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Non-covalent binding of some phenothiazine drugs to DNA

N.J. de Mol and J.P.A.C.M. Maanders

Department of Pharmacochemistry, Subfaculty of Pharmacy, State of University of Leiden, Gorlaeus Laboratories, Wassenaarseweg 76, P.O. Box 9502, 2300 RA Leiden (The Netherlands)

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Summary

The binding of a number of phenothiazine drugs (PHs) to calf thymus DNA was studied with equilibrium dialysis. The Scatchard plots reveal two types of binding. Relatively strong binding is observed at low PH concentration. For various PHs the apparent binding constants do not differ significantly, but the binding capacity of DNA varies with the 2-substituent: 2-chloro-PHs exhibit the highest extent of strong binding. For this binding type the DNA double-helix structure is essential, suggesting that (partial) intercalation of the PH-nucleus between two adjacent DNA base-pairs plays a role. At higher PH-concentrations the apparent binding constants become very small. In this PH-concentration range the structure of the side-chain of the PH appears to be of importance for the DNA-binding.

Compounds with a piperazine ring in the side-chain show cooperative binding in which next to electrostatic interactions also formation of aggregates may be important.

Introduction

The phenothiazines (PHs) with chlorpromazine (CPZ) as the most important representative, form a range of structurally related neuroleptic drugs which vary in 2- and 10-substituents.

A study of the interactions between PHs and DNA is relevant with regard to some aspects of the biological activity of these drugs. CPZ and also other PHs are notorious phototoxic drugs exhibiting also photo-induced genetic effects (Kelly-Garvert and Legator, 1973; Jose, 1979; Fujita et al., 1981a; Yonei and Todo, 1982; Mooibroek and Venema, 1982). Photo-induced covalent binding of CPZ to DNA

has also been reported (Kahn and Davis, 1970; Fujita et al., 1980, 1981b; Ben Hur et al., 1980). In these photo-induced phenomena complex formation of PHs to DNA previous to irradiation may be an important, stimulating factor, as in this case DNA is an excellent target for PHs in the excited state or for their photoproducts. Such DNA–drug complexes play also an important role in the photobiological activity of furocoumarins (de Mol et al., 1981). Reports about dark genetic effects are more exceptional and less explicit: Nielsen et al. (1969) reported a significantly higher frequency of chromosome gaps, breaks and hypodiploid cells in patients treated with perphenazine. Jenkins (1970) reported a higher incidence of chromatid breakage in human peripheral blood cultures derived from patients under PH treatment. These effects were thought to be connected with interference of CPZ with the structure of DNA, or with DNA metabolism. Contradictory to these reports, Cohen et al. (1969, 1972) reported that CPZ and some other PHs did not produce observable damage to the chromosomes of human leukocytes in vivo or in vitro.

Talukder (1975) also noticed no increase in incidence of chromosome aberrations in PH-treated human leukocytes. Recently (Kittle et al., 1981) it was suggested that interactions between CPZ and DNA might block the intercalation of benzo[α]pyrene metabolites in DNA, inducing in this way an inhibitory effect on the carcinogenicity of benzo[α]pyrene.

Thus far several reports are concerned with the actual physicochemical aspects of the dark interactions between PHs, especially CPZ, and nucleic acids (Ohnishi and McConnell, 1965; Waring, 1970; Yamabe, 1973; Kantesaria and Marfey, 1975; Porumb and Slade, 1976). Binding of CPZ via intercalation as a special type of interaction has remained controversial, also because of the non-planar conformation of the PH nucleus. In the present paper a study of the non-covalent binding of a number of PHs with calf thymus DNA is reported. The extent of DNA binding was determined from equilibrium dialysis experiments. The nature of the binding was further studied with denaturated DNA, viscosity measurements and UV-absorption changes. The relation between the structure of the investigated PH-drugs and the non-covalent DNA binding is discussed.

Materials and Methods

Materials

The following phenothiazines were pure (pharmaceutical quality) and used as purchased: chlorpromazine-HCl (CPZ), Specia; promethazine-HCl (PMZ), Brocacef. Triflupromazine-HCl (TPZ) and fluphenazine-2 HCl (FPZ) were gifts of Squibb. Prochlorperazine-bimethanesulfonate (PCP) was a gift of Rhône-Poulenc. DNA was highly polymerized sodium salt from calf thymus (Type I, Sigma Chemicals). $\epsilon_{(P)}^{260} = 6600\ l \cdot mol^{-1} \cdot cm^{-1}$. 0.1 SSC buffer was 0.015 M sodium chloride and 0.0015 M sodium citrate, pH 7.4. Buffer was made in double-distilled water.

Equilibrium dialysis

Solutions of PHs in 0.1 SSC buffer were made in a concentration range of 0.05–1

mM. For each experiment a fresh DNA solution was made in 0.1 SSC. To obtain a good fit of separate experiments on the Scatchard curve the nucleotide concentration of the DNA solution was determined according to Nakamura (1952). The nucleotide concentration was usually ~ 2 mM. Dialysis was performed with an Equilibrium Dialyser Dianorm (Diachema A.G.). Of each 5 ml dialysis cell one compartment was filled with 4 ml of the PH-solution and the other compartment with 4 ml DNA solution. The compartments were separated by a dialysis membrane (High Permeability, MW cut-off 10,000, diameter 63 mm; Diachema A.G.) which had been thoroughly shaken various times in 0.1 SSC. As in some cases the PH was too poorly soluble to reach a high r-value, the DNA was then dissolved in PH solution. To determine binding of PHs to the dialysis membrane, control experiments were performed, in which the DNA solution was substituted by 0.1 SSC. The dialyzer was placed in a thermostatted bath (37 ± 0.1°C). After ~ 4 h equilibrium was reached. The free-PH concentration in solution from the non-DNA compartment was determined spectrophotometrically (Zeiss PMQ III equipped with Obtilab Multiblank 171 and Multilog 311): PH, λ (nm), ϵ_λ ($l \cdot mol^{-1} \cdot cm^{-1}$); CPZ, 308, 3880; PMZ, 301, 3390; TPZ 307, 3550; FPZ, 310, 3470; PCP, 311, 4060. After correction for membrane-bound PH, the amount of DNA-bound PH was obtained. In the Scatchard plot the ratio of DNA-bound PH molecules to DNA nucleotides (r) is plotted vs r/c with c being the concentration of free PH.

Denaturated DNA was obtained by heating 10 ml of a ~ 2 mM DNA solution in boiling water during 10 minutes and rapidly cooling on ice.

Viscosity determinations

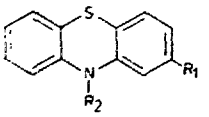
To obtained small DNA fragments for viscosity measurements, a DNA solution in 0.1 SSC with nucleotide concentration of ~ 3 mM was cooled on ice and sonicated during 3 min at amplitude 0.8 μm (MSE Ultrasonic Disintegrator 150 W). $\overline{M}$ of the DNA fragments was 6×10^5 dalton (determined according to Kantesaria and Marfey, 1975). Solution containing sonicated DNA (nucleotide concentration ~ 0.6 mM) and PH in a concentration range of 0–0.4 mM were made in 0.1 SSC. The viscosity of these solutions was measured in an Ubbelohde viscosimeter type OC at a temperature of 25 ± 0.05°C after 2 h incubation. The intrinsic viscosity (η) was calculated using the value of 0.36 for Huggins' coefficient (Kantesaria and Marfey, 1975).

Results and Discussion

In this study a number of clinically used PH-derivatives was investigated (Table 1). The results of the equilibrium dialysis experiments are represented in the Scatchard plots (Fig. 1). The Scatchard plots for CPZ–DNA binding (see also Fig. 2) and that for PMZ show a biphasic curve with relatively strong binding at low and weak binding at higher r-values. This type of Scatchard plot with relatively large differences in apparent binding constants (i.e. slope of the Scatchard curve) for various r-values, indicates that two binding types are involved (Dougherty and

TABLE I

STRUCTURE OF THE INVESTIGATED PHENOTHIAZINES



compound	R_1	R_2
chlorpromazine (CPZ)	Cl	$-CH_2-CH_2-CH_2-N(CH_3)(CH_3)$
promethazine (PMZ)	H	$-CH_2-CH(CH_3)-N(CH_3)(CH_3)$
triflupromazine (TPZ)	CF_3	$-CH_2-CH_2-CH_2-N(CH_3)(CH_3)$
fluphenazine (FPZ)	CF_3	$-(CH_2)_3-N$ piperazine $N-CH_2-CH_2OH$
prochlorperazine (PCP)	Cl	$-CH_2-CH_2-CH_2-N$ piperazine $N-CH_3$

Pigram, 1982). For the occurrence of the relatively strong binding at low r-values the double-helix structure of DNA is essential, as strong binding is not observed with denaturated DNA (Fig. 2). This suggests that (partial) intercalation of the phenothiazine nucleus is responsible for this type of binding. However, as the phenothiazine nucleus is folded along the S–N axis (folding angle from crystallographic data: 139.4° (McDowell, 1974), total insertion between two adjacent DNA base-pairs is not likely, because of steric hindrance. The apparent binding constants for the strong

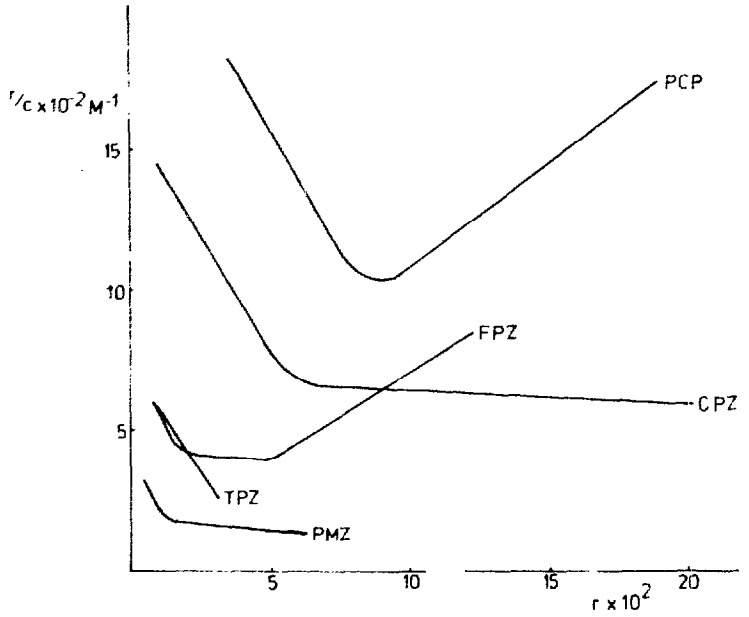


Fig. 1. Combined Scatchard plots of binding of some phenothiazine drugs to calf thymus DNA in 0.1 SSC at 37.0°C.

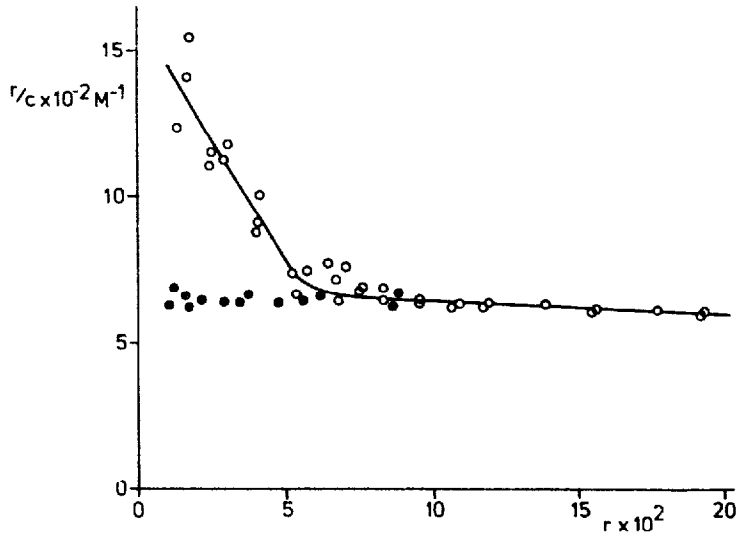


Fig. 2. Scatchard plots of CPZ binding to calf thymus DNA in 0.1 SSC at 37.0° C. ○, native DNA; ●, denatured DNA.

binding of the investigated PHs (Table 2) are much less than that for excellent intercalating species such as for instance acridines, which are structurally related to the PHs, but possess a flat aromatic nucleus: for the binding of proflavine (2,8-diaminoacridine sulphate) with herring sperm DNA an apparent binding constant of 1.3×10^6 M^{-1} is reported, corresponding to a change in standard free energy (ΔG) of 35.2 kJ (Peacocke and Skerrett, 1956). For CPZ with an apparent binding constant of 1.7×10^4 M^{-1}, ΔG is 24.3 kJ/mol (Kantesaria and Marfey (1975) reported ΔG for CPZ is 24.7 kJ/mol). However, in the DNA binding of acridines also a positive charge near or on the acridine nucleus is a contributing factor for DNA binding (Albert, 1975). The apparent binding constants for strong binding of the various investigated PHs do not show significant differences (Table 2). However,

TABLE 2

BINDING PARAMETERS FROM SCATCHARD PLOTS

Compound	Strong binding $k \times 10^{-4}$ (M^{-1})+S.D.	n	Weak binding $k \times 10^{-3}$ (M^{-1})±S.D.
chlorpromazine (CPZ)	1.6±0.2	18	0.5±0.1
promethazine (PMZ)	1.2±0.2	70	0.5±0.2
fluphenazine (FPZ)	1.9±0.3	65	0.4±0.1 and cooperative binding
triflupromazine (TPZ)	1.5±0.2	20 < n < 35	poor solubility
prochlorperazine (PCP)	1.4±0.2	12	cooperative binding

n = number of nucleotides/number of bound phenothiazine molecules when strong binding is maximum (taken from the inflection point of the Scatchard curve, n = 1/r).

the maximum number of occupied binding sites for strong binding (n), as derived from the inflection points of the Scatchard curves, varies markedly (Table 2 and Fig. 1).

Although the number of investigated compounds is limited, the Scatchard curves suggest some interesting relations between the structure and the DNA binding of the investigated phenothiazines. In the relatively strong binding at low r-value, the 2-substituent seems to be important: it appears that the two compounds with a 2-chlorosubstituent (CPZ and PCP) exhibit the largest binding capacity. The strong binding of the compounds with a 2-CF_3 group (TPZ and FPZ) is equal. If the substituent at the 2-position is important for the binding at low r-value, this means that especially the phenothiazine nucleus is involved in this binding. This supports the concept that (partial) intercalation is involved in this binding. In the weak binding process at higher r-value, the structure of the side-chain seems to be important. Compounds with a N,N-dimethyl amino alkyl side-chain show a small apparent binding constant of $\sim 0.5 \times 10^3$ M^{-1}, $\Delta G = 15.5$ kJ/mol. For TPZ no data at high r-values could be obtained because of poor solubility.

The Scatchard plots of PCP and FPZ, both with a longer, piperazine-containing side-chain, show an upward curve at higher r-value (Fig. 1). In the case of FPZ this upward curve starts at $r > 0.05$ after weak binding. An upward Scatchard curve is usually ascribed to cooperative binding (i.e. the attachment of further molecules is favoured by already bound molecules (Dougherty and Pigram, 1982)). The usually observed optimum in the Scatchard plot is not reached, because of precipitation of PH–DNA complexes at higher PH concentrations. This precipitation is a known phenomenon, connected with conformational changes in DNA, which are induced by interactions between negative DNA phosphate groups and the positive PH side-chain (see also Hele, 1963). From the plot of r vs the free-PH concentration (Fig. 3), the cooperative binding characteristics appear once more (Blake and Peacocke, 1968).

In the weak binding process at higher r-values, electrostatic interactions between the positively charged side-chain and negatively charged phosphate groups of DNA are likely to play a role. Such interactions are important in the DNA binding of amino alkyl-substituted dyes (Albert, 1975). Next to electrostatic interactions also aggregation of PHs with already bound drug molecules may occur especially in the case of cooperative binding. Dimer formation of PHs in solution is a known process (Borg and Cotzias, 1962; Felmeister et al., 1965). At r-values > 0.2 the UV spectrum of PCP in the presence of DNA shows a shift in λ_{max} from 311 to 314 nm with simultaneously a slight increase of molar absorptivity. The UV absorption spectrum of PCP without DNA being present did not show such characteristics but obeyed Beer's Law in the relevant concentration range (0–0.25 mM). This does not explicitly mean PCP does not form dimers. It has been reported that formation of CPZ-dimers in solution does not induce UV-spectral changes (Borg and Cotzias, 1962). So, if the cooperative binding is caused by aggregation, the nature of the aggregates must be distinct from dimers formed in solution.

The hydrodynamic behaviour of PH–DNA complexes was also studied. In Fig. 4 is shown the viscosity of CPZ- and PCP–DNA complexes in dependence of r. The

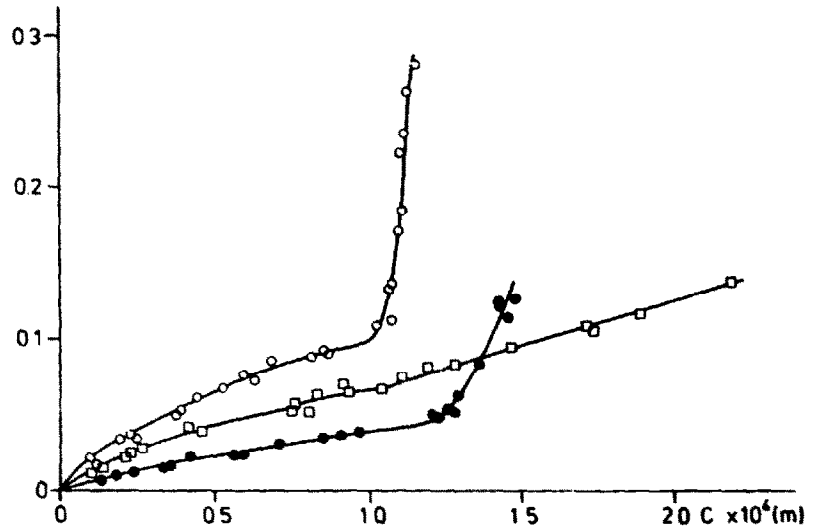


Fig. 3. Binding of PCP (○), FPZ (●) and CPZ (□) to calf thymus DNA in 0.1 SSC at 37°C. c = free-phenothiazine concentration.

initial increase of the viscosity at low r-values coincides with the strong binding process and is possibly caused by the intercalation-like nature of this binding: intercalation is supposed to proceed with a local unwinding of the DNA double-helix, which increases the contour length of the DNA fragments and hence the viscosity of the DNA solution (Drummond et al., 1966; Waring, 1970).

However, one should be careful to ascribe increased viscosity of DNA complexes to binding of merely the intercalation type (van Duuren et al., 1969). At higher

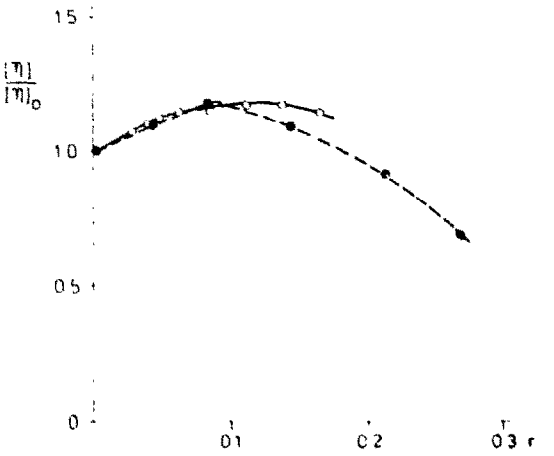


Fig. 4. Effect of DNA binding of CPZ (○) and PCP (●) on the viscosity of DNA in 0.1 SSC at 25.0°C. Nucleotide concentration = 0.63 mM. r-Values are obtained from the Scatchard plots by adjusting for the present nucleotide concentration assuming that r is determined only by the free-PH concentration and independent of the nucleotide concentration.

r-values the viscosity of the PH–DNA complexes decrease after an initial increase (Fig. 4). The inflection points coincide with the inflection points of the Scatchard plots. This illustrates the different influence of the binding mode on the hydrodynamic behaviour of the PH–DNA complexes. The decreased viscosity at higher r-values, especially for PCP, supports the hypothesis that the cooperative binding is caused by formation of aggregates to already bound PCP molecules: the aggregated molecules might contribute to a further decrease of the mutual repulsion between the charged phosphate groups. This causes a decrease in the contour length of the DNA fragments and hence a decreased viscosity. A comparable effect is reported by Drummond et al. (1966) who measured the influence of ionic strength of denaturated and native DNA.

In the case of PMZ no effect on DNA viscosity could be observed, probably because of the poor capability of this compound to bind to DNA. In conclusion, different types of binding of PHs to DNA can be distinguished. The amount with which each of these binding types contributes to the total DNA binding depends on the chemical structure of the PH-derivative. The 2-substituent on the phenothiazine nucleus seems to be important for the relatively strong binding at low r-values. For this binding the DNA double-helix structure is essential. These facts and the increased viscosity of the DNA–PH complexes at low r strongly suggest that (partial) intercalation of the PH-nucleus in the DNA is responsible for this binding. At higher r-values especially the side-chain seems to be involved in the DNA binding, for which electrostatic interactions and formation of aggregates might be important. The latter phenomenon can especially play a role in the cooperative binding found with PCP and FPZ.

The formation of complexes between PHs and DNA might be important for some of the biological effects of these drugs. The PHs are potent phototoxic and photomutagenic drugs and the closeness of these drugs and DNA makes the latter a likely target for photo-activated PH molecules or reactive intermediates derived from them.

Furthermore, CPZ may be metabolically converted to the intermediate CPZ radical cation (Sackett et al., 1981). This radical is stabilized by DNA (Ohnishi and McConnell, 1965; de Mol et al., 1983) and may induce genetic damage (de Mol et al., 1983). On the other hand, the interactions between PHs and DNA may also have beneficial effects. The present study might also have some relevance for the observed PH-induced decreases in mutagenicity of benzo[α]pyrene (Kittle et al., 1981). Although the mechanism of the anti-mutagenic activity is not known yet, it is possible that complexed phenothiazines block the intercalation of benzo[α]pyrene metabolites (Kittle et al., 1981).

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