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Multi-site inhibition of human plasma cholinesterase by cationic phenoxazine and phenothiazine dyes

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Abstract

Two cationic phenoxazine dyes, meldola blue (MB) and nile blue (NB), and the structurally related phenothiazine, methylene blue (MethB), were found to act as complex inhibitors of human plasma cholinesterase (butyrylcholinesterase, BChE). Studied at 25 °C, in 100 mM MOPS buffer (pH 8.0), with butyrylthiocholine as substrate, the kinetic pattern of inhibition indicated cooperative I binding at 2 sites. Intrinsic K' values ($\equiv [I]^2_{0.5}$ extrapolated to $[S] = 0$) for MB, NB and MethB were 0.64 ± 0.05, 0.085 ± 0.026 and 0.42 ± 0.04 μM, respectively. Under the same experimental conditions the dyes acted as single-occupancy, hyperbolic-mixed inhibitors of electric eel acetylcholinesterase (AChE), with $K_i = 0.035 \pm 0.010$, 0.026 ± 0.0034 and 0.017 ± 0.0063 μM (for MB, NB, MethB); α (coefficient of competitive interaction) = 1.8–2.4 and β (coefficient of noncompetitive interaction) = 0.15–0.28. The complexity of the BChE inhibitory effect of phenoxazine/phenothiazine dyes contrasted with that of conventional ChE inhibitors which cause single-occupancy ($n = 1$), competitive or mixed inhibition in both AChE and BChE and signaled novel modes of ligand interaction at (or remote from) the active site gorge of the latter enzyme.

Keywords: Acetylcholinesterase; Butyrylcholinesterase; Meldola blue; Methylene blue; Multi-site inhibition; Nile blue; Plasma cholinesterase

Butyrylcholinesterase (BChE, EC 3.1.1.8) is a serine hydrolase abundant in liver and plasma and expressed in a variety of other tissues including the central nervous system [1,2]. Originally presumed to serve mainly as a scavenger of xenobiotics, the enzyme has more recently been implicated in neurogenesis and overall brain function including the progression of Alzheimer's disease [3–6]. The latter connection, shared with acetylcholinesterase (AChE,[1] EC 3.1.1.7), has added momentum to research on both cholinesterases and on cholinesterase inhibitors as therapeutic agents [7,8].

Although encoded by distinct genes, BChE and AChE are similar in overall structure, catalytic mechanism and a general affinity for cationic ligands [1,9]. The similarities present practical advantages by allowing interchangeable use of the two enzymes in a number of applications such as monitoring or antidoting target xenobiotics [10–12]. The differences, specifically those that are reflected in a differential response to effectors, serve as important probes into active site topology and provide a basis for the design of specific inhibitors aimed at one or the other enzyme [13–15]. A wide spectrum of compounds is under study in search of critical structural features which affect selective inhibitory potential [13–19].

We have recently reported on the inhibition of human BChE by cationic triarylmethane dyes, implicating two independent modes of I-binding and inhibitory impact [20]. The following is an extension of the study to include the cationic phenoxazine/phenothiazine dyes, meldola blue and nile blue and methylene blue (Fig. 1). The dyes were

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[1] *Abbreviations used:* AChE, acetylcholinesterase; ATC, acetylthiocholine; BChE, butyrylcholinesterase (plasma cholinesterase); BTC, butyrylthiocholine; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); MB, meldola blue; MethB, methylene blue; MOPS, 3-(*N*-morpholino)propanesulfonic acid; n, binding stoichiometry; NB, nile blue.

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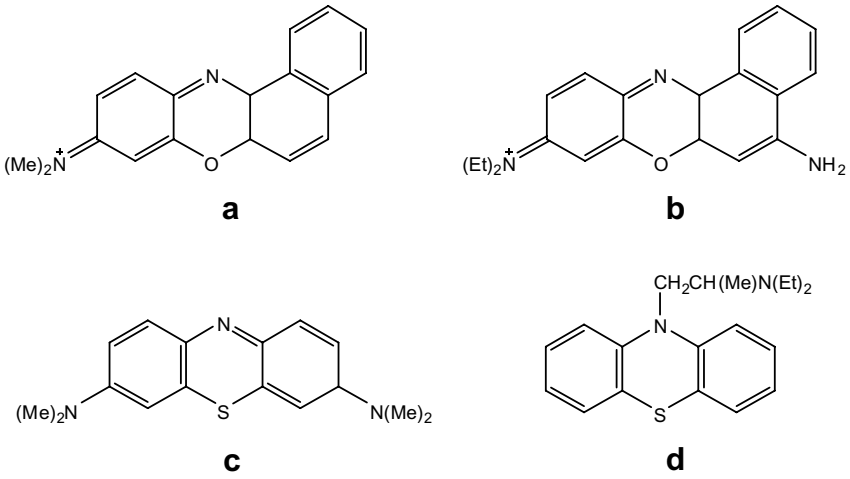

Fig. 1. The structures of the ligands studied. (a) Meldola blue, (b) nile blue, (c) methylene blue, (d) ethopropazine.

also tested on electric eel AChE for comparison. The results point to a inhibitory efficacy similar to that of previously studied ChE inhibitors. However, the kinetic patterns are distinct: The inhibition of BChE reveals a complex pattern and indicates cooperative I binding at more than one site. The impact on AChE is consistent with the single-occupancy mixed inhibitory scheme which has been found to apply to ChE inhibition in general, except for an atypical partial noncompetitive feature deriving from residual activity in the ternary (ESI) complex.

Materials and methods

Human plasma was obtained from the Blood Bank of Hacettepe University Hospitals. BChE (sp. act. ca. 200 U/mg protein) was purified by successive chromatography on DEAE–Trisacryl M and procainamide–Sepharose 4B [21]. Electric eel acetylcholinesterase (400 U/mg protein) was purchased from Sigma–Aldrich (USA). All remaining chemicals were of the highest purity available and were from Sigma–Aldrich or Merck (Germany). Stock solutions of dye (5 mM) were prepared in methanol.

Activity determinations

BChE and AChE were assayed at 25 °C, in 100 mM MOPS buffer (pH 8.0) containing 0.05–0.4 mM BTC or ATC as substrate, 0.125 mM DTNB and 0–5 μM ligand. The reactions were initiated by the addition of enzyme and monitored through the increase in A_{412}, using a Shimadzu 1601 PC spectrophotometer equipped with a Peltier unit. (The methanol content of the assay mixture, $\leqslant$2% v/v, was found to have no effect on enzyme activity.) *Initial rates were calculated* taking ε_{TNB} (412 nm) = 14.2 mM^{-1} cm^{-1} [22].

Checks on ligand and assay integrity

MB is highly reactive towards sulfhydryl groups [23] and could potentially interfere with the chromogenic reaction of DTNB with thiocholine. The assay system was checked for such interference by preincubating enzyme and BTC for 1 min and comparing the immediate A_{412} values observed upon the addition of DTNB ± MB. The two values agreed within ±1%.

Phenoxazines and phenothiazines are reportedly prone to dimerization (and higher-order aggregation) in aqueous solution [24,25]. The possibility of assay artifacts stemming from ligand aggregation was tested by comparing the fluorescence excitation and emission spectra of 0–6 μM MB and NB in 100 mM MOPS, pH 8.0. The spectra exhibited constant λ_{max} values and the fluorescence coefficients in the entire spectral range were invariant, pointing against any progressive association phenomena in the dye population within the specified concentration limits. Spectral measurements were made using a Shimadzu 5301 PC fluorescence spectrometer. The excitation spectra of MB were recorded using λ_{em} = 660 nm; emission spectra were recorded using λ_{exc} = 590 nm. The corresponding parameters in NB analyses were 680 and 630 nm. Slits = 10 nm (MB) and 5 nm (NB) each.

Software

The molecular volumes of the inhibitory ligands were estimated by use of the Molecular Modeling Pro Plus package program (ChemSW, USA). Nonlinear regression analyses were performed using the Statistica package (StatSoft, USA).

Results and discussion

The effect of MB, NB and MetB on BChE activity

All three dyes were found to cause total inhibition of BTC-hydrolizing activity. The inhibition was instant, as evidenced by the linearity of the A vs t traces during the 2-min assay period ($r^2 \geqslant 0.9983$) and the fact that preincubation of enzyme and inhibitor for 10 min and starting the assay by the addition of substrate yielded the same v_i values as the routine assay sequence where E was the final addend. *The data were initially processed with reference to the rapid equilibrium, linear-mixed inhibitory model generally found to apply to* cholinesterase inhibitors (Scheme 1; $\beta = 0$) [14,15,20].

Dixon plots (Fig. 2a) were nonlinear and pointed against Scheme 1. Hill plots according to Eq. (1) (Fig. 2b) indicated a minimum of 2 I-binding sites. The n values for MB, NB and MethB were 1.6 ± 0.27, 1.7 ± 0.19 and 1.6 ± 0.20,

$$\log[v/(v_o - v)] = -n\log[I] + \log K' \quad (1)$$

respectively. The data were then processed according to Scheme 2 (with $\beta = 0$) and the corresponding rapid equilib-