is approximately 28 mM and 10 mM respectively, a reservoir of this two highly water soluble sulfur components.[5-7] Hydrogen sulfide is easily oxidized by natural oxidant like oxygen, hydrogen peroxide in normal as well as sea water to sulfate, thiosulfate and molecular sulfur. Several mineral are deposited in the mines as sulfide, like iron pyrite (FeS_2) which on acidification releases H_2S.[6] Hydrogen sulfide can equilibrate between three forms in a pH-dependent manner: $H_2S \leftrightarrow HS^- + H^+ \leftrightarrow S^{2-} + H^+$ having a pK_a value of 6.9 for the first step and 17 for the second step.[7,8] Under physiological condition, the ratio of H_2S and HS- concentration is approximately 30% and 70%, respectively, whereas the sulfide (S^{2-}) concentration is insignificant.[7]

Biological oxidation of H_2S predominantly produces the elemental form of sulfur and polysulfur. Beside the available natural source, several microorganisms are known to produce H_2S in their respective biosynthetic pathways.[5] In the nature there are numerous sulfur fixing bacteria which carry out different aerobic and anaerobic reactions to balance the sulfur cycle in nature.[9-11]
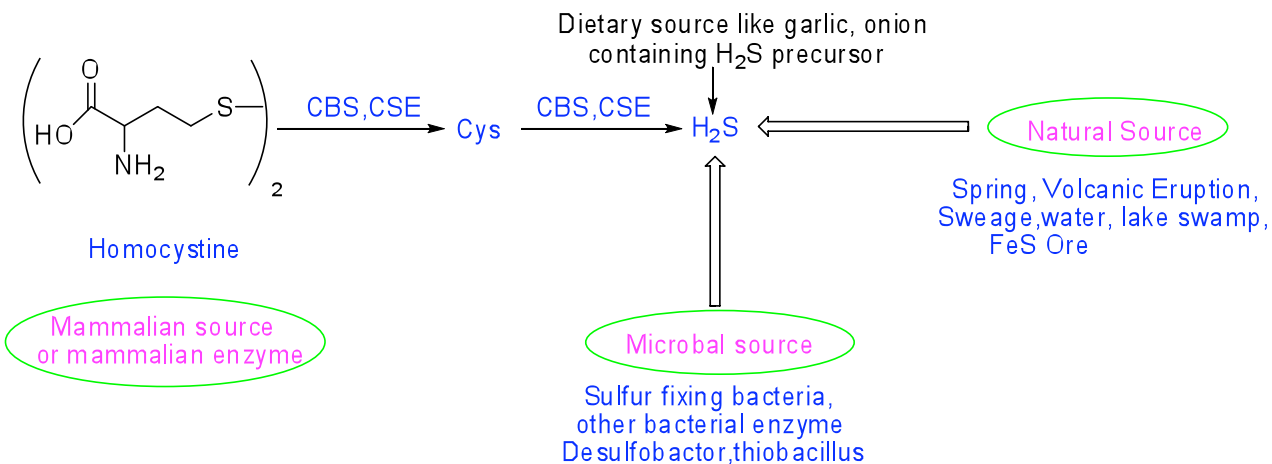
Scheme 1: Different source of H_2S, starting from natural resource to biological media.[5,12,13] CBS, CSE is cystathionine β-synthase and cystathionine-γ-lyase enzyme, can produce H_2S endogenously in the mammalian cell. Dietary garlic and onion contain poly sulfide and thiol component which can act as a precursor of H_2S production. H_2S is also found in hot spring water, volcanic eruption, ore and sewage area. Several microbes can produces this compound in various biosynthetic pathways.

Bacterial H_2S is produced from the desulfurization of sulfur rich amino acids like cysteine and methionine, sulfated polysaccharides and sulfolipids.[5] Microbes plays a significant role in the oxidation of H_2S to S_0 and SO_4^{2-} in wastewater management.[5] In mammalian system, various enzymes like cystathionine β-synthase (CBS), cystathionine γ-lyase, mercaptopyruvate sulfurtransferase (MST) and cysteine lyase catalyze the endogenous formation of H_2S by the desulfuration of cysteine (**Scheme 1**).[14-19] Most of this enzyme metabolizes cysteine and homo cysteine to produce H_2S. Starting from the nature, the existence and generation of H_2S in non-living and living system points the possibility of its presence as a very important component during prebiotic condition where it played an important substantial role in early stage of evaluation.[20]

4.2. Dual role of hydrogen sulfide (H_2S): a toxicant (hazard) during cell damage and as a potent therapeutics (beneficiary) in various cellular diseases:

For a long time, hydrogen sulfide has been viewed as toxic agent in the perspective of environment, food industry, public nuisance, sewage treatment system, etc. Inhalation of H_2S in low amount is known to trigger headache, dizziness, nausea, nasal congestion, respiratory irritation, inflammation, olfactory hyposmia, headache, pulmonary edema, corneal inflammation, respiratory paralysis, toxicity to olfactory system and even death.[21-23,22,24-27]

Several biofilter are used to optimize the hazards of H_2S generated in the industrial process.[28] H_2S toxicity involves the direct inhibition of cytochrome c oxidase, inhibition of aerobic metabolism with ATP depletion and lactic acid accumulation and tissue oxygen demand.[26,27,29] High solubility of H_2S in aqueous and lipid media enables it to diffuse through cell easily and is therefore readily oxidized. Here we will discuss about the positive sides of H_2S which so far remain unexplored. Given the further insight in research we can use this H_2S for our own benefit.

Beside mammals, hydrogen sulfide signaling is also known in low microorganisms like yeast.[1] In fermentation technology, H_2S is continuously produced from thiol containing amino acids.[30] But recent study shows the potential of H_2S as a new emerging gaseous signaling molecule and also its role as gasotransmitters like NO, CO.[13,31-34] H_2S signaling does not involve any receptor mediation or cGMP dependent pathways like NO and don't transit message between neurons.[34-36] Hydrogen sulfide is an important modulator in cardiovascular (CV) homeostasis and many of its other molecular targets inside the cell are not well known.[31,32] Hydrogen sulfide triggered vasorelaxation occurs via activation of K^+ (K_{ATP}) ion channel [31,34]

H_2S can also interact with metal dependent heme protein by binding to it as an axial ligand, thereby upregulating anti-inflammatory gene like heme oxygenase, other gene like vascular endothelial growth factor (VGEF), insulin like growth factor (IGF), several other receptor pathways and cause posttranslational modification of protein, reduction of disulfide bond.[12,37-39]

114
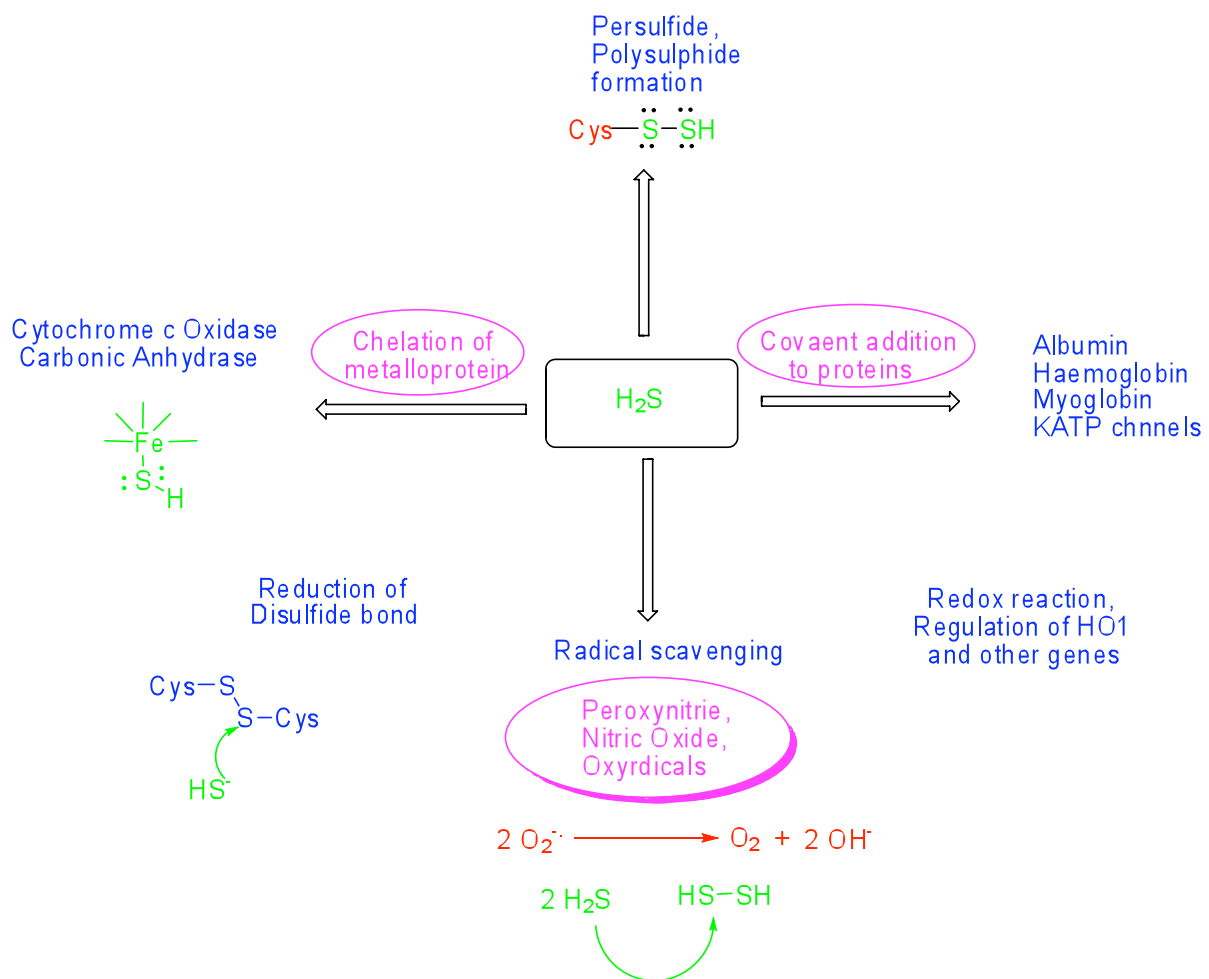
Scheme 2: The various biological effects of H_2S.[12] H_2S can act as antioxidant and ROS scavenging agent against NO, HOCl, HNO_2^- It can act as a reducing agent and maintain the redox status of cell. H_2S can covalently add to hemoglobin, K_{ATP} ion channel, albumin as a nucleophile and cause inhibition of cyt c oxidase enzyme by adding as an axial ligand to the metal centre. It can also regulate the gene expression of haem oxygenase 1 (HO1). Under oxidative condition, it can form hydrogen persulfide (H_2S_2) formation and persufide formation.

Cytoprotection and antioxidant properties of sulfide are greatly attributed to its behavior as redox controlling molecule like cysteine and glutathione in order to neutralize different ROS like peroxynitrite, HOCl and radicals (**Scheme 2**).[40,41] Hydrogen sulfide is produced in different mammalian cells and tissue types ranging from low micromolar to high micromolar concentrations. Dietary garlic which is a rich source of organic polysulfide is known to alleviate CV disease and reduce blood pressure.[42] Benavides has shown that organic polysulfides from garlic are converted to H_2S by red blood cell, thereby playing a significant role in vasculoprotection.[32]

Kimura and colleagues reported that hydrogen sulfide protect the neuron cells from oxidative damage by increasing the levels of the natural antioxidant, glutathione.[12,36] Intravenous injection of H_2S ameliorated the anti inflammatory drug (like ibuprofen) mediated gastrointestinal (GI) bleeding and ulcers.[36] Administration of H_2S (20 ppm to 60 ppm) in heart revived the suspended blood flow that occurred due to shock and inflammation.[36] Recently H_2S has drawn significant attention to scientist to use it for the patient as a therapy.

H_2S can create "hibernation like" state which causes a huge decrease in metabolite demand, less susceptibility to oxygen deprivation, lesser blood loss, lower heart beat and other stress.[36,43-47] This property can be exploited for the treatment of patients to survive longer from severe injuries and trauma by putting them in a hibernated like state.[36]

Mice administered with H_2S at low oxygen concentration survived for several hours compare to a control mouse (without H_2S administration) which survived only 20 minutes under oxygen tension.[12,13,36,46-48] It is therefore evident that H_2S can lower the oxygen demand and prevent the adverse effects happen due to hypoxic condition. Efforts are ongoing to use H_2S donors in different cases of animal model for the treatment of various aspects of cellular disease.[49-51]

4.3. Metabolism and oxidation of hydrogen sulfide and generation of reactive oxygen species:

H_2S metabolism is well studied both *in vivo* and *in vitro*. Major metabolites of H_2S are thiolsulfate ($S_2O_3^{2-}$), sulfur (S_0), ploysulfides (S_n), sulfate (SO_4^{2-}) and sulfite (SO_3^{2-}) (**Scheme 3**).[21,22,52,53] H_2S metabolites are relatively less toxic compared to NO which forms a toxic metabolite peroxynitrite. Sulfide concentrations were found to be different in various diseases. For example, plasma sulfide concentration dropped from 50 µM to 25 µM in coronary heart disease whereas in hemorrhagic shock plasma sulfide concentration increased from 30 µM to 40 µM.[54,55] Pharmacokinetic and metabolic studies were conducted by pre-exposing H_2S on human being.[56] Dose of 500 -1000 ppm were found to be fatal by immediate knock down of consciousness and does of 200 to 500 ppm resulted in loss of mobility due to gradual loss of consciousness.[57]

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H2S $\xrightarrow{\text{S}_0\text{, ploysulfides}}$ Thiosulfate $S_2O_3^{2-}$ → Sulfite SO_3^{2-}

($H_2S \leftrightarrow HS^-$)

TSR

SO

NADPH
2 GSH
GSSG
NADP+

CH_3SH

TSMT

CH_3SCH_3

Sulphate SO_4^{2-}

Urine, Blood
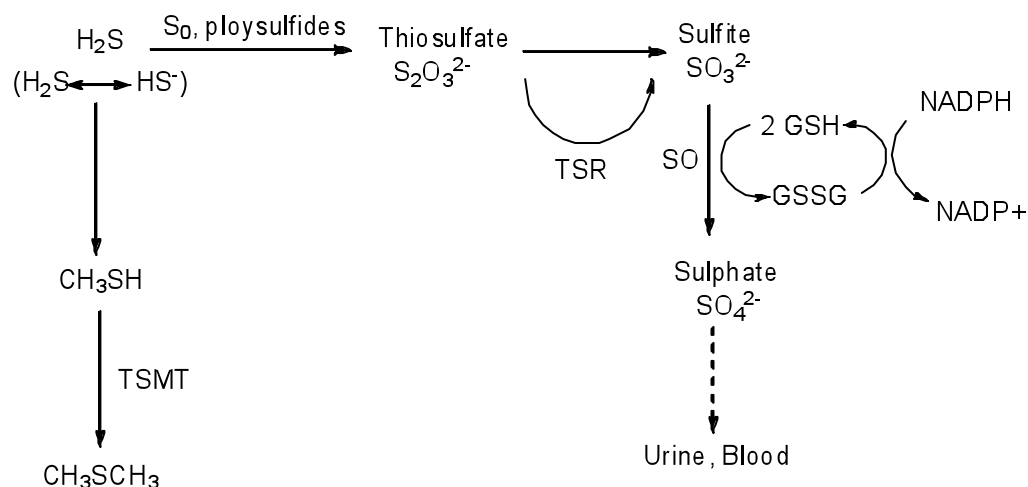
Scheme 3: The metabolism and oxidation pathways of H$_2$S *in vivo* and *in vitro*.[12,26] H$_2$S oxidation and metabolism leads to the formation of thiosulfate (S$_2$O$_3^{2-}$), sulfate (SO$_4^{2-}$), sulfite (SO$_3^{2-}$), ploysulfide (S$_n$), elemental sulfur (S$_0$). Thiol-S-methyl transferase can convert the H$_2$S to methyl and dimethyl mercaptans. H$_2$S can non-enzymatically produce thiosulfate which can be further oxidized to sulfite by thiosulfate reductase (TSR). Sulfite oxidase (SO) can further oxidize the sulfite to sulfate by a glutathione dependent process. Sulfate is eliminated through urine and H$_2$S is detoxified in this way.

Thiosulfate was found to be the major metabolite in the urine sample (4.6 to 11.5 μmol/l) as a biomarker of the H$_2$S poisoning.[56] Sulfate concentration in blood and urine are higher than endogenous thiosulfate and therefore metabolite S$_2$O$_3^{2-}$ is a major marker in case of H$_2$S poisoning.[58,59] Sulfide and polysulfide concentration in blood and urine are 30.4 μg/mL and 32.0 μg/mL) respectively found in case of fatal H$_2$S poisoning.

In tissue, these labile sulfur metabolites are present as "bound sulfur" and are extracted by excess thiols (like DTT) treatment.[60] It is difficult to identify any distinct pre-existing sulfide species present in tissue during analysis of labile sulfur products.[61] These sulfide products are determined using spectrophotometric and HPLC analysis with and without chemical derivatization of the labile sulfur.[61] Concentration of H_2S determined in human serum from this labile sulfur analysis with DTT is 1.16 ± 0.1 μM.[62] But the in vivo sulfide level varies based on the analytical techniques used to quantify sulfide from tissue, as metabolite. In general, H_2S metabolism is mediated through three pathways; oxidation, methylation and reaction with metal containing proteins like cytochrome c oxidase (**Scheme 3**).[22,26] Sometime, inhibition of cytochrome c oxidase by H_2S is treated as a better biomarker for *in vivo* H_2S exposure compare to labile sulfide level determination from tissue. The major oxidation products, sulfate and thiosulfate are excreted through urine and thus sulfide is detoxified.[26,56] Binding of sulfide to protein is observed when blood is treated with Na_2S, proposing a persulphides type intermediate.[12]

Cyanoalanine (BCA)   DL-propylargylglycine (PAG)
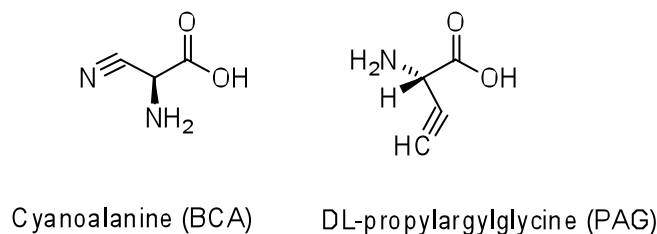
Figure 1: Structure of inhibitors of H_2S biosynthesis.[63]

Inhibitor of endogenous H_2S synthesis (inhibitors of CSE or CBS) such as DL-propargylglycine (PAG) (**Figure 1**) has been used in rat model of haemorrhagic shock and resulted in the recovery of arterial blood pressure.[12,64,65] Administration of H_2S inhibitor in case of inflammation model of rat, decreased the neutrophil infiltration, lung injury and pancreatic necrosis.[64,65] Yet from the point of view of rational drug development, inhibitors of H_2S production for mammalian cell is still far away from the actual drug molecule. Deficiency of CBS and CSE is known to increase the level of homocystine and create hypertension to endothelial cell.[12,66]

$$S^{2-} + 2M^{n+} \longrightarrow S^0 + 2M^{(n-1)+}$$
$$M^{(n-1)+} + O_2 \longrightarrow M^{n+} + O_2^-$$
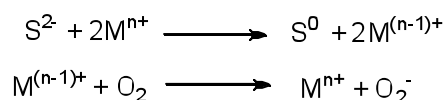
Scheme 4: A general scheme of H_2S mediated ROS generation.[24]

The other mechanism of H_2S toxicity and metabolism in vitro and in vivo is known to be the generation of ROS (**scheme 4**).[12,67] The process of H_2S generation and its metabolism also maintain the cellular redox status as well as O_2 levels.[68] Eghbal reported that H_2S metabolism is CYP450 dependent and H_2S induced the formation of ROS.[24]

Addition of CYP450 inhibitor prevented the formation of ROS by H_2S induction. They have used dichlorofluoroscein to measure the ROS generation from H_2S treated hepatocyte and H_2O_2 scavenger inhibited the ROS generation from H_2S toxicity.[24]

NaHS induced mitochondrial depolarization and decrease of mitochondrial membrane potential were evident by rhodamine 123 fluorescence.[24,69-72] Thiosulfate sulphutransferase is an enzyme involved in sulfide metabolism and could be possible candidate for antidote during H_2S detoxification.[73] Glutathione disulfide can also decrease the toxic effect of H_2S probably via reduction of disulfide group.[12] Not only H_2S mediated ROS generation is responsible for cytotoxicity, but also H_2S oxidation products and metabolite have the potential to trigger several biological responses.[73] Sulfite (SO_3^{2-}) is a metabolite and oxidation product of H_2S which exhibit antioxidant properties in vitro.[74] Neutrophils induced sulfite production occurs during pathogenic invasion and infection with bacterial endotoxin.[67] Sulfite concentration in the serum of acute pneumonia patients was observed to be quite high.[67] Hideki reported, that conversion of sulfide to sulfite by stimulated neutrophils is oxidative stress dependent and NADPH oxidase is required for the neutrophil mediated sulfite production.[67,75] Antioxidant like ascorbic acid inhibited the production of serum sulfite from H_2S and reinforces the oxidative pathways for sulfite generation in mammals.[67] Sulfite generation from H_2S presumably occurs at the site of inflammation and sulfite generation was found to be more than 5 μM within 30 minutes, a concentration is reported to effect kidneys and neuronal cell of mammals.[76] But most of the serum sulfite in humans is found to be protein bound and therefore proven to be less toxic.[67] Sulfite can also carry out oxidation in mitochondria and damage DNA in mammalian cells.[75] Thus a balance between sulfite and sulfide plays an important role during cell signaling and regulation at the inflammation site inside the cell.[77]

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4.4. Hypothesis:

Beside the several biological effects of H_2S, we were interested to see the impact of H_2S signaling during phosphorylation event of protein, as PTPase plays an important role in regulation of cellular phosphorylation status. In aortic smooth muscle cell, H_2S induced phosphorylation of ERK triggered apoptosis.[78] *In vitro* cell based assays have identified the H_2S-regulated genes and those are vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF) receptor and other receptor mediated growth factor genes.[12,37,79] Some of the cardiovascular remedy by H_2S has been proposed to be mediated by the phosphatidyl-inositol 3-kinase (PI3K)/Akt phosphorylation pathways.[50] The importance of endogenous sulfide in mitochondrial energy production as well as oxidative phosphorylation is not yet well explored. Cai reported that hydrogen sulfide triggers pro-angiogenesis by inducing Akt phosphorylation in endothelial cell.[50] Inhibitor of PI3K blocked the H_2S stimulated Akt phosphorylation and thus PI3K is the upstream regulator of Akt in H_2S stimulation pathways.[50] Akt plays an important role in various intracellular signaling pathways like angiogenesis, insulin signaling and Akt activation results in increasing endothelial cell growth, migration and other biological pathways.[12,50,80] As we know that Akt is a very important downstream regulator of insulin signal transduction pathways, we hypothesized that H_2S and its metabolite might target the PTPase and upergulate the phosphorylation of Akt.[80]

We have already seen that the H_2S metabolites generate ROS and some of them have significant role in biological process. As an example, thiosulfate ($S_2O_3^{2-}$) is known to modify protein containing cysteine as a catalytic residue.[81]

Nitric oxide can combine with superoxide anion to give a reactive metabolite peroxynitrite which can lead to various cellular damages.[82] We aimed to investigate whether, H_2S metabolite can similarly combine with ROS (H_2O_2, HOCl, NO) to generate reactive intermediates and see whether that intermediate has the potential to modify the phosphatase function. This will tell us whether H_2S signaling and its metabolism could play a potential role in endogenous regulation of PTPase inside the cell.

4.5. PTP1B activity against hydrogen sulfide and its metabolite:

We have screened the ability of some potential H_2S donor, sodium sulfide ($Na_2S.9H_2O$) and sodium hydrosulfide NaHS.xH_2O) against PTPase activity. We have used PTP1B as model PTPase for our study. We have found that high concentration (0.1 mM to 1 mM) of H_2S has no effect on enzyme activity.

All the assays were done in 50 mM 3,3-dimethyl glutarate buffer (pH 7.2) with chelating agent in order to rule out the possible contribution of the reactive oxygen species (ROS). The metabolites of H_2S (sulfate, thiosulfate, sulfite) were also screened against phosphatase activity.
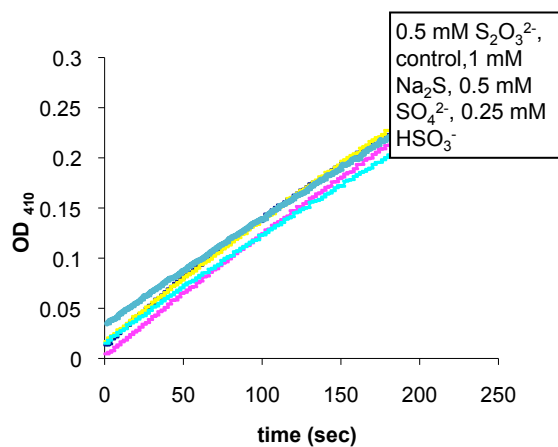
Figure 2: PTP1B (1 μM) was incubated with 1mM (Na$_2$S), 0.5 mM S$_2$O$_3^{2-}$, 0.5 mM SO$_4^{2-}$, 0.25 mM HSO$_3^-$ individually for 15 minutes, in a total volume of 100 μL reaction, containing 50 mM 3,3-dimethyl glutarate, 1 mM DTPA buffer (pH 7.2). After 15 minutes, an aliquot of 15 μL was withdrawn from the reaction mixture and added to a cuvette containing 1.0 mL solution of 20 mM pNPP substrate in 50 mM 3,3-dimethyl gluterate, 1 mM DTPA buffer (pH 7.2) and the remaining enzyme activity was measured from the substrate turn over and compared with respect to a control. No substantial loss of enzyme activity was observed with respect to a control.

The data clearly shows that neither H$_2$S nor its metabolites (SO$_4^{2-}$, SO$_3^{2-}$, S$_2$O$_3^{2-}$) has any efficiency to inactivate the PTP1B (**Figure 2**). In case of thiosulfate the absorbance window is slightly higher than control indicating the reducing behavior of the metabolite which is a favorable condition for the enzyme to stay active. When similar assay was done in absence of any chelating agent, metabolite bisulfite (HSO$_3^-$) has potential to inactivate the enzyme in a time dependent manner (**Figure 3A**).

3A.
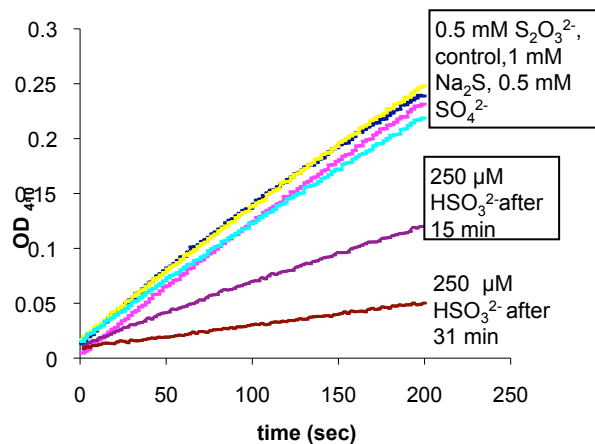
3B.
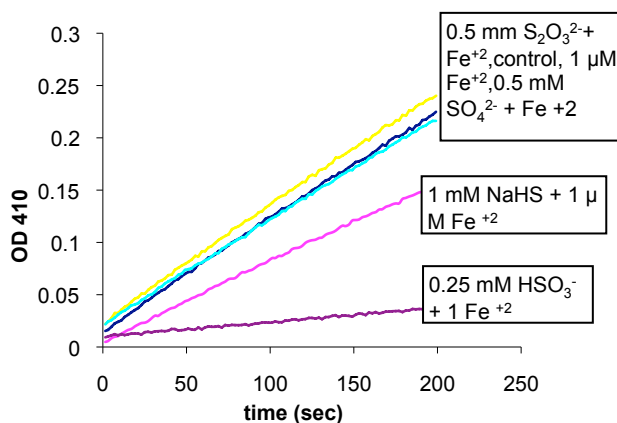
Figure 3: **(3A)** PTP1B (1 µM) was incubated with 1mM (Na$_2$S), 0.5 mM S$_2$O$_3^{2-}$, 0.5 mM SO$_4^{2-}$, 0.25 mM HSO$_3^-$ for 15 minutes, in a total volume of 100 µL reaction, containing 50 mM 3,3-dimethyl gluterate, buffer (pH 7.2) in absence of metal chelating agent DETAPAC. After 15 minutes an aliquot of 15 µL was withdrawn from the reaction mixture and added to a cuvette containing 1.0 mL solution of 20 mM pNPP substrate in 50 mM 3,3-dimethyl glutarate buffer (pH 7.2) and the remaining enzyme activity was measured from the substrate turn over and compared with respect to a control. Bisulfite (HSO$_3^-$) showed a time dependent inactivation in absence of metal chelating agent. **(3B)** Under identical assay condition as mentioned above, metal ion (1 µM Fe^{+2}) was added to the reaction and the time point measurement of PTPase activity was done by taking an aliquot of 15 µL and added to the assay buffer containing 20 mM pNPP substrate to measure the enzyme activity. Addition of exogenous Fe^{+2} ion caused the PTP1B inactivation by H$_2$S an HSO$_3^-$ whereas no inactivation was observed in case of other metabolite as well as control done similarly.

Even in presence of additional 1 μM Fe^{+2} ions, H_2S is a poor inactivator of PTP1B (**Figure 3B**). This result resembles with the existing literature, supporting the oxidation and generation of ROS by hydrogen sulfide and its metabolite (SO_3^- mainly) in presence of metal ion and under physiological condition.

4.6. Phosphatase inactivation by combination of H_2S metabolite with ROS:

We further investigated whether H_2S and its metabolite can combine with other ROS to generate reactive intermediates which can act as potential inactivator of PTP1B like peroxynitrite does and plays significant role as a third messenger inside cell.

We have found that most of the combined species of NaHS and other signaling agent have no significant effect upon PTP1B activity except NaHS and HOCl (50 μM) mixture (**Figure 4A**). Combined species of sulfate (SO_4^{2-}) with ROS don't have much impact on PTPase (**Figure 4B**). Except ($S_2O_3^{2-}$+ HOCl) mixture showed significant inhibition of PTPase activity (**Figure 5A**). The only metabolite that has remarkable effect with a combination of few biologically relevant ROS is sulfite (SO_3^2) (**Figure 5B**).

4A:
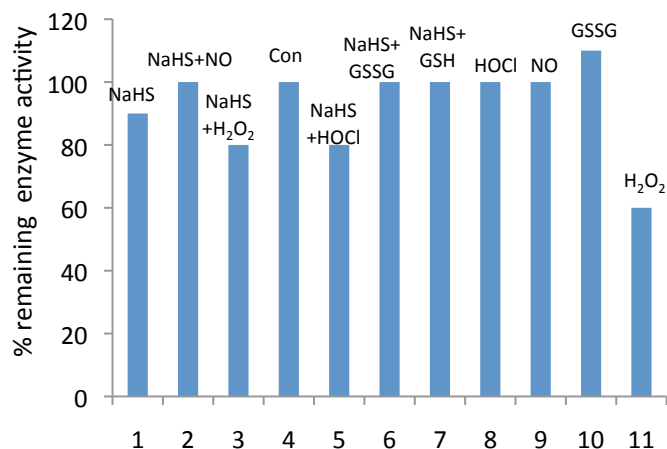
4B:
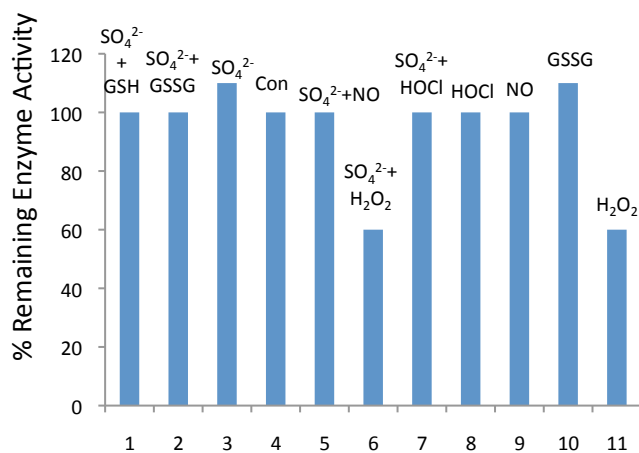
Figure 4: **A)** PTP1B (0.5 to 1.0 µM) was incubated with NaHS (200 µM) in five different reaction, in presence of GSH (1 mM), GSSG (0.5 mM), H_2O_2 (50 µM), HOCl (50 µM), NO (75 µM), respectively in 50 mM 3,3-dimemthyl glutarate buffer, 100 mM NaCl, 1mM DETPAC, pH 7.2 for 15 minute in order to give enough time to generate the reactive combine species. After 15 minute an aliquot of 15 µL was withdrawn and added to the same assay buffer containing 20 mM pNPP substrate to measure the phosphatase activity. No measurable inactivation was found in any case except some insignificant amount of inactivation was observed in case of H_2S and HOCl combination. **(B)** Similarly PTP1B was incubated with SO_4^{2-} (0.5 mM) in five different reaction, presence of GSH (1 mM), GSSG (0.5 mM), H_2O_2 (50 µM), HOCl (50 µM), NO (75 µM), respectively in 50 mM 3,3-dimemthyl glutarate buffer, 100 mM NaCl, 1mM DETPAC, pH 7.2 was carried out for 15 minute and after 15 minute an aliquot of 15 µL was withdrawn from the reaction mixture measure the phosphatase activity as described before. Abbreviation;con:control, GSH:glutathione,GSSG: glutathione disulfide. Individual control was done with GSSG (0.5 mM), H_2O_2 (50 µM), HOCl (50 µM), NO (75 µM). Data were generated in singlet (n=1) for preliminary study.

5A:
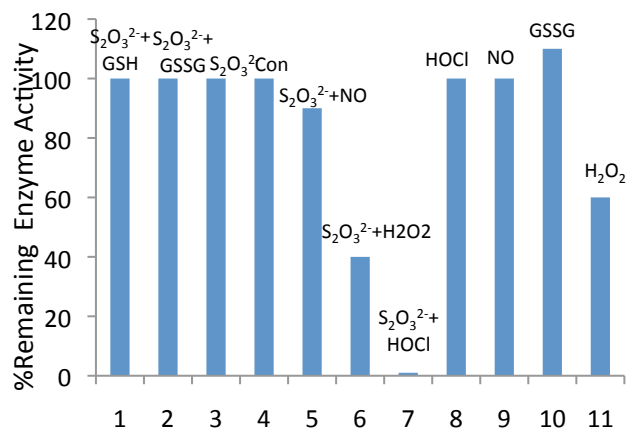
5B:
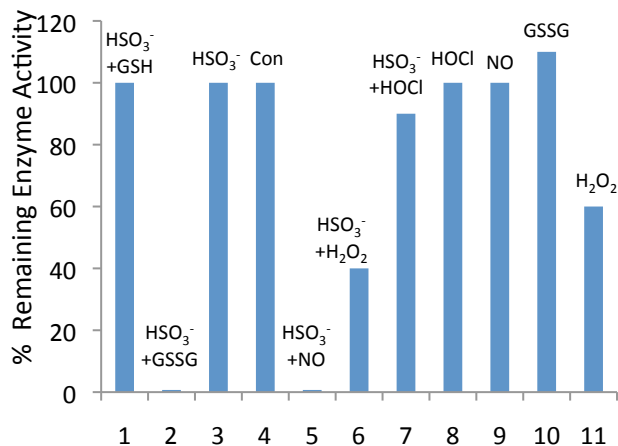
Figure 5: **(A)** PTP1B was incubated with $S_2O_3^{2-}$ (0.1 mM) in five different reaction, in presence of GSH (1 mM), GSSG (0.5 mM), H_2O_2 (50 µM), HOCl (50 µM), NO (75 µM), respectively in 50 mM 3,3-dimethyl glutarate buffer, 100 mM NaCl, 1mM DETPAC, (pH 7.2) for 15 minute. After 15 minute an aliquot of 15 µL was withdrawn and added to 1.0 mL of assay buffer containing 20 mM pNPP substrate to measure the phosphatase activity. No measurable inactivation was found in any case with respect to a control except $S_2O_3^{2-}$ (0.1 mM) & HOCl (50 µM) mixture completely abrogated the enzyme activity. **(B)** Similarly PTP1B was incubated with HSO_3^- (0.25 mM) in presence of GSH (1 mM), GSSG (0.5 mM), H_2O_2 (50 µM), HOCl (50 µM), NO (75 µM), respectively in 50 mM 3,3-dimemthyl gluterate buffer, 100 mM NaCl, 1mM DETPAC, for 15 minute and after 15 minute an aliquot of 15 µL was withdrawn and assayed for phosphatase activity. No measurable inactivation was found in most of the case, except HSO_3^- (0.25 mM)+ GSSG (0.5 mM) and HSO_3^- (0.25 mM)+ NO (75 µM) mixture completely abrogated the enzyme activity. A control with the individual signaling agent H_2O_2 (50 µM), HOCl (50 µM), NO (75 µM) and reducing equivalent GSH (1 mM), GSSG (0.5 mM) was done in order to count their individual contribution during co-operative inactivation. Abbreviation;con:control, GSH:glutathione,GSSG: glutathione disulfide. Data were generated in singlet (n=1) for preliminary study.

Finally we studied the contribution of polysulfide against PTPase activity. We have synthesized a precursor of polysulfur compound to carry out the study of possible effect of polysulfide upon PTPase activity. These polysulfides are very hard to dissolve under assay condition even with the aid of co-solvent due to its hydrophobic nature. Thus a precursor compound, 7-methyl benzopentathiepin (**Figure 6**) was synthesized to study the potential of endogenous polysulfide in the modulation of PTPase activity. Benzopentathiepin possesses rich biological properties and is known as an antitumor antibiotics, can cause DNA cleavage in presence of thiol via ROS mediation.[83]

7-Methylbenzo
-pentathiepin
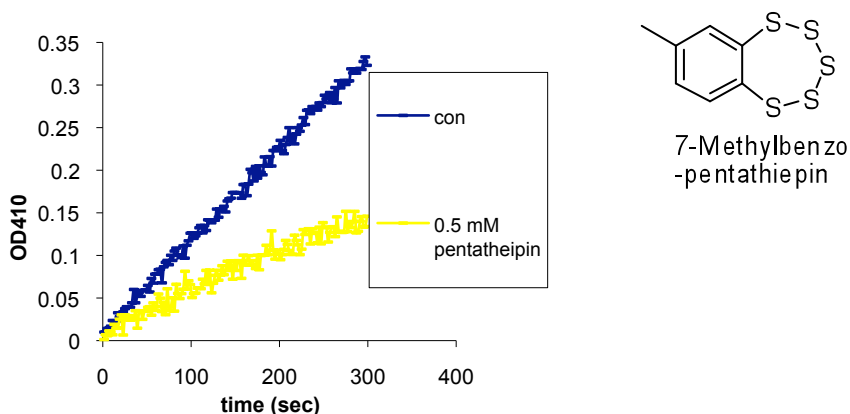
Figure 6: PTP1B (10 nM final) was added to a cuvette containing 500 µM benzopentatheipin and 20 mM pNPP substrate in 50 mM 3,3-dimemthyl glutarate buffer, 100 mM NaCl, 1mM DETPAC, pH 7.2 and enzyme inactivation was monitored continuously for 5 minute from the generation of p-nitrophenolate product by catalysis at 410 nm by UV-vis spectrometer. Identical control was done at the same time in absence of inactivator (only co-solvent DMF). Abbreviation; con:control.pentatheipin:7-methyl benzopentathiepin.

To our expectation we have found that benzopentathiepin (0.5 mM) shows time dependent inactivation of PTP1B in presence of 20 mM substrate (**Figure5**). This result clearly demonstrates that polysulfide whether generated from dietary source or H_2S metabolism has the potential to regulate the PTPase function.

4.7. Conclusion:

Our preliminary study clearly indicates the impact of endogenous H_2S signaling. Metabolites of H_2S could also modulate the PTPase function. As we have seen that PTPase plays a diverse role in several biological processes and cell signaling event. H_2S and its metabolite mediated individual or co-operative regulation of PTPs function could be important relevant to several physiological and pathophysiological condition of cell. Several dietary sources like garlic and onion contain the precursor compounds which are metabolized in the cell to produce H_2S and thereby dietary source of H_2S could be beneficial in certain physiological condition where both H_2S signaling and PTPase is involved in a concurring cellular process. But the exact chemical nature and the structure of the reactive species formed by co-operative combination of H_2S metabolite and other reactive oxygen species yet needs to be determined. Also the occurrence of H_2S signaling, availability of the H_2S metabolite and their presence at the same time during ROS mediated cell signaling yet needs to be determined.

Experimental Section:

Materials and methods.

    Reagents: All the other chemicals and reagents were of highest grade and were purchased from Sigma, Fischer Chemicals Company. Dilute stock of inhibitors were prepared freshly before the assay from the concentrated stock. Substrate, p-nitrophenolphosphate (pNPP), Glutathione (GSH), Glutathathione disulfide (Fluka), sodium sulfide ($Na_2S.9H_2O$), $NaHS.xH_2O$, sodium bisulfite ($NaHSO_3$), sodium sulfae (Na_2SO_4), elemental sulfur (S_8), 4-methyl-1,2-benzenedithiol (across chemical), Diethylenetriaminepentaacetic acid (DETAPAC from Fluka), HPLC grade solvents (methylene chloride, hexane, ethyl acetate) from Fischer, Silica mesh 60 from Merck for column chromatography, TLC plates 0.25 mm for monitoring the reaction, HOCl solution (10-13% available chlorine), NONOate (NO donor), 3,3-dimethyl gluterate, catalase, triethyl amine (Et_3N),were purchased from Sigma-Aldrich Chemical Company and 30 % H_2O_2 was purchased from Fischer Scientific Chemicals. Nuclear magnetic resonance spectra were recorded on Bruker 250 MHz spectrometer and in $CDCl_3$ unless, otherwise mentioned.

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Synthesis of 7-methyl benzopentathiepin: 4-methyl-1,2-benzenedithiol (**1**, 0.5 g, 3.2 mmol) was mixed with elemental sulfur (1.54 g, 48 mmol) and triethyl amine (0.09 mL, 0.64 mmol) in methylene chloride (5 mL) at room temperature. The reaction mixture was allowed to stir for 12 hour at room temperature.
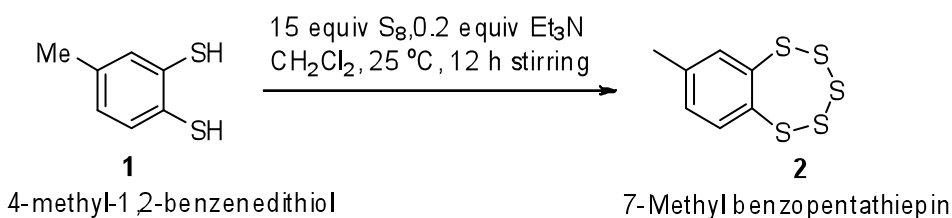
Scheme 5: Synthesis of 7-methyl benzopentathiepin.

The reaction mixture was filtered off and the filtrate was rotavaped. The thick yellow gum was treated with silica gel to make a dry powder and leaded onto a silica column packed in hexane. Flash column chromatography yielded the purified compound as a pale yellow solid **2** (0.31 g, 62 %). The spectroscopic data collected for this compound completely matched with the reported paper of one of our earlier group member.[83]

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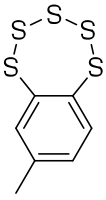
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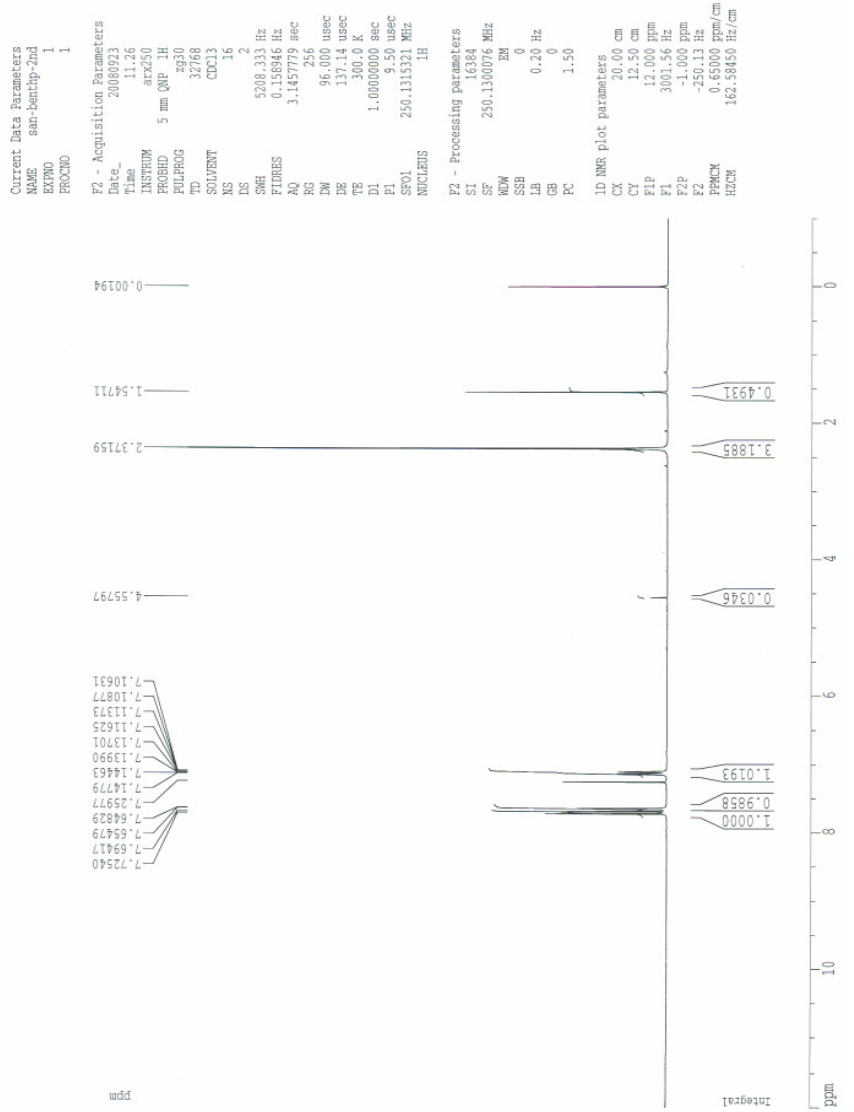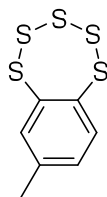
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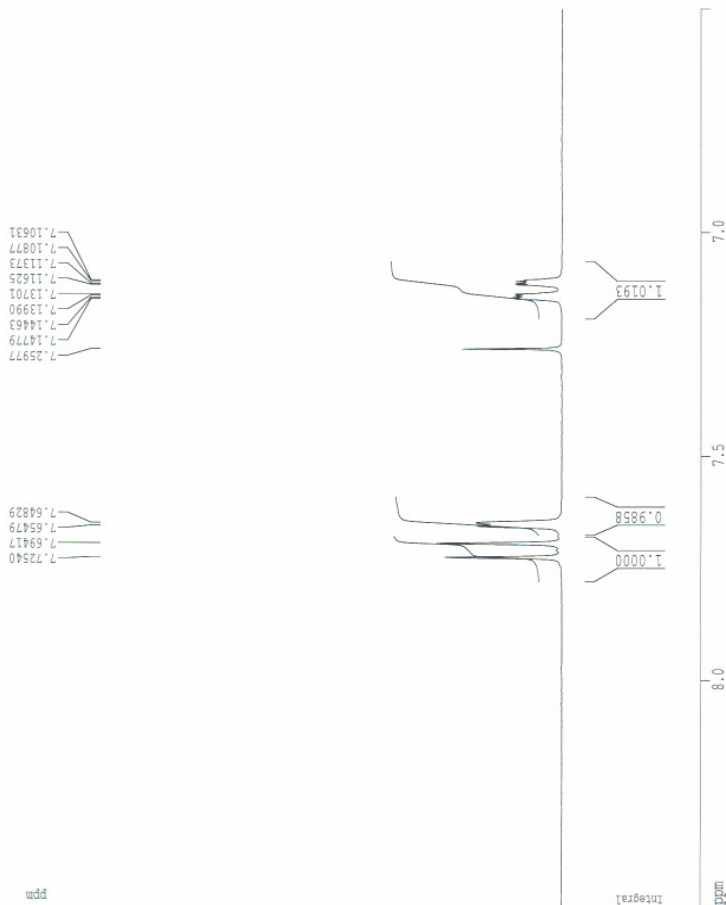
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Time   11.26
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PROBHD   5 mm QNP 1H
PULPROG   zg30
TD   32768
SOLVENT   CDCl3
NS   16
DS   2
SWH   5208.333 Hz
FIDRES   0.158946 Hz
AQ   3.1457779 sec
RG   256
DW   96.000 usec
DE   137.14 usec
TE   300.0 K
D1   1.00000000 sec
P1   9.50 usec
SFO1   250.1315321 MHz
NUCLEUS   1H

F2 - Processing parameters
SI   16384
SF   250.1300076 MHz
WDW   EM
SSB   0
LB   0.20 Hz
GB   0
PC   1.50

1D NMR plot parameters
CX   20.00 cm
CY   12.50 cm
F1P   8.500 ppm
F1   2126.10 Hz
F2P   6.500 ppm
F2   1625.84 Hz
PPMCM   0.10000 ppm/cm
HZCM   25.01300 Hz/cm

7.1063T
7.1087T
7.1137T
7.1625T
7.1370T
7.1390T
7.1446T
7.1477T
7.1597T

7.6082T
7.6547T
7.6941T
7.7254T

1.0193

0.9858
1.0000

ppm

Integral

7.0   7.5   8.0   ppm

140

7-Methylbenzo
-pentathiepin

san-benzotheipin the extra pure top spot from column 09.22.09

Current Data Parameters
NAME   san-bentbp-2nd
EXPNO   2
PROCNO   1

F2 - Acquisition Parameters
Date_   20080923
Time   11.34
INSTRUM   arx250
PROBHD   5 mm QNP 1H
PULPROG   zgdc30
TD   36864
SOLVENT   CDCl3
NS   161
DS   4
SWH   17241.379 Hz
FIDRES   0.467702 Hz
AQ   1.0691060 sec
RG   22800
DW   29.000 usec
DE   41.43 usec
TE   300.0 K
D12   0.00002000 sec
D15   23.00 dB
CPDPRG   waltz16
P31   103.00 usec
D1   2.00000000 sec
P1   8.00 usec
SFO1   62.9023694 MHz
NUCLEUS   13C
D11   0.03000000 sec

F2 - Processing parameters
SI   32768
SF   62.8952440 MHz
WDW   EM
SSB   0
LB   1.00 Hz
GB   0
PC   1.40

1D NMR plot parameters
CX   20.00 cm
CY   10.00 cm
F1P   160.000 ppm
F1   10063.24 Hz
F2P   0.000 ppm
F2   0.00 Hz
PPMCM   8.00000 ppm/cm
HZCM   503.16196 Hz/cm

20.929

77.465
76.956
76.449

140.958
136.797
136.005
130.789

ppm

141

7-Methylbenzo
-pentathiepin

san-benzotheipin the extra pure top spot from column 09.22.09

ppm

140.958

136.797
136.005

130.789
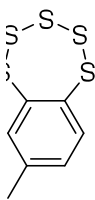
Current Data Parameters
NAME   san-benthp-2nd
EXPNO   2
PROCNO   1

F2 - Acquisition Parameters
Date_   20080923
Time   11.34
INSTRUM   arx250
PROBHD   5 mm QNP 1H
PULPROG   zgdc30
TD   36864
SOLVENT   CDCl3
NS   161
DS   4
SWH   17241.379 Hz
FIDRES   0.467702 Hz
AQ   1.0691060 sec
RG   22800
DW   29.000 usec
DE   41.43 usec
TE   300.0 K
D12   0.00002000 sec
DL5   23.00 dB
CPDPRG   waltz16
P31   103.00 usec
D1   2.00000000 sec
P1   8.00 usec
SFO1   62.9023694 MHz
NUCLEUS   13C
D11   0.03000000 sec
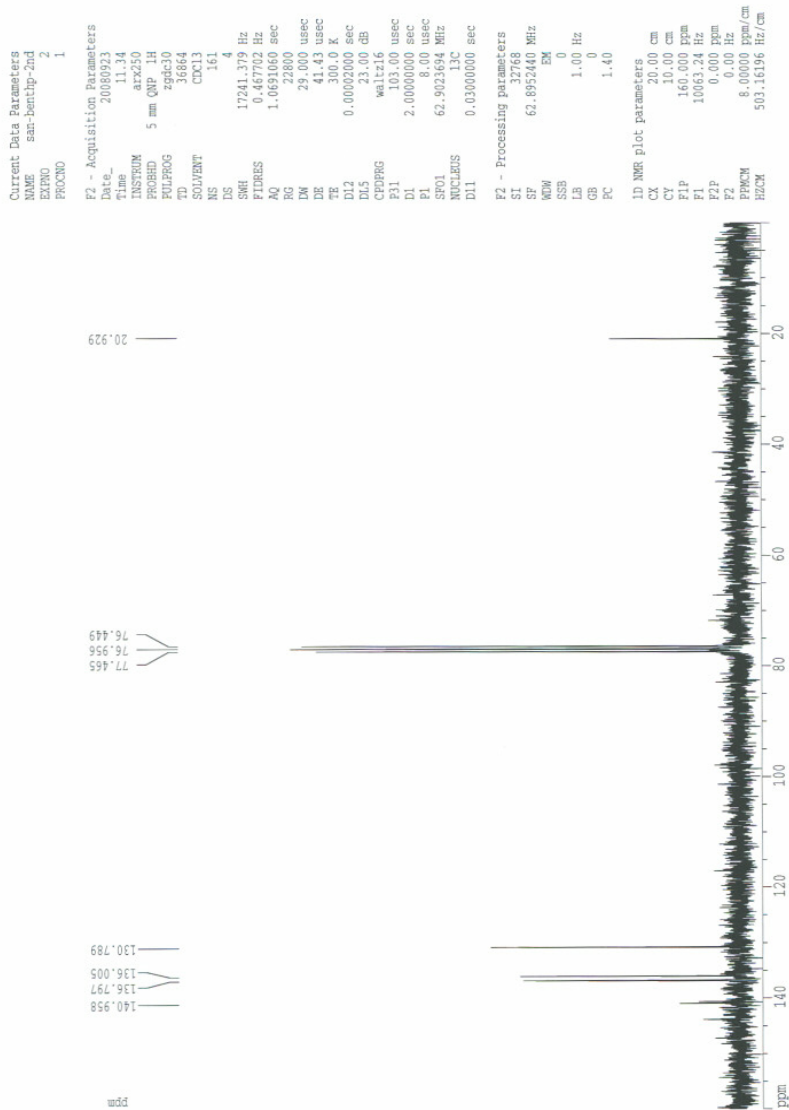
F2 - Processing parameters
SI   32768
SF   62.8952440 MHz
WDW   EM
SSB   0
LB   1.00 Hz
GB   0
PC   1.40

1D NMR plot parameters
CX   20.00 cm
CY   10.00 cm
F1P   150.000 ppm
F1   9434.29 Hz
F2P   120.000 ppm
F2   7547.43 Hz
PPMCM   1.50000 ppm/cm
HZCM   94.34286 Hz/cm

VITA

Sanjib Bhattacharyya was born in 1979 in Shyamnagar (near Calcutta), India. He studied his 10[th] grade in Mulajore Sitanath Pathsala in 1995 and 12[th] grade in Kanti chandra High school in 1997. After that he went to Ramakrishna Mission Vivekananda Centenary College (affiliated to University of Calcutta) in 1997 to complete his Bachelor study (B.Sc. Honors) in chemistry and earned the degree in 2000. He completed his M.Sc. Degree in chemistry from Indian Institute of Technology, Kharagpur, India in 2002. He has completed his Ph.D in 2009 from the University of Missouri, Columbia under the guidance of Prof. Kent S. Gates. He is now going to start a promising career in Nanomedicine in the laboratory of Dr. Priyabrata Mukherjee at Mayo Clinic in Rochester, Minnesota. Besides research, Sanjib loves to explore various types of music, story books and cooking different dishes.