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SHORT COMMUNICATIONS

Evaluation of the stoichiometry and strength of chloroquine–porphyrin interactions by difference spectroscopy

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The interaction of chloroquine with porphyrins assumed clinical significance with the discovery that hydroxyferriporphyrin inhibited the antimalarial action of the drug [1, 2]. Subsequently, it has been shown that complex formation with porphyrin also provides a plausible molecular basis for the therapeutic value of chloroquine administration to patients suffering from porphyria cutanea tarda [3, 4]. In view of this clinical relevance, it is surprising that no serious attempt has been made to define the stoichiometry and strength of the interaction, which is readily detected by difference spectroscopy [1, 3, 4]. The aim of the present investigation is to employ this method for quantitative characterization of chloroquine–porphyrin interactions in phosphate-buffered saline, pH 7.4.

Materials and methods

Uroporphyrin (free base), coproporphyrin and haematoporphyrin (both dihydrochlorides) were obtained from Sigma Chemical Co., St. Louis, MO, who also supplied the chloroquine diphosphate. Stock solutions (10 mM) of the porphyrins were prepared in 10 M HCl, and aliquots (30 μl) neutralized with 5 M NaOH (60 μl) before dilution to 3 ml with a phosphate-buffered saline (137 mM NaCl–1.5 mM KH_2PO_4–2.7 mM KCl–6.5 mM Na_2HPO_4, pH 7.4). Concentrations of the resultant porphyrin solutions (100 μM) were determined spectrophotometrically using conditions and extinction coefficients specified by the supplier: uroporphyrin in 0.5 M HCl, $\varepsilon_{mM} = 541$ at 405 nm; coproporphyrin in 0.1 M HCl, $\varepsilon_{mM} = 398$ at 400 nm; haematoporphyrin in 1.5 M HCl, $\varepsilon_{mM} = 317$ at 402 nm. Mixtures (3 ml) containing porphyrin (10 μM) and chloroquine (0–450 μM) in the phosphate-buffered saline were prepared in cuvettes which were then placed in the cell compartment of a Varian Superscan 3 spectrophotometer, thermostatically maintained at 25°. Difference spectra were recorded using an identical concentration (10 μM) of the appropriate chloroquine-free porphyrin solution in the reference compartment. The magnitude of the peak spectral difference, ΔA, which occurred in the wavelength region 412–416 nm [1, 3, 4], showed a rectangular hyperbolic dependence upon chloroquine concentration [4]. This feature of the difference spectroscopy results signifies (i) linear dependence of the absorbance change (ΔA) upon the fraction of porphyrin sites that have interacted with chloroquine, and (ii) the sufficiency of a single intrinsic [5] dissociation constant, K_d, to describe the interaction of chloroquine with n equivalent and independent sites on the porphyrin. By the law of mass action it may readily be shown [6, 7] that for such a system the total concentrations of porphyrin (acceptor), $\bar{m}_A$, and chloroquine (ligand), $\bar{m}_S$, are related by

$$\bar{m}_S/\alpha = n\bar{m}_A + K_d/(1-\alpha) \qquad (1)$$

where α, the fractional saturation of porphyrin sites, may be obtained from the ratio of the observed absorbance difference (ΔA) to the maximal difference (ΔA_m) corresponding to site-saturation with chloroquine. In experiments with a fixed total concentration of porphyrin, the stoichiometry (n) and the dissociation constant (K_d) may thus be inferred from the intercept and slope, respectively, of a plot of $\bar{m}_S/\alpha$ versus $1/(1-\alpha)$.

Results and discussion

Figure 1 displays, in Scatchard format, the difference spectroscopy results obtained for the interactions of chloroquine with uroporphyrin (circles) coproporphyrin (squares) and haematoporphyrin (triangles) in phosphate-buffered saline, pH 7.4. Open symbols refer to preliminary plots with the total chloroquine concentration, $\bar{m}_S$, used as a first approximation to the required quantity, m_S, the free ligand concentration, any curvilinearity of these plots being a manifestation of this approximation rather than an indication of non-equivalent binding sites on the porphyrins. Nevertheless, such plots enable reasonable first estimates of ΔA_m, and hence of α ($= \Delta A/\Delta A_m$), to be made for use in equation (1), which in turn yields first estimates of n for uroporphyrin and coproporphyrin: the much weaker interaction of chloroquine with haematoporphyrin precludes such designation of n. The solid symbols in Fig. 1 represent replots of the results, for uroporphyrin and coproporphyrin, with the free ligand concentration determined from the expression $m_S = (\bar{m}_S - \alpha\bar{m}_A)$. From these Scatchard plots, which are indeed linear, it is evident that the maximal absorbance change, ΔA_m, for the uroporphyrin–chloroquine interaction is approximately 50% greater than that for the binding of the drug to coproporphyrin. However, this variation in ΔA_m is not the consequence of a difference in the stoichiometry of the two interactions, as is evident from Fig. 2, which presents the results in the format suggested by equation (1). The fact that the results are describable in terms of a common

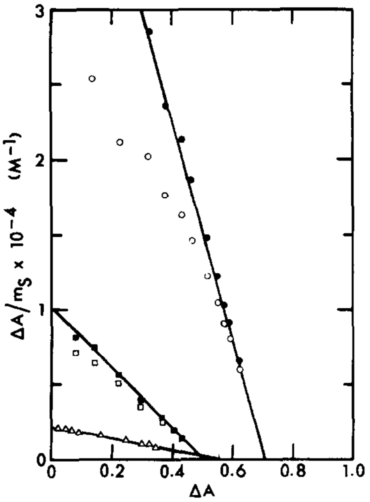

Fig. 1. Scatchard plots of difference spectroscopy results on the interaction of chloroquine with uroporphyrin (○, ●), coproporphyrin (□, ■) and haematoporphyrin (△) in phosphate-buffered saline, pH 7.4. Open symbols denote the plot obtained with the total chloroquine concentration ($\bar{m}_S$) taken as an initial estimate of m_S, the free concentration.

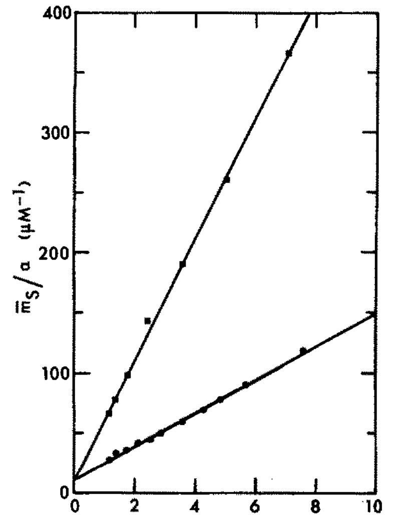

Fig. 2. Evaluation of the stoichiometry and strength of the interactions of chloroquine with uroporphyrin (●) and coproporphyrin (■), the results from Fig. 1 being plotted according to equation 1 with $\alpha = \Delta A/\Delta A_m$.

ordinate intercept of 10 μM, corresponding to the product $n\bar{m}_A$ in these experiments with 10 μM porphyrin, clearly signifies a 1:1 stoichiometry for the two porphyrin–chloroquine complexes. Least-squares calculations yield n = 1.0 (±0.1) and K_d = 14.0 (±0.3) μM for the uroporphyrin interaction; and n = 1.0 (±0.3) and K_d = 50 (±1) μM for the coproporphyrin–chloroquine system. The greater uncertainty in the estimate of n for the latter system reflects the fact that $\bar{m}_A << K_d$ for the coproporphyrin–chloroquine system [6, 7]. We note that the present results confirm the assumed value of unity for n in an earlier study of the coproporphyrin–chloroquine system [4], and hence validate the consequent value of 1 μM for K_d under conditions much lower in regard to ionic strength (0.05 M Tris–HCl, pH 7.2). The much smaller dissociation constant under those conditions indicates that an electrostatic interaction between the positively charged chloroquine molecule and the porphyrin carboxylate groups is a contributing factor to the strength of complex formation.

The most important feature of this investigation is the use of difference spectroscopy to elucidate the stoichiometry of complex formation between chloroquine and two porphyrins, the major difference between which is the existence of four and eight carboxyl groups on coproporphyrin and uroporphyrin respectively. Thus, although electrostatic factors affect the extent of the spectral changes [1] and the strength of the interactions (as discussed above in relation to the coproporphyrin–chloroquine system), the stoichiometry of the interaction between the cationic chloroquine and porphyrin is unaffected by the presence of the additional four negatively charged carboxyl groups on uroporphyrin: the strength of the interaction is, understandably, increased some threefold. Finally, although the stoichiometry cannot be established unequivocally for the interaction of chloroquine with haematoporphyrin, which possesses two carboxyl groups, the results are certainly consistent with a value of unity for n. Moreover, from the slope of the Scatchard plot (Fig. 1), the interaction is still weaker (K_d = 250 (±8) μM), a finding that also fits in with the concept that the number of negatively charged carboxyl groups on the tetrapyrrole structure has an important bearing on the strength of chloroquine–porphyrin interactions.

It is hoped that these quantitative investigations may lead to a better understanding of the therapeutic use of chloroquine in the treatment of porphyrias [3]; and also to an awareness of potential effects in the use of haematoporphyrin derivatives (HPD) in conjunction with phototherapy [9] to treat cancer in subjects also receiving antimalarial doses of chloroquine.

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Aging and stereospecific reactivation of mouse erythrocyte and brain acetylcholinesterases inhibited by soman

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Certain bispyridinium mono-oximes, so-called Hagedorn oximes (see Fig. 1 for chemical structures of oximes), when combined with atropine, have been reported as effective antidotes against intoxication by the nerve agent soman (1,2,2-trimethylpropyl methylphosphonofluoridate) in rodents [1–5] and dogs [6]. These oximes, although being effective in restoring *in vitro* the neuromuscular function of soman-poisoned rat diaphragm, hardly show any activity in restoring the neuromuscular function of human intercostal muscle after *in vitro* incubation with soman [7, 8]. In order to get more insight into the mechanism underlying the differences in effectiveness of the oximes between various