

단 신

아릴옥시렌에 의한 단백질 티로신 인산가수분해효소의 비활성화

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Inactivation of Protein Tyrosine Phosphatases by Aryloxiranes

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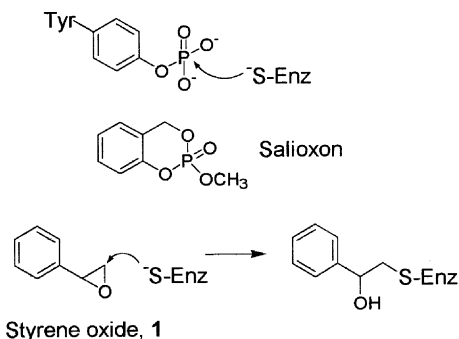
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Protein tyrosine phosphatases (PTPases) are a family of enzymes that hydrolyze phosphate moiety from a phosphotyrosine residue and therefore counteract the activities of protein tyrosine kinases.^{1,2} The regulation of phosphorylation levels on tyrosine of cellular proteins are important in a variety of biological processes and it is maintained by the dynamic balance of the opposing activities of PTPases and protein tyrosine kinases. Abnormal activities of these two classes of enzymes are often associated with diseases and, therefore, a lot of research is recently focused on the development of PTPase inhibitors.^{3,4} Here we report a new structural motif for active site-directed inactivators of PTPases.



In the first step of the PTPase catalyzed reaction, thiolate of active site Cys acts as a nucleophile and

forms a covalent bond between P and S atoms with a concomitant departure of the aryl oxide moiety from the phosphate.¹ Substitution of a electrophilic functional group for the phosphate moiety may be one of the possible ways for the design of irreversible inhibitors. Previously we reported salioxon as an inactivator of PTPases.⁵ Salioxon is a cyclized form of phenyl phosphate which is a substrate of PTPases and the active site nucleophile probably reacts with the cyclic phosphate and the enzyme is irreversibly inactivated. It might be envisioned that the oxirane of styrene oxide can react with the active site Cys of PTPases and permanently inactivates it. To examine this possibility, we evaluated the inhibitory activities of styrene oxide and its structural analogues toward PTPases.

In initial experiments, PTPase activities were measured in the presence of styrene oxide for five PTPases, including a human PTP1B, the catalytic domain of human SHP-1 (SHP1-cat), a *Yersinia* PTPase (YOP), a yeast PTP1 (YPTP1), and the cytosolic domain of CD45 (CD45-cyto).⁵⁻⁷ When the PTPases were preincubated with 20 mM styrene oxide for 10 min before initiation of the enzyme reaction by addition of the substrate, *p*-nitrophenyl phosphate (pNPP, 10 mM), various levels of the PTPase inhibition were observed; 80% inhibition for PTP1B,

Table 1. Percent activities of PTPases measured after incubation with styrene oxide (20 mM) for 10 minutes^a

	PTPIB	SHPI-cat	YOP	YPTPI	CD45-cyto
% Activity	21±4	15±2	76±4	96±2	91±1

^aData reported are the mean±SD from four independent experiments except YOP which is an average of two. Ethanolic solution (200 mM) of styrene oxide was added to the mixture containing PTPases and, after 10 minutes at 298K, the reaction was initiated by addition of pNPP. Final reaction mixture contained 100 mM Hepes (pH 7.0), 5 mM EDTA, 10 mM DTT, 1 mg/mL bovine serum albumin, 10% EtOH, 20 mM styrene oxide, nanomolar ranges of PTPases, and 10 mM pNPP.

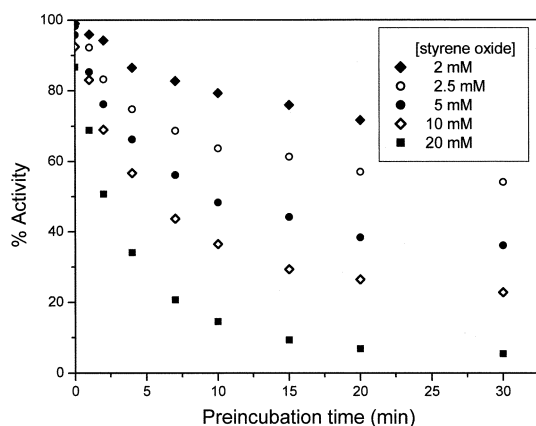


Fig. 1. Time- and concentration-dependent inactivation of PTPase by styrene oxide. Residual PTPase activities following incubation with various concentrations of styrene oxide are presented (pNPP concentration was 10 mM). The data are averages of three independent experiments and error bars are not shown in the figure to avoid complication.

85% for SHPI-cat, 25% for YOP, and less than 10% inhibition for YPTPI and CD45-cyto (Table 1).

Further experiments with PTPase with variations of preincubation time and inhibitor concentrations revealed that PTPase inhibition is time- and concentration-dependent (Fig. 1). The time-dependency of inhibition indicates that the enzyme is irreversibly inhibited. The inactivation mode, however, is not a simple first order and most likely biphasic. Similar pattern is preceded for a *Yersinia* PTPase YOP and enzyme aggregation during inactivation was suggested as a possible reason for the biphasic kinetics.⁸ The molecular basis for the complex kinetic pattern in this report is yet to be studied.

To show that the loss of the enzyme activity is irreversible PTPase was dialyzed overnight at 4 °C against the buffer devoid of styrene oxide. The PTPase was not reactivated by dialysis supporting

the irreversible nature of the inhibition. During this process, uninhibited PTPase partially lost its enzyme activity but retained 20% of the activity. Similar result was obtained when styrene oxide in the enzyme reaction mixture was removed by centrifugation through a membrane filter. The inactivated PTPase was concentrated to one third of the original volume by centrifugal filter (30 kD molecular weight cut, Millipore) and diluted to the original volume with the buffer without styrene oxide. Repetition of this process five times did not restore the PTPase activity to a detectable extent. In parallel experiment, uninhibited PTPase retained 20% of the activity.

To avoid unanticipated effect of the partial loss of the PTPase activity during dialysis or concentration/dilution, separate experiment was performed where uninhibited PTPase fully retains its activity. When the inactivated PTPase was diluted 50-fold with the reaction buffer without styrene oxide before the enzyme reaction, no restoration of the PTPase activity was observed. During the similar process, the uninhibited PTPase was stable with no loss of the activity. This result proves that the inhibition is irreversible.

Fig. 2 depicts the time courses of the reaction in the absence of or presence of styrene oxide at two different pNPP concentrations (0.5 mM for Fig. 2A and 10 mM for Fig. 2B). In the absence of styrene oxide, a straight line was observed from the plot of absorbance at 405 nm vs. time. Fig. 2A shows that, in the presence of styrene oxide, the rate of p-nitrophenolate formation decreased with time. This observation provides another evidence for the formation of a covalent bond between the enzyme and the inactivator. Same experiment at 20-fold higher pNPP concentration (10 mM) exhibited reduced

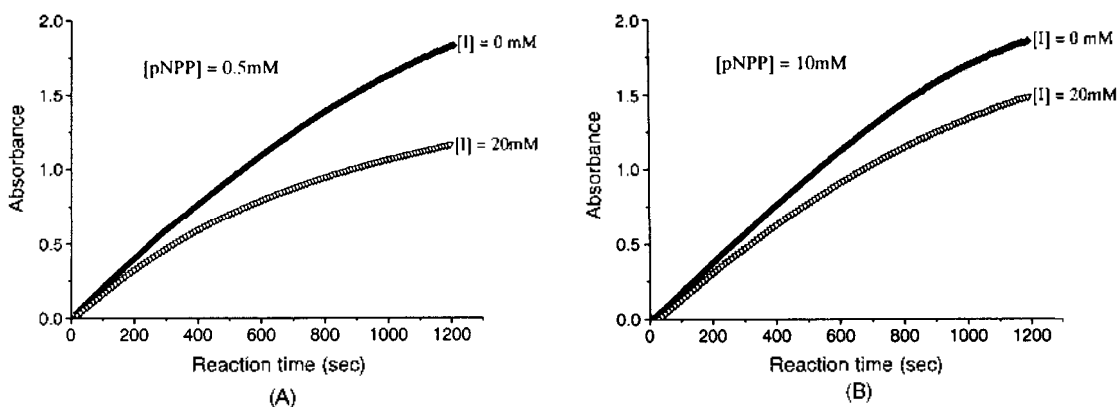


Fig. 2. Time course of the reaction of PTP1B in the absence or presence of styrene oxide. PTP1B (23 nM for A and 12 nM for B) was incubated at 298K with pNPP (0.5 mM for A and 10 mM for B) in 100 mM Hepes (pH 7.0), 5 mM EDTA, 10mM DTT, 1 mg/ml bovine serum albumin, and 10% EtOH or 20 mM styrene oxide in EtOH. The reactions were continuously monitored at 405 nm.

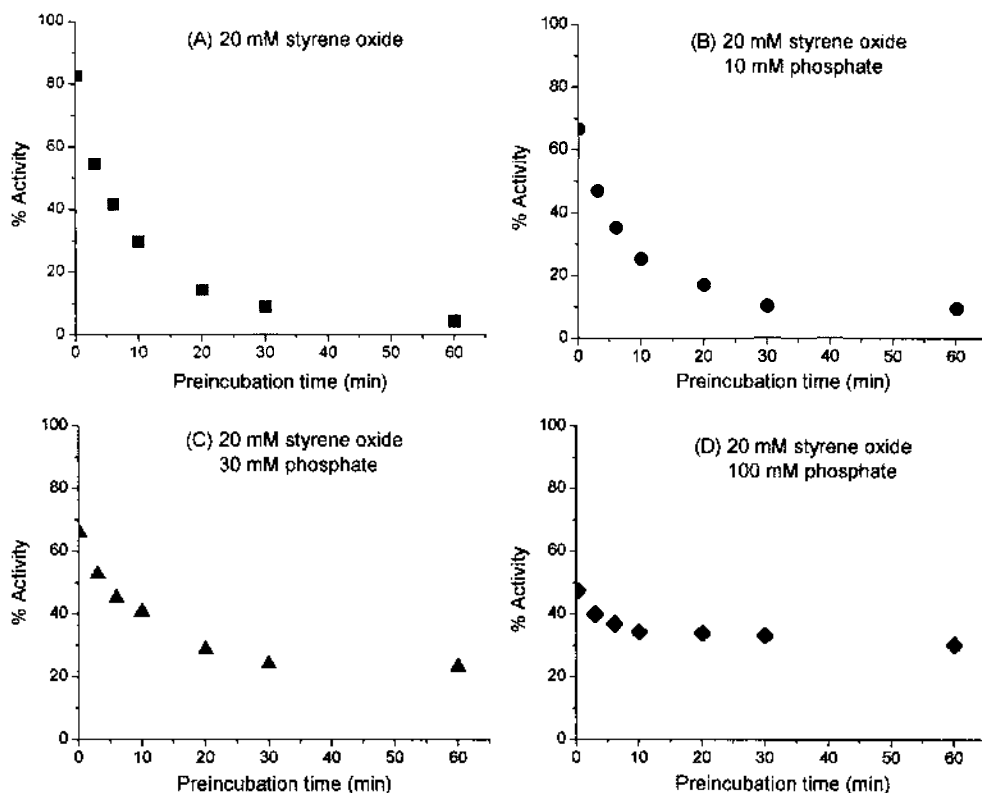


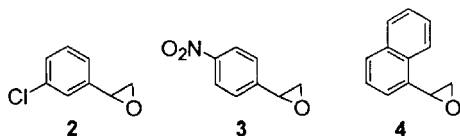
Fig. 3. Time-dependent inactivation of PTP1B by styrene oxide in the absence (A) or in the presence of inorganic phosphate (10 mM for B, 30 mM for C, 100 mM for D). Residual PTPase activities following incubation with 20 mM styrene oxide are presented. Enzyme activity in the absence of styrene oxide was not shown but it remained constant during this experiment.

inactivation rate (Fig. 2B). Because the protective effect of the pNPP against the inactivation by sty-

rene oxide is not negligible, if not obvious, it might be envisioned that the active site of the enzyme is

involved in the inactivation process.

To further study this issue, the time dependency of the inactivation was examined in the presence of various concentrations of inorganic phosphate (Fig. 3). Inorganic phosphate is generally considered as an active site-directed inhibitor of PTPases even though it is not yet definitely proved. Significant reduction of the inactivation rates was observed as the phosphate concentration increases and, in the presence of 100 mM phosphate, the enzyme is efficiently protected against inactivation suggesting that styrene oxide acts at the active site of PTP1B. A possible scenario is a nucleophilic attack at the carbon atom of the oxirane of styrene oxide by the active site Cys in the PTPase resulting in the formation of a covalent enzyme-inactivator complex with a concurrent opening of the oxirane ring.



To examine the effect of structural variation of the aromatic rings of the oxiranes on the potency of the inactivator, we synthesized aryloxiranes containing aromatic rings of different structures (Compound **2-4**). They were synthesized from the corresponding aldehydes by insertion of methylene groups using sulfur ylides.⁹ These compounds were tested for PTP1B because PTP1B is a well known therapeutic target for type II diabetes¹⁰ and the activity diminution of PTP1B was more significant than those of other PTPases tested (Table 1). As shown in Table 2, substitution of naphthalene ring for the phenyl group of styrene oxide afforded improved result. It was, however, problematic to obtain exact kinetic data for **4** because of its low

solubility in water. The potency of the inactivator is still not satisfactory in that the inhibitor concentration for the half-inactivation of PTP1B is in millimolar range. This study, however, provides a novel structural motif for the development of PTPase inactivators and further modifications and derivatizations of the aromatic ring and the oxirane ring may improve the potency of the inactivator. It may be important to introduce a functional group that can mimic the phosphate moiety of phosphotyrosine to increase the affinity toward PTPases. Introduction of carboxyl group at the oxirane ring might be a way and these studies are currently in progress.

EXPERIMENTAL

Chemicals were from Aldrich or Sigma. Styrene oxide were racemic mixture and it was obtained from Aldrich. YOP PTPase was purchased from New England BioLabs (Beverly, USA). PTP1B, SHP1-cat, YPTP1, and CD45-cyto PTPases were overexpressed in *E. coli* and purified as previously reported.¹¹⁻¹³ Absorbances were recorded by UV/VIS spectrophotometer (Metertek SP-830).

General procedure for the syntheses of aryloxiranes.⁹ In a round-bottom flask was introduced 450 mg of 55-65% NaI-oil dispersion and the oil was removed by washing with petroleum ether. Anhydrous DMSO (6 mL) was then introduced and the mixture was stirred at 70°C for 30 min. TIF (6 mL) was added to the reaction flask and it was cooled with ice bath. (CH₃)₂I (2.40 g, 12 mmol) in anh. DMSO (11 mL) was added over a few minutes. Aryl aldehyde (5.0 mmol) in THF (3 mL) was then introduced during 1 min. The ice bath was removed and, after stirring overnight, water (20 mL) was

Table 2. Percent activities of PTP1B measured after incubation with inactivators (2 mM) for 10 minutes^a

	1	2	3	4 ^b	4 (0.1 mM)
% Activity	85±1	81±2	68±2	29±2	92±2

^aData reported are the mean±SD from four independent experiments. The reaction was performed as described in Table 1 ([PTP1B] = 67 nM).

^bPrecipitates were formed as soon as ethanolic solution of **4** was added to the reaction mixture. Working concentration of **4** is therefore much lower than 2 mM.

added. Extraction with Et₂O (50 mL × 3), concentration afforded epoxides contaminated with 20-30% of starting material. Because the epoxide and starting material was indistinguishable on TLC, the crude product was dissolved in 20 mL of MeOH and treated with NaBH₄ (150 mg) for 1 h to convert the aldehyde to alcohol. Column chromatography of the resulting mixture afforded epoxides typically in about 40% yield. The yields were not maximized.

PTPase Inhibitor Assay. For PTPase assay, inhibitor (5 μL in EtOH) was added to a mixture containing enzyme (5 μL), 5x reaction buffer (10 μL, 500 mM Hepes, 25 mM EDTA, 50 mM DTT, pH 7.0) and water (25 μL). Typical enzyme concentration in the reaction mixture was 130 nM. The mixture was incubated at 25 °C for an appropriate preincubation time and pNPP (100 mM, 5 μL) was added to initiate the enzyme reaction. After incubation at 25 °C for 2-5 min, the reaction was quenched by addition of 0.9 mL of 0.5 M NaOH and the progress of the reaction was determined for the formation of *p*-nitrophenolate by measuring the absorbances at 405 nm.

For the continuous assay, the reaction was initiated by addition of PTPase (5 μL) to the fresh mixture of pNPP (5 μL), inhibitor (5 μL), 5x reaction buffer (10 μL) and water (25 μL). The progress of the reaction was monitored by recording the absorbance at 405nm without addition of the NaOH solution. Enzyme concentration in the reaction mixture was 12 or 23 nM (See Fig. 2 legends).

For dilution assay, styrene oxide (100 μL, 200 mM in EtOH) was added to a mixture of PTP1B (0.75 mg/mL, 100 μL), 5x reaction buffer (200 μL, 500 mM Hepes, 50 mM DTT, pH 7.0), bovine serum albumin (10 mg/mL, 100 μL), and water (500 μL). To check the enzyme inactivation, forty five μL of the mixture was taken occasionally and pNPP (100 mM, 5 μL) was used for the reaction. When the PTPase activity was almost abolished, appropriate amounts

of the mixture was diluted with a buffer (100 mM Hepes, 10 mM DTT, 1 mg/mL bovine serum albumin, 10% EtOH, pH 7.0) and assayed using pNPP to a final concentration of 10 mM.

The assays in the presence of inorganic phosphate were performed as the procedure described at the first part of assay method except that appropriate concentrations of the phosphate buffer (pH 7.0) was substituted for the same concentrations of Hepes buffer.

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REFERENCES

1. Zhang, Z.-Y.; Dixon, J. E. *Adv. Enzymol.* **1994**, *68*, 1.
2. Denu, J. M.; Dixon, J. E. *Curr. Opin. Chem. Biol.* **1998**, *2*, 633.
3. Hunter, T. *Cell* **1995**, *80*, 225.
4. Burke, T. R. Jr.; Zhang, Z.-Y. *Biopolymers* **1998**, *47*, 225.
5. Cho, H.; Park, J.; Yung, D.; Ham, S. W. *Bull. Korean Chem. Soc.* **2000**, *21*, 515.
6. Han, J.; Kwon, M.; Cho, H. *J. Biochem. Mol. Biol.* **1998**, *31*, 135.
7. Cho, H.; Ramer, S. E.; Itoh, M.; Kitas, E.; Bannwarth, W.; Burn, P.; Saito, H.; Walsh, C. T. *Biochemistry* **1992**, *31*, 133.
8. Taylor, W. P.; Zhang, Z.-Y.; Widlanski, T. S. *Bioorg. Med. Chem. Lett.* **1996**, *4*, 1515.
9. Duncan, W. G.; Colwell, W. T.; Scott, C. R.; Henry, D. W. *J. Med. Chem.* **1968**, *11*, 1221.
10. Elchebly, M.; Cheng, A.; Tremblay, M. L. *J. Mol. Med.* **2000**, *78*, 473.
11. Kwon, M.; Oh, M.; Han, J.; Cho, H. *J. Biochem. Mol. Biol.* **1996**, *29*, 386.
12. Cho, H.; Krishnaraj, R.; Itoh, M.; Kitas, E.; Bannwarth, W.; Saito, H.; Walsh, C. T. *Protein Sci.* **1993**, *2*, 977.
13. Pei, D.; Neel, B. G.; Walsh, C. T. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 1092.