Nuclear Magnetic Resonance Studies of the Interaction of Chloroquine Diphosphate with Adenosine 5′-Phosphate and Other Nucleotides

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SUMMARY

The interaction of the antimalarial drug chloroquine diphosphate, 7-chloro-4-(4-diethylamino-1-methylbutylamino)quinoline diphosphate, with nucleotides has been studied by means of nuclear magnetic resonance spectroscopy. Certain of the proton resonances of the drug and the nucleotides are shifted as a result of the interaction.

This study shows that ring interactions occur in mixtures of chloroquine diphosphate and purine nucleotides, but appear to be minimal in drug mixtures containing pyrimidine nucleotides. Adenosine 5′-triphosphate, in contrast to adenosine 5′-phosphate, causes shifts of the chloroquine side chain proton signals in addition to those of the ring, suggesting an electrostatic interaction due to the presence of the additional phosphate groups. Interactions are decreased sharply at elevated temperatures. The differences observed between the shifts in proton signals of chloroquine in chloroquine-nucleotide mixtures and those observed at high concentrations of the drug alone suggest that the major interactions are between drug and nucleotide.

INTRODUCTION

The world-wide threat of malaria continues to stimulate interest in the mode of action of antimalarial drugs and a search for new and more effective agents. At present, chloroquine, 7-chloro-4-(4-diethylamino-1-methylbutylamino) quinoline, is the drug being used and studied most extensively. Irvin, Irvin, and Parker first suggested in 1949 (1) that this compound interacts with nucleic acids, and recent work has focused on this interaction as a possible mechanism of drug action (2–4).

Cohen and Yielding studied the mechanism of chloroquine-DNA interaction spectrophotometrically and proposed that binding involves both electrostatic attraction between the protonated chloroquine ring and the anionic phosphate groups of DNA, and a more specific interaction between the aromatic rings of chloroquine and the nucleotides. These studies indicated a preferential interaction with the purines (both adenine and guanine) in the DNA chain. Blodgett and Yielding (5), in a study of drug binding to polyadenylate and polyguanylate, found that chloroquine binds to these two purine polymers equally well.

Hahn *et al.* (4) also studied the mode of action of chloroquine on DNA and related polymers. They proposed that the 7-chloroquinoline ring of the drug binds preferentially to guanine in DNA, and that the diamino aliphatic chain in chloroquine is bound to the phosphoric acid backbone. They also concluded from viscosity measurements, sedimentation experiments, and dichroism data that the chloroquine-DNA complex is formed by intercalation of the ring systems.

During recent years nuclear magnetic resonance spectroscopy has been used to investigate the structure (6), conformation (7), and inter- and intramolecular interaction of nucleosides and nucleotides (8–

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10). In these studies, the influence of pH, temperature, concentration, and ring stacking of the bases on the chemical shift has been considered.

We decided, therefore, to study the interaction of chloroquine with nucleotides by NMR spectroscopy: (a) to provide direct evidence concerning ring-ring interactions between chloroquine and nucleotides; (b) to investigate the nucleotide specificity with respect to purine (adenine and guanine) and pyrimidine (thymine and cytosine) bases, and the influence of the degree of phosphorylation (nucleoside mono- and triphosphates); and (c) to observe the effect of temperature on the chloroquine-nucleotide interaction.

METHODS

NMR spectra were recorded with a Varian A-60A spectrometer equipped with a variable temperature probe and the V-6040 variable temperature controller, and with a Jeolco JNM-C-60H spectrometer. Normal probe temperature for the Varian instrument was 41° ± 1°, and for the Jeolco machine, 23° ± 1°. Chemical shifts were measured from TSPS,[1] used as an internal standard. These measurements were accurate to ±0.02 ppm.

Determination of apparent pH values in D_2O solvent were made with a digital direct-reading Sargent pH meter, model DR S-30000, equipped with a Sargent miniature combination S-30070-10 electrode.

Materials. The nucleotides were obtained from Sigma Chemical Company as sodium or disodium salts and were used without further purification.

Chloroquine phosphate was a product of Sterling-Winthrop Research Institute; D_2O, of Volk Radiochemical Company; and TSPS, of Merck and Company, Darmstadt, West Germany.

Sample preparation. Samples were dissolved in D_2O which contained about 3 drops of a 1% internal standard solution per milliliter. The apparent pH values of the different sample solutions were in the range 7.6–8.0. A 2 M KOD solution was used to adjust the apparent pH. Chemical shifts are reported in parts per million relative to TSPS.

[1] The abbreviation used is: TSPS, 3-(trimethylsilyl)propanesulfonic acid sodium salt.

RESULTS

Analysis of spectra. Figure 1 is a spectrum of 10% chloroquine phosphate in D_2O at an apparent pH of 7.6, showing the proton assignments which were made.

The group of five bands in high field between 1.25 and 1.57 ppm is due to 9 protons. These bands were assigned to the three methyl groups on the side chain (*d*) on the basis of the following reasoning. The two methyl groups adjacent to methylenes should be magnetically equivalent, and their resonance signal should be split into a triplet. The signal of the methyl group adjacent to the methyne should appear as a doublet. Thus, the total signal from all the methyl groups should be a quintet, as was observed. If the resonance lines are numbered from high to low field, then lines *1*, *2*, and *4* would seem to be the triplet of the methyl groups adjacent to methylene, and bands *3* and *5*, the doublet of the single methyl group adjacent to methyne. The coupling constants are 7.5 Hz for the triplet and 7.0 Hz for the doublet.

The broad band from 1.75 to 2.08 ppm is due to 4 protons. It was assigned to the two methylene groups (*b*) between the methyne (*a*) and one of the methylenes (*c*). This assignment seemed reasonable, since these methylene groups are the only remaining unassigned aliphatic groups *not* adjacent to nitrogen, and their signal thus should appear at high field.

The quartet between 3.13 and 3.50 ppm, produced by 6 protons, was assigned to the three methylene groups (*c*) attached to the nitrogen of the side chain. The signal should be shifted downfield with respect to the signal of the other methylenes, owing to the proximity of nitrogen. The coupling constant of the quartet, 7.5 Hz, agrees with the coupling constant of the adjacent methyl groups. Coupling of one of these methylenes with the methylene (*b*) should produce a triplet, which apparently is not discernible because of overlapping. The strong absorption at 4.88 ppm is an HDO

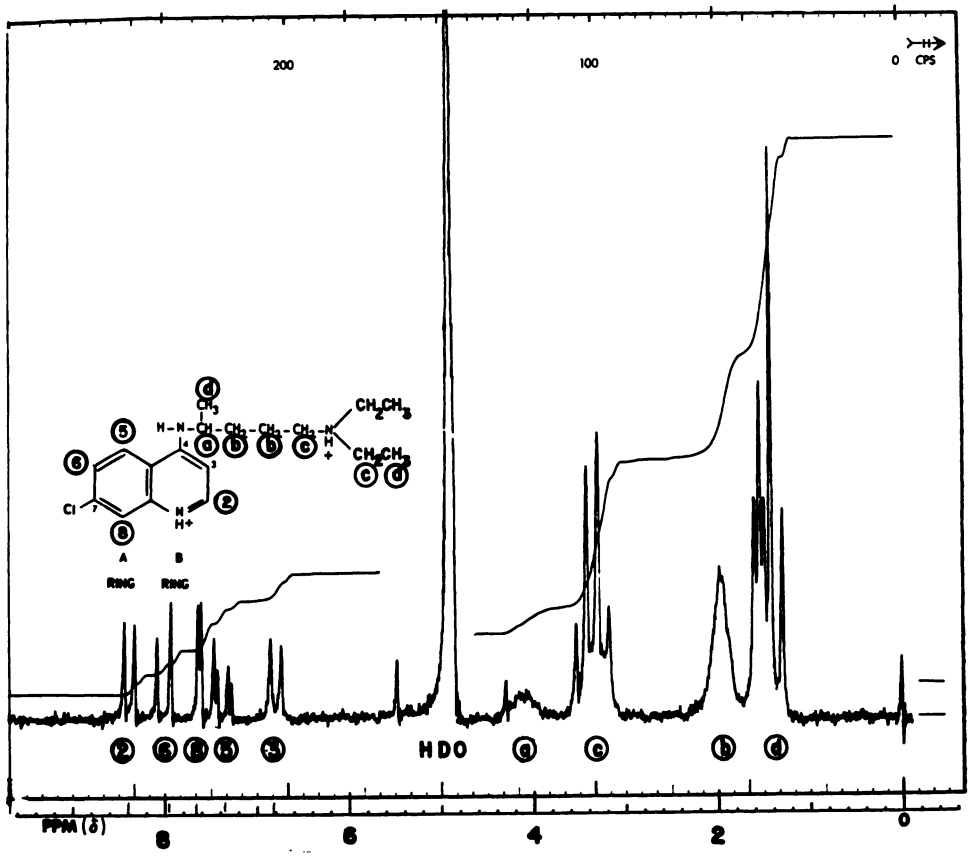

FIG. 1. *NMR spectrum of 10% chloroquine phosphate in D_2O at an apparent pH of 7.6*

resonance, a result of exchange with the amine and phosphoric acid protons of chloroquine phosphate.

The remaining bands are located in the low field region commonly associated with aromatic protons and have been given the following assignments: the doublet at 6.67–6.78 ppm is the $H_{(3)}$ proton. Its signal is split by coupling with the $H_{(2)}$ proton ($J_{23} = 7$ Hz, typical for *ortho* coupling). Deshielding by the adjacent quinoline nitrogen shifts the $H_{(2)}$ signal to 8.25–8.36 ppm. Bands from 7.20 to 7.38 ppm can be attributed to the $H_{(5)}$ proton, which is coupled with the $H_{(6)}$ proton at 7.85–8.00 ppm with a coupling constant $J_{56} = 9$ Hz, and with the $H_{(8)}$ proton at 7.52–7.55 ppm with $J_{58} = 2$ Hz, typical of protons *para* to each other.

The spectra of the nucleotides were interpreted according to the assignments of nucleotide proton resonances by Jardetzky and Wade-Jardetzky (7).

NMR spectra were used to observe the interaction of chloroquine with different nucleotides by examining the well-resolved resonance lines of the ring protons and of the ribose-H'_1 proton of the nucleotides, and by studying chemical shifts of chloroquine ring and side chain proton lines.

Interaction of purine nucleotides and chloroquine. Table 1 summarizes the effects of added chloroquine on the NMR spectrum of AMP at 11°, 41°, and 93°. As shown, all nucleotide ring protons and the H'_1 were shifted upfield. Interaction was strong at 11°, reduced at 41°, and not observable at 93°. Similar results were obtained with ATP and GMP.

The addition of AMP to chloroquine solutions shifted the signals of both the A ring (0.15 ppm) and the B ring (0.10 ppm)

TABLE 1

Effects of 0.058 M chloroquine on NMR spectrum of 0.071 M AMP

The experiment was performed at an apparent pH of 7.8–8.0. Differences for each proton are given as chemical shift (with respect to TSPS) in the mixture minus the shift of the proton in AMP alone.

Temperature	AMP proton shift difference in presence of chloroquine		
	ΔH_8	ΔH_2	$\Delta H'_1$
	ppm	*ppm*	*ppm*
11°	−0.13	−0.24	−0.20
41°	−0.10	−0.15	−0.12
93°	±0	±0	±0

of the drug as summarized in Table 2. Again, the interaction, as measured by the chemical shifts, was less at higher temperatures. Side chain proton signals were not shifted by the addition of AMP. However, the addition of ATP produced weak deshielding of the side chain protons of chloroquine, and the methyl protons (*d*) coalesced from a sharp quartet to a broad triplet. The ring proton signals of chloroquine were shifted to the same extent by the addition of ATP and AMP. These results indicate that both AMP and ATP interact with chloroquine by ring stacking, but that only ATP has significant interaction with the chloroquine side chain.

dAMP was indistinguishable from AMP in its chloroquine interactions. GMP produced shifts similar to those observed for AMP.

Interaction of pyrimidine nucleotides and chloroquine. As shown in Table 3, the addition of UMP and of CMP to chloroquine produced only minimal shifts in chloroquine proton signals. Changes in absorption of nucleotide ring protons (Table 4) were also minimal under these conditions and in no instance were greater than 0.06 ppm. Thus, the interaction between the drug and pyrimidine nucleotides appeared to be considerably less than that with purines, as proposed on the basis of ultraviolet spectra (3). However, the NMR data do not definitely rule out the possibility of chloroquine–pyrimidine interactions. Since the pyrimidines are not aromatic and do not have ring current magnetic anisotropy, small chemical shifts do not necessarily mean weak interactions (9).

Effect of concentration on NMR spectra of AMP and chloroquine. In evaluating the mode of stacking in mixtures of nucleotides and chloroquine, the possibility of chloroquine-chloroquine and nucleotide-nucleotide interactions must be considered. Accordingly, the effects of concentration on the spectra of these two separate components are shown in Tables 5 and 6. The concentration-dependent shift in the ring protons observed for both compounds suggests self-aggregation at high concentration. This is in agreement with previous reports of stacking of purines in solution (11, 10). It may also be noted that high concentrations of chloroquine alone produce downfield shifts in the signals of side chain and B-ring protons and only a slight shift

TABLE 2

Effects of 0.071 M AMP on NMR spectrum of 0.058 M chloroquine

The experiment was performed at an apparent pH of 7.8–8.0. Differences for each proton are given as chemcal shift (with respect to TSPS) in the mixture minus the shift of the proton in chloroquine alone.

Temperature	Chloroquine proton shift difference in presence of AMP				
	ΔH_2	ΔH_3	ΔH_5	ΔH_6	ΔH_8
	ppm	*ppm*	*ppm*	*ppm*	*ppm*
11°	−0.10	−0.13	−0.17	−0.11	−0.14
41°	−0.08	−0.12	−0.15	−0.14	−0.14
82.5°	−0.03	−0.05	−0.04	−0.05	−0.06
93°	±0	−0.07	−0.07	−0.06	−0.05

TABLE 3

Comparison of effects of different nucleotides on NMR spectrum of chloroquine

The experiments were performed at an apparent pH of 7.8–8.0 and a temperature of 41°. Differences for each chloroquine proton are given as chemical shift (with respect to TSPS) in the mixture minus the shift of the proton in chloroquine alone.

Nucleotide	Chloroquine proton shift difference				
	ΔH_2	ΔH_3	ΔH_5	ΔH_6	ΔH_8
	ppm	*ppm*	*ppm*	*ppm*	*ppm*
AMP	−0.08	−0.12	−0.15	−0.14	−0.14
GMP	−0.07	−0.05	−0.08	−0.05	−0.08
UMP	±0	±0	−0.02		+0.02
CMP	−0.03	−0.05	+0.02		±0

to higher field of the A-ring protons, in contrast to chloroquine-nucleotide mixtures. The downfield shift of the side chain protons cannot be definitely interpreted at present.

DISCUSSION

The results provide direct evidence that ring interactions occur when chloroquine is mixed with solutions of purine nucleotides. In contrast, interaction is minimal in drug mixtures containing pyrimidine nucleotides, in accord with previous conclusions (3). The nucleoside monophosphates (GMP and AMP) cause upfield shifts in ring protons of chloroquine, while ATP also causes shifting of the chloroquine side chain protons, suggesting electrostatic interaction due to the presence of the additional phosphates. Previous work using absorption spectroscopy also distinguished between the monophosphate and the diphosphate (3).

TABLE 4

Effects of chloroquine on NMR spectra of different nucleotides

The experiments were performed at an apparent pH of 7.8–8.0 and a temperature of 41°. Differences for each nucleotide are given as chemical shift (with respect to TSPS) in the mixture minus the shift of the proton in nucleotide alone.

Nucleotide	Nucleotide proton signal shift difference		
	ΔH_8 (H_6)[a]	ΔH_2 (H_5)	$\Delta H'_1$
	ppm	*ppm*	*ppm*
AMP	−0.10	−0.15	−0.11
GMP	−0.10		−0.12
UMP	−0.03	−0.06	−0.03
CMP	−0.05	−0.06	−0.03

[a] Parenthetical expressions refer to UMP and CMP, respectively.

Interactions are decreased sharply at elevated temperatures. This observation is significant in view of previous reports attributing effects of elevated temperature on DNA-chloroquine binding simply to changes in DNA structure (4). No doubt, the effects of temperature on solvent structure are important in any ring interaction phenomenon and must be taken into account in deducing a binding model.

The key question is whether the ring interactions occur between the drug and nucleotides (heterogeneous stacking) or simply between like species (purine to purine and drug to drug—homogeneous stacking) following charge interactions between the phosphate and the positively charged chloroquine molecule. The differences observed between the proton shifts of chloroquine in chloroquine-nucleotide mixtures, and those due to high concentrations of the drug, suggest that the major interactions are between drug and nucleotides. The results are consistent with the contention that there is a specific interaction between the chloroquine ring and the purine ring. This ring interaction is not changed even when the chloroquine side chain interacts with the phosphates of ATP. For acridine binding to DNA it has been proposed that heterogeneous and homogen-

TABLE 5

Effect of chloroquine concentration on its NMR spectrum

The experiments were performed at an apparent pH of 7.8–8.0 and a temperature of 41°. Differences for each proton are given as the chemical shift (with respect to TSPS) in 0.290 M and 0.580 M chloroquine minus the shift of the proton in 0.058 M chloroquine.

	Chloroquine proton signal difference							
Chloroquine concentration	ΔCH_3—C (d)	ΔCH_2—C (b)	ΔCH_2—N (c)	ΔH_2	ΔH_3	ΔH_5	ΔH_6	ΔH_8
M	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*
0.290	+0.13	+0.14	+0.14	+0.07	±0	−0.13	−0.17	−0.12
0.580	+0.23	+0.20	+0.21	+0.12	+0.12	−0.16	−0.07	−0.15

eous binding are both important (12). Although the same may be true for the aminoquinolines, it is not supported by the present data.

TABLE 6

Effect of AMP concentration on its NMR spectrum

The experiment was performed at an apparent pH of 7.8–8.0 and a temperature of 41°. The difference for each proton is given as the chemical shift (with respect to TSPS) in 0.710 M AMP minus the shift of the proton in 0.071 M AMP.

	AMP proton signal shift difference		
AMP concentration	ΔH_8	ΔH_2	$\Delta H'_1$
M	*ppm*	*ppm*	*ppm*
0.710	±0	−0.11	+0.03

REFERENCES

1. J. L. Irvin, E. M. Irvin and F. S. Parker, *Science* **110**, 426 (1949).
2. S. N. Cohen and K. L. Yielding, *Proc. Nat. Acad. Sci. U. S. A.* **54**, 521 (1965).
3. S. N. Cohen and K. L. Yielding, *J. Biol. Chem.* **240**, 3123 (1965).
4. F. E. Hahn, R. L. O'Briend, J. Ciak, J. L. Allison and J. G. Olenick, *Mil. Med. (Suppl).* 1071 (1966).
5. L. W. Blodgett and K. L. Yielding, *Biochim. Biophys. Acta* **169**, 451 (1968).
6. C. D. Jardetzky and O. Jardetzky, *J. Amer. Chem. Soc.* **82**, 222 (1960).
7. O. Jardetzky and N. G. Wade-Jardetzky, *J. Biol. Chem.* **241**, 85 (1966).
8. S. I. Chan, M. P. Schweizer, P. O. P. Ts'o and G. K. Helmkamp, *J. Amer. Chem. Soc.* **86**, 4182 (1964).
9. M. P. Schweizer, S. I. Chan and P. O. P. Ts'o, *J. Amer. Chem. Soc.* **87**, 5241 (1965).
10. M. P. Schweizer, A. D. Broom, P. O. P. Ts'o and D. P. Hollis, *J. Amer. Chem. Soc.* **90**, 1042 (1968).
11. K. H. Scheit, F. Cramer and A. Franke, *Biochim. Biophys. Acta* **145**, 21 (1967).
12. D. F. Bradley and M. K. Wolf, *Proc. Nat. Acad. Sci. U. S. A.* **45**, 944 (1959).