Mapping the Binding Site of Colchicinoids on β-Tubulin

2-CHLOROACETYL-2-DEMETHYLTHIOCOLCHICINE COVALENTLY REACTS PREDOMINANTLY WITH CYSTEINE 239 AND SECONDARILY WITH CYSTEINE 354*

Ruoli Bai‡, David G. Covell§, Xue-Feng Pei¶, John B. Ewellí, Nga Y. Nguyení, Arnold Brossií, and Ernest Hamel‡**

From the Screening Technologies Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, NCI-Frederick Cancer Research and Development Center, Frederick, Maryland 21702, the Laboratory of Experimental and Computational Biology, NCI-Frederick Cancer Research and Development Center, Science Applications International Corporation-Frederick, Frederick, Maryland 21702, the Laboratory of Structural Biology, NIDDK, National Institutes of Health, Bethesda, Maryland 20892, and the Facility for Biotechnology Resources, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland 20892

2-Chloroacetyl-2-demethylthiocolchicine (2CTC) and 3-chloroacetyl-3-demethylthiocolchicine (3CTC) resemble colchicine in binding to tubulin and react covalently with β-tubulin, forming adducts with cysteine residues 239 and 354. The adducts at Cys-239 are less stable than those at Cys-354 during formic acid digestion. Extrapolating to zero time, the Cys-239 to Cys-354 adduct ratio is 77:23 for 2CTC and 27:73 for 3CTC. Using energy minimization modeling to dock colchicinoids into the electron crystallographic model of β-tubulin in protofilaments (Nogales, E., Wolf, S. G., and Downing, K. H. (1998) Nature 391, 199–203), we found two potential binding sites. At one, entirely encompassed within β-tubulin, the C2- and C3-oxygen atoms of 2CTC and 3CTC overlapped poorly with those of colchicine and thiocolchicine, but distances from the reactive carbon atoms of the analogs to the sulfur atoms of the cysteine residues were qualitatively consistent with reactivity. The other potential binding site was located at the α/β interface. Here, the oxygen atoms of the analogs overlapped well with those of colchicine, but relative distances of the reactive carbons to the cysteine sulfur atoms did not correlate with the observed reactivity. A significant conformational change must occur in the colchicine binding site of tubulin in the transition from the unpolymerized to the polymerized state.

Despite the interaction of tubulin with a large number of drugs that inhibit or promote its assembly into microtubules, precise definition of drug binding sites on the protein has not been possible. This is a consequence of the lack of success in crystallizing the protein, probably because of its sequence and post-translational heterogeneity, its instability, and its tendency to form oligomers and polymers of highly aberrant morphology in the presence of many of these drugs. The recent electron crystallographic determination of a relatively detailed structure for zinc-induced antiparallel tubulin protofilaments has provided insights into the paclitaxel/docetaxel site on these protofilaments, since docetaxel was used to enhance their stability during data accumulation (1), and provided a scaffold on which to model other drug sites (2). A limitation in such analysis, however, is that drugs that inhibit assembly have limited ability to bind to tubulin polymers containing linear protofilaments.

Alternative approaches to obtain preliminary information about drug binding sites have included “direct” photocross-linking (e.g. Ref. 3), analog photoaffinity labeling (e.g. Ref. 4), and cross-link formation with chemically reactive analogs that contain biological activity (e.g. Ref. 5). In the first method, a drug-tubulin complex is exposed to light of an appropriate wavelength, and ligand-protein cross-link formation is evaluated. In the second, an active drug analog containing a photo-reactive moiety is prepared and bound to tubulin, and cross-link formation is induced by exposure of the complex to light of an appropriate wavelength. In the third method, an active analog with a chemically reactive moiety is prepared and bound to tubulin, and cross-link formation occurs either spontaneously or, in principle, following a rapid change in reaction conditions. Generally, the reactive ligand is radiolabeled to permit quantitation of the reaction and identification of peptides in the protein involved in cross-link formation. In all cases, there are two major problems. The first is the potential for nonspecific protein alkylation by the ligand, which is generally excluded by demonstrating that excess nonreactive ligand substantially inhibits the covalent reaction. The second is that adequate radiolabel participates in cross-link formation to permit identification of the tubulin subunit(s) and the ligand region of the subunit, and, ideally, the specific amino acid residue(s) involved.

The tubulin-colchicine interaction has attracted a great deal of attention, probably because of its unusual chemical characteristics (for a review, see Ref. 6). The drug (structure in Fig. 1) binds exceptionally slowly, but noncovalently, to tubulin, and, once formed, the drug-protein complex is highly stable. Although some have described the association reaction as “essentially irreversible” (6), the dissociation reaction has been carefully studied, and the half-life of the tubulin-colchicine complex varies from 14 to 77 h at 37 °C, depending on precise reaction conditions. In addition, the interaction of tubulin with colchicine appears to involve significant changes in conformation in both the drug and the protein.

Photoaffinity analogs of colchicine have reacted predominantly with α-tubulin or with both subunits (7, 8). In contrast, direct photoaffinity labeling, with irradiation at 350 nm (the
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absorbance maximum of the tropolonic C ring of colchicinoids), resulted in strongly preferential labeling of $\beta$-tubulin (3, 9). A cross-link was formed between radiolabeled colchicine and amino acid(s) in peptide sequence 1–36 or peptide sequence 214–241, but not with both peptides.

Our own approach has been to place the small chloroaetyl group (about 3 Å in length) at various locations in analogs of colchicine and thiocolchicine. When placed in the side chain or amino acid(s) in peptide sequence 1–36 or peptide sequence 214–241, but not with both peptides.

In the present study, we demonstrate the reactivity of 2CTC predominantly with cysteine 239 but secondarily with cysteine 354 as well. We also attempted to construct a model for the A ring subsite of colchicine based on quantitative differences in the reactivity of 2CTC and 3CTC with the two cysteine residues and on the electron crystallographic model of tubulin (1).

EXPERIMENTAL PROCEDURES

Materials—Preparation of electrophoretically homogeneous bovine brain tubulin (10) and [$^{14}$C]2CTC (11) were described previously. Specific activity of the [$^{14}$C]2CTC was 40 cpm/pmol. Decylagarose was from ICN Immunobiologicals; CNBr and N-ethylmaleimide were from Sigma; podophyllotoxin and formic acid were from Aldrich; “sequencing grade” trypsin and “sequencing grade” EP-GC (Staphylococcus aureus V8) were from Roche Molecular Biochemicals; and precast Tricine-16% acrylamide polacyrylamide gels and PVDF membranes were from Novex. Kodak Biomax MR film was used for preparation of autoradiographs.

Preparation of Tubulin Derivatized with [$^{14}$C]2CTC and Separation of $\alpha$- and $\beta$-Tubulin Subunits by Decylagarose Chromatography—Reaction mixtures contained 25 $\mu$g (2.5 mg/ml) tubulin, 25 $\mu$g [$^{14}$C]2CTC, podophyllotoxin as indicated, 1.0 M monosodium glutamate, 0.1 M sodium phosphate (pH 7.0), 0.1 mM GDP, and 0.5 mM MgCl$_2$. Incubation was for 30 min at 37 °C, and the reaction was stopped by adding N-ethylmaleimide to a final concentration of 5 mM. The mixture was left overnight at 4 °C, but precipitation of the tubulin was usually incomplete. The mixture was made 5% (v/v) in trichloroacetic acid, and, after an additional 30 min at 0 °C, the precipitated protein was harvested by centrifugation. The pellet was dissolved in a solution containing 4 M guanidine hydrochloride and 2 M NaCl (adjusted to pH 5.0 with HCl) and applied to a column of decylagarose (12). Chromatography and analysis of protein peaks by SDS-PAGE was performed as described previously (5). Stoichiometry of [$^{14}$C]2CTC associated with the $\beta$-tubulin peak was 0.17, and stoichiometry of that associated with the $\alpha$-tubulin peak was 0.03. Only $\beta$-tubulin of least 90% purity was used in further studies, except in the podophyllotoxin inhibition study, in which unresolved tubulin was used.

Chemical and Enzymatic Digestions of $\beta$-Tubulin Cross-linked to [$^{14}$C]2CTC—The [$^{14}$C]2CTC-$\beta$-tubulin was digested at 37 °C in the dark either with 75% formic acid for 96 h (13) or with CNBr (20 mg/ml) in 70% formic acid for 24 h. The formic acid and, if present, CNBr were removed by lyophilization, and the residue was washed twice with water, which was removed each time by lyophilization.

For enzymatic digestions, the [$^{14}$C]2CTC-$\beta$-tubulin was dissolved in 1.0 M Tris (pH 8.0 with HCl). The tubulin solutions were diluted 10-fold into 50 mM ammonium acetate (pH 4.0) for EP-GC or into water for trypsin, and the appropriate enzyme was added at an enzyme/substrate ratio of 1:50. The resulting reaction mixtures were incubated for 24 h at 37 °C in the dark. At the end of the incubation, about 75% of the water in the samples was removed by lyophilization.

Peptide Purification—Peptide separation was by SDS-PAGE on

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Fig. 1. Structures of colchicine, thiocolchicine, 2CTC, and 3CTC. In the diagrams of 2CTC and 3CTC, the radiolabeled carbons are indicated by the arrowheads. The compounds are shown in the preferred dS-7S configuration (38).

The abbreviations used are: 2CTC, 2-chloroacetyl-2-demethylthio-colchicine; [$^{14}$C]2CTC, 2-chloroacetyl-[14C]carbonyl)-2-demethylthio-colchicine; 3CTC, 3-chloroaetyl-3-demethylthiocolchicine; [$^{14}$C]3CTC, 3-(chloroethyl-[14C]carbonyl)-3-demethylthiocolchicine; HPLC, high performance liquid chromatography; PVDF, polyvinylidene difluoride; EBI, N,N'-ethylenebis(sodiumetamide; EP-GC, endoproteinase Glu-C; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)glycine. 

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Novex precast gels, with the peptide solution to be analyzed dissolved in the Tricine-SDS sample buffer solution provided by Novex. Following electrophoresis, the separated peptides were transferred from the gel to a PVDF membrane (pore size, 0.2 μm) with an Entrapex semidry transblot system (1 h, 100 V). The membrane was stained with Coomassie Blue R250 and autoradiographed (24–72 h exposure). Radiolabeled peptides were cut from the membrane for sequencing.

**Sequence Analysis**—Automated Edman degradation for determination of amino acid sequence was performed with an Applied Biosystems model 494A Protein Sequenator. Identification of phenylthiohydantoin-derivatives was carried out with an Applied Biosystems model 140C Microsequenator System and model 780A Programmable Absorbance Detector. Identification of radiolabeled amino acid residues was performed by the University of Virginia Biomolecular Research Facility. Following each cycle of Edman degradation, the sample stream was analyzed by liquid scintillation counting for radiolabel instead of analyzed by HPLC to identify the derivatized amino acid residue.

**Molecular Modeling**—A two-stage modeling analysis was used, first to identify candidate binding sites on the tubulin dimer and, second, to dock the molecular structures of colchicinoids into these sites. The first procedure probes the Cα coordinates of the tubulin dimer (1) to determine exterior positions that are most likely to be found within a binding interface. This analysis uses information about local geometry and chemical composition of subregions of the target surface for selecting candidate sites. Relative rankings of these potential interaction sites are based on a scoring scheme derived from a statistical analysis of all known protein-ligand complexes. In applying this method to analysis of new crystal complexes, we have found that the correct ligand interface is found within the top 5% of candidate binding sites. This method has also been shown to correctly identify ligand binding sites for a wide range of proteins and ligands (14, 15). For a complete description of this procedure see Ref. 16.

The second stage of the analysis involves docking the test ligands at candidate binding sites. The initial docking is based exclusively on geometric considerations. This step uses a "geometric hashing technique" that has been found to rapidly determine a family of possible binding geometries for each ligand (17). Each of these possible binding arrangements is further refined to determine those positions with the maximum binding strength between ligand and target protein. A previously published model of ligand binding (18) was used to select the best binding geometries. This model is based on the atomic preferences of adjacent surfaces buried within a binding interface (18). The model has been shown to predict accurately ligand binding strengths and assess the relative contributions of atomic interactions within a binding interface. Moreover, the model has been extended as an adjunct to computational docking (19), has proven effective for identifying ligands active against Ncp7 targets (20), and has been useful in providing testable hypotheses about the modes of action of candidate inhibitors for a variety of enzymes (21–23).

The final stage of docking was obtained from successive in vacuo molecular dynamics and energy minimization calculations using the CVFF91 force field within Discover5.0 (Molecular Simulations, Inc., San Diego CA), based on the candidate geometries obtained from steps one and two, as outlined above. This final step resulted in small changes in geometries, both in the ligand and in the target protein, primarily to eliminate energetically unfavorable van der Waals interactions. These dynamics and minimization steps were performed repeatedly to achieve the final geometries. Exploration of these final geometries, which were used for our analysis, indicates trapping in a local energy minimum. The resulting geometries did not significantly alter the starting geometries of each ligand and were acceptable within the 3.6-A resolution of the electron crystallographic structure of tubulin (1).

**RESULTS**

In our initial characterization of the interactions of 2CTC and 3CTC with tubulin (24), we found that these interactions were similar to that of colchicine with tubulin (slow, temperature-dependent binding; similar quantitative inhibitory effects on polymerization; similar binding stoichiometries), and both compounds were competitive inhibitors of the binding of [3H]colchicine to tubulin (apparent Kd values of about 3 μM). Unlike colchicine, however, they formed a covalent bond with β-tubulin, and bond formation, as well as the initial binding reaction, was strongly inhibited by podophyllotoxin. The major difference between 2CTC and 3CTC was in the covalent reactions, which were studied most extensively with the compounds at 5 μM and tubulin at 20 μM. With 3CTC, the covalent reaction occurred almost simultaneously with binding, and about 57% of the bound drug formed a covalent bond with tubulin. With 2CTC, covalent bond formation was much slower than the binding reaction. After 30 min, about 26% and after 1 h 30% of the bound drug had covalently reacted with the tubulin. Finally, with superstoichiometric concentrations of both 2CTC and 3CTC the covalent reactions were more extensive, but there was a significant reduction in the apparent specificity of the covalent reactions (i.e. a smaller proportion of covalent bond formation was inhibited by podophyllotoxin).

Because of the more extensive covalent reaction with 3CTC, we initially studied it in detail (5), purifying the alkylated β-tubulin by decylagarose chromatography. Analysis of CNBr peptide digests resolved by HPLC was consistent with alkylation of Cys-354 and Cys-239 in roughly a 2:1 ratio, but formic acid peptide digests resolved by SDS-PAGE indicated a 9:1 ratio.

In the studies presented here, we used 75% formic acid digestion for the initial analysis of [14C]2CTC-containing peptides. The primary cleavage site under the condition used is aspartylproline (13), and there are two such sites in β-tubulin (positions 31/32 and 304/305). Rao et al. (4) termed the three resulting peptides A1 (residues 1–31), A2 (residues 32–304), and A3 (residues 305–445), and these peptides migrate as expected upon SDS-PAGE. In our hands, there are also secondary cleavage sites of β-tubulin in formic acid (5), but the amino acid sequences of the less prominent bands suggested that secondary cleavage occurred subsequent to hydrolysis of the aspartylproline bonds (also see below).

In an initial experiment with unresolved tubulin following its interaction with [14C]2CTC, we observed heavy labeling of both A2 and A3 by [14C]2CTC. Formation of both radiolabeled peptides was abolished in the presence of podophyllotoxin (data not presented), consistent with our previous observations (24).

Thus encouraged, we separated α- and β-tubulin on decylagarose following the reaction with [14C]2CTC prior to formic acid digestion. After formic acid treatment, the protein digest was subjected to SDS-PAGE, and the peptides on the gel were electrotransferred to a PVDF membrane, which was stained and autoradiographed. Track I in Fig. 2A shows the Coomassie Blue stain pattern obtained, with peptides A1, A2, and A3 indicated. Track II is the autoradiogram of the stained track I, showing the heavily labeled A2 and A3, with a number of minor...
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Table I

Amino acid sequence analysis of the major radiolabeled peptides derived from tubulin cross-linked to [14C]2CTC

| Cycle no. | Formic acid peptides | CNBr peptides | EP-GC peptides |
|-----------|----------------------|---------------|----------------|
|           | A2                   | A3            | a              | b              | a     | b     |
| 1         | Pro-32               | Pro-305       | Ser-234        | Leu-331        | XX    | Thr-199 |
| 2         | Thr                  | Arg           | Gly            | Asn            | Ile   | Tyr   |
| 3         | Gly                  | His           | Val            | Thr            | Gln   | Asn   |
| 4         | Ser                  | Gly           | Thr            | Asn            | Gln   | Asn   |
| 5         | Tyr                  | Arg           | X              | Lys            | Val   | Asn   |
| 6         | His                  | Tyr           | Leu            | Asn            | Lys   | Glu   |
| 7         | Gly                  | Leu           | Thr            | Asn            | Thr   | Ala   |
| 8         | Asp                  | Thr           | Phe            | Tyr            | Ala   | Tyr   |
| 9         | Ser                  | Val           | Val            | Pro            | Val   | Asp   |
| 10        | Asp                  | Ala           | Pro            | X              | Asp   | Arg   |
| 11        | Leu                  |                |                |                |       |       |
| 12        | Gln                  |                |                |                |       |       |
| 13        | Leu                  |                |                |                |       |       |
| 14        | Glu                  |                |                |                |       |       |
| 15        | Arg                  |                |                |                |       |       |
| 16        |                    |                |                |                |       |       |
| 17        |                    |                |                |                |       |       |

Fig. 2B and the sequence data obtained from the peptide suggested that the secondary reaction was more substantial than we had previously thought. At the same time, the apparent identity of the CNBr peptides obtained from the [14C]3CTC-reacted β-tubulin and the [14C]2CTC-reacted β-tubulin strongly indicated that the two colchicine analogs alkylated the same cysteine residues in different proportions.

The microsequencing Edman degradation procedure uses too little material for direct identification of the radiolabeled amino acid residue on peptides embedded in PVDF membranes (the degradation steps are performed directly on the membrane slice). In previous studies (5, 25), we have found it possible to perform an appropriate digestion of the entire polypeptide, subject the peptide mixture to sequential Edman degradation, and count the outflow of each cycle to obtain evidence to identify the specific amino acid residue that had been alkylated. Considering the two radiolabeled peptides generated by CNBr digestion (234–257 and 331–363), a total of β-tubulin-[14C]2CTC cyanogen bromide digest should yield a peak of radiolabel at the sixth degradation cycle if the expected Cys-239 were radiolabeled (radiolabel cross-linked to Cys-354 would not appear for 23 cycles). Such an experiment was performed, with the outflow of 10 cycles counted. We obtained the expected result (Fig. 3A) and conclude that the major alkylation of β-tubulin by 2CTC occurs at Cys-239.

Table II

Relative apparent reactivity of Cys-239 and Cys-354 with 2CTC and 3CTC

| Digestion method | [14C]2CTC | [14C]3CTC |
|------------------|-----------|-----------|
| Densitometry data |           |           |
| Formic acid (Fig. 2A) | 0.7 | 0.1 |
| Formic acid (overall) | 1.2 ± 0.5 | 0.18 ± 0.1 |
| CNBr (Fig. 2B) | 2 | 0.4 |
| CNBr (overall) | 1.8 ± 0.4 | 0.43 ± 0.02 |

| Analog | Calculated/stoichiometry |
|--------|--------------------------|
|        | Total | At Cys-239 | At Cys-354 |
| 2CTC   | 0.17 | 0.12     | 0.05 (0.04) |
| 3CTC   | 0.32 | 0.11     | 0.21 (0.16) |

*See “Discussion.”

Peptides sequenced are those shown in Fig. 2. X indicates the cysteine positions in the sequences, and XX the tryptophan residues. These residues cannot be identified following Edman degradation, and in the actual sequence studies no definitive amino acid assignment could be made. Other positions for which no definitive assignment could be made are indicated by the absence of an entry. Sequencing was performed by automated Edman degradation on an Applied Biosystems model 494A Protein Sequenator. Identification of phenylthiohydantoin amino acid derivatives was performed with an Applied Biosystems model 140C Microgradient System and model 785A Programmable Absorbance Detector.

radiolabeled bands between A2 and A3 and between A3 and A1. These presumptive assignments were confirmed by sequential Edman degradation for 15 cycles in the case of A2 and 10 cycles for A3 (Table I). In addition, sequence analysis was performed on minor radiolabeled bands running between A2 and A3 and between A3 and A1. In the former case, these were found to have the same amino-terminal sequence as A2 and, in the latter, the same amino-terminal sequence as A3 (data not presented). For comparison, we also include as track II in Fig. 2A an autoradiogram of formic acid-digested β-tubulin following an identical incubation with [14C]3CTC. As previously (5), there was little radiolabel in A2, in contrast to the heavy radiolabel in A3 and A3 fragments. Densitometric analysis of gels II and III indicated that the ratio of A2 + fragments to A3 + fragments was about 0.7:1 following reaction with 2CTC and 0:1:1 following reaction with 3CTC (see Table II).

To better define the two reactive amino acids following the covalent interaction of β-tubulin with [14C]2CTC, we next digested the decylagarose-isolated protein with CNBr, with subsequent PAGE and electrophoresis of the peptides to PVDF. An autoradiogram from a typical experiment is shown as track I in Fig. 2B. Reproducibly, only two radiolabeled bands were observed, with the upper “peptide a” more heavily labeled than the lower “peptide b”. Sequential Edman degradation of peptides a and b yielded sequences consistent with CNBr-derived peptides spanning residues 234–257 (for 10 cycles) and 331–363 (17 cycles), respectively. The former peptide is entirely within the A2 peptide obtained with formic acid, and the latter is within the A3 peptide.

We also reevaluated β-tubulin following its reaction with [14C]3CTC and CNBr digestion by the PAGE-electrotransfer methodology. A typical autoradiogram is shown as track II in Fig. 2B. Reproducibly, only two radiolabeled bands were observed in the form of peptide a and b peptides in the two tracks shown in Fig. 2B were 2:1 for the 2CTC sample and 0:4:1 for the 3CTC sample, as determined by densitometry (summarized in Table II).

In our previous study, we had clearly established that the bound [14C]3CTC reacted primarily with Cys-354 of β-tubulin, but we had also obtained preliminary data consistent with a secondary reaction of bound 3CTC with Cys-239 (5). However, the autoradiogram of the electroblot shown as track II in...
To confirm that 2CTC alkylation of β-tubulin also occurred at Cys-354, we turned to enzymatic digestion of β-tubulin that had reacted with the drug. A single experiment with EP-GC was performed. The peptide digest was subjected to SDS-PAGE and electrotransfer to PVDF, and the autoradiogram obtained is presented in Fig. 2C. Two closely spaced radiolabeled peptides were observed, and the sequences obtained from the minor, upper peptide a and the major, lower peptide b are presented in Table I. Consistent with EP-GC digestion, the sequence of the minor peptide, through 11 cycles, resulted from cleavage between Glu-343 and Trp-344 (the carboxyl terminus of this peptide could be at Asp-355 but most likely is at Glu-376, Glu-383, or Glu-401, based on the apparent size of the peptide). This result further narrowed the location of the secondary alkylation site of 2CTC to the β-tubulin sequence spanning amino acid residues 344–363 (the amino terminus of the CNBr secondary peptide).

The major EP-GC peptide, however, yielded an unexpected sequence, beginning at Thr-199, indicating cleavage after Glu-198. Three potential downstream cleavage sites (Asp-203, Glu-205, and Asp-209) are included in the sequence obtained, and an additional potential site at Asp-224 must have also been skipped for this peptide to include Cys-239. Based on peptide size, we assume that the carboxyl cleavage site was Asp-249, since the next potential residue is Glu-288.

We next examined trypsin, but only very small peptides were generated from β-tubulin that had reacted with [14C]2CTC, and resolution by SDS-PAGE was minimal. On most electropherograms, a single radiolabeled peptide band was observed, but sequence analysis indicated it was very heterogeneous. However, when the entire β-digest was subjected to sequential degradation and the outflow stream was counted, a dramatic radiolabeled peak was observed at the fourth of 14 cycles (Fig. 3B). This is the expected result for radiolabel at Cys-354, for trypsic cleavage should occur at Lys-350. (The tryptic peptide containing Cys-239 should begin with Leu-217, so no radiolabel should derive from this peptide during the 14 cycles examined.)

_Molecular Modeling—_The electron crystallographic coordinates of the αβ-tubulin dimer (1) were analyzed for candidate binding sites for colchicine. As described above, the procedure consists of scoring the solvent-accessible surface of the tubulin dimer for cavities with strong ligand binding features. This method was developed by a detailed examination of multiple crystallographically available ligand-receptor complexes, with subsequent determination of residue types most likely to be found in a binding interface. Subsequent testing of this method against newly available crystal complexes has shown that the correct ligand binding site is found within the topmost candidate sites identified by the method. Regions on the tubulin dimer that were identified as candidate binding sites were then subjected to computational docking with colchicine, thio-colchicine, 2CTC, and 3CTC. The final docking arrangement was obtained by sampling the lowest energy geometries from successive _in vacuo_ molecular dynamics and minimization calculations. In these simulations, both ligand and target were allowed to relax to their lowest energy configurations.

In the initial step, the entire surface of the tubulin dimer was scanned for candidate binding sites. The analysis yielded sites that were located at the plus and minus ends of the dimer, in a region entirely within β-tubulin near the paclitaxel binding site, and at the α/β interface. The two highest scoring sites for colchicine binding were the latter two, and both were located near the Cys-239/Cys-354 region of β-tubulin. The sites at the plus and minus ends of the dimer were not examined further, since they were distant from the two reactive cysteine residues. The site contained within β-tubulin we term “site A” (cf. Ref. 2), and the interface site we term “site B.” Fig. 4 shows a colchicine molecule docked into each of these alternative potential binding sites (we emphasize that this figure does not mean to imply two independent binding sites for colchicine), with the left-hand panel showing the solvent accessible surface and the right-hand panel the peptide backbone as a ribbon diagram. Note that site B is most consistent with data obtained with photoactive colchicine analogs, where covalent interactions with both α- and β-tubulin were observed (7, 8).

The amino acid residues of β-tubulin in closest contact with colchicinoids bound in Site A were His-227 and Phe-270 (A ring), Val-23 and Ala-231 (B ring), and Asp-26, Tyr-36, and Phe-242 (C ring). For site B, β-tubulin residues in closest contact with bound colchicinoids were Tyr-36 (A ring) and Arg-2 (C ring), and α-tubulin residues were Asp-76 (B ring) and Thr-73 (C ring).

In Fig. 5, we show the relationship of the site A (A) and site B (B) docked colchicine molecules to the peptides identified by Uppuluri et al. (9) following direct photoaffinity labeling of tubulin by colchicine. In both panels, the backbone of the peptide containing residues 1–36 is shown in white, while the peptide containing residues 214–241 is shown in orange. In addition, the side chains of Cys-239 and Cys-354 are shown in both panels, with the sulfur atom of the former colored light blue and of the latter colored yellow. In Table III, we present the distances for both potential binding sites between the C2 and C3 oxygen atoms of colchicine and the Cys-239 and Cys-354 sulfur atoms. Although the backbone of the residue 214–241 peptide appears to be in closer contact with colchicine docked in site A than in site B, van der Waals distances measured from colchicine docked in the sites to any atom of the peptide, including the side chains, did not allow us to choose between sites A and B. Colchicine docked in site A was 2.8 Å from residue 33 and 2.1–2.5 Å from residues 231, 234, and 239. Colchicine docked in site B was 1.6–2.6 Å from residues 2, 3, and 36 and 2.1–2.3 Å from residues 240 and 241.

In addition, in examining this region of the model in detail, we noted that for potential binding site A several amino acid side chains formed a significant barrier between the sulfur...
atoms of Cys-239 and Cys-354 and between colchicine and the sulfur atom of Cys-354, and, conversely, for potential binding site B these side chains formed a barrier between the bound colchicine and the sulfur atom of Cys-239 (not shown). This aspect of the model appears to be inconsistent with the ready cross-linking of Cys-239 and Cys-354 in unpolymerized tubulin that occurs with EBI and the extensive inhibition of this intercysteine cross-link formation by colchicine site drugs (26, 27). A significant conformational change may therefore occur in this region of the molecule when tubulin αβ-dimers polymerize into protofilaments.

When thiocolchicine was modeled into both sites by energy minimization, its position did not differ greatly from that of colchicine (Table III), consistent with its similar properties in binding to tubulin (28, 29). However, neither the C2-oxygen atom of 2CTC nor the C3-oxygen atom of 3CTC overlapped closely onto the C2- and C3-oxygen atoms, respectively, of colchicine when these molecules were positioned into site A by energy minimization (Fig. 6). These shifts in position, however, did bring the reactive carbon atoms of the chloroacetyl groups relatively close to the two sulfur atoms, and the relative distances (shown in Table III) did correlate qualitatively with the relative reactivities of the two cysteines that we have observed. However, in this modeling method the amino acid side chains blocking access to the sulfur atom of Cys-354 were not substantially repositioned, so that this sulfur atom remains shielded from the chloroacetyl moieties and therefore should not react with them.

For potential binding site B, the energy minimization modeling again showed negligible differences between colchicine and thiocolchicine (Table III), and in this binding site the C2-oxygen atom of 2CTC and the C3-oxygen atom of 3CTC more closely overlapped with the corresponding oxygen atoms in colchicine (Fig. 7). However, the distances from the reactive carbons of both analogs to the sulfur atoms of Cys-239 and Cys-354 were all nearly identical (Table III). In addition, the Cys-239 sulfur atom remained shielded from the chloroacetyl moieties of 2CTC and 3CTC by amino acid side chains. In Fig. 7, we also show a portion of the α-tubulin peptide backbone, and it is notable how the B ring side chain of the colchicinoids has similar proximity to both tubulin subunits. Note also that in site B the colchicinoid C ring overall is in closer contact with α-tubulin than with β-tubulin.

The poor overlap of the colchicinoids in site A raised the
question of whether the iterative energy minimizations had resulted in a significant change in drug conformation in fitting the compounds into this site. Comparison of \textit{in vacuo} drug structures with those bound in sites A and B demonstrated that this was not the case, as shown in Fig. 8 for colchicine, 2CTC, and 3CTC. Each compound is shown in energy-minimized unbound conformations superimposed with the conformations of the compound bound in site A and in site B. There thus appears to be a shift of the 2CTC and 3CTC molecules relative to colchicine when energy-minimized structures are bound in site A.

This poor overlap of the C2 and C3 oxygen atoms of 2CTC and 3CTC with those of colchicine in site A remained of concern to us. Our original rationale in undertaking this study was that the reactivity of the analogs with tubulin amino acid residues should provide insight into the location of these two oxygen atoms of colchicine in its binding site, having made the assumption that the molecular conformations of the unbound drugs would be almost identical. We therefore confirmed this original assumption by performing an energy minimization comparison of unbound 2CTC, 3CTC, thiocolchicine, and colchicine. Fig. 9 shows the nearly complete overlap of common structural elements, including the C2 and C3 oxygen atoms.

### DISCUSSION

Our goal in this project was to attempt to model colchicine (and closely related structural analogs) into a theoretical binding site on tubulin. Ludueña and colleagues (26, 27) have shown that EBI cross-links \( \beta \)-Cys-239 and \( \beta \)-Cys-354 with high specificity. Long carbon bridges between the two iodoacetamide moieties almost eliminated cross-link formation, leading to the conclusion that the two sulfur atoms were about 8 Å apart; and formation of this cross-link is potently inhibited by colchicine site drugs. In our previous study with \(^{14}\text{C}\)3CTC (5), we had concluded, largely based on results of formic acid digestion, that \( \beta \)-Cys-354 was the primary alkylation site in tubulin for this analog, although we also noted a minor reaction at \( \beta \)-Cys-239. In our current studies with \(^{14}\text{C}\)2CTC, alkylation was greater at Cys-239 than at Cys-354, and this was most apparent following digestion with CNBr or endoproteinases. The apparent reactivity of 2CTC with Cys-239 was reduced following formic acid digestion compared with other methods, and this caused us to reevaluate the reactivity of 3CTC. The apparent relative reactivity of the two cysteine residues with both colchicine analogs is summarized in Table II, which summarizes both the autoradiograms shown in Fig. 2 and average results obtained from multiple formic acid and CNBr digestions.

It is clear that with both analogs the cross-link to Cys-239 is relatively labile in the formic acid digestion, and we assume that this is due to the prolonged 4-day incubation. Since the 1-day incubation with CNBr occurs at nearly the same formic acid concentration, it is not unreasonable to extrapolate the ratio of Cys-239 to Cys-354 modification back to zero time by drawing straight lines through the 1- and 4-day time points. When this is done (not shown), one obtains a 77/23 distribution of Cys-239/Cys-354 modification for 2CTC and a 27/73 distribution for 3CTC.

The obvious conclusion from these results is that the C2-oxygen atom of colchicinoids is closer to Cys-239 and the C3-oxygen atom is closer to Cys-354, assuming that in all cases the covalent reactions involve a nucleophilic attack of electrons of the cysteine sulfur atom on the chloroacetyl group with displacement of the chlorine atom. There are additional quantitative aspects of the two drug-tubulin interactions that should be considered.

Our initial observations that 3CTC reacted covalently with tubulin virtually as fast as it bound while the 2CTC covalent reaction lagged behind binding (24) were reflected in the stoichiometry of covalent drug bound to \( \beta \)-tubulin isolated by preparative decylagarose chromatography for the sequencing studies. The average value for \(^{14}\text{C}\)3CTC was 0.32 mol of drug/mol of tubulin (5), and for \(^{14}\text{C}\)2CTC it was 0.17 mol/mol (see “Experimental Procedures”). Multiplying these values by the zero time distributions, one can conclude that with 2CTC there is 0.12 mol/mol of \(^{14}\text{C}\)labeled adduct at Cys-239 and only 0.05 mol/mol at Cys-354; and with 3CTC there is 0.11 mol/mol at Cys-239 as compared with 0.21 mol/mol at Cys-354 (Table II).

There is, however, a further complication in these calculations, in that there are four isotypes of \( \beta \)-tubulin in bovine brain. One of these, \( \beta_{11} \), has a serine residue instead of cysteine at position 239, and this isotype represents about 25% of total brain \( \beta \)-tubulin (30). Assuming that 2CTC and 3CTC bind equivalently to all isotypes and that the covalent reactions occur equally (however, see below), then the relative reactivity of Cys-354 needs to be corrected for the absence of Cys-239 in \( \beta_{11} \)-tubulin. This reduces the relative stoichiometry of 2CTC cross-linked to Cys-354 to 0.04 and of 3CTC to 0.16.

Let us assume these relative stoichiometries correlate with distances between the C2- and C3-oxygen atoms and the cysteine sulfur atoms. If this is true, then (i) the C2-oxygen is about 3 times as far from the Cys-354 sulfur as the Cys-239 sulfur, (ii) the C3-oxygen is about 50% farther from the Cys-239 sulfur than the Cys-354 sulfur, (iii) both oxygens are nearly equidistant from the Cys-239 sulfur, and (iv) the C2-oxygen should be about 4 times farther than the C3-oxygen from the Cys-354 sulfur.

However, when we used energy minimization programs to model colchicine and the analogs into the electron crystallographic model of \( \beta \)-tubulin (1), we obtained entirely unexpected results, largely inconsistent with the above predictions (see Table III). For potential binding site A, colchicine and thiocolchicine were relatively close to the components of the two peptide sequences (one of which included Cys-239) that reacted with \(^{3}\text{H}\)colchicine following direct photoaffinity labeling (9), but Cys-354 was somewhat more distant, and direct access to

| TABLE III Calculated intermolecular distances from best fit models |
|----------------------|----------------------|
|                       | To Cys-239 sulfur atom | To Cys-354 sulfur atom |
| Potential binding site A |                         |                        |
| Colchicine*           |                        |                        |
| From C2 oxygen atom   | 5.6                    | 9.0                    |
| From C3 oxygen atom   | 8.3                    | 11.7                   |
| Thiocolchicine*       |                        |                        |
| From C2 oxygen atom   | 5.6                    | 9.2                    |
| From C3 oxygen atom   | 8.3                    | 11.9                   |
| 2CTC*                |                        |                        |
| From C19              | 5.1                    | 5.4                    |
| 3CTC*                |                        |                        |
| From C20              | 5.9                    | 4.7                    |
| Potential binding site B |                         |                        |
| Colchicine*           |                        |                        |
| From C2 oxygen atom   | 11.1                   | 9.4                    |
| From C3 oxygen atom   | 10.0                   | 11.1                   |
| Thiocolchicine*       |                        |                        |
| From C2 oxygen atom   | 11.1                   | 9.4                    |
| From C3 oxygen atom   | 10.0                   | 11.1                   |
| 2CTC*                |                        |                        |
| From C19              | 13.8                   | 10.9                   |
| 3CTC*                |                        |                        |
| From C20              | 11.3                   | 11.2                   |

* Numbering as shown in Fig. 1.
its sulfur atom was blocked by several amino acid side chains. Moreover, the A rings of 2CTC and 3CTC differed significantly in location from the A rings of colchicine and thiocolchicine following modeling by energy minimization into site A (despite the nearly identical biochemical properties of the four colchicinoids and the equivalent conformations of the unbound molecules). This could indicate a remarkable degree of plasticity in the colchicine site in its accommodation of structurally similar compounds, and the larger site A could probably accommodate the significant structural variability in ligands known to bind at the colchicine site. Finally, the reactive carbon atoms of 2CTC and 3CTC were relatively close to the sulfur atoms of Cys-239 and Cys-354.

For site B, colchicine remains in close proximity to components of the peptides cross-linked to the drug in the direct photoaffinity study (9), and site B is consistent with the published subunit reactivity in the analog photoaffinity studies (7, 8). Moreover, in site B there is greater similarity in the binding footprints of 2CTC and 3CTC to that of colchicine. In this model, however, the reactive carbon atoms in the chloroacetyl groups are more distant from the cysteine sulfur atoms, and it is now the sulfur atom of Cys-239 that is shielded by amino acid...
side chains. In addition, binding site B is smaller and would appear to be more restrictive in the structural diversity it would tolerate in potential ligands.

Alternatively, the colchicine site may undergo significant conformational change from αβ-dimer to protofilament, suggested by the failure of polymer to bind colchicine (31, 32), the failure of dimer to bind paclitaxel (33), and the ease with which EBI cross-links Cys-239 and Cys-354 (26, 27) in the dimer. The above discussion, as well as the electron crystallographic model (1), assumes no differences between the different iso-

isotypes (βII, 58%; βIII, 25%; and βIV, 13%; data from Ref. 30) reacts with colchicine (34, 35) and colchicine analogs (36, 37) with different kinetics and different affinities. It is even possible, although probably far fetched, that only one isotype (e.g. tubulin containing βII) reacts at Cys-354 and another (e.g. βII) reacts at Cys-239.

Our findings with [14C]2CTC and [14C]3CTC allow us to make one additional speculation. Although thiocolchicine (28, 29) and 2CTC and 3CTC (24) bind to tubulin more rapidly than colchicine, these analogs share with colchicine a relatively slow binding reaction. Since, within the measured time frame, 3CTC reacts covalently with β-tubulin as fast as it binds, it is likely that the spatial relationship between Cys-354 and Cys-354 and the C3-substituent is relatively static, at least under the reaction conditions used. However, the covalent reaction of 2CTC with β-tubulin increases with time of incubation, lagging well behind the binding reaction. Perhaps this is due to the changes in the conformation of drug or tubulin previously documented by other workers (6). This implies that such conformational changes would include moving the C2-substituent closer to Cys-239 and/or Cys-354.

Finally, we should point out an apparent limitation to molecular modeling approaches that was revealed as our studies progressed. Although there is no evidence for more than one high affinity site for colchicine binding to tubulin (6), we initially observed four candidate binding sites that merited exploration. Only two could be eliminated because they were inconsistent with our biochemical data, and neither remaining site is entirely satisfactory without postulating conformational changes that might occur as tubulin alternates between the polymerized and unpolymerized states and/or responds to binding of the ligand. While modeling methods similar to those we used here have found widespread application in areas of rational drug discovery and design, it is clear that additional validation steps for predicted docking geometries will be necessary. Computational results can be especially valuable, however, in providing a scaffold for hypothesis generation and data interpretation.

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Ruoli Bai, David G. Covell, Xue-Feng Pei, John B. Ewell, Nga Y. Nguyen, Arnold Brossi and Ernest Hamel

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