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Unusual Binding Properties of B-ring Analogs of Colchicine Controlled by B-ring Side Chain

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Bhabatarak Bhattacharyya **

Abstract

In this study, we have shown that quantum yield, fluorescence property, activation energy and pH dependence of aminocolchicinoids binding with tubulin are controlled at different points of B-ring and its side chain at C-7 position. The substituent at the C-7 position of the B-ring seems to dictate the relative quantum yield of the colchicinoid-tubulin complexes. We synthesized two compounds, $NHCSCH_3$-DAAC and in $NHCH_2COCH_3$-DAAC. Mechanism of induction of fluorescence was elaborately discussed with these compounds. From the X-ray structure of tubulin-damacolchicine complex, it is known that A- and C-ring of colchicine bind at β-tubulin whereas C-7 side chain of B-ring interacts with α-tubulin. Here we have shown that although the colchicinoid molecules bind essentially at β-tubulin, β-C-terminus has no role on colchicinoids binding with tubulin. But binding is significantly less for both C-termini depleted tubulin ($\alpha_s\beta_s$), which can be explained by a inter-subunit communication through the 'tail-body' interaction. Moreover, we observed that the activation energy of $NHCSCH_3$-DAAC and $NHCH_2COCH_3$-DAAC are low and independent of pH. Under identical conditions, the activation energy of $N(Me)COCF_3$-DAAC is high, like colchicine and shows pH dependency. The addition of acetyl group in colchicine causes dramatic decrease in off-rate constant from DAAC. These results indicate that the oxygen atom plays a very significant role which is still unknown to us. It may also possible that the amino and carbonyl groups of B-ring side chain are involved in making important contacts with protein in complex form.

Keywords:

Tubulin,
Colchicine,
B-ring analogs,
pH effect,
Quantum Yield

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1. Introduction

Colchicine is oldest among antimitotic drugs whose binding with tubulin is well studied. Other well-studied drugs are taxol and vinblastine, which bind tubulin at different sites. Structures of tubulin with all these drugs are known [1, 2, 3]. While taxol and vinblastine are well-established drugs used for the treatment of cancer, colchicine is toxic and not acceptable as a drug. However, due to immense therapeutic importance of colchicine, a large number of colchicine analogs are being synthesized and tested for biological activities. From the X-ray structure of tubulin-damacolchicine complex (PDB ID: 1SA0) [3], it is known that A- and C-ring of colchicine bind at β-tubulin whereas C-7 side chain of B-ring interacts with α-tubulin. Using 1SA0 as template, Nguyen *et al.* performed docking and molecular simulation and determine the attachment points (pharmacophoric) of bound colchicine to tubulin [4]. They described the A- and C-ring as pharmacophoric attachment point of colchicine, but not the B-ring and its side chain at C-7 position due to lack of suffient

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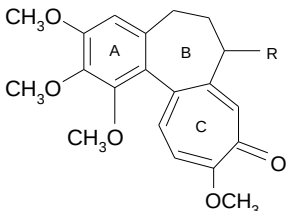
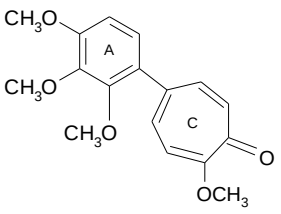
data. Biochemical studies have also established that colchicine analogs modified at, or depleted of the B-ring retains potent antimitotic activity, self-inhibitory activity and the binding activity of tubulin at the colchicine site [5]. Nevertheless the presence of B-ring alone or substituents at C-7 position influences the on-rate, off-rate, activation energy, reversibility, pH-dependency and the quantum yield of the complexes of the drug with tubulin [6, 7, 8].

The substituent at the C-7 position of the B-ring seems to dictate the relative quantum yield of the colchicinoid-tubulin complexes. Thus, if the C-7 substituent is an amine or an alkyl amine, then the quantum yield decreased in the following order: R= $H>NH_2>>NH.CH_3>N(CH_3)_2$. The low quantum yield of aminoallocolchicinoids in solvents and bound to tubulin was explained by the exciplex formation between the lone pair of electrons on the nitrogen atom and the allocolchicinoids π–system, which leads to quenching of allocolchicinoid's fluorescence [7]. It has been suggested that the similar mechanism is also operating in case of aminocolchicinoid system of compounds [7]. The obvious question is then why the colchicine molecule (which contains an amide $-NHCOCH_3$ group at C-7 position) fluoresces in presence of tubulin? Is it because the carbonyl oxygen prevents lone pair of nitrogen to interact with the π–system of colchicinoids? Then what happens to fluorescence, when a methylene group ($>CH_2$) is introduced in between nitrogen atom and the carbonyl group or when a less electronegative element sulphur atom is added as a thioamide ($-NHCSCH_3$) at C-7 position? In this paper, we have investigated fluorescence properties of such compounds and tried to answer the above questions.

We have reported that the binding of colchicine and its B-ring analogs (with C-7 substituents) to tubulin are pH sensitive and have high activation energy [8]. Under identical conditions, the binding of analogs without C-7 substituent is pH independent and has lower activation energy. Like fluorescence, the on-rate and the off-rate of colchicine-tubulin interactions are significantly low when compared with that of aminocolchicinoids [9]. These unusual properties of colchicine-tubulin interaction emphasize the role of oxygen atom at that particular position of B-ring side-chain. We, therefore, synthesized a compound, i.e., $NHCSCH_3$-DAAC, where sulphur atom replaced oxygen at the same position. In another analog, i.e., in $NHCH_2COCH_3$-DAAC, the position of carbonyl oxygen is shifted by a methylene ($>CH_2$) group (Figure 1) In this study, we have determined on-rate, activation energy, off-rate, reversibility and pH dependence of these aminocolchicinoids to explore the binding properties as well as mechanism of fluorescence of these B-ring colchicinoid analogs. Through our studies, interesting outcomes of the compounds have been emerging out which will be discussed in details in this communication.



R = H : DAAC: Deacetamido colchicine
R = $NHCOCH_3$: Colchicine
R = $NHCH_2COCH_3$: $NHCH_2COCH_3$- DAAC
R = $NHCSCH_3$: $NHCSCH_3$-DAAC
R = $N(Me)COCF_3$: $N(Me)COCF_3$-DAAC



AC
[2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone]

Figure 1. Structure of Colchicine and its B-ring analogs.

2. Materials and methods

Materials

PIPES, EGTA, GTP, and colchicine were purchased from Sigma. Other reagents were of analytical grade. $NHCH_2COCH_3$-DAAC and $NHCSCH_3$-DAAC were synthesized from deacetyl colchicine. The purity and identity of the compounds were determined using ^1H NMR and TLC analysis.

Drugs

A stock solution was made with 100% DMSO and the concentration of drugs was determined spectrophotometrically by use of an extinction coefficient in PEM buffer (50 mM PIPES, pH 6.9, 1 mM

EGTA, 0.5 mM $MgCl_2$). The extinction coefficients were as follows: $NHCH_2COCH_3$-DAAC at 352 nm, ε = 15785 $M^{-1}cm^{-1}$, $NHCSCH_3$-DAAC at 354 nm, ε = 14271 $M^{-1}cm^{-1}$, respectively.

Tubulin isolation and estimation

Pure tubulin was isolated from microtubular proteins by two additional cycles of temperature-dependent polymerization and depolymerization using 1 M glutamate buffer for assembly [10]. The protein was stored at –70°C. The protein concentration was determined by the method of Lowry *et al*. [11] using bovine serum albumin as standard.

Preparation of $\alpha\beta_s$ and $\alpha_s\beta_s$

Tubulin (50 μM) in 100 mM MES assembly buffer containing 3.4 (M) glycerol, 5 mM $MgCl_2$ and 1 mM GTP was preincubated at 37°C for 40 mins and then treated with 1:100 (w/w) subtilisin for 10 mins. The reaction was terminated by the addition of 1% by volume of 1% (w/v) PMSF in DMSO. The product $\alpha\beta_s$ is termed as hybrid tubulin**.** Digestion at 30°C cleaved C-terminus of both the subunits resulting in tubulin S ($\alpha_s\beta_s$) [12].

Polymerization Inhibition

Polymerization was initiated using 10% dimethyl sulfoxide (Me_2SO) and the turbidity was measured by the absorbance at 410 nm rather than at the usual 360 nm. A Shimadzu UV-160 double beam spectrophotometer, fitted with a temperature controlled circulating water bath accurate to ±0.2°C is used for this purpose.

Quantum Yield

Fluorescence quantum yield of the colchicinoid-tubulin complexes were calculated by comparison with the quantum yield of quinine sulphate in 0.1 M H_2SO_4. Solutions containing 30 μM tubulin and 3 μM ligand in PEM buffer were incubated for 1.0 h at 37°C. The absorbance of the tubulin-colchicinoids complexes was maintained below 0.05 at 350 nm for fluorescence measurement. The fluorescence spectra were measured at an excitation wavelength of 350 nm at 25°C in a Hitachi F-4000 spectrofluorimeter. The excitation and emission band passes were 10 and 5 nm respectively. The quantum yield of quinine sulphate was taken to be 0.547 at 25°C [13**].** The quantum yield of colchicinoid-tubulin complexes were calculated by comparing the absorbance at the exciting wavelength and the area of the emission spectra.

Association kinetics

The kinetics of the association of colchicinoids with tubulin was measured under pseudo-first order conditions using spectrofluorimeter. Concentrations of tubulin and colchicinoids were 1 and 20 μM, respectively. The ligand was added to tubulin solution, and emission at 335 nm was measured upon excitation at 280 nm (excitation and emission slits = 5 nm). The quenching data were analyzed according to Pyles and Hastie [7] using the following biexponential equation:

$$F = Ae^{-k1}t + Be^{-k2}t + C \qquad \text{(Eq. 1)}$$

where F is the fluorescence of the ligand-tubulin complex at time t, A and B are the amplitudes for the fast and slow phases, k_1 and k_2 are the pseudo-first-order rate constant for these two phases, respectively, and C is an integration constant. The data were analyzed using the software, Microcal Origin 5.0. For all the tubulin-colchicinoid complexes, the amplitude of the slow phase was not analyzed further. The apparent-second-order rate-constants (k_{on}) were determined at different temperature ranging from 25 to 37°C.

Activation energy

The association rate constant (k_{on}) was determined at different temperatures ranging from 25° to 37°C, and the activation energy (E_a) was calculated by plotting ln k_{on} against 1/T according to the Arrhenious equation,

$$k_{on} = Aexp\left(-\frac{E_a}{RT}\right)$$

where A is the preexponential factor.

Reversibility

To investigate whether these colchicinoids bind tubulin irreversibility or not, time dependence binding was monitored spectrofluorimetrically at 37°C when 5 μM tubulin was mixed with 5 μM of colchicinoids. After saturation, excess amount of podophyllotoxin (100 μM) was added to the reaction mixture and binding was monitored.

Dissociation kinetics

The dissociation of the colchicinoid-tubulin complexes were measured by monitoring the time-dependent increase of intrinsic protein fluorescence as the ligands were released from its binding site on tubulin upon a 300-fold dilution of the complex [14]. This process was described as a single first-order reaction. The rate constant of this process was determined using the relation:

$$\ln(F_{max} - F_t) = k_{off} \times t + constant \quad \text{(Eq.2)}$$

where F_{max} and F_t are the maximum intrinsic protein fluorescence intensity at infinite time and at time t, respectively, and k_{off} is the first-order dissociation rate constant. The dissociation rate constant was determined at 37 °C.

Scatchard analysis

We determined the affinity constant and stoichiometry for colchicine analogues with tubulin using a conventional Scatchard analysis [15].

$$\frac{r}{c} = nK - Kr \quad \text{(Eq.3)}$$

where r is the number of moles of drug bound per mole of tubulin, C is the free drug concentration, K is the affinity constant, and n is the number of drug binding sites on tubulin. We performed a reverse titration using 3 μM of the colchicinoids with increasing concentration of tubulin and a standard curve was obtained when 1/fluorescence was plotted against 1/[Protein]. From this curve, the fluorescence intensity corresponding to the 3 μM ligand-tubulin complex was determined. To generate the binding isotherm, colchicinoids (0.5-24 μM) were added to 3 μM tubulin and each sample was incubated at 37°C for 60 mins.

Affinity constant by quenching method

Affinity constant was also determined by considering quenching of protein fluorescence, as $NHCH_2COCH_3$-DAAC and $NHCSCH_3$-DAAC will have low quantum yield value. For this purpose, 1 μM tubulin was titrated with $NHCH_2COCH_3$-DAAC, $NHCSCH_3$-DAAC, and $N(Me)COCF_3$-DAAC (1-40 μM) in separate test tubes, incubated at 37ºC for 60 mins. Fluorescence was measured with excitation and emission wavelength of 280 and 335 nm respectively. Fluorescence data was corrected for inner filter effect according to the equation of Lakowicz [16]:

$$F_{cor} = F_{obs}\{antilog\ (A_{ex} + A_{em})/2\} \quad \text{(Eq.4)}$$

where A_{ex} and A_{em} are the absorbance at the excitation and the emission wave length, respectively.

Binding study at different pH

Binding of colchicinoids to tubulin at different pH was monitored using spectrofluorimeter. The tubulin concentration was 3 μM, and the drug concentration was 15 μM in all cases. We constructed a standard curve by titrating 3 μM of drug with increasing amounts of tubulin until the corrected fluorescence due to bound ligand reached saturation. This type of standard curves was generated for all these analogs at different pH values. The amount of drug-protein complex formed was determined from the respective standard curve. Complexes were excited at their absorption maxima and the emission spectra were taken at the corresponding emission maxima.

Binding of colchicinoids with αβ, $\alpha\beta_s$ and $\alpha_s\beta_s$ tubulin

We observed the binding fluorescence of colchicine, DAAC (devoid of B-ring side chain), AC (devoid of B-ring), and these three colchicinoids using 5 μM of desired protein (αβ, $\alpha\beta_s$ and $\alpha_s\beta_s$**)** with 10 μM of colchicine analogues at neutral pH (pH = 7.0).

3. Results and Discussion

Fluorescence of colchicinoids bound to tubulin

Aminocolchicinoids examined in this study are shown in Figure 2. Figure 2A contains colchicine, DAAC and AC compound and their fluorescence with excess tubulin. Similarly, Figure B contains DAAC, NH_2-DAAC, NH(Me)-DAAC and $N(Me)_2$-DAAC and their fluorescence with excess tubulin. Colchicine, $NHCH_2COCH_3$-DAAC, $N(Me)COCF_3$-DAAC and $NHCSCH_3$- DAAC and their fluorescence with excess tubulin are shown in Figure 2C.

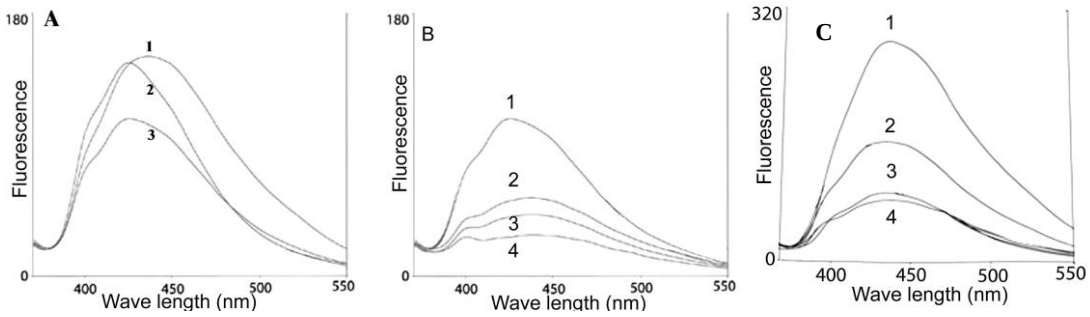


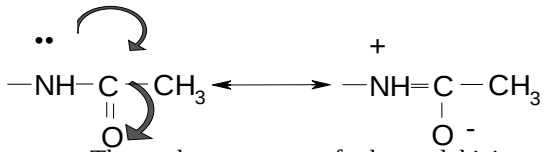
Figure 2. Emission spectra of aminocol-chicinoids with excess tubulin are shown. (A) colchicine (1), AC (2) and DAAC (3), **(B)** DAAC (1), NH_2-DAAC (2), NH(Me)-DAAC (3) and $N(Me)_2$-DAAC (4), **(C)** $N(Me)COCF_3$-DAAC (1), colchicine (2) $NHCSCH_3$-DAAC (3), and $NHCH_2COCH_3$-DAAC (4) are shown.

Biochemical studies have established that AC compound contains minimum structural requirement for the high affinity binding with [14]. It fluoresces in presence of tubulin. Similarly, DAAC also fluoresces in presence of tubulin as shown in Figure 2A. Quantum yield of these two colchicinoids are determined with 10 times excess tubulin and are shown in Table 1. These results established that B-ring or its side chain at C-7 position is not essential for the promotion of fluorescence of colchicinoids in presence of tubulin. While the B-ring side chain is not essential for the promotion of fluorescence of colchicinoids, the presence of side chain at C-7 position may significantly diminish the fluorescence properties of aminocolchicinoids. The quantum yield of the aminocolchicinoids decreased with amine substitution at the C-7 position in the following order : R=H>NH_2> $NHCH_3$>$N(CH_3)_2$. The quantum yield of allocolchicinoids with tubulin follows the similar trend [7]. The low quantum yield of the aminocolchicinoids in solvents and bound to tubulin was explained by the exciplex formation between the lone pair of electrons on the nitrogen atom and the allocolchicinoids π–system, which leads to the quenching of the fluorescence. Alkyl amine analog of aminocolchicinoids is less fluorescent compare to amine analog. Similarly, dialkyl derivative [$N(Me)_2$-DAAC] is less fluorescent than monoalkyl derivative [$NH(CH_3)$-DAAC]. This is because positive inductive effect of methyl group. This decrease in quantum yield of aminocolchicinoid follows the same trend as was observed in case of allocolchicinoid [7]. As suggested, we also believe that similar mechanism occurs in both series.

Table 1
Quantum yield of colchicinoid-tubulin complexes at 25°C

Name of the ligand	Colchicine	DAAC	AC	$N(Me)COCF_3$-DAAC	$NHCH_2COCH_3$-DAAC	$NHCSCH_3$-DAAC	NH_2-DAAC	NH(Me)-DAAC	$N(Me)_2$-DAAC
Quantum Yield (10^2)	1.4	0.75	0.95	2.0	0.69	0.71	0.48	0.45	0.42

If the quantum yield of the aminocolchicinoids decreases due to the presence of methyl (-CH_3) group at the nitrogen atom, then the presence of an electron-withdrawing group like oxygen (>C=O) should promote the fluorescence. This is exactly the case as colchicine ($NH.CO.CH_3$-DAAC) is highly fluorescent. It is to be noted that the presence of >C=O adjacent to nitrogen atom imparts special property on it. Thus, colchicine ($NH.CO.CH_3$-DAAC) is highly soluble in water; alkyl amine analogs ($NH.CH_3$-DAAC) are weakly soluble indicating that the solvation property of these molecules is very much influenced by the side-chain property at C-7 position. The presence of lone pair of electrons of nitrogen will significantly influence the carbonyl oxygen of colchicine side chain as follows:



Thus, the oxygen of the colchicine side chain and the electron rich nitrogen atom in aminocolchicinoids can serve as potential electron donor in making hydrogen bond with surroundings. It is possible that the amino and carbonyl groups are involved in making important contacts with the protein in the complex form. To verify the above-mentioned possibility, we synthesized an aminocolchicinoid analog with $NH.CH_2.CO.CH_3$ side chain at C-7 position. The presence of methylene group (-CH_2-) in between nitrogen and carbonyl should prevent the movement the electron from the nitrogen atom towards the carbonyl group

and hence the fluorescence should decrease compare to that of colchicine. This is indeed the case as shown in Figure 2C. We have seen the role of carbonyl group in promoting the fluorescence of colchicine upon binding to tubulin. These results confirmed that the electron-withdrawing group adjacent to nitrogen atom would promote fluorescence whereas group contributing towards positive inductive effect (electron donating group) would exhibit poor fluorescence. This phenomenon of inductive effect is further confirmed by designing an aminocolchicinoid analog having $–N(Me)COCF_3$ instead of $–NHCOCH_3$ at C-7 position which will withdraw the lone pair of electron of nitrogen atom more efficiently. The fluorescence emission spectra of $N(Me)COCF_3$-DAAC with tubulin show significant enhancement in quantum yield (Table 1) when compared with that of colchicine. Finally, we synthesize another analog with thiomide ($-NHCSCH_3$) side chain ($NHCSCH_3$-DAAC). This compound fluoresces poorly when compared to tubulin as shown in Table 1 and Figure 2C. Compared to oxygen, sulphur is less electronegative and therefore will withdraw lone pair of electron less efficiently.

Inter subunit communication through the 'Tail-body' interaction

From X-ray crystal structure of damacolchicine-tubulin complex [2], it is clear that A and C ring of colchicine completely resides in β–tubulin, while the B-ring shares both α and β-tubulin, its side chain at C-7 position interacts with α-tubulin only. Chromophore or the ring responsible for colchicine fluorescence is C-ring and its conjugation with the A-ring. However, we saw that the fluorescence of colchicinoids is also controlled at B-ring side chain at C-7 position. Since the side chain at C-7 position is in contact of α–tubulin, we are interested to check whether α–tubulin has any role in promoting colchicine fluorescence.

In the present study, we have chosen AC compound, which resides completely in β-tubulin, and DAAC that shares both α and β-tubulin. Third group of compound contains colchicine ($NHCOCH_3$-DAAC), $NHCH_2CO\text{-}CH_3$-DAAC, $N(Me)COCF_3$-DAAC, and $NHCSCH_3$-DAAC. Side chains of all these compounds make contact with α–tubulin. We measured fluorescence emission spectra of these compounds with native tubulin (αβ), hybrid tubulin ($\alpha\beta_s$), and tubulin S ($(\alpha_s\beta)_s$,. Results of such experiments are shown in Figure 3. Results are surprising as the fluorescence emission spectra of all these compounds follow very similar trend irrespective of the presence and absence of B-ring and its side chain. These results indicate that although the colchicinoid molecules bind essentially at β-tubulin, but β-C-terminus has no role on colchicinoids binding with tubulin as their digestion has little effect on fluorescence. More surprisingly we observed that the fluorescence emission spectra of all these compounds with $\alpha_s\beta_s$-tubulin is significantly less compared to that of αβ and $\alpha\beta_s$ tubulin. The presence of side chain or B-ring or absence of B-ring has no distinction. These could be due to the differences in the quantum yield of complexes with two types of tubulin. We therefore determined quantum yield of all these compounds with αβ and $\alpha_s\beta_s$ tubulin and there was 50% reduction in the quantum yield values.

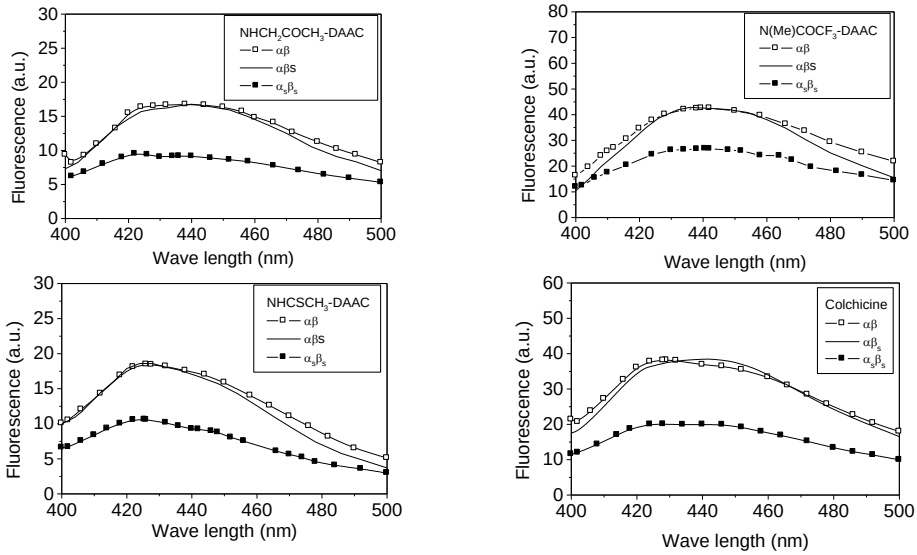


Figure 3. Emission spectra of different colchicinoids with αβ, $\alpha\beta_s$, and $\alpha_s\beta_s$-tubulin at 37ºCEffect of pH on the activation energy of colchicinoid-tubulin interaction

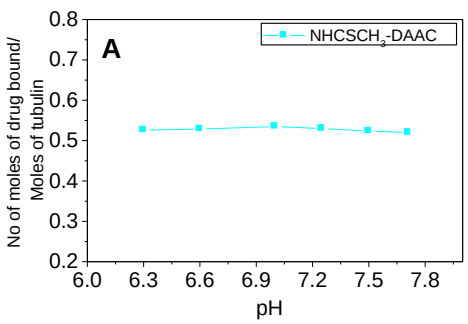
We have shown that the binding of colchicine and its B-ring analogs (with C-7 substituent) to tubulin are pH sensitive and have high activation energy [8]. Under identical conditions, the binding of analogs without C-7 substituent is pH independent and has lower activation energy. These results indicate

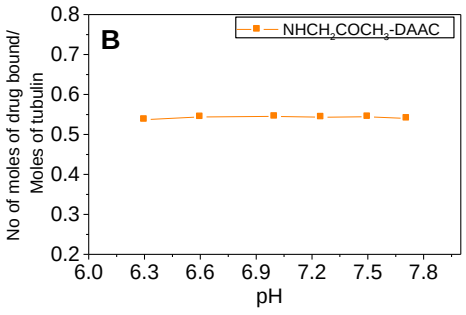
that B-ring side chain at C-7 acts as an indicator to monitor the changes in the ionization state and associated structural changes of tubulin due to the pH changes. We have tested the pH sensitivity of colchicine analogs used in this study such as: $NHCSCH_3$-DAAC (thiomide side chain), $NHCH_2COCH_3$-DAAC, and $N(Me)COCF_3$-DAAC upon binding with tubulin. Surprisingly, we observed that while $N(Me)COCF_3$-DAAC shows pH-dependency binding like colchicine, both $NHCSCH_3$-DAAC and $NHCH_2COCH_3$-DAAC binding to αβ-tubulin was little influenced by pH in the range of 6.0-8.0 (Figure 4). We also measured activation energy of $NHCSCH_3$-DAAC, $NHCH_2COCH_3$-DAAC, and $N(Me)COCF_3$-DAAC in this pH range of 6.0-8.0 (Figure 5). We observed that the activation energy of $NHCSCH_3$-DAAC and $NHCH_2COCH_3$-DAAC are around (13.45±0.10) Kcal/mole and (14.58±0.35) Kcal/mole at pH = 7.0 (Table 2) and both values are independent of pH (Figure 5). Under identical conditions, the activation energy of $N(Me)COCF_3$-DAAC is (21.20±0.78) Kcal /mole and shows pH dependency (Figure 5). β–C-terminus-truncated tubulin ($\alpha\beta_s$) with colchicine shows similar pH sensitivity and activation energy like native tubulin (αβ). Removal of the C-termini of both subunits of tubulin ($\alpha_s\beta_s$) or the specific binding of P_2 peptide to the C-terminus of α-tubulin causes a colchicine tubulin interaction independent of pH with low activation energy (data not shown). Although tubulin dimer structure with damacolchicine shows that the B-ring side chain is inside the α-tubulin, nevertheless the C-terminal α–tail is too far from the B-ring side chain to have a possible interaction between them. This is the first time, two B-ring C-7 analogs of colchicine have been identified, which bind to tubulin pH independently and behave like AC (no B-ring) or DAAC (B-ring without side chain at C-7 position). It is to be noted that colchicine fluorescein, which contains sulphur atom at the same position as $NHCSCH_3$-DAAC, also shows lower activation energy (~13 kcal/mole) and pH-independent binding with tubulin (data not shown). From these experiments, it appears that the position of oxygen atom in colchicine molecule is a crucial point where substituent of sulphur or incorporation of $>CH_2$ group in between carbonyl and oxygen atom produces compound, which exhibits very different binding mechanism with tubulin.

Table 2

Association and dissociation rate constants			
Ligand	**k_{on} ($M^{-1}s^{-1}$) [a]**	**E_a (kcal mol^{-1}) [b]**	**k_{off} x 10^3 (s^{-1})**
Colchicine	130	20.4 ± 0.3	(0.018)[c]
$NHCH_2COCH_3$-DAAC	241.55	14.58 ± 0.35	2.93
$NHCSCH_3$-DAAC	315.66	13.45 ± 0.19	2.15
$N(Me)COCF_3$-DAAC	123.27	21.20 ± 0.78	ND
AC [d]	52000.0	13	60

[a] Association rate constant determined at 37 °C.
[b] E_a, the activation energy.
[c] From Diaz *et al.*, 1997.
[d] From Bane *et al.*, 1984.





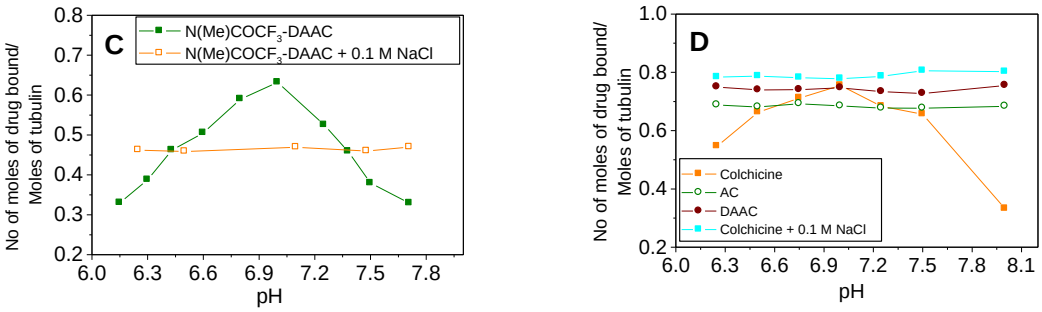


Figure 4. Effect of pH of the different colchicinoids binding to tubulin.

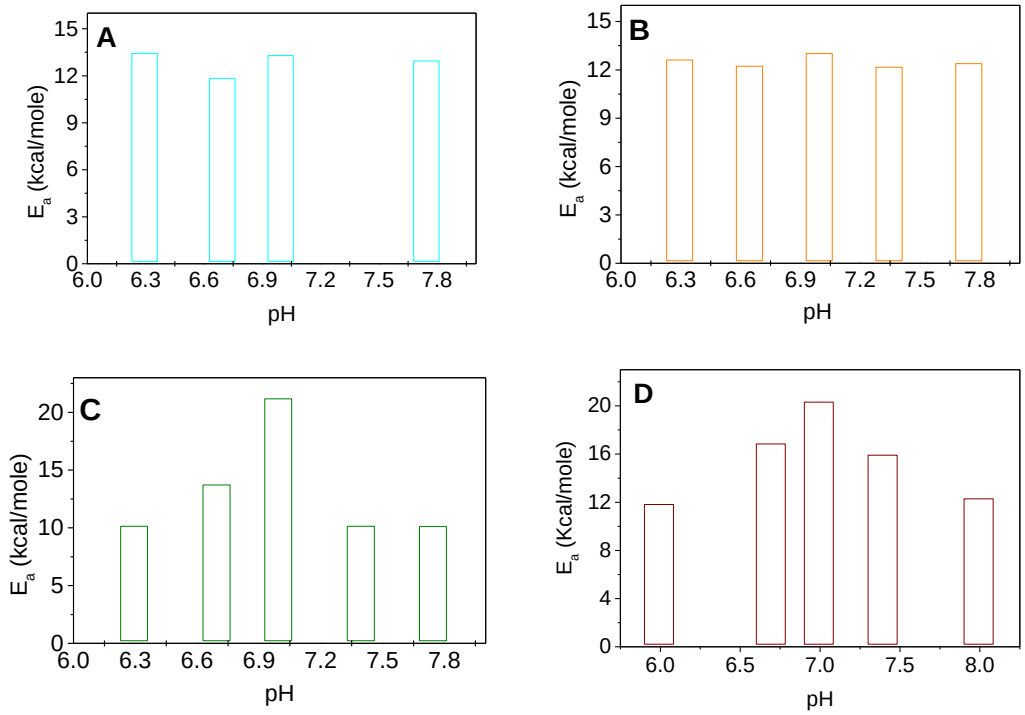


Figure 5. Effect of pH on activation energy of different colchicine analogs (A) NHCOCH$_3$-DAAC, (B) NHCSCH$_3$-DAAC, (C) N(Me)COCH$_3$ -DAAC, and (D) Colchicine.

Dissociation constant and reversibility

In this study, we have shown that quantum yield, fluorescence property, activation energy and pH dependence of aminocolchicinoids binding with tubulin are controlled at different points of B-ring and its side chain at C-7 position. Previous studies have reported that the on-rate and the off-rate of aminocolchicinoids binding to tubulin are being controlled at different points of analog structure. Thus, the on-rate and off-rate is highest for AC-tubulin interactions compared to aminocolchicinoids which follows the sequence as shown [R = H>>NH_2 ~ NH(Me) ~ $N(Me)_2$] [9]. The on-rate of DAAC-tubulin interaction is ~$3x10^3$ $M^{-1}S^{-1}$, it decreases 5-6 times upon substitution at C-7 position. Thus, the on-rates of NH_2-DAAC, NH(Me)-DAAC and $N(Me)_2$-DAAC are about (4-6)$x10^2$ $M^{-1}S^{-1}$ [9].Here, the first substitution affects on rate and further substitution of methyl group have no effect. Again, about 5-6 times lowering of on-rate occurs when acetyl group (>$COCH_3$) is added as in case of colchicine. The on-rate of colchicine binding is around 130 $M^{-1}S^{-1}$ (Table 2). Unlike on-rate, the off-rate remains more or less unchanged upon introduction of -NH_2 group at C-7 position as in case of NH_2-DAAC [9]. The addition of acetyl group as in case of colchicine (NH.CO.CH_3- DAAC), however, causes dramatic decreases in the off-rate from ~ $3x10^{-3}$ S^{-1} (for DAAC) to (5-9) x 10^{-6} S^{-1} (colchicine). Is this the property of oxygen atom at this particular position responsible for such observation? We have, therefore, synthesized two compounds NHCSCH$_3$-DAAC and NHCH$_2$COCH$_3$-DAAC and measured their off-rate fluorometrically. Surprisingly, the off-rates are $2.1x10^{-3}$ S^{-1} and $2.9x10^{-3}$

S^{-1} for $NHCH_2COCH_3$-DAAC and $NHCSCH_3$-DAAC respectively which are very similar values obtained for NH_2-DAAC, NH(Me)-DAAC and $N(Me)_2$-DAAC. These results indicate that the oxygen atom plays a very significant role, which is still unknown to us. This is possible that the oxygen of carbonyl side chain and nitrogen atom in aminocolchicinoids can serve as potential electron donor in making hydrogen with surrounding. It is also possible that the amino and carbonyl groups are involved in making important contacts with protein in complex form.

Finally, we measured the reversibility of both compounds and are shown in Figure 7. For this experiment, we first prepared complex of 5 μM tubulin and 5 μM colchicinoid analog at 37°C. After saturation, 100 μM podophyllotoxin was added to the complex. Fluorescence was measured with time till plateau was reached. Under identical condition, colchicine and AC were tested. While the addition of 100 μM podophyllotoxin to colchicine-tubulin complex causes no decrease in fluorescence, there are decrease of fluorescence 32% and 22% in case of $NHCH_2COCH_3$-DAAC and $NHCSCH_3$-DAAC respectively.

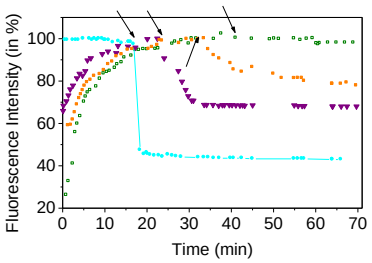


Figure 7. Effect of addition of podophyllotoxin (100 μM) to tubulin-colchicinoid complexes (5 μM) after saturation. Considering colchicine-tubulin binding (□) as irreversible, AC (●), NHCH2COCH3-DAAC (▼) and NHCSCH3-DAAC (■) show 57%, 32%, and 22% reversible binding.

Affinity constant and polymerization inhibition

We determined the stoichiometry and affinity constant for colchicinoids with tubulin using Scatchard analysis (Figure 8) as well as from tryptophan fluorescence quenching of tubulin (Figure 9). The affinity constant at 37°C for $NHCH_2COCH_3$-DAAC, $NHCSCH_3$-DAAC and $N(Me)COCF_3$-DAAC binding to tubulin were represented in Table 3. From scatchard plot, the affinity constant of $NHCH_2COCH_3$-DAAC, $NHCSCH_3$-DAAC and $N(Me)COCF_3$-DAAC towards tubulin were found to be 2.4, 5.2, and 3.4 x 10^5 M^{-1} respectively. Under the same experimental condition, colchicine possesses affinity constant value of 5.84 x 10^5 M^{-1}.

Equilibrium constants (K_a) have also been calculated using the following equation:

$$K_a = \frac{k_{on}}{k_{off}} \qquad \text{(Eq. 5)}$$

where k_{on} and k_{off} are the apparent second-order association rate constant and first-order dissociation rate constant, respectively.

However, the affinity constant for these colchicinoids as calculated from the kinetic parameters were very close to that of calculated from Scatchard analysis. Since, $NHCH_2COCH_3$ -DAAC, $NHCSCH_3$-DAAC have low quantum yield value, we also determined the affinity constant value by tryptophan quenching. Here we get almost identical K_a value. The results were represented in Table 3.

Table 3

Affinity constant and activation energy of B-ring analogs of colchicine binding to tubulin			
Name of the ligand	**[a] K_a x 10^5(M^{-1})**	**[b] K_a x 10^5(M^{-1})**	**[c] K_a x 10^5(M^{-1})**
Colchicine	5.84	-	-
$NHCH_2COCH_3$-DAAC	2.4	0.88	0.82
$NHCSCH_3$-DAAC	5.2	1.71	1.47
$N(Me)COCF_3$-DAAC	3.4	1.6	0.14
[a] Affinity constant determined from Scatchard plot at 37ºC. [b] Affinity constant determined from tryptophan quenching at 37ºC. [c] Affinity constant determined from $K_{eq} = k_{on}/k_{off}$ at 37ºC			

We tested the inhibition of tubulin polymerization of these colchicinoids using dimethyl sulfoxide (Me_2SO) to check their potency. *In vitro*, all colchicinoids show progressive concentration-dependent inhibition of tubulin self-assembly with an IC_{50} of 4.6 μM, 9.96 μM and 5.4 μM for $NHCSCH_3$-DAAC, $NHCH_2COCH_3$-DAAC, and $N(Me)COCF_3$-DAAC respectively (Table 4). Under the same experimental condition, colchicine was found to inhibit tubulin self-assembly with an IC_{50} of about 4.58 μM.

Table 4

IC_{50} of colchicinoids towards tubulin binding	
Name of the Drugs	**IC_{50} (μM)**
Colchicine	4.58
[$NHCH_2COCH_3$-DAAC]	9.96
[$NHCSCH_3$-DAAC]	4.6
[$N(Me)COCF_3$-DAAC]	5.4

4. Conclusion

In our study, we attempted to generate some B-ring colchicinoids from which some interesting binding information regarding the B-ring and C-7 side chain are spurring out of it. From our work, it has been noticed that pH dependency or high activation energy or high quantum yield of colchicinoids are consequences of ionic interaction between α-chain C-terminus and B-ring C-7 side chain carbonyl group of drug molecule.

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