Biophysical Chemistry 22 (1985) 83–87
Elsevier

BPC 00926

SELECTIVE ^{1}H-NMR RELAXATION INVESTIGATIONS OF MEMBRANE-BOUND DRUGS IN VITRO

1. COLCHICINE

Gianni VALENSIN, Andrea LEPRI and Elena GAGGELLI

Department of Chemistry, University of Siena Pian dei Mantellini 44, 53100 Siena, Italy

Received 29th November 1984
Accepted 19th March 1985

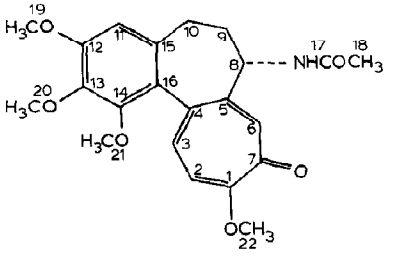
Key words: ^{1}H-NMR; Drug binding; Colchicine

Binding of colchicine to dipalmitoylphosphatidylcholine bilayer vesicles was detected by measuring the ^{1}H-NMR selective spin-lattice relaxation rates of the low-field protons of colchicine. From the temperature dependence of the selective rates, preferential binding was observed above the temperature of transition. In the same way, binding of colchicine to red blood cells was detected and the equilibrium constant determined. Binding to the lipid matrix of red blood cells accounted only partially for the binding of colchicine to whole cells.

1. Introduction

It has been recently shown [1,2] that measuring ^{1}H-NMR spin-lattice relaxation rates after selective excitation of properly chosen resonances is a very suitable way of delineating binding interactions between relatively small 'NMR-visible' drugs and macromolecular receptors. The method stems from the fact that the selective relaxation rate is a linear function of the motional correlation time, τ_c, which progressively increases even after molecular motions have slowed down to the $\omega_0 \tau_c \gg 1$ region. As a consequence, provided fast exchange between free and bound environments holds, dynamic and/or structural features of the bound site can be detected in the bulk. The selective irradiation method has been indeed applied to systems involving enzymes [1], membranes and, even whole cells [2].

In the present report selective irradiation methods have been used for investigating the interaction of colchicine (I) with membrane models and red blood cells. In fact, colchicine, the main alkaloid of *Colchicum autumnale* and other species of Lyliaceae, is a remarkable biologically active substance possessing anti-inflammatory, antimitotic and tumor-inhibiting activities. Previous experiments have shown that the alkaloid drug affects the lateral mobility of membrane components [3,4], suggesting either intercalation into the lipid bilayer or binding to membrane protein [5]. ESR studies, using spin-labelled phosphatidylcholine liposomes, have excluded intercalation into the lipid bilayer as in the case of cholesterol [6], but extensive investigation of the binding interaction has not yet been reported. We show herein that ^{1}H-NMR selective relaxation methods allow suitable delineation of the binding features of col-



0301-4622/85/$03.30

chicine with phosphatidylcholine bilayers as well as with red blood cells.

2. Materials and methods

Colchicine and synthetic L-α-dipalmitoylphosphatidylcholine (DPPC) were supplied by Sigma Chemical Co. and used without further purification. Solutions of colchicine were obtained either in 99.95% ^2H_2O (Merck) or in deuterated phosphate buffer. Sonicated DPPC vesicles were prepared from a 50 mg/ml suspension in 10 mM Tris-chloride and 10 mM EDTA solution. The suspension was sonicated for 10 min at relatively low power and the sonicator chamber was maintained at 50°C throughout the sonification. The sonicated suspension was centrifuged for 30 min at 45 000 rpm at 45°C; the zone containing the clear supernatant was removed and stored at 50°C.

Human group O red blood cells were separated from freshly drawn blood containing 0.1 M sodium citrate, washed in isotonic phosphate buffer, centrifuged for 15 min at 2000 rpm, and suspended in deuterated phosphate buffer. Erythrocyte ghosts were prepared using the method of Dodge et al. [7].

The NMR measurements were performed using a Varian XL-200 NMR spectrometer in the pulsed FT mode. The temperature was controlled at ±1°C. The non-selective proton spin-lattice relaxation rates, R^{ns}, were measured by using the inversion recovery pulse sequence $(\pi - \tau - \pi/2 - t)_n$. The selective proton spin-lattice relaxation rates, R^s, were measured in the initial rate approximation [8] by giving a selective π pulse with the proton decoupler at the selected frequency for a relatively long time (19–21 ms). After the time τ, a non-selective $\pi/2$ pulse was given to detect the longitudinal magnetization. The R^{ns} or R^s values were obtained from a three-parameter exponential regression analysis of the recovery curve for longitudinal magnetization.

3. Results and discussion

The theoretical approach to selective relaxation methods has been thoroughly reported elsewhere [1,8,9] and only the most relevant equations will be summarized herein. Typical selective relaxation rate measurements are shown in fig. 1 and the whole relaxation data for the low-field protons of colchicine are summarized in table 1.

For a pure dipole-dipole ^1H-^1H relaxation pathway, R^{ns} and R^s of any proton i are given by [9]:

$$R_i^{ns} = \sum_{i \neq j} \rho_{ij} + \sum_{i \neq j} \sigma_{ij} \tag{1}$$

$$R_i^{s} = \sum_{i \neq j} \rho_{ij} \tag{2}$$

where ρ_{ij} and σ_{ij} are the direct relaxation and the cross-relaxation terms for a proton pair:

$$\rho_{ij} = \tfrac{1}{10} \frac{\hbar^2 \gamma_H^4}{r_{ij}^6} \left\{ \frac{3\tau_c}{1 + \omega_0^2 \tau_c^2} + \frac{6\tau_c}{1 + 4\omega_0^2 \tau_c^2} + \tau_c \right\} \tag{3}$$

$$\sigma_{ij} = \tfrac{1}{10} \frac{\hbar^2 \gamma_H^4}{r_{ij}^6} \left\{ \frac{6\tau_c}{1 + 4\omega_0^2 \tau_c^2} - \tau_c \right\} \tag{4}$$

where r_{ij} is the proton-proton distance. It has been shown [8] that a pure dipolar mechanism yields, for a given proton pair, $R^{ns}/R^s = 1.5$ in the extreme narrowing region ($\omega_0 \tau_c \ll 1$). R^{ns}/R^s values lower than 1.5 are expected either outside the extreme narrowing region or in the presence of effective relaxation mechanisms other than the dipolar one. Since 'other' relaxation mechanisms are not likely to be effective for ring protons of the colchicine molecule (spin-rotational interaction may be relevant only for the methyls), the data in table 1 suggest that the $\omega_0 \tau_c \gtrsim 1$ condition applies, allowing evaluation of the modulating correlation time [1] ($\tau_c = 2.6 \times 10^{-10}$ s at 310 K). However, the R^{ns}/R^s values are not significantly different from 1.5 such that the calculated τ_c should be taken as an upper limit. Moreover, in the R^s measurements it is seldom possible to avoid some reduction of the intensity of a certain resonance when irradiating a nearby NMR line with consequent reduction of the R^{ns}/R^s value. Nevertheless, the above-reported τ_c is in very good agreement with that calculated for the ring carbons from ^{13}C spin-lattice relaxation rates [10], as reported in table 2. Since colchicine is a rather rigid

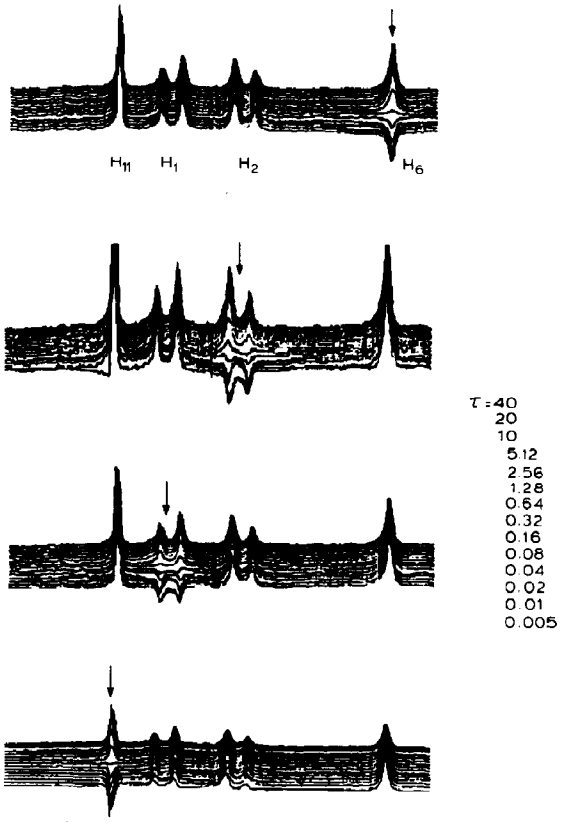


Fig. 1. Selective partially relaxed spectra of selected protons of colchicine (0.1 mol dm^{-3}) in ^2H_2O at pH = 7.0 and T = 298 K. Assignments are reported in the top spectrum. The arrow indicates the frequency of selective irradiation.

structure, the correlation time calculated for C_8 and C^{10} can be identified with the molecular reorientational time whereas the correlation time of the methyl groups reflects their relatively free motion.

Table 1

Non-selective and selective spin-lattice relaxation rates of selected protons of colchicine (0.1 mol dm^{-3}) in ^2H_2O at pH = 7.0 and T = 298 K

± figures denote approx. 95% confidence limits of the regression analysis.

Resonance	R^{ns} (s^{-1})	R^s (s^{-1})	R^{ns}/R^s
H_{11}	1.29±0.05	0.89±0.04	1.45±0.12
H_3	2.96±0.14	2.07±0.06	1.43±0.11
H_2	3.95±0.19	2.72±0.12	1.45±0.14
H_6	4.35±0.18	3.02±0.13	1.44±0.13

Table 2

Spin-lattice relaxation rates of selected carbons of colchicine (0.1 mol dm^{-3}) in ^2H_2O at pH = 7.0 and T = 298 K

± figures denote approx. 95% confidence limits of the regression analysis.

	No. attached protons (n)	R_1/n (s^{-1})	τ_c ($\times 10^{10}$) (s)
C_8	1	6.14±0.41	2.28±0.15
C_{10}	2	4.67±0.25	1.74±0.09
C_{18}	3	0.44±0.04	0.16±0.01
C_{21}	3	0.45±0.04	0.17±0.02

The effects of adding different preparations to the colchicine solution are summarized in table 3. As expected, the R^{ns} values were quite unaffected by the addition of whichever preparation, while the R^s values were greatly enhanced for all the low-field protons of colchicine, almost without any differential behavior. Binding of a certain fraction of colchicine molecules, rather than viscosity effects, can be suitably suggested by the observed R^s enhancements. In fact, the largest R^{ns}/R^s value, obtained upon addition of the biological preparation, would suggest a correlation time almost one order of magnitude longer than that of colchicine in aqueous solution [1]. In contrast, fast exchange of colchicine between environments characterized by very different motional freedom can easily account for the observed change in R^s. The following equation can therefore be suggested to apply:

$$R^s_{obs} = p_f R^s_f + p_b R^s_b \qquad (5)$$

where f and b refer to free and bound sites and p_f and p_b are the fractions of colchicine molecules in the free and bound sites ($p_f + p_b = 1$), respectively. As a consequence, differences in R^s_{obs} upon addition of different biological samples (table 3) arise either from p_b (which means difference in the apparent equilibrium constant) or from R^s_b (which means difference in motional features and/or in relaxation mechanism at the bound site). The most straightforward interpretation of the data in table 3 is that colchicine binds to the phospholipid matrix and that the colchicine-lipid interaction only partially accounts for the binding interaction with red blood cells. Namely the much more pronounced R^s enhancement upon addition of red blood cells is worth considering. The rigid

Table 3

Non-selective and selective spin-lattice relaxation rates (in s^{-1}) of colchicine (0.1 mol dm^{-3}) at pH = 7.0 after the addition of 0.1 ml of different biological preparations

Preparation added	H_{11}		H_3		H_2		H_6	
	R^{ns}	R^s	R^{ns}	R^s	R^{ns}	R^s	R^{ns}	R^s
–	1.29	0.89	2.96	2.07	3.95	2.72	4.35	3.02
DPPC vesicles	1.31	1.18	3.08	2.78	3.97	3.65	4.31	3.97
Red blood cells (4%)	1.42	1.51	3.01	3.48	4.07	4.56	4.36	5.13
Red blood cell ghosts (4%)	1.34	1.21	3.06	2.81	4.01	3.72	4.30	3.89

structure of the colchicine molecule, such that no differential R^s enhancement could be observed, does not allow inferences about the binding sites: protons in the three rings become affected to the same extent by the presence of macromolecular receptors. However, the relevance of detecting the binding interaction at very low concentration of receptor sites is worth underlining.

Once the lipid-colchicine binding interaction was established further details were gained from the temperature dependence of the R^s enhancement, as shown in fig. 2. A relatively smooth decrease in ΔR^s was apparent on raising the temperature which can be accounted for either by increased molecular tumbling motions or, more probably, release of a certain fraction of bound colchicine molecules. The break-down of the ΔR^s vs. T plots in relation to the temperature of transition from gel to liquid crystal of the hydrocarbon chains is likely to reflect the change in motional freedom of the lipid matrix, suggesting tight binding of the colchicine molecule.

The method of selective relaxation was also found to allow evaluation of the apparent equilibrium constant, as will be exemplified for the binding interaction between colchicine and red blood cells. 1:1 interaction will be assumed for simplicity, but from the final result it will be evident that the evaluated K is independent of such an assumption. For a 1:1 interaction the binding equilibrium can be schematized as (RBC, red blood cells):

$$C + RBC \rightleftarrows RBC\text{-}C \tag{6}$$

The apparent equilibrium constant is given by

$$K = \frac{[RBC\text{-}C]}{[C][RBC]} = \frac{[RBC\text{-}C]}{[C]\{[RBC]_0 - [RBC\text{-}C]\}} \tag{7}$$

where [C] is the concentration of free colchicine and $[RBC]_0$ represents the initial concentration of red blood cells.

The fraction of bound colchicine, p_b, is given by

$$p_b = \frac{[RBC\text{-}C]}{[C] + [RBC\text{-}C]} \approx \frac{[RBC\text{-}C]}{[C]} \tag{8}$$

Eq. 7 can be rearranged in the following way

$$[RBC\text{-}C] = \frac{K[C][RBC]_0}{1 + K[C]} \tag{9}$$

Substituting eq. 9 into eqs. 5 and 8 yields

$$\frac{1}{\Delta R^s} = \left(\frac{1}{K} + [C]\right)\frac{1}{R_b^s[RBC]_0} \tag{10}$$

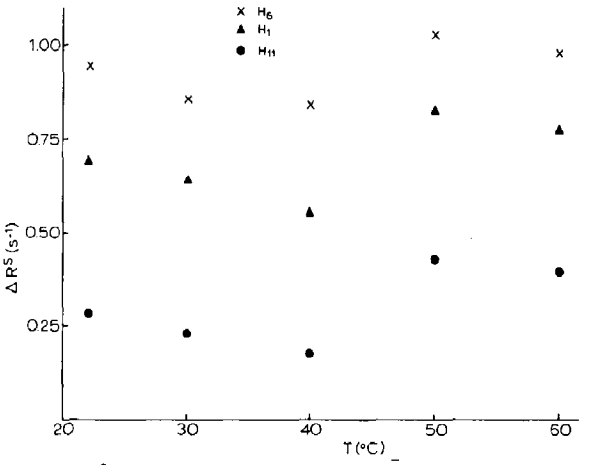


Fig. 2. ΔR^2 vs. T plot for selected protons of colchicine (0.1 mol dm^{-3}) in ^2H_2O at pH = 7.0 in the presence of DPPC vesicles.

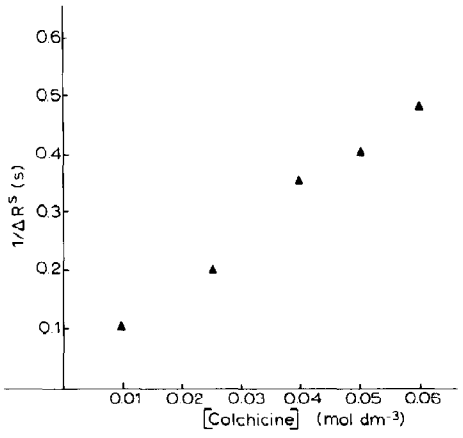


Fig. 3. $1/\Delta R^s$ vs [colchicine] plot for the H_2 protons in the presence of red blood cells.

Eq. 10 states that extrapolating the plot of $1/\Delta R^s$ vs. [C] to zero allows evaluation of K ($1/\Delta R^s$ is zero when $[C] = -1/K$). A typical plot is shown in fig. 3 for the H_2 proton of colchicine: the linear relationship is evident, allowing straightforward extrapolation to $1/\Delta R^s = 0$. The existence of n receptor sites per cell for the ligand affects the linearity of the plot only slightly, since, in that case, the following equation can be easily derived:

$$\frac{1}{\Delta R^s} = \left(\frac{1}{K[C]^{n-1}} + [C]^n \right) \frac{1}{R_b^s [RBC]_0} \qquad (11)$$

However, extrapolation to $1/\Delta R^s = 0$ yields a certain value of [C] from which K can be obtained if n is known or vice versa ($1/\Delta R^s$ is zero when $[C]^n = -1/K$).

It can be concluded that measuring the selective proton spin-lattice relaxation rates of the low-field protons of colchicine allows detection of binding interactions with different macromolecular receptors. The following further considerations can be made.

(i) Colchicine binds to phosphatidylcholine as well as to the lipid matrix of red blood cells;

(ii) The colchicine-lipid interaction does not completely account for the binding of colchicine to intact red blood cells;

(iii) The binding brings about enhancement of the selective spin-lattice relaxation rates of protons located in different parts of the colchicine molecule, such that the binding site cannot be delineated;

(iv) The R^s enhancement 'senses' the gel-to-liquid transition on the lipid matrix, reflecting the difference in molecular motions;

(v) The plot of $1/\Delta R^s$ vs. [C] yields a function of the apparent equilibrium association constant (K) and of the number of receptor sites per cell (n), from which K can be evaluated if n is known or vice versa.

References

1 G. Valensin, T. Kushnir and G. Navon, J. Magn. Reson. 46 (1982) 23.
2 G. Valensin, A. Casini, A. Lepri and E. Gaggelli, Biophys. Chem. 17 (1983) 297.
3 R.D. Berlin, J.M. Oliver, T.E. Ukena and H.H. Yin, Nature 247 (1974) 45.
4 S. DePetris, Nature 250 (1974) 54.
5 F. Wunderlich, R. Müller and V. Speth, Science 182 (1973) 1136.
6 L.D. Altstiel and F.R. Landsberger, Nature 269 (1977) 70.
7 J.T. Dodge, C. Mitchell and D.J. Hanahan, Arch. Biochem. Biophys. 100 (1963) 119.
8 R. Freeman, H.D.W. Hill, B.L. Tomlinson and L.D. Hall, J. Chem. Phys. 61 (1974) 4466.
9 J.H. Noggle and R.E. Schirmer, The nuclear Overhauser effect (Academic Press, New York, 1971).
10 G.C. Nelson and G.L. Levy, Carbon-13 nuclear magnetic resonance for organic chemists (Wiley-Interscience, New York, 1972) p. 187.