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BINDING OF COLCHICINE AND LUMICOLCHICINE TO COMPONENTS IN PLANT EXTRACTS

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Key Word Index—*Heracleum mantegazzianum*; Umbelliferae; giant hogweed; binding assays; colchicine; lumi-isomers.

Abstract—Irradiation of dilute, aqueous solutions of colchicine and of colcemid by long wavelength UV resulted, in each case, in a mixture of three major lumi-products whose relative concentrations depended upon the degree of UV exposure. Components in plant extracts, after concentration by ammonium sulphate precipitation, bound the drugs and their lumi-derivatives. Consideration of the different ratios of colchicine- to lumicolchicine-binding activity in different fractions of a plant extract, and after various enzymatic and temperature treatments of the plant preparations, suggest the involvement of different binding sites for the various forms of the alkaloid.

INTRODUCTION

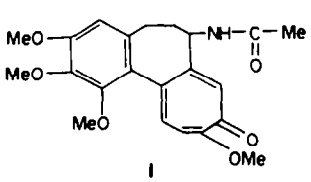
Many of the biological effects of the antimitotic alkaloid, colchicine, derive from its action on microtubules [reviews 1, 2]. The drug affects microtubules by binding to soluble subunits which are thought to be in equilibrium with certain types of microtubules. The binding is specific for colchicine (**1**); lumicolchicine (**2**), the mixture of isomers produced by exposure of the drug to sunlight [3], does not disrupt microtubules nor bind to their subunits [4–6]. This effect of colchicine has been of value in isolating and characterising tubulin, the major protein subunit of microtubules, and the binding of colchicine-[methoxyl-^{3}H] by biological preparations is used, with certain provisos [2], as a diagnostic test for the preliminary identification of tubulin in animal cells.

However, colchicine has other physiological effects which are thought not to be related to direct interaction with tubulin [see 7]. Many of these effects involve the cell membrane [8] or membrane-related processes [9, 10] and are characterised by the facts that: (a) lumicolchicine is as effective as colchicine; (b) the effects are seen at high (greater than 10^{-5} M) concentrations of the drug.

In plants, inhibition of physiological processes is achieved only with relatively high concentrations of colchicine [11–15]. Furthermore, while plant preparations do bind colchicine, lumicolchicine is also bound [16]. Thus, with plant tissues, difficulty arises not only in ascribing many of the effects of colchicine directly to actions on microtubules, but also in unequivocally detecting tubulin by assaying the colchicine-binding activity of plant preparations. In this report, the binding of colchicine and of its lumi-derivatives by components in plant extracts are shown to be distinct phenomena. The binding of the lumi-isomers is discussed with regard to its possible biological significance and to its interference in practice with colchicine-binding assays for the detection of plant tubulin.

RESULTS

Effects of UV irradiation on colchicine. Aqueous solutions of colchicine-[ring C methoxyl-^{3}H] at 3.6×10^{-4} M were irradiated in sealed capillaries for various times and then analysed by TLC. Table 1 shows the ^{3}H in each R_f zone of the chromatograms as a percentage of the total radioactivity applied in each sample. The initial effect of UV was the production of two lumicolchicines; continued irradiation resulted in the appearance of a third lumicolchicine, detected as ^{3}H remaining at the origin of the chromatogram. Irradiation over a period of days produced samples in which a maximum of 36% of the total ^{3}H was retained at the origin. The use of MeOH as chromatographic developing solvent [17, 18], in our hands, resulted in poor separation between colchicine and lumicolchicine, and did not resolve lumicolchicine into more than one component. Exposure of solutions of colcemid-[ring C methoxyl-^{3}H] (**3**) to UV gave a qualitatively similar chromatographic pattern of ^{3}H distribution, including the initial appearance of two mobile lumicolcemids followed by the subsequent appearance of ^{3}H retained at the origin. These patterns of lumi-product formation did not occur in



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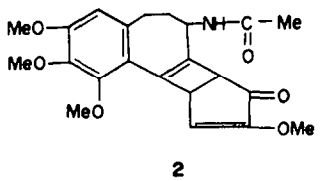

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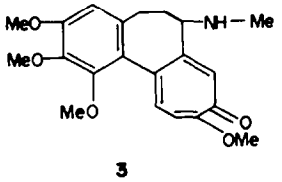

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Table 1. Distribution of ^3H after TLC of colchicine samples exposed to UV for various lengths of time

	^3H in each zone, as % total applied in each sample				
$R_f \times 100$	Colchicine	UV 15 min	UV 45 min	UV 5 hr	UV 1 day
100	0.1	0.2	0.2	0.5	0.2
92	0.1	0.5	0.2	0.5	0.2
83	0.5	1.0	2.0	1.0	1.0
75	5.5	55.0	73.0	78.0	76.0
65	0.5	1.0	0.3	2.0	1.4
55	3.0	6.0	10.0	10.0	9.8
45	0.5	1.0	0.2	0.2	0.6
35	88.0	32.0	11.0	0.3	0.4
25	0.4	0.8	0.3	0.3	0.2
15	0.4	0.5	0.3	0.4	0.2
5	1.0	2.0	2.5	6.8	9.8

R_f values correspond to leading edges of zones; solvent = C_6H_6–EtOAcEt$_2$ NH (5:4:1).

alkaloid samples during their manipulation under fluorescent laboratory lighting, nor during incubation of drug with plant preparations.

Drug binding to components in plant extracts. A standard method for assaying colchicine-binding activity utilises DEAE-impregnated filter discs to separate free colchicine from the protein-bound alkaloid [19]. The behaviour of the alkaloids and their lumi-products in the DEAE disc assay, and their binding to various biological samples, are shown in Table 2. Colchicine bound to components in the 30–50% saturated $(NH_4)_2SO_4$ fraction of an extract of *Heracleum* vascular tissue. Limited UV preirradiation of the drug reduced the degree of its interaction with the plant components, but more prolonged exposure of the drug to UV resulted in lumi-products which showed significant binding to the plant preparations and to DEAE itself. However, the most marked change in the degree of interaction between alkaloid and plant components resulted from aging of the plant preparations. After storage in buffer at 3°, plant samples showed increased binding of all forms of the alkaloid, and particularly of the lumi-forms; there was markedly high binding of lumicolcemid.

Results from an equivalent assay of the alkaloid-binding activity of a tubulin-containing fraction, prepared by the batch procedure from pig brain [20], are also included in Table 2, to demonstrate the very large differences in levels of colchicine-binding activities between these plant and animal preparations and to show that there was no significant binding of the lumi-derivatives by a tubulin preparation. Other experiments showed that denaturation of tubulin by a variety of methods, including heating in the presence of plant extract, did not confer on tubulin the ability to bind lumi-drugs.

The binding of lumi-isomers can also be assayed by gel filtration. After incubation of a plant sample with alkaloid-[^3H] and passage through Sephadex G-100, the bound ^3H was eluted just behind the void volume of the column. Again, higher binding was observed with longer exposure of the ligand to UV irradiation.

Distinction between colchicine and lumicolchicine binding. Attempts were made to separate possible plant components which specifically bound colchicine from any responsible for binding only lumicolchicine, by fractionation of an extract of *Heracleum* vascular tissue using a procedure originally designed to purify tubulin [20]. The fractions described in Table 3 were resolubilised in extraction buffer, and 0.5 ml samples of each were incubated with either colchicine-[^3H] or lumicolchicine-[^3H] (1 day UV) at 2.5×10^{-7} M. The results show that complete separation of components responsible for binding either colchicine or lumicolchicine was not achieved. However, there were marked differences in the ratios of colchicine:lumicolchicine activity over the fractionation range, indicating that more than one component was responsible for binding.

The binding of colchicine and of lumicolchicine were also differentially influenced by other treatments. While the temperature of incubation with the drug enhanced both types of binding, the level of binding of lumicolchicine showed a greater and more linear response to increasing temperature (Fig. 1). Exogeneously added hydrolytic enzymes also had differential effects on the relative levels of binding. Samples of a standard 30–50% saturated $(NH_4)_2SO_4$ fraction, in 0.1 M buffer, pH 6.8, were pre-incubated at 25° for 20 min, either alone or in the presence of a hydrolytic enzyme, before being routinely assayed for drug-binding activity (Table 4). While the components responsible for each type of binding

Table 2. Binding in the DEAE disc assay of colchicine-[^3H] and colcemid-[^3H] before and after exposure to UV irradiation

	cpm bound per disc				
Preparation	Colchichine	Colchicine × UV 60 min	Colchicine × UV 1 day	Colcemid	Colcemid × UV 1 day
Buffer alone	205	192	686	553	640
Fresh plant	1440	696	1850	1860	4480
Stored plant	1850	890	2850	2530	8870
Tubulin	45000	1680	1896	28300	894

Colchicine-[^3H]: 690 mCi/mmol, irradiated at 3.6×10^{-4} M, incubated at 7.2×10^{-7} M; Colcemid-[^3H]: 8.96 Ci/mmol, irradiated at 2.7×10^{-5} M, incubated at 8×10^{-8} M; Tubulin: 2.3 mg/ml, from fresh pig brain via the batch procedure; Plant: fraction of *Heracleum* vascular extract precipitating at 30–50% saturated $(NH_4)_2SO_4$; resuspended in extraction buffer at 2.6 mg protein/ml; Used either freshly prepared or after 2 days storage at 3°; Incubation mixture: 0.5 ml vol; triplicate 0.1 ml samples applied to discs; results expressed as average cpm retained after 7 × 50 ml washes.

Table 3. Binding of colchicine-[^{3}H] and of lumicolchicine-[^{3}H] by samples from a fractionated extract of *Heracleum* tissue

Fraction	Protein mg/ml	Bound ^{3}H-cpm Colchicine total cpm	Colchicine cpm/mg protein	Lumicolchicine total cpm	Lumicolchicine cpm/mg protein	Binding ratio colchicine: lumicolchicine
$(NH_4)_2SO_4$ satd: 0–15%	0.2	0	0	0	0	—
15–30%	0.6	0	0	0	0	—
A-30–45%	4.6	26800	1940	27800	2060	1.0
45–60%	5.4	17700	1050	25200	1560	0.7
60–75%	3.8	5400	470	13500	1180	0.4
DEAE adsorption of A:						
not adsorbed	2.8	1640	585	5570	1990	0.3
eluted 0.4 M KCl	0.8	4150	5200	4450	5560	0.9
eluted 0.8 M KCl	0.6	4850	8100	2850	4800	1.7

30 g fr. wt vascular tissue; fractions resuspended in 3 ml extraction buffer; 1 ml of fraction 30–45% satd salt added to 1 ml Sephadex A-50; gel eluted with 1 ml each of 0.4 M and 0.8 M KCl; the 3 DEAE fractions resuspended in 1 ml buffer after reprecipitation with 50% satd $(NH_4)_2SO_4$ ref. [20]; 0.5 ml samples of all fractions incubated at 37° with 2.5×10^{-7} M ^{3}H-alkaloid (^{3}H-Lclc, 1 day UV) and triplicate 0.1 ml vol assayed by disc method. Results calculated from average ^{3}H-cpm retained on discs, after subtraction of buffer control cpm.

were both susceptible to proteinases, colchicine-binding activity was most affected by trypsin but the level of lumicolchicine-binding was most markedly lowered by nonspecific bacterial protease. (Pre-incubation with pectinase enhanced binding, particularly of colchicine, suggesting that contaminating pectic substances may mask colchicine-binding sites.) Furthermore, only the colchicine-binding reaction was enhanced by the inclusion of vinblastine at 10^{-3} M, in the incubation medium (Table 4).

DISCUSSION

From described R_f values [21], the mobile lumicolchicines may be tentatively identified as γ-lumicolchicine (R_f 0.55) and as β-lumicolchicine (R_f 0.75). The chromatographically immobile material appearing in samples after more prolonged UV treatment was probably α-lumicolchicine, the water-insoluble [22] dimer of β-lumicolchicine [23], previously described as difficult to prepare using solar radiation [24]. The changing ratios of lumiproducts with increasing exposure to UV may explain differences [3, 22] in previously reported ratios of lumicolchicines produced in response to solar radiation.

In agreement with other studies [16, 25, 26], it has been demonstrated that the fraction of a plant extract precipitating at 50% $(NH_4)_2SO_4$ saturation contains components which bind colchicine and, further, that this colchicine-binding activity can be reduced by limited pre-exposure of the drug to UV radiation. However, such plant fractions also bind the lumi-derivatives of colchicine and colcemid, to increasing extents depending both on more prolonged UV irradiation of the drug and on the denaturing pretreatment of the plant sample. The increase in level of lumicolchicine-binding with increasing exposure of the drug to UV, would suggest that the more slowly forming α-lumicolchicine may be the major

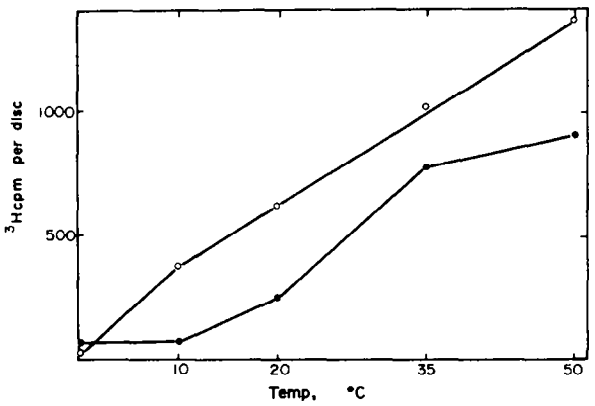

Fig. 1. Effects of temperature on the ability of a plant preparation to bind colchicine-[^{3}H] or lumicolchicine-[^{3}H]. Standard 30–50% $(NH_4)_2SO_4$ fraction at 3.4 mg protein/ml; incubated with 2.5×10^{-7} M ^{3}H-alkaloid at specified temperatures; triplicate 0.1 ml samples applied to DEAE discs at 3° followed by 7 × 50 ml washes with ice-cold 0.01 M buffer: ●——● colchicine-[^{3}H]; ○——○ lumicolchicine-[^{3}H]. Abscissa: temperature; Ordinate: bound ^{3}H as cpm/disc (average after subtraction of cpm retained in buffer alone).

Table 4. Effects of pre-incubation with various enzymes, and of vinblastine, on the ability of components in plant preparations to bind colchicine and lumicolchicine

Treatment	Bound ^{3}H, % control: Colchicine	Lumicolchicine
(A) Enzyme digestion:		
No enzyme	100	100
Trypsin	35	67
Protease	49	21
Pectinase	132	105
Cellulase, amylase, β-glucoronidase, DNase, RNase —no effects.		
(B) 10^{-3} M vinblastine	148	98

Standard preparations of 30–50% satd. $(NH_4)_2SO_4$ fraction of *Heracleum* vascular extract, at 5.6 mg protein/ml; pre-incubated at 25° × 30 min ± 1 mg/ml enzyme in Pi buffer, pH 6.8; protease = Sigma Type VI; ^{3}H-alkaloid added to 2.5×10^{-7} M; after further standard incubation, assayed in triplicate by disc method. Results expressed as % control, calculated after subtraction of background ^{3}H cpm.

species of lumicolchicine to be bound. The particularly high binding of lumicolcemid may be of significance in relation to a suggested [18] possible difference between plant and animal tubulin: preparations from yeast cells bound colcemid with an affinity an order of magnitude greater than that for colchicine. However, the purity of that colcemid was chromatographically monitored with MeOH as developing solvent, a system we found to distinguish poorly between drug and lumi-derivative. In extracts of water-mould, no site with a binding affinity greater for colcemid than for colchicine was observed [25].

The differences in levels of colchicine and lumicolchicine binding during fractionation of a plant extract, and in response to various incubation treatments, indicate that the binding reactions are separate phenomena involving sites located on different cellular moieties. We suggest that plant extracts prepared by these procedures possess a variety of sites with the ability to bind colchicine. One class of such sites shows some of the characteristics of the colchicine-binding activity of tubulin, including enhancement by the presence of vinblastine [5]. Other, more stable, sites which have a non-specific affinity for both colchicine and its lumi-isomer may also exist. This second class of sites would be more accessible *in vitro* at elevated temperatures, like the cytochrome-binding sites of mitochondrial membranes [27], and may originate from membrane components [28]. It has been reported that extracts of water-mould contain more than one type of site which differ in stability and in affinity for colchicine [25]. The presence of a variety of types of potential colchicine-binding sites in plant tissues may be of biological significance in relation to the relative insensitivity of plant processes towards colchicine, whereby the interaction of colchicine with physiologically important sites is impeded. Such sequestering of exogenously applied drugs has been suggested to be an element in the resistance of hamster cells to colchicine [29].

Of more immediate practical concern to studies of this nature, however, are the experimental difficulties raised by the fact that binding of the non-specific type will complicate the straightforward use of colchicine-[OMe-^{3}H] for the identification and quantification of plant tubulin. Experiments to characterise and isolate sites interacting specifically with colchicine-[^{3}H] may require the presence in the medium of unlabelled lumicolchicine during the extraction and assay stages, to saturate the non-specific sites. The saturating concentrations of lumi-drug will not only require determination for each type of tissue, but also for the degree of non-specific site exposure engendered by the particular procedure employed. Meanwhile, previous work on colchicine-binding, and particularly colcemid-binding, in material from plant sources may need re-evaluation in terms of the degree of specificity and probable component involved.

EXPERIMENTAL

Samples of tubulin were prepared from pig brain by the batch procedure [20] and stored at −20°. Methods of preparing the plant samples and of assaying their drug-binding activities are described more fully in ref. [16]. All operations, except incubations with alkaloids were carried out at 2°. Isolated vascular tissue of *Heracleum mantegazzianum* Somm. et Lev. was homogenised in a mortar in 0.1 M Pi buffer, pH 6.8, containing 0.01 M $MgCl_2$, using 40 g tissue/100 ml buffer. After centrifugation at 25000 *g* for 20 min, the supernatant fraction was treated with $(NH_4)_2SO_4$ and the fraction precipitating between 30–50% saturation was resuspended in extraction buffer and routinely used at specified protein concn as the standard plant preparation. Preparations (routinely freshly prepared) were incubated at 37° for 1 hr with specified concn of alkaloid-[^{3}H] and the binding activity was then assayed either by gel filtration on Sephadex G-100, or by applying triplicate aliquots of the incubation mixture to DEAE ion exchange paper [5, 19, 20]. Radioactivity in the appropriate column eluate fraction, or retained on the ion exchange discs after 7 × 50 ml washes with cold 0.01 M buffer, was detected by liquid scintillation counting in Instagel (Packard Co.). All expts were repeated at least ×3 and the patterns of results were reproducible between expts. The data reported are from single, typical expts, and in the case of the DEAE paper assay method, represent averages of triplicate assays of incubation mixture.

Colchicine-[ring C OMe-^{3}H] (690 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, and colcemid-[ring C OMe-^{3}H] (8.96 Ci/mmol) from NEN Chemicals, GmbH, W. Germany. Lumi-derivatives of the drugs were prepared by exposure of colchicine-[^{3}H] (3.6×10^{-4} M in H_2O) or colcemid-[^{3}H] (2.7×10^{-5} M in 50% EtOH) in sealed capillary tubes to a long wavelength UV lamp at a distance of 5 cm. Conversion to the lumi-derivatives was monitored by TLC in light-shielded tanks on 0.25 mm layers of Si gel in C_6H_6–EtOAc–Et_2NH (5:4:1) [21]. After chromatography, ^{3}H-samples were located by elution of R_f zones in Instagel for scintillation counting. Unlabelled samples were visualised under UV down to level of 10^{-1} μmol colchicine per 0.3 cm spot. The level of visual detection could be improved 10-fold by the use of fluorescent Si gel. Protein was assayed by the method of ref. [30] with BSA as standard.

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