Evidence for an Alternative Pathway for Colchicine Binding to Tubulin, Based on the Binding Kinetics of the Constituent Rings*

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The kinetics of tropolone methyl ether binding to tubulin were measured by following the loss of colchicine binding capacity upon preincubation of tubulin with tropolone methyl ether. At 25 °C a bimolecular association rate constant of 2.7 (±0.2) M⁻¹ min⁻¹ was determined, and from the temperature dependence an activation energy of 37 (±8) kJ·mol⁻¹ was calculated.

By displacement experiments a dissociation rate constant of 2.9 (±0.6) × 10⁻⁵ min⁻¹ was determined at 25 °C.

The effect of 3',4',5'-trimethoxyacetophenone (TMA) is 2-fold. TMA reduces the apparent association rate constant of colchicine, indicating that it equilibrates very rapidly and reversibly with the colchicine binding site. From this reduction the binding constant for TMA can be obtained. At 25 °C a value of 112 (±13) M⁻¹ is estimated. The binding of TMA is practically thermoneutral.

Preincubation of tubulin with TMA over 30 min not only reduces the subsequent binding rate constant of colchicine but also the amplitude. This indicates that TMA also binds slowly in a second mode or site.

Stopped-flow kinetic studies reveal that fast TMA binding competes for the initial binding of colchicine. From these results it is concluded that colchicine binds initially with its trimethoxybenzene ring and in a subsequent step with the tropolone ring.

Colchicine is a well known cytostatic agent because of its effective inhibition of the assembly of microtubules and consequently its inhibition of cell division.

The binding of colchicine and related molecules to tubulin has been studied in great detail. Equilibrium measurements were performed on those molecules as well as on constituent separated rings (Bhattacharyya and Wolff, 1974; Andreu and Timasheff, 1982a, 1982b; Medrano et al., 1989). The binding of colchicine to tubulin results in the formation a fluorescent tubulin-colchicine complex, which allows the reaction to be followed continuously. Stopped-flow kinetic studies allowed the binding mechanism to be dissected into two steps: a fast initial binding of relatively low affinity followed by a rather slow conformational change of the initial complex (Garland, 1978; Lambeir and Engelborghs, 1981). (See Scheme 1, where K₁ is the association constant for the initial binding and k₁ and k₂ are the association and the dissociation rate constants of the final complex.)

The analysis of the kinetics is further complicated by the presence of parallel reactions, which were initially interpreted as either belonging to two major tubulin isoforms or to two states in slow equilibrium. Later it was shown that the two parallel phases are caused by separatable tubulin isoforms (Banerjee and Ludueña, 1987, 1991).

The temperature dependence of the equilibrium constants and the kinetic parameters obtained from stopped-flow experiments allowed the determination of the thermodynamic parameters of the individual steps (Lambeir and Engelborghs, 1981) i.e. the reaction enthalpy of the initial fast equilibrium and the activation parameters for the second step. The dissociation rate constant k₂ was measured by displacement experiments, and its activation energy was determined by Díaz and Andreu (1991).

Andreu and Timasheff (1982a) studied the tubulin binding properties of tropolone methyl ether and mescaline, two constituent rings of colchicine. These authors found that the thermodynamic parameters for the binding of tropolone methyl ether were similar to those for the initial complex formation of colchicine, as obtained by the stopped-flow kinetics. The following mechanism was therefore proposed. In a fast preequilibrium colchicine first binds to tubulin with its tropolone moiety. The second step consists of a slow conformational change of the initial complex, which makes a specific site available for the trimethoxyphenyl moiety.

On the basis of this two-site model, the behavior of the colchicine analog MTC* is expected to be analogous to that of colchicine. MTC and colchicine have their trimethoxyphenyl and tropolone moieties in common, MTC only lacking the double bridge between them. Stopped-flow experiments with this compound reveal a different thermodynamic behav-

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1 The abbreviations used are: MTC, 2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one; Mes, 4-morpholinethanesulfonic acid; TMA, 3',4',5'-trimethoxyacetophenone; TME, tropolone methyl ether.

T + C \xrightarrow{K} TC \xrightarrow{k_1} TC*

SCHEME 1
ior for the initial low affinity binding (Engelborghs and Fitzgerald, 1986, 1987). Although the initial binding of colchicine (to the fast binding tubulin isoform) is exothermic (\( \Delta H^\circ = -33 \pm 12 \text{ kJ} \cdot \text{mol}^{-1} \)), the initial binding of MTC is almost thermoneutral (\( \Delta H^\circ = -1 \pm 1.6 \text{ kJ} \cdot \text{mol}^{-1} \)). For the slow binding tubulin isoform it is even endothermic (\( \Delta H^\circ = 24 \pm 7 \text{ kJ} \cdot \text{mol}^{-1} \)).

The different behavior of colchicine and MTC seems to question the simple two-site model. Recently a suggestion has been proposed by Andreu et al. (1991) to reconcile these results with the two-site model. The deviating behavior of MTC is suggested to be caused by the (unproven) possibility that MTC may enter its binding site in either of two ways: with either the A- or the C-ring first.

To test further the pathway of colchicine binding, we studied the kinetics of the binding of the two constituent rings (or analogs) and their influence on each other and on the binding of colchicine and MTC.

**MATERIALS AND METHODS**

Microtubule protein was purified from pig brain homogenates by two cycles of temperature-dependent assembly/disassembly according to the method of Shedlanski et al. (1975) and modified as described previously (Engelborghs et al., 1977). Glycerol was added only in the first cycle to increase the yield. This preparation contained about 15% of microtubule-associated proteins. Protein concentrations were estimated by the procedure of Bradford (1976).

Pure tubulin was obtained by phosphocellulose chromatography (Whatman P11) according to Weingarten et al. (1975), and gel filtration chromatography on Sephadex G-25 in Mes buffer. Its purity was checked by sodium dodecyl sulfate electrophoresis. The concentration of pure tubulin-GTPyS and free nucleotide is determined by two-component analysis using the measured absorption at 278 and 255 nm, respectively, allowing the calculation of an activation energy of 37 ± 3 kJ.mol\(^{-1}\) (Engelborghs, 1981). It is closer to the barrier of 58 kJ.mol\(^{-1}\) reported for the initial low affinity binding (Engelborghs and Fitzgerald, 1986, 1987).

MTC was added to a final concentration of 1 mM to prevent polymerization. The buffer was adjusted to 5% (v/v) dimethyl sulfoxide. The MTC concentration was determined spectrophotometrically with an extinction coefficient of 18.8 mM\(^{-1}\) cm\(^{-1}\) at 350 nm (Fitzgerald, 1976). TMA was purchased from Janssen Chimica. MTC was prepared and purified as described previously (Fitzgerald, 1976).

**RESULTS**

**Kinetics of TME Binding to Tubulin**—That the binding of TME to tubulin is very slow was noted by Andreu et al. (1982a). We have studied the binding kinetics by incubating tubulin with TME for increasing amounts of time. After different intervals of incubation with TME, the concentration of remaining free tubulin binding sites was determined from the amplitude of the fluorescence increase observed upon the binding of colchicine. A concentration of 0.6 mM colchicine and 5 \( \mu \text{M} \) tubulin was used and the reaction followed for 450 s to allow for a plateau to develop. At a 0.6 mM colchicine concentration and at 25 °C, the rate constant is 0.03 s\(^{-1}\) for the fast phase and 0.01 s\(^{-1}\) for the slow phase (Lambeir and Engelborghs, 1981). In the experiments presented here the two phases were difficult to resolve, and individual amplitudes were obtained with relative errors. Therefore only the total amplitude was used. Relative amplitudes were calculated with respect to the amplitude obtained in the presence of the same amount of TME but at time zero. In this way inner filter effects caused by TME were automatically taken into account. The decrease of the amplitude of colchicine binding (= loss of free colchicine binding sites) can be fitted to a single exponential curve, as shown in Fig. 1, giving pseudo first-order rate constants for TME binding. From their concentration dependence a bimolecular rate constant of 2.7 ± 0.2 M\(^{-1}\) s\(^{-1}\) at 25 °C can be calculated (Fig. 2). A control experiment performed in the absence of TME showed that no irreversible loss of colchicine binding sites occurred in the time span of the experiments.

These preincubation experiments were repeated at several temperatures; when the logarithm of the rate constants was plotted versus 1/\( T \), a linear Arrhenius plot was obtained which allows the calculation of an activation energy of 37 ± 8 kJ.mol\(^{-1}\) (Fig. 3). This activation energy is quite high, but it is much lower than the barrier of about 100 kJ.mol\(^{-1}\) crossed during the second step of colchicine binding (Lambeir and Engelborghs, 1981). It is closer to the barrier of 58 kJ.mol\(^{-1}\) for MTC binding in the fast phase and has the same value as the amplitude of the fluorescence increase obtained upon the binding of colchicine.

The different experiments were done with pure tubulin in a buffer consisting of 50 mM Mes, 70 mM NaCl, 1 mM MgCl\(_2\), 1 mM EGTA, 1 mM NaN\(_3\), adjusted to pH 6.4 with NaOH. The ion strength of this buffer is 0.1 M. GDP is added to a final concentration of 1 mM to prevent polymerization. The buffer was adjusted to 5% (v/v) dimethyl sulfoxide for solubility reasons of TMA and MTC (also in the case of the appropriate blanks).

Colchicine was purchased from Aldrich Chemical Co. Its concentration was determined spectrophotometrically with \( \varepsilon_{280} = 16.6 \text{ mM}^{-1} \text{cm}^{-1} \). TMA was purchased from Janssen Chimica. MTC was prepared and purified as described previously (Fitzgerald, 1976). Stock solutions of MTC in dimethyl sulfoxide were obtained with an extinction coefficient of 18.8 mM\(^{-1}\) cm\(^{-1}\) at 350 nm (Bane et al., 1984).

TME was prepared by one of us (T. J. F.) as described by Andreu and Timasheff (1982a), and its purity and structure were checked by chromatography and NMR. An extinction coefficient of \( \varepsilon_{280} = 25.9 \text{ mM}^{-1} \text{cm}^{-1} \) was used (Andreu and Timasheff, 1982a).

The binding of colchicine in the presence of TMA and/or TME was studied in a SPEX spectrofluorometer. To reduce the inner filter effects of TMA and especially of TME, the excitation monochromator (SPEX 1861 Minimita 2) was set at 390 nm. The emission monochromator (SPEX 1680 double spectrometer using two grids of 1,200 lines/mm each) was set at 435 nm.

Fast kinetics of the binding were measured in a stopped-flow instrument, especially designed for fluorescence measurements and built in the laboratory. A Hamamatsu superquart mercury-xenon 150-watt arc lamp (L2482) was used. The excitation monochromator was set at 390 nm. The optical pathway is 2 mm and is perpendicular to the flow direction. The light beam, however, is 8 mm wide along the flow direction. Emission was collected over a wide angle so that a large part of the front surface fluorescence was collected as well. A Kodak Wratten filter 2B (cut off at 395 nm) was used in the emission pathway. The dead time of the instrument was determined with the reaction of N-bromosuccinimide with N-acetyltryptophanamide and was found to be about 1.5 ms (Peterman, 1979). All fittings were done with Sigmaplot\textsuperscript{TM}.

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**Fig. 1. Loss of free colchicine binding sites after preincubation of tubulin with TME at 25 °C.** Tubulin at 5 \( \mu \text{M} \) was incubated with 5 mM (O) or 15 mM (O) TME. After a given incubation time (abscissa) colchicine was added at 0.6 mM, and colchicine binding was followed by fluorescence measurements at 25 °C during 450 s. The fluorescence amplitude is a relative measure for the remaining free binding sites and is plotted versus the incubation time of TME. The amplitude decreases with time. The lines are the fitted exponential curves.
concentrations could be fitted with a binding isotherm, giving a binding constant of 60 M\(^{-1}\).

We therefore interpret this extremely slow colchicine binding. (The fast and slow phases of direct colchicine binding, the second increase is extremely slow, and is not observed in the absence of bound TME. It is therefore attributed to the displacement of bound TME. From this phase the dissociation rate constant of TME can be determined. At 25 °C a value of 2.9 (±0.6) × 10\(^{-2}\) min\(^{-1}\) is found.

The barrier of 39 kJ·mol\(^{-1}\) for MTC binding in the slow phase (Engelborghs and Fitzgerald, 1986).

On a much longer time scale (3,600 s instead of 450 s) an additional and extremely slow fluorescence increase appears in the samples that had prebound TME (Fig. 4). At 25 °C the rate constant of this slow phase is 2.9 (±0.6) × 10\(^{-2}\) min\(^{-1}\). This is much too slow to be caused by the second phase of colchicine binding. (The fast and slow phases of direct colchicine binding occur together within the first 450 s.) Moreover, this slow phase appears only with the samples preincubated with TME. We therefore interpret this extremely slow phase as being caused by the displacement of bound TME in the presence of an excess colchicine. The rate constant of this extremely slow phase is therefore the rate constant of TME dissociation. (These experiments were not done at different temperatures.)

Equilibrium Constants for TME Binding—These could be deduced from the determination of the concentration of remaining free sites after sufficiently long incubation times (with TME) to establish the equilibrium. The data at different concentrations could be fitted with a binding isotherm, giving a binding constant of 60 (±7) M\(^{-1}\) at 25 °C (data not shown).

Also from the association and dissociation rate constant at 25 °C an equilibrium value of 94 (±11) M\(^{-1}\) could be calculated.

Kinetics of the Association of TMA to Tubulin—When tubulin is allowed to react with a mixture of TMA and colchicine, a reduced rate constant of colchicine binding is observed as compared with the blank experiment in the absence of TMA (only the fast phase is analyzed). The effect on the amplitude is negligible. This indicates that TMA equilibrates very quickly with tubulin and kinetically inhibits the binding of colchicine. The fractional reduction of the rate constant for colchicine binding can be used as a measure of the saturation with TMA (see Equation 11 in Appendix 1). From the dependence on the concentration of TMA (Fig. 5) a binding constant of 112 (±13) M\(^{-1}\) can be determined. Unfortunately these experiments are limited to concentrations of TMA below 10 mM because of the limited solubility of TMA, and therefore only points below 50% saturation could be determined. When these experiments (at a limited set of concentrations) were repeated at several temperatures (Table I), it turns out that the binding of TMA is almost thermoneutral, within the experimental error.

When tubulin (5 μM) is preincubated with TMA (for 30 min) and then the binding of colchicine is studied, the ampli-
The association rate constant for fast colchicine binding at 0.6 mM colchicine was measured in the absence (k_{blank}) and in the presence (k_{obs}) of different concentrations of TMA and at different temperatures. The ratio k_{obs}/k_{blank} gives the degree of saturation of the binding site and allows the calculation of the association constant.

| Temperature (°C) | [TMA] (mM) | k_{obs}/k_{blank} | k_{o} (M^{-1} s^{-1}) |
|------------------|-----------|-------------------|-----------------------|
| 20               | 4         | 0.78              | 70                    |
| 25               | 4         | 0.76              | 79                    |
| 30               | 8         | 0.59              | 80                    |
| 30               | 4         | 0.79              | 66                    |
| 35               | 10        | 0.61              | 64                    |
| 35               | 4         | 0.73              | 92                    |

Are TMA and TME Binding to Different Sites?—TMA is clearly an analog of the trimethoxybenzene-(A)-ring of colchicine and is therefore supposed to bind to the A-site on tubulin. However, Meurano et al. (1989) studied the binding of a biphenyl analog of colchicine that carries the acetophenon group instead of the tropolone ring. Therefore TMA might also bind to the tropolone site. If the fast binding of TMA would be at the tropolone site, we would expect that TMA would inhibit the binding of TME in the same way as it inhibits colchicine binding. Therefore tubulin was preincubated with TMS and TMA together (for 30 min), and then colchicine was bound (Table II). Clearly the binding of TMA has not decreased the effect of TME. In fact the effects of TMA and TME on the amplitudes were purely cumulative.

\[ \gamma_{TMA+TME} = \gamma_{TMA} \cdot \gamma_{TME} \]  

(Eq. 1)

where \( \gamma \) is the ratio of the amplitude observed in the presence of X, relative to the blank (absence of X). The experimental value of \( \gamma_{TMA+TME} \) is 0.48 (see Table II) is closer to the value of 0.51 for the noncompetitive case, as compared with 0.55 of the competitive case (see Appendix 2). However, the differences are too small to be absolutely conclusive.

Recently it has been shown that the binding of the drug MDL 27048, which has an A-ring in common with colchicine, is slowed down by TMA, whereas TME has no effect on the binding of the drug (Silence et al., 1989). This proves that the fast effect of TMA occurs at a site that is not overlapping with the TME site.

Concentration Dependence of the Kinetics of Colchicine Binding to Tubulin in the Presence of TMA—Using the stopped-flow method, the binding of colchicine was studied at increasing concentrations, in the presence of a fixed concentration of TMA (5 and 10 mM). The evolution of the apparent association rate constant (at 30 °C) as a function of the colchicine concentration is illustrated in Fig. 6. Only the fast phase is considered here. The same effects on the observed rate constants were obtained when tubulin was preincubated with TMA or when TMA was added together with colchicine. From Fig. 6 it is clear that the observed association rate constant is less pronounced at higher colchicine concentrations indicating competition for the first site.

**Figure 6.** Colchicine binding kinetics in the presence of TMA. Observed rate constant (k_{obs} in s^{-1}) of the fast phase of colchicine binding, as a function of colchicine (C) concentration, in the absence (0) and the presence (V) of 5 mM TMA and (C) 19 mM TMA. (Tubulin concentration was 10 μM). Note that the relative reduction of the observed rate constant is less pronounced at higher colchicine concentrations indicating competition for the first site.

**DISCUSSION**

Colchicine binding to tubulin is described by a two-step mechanism: a fast preequilibrium is followed by a slow conformational change in the initial complex. A bifunctional ligand model was formulated by Andreu and Timasheff (1982a, 1982b). This model specifies that the initial complex formation is competitively inhibited by TMA. Similar results are obtained using TMC instead of colchicine. These curves clearly indicate that the fast phase of colchicine is certainly not reduced and that TMA acts like a substance of type A, which competes for the fast binding of colchicine at its initial site (see Appendix 2).
Kinetics of Colchicine Binding to Tubulin

Timasheff used the gel chromatography technique of Hummel and Dreyer (1962), and therefore made a direct binding study. Here an indirect fluorescence titration is performed.

The molecule TMA behaves rather differently. It reduces the apparent rate constant for the binding of colchicine or MTC, indicating that a fast and competitive equilibration is taking place. After preincubation of tubulin with TMA, it becomes clear that a slowly exchanging state is also being formed. TMA shows therefore a behavior that is reminiscent of colchicine itself: fast binding followed by a switch to a slow exchanging state.

That TMA and TME act at different sites is proven by the fact that the binding of the drug MDL 27048, which has an A-type methoxybenzene ring, is slowed down by TMA and not influenced at all by TME (Silence et al., 1993).

The fast inhibition by TMA of the colchicine binding kinetics depends on the colchicine concentration in a way that is indicative for a fast competition with the first step of colchicine binding only (see Appendix 1).

The results obtained in this study strongly suggest that colchicine binds first through the trimethoxybenzene ring and subsequently through the tropolone ring. The fact that TMA binds very quickly while TME binds very slowly already is a strong indication that fast binding of TMA resembles better the initial fast equilibration of tubulin with colchicine.

Also the binding constant of TMA (112 M<sup>-1</sup> at 25 °C) is of the same order of magnitude as the values found for the first binding of colchicine (220 M<sup>-1</sup>) and MTC (273 M<sup>-1</sup>) determined previously (Lambeir and Engelborghs, 1981; Engelborghs and Fitzgerald, 1986, 1987).

The slow binding kinetics of the single TME ring is very remarkable. In fact colchicine binds even faster than the single ring TME, although the activation enthalpy change ΔH<sub>f</sub> is smaller for TME (37 ± 8 kJ·mol<sup>-1</sup>) than for colchicine (100 kJ·mol<sup>-1</sup>). For MTC binding the activation enthalpy changes are more comparable to TME: 58 kJ·mol<sup>-1</sup> for the fast phase and 39 for the slow phase. This increasing activation enthalpy change probably reflects an increasing amount of structural deformation necessary for binding into the C-site. The fact that MTC and even colchicine bind faster, despite the higher activation enthalpy, clearly proves their entropic advantage as compared with TME, partly because of the initial low affinity binding of colchicine and MTC through the trimethoxybenzene moiety.

The similarity of the thermodynamic parameters of TME binding and of the initial binding of colchicine now becomes very intriguing. Equally intriguing is the difference between the thermodynamic parameters of N-acetylmescalin binding and the initial binding of colchicine (Andreu and Timasheff, 1982a; Lambeir and Engelborghs, 1981). In our opinion, these differences indicate that the orientation of a constituent ring of colchicine in its binding site on tubulin is different when it binds as a single ring or as a part of colchicine. An indication for this hypothesis could be that TME binding does not lead to tubulin fluorescence quenching, whereas its extinction coefficient at 350 nm is still 42% of that of colchicine. It should also be taken into account that equilibrium measurements are always made on the mixture of the different tubulin isoforms, which have different thermodynamic parameters as shown by the kinetic measurements.

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Appendix 1

Kinetic Equations for Competition of Constituent Rings of Colchicine for the First or the Second Subsite

Taking into account the fact that colchicine binds almost irreversibly to tubulin within the measuring time (K<sub>1,2</sub> being of the order of 10<sup>-4</sup> s<sup>-1</sup>), the rate equation for the binding can be expressed as

\[ \frac{d[T C^*]}{dt} = k_2 \cdot [T C] \]  
(Eq. 2)

with

\[ [T C] = ([T]<sub>tot</sub> - [T C*]) \cdot K<sub>1</sub>[C]/(1 + K<sub>1</sub>[C]) \]  
(Eq. 3)

where [T]<sub>tot</sub> is the total concentration of tubulin in the solution. Therefore the observed rate constant is as follows.

\[ k_{obs} = k_2 \cdot K<sub>1</sub>[C]/(1 + K<sub>1</sub>[C]) \]  
(Eq. 4)

Binding colchicine to tubulin in the presence of a molecule (B) that equilibrates rapidly with the second subsite and does not interfere with the initial binding of colchicine can be represented by Scheme 2.

\[ T + C \rightarrow K_1 \rightarrow T C^* + B \]  
(Eq. 5)

with

\[ [T C] = ([T]<sub>tot</sub> - [T C*]) \cdot K<sub>1</sub>[C]/(1 + K<sub>1</sub>[C]) \]  
(Eq. 6)

and

\[ k_{obs} = k_2 \cdot K<sub>1</sub>[C]/(1 + K<sub>1</sub>[C])((1 + K<sub>1</sub>[B]) \]  
(Eq. 7)

In contrast, when a molecule (A) rapidly and reversibly competes with the initial tubulin-colchicine complex formation, its influence can be described with Scheme 3.

\[ T + A + C \rightarrow K_1 \rightarrow TC + A^* \rightarrow k_2 \rightarrow TC^* + A \]  
(Eq. 8)

with

\[ [T C] = ([T]<sub>tot</sub> - [T C*]) \cdot K<sub>1</sub>[C]/(1 + K<sub>1</sub>[C] + K<sub>4</sub>[A]) \]  
(Eq. 9)

and

\[ k_{obs} = k_2 \cdot K<sub>1</sub>[C]/(1 + K<sub>1</sub>[C] + K<sub>4</sub>[A]) \]  
(Eq. 10)

Note that the effect of A decreases with an increasing concentration of C, whereas the effect of B is independent of C.

At low colchicine concentrations, when K<sub>1</sub>[C] << 1, both situations reduce to the same formula.

\[ k_{obs} = k_2 \cdot K<sub>1</sub>[C]/(1 + K<sub>1</sub>[X]) \]  
(Eq. 11)

where X is either A or B.
After preincubation of tubulin with TMA or TME (separately) the remaining amplitude of colchicine binding is the amplitude of the blank multiplied with the following factor.

\[ \gamma_{\text{TMA}} = \frac{1}{1 + K_{\text{TMA}} \cdot [\text{TMA}]} \]  

(Eq. 12)

and

\[ \gamma_{\text{TME}} = \frac{1}{1 + K_{\text{TME}} \cdot [\text{TME}]} \]  

(Eq. 13)

Experimentally it is observed that after preincubation of tubulin with TMA and TME together, the remaining amplitude is the following product.

\[ \gamma_{\text{TMA+TME}} = \gamma_{\text{TMA}} \cdot \gamma_{\text{TME}} \]  

(Eq. 14)

This can be worked out.

\[ \gamma_{\text{TMA+TME}} = \frac{1}{(1 + K_{\text{TMA}}[\text{TMA}] + K_{\text{TME}}[\text{TME}] + K_{\text{TMA}}K_{\text{TME}}[\text{TMA}][\text{TME}])} \]  

(Eq. 15)

The denominator of this fraction is clearly the partition function for noncompetitive binding to two independent sites. For a competitive binding the denominator would be as follows.

\[ 1 + K_{\text{TMA}}[\text{TMA}] + K_{\text{TME}}[\text{TME}] \]  

(Eq. 16)

In that case one can easily calculate the following.

\[ \gamma_{\text{TMA+TME}} = \frac{\gamma_{\text{TMA}} + \gamma_{\text{TME}} - 1}{-1} \]  

(Eq. 17)

Applying these formulas to the data of Table II shows that the calculated value of \( \gamma_{\text{TMA+TME}} \) would be 0.55 for the competitive and 0.51 for the noncompetitive case.

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