Volume 218, number 1, 102–106 FEB 04796 June 1987

Properties of B-ring analogues of colchicine

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Received 16 April 1987

Absorption spectra of colchicine and its analogues are affected by the presence of the B-ring, although it is not part of the chromophore (C-ring). Thus, 2-methoxy-5-(2′,3′4′-trimethoxyphenyl)tropone has absorption maxima at 341 nm, whereas that of desacetamidocolchicine is at 353 nm. A similar red shift in the λ_{max} of colchicine, desacetamidocolchicine and 2-methoxy-(2′,3′,4′-trimethoxyphenyl)tropone also occurs when they are immobilized in the binding site to tubulin or in pure glycerol. We also observed that the B-ring of colchicine alone or with substituent does not affect the UV-induced rearrangement of colchicine to lumicolchicine. However, in the absence of the B-ring, as in the case of 2-methoxy-5-(2′,3′4′-trimethoxyphenyl)tropone, the rearrangement reaction of the C-ring slows down significantly.

Colchicine analog; Tubulin; Absorption spectrum

1. INTRODUCTION

The importance of the B-ring of colchicine in its binding to tubulin has been emphasized by different laboratories [1–8]. It has been observed that several properties of the colchicine-tubulin interaction such as the association rate, reversibility and the promotion of drug fluorescence are related to the B-ring of colchicine [7]. Recently significant differences in the binding and thermodynamic parameters such as activation energy, entropy and enthalpy have been reported for colchicine and 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone (called AC because it lacks the B-ring of colchicine, see fig.1) [5,6]. Studies on B-ring analogues of colchicine are also interesting from the clinical point of view. Thus, Capraro and Brossi [8] synthesized a variety of interesting B-ring analogues to determine the structural entities needed for optimal biological effects such as tubulin-binding activity and antileukemic activity with minimum level of toxicity.

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This B-ring is a seven-membered saturated ring of colchicine (with a side chain at the C-7 position, see fig.1) and has no chromophore to show absorption in the visible region. Here, we observed that the presence of the B-ring has a significant effect on the absorption spectra of the C-ring as well as on the photo-induced rearrangement of the C-ring.

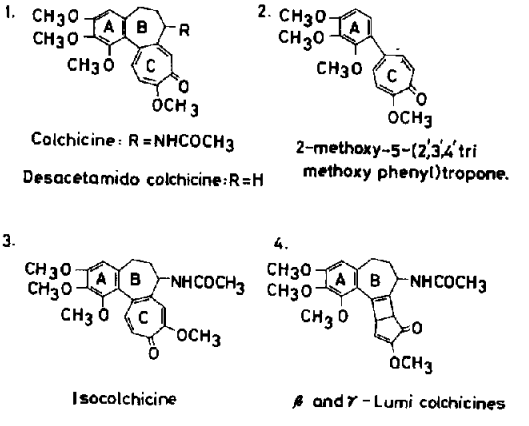

Fig.1. Structure of colchicine and its analogues.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

2. MATERIALS AND METHODS

Colchicine and GTP were products of Sigma. Desacetamidocolchicine and 2-methoxy-5-(2′,3′, 4′-trimethoxyphenyl)tropone were kind gifts of Dr T.J. Fitzgerald, Florida A and M University. All other chemicals used were reagent grade.

Tubulin was prepared by phosphocellulose chromatography of goat brain microtubule protein, prepared by two cycles of temperature-dependent polymerization [9]. Protein was stored in liquid nitrogen in tubulin assembly buffer [0.1 M Mes (pH 7.0), 0.5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM GTP]. The same assembly buffer without GTP was used for binding experiments to prevent polymerization. Protein was estimated according to Lowry et al. [10] using BSA as a standard.

Absorption spectra were obtained on a Cary model 17D recording spectrophotometer. For ultraviolet irradiation of free drug, each compound dissolved in 95% ethanol in a 1 ml quartz cuvette (1 cm light path) was placed exactly 2.5 cm from the UV lamp (model UV 4-11; Ultraviolet Products, San Gabriel, CA) in a dark room. After each irradiation the absorbance was measured at 350 nm. Bound drug was irradiated under identical conditions after the drug-tubulin complex was formed on incubation at 37°C for 30 min. Fluorescence measurements were made with a Perkin-Elmer MPF 44B spectrofluorometer.

3. RESULTS AND DISCUSSION

3.1. *Effect of the B-ring on the absorption spectra of the C-ring of colchicine*

Absorption spectra of colchicine and its analogues can be divided into two parts: a UV part ranging from 200 to 300 nm having a high extinction coefficient (ϵ) of 10^4–10^5 and the other overlap both UV and visible regions ranging from 300 to 400 nm and a little weaker in intensity ($\epsilon \sim 1.5$–1.8×10^4). Colchicine and all its analogues discussed here have C = O (at the C-ring) and C = C (of the A- and C-rings) conjugated with each other. Two absorption bands observed in the case of colchicine (fig.2A) are related on π-electrons extending from the A- to C-ring and are strong in intensity ($\epsilon > 10^4$) and no band related to the

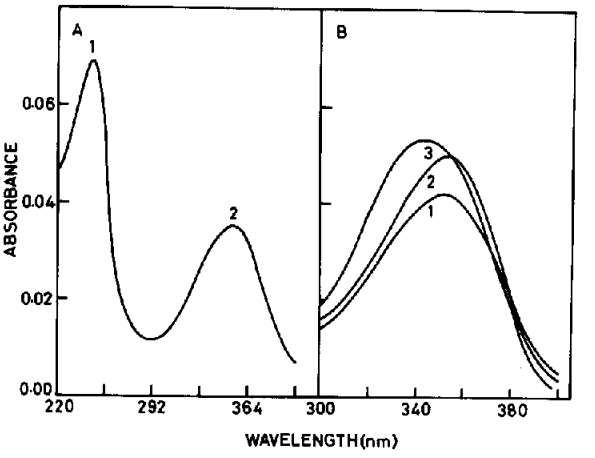

Fig.2. (A) Ultraviolet absorption spectra of colchicine (2.27×10^{-6} M) in water. Absorption maxima: λ_{max_1} = 247 nm; λ_{max_2} = 353 nm. (B) Absorption spectra of colchicine (2.7×10^{-6} M), desacetamidocolchicine (2.8×10^{-6} M) and AC compound (3×10^{-6} M) in assembly buffer. (1) Colchicine, λ_{max} = 353 nm; (2) desacetamidocolchicine, λ_{max} = 353 nm; (3) AC compound, λ_{max} = 341 nm.

nonbonding molecular orbital, largely localized on the oxygen atom with weaker intensity ($\epsilon \sim 10^1$) is seen. Band 2 represents the absorption property of the C-ring. Fig.2B shows the visible absorption spectra of colchicine, desacetamidocolchicine and AC in water. The values of the extinction coefficients are very similar for each compound. Colchicine and desacetamidocolchicine have absorption maxima at 353 nm whereas in the case of AC, it is at 341 nm. This significant red shift (341 to 353 nm) in the absorption spectra of desacetamidocolchicine or chicine is due to the presence of an alicyclic B-ring in the molecule. The question which thus arose was how the presence of this ring which is not a part of the conjugation system of the chromophore caused this shift in the absorption maxima. It is known that the intensities and positions of peaks depend on the length of the conjugated system: the longer such a system, the longer the wavelength of the absorption and the larger the extinction coefficient. In the case of colchicine or desacetamidocolchicine, the presence of the B-ring does not increase the length of the conjugated system. One possibility is that the π-electron system of the A- and C-rings in the case of AC is prevented from achieving coplanarity, thus

the degree of overlap of the π-electron system of two rings will be diminished, resulting in the blue shift in the absorption spectra. Inspection of the structure of AC by dreiding model clearly reveals that the A- and C-rings in AC prefer to remain above about 60° with respect to each other to minimize the steric interaction between the C_1 methoxyl and C_{12} hydrogen (fig.3). In the case of colchicine and desacetamidocolchicine the dihedral angle defined by the planar A-ring and by carbons C_{7a}, C_{11}, C_{12} and C_{12a} of the 'tube-shaped' methoxytropone, i.e. ring-C, is approx. 53° (fig.3). Colchicine exists in this conformation both in crystal [11,12] and in solution [13]. It has been suggested by Detrich et al. [14] as well as by Bane et al. [6] that colchicine is converted to conformer 2 (fig.3) when bound to tubulin. Conformer 2 is related to conformer 1 by a boat-boat interconversion of the troponoid C-ring and has a considerably smaller dihedral angle i.e. 19° (fig.3). This reduction of the dihedral angle in conformer 2 is expected to favour the extended conjugation and lead to the red shift in the absorption spectra (fig.4A,B). A similar change in conformation is expected when AC is bound to tubulin which might lead to the red shift in the absorption spectra (fig.4C). Recently, it has been demonstrated that colchicine, which shows fluorescence when binding tubulin, is mainly due to the immobilization of the drug in the binding site and this response to immobilization of colchicine depends in part on the partially flexible nature of the drug [15]. Thus, the conclusion of the colchicine fluorescence was based upon the findings that colchicine shows a remarkable fluorescence in pure glycerol and this fluorescence decreases with decreasing viscosity of

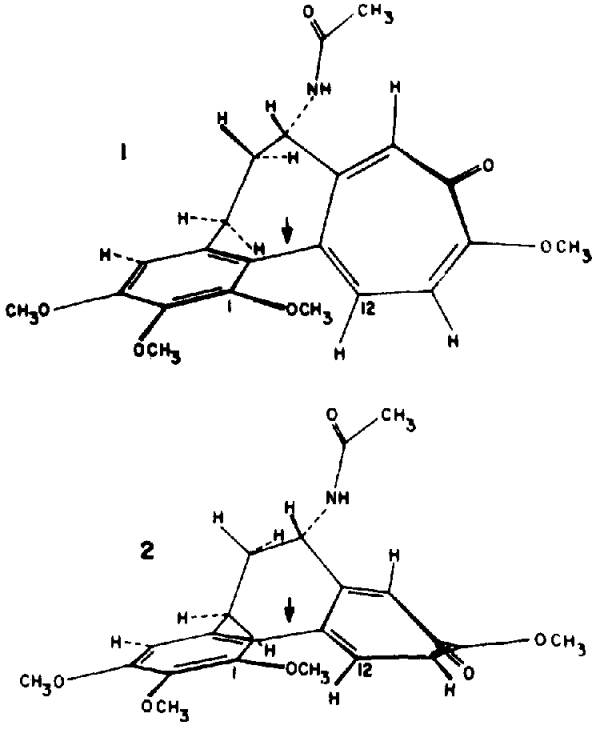

Fig.3. Multiple conformations of colchicine. Four different conformations are possible. Conformation 2 shown above is related to conformation 1 by a boat-boat interconversion of the troponoid C-ring. The dihedral angle between the plane defined by C_{7a} C_{11} C_{12} and C_{12a} of the C-ring and the planar A-ring is about 53°. This dihedral angle is reduced to 19° in conformation 2. Two other conformations of colchicine are possible which are related to the shown C-ring boat-boat conformers by atropisomerism (about the biaryl bond ⇓). Although such atropisomerism leads to change the chirality of the biaryl moieties, there is no change in the dihedral angles. (Reproduced with permission from the American Chemical Society.)

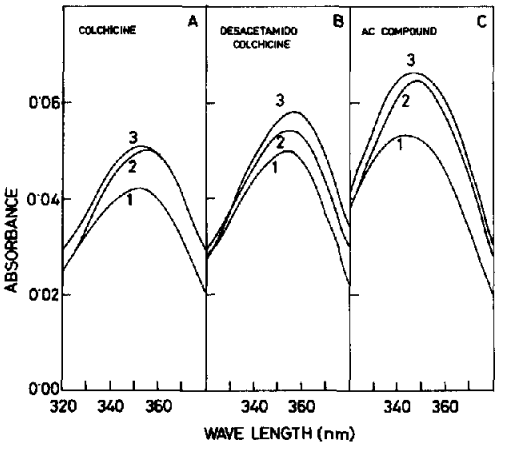

Fig.4. Absorption spectra of colchicine, desacetamidocolchicine and AC compound in assembly buffer (1), in 100% glycerol (2) and in the presence of tubulin (3). In (3), the drugs are incubated with a 5-fold excess of tubulin (15 μM) in assembly buffer without GTP at 37°C for 30 min. (A) Colchicine (2.7×10^{-6} M). λ_{max}: (1) 353 nm, (2) 358 nm, (3) 354 nm. (B) Desacetamidocolchicine (2.8×10^{-6} M). λ_{max}: (1) 353 nm, (2) 355 nm, (3) 356 nm. (C) AC compound (3×10^{-6} M). λ_{max}: (1) 341 nm, (2) 348 nm, (3) 346 nm.

the medium. We feel that immobilization is also partly responsible for the differences in absorption maxima of AC and colchicine. Fig.4A shows the visible absorption spectra of colchicine when immobilized to its binding site at tubulin or in pure glycerol. In both cases a significant red shift of the absorption maxima is observed. Similar red shifts in λ_{max} are observed in the case of desacetamidocolchicine and AC when they are bound to tubulin or present in pure glycerol (fig.4B,C). Partial immobilization can also be achieved by lowering the solution temperature. Thus, it was shown that colchicine fluoresces in glass of ether, isopentane, ethanol (5:5:2) at 77° and even in ice in a temperature-dependent manner [16]. Here, we observed that the absorption maxima of colchicine in aqueous solution (25°C) change from 353 to 355 nm when the temperature is lowered to 4°C. A similar red shift is observed in the case of AC. In addition to immobilization, lowering of temperature and addition of glycerol might also favour the formation of conformer 2 from conformer 1 (fig.3).

3.2. *Effect of the B-ring on photo-induced rearrangement of the C-ring*

Ultraviolet irradiation causes the conversion of colchicine to β- and γ-lumicolchicine [17–20]. This photo-induced rearrangement occurs at the C-ring part of colchicine (fig.1). It has been reported that the positions of the groups present on the C-ring affect the rate of the rearrangement reaction [20]. A marked effect is observed when the positions of the carbonyl and methoxy group in the C-ring are interchanged. Thus, isocolchicine shows remarkable stability in the presence of ultraviolet light [20]. The effect of a B-ring substituent at the C-7 position has been found to be less significant. In fact, when the structures of colchicine and lumicolchicine are compared (fig.1), no direct involvement of the B-ring in the rearrangement is noticed. However, here we observed that the absence of the B-ring from colchicine results in a compound which is very stable towards ultraviolet irradiation. Thus the degree of conversion of AC into its lumicolchicine derivative is much less than that of colchicine. Since the conversion of col-

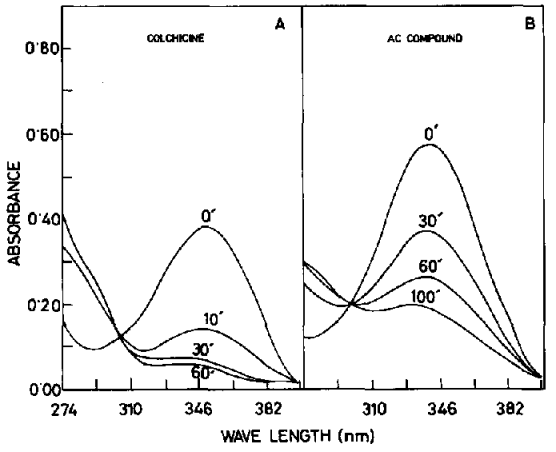

Fig.5. Absorption spectra of colchicine and AC compound at different time intervals during irradiation with ultraviolet light. The irradiation experiment was carried out as described in section 2. (A) Colchicine, isosbestic point, 303 nm. (B) AC compound, isosbestic point, 292 nm.

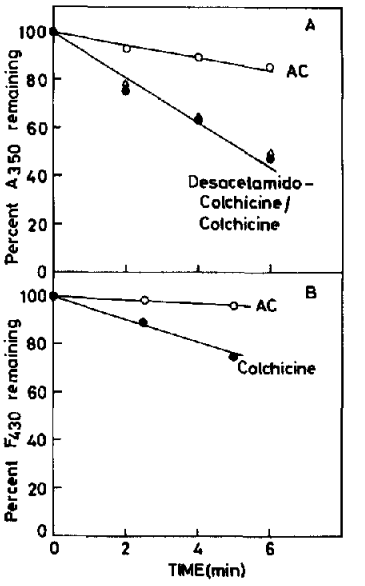

Fig.6. (A) Time kinetics of ultraviolet-induced rearrangement of colchicine, desacetamidocolchicine and AC compound in 95% ethanol. The ultraviolet irradiation experiment was done as described in section 2. (B) Time course of release of drug from drug-tubulin complex (measured by decrease in fluorescence at 430 nm) during irradiation with ultraviolet light. 2.5 μM tubulin was incubated with 5 μM drug at 37°C for 30 min. The resultant drug-tubulin complex was irradiated for the indicated times and the fluorescence at 430 nm was measured.

chicine or its analogs into their corresponding lumicolchicine derivative takes place with the loss of the chromophore C-ring (which has absorption maxima at 350–353 nm) this photochemical conversion was followed by measuring the decrease in absorbance at 350 nm. Fig.5 shows a typical irradiation experiment with colchicine and AC. It can be seen that AC has much slower breakdown than colchicine and 30 min irradiation caused a 33 and 80% decrease in absorbance (at their corresponding λ_{max}), respectively. The isosbestic point, which is a proof for the authenticity of a compound, has been found to be at 303 and 292 nm for colchicine and AC, respectively. Desacetamidocolchicine, a compound having the B-ring but no substitution at the C-7 position, has been found to be equally susceptible to colchicine (fig.6A). All these irradiation experiments were performed by dissolving drug in 95% ethanol.

It has been reported by Wilson and Friedkin [20] that the positions of the carbonyl and methoxy groups present at the C-ring affect the rate of the ultraviolet-induced rearrangement reaction. Thus, compared to colchicine, isocolchicine shows remarkable stability towards ultraviolet irradiation. Here we observed that, in comparison to colchicine and desacetamidocolchicine, AC is stable towards ultraviolet irradiation. Obviously, it is interesting to know whether this increased stability of AC towards irradiation is due to the presence of its isocolchicine form in solution. AC can assume the isocolchicine form very easily due to its free rotation around the single bond between the A- and C-rings. To answer this question we have irradiated tubulin-AC complex where AC should be in the colchicine form (since the isocolchicine form does not bind tubulin). The results of such an experiment are shown in fig.6B. Since AC and colchicine fluoresce only when bound to tubulin, the drug-tubulin complexes were irradiated for different times and the fluorescence was measured after each irradiation. Here, it has also been observed that AC bound to tubulin is affected much less by ultraviolet light compared to colchicine bound to tubulin. Here, the rate of rearrangement of colchicine bound to tubulin is slightly lower compared to its free state (fig.6A). This could be due to the difference in the environment in which the drug is present. In fact, we observed that the rate of rearrangement of colchicine into lumicolchicine varies in different solvents studied, i.e. water, ethanol, DMSO and dioxane, etc. However, no correlation is observed between the rate of rearrangement and the solvent polarity.

REFERENCES

[1] Fitzgerald, T.J. (1976) Biochem. Pharmacol. 25, 1383–1387.
[2] Banerjee, A.C. and Bhattacharyya, B. (1979) FEBS Lett. 99, 333–336.
[3] Ray, K., Bhattacharyya, B. and Biswas, B.B. (1981) J. Biol. Chem. 256, 6241–6244.
[4] Chaudhury, G.G., Banerjee, A., Bhattacharyya, B. and Biswas, B.B. (1983) FEBS Lett. 161, 55–59.
[5] Adreu, J.M., Gorbunoff, M.J., Lee, J.C. and Timasheff, S.N. (1984) Biochemistry 23, 1742–1752.
[6] Bane, S., Puett, D., McDonald, T.L. and Williams, R.C. (1984) J. Biol. Chem. 259, 7391–7398.
[7] Bhattacharyya, B., Howard, R., Maity, S.N., Brossi, A., Sharma, P.N. and Wolff, J. (1986) Proc. Natl. Acad. Sci. USA 83, 2052–2055.
[8] Capraro, H.G. and Brossi, A. (1984) in: The Alkaloids (Brossi, A. ed.) pp.1–70, Academic Press, New York.
[9] Sloboda, R.D. and Rosenbaum, J.L. (1982) Methods Enzymol. 85, 409–415.
[10] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[11] Rossi, M., Link, J. and Lee, J.C. (1984) Arch. Biochem. Biophys. 231, 470–476.
[12] Margulis, T.N. (1974) J. Am. Chem. Soc. 96, 899–902.
[13] MacDonald, T.L. and Reagan, D.R. (1980) Abstracts of the American Chemical Society Second Chemical Congress of the North American Continent, Las Vegas, NV.
[14] Detrich, H.W., Williams, Robley, C., Macdonald, T.L., Wilson, L. and Puett, D. (1981) Biochemistry 20, 5999–6005.
[15] Bhattacharyya, B. and Wolff, J. (1984) J. Biol. Chem. 259, 11836–11843.
[16] Letterier, F. and Rieger, F. (1975) CRSA Trav. Sci. 5, 224–225.
[17] Grewe, R. and Wolff, W. (1951) Chem. Ber. 84, 621.
[18] Gardner, P.D., Brandon, R.L. and Haynes, G.R. (1957) J. Am. Chem. Soc. 79, 6334.
[19] Forbes, E.J. (1955) J. Chem. Soc., 3864.
[20] Wilson, L. and Friedkin, M. (1966) Biochemistry 5, 2463–2468.