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Evans Blue and other dyes as protein tyrosine phosphatase inhibitors

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Abstract—Commonly used dyes including Evans Blue and Trypan Blue were examined for their inhibitory activities against protein tyrosine phosphatases (PTPases), all of them showed inhibition of PTPases with different potencies. Of the 13 dyes tested, four exhibited IC_{50} value of less than 10 μM, Evans Blue lowest IC_{50} of 1.3 μM against PTP1B. Care must be taken in the use of dyes for clinical or biochemical experiments to avoid unwanted side effects. Some of the low molecular weight dyes might be useful as lead compounds for the development of potent and selective PTPase inhibitors.

Dyes are used primarily to introduce colors in life but their use has been extended to other areas such as biochemical research and clinical medicine. Not to mention Gram's reagent (Crystal Violet) for classification of bacteria, Trypan Blue and Hoechst are only a few examples of dyes indispensible in cell biological study. Dye-derived chemicals like salvarsan and suramin have been used as antimicrobial drugs.[1] Antimalarial drugs quinacrine and chloroquine as well as antihistamine and antipsychotic drugs such as promethazine and chlorpromazine are modified versions of Methylene Blue dye.[1,2] Clinical use of dyes is further extended to a diverse of diseases ranging from dementia to cancer.[1] Recent studies revealed part of the biological effects of dyes like inhibitory activities against cellular enzymes. Triosephosphate isomerase is inhibited by several sulfonated dyes and alcohol dehydrogenase by Vilmafix Blue A-R dye.[3,4] Antitrypanosomal drug suramin has been shown to inhibit glycolytic enzymes and glycosylphosphatidylinositol phospholipase D.[3,5,6] Trypan Blue and Evans Blue are P2-purinoceptor antagonists and Evans Blue inhibits the activities of nucleotide-binding enzymes such as DNA and RNA polymerases, DNA primase and viral reverse transcriptase.[7–9] Also reported were the inhibition of apoptotic cell death, vesicular glutamate transport and the stimulation of large-conductance Ca^{2+}-activated K^+ channels by Evans Blue.[10–15]

Recently, it was found that dye-related compounds suramin and aurintricarboxylic acid are potent inhibitors of protein tyrosine phosphatases (PTPases).[16–18] These observations, together with the consideration that PTPase family of enzymes is a promising target for the treatment of human diseases, prompted us to examine the effect of various dyes on the activities of PTPases. Examined were 13 arbitrarily selected dyes against human PTPase 1B (PTP1B), membrane proximal catalytic domain of human LAR (LAR-D1) and YPTP1 from *Saccharomyces cerevisiae*.[18] Initial enzyme assay proved that all the dyes tested behave as inhibitors of the PTPases with different potencies. Evans Blue was the most potent inhibitor of PTP1B and YPTP1 with the concentrations for half-maximal inhibition (IC_{50}) of 1.3 μM and 1.2 μM, respectively. Trypan Blue, Senda Chrome AL and Chromotropate inhibited these enzymes with IC_{50} value less than 10 μM (Table 1). Evans Blue was ca. 50-fold selective for PTP1B and YPTP1 against LAR-D1. Trypan Blue and Chromotropate exhibited 20–90-fold selectivity against LAR-D1. Allura Red AC, Allizarin Red S, Carmine and Shikonin are inhibitors of medium potency with IC_{50} <40 μM for at least one of the PTPases. Eriochrome Red B, Mordant Orange 1, Pamoic Acid, Fluorescein and Ponceau 6R are weak inhibitors with hundreds μM of

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IC_{50}. In most cases, dyes were significanty less effective against LAR-D1 (Fig. 1).

To investigate the mode of inhibition by the dyes, steady-state kinetic experiments of PTP1B and YPTP1 were performed for Chromotropate and Evans Blue. As for the inhibition by Chromotropate, the Lineweaver–Burk plot is comprised of lines that intersect on the y-axis characteristic for competitive inhibition for both the enzymes (Fig. 2A). On the other hand, Evans Blue exhibited a line pattern characteristic for mixed type inhibition for both YPTP1 and PTP1B (Fig. 2B). These results indicate that Chromotropate, but not Evans Blue, competes with the substrate for the binding on these enzymes. In addition, the replots of the slope versus inhibitor concentration were hyperbolic in both cases for PTP1B as well as YPTP1 indicating that binding of the dyes to PTPases is not a simple process (Fig. 2A and B, insets). To study further the mode of inhibition by the dyes, we measured the residual activity of YPTP1 after the enzyme was preincubated with Chromotropate or Evans Blue for different time periods. As shown in Figure 3, the inhibition of YPTP1 by Evans Blue was dependent on the preincabation time indicating that Evans Blue is a slow-binding inhibitor of YPTP1. Similar behavior was also observed for Chromotropate (data not shown). When the YPTP1-catalyzed reaction was monitored continuously in the presence of Evans Blue, the reaction progress curve exhibited time-dependent decrease of the reaction rate providing another evidence for the slow-binding behavior of the dye (data not shown).

Senda Chrome AL showed PTP1B selectivity against LAR-D1 and YPTP1 of >200-fold and 6-fold respectively. Considering that many other dyes did not discriminate PTP1B and YPTP1 effeiciently, the selectivity exhibited by Senda Chrome AL advocated the dye as a good model for the design of selective PTP1B inhibitors. Also noteworthy is that Senda Chrome AL inhibited PTP1B with IC_{50} value of 5.2 μM compared to a structurally related compound methylenedisalicylic acid which, in its chemically pure form, inhibited PTP1B with IC_{50} of 3600 μM (S. Shrestha, et al. unpublished results). Structural basis for the surprising difference in inhibitory potencies is yet to be studied. Another dye with a low molecular weight, Chromotropate, exhibited potent inhibitory activity (IC_{50} <10 μM) against PTP1B and YPTP1. The dye attracted our attention because it is a competitive inhibitor of PTPases and also it contains free hydroxyl groups to introduce additional structural features without a significant change of the core structure. Derivatization of Chromotropate might lead to the identification of more potent and selective PTPase inhibitors and this project is in progress.

It is also interesting to find that Evans Blue is a potent inhibitor of PTPases because this dye has been known

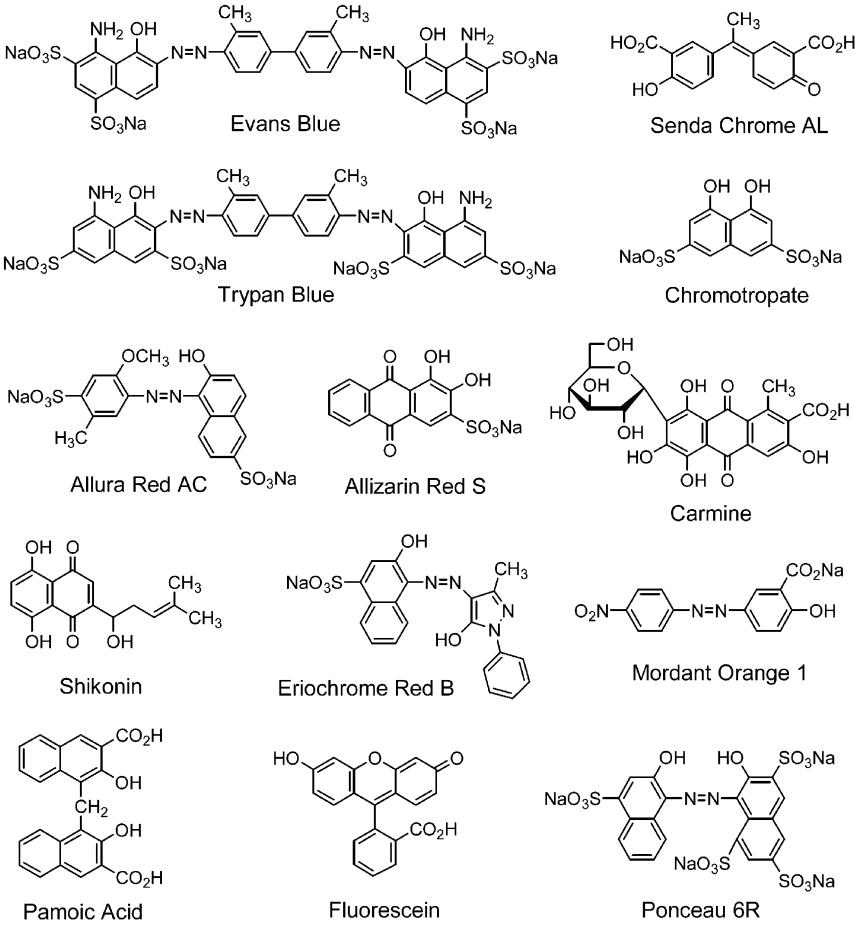

Figure 1. Structures of the dyes used in this study.

Table 1. Inhibition of PTPases by various dyes[a]

Compounds[b]	IC_{50} (μM)[c]		
	PTP1B[d]	LAR-D1[d]	YPTP1[d]
Evans blue	1.3±0.2	61±5	1.2±0.2
Trypan blue	3.9±0.3	170±45	7.4±1.0
Senda chrome AL	5.2±0.7	>1000	30±3
Chromotropate	9.7±0.9	570±70	6.5±0.5
Allura red AC	33±6	90±2.4	410±80
Allizarin red S	40±4.5	82±5	48±8.5
Carmine	26±0.8	>1000[e]	15±0.3
Shikonin	25±4[e]	>1000[e]	70±5[e]
Erichrome red B	150±30	660±230	140±20
Mordant orange 1	210±78	>1000	630±43
Pamoic acid	310±29	>1000	>1000
Fluorescein	600±26[e]	>1000[e]	850±20[e]
Ponceau 6R	460±110	460±200	110±30

[a] For inhibition assay, inhibitor (5 μL in H_2O or DMSO) was added to a mixture containing enzyme (5 μL), 5× reaction buffer (10 μL, 0.5 M Hepes, 25 mM EDTA, 50 mM DTT, pH 7.0) and water (25 μL) and it was incubated at 37 °C for 10 min. The reaction was initiated by addition of *p*-nitrophenyl phosphate (*p*NPP) (5 μL, 20 mM) and, after 5 min at 37 °C, the reaction was quenched by addition of NaOH solution (950 μL, 0.5 M). The progress of the reaction was determined for the formation of *p*-nitrophenolate by measuring the absorbance at 405 nm. The quantity of enzymes used for typical 50 μL reaction was 200 ng for PTP1B, 30 ng for YPTP1 and 1.25 units (manufacturer's definition) for LAR-D1.

[b] Evans Blue and Allizarin Red S were purchased from Aldrich (Milwaukee, USA) and other were from TCI (Tokyo, Japan). They were used without further purification.

[c] IC_{50} values were usually derived from double experiments using a range of inhibitor concentrations.

[d] PTP1B and YPTP1 were expressed in *E. coli* expression systems and purified as described.[18] LAR-D1 was purchased from New England Biolabs (Beverly, USA). The enzymes were diluted before use to an appropriate concentration by enzyme dilution buffer (25 mM Hepes, 5 mM EDTA, 1 mM DTT, 1 mg/mL BSA, pH 7.3).

[e] These dyes were dissolved in DMSO for inhibition assay. Others were dissolved in H_2O.

to prevent apoptosis in certain cell types with unknown mechanism of action.[19] Evans Blue is known to bind certain membrane proteins and this property could be responsible for the antiapoptotic effect of the dye. On the other hand, it might be imagined that Evans Blue inhibits cellular PTPase(s) to thwart the progress of apoptotic pathway and, indeed, precedented were the observations of suppression of apoptosis by down-regulation of PTPase activity.[20–24] To inhibit the action of endogenous PTPase(s), however, the dye must be transported into the cytoplasm. Even though Evans Blue is generally considered to be membrane impermeable,[25] it may not be absolutely true in certain specific conditions or when the cells were treated for long periods of time. For example, Trypan Blue, structurally analogous to Evans Blue and generally regarded as membrane impermeable, permeated into the cells treated with certain chemicals, heat or a toxin.[26–28] Despite of these observations, the correlation of the PTPase inhibition and antiapoptotic effect of Evans Blue is not evident at this stage. Rather, this issue is better to be considered in regard that dyes, which are commonly used for medical purposes or biochemical experiments, inhibit PTPases in vitro and their use might cause unwanted or biased results. Evans Blue has been used for some time in the colorimetric determination of blood volume, as well as in the outlining of the lymphatics for lymphangiograms.[29] Trypan Blue is widely used as an indicator of membrane integrity and to identify nonviable cells. Methylene Blue has been used for diagnostic and therapeutic purposes but its potential toxicity was reported recently.[30] Listed are only a few examples of clinical or biochemical use of dyes.

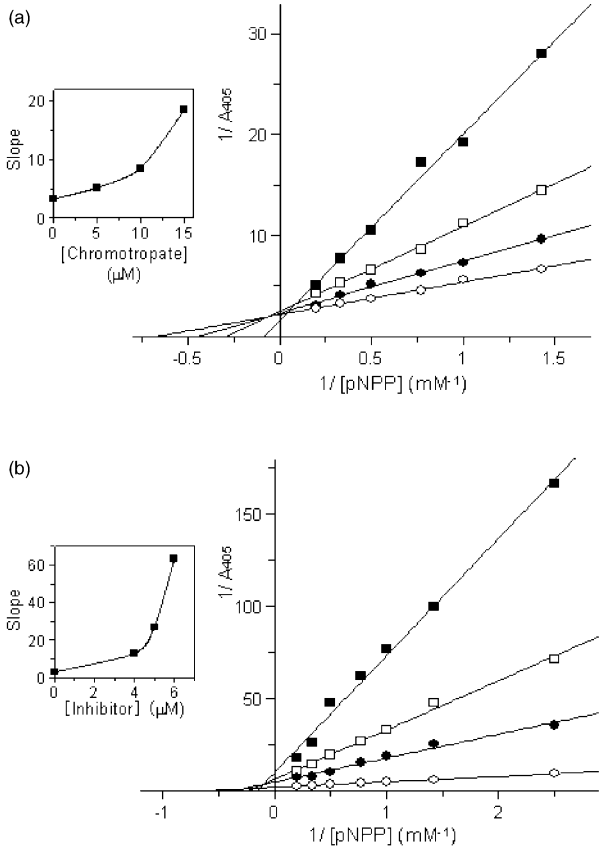

Figure 2. Lineweaver–Burk analysis for YPTP1 catalyzed reactions in the presence of Chromotropate or Evans Blue. Phosphatase activity was measured against *p*NPP in the presence of (A) Chromotropate; none (○), 5 μM (●), 10 μM (□) and 15 μM (■) or (B) Evans Blue; none (○), 4.0 μM (●), 5.0 μM (□) and 6.0 μM (■). Insets are secondary plots of the slope vs. dye concentration. Similar results were obtained for PTP1B with both Chromotropate and Evans Blue.

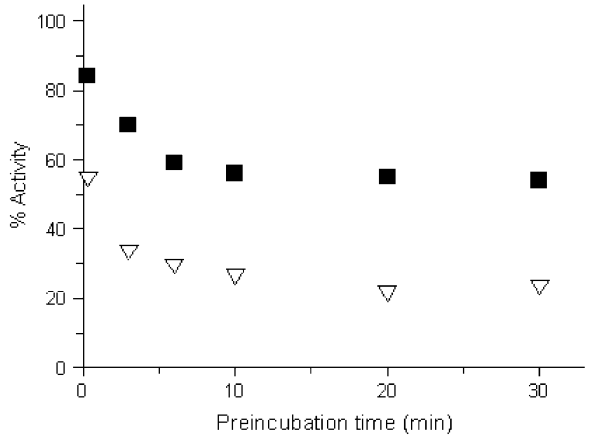

Figure 3. Time dependent inactivation of YPTP1 by Evans Blue. Percent residual phosphatase activity was determined after preincubation of YPTP1 and Evans Blue (2 μM, ■; 4 μM, ▽) for 20 s, 3, 6, 10, 20 and 30 min before initiation of the enzyme reaction by addition of the substrate, *p*NPP.

In summary, we have shown that commonly used dyes including Evans Blue are capable of inhibiting PTPases. Some of them might be useful as lead compounds for the development of potent and selective PTPase inhibitors. Furthermore, the inhibitory activities of dyes necessitate careful consideration in their use for clinical and biochemical experiments.

Acknowledgements

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