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# Structure–activity relationships for protein binding of a series of basic non-steroidal anti-inflammatory drugs

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Most non-steroidal anti-inflammatory drugs (NSAID)* are acidic compounds which are highly bound in plasma. They bind mainly to one of two specific drug-binding sites on human serum albumin (HSA), namely sites I and II [1, 2]. Basic drugs bind primarily to $\alpha_1$-acid glycoprotein or to lipoproteins in plasma [3]. However, we have shown recently that GP53,633, a basic NSAID, can bind to site I providing it is present in the unionised form [4]. The present communication reports studies on the structure–activity relationships for binding of a series of basic NSAID structurally related to GP53,633 (Fig. 1).

## *Materials and Methods*

GP53,633 and its analogues studied were donated by Ciba–Geiby Limited (Australia), who also supplied $^{14}C$-labelled GP53,633. $^{14}C$-Labelled warfarin was purchased from the Radiochemical Centre (Amersham, U.K.). $^{14}C$-Labelled ibuprofen and flurbiprofen were gifts from the Boots Co. Ltd. (Australia). The radiochemical purities of these compounds as stated by the suppliers were greater than 99%. In each case, the radiochemical purity of free drug (on the buffer side after equilibrium dialysis against human serum) was better than 98% [4].

HSA, essentially fatty acid free (Lot No. 110F-9350), 5-dimethylaminonaphthalene-1-sulfonamide (DNSA) and dansylsarcosine were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). The essentially fatty acid free HSA contained 0.04 mol fatty acid/mol of albumin, as determined by the method of Duncombe [5]. The *Mr* of albumin was taken as 66,500 [6]. Serum was obtained from Ortho Diagnostic Inc. (Lot No. 5S-225) and had a fatty acid content of 0.9 mol fatty acid/mol HSA.

All experiments were performed using 0.1 M sodium phosphate buffer, pH 7.4, containing 0.9% (w/v) NaCl, unless otherwise stated. Fluorescent probe techniques were used to study the displacement of marker probes (DNSA, warfarin and dansylsarcosine) binding to specific binding sites on HSA as previously described [7]. Fluorescence measurements were made at room temperature (22°) using a Perkin–Elmer model MP-3000 spectrofluorometer. Binding was performed by equilibrium dialysis using a Dianorm® apparatus at 37° as described earlier [4]. The concentrations of GP53,633 analogues after dialysis were analysed by HPLC. The mobile phase consisted of acetonitrile and 10 mM trisodium citrate, pH 3.0 (30:70, v/v). The flow rate was 2 mL/min. Absorbance was monitored at a wavelength of 254 nm. Sample (0.2 mL) was mixed with 0.1 mL of acetonitrile and centrifuged for 2 min. An aliquot of 0.07 mL of clear supernatant was injected onto the HPLC column (Waters $\mu$-Bondapak C-18 reversed-phase). The concentration of compound was determined by comparison of the peak height with a calibration curve for each compound.

The $pK_a$ of GP53,633 and its analogues was measured by the UV titration method at a wavelength of 200 nm [4]. The change in UV absorbance with pH indicates the ionisation of a titratable chromophore, probably the imidazole moiety. The *n*-octanol/water partition coefficients of GP53,633 and its analogues were determined by a flask-shaking method [8], using 0.1 M NaOH. After partitioning and centrifugation, concentrations of the compounds were analysed by the HPLC method described earlier.

## *Results and Discussion*

*Binding of GP*53,633 *analogues to HSA and diluted serum*. Eight compounds having chemical structures similar to GP53,633 were studied (Fig. 1). Compounds **1** and **2** are GP53,633 and its known metabolite CGP8716 respectively. Apparent $pK_a$ values for **2**, **3**, **4** and **7** were determined by UV absorption and were all found to be very similar to that for GP53,633 (pH 6.4). The apparent $pK_a$ for **9**, however, was higher ($pK_a$ of 7.1).

Figure 1 illustrates the binding of GP53,633 analogues to pure HSA and to diluted serum at the same albumin concentration of 100 $\mu$M. Binding of phenylbutazone is also shown for comparison. The binding to HSA of GP53,633 and most of its analogues was generally greater than that of phenylbutazone. This suggests that the analogues would be highly protein bound in plasma with albumin as the major binding protein. The binding of all compounds was higher with serum than with fatty acid free HSA and the HSA to serum free fraction ratios (HSA/Serum) ranged from 3.78 (**7**) to 1.04 (**9**). This suggests that, as with GP53,633 [4], the binding of the analogues is increased by the presence of fatty acids.

*Structure–activity relationships for binding of GP*53,633 *analogues*. The binding of GP53,633 and most of its

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* Abbreviations: NSAID, non-steroidal anti-inflammatory drugs; HSA, human serum albumin; GP53,633, 2-*tert*,butyl-4(5)phenyl-5(4)-(3-pyridyl)-imidazole (Ciba–Geigy); and DNSA, 5-dimethylaminoaphthalene-1-sulfonamide.

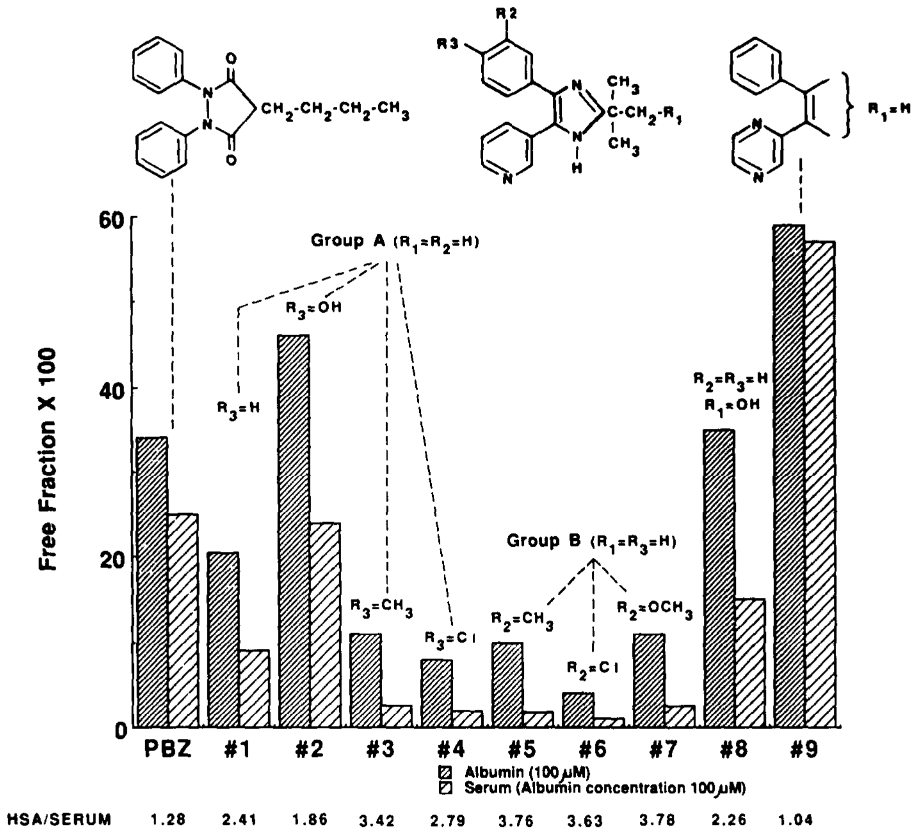

Fig. 1. Binding of phenylbutazone (PBZ) and GP53,633 analogues to albumin. Essentially fatty acid free HSA (100 μM) and serum diluted to an albumin concentration of 100 μM were used. The structures of the compounds are also shown. They were each added into the buffer side at an initial concentration of 50 μM and dialysed against albumin solution. Essentially fatty acid free HSA contained 0.04 mol fatty acid/mol albumin. The serum contained free fatty acids at a molar concentration ratio with albumin of 0.9:1.

Table 1. Displacement of marker drugs for sites I and II by GP53,633 analogues as measured by dialysis

| | Free fraction as % control | | | |
|---|---|---|---|---|
| | Site I | | Site II | |
| Compound | Warfarin | GP53,633 | Ibuprofen | Flurbiprofen |
| **1** (GP53,633) | 144 | — | 64 | 83 |
| **2** (CGP8716) | 121 | 150 | 81 | 96 |
| **3** | 166 | 199 | 71 | 97 |
| **4** | 152 | 172 | 82 | 94 |
| **5** | 161 | 227 | 64 | 91 |
| **6** | 181 | 238 | 64 | 85 |
| **7** | 173 | 232 | 72 | 99 |
| **8** | 134 | 177 | 85 | 89 |
| **9** | 114 | 142 | 90 | 95 |

Essentially fatty acid free HSA (fatty acid content 0.04 mol/mol albumin) was used. HSA (100 μM) was dialysed against buffer containing 10 μM warfarin, GP53,633, ibuprofen or flurbiprofen with or without the addition of other compounds (100 μM). The free fractions of the marker drugs in the absence of other compounds were: 0.073 (warfarin), 0.145 (GP53,633), 0.029 (ibuprofen) and 0.007 (flurbiprofen). Numbers of the compounds refer to those in Fig. 1.

analogues was markedly greater than that of phenylbutazone (Fig. 1), suggesting that the bulkier alkyl aliphatic side chain (*tert.*butyl) and/or the substitution of an imidazole moiety for the pyrazolidine dione ring may increase binding to site I on HSA. Substitution of —OH for —H either on the phenyl ring or on the alkyl side chain, decreased the binding to HSA (i.e. **2** and **8**). This may be due to a decrease in hydrophobicity or an increase in hydrogen bonding of the compounds containing an —OH group with the solvent water molecules.

Methyl ($—CH_3$) substitution at position R3 or R2 on the phenyl ring (i.e. compounds **3** and **5**) increased the binding to albumin when compared to the parent compound GP53,633. A substitution of a —Cl group at either the meta- or para-positions on the phenyl ring resulted in an increase in binding as shown with compounds **4** and **6**. The substitution of $—CH_3$ or —Cl on the phenyl ring had little effect on binding. Compound **7** with a methoxy ($—OCH_3$) group had a similar degree of binding to **5**, suggesting that methoxy and methyl substituents have similar effects on binding.

Of interest, compound **9** has a structure similar to GP53,633, except that it has a pyrazine ring instead of a pyridyl ring. The degree of binding of **9** to HSA was much lower than that of GP53,633. This may be explained by the greater apparent $pK_a$ value of 7.1 for **9**, resulting in a reduction of the unionised form at pH 7.4, as it has been shown that only the *unionised* form of GP53,633 binds to albumin [4].

*Binding site for GP*53,633 *analogues*. The patterns of displacement of fluorescent probes by the analogues were similar to that for GP53,633 itself [4]. All of the analogues caused a marked decrease in the fluorescence of site I probes (warfarin and DNSA) but none produced a significant change in the fluorescence of dansylsarcosine. These results are consistent with data obtained from equilibrium dialysis studies (Table 1). All of these compounds added to HSA at a concentration ratio of 1:1 with albumin caused a significant displacement of the site I marker drugs, warfarin and GP53,633, whereas there was no change or a slight increase in the binding of ibuprofen and flurbiprofen at site II. These results, therefore, indicate that all of the GP53,633 analogues are bound primarily to site I on HSA.

*Binding of GP*53,633 *analogues and hydrophobicity*. Many investigators have reported the importance of hydrophobicity in drug binding to albumin [9–16] and have suggested that binding energy is governed mainly by hydrophobic interactions. There was a good correlation ($r = -0.958$; $P < 0.001$) between the degree of binding of the analogues and their hydrophobicity as measured by log partition coefficients of the unionised species (Fig. 2). There were also good correlations between hydrophobicity (log P) and ability to displace the site I marker drugs, warfarin ($r = 0.922$; $P < 0.005$) and GP53,633 ($r = 0.875$; $P < 0.05$). This suggests that, as with other classes of drugs, hydrophobic interactions play an important role in the binding of GP53,633 analogues to albumin. The pH titration data suggest that these compounds are largely unionised at physiological pH so that electrostatic interactions are not likely to be involved in binding.

The results presented in this report show that the basic NSAID, GP53,633 and its analogues all bind to albumin at drug binding site I. The fluorescent probe and dialysis studies and the effects of fatty acids and pH on the binding of GP53,633 [4] are all consistent with this view. Binding at this site is usually associated with acidic drugs but the results show that basic compounds can also bind with high affinity to site I as long as they are in the *unionised* form. A negative charge is not necessary for binding but a positive charge precludes binding at this site and this is consistent with the presence of a cationic group on the protein surface close to or within site I. From this it follows that the major part of the binding energy at site I is due to hydrophobic interactions, although for acidic drugs electrostatic interactions may also play some role [4, 17].

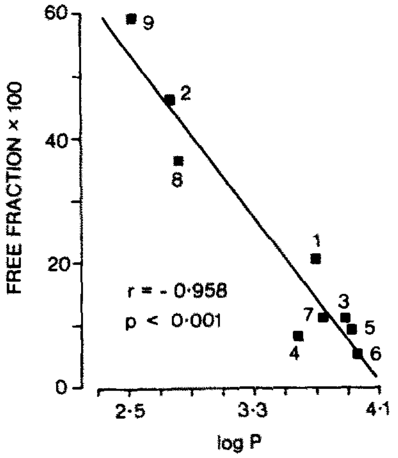

Fig. 2. Correlation between the free fraction of GP53,633 analogues and log partition coefficient (log P). Free fraction values in HSA (100 μM) are those from Fig. 1. Partition coefficients were measured in *n*-octanol/water (pH 11). Numbers of the compounds refer to those in Fig. 1 and *r* is the correlation coefficient.

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# Inhibition of serine esterases in different rat tissues following inhalation of soman

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The acute toxic effects of organophosphates (OPs) are due to inhibition of acetylcholinesterase (AChE) (EC 3.1.1.7). The inhibition of other serine esterases, such as butyrylcholinesterase (BuChE) (EC 3.1.1.8) and carboxylesterase (CarbE) (EC 3.1.1.1) does not induce any known physiological alterations. Recently, BuChE has been shown to coregulate acetylcholine lifetime in canine trachealis muscle and may therefore in some tissues play a role in the breakdown of acetylcholine (ACh) [1]. BuChE and especially CarbE may, however, be important for detoxification of low doses of OPs in rodents, since they have a high plasma concentration of CarbE [2–4].

A good correlation between the concentration of plasma CarbE and the LD$_{50}$ of the OP soman in the developing rat has previously been shown [5]. Furthermore, injection of partially purified rat liver CarbE into 14-day-old rats increased the tolerance to soman, indicating that CarbE in plasma may be of great importance for the detoxification of organophosphorus compounds. CarbE may thereby function as a very important barrier which limits the distribution of the toxic agent to vital organs [6], since the difference in plasma concentration of CarbE between different species correlates with the difference in LD$_{50}$ [7].

The aim of the present work was to elucidate whether CarbE in respiratory tissue and plasma plays an important role in detoxification of soman during inhalation exposure.

## *Materials and Methods*

*Chemicals.* [1-$^{14}$C]Acetylcholine chloride ([$^{14}$C]ACh) was purchased from Amersham International (Bucks, U.K.). Ethopropazine (10-[2-(diethylamino) propyl]-phenothiazine) and 4-nitrophenyl butyrate were from the Sigma Chemical Co. (Poole, U.K.). Soman (*O*-[1,2,2-trimethyl-propyl]-methyl-phosphonofluoridate), assessed to be more than 99% pure by nuclear magnetic resonance spectroscopy, was synthesized in our laboratory. All other chemicals were of analytical laboratory reagent grade.

*Inhalation method.* Whole body exposures of male Wistar rats (200–300 g) (Møllegard, Copenhagen) to sub-acute concentrations of the acetylcholinesterase inhibitor soman were carried out in a dynamic inhalation system designed specifically for the exposure of small rodents to highly toxic gases [8]. Two rats were exposed simultaneously in a glass chamber of 2200 mL, the atmospheric concentration of soman was measured by gas chromatography (Carlo Erba, HRGC 5160) with a nitrogen/phosphorus detector.

No symptoms of poisoning were observed during the inhalation period.

*Enzyme activity assays.* The total cholinesterase (ChE) activity was determined by the radiochemical method of Sterri and Fonnum [9] at 30°. The AChE activity was measured after inhibition of BuChE by 0.2 mM ethopropazine [10]. The CarbE activity was measured by the spectrophotometric method of Ljungquist and Augustinsson [11] with modifications [12]. The protein concentration was determined by the method of Lowry *et al.* [13].

Means and standard error of the mean (SEM) were calculated for all data. The Student's *t*-test was used to assess the significance of the differences between data groups.

## *Results and Discussion*

The airways and lungs are the first tissues exposed to toxic gases and vapours and are also the primary uptake sites for some OPs. The results from this study show that long-term exposure to low concentrations of soman primarily inhibits the cholinesterases of the respiratory tissue, plasma and erythrocytes and the CarbE of plasma and airways (Table 1). Although there were no symptoms