Mechanism of Binding of the New Antimitotic Drug MDL 27048 to the Colchicine Site of Tubulin: Equilibrium Studies†

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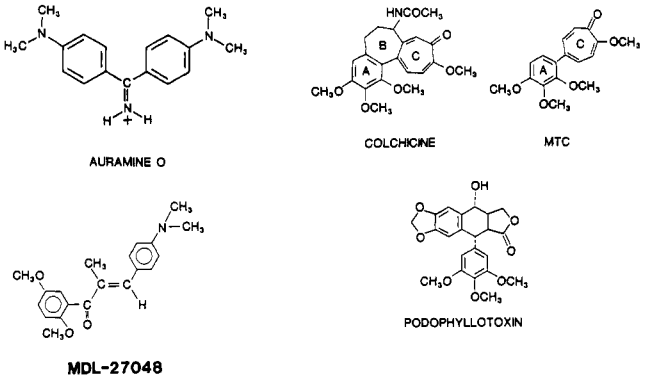
ABSTRACT: MDL 27048 [*trans*-1-(2,5-dimethoxyphenyl)-3-[4-(dimethylamino)phenyl]-2-methyl-2-propen-1-one] fluoresces when bound to tubulin but not in solution. This effect has been investigated and found to be mimicked by viscous solvents. Therefore, MDL 27048 appears to be a fluorescent compound whose intramolecular rotational relaxation varies as a function of microenvironment viscosity. The binding parameters of MDL 27048 to tubulin have been firmly established by fluorescence of the ligand, quenching of the protein fluorescence, and gel equilibrium chromatography. The apparent binding equilibrium constant was $(2.75 \pm 0.45) \times 10^6$ M^{-1}, and the binding site number was 0.81 ± 0.12 (10 mM sodium phosphate–0.1 mM GTP, pH 7.0, at 25 °C). The binding is exothermic. The binding of MDL 27048 overlaps the colchicine and podophyllotoxin binding sites. Binding of MDL 27048 to the colchicine site was also measured by competition with MTC [2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one], a well-characterized reversibly binding probe of the colchicine site [Andreu et al. (1984) *Biochemistry 23*, 1742–1752; Bane et al., (1984) *J. Biol. Chem. 259*, 7391–7398]. In contrast with close analogues of colchicine, MDL 27048 and podophyllotoxin neither affected the far-ultraviolet circular dichroism spectrum of tubulin, within experimental error, nor induced tubulin GTPase activity. Like podophyllotoxin, an excess of MDL 27048 over tubulin induced no abnormal cooperative polymerization of tubulin, which is characteristic of colchicine binding. It is proposed that the dimethoxyphenyl moiety of MDL 27048 binds to the ring A (trimethoxyphenyl) subsite of tubulin, which is common to colchicine and podophyllotoxin, while the remaining part of the MDL 27048 compound might generate a new contact on the protein.

In a previous paper (Peyrot et al., 1989) we showed that MDL 27048[1] (Chart I) constitutes a powerful specific antimitotic drug, which binds rapidly and reversibly to tubulin and inhibits cellular microtubules and mitosis. Previous binding measurements were performed by difference absorption and fluorescence spectroscopy of the ligand. They indicated high affinity binding to one site per tubulin heterodimer, as for colchicine and its analogues (Chart I). On the other hand, we reported that podophyllotoxin (Chart I) displaces MDL 27048 from its binding site, suggesting that the trimethoxyphenyl ring of podophyllotoxin might bind at or near the dimethoxybenzene ring site of MDL 27048 (Peyrot et al., 1989). For in vitro inhibition of polymerization, results showed that MDL 27048 substoichiometrically inhibited the assembly of microtubules, as with colchicine or podophyllotoxin. Furthermore at high concentration, MDL 27048, like colchicine, does not disrupt preformed microtubules. Hence, we proposed that MDL 27048 constituted a new type of antitubulin agent, which might overlap the colchicine-binding site. Preclinical studies of this compound are being undertaken (Sunkara et al., 1991).

Chart I



Podophyllotoxin competes with colchicine binding through the trimethoxybenzene ring which is common to both alkaloids (Cortese et al., 1977; Andreu & Timasheff, 1982a). Therefore, we have characterized the binding of MDL 27048 to the tubulin–colchicine complex and the effects of MTC (Chart I) and podophyllotoxin on MDL 27048 binding.

The other purposes of this study were (i) to further characterize the MDL 27048–tubulin interaction by means

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[1] Abbreviations: DCVJ, 9-(dicyanovinyl)julolodine; CD, circular dichroism; MDL 27048, *trans*-1-(2,5-dimethoxyphenyl)-3-[4-(dimethylamino)phenyl]-2-methyl-2-propen-1-one; MTC, 2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one; PG buffer, 10 mM sodium phosphate–0.1 mM GTP, pH 7.0.

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of fluorescence and equilibrium gel chromatography and (ii) to explore the effects of MDL 27048 binding on the structure of purified tubulin and its in vitro self-association processes. The present study also shows that, in high viscosity solvents, MDL 27048 becomes fluorescent like 9-(dicyanovinyl)-julolidine (DCVJ) (Kung & Reed, 1989).

MATERIALS AND METHODS

Protein

Tubulin was purified from calf brains and stored in liquid nitrogen. It was prepared for use, and its concentration was measured spectrophotometrically with the extinction coefficients $\epsilon_{276nm} = 1.16$ L g^{-1} cm^{-1} (scattering-corrected absorbance in neutral aqueous buffer), $\epsilon_{275nm} = 1.07$ L g^{-1} cm^{-1} (0.5% sodium dodecyl sulfate in neutral aqueous buffer), and $\epsilon_{275nm} = 1.09$ L g^{-1} cm^{-1} (6 M guanidine hydrochloride) as reported (Weisenberg et al., 1968; Lee et al., 1973; Andreu et al., 1984). The tubulin–colchicine complex was prepared as described (Andreu & Timasheff, 1982). All experiments were done in PG buffer,[1] at 25 °C, except where indicated.

Chemicals

Podophyllotoxin was from Aldrich Chemical Co. MDL 27048 was a gift from Merrell Dow Laboratory, and its concentration was measured spectrophotometrically with the extinction coefficient $\epsilon_{398nm} = 21000 \pm 1100$ M^{-1} cm^{-1}. 2-Methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one (MTC)[1] was a gift from Dr. T. J. Fitzgerald, and its concentration was measured spectrophotometrically with the extinction coefficient $\epsilon_{343nm} = 17600$ M^{-1} cm^{-1} (Andreu et al., 1984). GTP disodium salt, colchicine, and glycerol were from Fluka. [γ-^{32}P]GTP triethylammonium salt (10 mCi/mmol) was from Amersham International.

Binding Measurements

Binding measurements were performed by the equilibrium gel chromatography technique of Hummel and Dreyer (1962), as applied to tubulin (Medrano et al., 1989) and by fluorometric titration.

Gel Chromatography. A total of 0.6 mL of a solution containing 9 μM tubulin and a known total concentration of ligand in PG buffer was prepared and applied immediately to a column equilibrated with the same buffer of identical ligand concentration. Bio-Gel P4 columns (0.9 × 15 cm) at 25 and 6 °C and syringe pumps were employed (MDL 27048 adsorbs to Sephadex G 25 and to plastic tubing). The protein and ligand concentrations in the column effluent were measured spectrophotometrically. This procedure frequently relies on the measurement of small absorbance increments due to bound ligand and is therefore subject to several potential sources of error [see Andreu et al. (1984)]. Hence, the ligand and protein measurements were done after adding a small volume of concentrated sodium dodecyl sulfate to each fraction (final detergent concentration of 1%). This procedure displaced the protein–ligand interaction and solubilized any aggregated protein. In our case, the ligand concentration was measured at 400 nm; the MDL 27048 extinction coefficient in 1% sodium dodecyl sulfate (neutral aqueous buffer) was 29800 ± 1000 M^{-1} cm^{-1}. Similar experiments were performed with the tubulin–colchicine complex substituted for tubulin. The concentrations of ligands and protein in the column effluents with 1% SDS were determined as follows: the protein concentration in the tubulin–colchicine complex was measured spectrophotometrically using an extinction coefficient of $\epsilon_{275nm} = 1.14$ L g^{-1} cm^{-1}, and the colchicine concentration was calculated on the basis of 0.97 mol of colchicine bound/mol of tubulin (Andreu & Timasheff, 1982a). The absorbance measurement at 400 nm was corrected for the contribution of bound colchicine by using a colchicine extinction coefficient of 1550 M^{-1} cm^{-1} (400 nm, in 1% SDS buffer).

The binding equilibrium constant and stoichiometry were obtained from Scatchard plots of the data.

Fluorometric Titrations. The fluorescence of free ligand was measured, and this value, practically negligible for MDL, was subtracted. A concentration of ligand giving no appreciable inner filter effect (absorption <0.05) was titrated with tubulin until fluorescence due to bound ligand reached saturation. This procedure yield the fluorescence intensity of bound ligand (i.e., 20 ± 0.5 units/10^{-6} M bound MDL 27048 in PG buffer at pH 7.0 and 25 °C with excitation at 400 nm and emission at 480 nm, in the Kontron SFM 25 fluorimeter). Then, aliquots of a protein solution were titrated with known total concentrations of ligand. Inner filter corrections were made by the procedure of Mertens and Kagi (1981). The free-ligand concentration was taken as the difference between total and bound concentrations. The values of the binding equilibrium constant and the number of sites were obtained from Scatchard plots.

Quenching of the intrinsic protein fluorescence by ligand was also employed to estimate the binding parameters. The maximal fluorescence quenching by an excess of ligand was measured, correcting for the inner filter effect as described above. Then, the fraction of sites occupied, X, was considered equal to the fraction of the maximal quenching effect at a given total ligand concentration. The binding equilibrium constant was determined by using the relationship

$$1/(1 - X) = -nK_a[P_o] + K_a([L_o]/X)$$

(Guilbault, 1973), where $[P_o]$ and $[L_o]$ are the total protein and ligand concentrations, K_a is the apparent equilibrium binding constant, and n is the number of binding sites.

Indirect Binding Measurement by Competition with a Well-Characterized Reference Ligand. In the presence study, the reference ligand was MTC, whose binding had been measured fluorometrically ($K_a = 4.6 \times 10^5$ M^{-1} under identical conditions; Andreu et al., 1984). The method used (Peyrot and Sarrazin, unpublished) is outlined below.

The fractional saturation of the binding sites, P, for the reference ligand A (here MTC) (defined as $\alpha(A) = [PA]/[P_o]$) and for the studied ligand B were measured ($\beta(B) = [PB]/[P_o]$). Assuming a 1:1 stoichiometry when a competition occurs, we have

$$P + A \rightleftharpoons PA \quad (1)$$

$$P + B \rightleftharpoons PB \quad (2)$$

giving the following expressions

$$K_1 = [PA]/[P][A] \quad (3)$$

$$K_2 = [PB]/[P][B] \quad (4)$$

$$[A] = [A_o] - [PA] \quad (5)$$

$$[B] = [B_o] - [PB] \quad (6)$$

$$[P] = [P_o] - [PA] - [PB] = [P_o](1 - \alpha(A) - \beta(B)) \quad (7)$$

Equations 3–7 lead to

$$[A][PB] = (K_2/K_1)[B][PA]$$

or more explicitly

$$[MTC]_{free} \times [MDL]_{bound} = (K_2/K_1)[MDL]_{free} \times [MTC]_{bound} \quad (8)$$

A plot of $[MTC]_{free} \times [MDL]_{bound}$ versus $[MDL]_{free} \times [MTC]_{bound}$ gives a straight line with a slope equal to the association constant ratio.

Fluorescence spectra demonstrate the competition between MTC and MDL 27048 for the same tubulin binding site (for an example see Figure 7). Each spectrum may be considered as a linear combination of bound MDL and MTC spectra and buffer–protein background. The latter contribution cannot be neglected; it was particularly important in the wavelength region of bound MTC emission; however, the fluorescence of the free ligands was negligible. Using the input/output facilities of the Kontron SFM fluorimeter, we stored each spectrum in a computer file (250 points per file). For each observed spectrum, S_{obs}, we may write the following expression:

$$S_{obs,i} = A \times S_{MDLbound,i} + B \times S_{MTCbound,i} + C \times S_{buffer\ protein,i}$$

i.e., at each wavelength, i, the observed fluorescence intensity, S_{obs}, is a linear combination of the three reference contributions. In our conditions, at constant protein concentration, $C \times S_{buffer\ protein}$ was constant, the equation became a two-component fit. Using a nonlinear regression minimizing the sum of squared deviations with a Gauss–Newton method, we obtained the best fitting coefficients A and B (for an example see the inset of Figure 7). As we knew the complex concentrations responsible for the reference spectra (spectra 1, 2, and 4 in the inset of Figure 7), we then translated the A and B coefficients into complex concentrations for each competition spectrum. The free ligand concentration was determined by calculating the difference from the known total ligand value. The calculated values were plotted according to eq 8.

Miscellaneous Procedures

Ligand-Induced GTPase Activity. It was assayed by the release of [^{32}P]phosphate in 10 mM sodium phosphate–1 mM $MgCl_2$ buffer, pH 7.0, containing 0.1 mM [γ-^{32}P]GTP, at 37 °C. Tubulin purified by the modified Weisenberg procedure was subjected to a gel filtration chromatography (Sephacryl S-300 HR) to eliminate the ligand-independent GTPase activity (Andreu et al., 1991). The assay was done as described (Andreu & Timasheff, 1981), except that the enzyme reaction was stopped after 30 min by addition of 0.45 mL of perchloric acid (0.25 N). The samples were counted in a Beckman LS-1701 liquid scintillation counter.

Sedimentation Velocity. Sedimentation velocity experiments were performed with a Beckman Model E analytical ultracentrifuge. Identical samples, with and without ligand, were run simultaneously in double-sector cells in an An-D rotor at 60000 rpm or 48000 rpm at 20 °C.

Self-Assembly of Tubulin into Abnormal Polymers. This reaction in the presence of colchicine, podophyllotoxin and MDL 27048 was followed turbidimetrically at 500 nm, at 37 and 42 °C, in PG, 16 mM $MgCl_2$, buffer, pH 7.0, using a Beckman DU 70 spectrophotometer. The polymers formed with colchicine were fixed with 0.5% glutaraldehyde, adsorbed to Formvar-coated grids, negatively stained with 1.5% uranyl acetate, and examined under a Philips EM 400 electron microscope (Service Commun de Microscopie Electronique, Faculte des Sciences, Marseille, France).

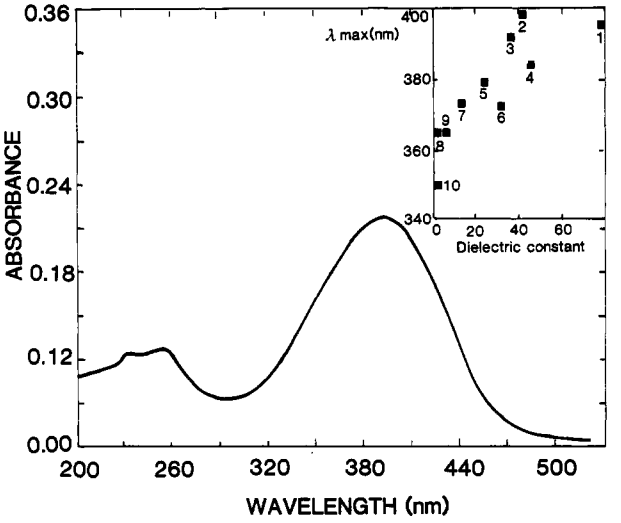

FIGURE 1: Absorption spectrum of 14 μM MDL 27048 in PG buffer, pH 7.0 at 25 °C. The inset shows the measured absorption maxima, λ_{max} (nm), of MDL 27048 plotted against the dielectric constants of solvents of varying polarity at 25 °C: (1) water, (2) glycerol, (3) ethylene glycol, (4) dimethyl sulfoxide, (5) ethanol, (6) methanol, (7) hexanol, (8) ethyl acetate, (9) carbon tetrachloride, and (10) hexane.

Spectroscopic Measurements. Fluorescence measurements and uncorrected spectra were obtained with a Kontron SFM 25 spectrofluorimeter operating at excitation and emission wavelengths of 400 or 340 and 480 nm, respectively, with slit widths of 5/5 nm, and also with a Shimadzu RF 540 spectrofluorimeter with slit widths of 2/10 nm. A neutral Kodak Wratten filter (ND200) was used to avoid photobleaching of MDL 27048. When quenching of the intrinsic protein fluorescence by ligand was measured, the excitation and emission wavelengths were 295 or 280 and 330 nm, respectively, with slit widths of 5/5 nm.

Circular dichroism spectra were obtained with a Rousell-Jouan dicrograph II, in 0.1- and 1.0-cm cells at 25 ± 0.5 °C.

Microcalorimetric Measurements. Microcalorimetric measurements were performed with a flow calorimeter (LKB Bioactivity Monitor 2277; Gilli et al., 1990) at 25 °C in PG buffer, pH 7. Before each experiment, the baseline was established by pumping a ligand buffer solution into the circuit of the calorimeter at a flow rate of 22.5 mL/h with a syringe pump and by pumping the buffer alone into the second circuit at the same flow rate. The sensitivity was 10 μW full-scale, with a background noise of 0.1 μW. Tubulin was equilibrated with the experimental buffer by gel filtration, and 1 mL was injected into the buffer circuit while the ligand solution was pumped into the first circuit. Data were corrected for dilution heat values.

Microcalorimetric measurements were also performed employing a LKB batch microcalorimeter and correcting for the protein dilution and mixing heat effects (Menendez et al., 1989). Because of the low solubility of MDL 27048, the ligand was saturated with an excess of protein, as for allocolchicine (Menendez et al., 1989).

RESULTS

Spectral Absorption and Fluorescence Properties of MDL 27048. MDL 27048, whose structure is shown in Chart I, had an absorption maximum in the violet region of the spectrum (400 nm) (Figure 1) characteristic of a $\pi \rightarrow \pi^*$ charge-transfer transition. Generally, the excited π^* state

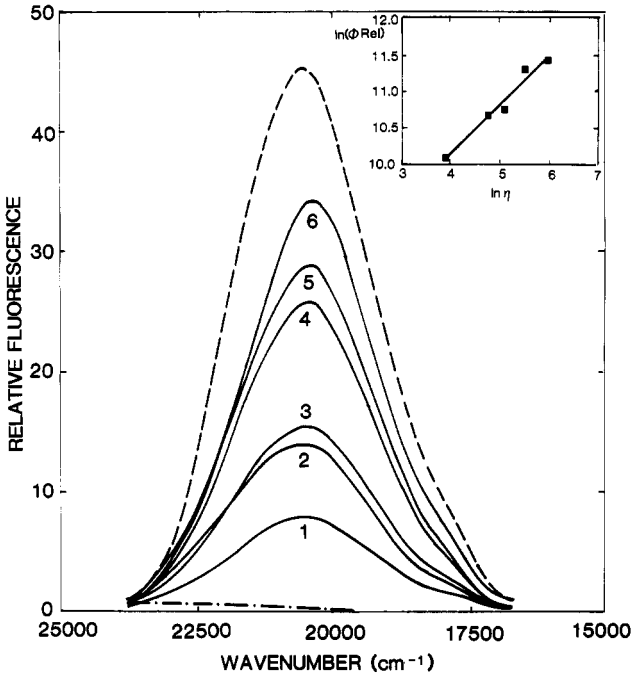

FIGURE 2: Fluorescence emission spectra of 10 μM MDL 27048 in different solvent conditions. (– · –) Fluorescence emission spectrum of MDL 27048 in ethyl acetate and (—) in ethylene glycol/glycerol (v/v) mixtures of varying viscosity at 25 °C: (1) 7:3, η = 49 cP; (2) 5:5, η = 115 cP; (3) 4:6, η = 163 cP; (4) 3:7, η = 245 cP; (5) 2:8, η = 391 cP; (6) in glycerol 100%. (– –) Fluorescence emission spectra of 3.7 μM tubulin with 10 μM MDL 27048 displayed on a 3×-reduced scale. The excitation wavelength was 400 nm. In ethyl acetate, excitation was at 365 nm. The inset shows the relationship between relative quantum yield (Φ_{rel}, defined here as the integral of the uncorrected fluorescence intensity, in relative units, over wavenumber) and viscosity. The slope of the line is 0.66, a value which coincides with the $^2/_3$ power viscosity dependence of the quantum yield reported by Förster and Hoffman (1971).

receives predominant contribution from highly polar structures such as R_2 C^+–O^- and is consequently more polar than the ground state. Such structures with separated charges are stabilized in polar solvents (Jaffé & Orchin, 1964). The inset of Figure 1 shows that the energy of the transition depends strongly on solvent dielectric constant, i.e., polarity, which is characteristic of charge transfer. For the $\pi \rightarrow \pi^*$ transition of compounds like ketones, the hydrogen bond with solvent persists in both the ground and the excited states, and the stabilization of the more polar excited state leads to a red shift by polar solvents.

The fluorescence of MDL 27048 in neutral buffer or apolar solvent was extremely poor, as would be expected for completely unrestricted torsional rotation of the fluorophore (Figure 2). In presence of tubulin [or bovine serum albumin, see, Peyrot et al. (1989)], or glycerol (see Figure 2), the fluorescence intensity increased dramatically. Figure 2 also shows the emission spectra of MDL 27048 in several mixtures of ethylene glycol/glycerol of varying viscosity. The dielectric constant was virtually unchanged [from 38.6 to 41.4, while viscosity increased from 49 to 391 cP, Kung and Reed (1989)]. The fluorescence quantum yield increased with viscosity (see inset of Figure 2).

Binding Equilibrium Parameters. (A) Measurement of Binding by Fluorescence Spectroscopy of Ligand.[2] As shown

[2] In our previous report (Peyrot et al., 1989), the fluorescence measurements were affected by possible errors due to unnoticed photolysis of MDL 27048 which have now been avoided, and the binding has been reexamined.

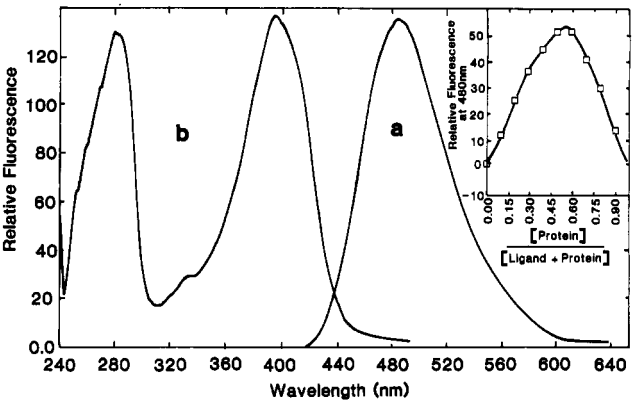

FIGURE 3: Fluorescence changes produced by the MDL 27048–tubulin interaction. (a) Uncorrected fluorescence emission (excitation at 400 nm); (b) uncorrected excitation (emission at 485 nm). The higher energy band may be affected by variations in lamp intensity. Spectra of a solution contain 4 μM MDL 27048 and 4 μM tubulin (the ligand alone gave no fluorescence in this experiment). The inset shows fluorescence variations of the bound ligand in solutions in which the sum of the total concentration of ligand and protein was maintained at 6.6 μM, while varying their proportions, which is well above the dissociation constant and, therefore, gives a semiquantitative indication of the binding stoichiometry (Ward, 1985).

above, the fluorescence intensity of MDL 27048 increased in presence of tubulin. The ligand–tubulin complex (Figure 3) had excitation maxima at 400 nm and around 280 nm and emission maximum at 485 nm. The inset of Figure 3 shows a continuous-variation experiment. The maximum fluorescence occurred at a protein mole fraction close to 0.5 (see legend), suggesting the possible formation of a 1:1 complex between tubulin and MDL 27048 (Asmus, 1961; Ward, 1985). Fluorimetric titrations of tubulin with various MDL 27048 concentrations were performed as described under Materials and Methods. The equilibrium parameters were respectively (3.35 ± 0.7) × 10^6 M^{-1} and 0.66 ± 0.10 for the apparent binding constant and number of binding sites (four different determinations with three different tubulin preparations).

(B) Quenching of Tubulin Fluorescence by MDL 27048. When excited at 295 nm, tubulin showed a characteristic fluorescence emission spectrum with a maximum at 335 nm. Excitation at 295 nm was selected to minimize the emission due to tyrosine residues, since it is known to selectively excite tryptophan residues in protein (Lakowicz, 1983). As shown in Figure 4, when MDL 27048 was added to tubulin we observed a decrease in emission, and the appearance of the spectrum at 480 nm, which is characteristic of MDL 27048 bound to tubulin. These findings were taken as indicating an energy transfer between tryptophan and bound MDL 27048. Similar experiments with excitation at 280 nm also showed quenching of tubulin fluorescence. The spectra showed an isoemissive point at 430 nm. The fluorescence quenching by MDL 27048 did not affect the position of the maximum wavelength, indicating that there is no strong polarity modification around the tryptophanyl residues as a result of the binding of MDL 27048.

The inset of Figure 4 shows the analysis of the protein fluorescence quenching titration with MDL 27048. The average data, obtained with different tubulin preparations, gave an apparent association constant of (2.80 ± 0.50) × 10^6 M^{-1} and an apparent number of binding sites of 0.80 ± 0.25.

(C) Binding Equilibrium Parameters by Gel Chromatography. To measure binding by a direct procedure not relying on any assumptions, the quantitative characterization of the binding equilibrium of MDL 27048 to tubulin was performed by the equilibrium gel chromatography technique of Hummel

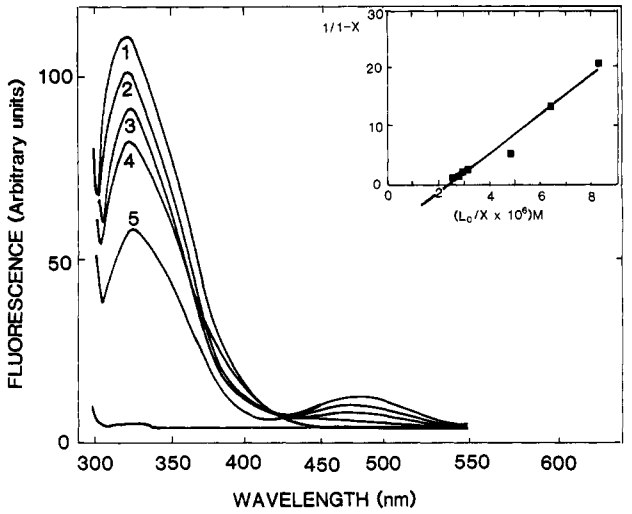

FIGURE 4: Fluorescence changes produced by the tubulin–MDL 27048 interaction: tubulin at 2 μM in PG buffer, pH 7.0, was mixed with 0 (spectrum 1), 0.4 (spectrum 2), 0.8 (spectrum 3), 1.2 (spectrum 4), and 5.5 (spectrum 5) μM MDL 27048. Excitation wavelength was 295 nm. The inset shows protein fluorescence quenching titration of the binding of MDL 27048 to 2 μM tubulin at 25 °C. Emission wavelength was set at 335 nm (see Materials and Methods). These data give an apparent association constant of (3.30 ± 0.60) × 10^6 M^{-1} and a number of binding sites of 1.

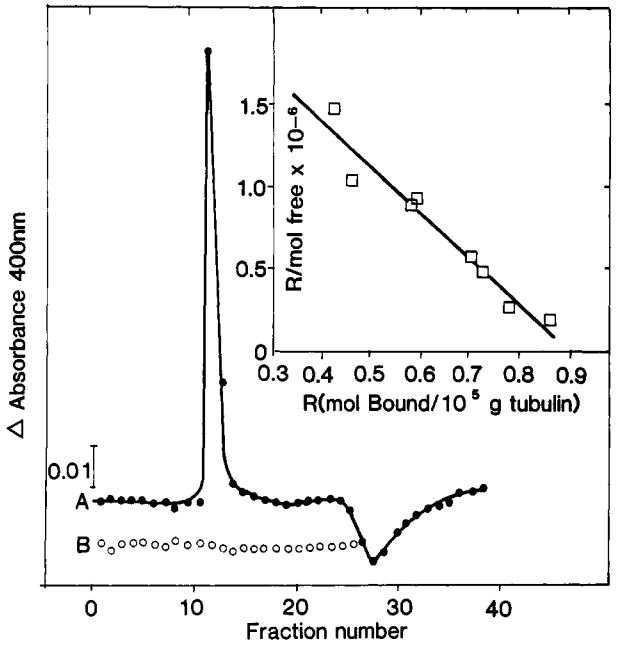

FIGURE 5: Gel chromatography of tubulin in columns equilibrated with MDL 27048. Profile A: A total of 0.6 mL of solution containing 9.2 μM tubulin was chromatographed in a column equilibrated with 3.13 μM free MDL 27048 in PG buffer, pH 7.0, 25 °C; absorbance measurements were done as described under Materials and Methods. Profile B: A total of 0.6 mL of solution containing 2.7 μM tubulin was chromatographed in a column equilibrated with 4.98 μM MDL 27048 in PG buffer containing 2% SDS. The Bio-Gel P4 column size was 0.9 × 18 cm, the syringe pump flow rate was 12 mL/h, and fractions were 1 mL. The inset shows a Scatchard plot of the equilibrium gel chromatography measurements.

and Dreyer (1962). Figure 5, profile A, shows a typical elution profile. The good separation of the peak and trough indicated the attainment of binding equilibrium. As shown by profile B, an identical experiment was performed with 2% sodium dodecyl sulfate; no MDL 27048 binding was detected, indicating complete dissociation and reversibility of the tubulin–MDL 27048 complex. A linear regression of a Scatchard plot of the binding data gave (inset) 0.91 ± 0.07

Table I: Binding of MDL 27048 to Tubulin

	K_a × 10^{-6} M^{-1} at 25 °C	n
gel chromatography	(2.70 ± 0.60)	0.90 ± 0.02
	(3.50 ± 0.75)[a]	0.80 ± 0.05
ligand fluorescence	(3.35 ± 0.60)	0.67 ± 0.10
	(4.50 ± 0.30)[b]	0.67 ± 0.17
protein fluorescence quenching	(2.80 ± 0.50)	0.80 ± 0.25
ligand fluorescence competition	(2.80 ± 0.15)	ND[d]
ligand difference absorption spectrophotometry	(2.10 ± 0.40)[c]	0.96 ± 0.10

[a] At 6 °C. [b] At 12 °C. [c] Peyrot et al. (1989) using phosphocellulose-purified tubulin. [d] ND, information not determined by this procedure.

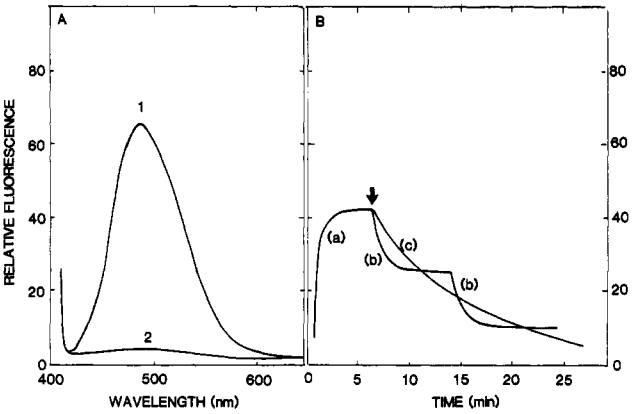

FIGURE 6: (Panel A) Inhibition by colchicine of the MDL 27048 binding to tubulin at 25 °C (tubulin–colchicine complex was prepared as described in Materials and Methods). (1) Emission spectrum of 3 μM tubulin and 7 μM 27048. (2) Emission spectrum of 3 μM tubulin–colchicine complex and 7 μM MDL 27048 (excitation wavelength was 400 nm). (Panel B) Fluorescence time course of 4 μM MDL 27048 binding to tubulin (1.72 μM) at 25 °C (excitation wavelength was 400 nm). At the time indicated by the arrow, 20 μM MTC (trace b) or 20 μM podophyllotoxin was added (trace c). In trace b 100 μM MTC was added again after 10 min.

sites and an apparent binding equilibrium constant K_a = (2.73 ± 0.66) × 10^6 M^{-1}. Table I summarizes the binding parameters determined by the different methods. The average value of the binding equilibrium constants from the different methods yielded an apparent standard free energy change, $\Delta G°_{app}$ = −8.8 ± 0.1 Kcal mol^{-1} for the binding of MDL 27048 to tubulin in PG buffer at 25 °C.

(D) Determination of the Enthalpy of Binding. Microcalorimetric measurements were performed by both flow and batch procedures (Materials and Methods). The binding of MDL 27048 to tubulin equilibrated in PG buffer, at 25 °C, is exothermic, with an enthalpy change $\Delta H°$ = −6.2 ± 1.6 Kcal/mol of complex.

Specificity of the Interaction. It has been shown (Peyrot et al., 1989) that MDL 27048 affects the colchicine binding site. It was of interest to know whether (i) the binding of MDL 27048 is specifically directed to the colchicine site or (ii) its binding loci on the protein is not directly involved in the binding of colchicine and analogues.

The interaction of MDL 27048 with tubulin in the presence of colchicine is examined in the experiment depicted in Figure 6A. Trace 1 is the fluorescence spectrum when the unliganded tubulin was used, while trace 2 is the result with the tubulin–colchicine complex. Their simplest interpretation is that MDL 27048 does not bind to the tubulin–colchicine complex. To obtain further evidence of the inhibition of MDL 27048 binding by colchicine, we performed experiments similar to those of the column binding measurements described above but with the tubulin–colchicine complex substituted for tubulin. A very small ligand (MDL) absorption increment in the protein

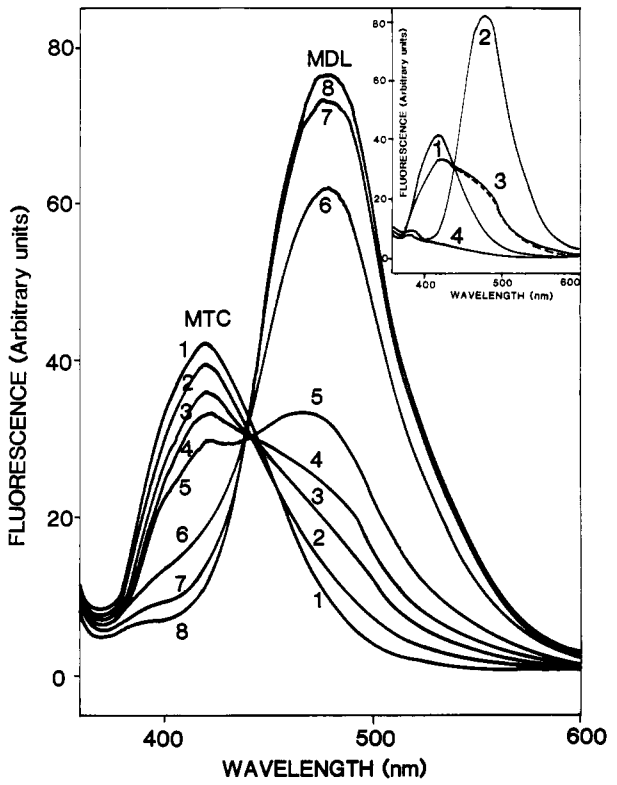

FIGURE 7: Inhibition of MTC binding to tubulin by MDL 27048. This figure shows spectra of the equilibrium competition between MTC (6.2 μM) and MDL 27048. Various MDL 27048 concentrations were added to the mixture of tubulin and MTC. (1) 6.2 μM MTC alone, (2) MTC–tubulin complex plus 0.11 μM MDL 27048, (3) MTC–tubulin complex with 0.34 μM MDL 27048, (4) MTC–tubulin complex with 0.60 μM MDL 27048, (5) MTC–tubulin complex with 1.10 μM MDL 27048, (6) MTC–tubulin complex with 3.10 μM MDL 27048 , (7) MTC–tubulin complex with 5.10 μM MDL 27048, and (8) MTC–tubulin complex with 10 μM MDL 27048. The tubulin concentration was 5 μM. The excitation wavelength was 340 nm. The inset shows (1) spectrum of MTC 6 μM alone, (2) spectrum of MDL 6 μM, alone, (3) experimental (—) and calculated spectra (- -) of the equilibrium competition between MTC (6 μM) and MDL 27048 (0.60 μM), and (4) buffer–protein background.

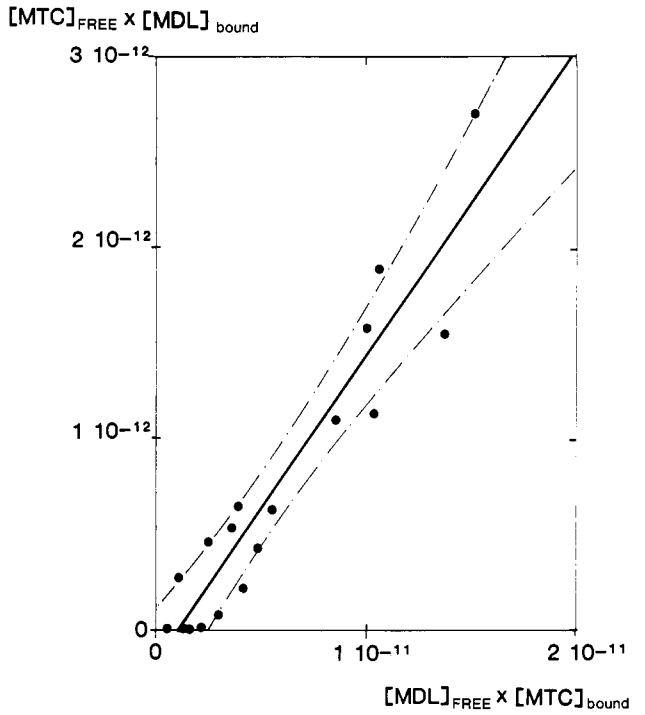

FIGURE 8: Determination of the apparent binding constant of MDL 27048 by competition with MTC. Each point represents a measurement of competition between MDL 27048 and MTC.

elution peak was observed, indicating that binding of MDL 27048 (7 μM) to the tubulin–colchicine complex (5 μM) was essentially inhibited. This binding was characterized by a R (mol of ligand/10^5 g of tubulin) value of 0.07 ± 0.02. We also examined the equilibrium binding of MDL 27048 to tubulin in columns equilibrated with 2 μM MDL 27048 and 200 μM podophyllotoxin. This interaction was characterized by a measured R value of 0.04 ± 0.02.

Figure 6B shows a time-course of association of MDL 27048 to tubulin (trace a). The dissociation of the tubulin–MDL 27048 complex was checked by displacement with podophyllotoxin and MTC, which provides an additional criterion of specificity. The results are shown in Figure 6B, traces b and c. The observed dissociation rates and differences in plateau values are a function of kinetic and binding equilibrium constants of the three compounds.

The equilibrium binding of MDL 27048 to the colchicine site was quantitatively examined by competition with MTC. The inhibition of the equilibrium binding of MTC by MDL 27048 is displayed in Figures 7 and 8. Figure 7 shows the disappearance of the MTC–tubulin complex emission at 425 nm as the concentration of MDL 27048 increases. The formation of the MDL 27048–tubulin complex was detected at 480 nm, which is the expected wavelength. An isoemissive point was observed at 440 nm. The order of addition of the two ligands did not modify the results. The competition data were analyzed as described under Materials and Methods, and the result is shown in Figure 8. Linear regression gave a slope value of 6.124, and assuming a K_a value of 4.6×10^5 M^{-1} for MTC (Andreu et al., 1984), the K_a value of MDL 27048 was 2.80×10^6 M^{-1}.

Effects of MDL 27048 Binding on Conformational Parameters and Self-Associations of Tubulins. Since colchicine is believed to induce a structural change in tubulin, one manifested by a small pertubation of the protein circular dichroism at 217–225 nm region and the appearance of GTPase activity (Andreu & Timasheff 1982a), these properties were examined in tubulin liganded to MDL 27048. Examination of the far-ultraviolet circular dichroism spectra (215–250 nm) of tubulin (3 μM) unliganded and in the presence of MDL 27048 (8 μM) showed that MDL 27048 had no significant effects. We did not detect MDL 27048 (25 μM) circular dichroic band in the 300–480 nm region both in the absence and in the presence of tubulin (5 μM). Examination of ligand-induced GTPase activity of tubulin indicated a nil value within experimental error for 100 μM MDL 27048 and 100 μM podophyllotoxin, in comparison with 100 μM colchicine in the same experiment.

Addition of 100 μM MDL 27048 did not induce significant changes in the sedimentation velocity of tubulin (8 mg mL^{-1}) in PG buffer, pH 7.0 at 20 °C, indicating the absence of ligand-induced tubulin self-association under these conditions. This observation indicates that the binding is independent of protein concentration. In the presence of Mg^{2+}, tubulin–GDP undergoes a self-association reaction with the formation of double rings characterized by a bimodal Schlieren profile (Howard & Timasheff, 1986). Under these conditions (10 mM phosphate buffer, 6 mM $MgCl_2$, 1 mM GDP, pH 6.7, 20 °C, 6.5 mg mL^{-1} tubulin), an excess of 100 μM MDL 27048 did not induce major changes, such as had been reported for colchicine or MTC (Andreu et al., 1983, 1984).

The formation of anomalous tubulin polymers, with a critical concentration of 1 mg mL^{-1}, by stoichiometric binding of the drug to $\alpha\beta$-tubulin is characteristic of colchicine (Saltarelli

Table II: Thermodynamic Parameters of Binding of MDL 27048 and Other Ligands to Tubulin

ligand	T, °C	ΔG_{app}, Kcal mol^{-1}	ΔH_{app}, Kcal mol^{-1}	ΔS_{app}, cal mol^{-1} K^{-1}
MDL 27048[a]	25	−8.8 ± 0.1	−6.2 ± 1.6	8.7 ± 5.3
MTC[b]	25	−7.7 ± 0.1	−4.6 ± 0.2	10.4 ± 0.7
colchicine[c]	25	−9.6 ± 0.1	−5.0 ± 0.5	15.5 ± 1.6
podophyllotoxin[d]	37	−8.8		

[a] This study. [b] Menendez et al. (1989). [c] Diaz and Andreu (1991) and Menendez et al. (1989). [d] Cortese and Wolff (1977).

& Pantaloni, 1982; Andreu & Timasheff, 1982b) and its bifunctional analogues (Andreu et al., 1991). An excess of MDL 27048 (100 μM) or podophyllotoxin (100 μM) did not induce an increase in turbidity in PG buffer and 16 mM $MgCl_2$, pH 7.0, when the tubulin (2.5 mg mL^{-1}) solution was heated to 37 °C and even at 42 °C. Colchicine (100 μM), in the same medium, induced the formation of anomalous polymers.

DISCUSSION

Binding of MDL 27048 to Colchicine Site of Tubulin. The interaction of MDL 27048 with purified Weisenberg-tubulin has been firmly established (see Table I). The K_a value obtained with equilibrium gel chromatography was highly similar to those obtained with the indirect binding measurements. The different results and those with difference absorption spectroscopy (results obtained with PC–tubulin) were highly correlated.

MDL 27048 inhibits the binding of the well-characterized reference ligand MTC and, reciprocally, MTC inhibits the binding of MDL 27048. Using a simple graphical analysis of the competition measurements, we found a MDL 27048 K_a value identical with that obtained by gel equilibrium measurements. Colchicine and podophyllotoxin inhibit the binding of MDL 27048 to tubulin. MDL 27048 binds reversibly to tubulin much more rapidly than colchicine, but more slowly than MTC [the emission intensity of MTC reached 90% of its maximal value in less than 1 min (Andreu et al., 1984)].

Quenching of tubulin fluorescence suggests that MDL 27048 binds to tubulin close to a fluorophore, i.e., tryptophan. A similar result was reported for colchicine (Andreu & Timasheff, 1982a), but no quenching was observed for podophyllotoxin.

Let us now compare the thermodynamic parameters of binding of MDL 27048 to other colchicine site ligands. Although the temperature dependence of the binding of MDL 27048 (Table I) suggests a slightly exothermic binding, the enthalpy change is best measured by calorimetry, as discussed elsewhere (Menendez et al., 1989; Timasheff et al., 1991). As compared in Table II, MDL 27048 binds with intermediate free energy change between MTC and colchicine; the apparent enthalpy changes of the three ligands are not significantly different within experimental error. The three compounds have significant entropic contributions to the binding free energy change.

Fluorescence Properties of MDL 27048 upon Binding to Tubulin. The quantum yield of MDL 27048 does not appear strongly dependent on the dielectric constant (the emission intensity of MDL 27048 in organic solvents is very poor), but rather on the intermolecular rotational relaxation of the molecule itself. For this kind of molecule, any process which restricts the relaxation field will result in an increase in fluorescence intensity (Förster & Hoffman, 1971; Kung & Reed, 1989).

Colchicine binding to tubulin enhances its fluorescence intensity. By comparison to glycerol solutions, Bhattacharyya and Wolff (1984) suggested that this fluorescence increase is essentially due to the "immobilization" of the drug upon binding. However, very recent detailed spectroscopic studies of colchicinoids indicate that the fluorescence properties in glycerol may not be reliable indicators of the state of the tubulin-bound ligands. (Pyles et al., 1992; Pyles & Hastie, 1992). Specifically, there is evidence of exciplex formation between the C-7 amide nitrogen and the ring C π system, and glycerol may increase fluorescence both by viscosity and by hydrogen bonding to the electrons of the nitrogen. On the other hand, the viscosity- or binding-dependent fluorescence enhancements exhibited by DCVJ may be interpreted as resulting from a restriction of the internal rotation of aromatic groups, resulting from highly viscous medium or the binding to tubulin (Kung & Reed, 1989). Another example of immobilization is found in the interaction of Auramine O with alcohol dehydrogenase (Conrad et al., 1970). Furthermore, a fluorescence increment of this compound is not observed in water, ethanol, or benzene but is in viscous solutions (Oster & Nishijima, 1956). The viscosity-dependent fluorescence exhibited by Auramine O apparently results from a restriction of internal rotation of the two phenyldimethylamine groups (see Chart I). MDL 27048 is also substituted by a phenyldimethylamine at carbon 3. Consequently, one may attribute the viscosity or binding enhancement of fluorescence of MDL 27048 to a restriction of the free rotation of the dimethylamine or the phenyldimethylamine group, although other effects should not be ruled out.

Consequences of Binding of MDL 27048 to Tubulin. As reported by Andreu et al. (1991), all the biphenyl ligands that bind to the colchicine tubulin site induce GTPase activity. For colchicine and its analogoues, binding to both the ring A and the ring C subsites seems to be required. Podophyllotoxin binds to the ring A locus and does not induce GTPase activity. Presently, there is no evidence that podophyllotoxin might bind to the ring C locus. Like podophyllotoxin, MDL 27048 induces no GTPase activity.

Colchicine, MTC, and other analogues induced a weak perturbation in tubulin circular dichroism. On the contrary, podophyllotoxin has no significant effects in the tubulin CD (Andreu & Timasheff, 1982a). Similar results were observed with MDL 27048.

The formation of anomalous tubulin polymers at 37 °C by stoichiometric binding seems to be related to the ability of the ligand to be anchored in a proper orientation at the ring C subsite of tubulin (Andreu et al., 1991). Like podophyllotoxin, MDL 27048 is unable to promote this abnormal polymerization of tubulin, even at higher temperature (42 °C). The formation of microtubules from tubulin or microtubule proteins was inhibited by substoichiometric amounts of MDL 27048. The half-inhibitory concentrations were respectively 1 and 0.6 μM for MDL 27048 [see Peyrot et al. (1989)] and podophyllotoxin (Loïke et al., 1978).

The consequences of the binding of MDL 27048 to tubulin indicate that this ligand overlaps only in part the colchicine–MTC site. From the available data, it cannot be ascertained whether MDL 27048 overlaps the podophyllotoxin site fully or only in part. Since the part common to the structures of

[3] Single-ring analogues of ring A might be expected to weakly inhibit the binding of MDL 27048. However, the effect of *N*-acetyl mescaline on the binding of colchicine was only marginal (Andreu & Timasheff (1982c), and we have not observed an inhibition of binding of MDL 27048 or of MTC binding by this low affinity ligand.

MDL 27048, colchicine–MTC, and podophyllotoxin is the methoxybenzene ring, the simplest interpretation is that this part of the ligand constitutes a point of attachment to the protein-binding site, i.e., MDL 27048 binds to the methoxybenzene subsite of the colchicine binding site (Andreu & Timasheff, 1982a; Andreu et al., 1991).[3] It is attractive to think that the other part of the MDL 27048 structure might generate a new productive contact with a locus in the protein not involved in colchicine binding.

Finally, the kinetics of the association and dissociation, of MDL 27048 with tubulin were monitored by the modification in ligand emission fluorescence intensity. The kinetics are more adequately studied under pseudo-first-order conditions by stopped flow methods, and the results are the subject of the following paper.

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