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Colchicine Photosensitizes Covalent Tubulin Dimerization

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ABSTRACT: Pure rat brain tubulin can be cross-linked by ultraviolet irradiation of tubulin–colchicine complexes at the high-wavelength maximum of colchicine to form covalent dimers > trimers > tetramers. With colchicine concentrations $\sim 3 \times 10^{-4}$ M (mole ratio to tubulin 3–12) and irradiation for 5–10 min at 95–109 mW/cm^2, the yield of dimers is 11–17% and of trimers is 4–6% of the total tubulin. The oligomers show polydispersity and anomalously high apparent molecular masses that converge toward expected values in low-density gels. Maximal dimer yields are obtained with MTC and the decreasing photosensitizing potency is MTC > colchicine > colchicide > isocolchicine > thiocolchicine. Single-ring troponoids also promote dimerization. Evidence is presented suggesting that the initial, low-affinity, binding step of colchicine and its analogues is sufficient to photosensitize tubulin dimerization.

Covalent labeling of tubulin with [^{3}H]colchicine can be accomplished by ultraviolet irradiation of the tropolone moiety of the drug at ≈353 nm (Wolff et al., 1991). The label is found primarily in the β-monomer, but some labeling of the α-subunit can be induced under mild denaturing conditions suggesting that the drug might span the α/β subunit interface. This suggested that irradiation of bound colchicine might covalently cross-link the two subunits, and we have investigated this possibility. Earlier studies, employing various chemical cross-linking agents, had shown that covalent tubulin oligomers could be formed in good yield. Thus, Luduena et al. (1977), using chick brain tubulin, showed that $\alpha\beta$, as well as homologous dimers, could be identified by mobility and double-isotope methods; Galella and Smith (1982) found that oligomers up to hexamers were formed from tubulin treated with various bifunctional imido esters having spacers of 5–10 Å. Subsequently, it was found that hexanedione cross-linked tubulin to dimers and larger forms (Boekelheide, 1987; Sioussat & Boekelheide, 1989).

Ultraviolet irradiation of tubulin at low wavelengths has long been known to lead to destruction of mitotic spindles, etc., which is thought to result from conformational changes in

tubulin as shown by circular dichroic and ultraviolet spectra, loss of SH groups, and cross-linking (Zaremba et al., 1984). However, the covalent dimerization we report here results from irradiation of the tropolone moiety of colchicine at wavelengths that are not directly absorbed by proteins without colchicine. Irradiation without colchicine at these wavelengths does not cause dimerization. Such effects can, therefore, be considered to be colchicine-sensitized photodimerization of tubulin.

Experimental Procedures

Methods. Rat brain tubulin was prepared by temperature-dependent polymerization–depolymerization cycles followed by treatment with 1.0 M sodium glutamate (Hammel & Lin, 1984). It was >98% pure in overloaded SDS[1] gels and was kept in liquid N_2 until just before use. Residual guanidine nucleotides were not removed, but all subsequent reactions were carried out in the absence of GTP to prevent polymerization. Except where noted, mixtures were incubated in assembly buffer (100 mM MES, pH 6.85, 1 mM $MgCl_2$, 1 mM EGTA) at 37 °C in the dark for 30–60 min and were then irradiated at 4 °C by a water-cooled Osram high-pressure mercury lamp at 90–100 W of lamp power. Delivered power was measured with a Spectroline DM365N radiometer and a neutral filter. To protect the protein and to filter out infrared components, all irradiations were carried out under 2 cm of a 20% $CuSO_4 \cdot 5H_2O$ solution (Wolff et al., 1991; Kasha, 1949) which permitted <1% transmission at 305 nm, 50% transmission at 322 nm, and 97% transmission at 353 nm, the absorption maximum of colchicine. The samples were irradiated in cooled polyethylene Eppendorf tubes, generally in a volume of 20 μL. Irradiation times were varied as described in the text. Samples were then boiled for 2–3 min in an SDS/mercaptoethanol loading solution, cooled, and electrophoresed on 8% SDS/polyacrylamide gels (bonded on GelBond sheets unless otherwise noted) at 20 mA until 15–20 min beyond the dye-expulsion time, unless otherwise noted. In some experiments the mercaptoethanol was deleted. After being stained and destained, gels were soaked in 5% glycerol in water for 6–15 h, dried, and covered with a plastic sheet. They were then scanned in a Beckman Model DU-8 gel scanner, and the machine-computed areas were used to calculate the percent of distribution on each lane. The linearity of the scanner response was checked for the range of the rather high protein concentrations used in this study. The response was linear ±10% (expressed as the fraction present as dimers) over a range of 10–50 μg of total protein per lane. Moreover, several gels were rescanned after one year and yielded identical results.

Transverse gradient gels were prepared by forming linear 4–9% polyacrylamide gradients at right angles to the running direction. Stacking and loading gels were then applied as usual, and alternate sample and standard lanes were electrophoresed across the whole gradient to permit analysis according to Ferguson (Andrews, 1986).

Materials. Commercial chemicals were of the highest purity available. MTC [2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)-tropone] was synthesized by Dr. T. Fitzgerald, Flordia A&M University. Colchicoside was a gift of Dr. C. Chignell; colchicide, thiocolchicine, and isocolchicine were generously provided by Dr. Arnold Brossi of our institute. Tropolone was from Aldrich, and 2-methoxy-5-nitrotropone was from American Tokyo Kasai.

[1] Abbreviations: MTC, 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

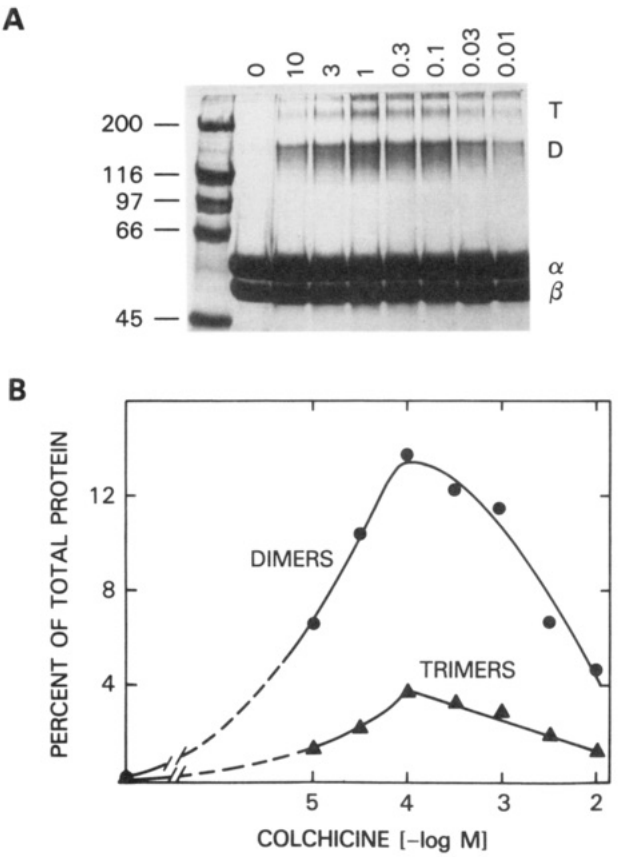

FIGURE 1: The effect of colchicine concentration on dimer formation by irradiation of rat brain tubulin. (A) A 50 μM concentration of rat brain tubulin was incubated with colchicine for 30 min at 37 °C, irradiated for 5 min at 109 mW/cm², and electrophoresed on an 8% gel. Colchicine concentrations (millimolar) are listed above each lane. α and β stand for the tubulin monomers; D and T stand for the presumptive dimers and trimers. Numbers refer to marker proteins in kilodaltons. (B) Densitometric scans of above Coomassie-stained gel.

Results

Under the present incubation conditions, reversibly bound colchicine amounts to 0.7–0.8 mol/mol of dimer (Bhattacharyya & Wolff, 1974). However, irradiation for covalent binding conditions is competed for by photoisomerization of the colchicine to lumicolchicine and, hence, yields are expected to be much lower (Wolff et al., 1991). Moreover, the nature of the binding reaction for dimerization may not be entirely the same as for formation of the covalent colchicine–tubulin adduct. Thus, the correlation between reversible or covalent binding and dimerization is difficult to quantify. Nevertheless, many other aspects of the photosensitized dimerization of tubulin can be studied.

The yield of dimers (and higher oligomers) depends on the concentration of colchicine present during irradiation, attaining a maximum at $(1–3) \times 10^{-4}$ M colchicine in various experiments when the tubulin concentration was 50 μM (that is, at a mole ratio of 4–12 for colchicine to tubulin). A typical example is depicted in Figure 1A with the corresponding scanning results for the total dimer and trimer peaks in Figure 1B. Similar maxima were observed for presumptive trimer formation; the presumptive tetramer bands were often too weak for quantitation at these protein concentrations. It should be noted that the concentrations of colchicine yielding maximal cross-linking are higher than required for saturation of the binding site. This is due to the rapid conversion of colchicine to lumicolchicine under ultraviolet light (Wilson & Friedkin, 1966). Thus, the concentration of colchicine decreases rapidly

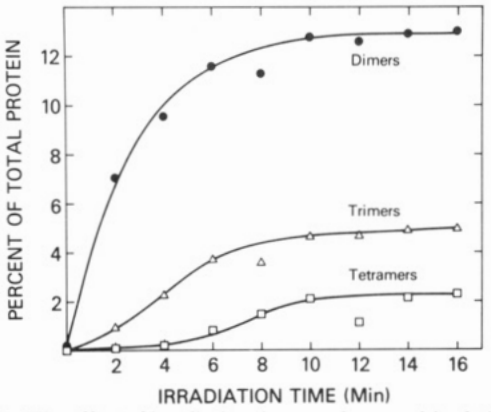

FIGURE 2: The effect of irradiation time on photosensitized oligomer formation in tubulin by colchicine. A 30 μM conentration of tubulin and 3×10^{-4} M colchicine were incubated at 37 °C for 50 min in assembly buffer and irradiated at 98 mW/cm^2 for the times indicated, electrophoresed on an 8% SDS gel, and scanned.

during the period of irradiation. However, as will be shown below, these are not nonspecific effects, since a nonbinding colchicine analogue does not promote dimerization when irradiated together with tubulin. The decrease in dimer formation occurring at high colchicine concentrations is not understood; it may be partly due to the inner filter effect and also to colchicine dimer formation at the high concentrations (Engelborghs, 1981). When the colchicine/tubulin ratio was held constant at 2.4 or 4.8 with 3×10^{-4} M colchicine, there was little or no effect of varying the tubulin concentration on the yield of dimer (data not shown). Three different rat brain tubulin preparations and one beef brain tubulin preparation, when irradiated with 3×10^{-4} M colchicine for 10 min at 108 mW/cm^2, yielded approximately 11–17% of total protein as dimers and 4–6% as trimers.

It is apparent from Figure 1A that the dimer (D) area is complex. With longer electrophoresis, we could invariably see five dimer bands. Gels in which disulfide bonds had *not* been reduced before electrophoresis contained higher percentages of protein as dimer, although they appeared as a smear (e.g., for colchicine, 10.6% dimer when reduced and 13.8% dimer when not reduced). However, the bulk of the dimer bonds was of another type. We assume that the two extra bands in addition to the expected $\alpha\beta$, $\alpha\alpha$, and $\beta\beta$ dimers arise from cross-linking of isoforms of tubulin, but this has not been established. This degree of polydispersity of dimers has also been observed in tubulin cross-linked with hexanedione (Sioussat & Boekelheide, 1989).

The effect of time of irradiation at a constant power of 98 mW/cm^2 is depicted in Figure 2. Maximum yields of oligomers were attained at ~10 min of irradiation and then plateaued or declined slightly at longer irradiation times when the colchicine/tubulin mole ratio was 10 (Figure 2). The maximum yields of oligomers (presumptive dimer, trimer, and tetramer) amounted to ≈20% of the protein present on the gel. As judged by the ratios of dimer/trimer, etc., dimer formation precedes trimer formation and trimer formation precedes tetramer formation (Figure 2).

Oligomer Size. Up to now, we have assumed that the next larger species present above monomer was the covalent dimer. However, from protein standards, the apparent molecular mass of this presumptive dimer was ~150 kDa on 8% gels. To distinguish anomalous migration on gels from trimers, it was necessary to eliminate sieving effects (Andrews, 1986) by extrapolation of gel mobilities to zero gel densities. It is apparent from Figure 3, which depicts a 4–9% SDS transverse gradient gel (constructed at right angles to the running direction), that the slope of the line connecting the midpoints of the presumptive dimer peaks is steeper than that of the nearest marker protein of 116 kDa and approaches it at 4% gel density. Thus, at 9% SDS the apparent molecular mass is ~180 kDa and extrapolates to ~100 kDa at zero gel density. A similar slope difference is seen for the presumptive trimer, which crosses the line connecting the 200-kDa marker and approaches an extrapolated apparent molecular mass of ~145 kDa. The same trend is apparent for the presumptive tetramer bands, but suitable markers were not available. It seems safe to conclude that the protein bands identified as presumptive dimers, timers, and tetramers are, in fact, likely to be just that. Similar size anomalies were noted in tubulin dimers cross-linked with hexanedione (Boekelheide, 1987) but not after cross-linking with a carbodiimide (Mejillano & Himes, 1991). It is, however, not clear at present whether or not the conformation of the dimers and trimers is the only factor contributing to the apparent high molecular masses.

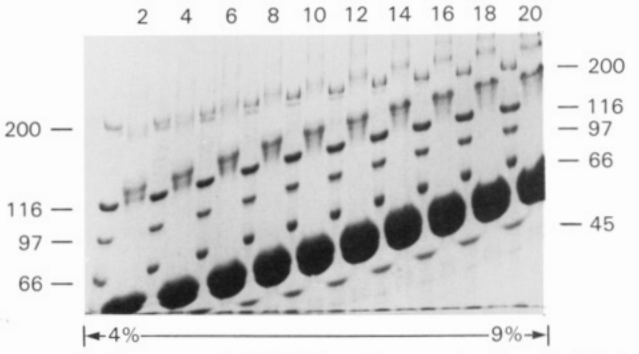

FIGURE 3: Transverse 4–9% SDS gradient acrylamide gel with alternating overloaded sample lanes (even numbers) and protein standard lanes at 200, 116, 97, 66, and 45 kDa (odd numbers). Note that the slope of the dimer "band" approaches the 116-kDa marker at 4% gel density and that the trimer peak crosses the 200-kDa marker. The α and β monomer peaks do not separate because of overloading.

Table I: Potency of Colchicinoids in Promoting Tubulin Dimerization

compound[a]	concn for 7% dimer yield (M)
MTC	6×10^{-6}
colchicine	$(2–4) \times 10^{-5}$
colchicide	8×10^{-5}
isocolchicine	3×10^{-4}
thiocolchicine	4×10^{-3}
2-methoxy-5-nitrotropone	1×10^{-3}
tropolone	2.5×10^{-3}

[a] Refer to Figure 4 for structures.

Analogues. A number of analogues of differing chemical properties bind at the colchicine binding site of tubulin. We have selected several of these absorbing beyond the protein spectrum to test the requirements for photosensitized dimerization (Figure 4). Thiocolchicine, which has a thiomethyl group in the 10-position, was less than one-tenth as potent in promoting covalent dimer formation as was colchicine (Figure 5). Although it binds well to tubulin (Chabin & Hastie, 1990), it fluoresces poorly, presumably because of rapid relaxation by nonradiative pathways (Figure 5). On the other hand, colchicide, lacking the 10-methoxy group (Figure 4), promoted photodimerization efficiently (Table I). Not surprisingly, colchicoside, which is sterically prevented from binding to the site, does not have significant antimitotic activity and fails to block colchicine binding (Zweig & Chignell, 1973), had virtually no ability (<0.5%) to promote subunit cross-linking of concentrations where colchicine was highly effective. At very high concentrations (2.5 mM), some dimer formation occurred. It is not clear whether this is due to intrinsic activity

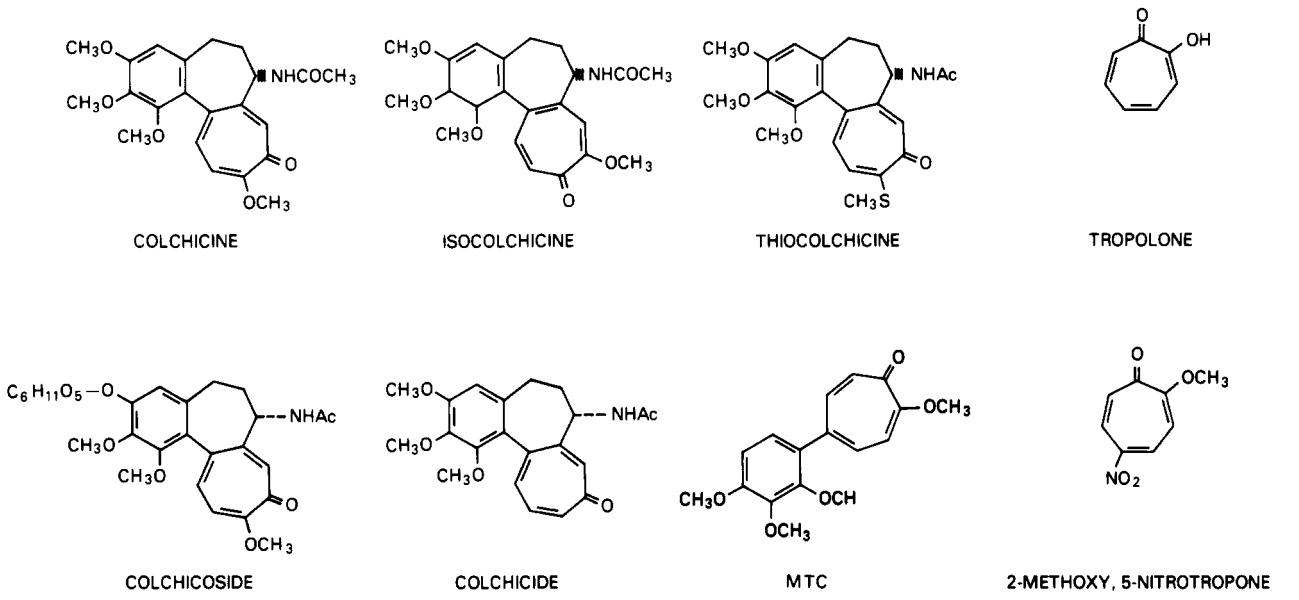

FIGURE 4: Colchicine analogues used in the photodimerization studies.

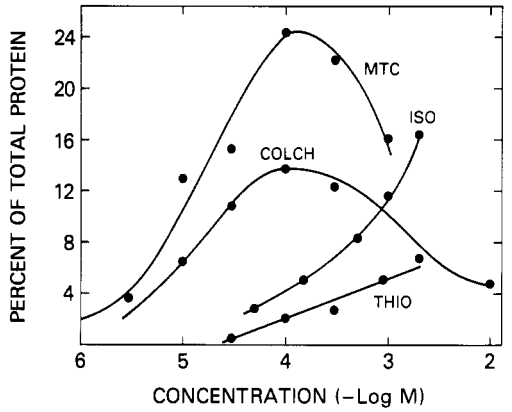

FIGURE 5: Covalent tubulin dimer formation promoted by various colchicine analogues. A 50 μM concentration of tubulin in assembly buffer was incubated for 30 min at 37 °C, irradiated at 90 W of lamp power for 5 min, electrophoresed on 8% SDS gels, and scanned. The areas for the various dimers are pooled and listed as the percent of total stained protein in each lane. Abbreviations: colch, colchicine; iso, isocolchicine; thio, thiocolchicine.

or may be explained by a <1% contamination of the glucoside with the parent compound. These results suggest that some form of binding is necessary for the stimulation of cross-linking.

MTC, lacking the B-ring of colchicine, which binds to tubulin in a manner like that of colchicine with approximately the same affinity constant but with more rapid kinetics, proved to be the most potent promoter of cross-linking of the subunits. This was observed in more than a dozen experiments and was true for the formation of dimers (Figure 5) and trimers and tetramers (data not shown). While the absence of the B-ring may contribute to this greater potency, we are inclined to the view that the much slower photoisomerization to the corresponding "lumi" derivative (Maity & Bhattacharyya, 1987) was largely responsible for the greater cross-linking efficiency.

Single-ring troponoids such as tropolone and 2-methoxy-5-nitrotropone [both of which are known to interact with tubulin (Watanabe & Flavin, 1973; Bhattacharyya & Wolff, 1974; Cortese et al., 1977)] photosensitized dimer formation as a linear function of concentration up to 3 mM. The 2-methoxy-5-nitrotropone was slightly more potent than tropolone (Table I). This suggests that these single-ring compounds bind to part of the colchicine binding site as has previously been established by other criteria (Bhattacharyya & Wolff, 1974; Cortese et al., 1977; Andreu & Timasheft, 1982), but the mechanism by which these single-ring compounds cross-link remains to be explored. The relative potencies of the compounds tested are listed in Table I. Because of the low activities of some compounds, the comparison is given for the initial concentration required to attain 7% dimer formation under otherwise similar conditions. The steric requirements for photosensitizing dimerization are thus moderately flexible, but they probably include both occupancy of the tropolone portion of the binding site and the ability to make the excited-state energy available for cross-linking.

Table II: Incubation Requirement[a] for Photo-Cross-Linking

drug	incubation time (min)	dimer (% of total protein)	trimer (% of total protein)
control (no drug)	45	0.2	0
colchicine	45	12.0	3.9
colchicine	0	4.6	1.1
MTC	45	15.4	7.2
MTC	0	15.6	7.4

[a] A 120 μM concentration of rat brain tubulin was incubated with the drugs in assembly buffer as indicated and irradiated for 5 min at 98 mW/cm^2. Analysis was on 8% SDS–polyacrylamide gels. Samples from 0 min of incubation were kept at 4 °C throughout the experiment.

Colchicine binding has been considered a two-step process (Garland, 1978; Lambeir & Engelborghs, 1981) characterized by a rapid, reversible, first-order, low-affinity step followed by a slow, pseudoirreversible, zero-order rearrangement in both the binding site and the ligand, producing the higher affinity generally measured. A similar mechanism is thought to govern the binding of MTC to tubulin (Baine et al., 1984; Engelborghs & Fitzgerald, 1987). In order to test which of these two steps was primarily responsible for photo-cross-linking, two approaches were used: (1) the requirement for preincubation of colchicine with tubulin and (2) the use of isocolchicine, which is thought to be capable only of the first, rapid step in binding (Hastie et al., 1989). Most of the results presented here have used preincubation followed by irradiation. We were, therefore, surprised that, when high-affinity binding was inhibited (0 °C and no preincubation), colchicine still enhanced cross-linking to dimers and other oligomers, although it was less than with preincubation (Table II). The MTC compound binds tubulin very rapidly as measured by fluorescence enhancement. It also promoted cross-linking without preincu-

bation and at 0 °C, but it did so with nearly the same efficiency as after preincubation at 37 °C (Table II). This suggested that the rapid, low-affinity binding step might be all that is required for photosensitization.

To confirm the hypothesis that the rapid, low-affinity binding step may be all that is required for cross-linking, we used isocolchicine, which cannot undergo the biaryl conversion to the near-planar state associated with high-affinity binding (Hastie et al., 1989; Detrich et al., 1981; Brossi et al., 1988) and has an association rate constant similar to that for the first binding step of colchicine binding (Hastie et al., 1989). As shown in Figure 5, isocholchicine, though less efficient than colchicine, promoted covalent dimer formation and yielded *more* dimer than did colchicine at high concentrations. This may be due, in part, to the fact that isocolchicine is substantially more stable to 350-nm light (Wilson & Friedkin, 1966). Our presumptive conclusion is, therefore, that the low-affinity binding step is sufficient to promote photosensitized dimerization of tubulin.

Discussion

Large spacers on chemical cross-linkers or photo-cross-linkers may make it difficult to ascertain the correct proximity of adjacent protein domains or subunits and may lead to erroneous localization of ligand binding sites when these are located at subunit interfaces. This was recently shown to be the case for direct photolabeling of tubulin with unmodified colchicine (Wolff et al., 1991). The drug was found to bind preferentially to β-tubulin, but near enough to the α/β interface to account for labeling of α-tubulin under "unnatural" conditions. It was not surprising, therefore, that cross-linking of tubulin monomers could be observed when the colchicine–tubulin complex was irradiated by near-ultraviolet light. We have exploited this observation in the present study to investigate the mechanisms of this photosensitized dimerization.

The rapid photoisomerization of colchicine to lumicolchicine under ultraviolet irradiation is an intrinsic limit to the efficiency of the cross-linking of tubulin monomers and requires the use of excess colchicine. It has been known for a long time that bound colchicine is released from the binding site during near-ultraviolet irradiation (Borisy et al., 1972; Amrhein & Filner, 1973; Bryan, 1975). This is accompanied by a loss of the colchicine binding site, whereas the ability to assemble to microtubules is maintained. The cross-linking reaction is shown here to attain maximum yields of dimer (as a percent of the total protein on the gel) of ~17%, 5% trimer, and several percent tetramer. The yield is a function of the colchicine/tubulin ratio, the time of irradiation, and the condition of the tubulin. Stability of the drug toward competing photoisomerization reactions also influences the yield. For example, MTC attains maximum yields of oligomers that are as much as 50% greater than colchicine (Figure 5). We believe this to be due, at least in part, to the greater stability of MTC to photoisomerization (Maity & Bhattacharyya, 1987); similar stability considerations apply to the cross-linking promoted by isocolchicine (Wilson & Friedkin, 1966).

There is, however, a surprising latitude in the structural requirements in the C-ring. Thus, colchicide (lacking the 10-methoxy group) shows good photosensitizing activity as does isocholchicine (discussed below), whereas thiocolchicine (with a 10-thiomethyl group) is only weakly active despite the fact that it binds to tubulin with an affinity similar to that of colchicine. Whether or not this is due to its higher absorption maximum ($\lambda_{max} \approx 380$ nm), different relaxation from the excited state, or other factors is not known. Finally, single-ring troponoids, such as 2-methoxy-5-nitrotropone and tropolone, are able to promote dimer formation. Although extensive concentration studies were not carried out, the decreasing order of photosensitizing potency appears to be MTC > colchicine > colchicide > isocolchicine > 2-methoxy-5-nitrotropone ≥ tropolone > thiocolchicine.

The kinetics of colchicine binding to tubulin are complex and can be divided into two steps: a rapid, rather low-affinity binding reaction and a slow process believed to involve conformational changes in both colchicine and tubulin (Garland, 1978; Lambeir & Engelborghs, 12981; Baine et al., 1984; Engelborghs & Fitzgerald, 1987; Detrich et al., 1981; Brossi et al., 1988; Sackett et al., 1989). Because preincubation of colchicine with tubulin was not absolutely required to achieve photosensitized dimer fomration (Table I), the possibility existed that cross-linking could result from colchicine occupancy of the low-affinity component of the binding site represented by the rapid, first kinetic step. This hypothesis is strengthened by the finding that isocolchicine, which shows the low-affinity binding only (Hastie et al., 1989), also promoted tubulin dimerization when it was irradiated at the tropolone absorption maximum. That this was not simply nonspecific photosensitization was shown when the binding site was occupied by podophyllotoxin (Wolff et al., 1991; Cortese et al., 1977) which does not absorb at the tropolone wavelength but blocks colchicine binding. Under these conditions colchicine-induced photosensitization was virtually abolished, as was MTC-induced photosensitization. Moreover, since colchicoside did not photosensitize dimerization, it is clear that the mere presence of a colchicine analogue in solution (i.e., not bound) is insufficient to promote the process. Together, these data provide evidence that occupancy of that part of the colchicine binding site involved in the rapid, low-affinity kinetic step for the binding of colchicine, MTC, or isocolchicine is sufficient to photosensitize tubulin dimerization.

Acknowledgments

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Registry No. MTC, 60423-21-4; colchicine, 64-86-8; isocolchicine, 518-12-7; thiocolchicine, 2730-71-4; tropolone, 533-75-5; colchicoside, 477-29-2; colchicide, 518-15-0; 2-methoxy-5-nitrotropone, 14628-90-1.

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Errors in RNA NOESY Distance Measurements in Chimeric and Hybrid Duplexes: Differences in RNA and DNA Proton Relaxation†

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ABSTRACT: Nuclear magnetic resonance experiments reveal that the base H8/H6 protons of oligoribonucleotides (RNA) have T_1 relaxation times that are distinctly longer than those of oligodeoxyribonucleotides (DNA). Similarly, the T_1 values for the RNA H1′ protons are approximately twice those of the corresponding DNA H1′ protons. These relaxation differences persist in single duplexes containing covalently linked RNA and DNA segments and cause serious overestimation of distances involving RNA protons in typical NOESY spectra collected with a duty cycle of 2-3 s. NMR and circular dichroism experiments indicate that the segments of RNA maintain their A-form geometry even in the interior of DNA-RNA-DNA chimeric duplexes, suggesting that the relaxation times are correlated with the type of helix topology. The difference in local proton density is the major cause of the longer nonselective T_1s of RNA compared to DNA, although small differences in internal motion cannot be completely ruled out. Fortunately, any internal motion differences that might exist are shown to be too small to affect cross-relaxation rates, and therefore reliable distance data can be obtained from time-dependent NOESY data sets provided an adequately long relaxation delay is used. In hybrid or chimeric RNA-DNA duplexes, if the longer RNA relaxation times are not taken into account in the recycle delay of NOESY pulse sequences, serious errors in measuring RNA proton distances are introduced.

Modern molecular biology research has greatly expanded our knowledge of RNA function in recent years (Hoffman, 1991). Like proteins, RNA performs a wide variety of structural and functional roles, ranging from transfer RNA molecules, with their distinctive cloverleaf structures, to the replicating "killer toxin"-encoding double-stranded yeast RNA (Wickner et al., 1986). In addition, RNA carries the genetic transcript as messenger RNA, is found in association with proteins [e.g., ribosomes, spliceosomes, and RNaseP (Guerrier-Takada et al., 1983)], and has been found to self-splice and act as a catalyst (Cech & Bass, 1986). It also plays a role in gene regulation as antisense RNA (Green et al., 1986) and is the genetic material of many families of viruses (e.g., retroviruses). Recent advances in solid-state phosphoramidite chemistry have made it possible to routinely synthesize very pure RNA oligomers (Ogilvie et al., 1988) in NMR quantities (Chou et al., 1989). Thus defined sequence DNA-RNA hybrids are now amenable to structural investigation using NMR techniques. Indeed, because the strategies for DNA and RNA synthesis are compatible, one can study not only

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