Differential Association of T-2 and T-2 Tetraol with Mammalian Cells

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ABSTRACT

The interactions of T-2 and its metabolite T-2 tetraol (hereafter tetraol) with CHO (Chinese hamster ovary cells) and CHO ribosomes were studied. T-2 was about 300-fold more potent at inhibiting protein synthesis in CHO than was tetraol. Association of T-2 with CHO was highly specific and achieved a maximum at a concentration producing complete inhibition of protein synthesis. Association of tetraol with CHO was of low specificity, but the specific fraction did correlate with the dose-response curve for protein synthesis inhibition. Binding of both T-2 and tetraol to isolated CHO ribosomes was quantitatively similar and highly specific. With isolated ribosomes, each toxin competed effectively for the binding of the other. Using intact cells, tetraol competed for T-2 cell association, but not the converse. The kinetics at physiological temperature for total and specific T-2 cell association were much more rapid than those for tetraol. Furthermore, the rate of tetraol-cell association was indistinguishable from the rate for cellular uptake of tritiated water. At 0°C, there was a substantial association of T-2 with cells, whereas none was observed with tetraol. The kinetics of dissociation of both toxins from CHO were similar. We conclude that T-2 rapidly crosses the cell membrane of cells and binds to the intracellular target, the ribosomes. In contrast, tetraol is taken up by the cell much more slowly, and many more toxin molecules are found in the cell than there are ribosomes. It would appear that the main physical property of the toxins that brings about these results is the relative hydrophobicities of the molecules. Thus, we suggest that one factor determining the potency of a trichothecene toxin may be its lipophilicity.

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ABBREVIATIONS: CHO, Chinese hamster ovary cells; tetraol, T-2 tetraol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HBSS, Hanks’ balanced salt solution.
CCI-61). Cells were maintained in 75-cm² T-flasks (Costar no. 3075) with Earle's minimal essential medium, 10% fetal bovine serum and 50 μg/ml of gentamicin.

**Media and sera.** All media, vitamins, antibiotics and amino acids were obtained from Grand Island Biological Co. (Grand Island, NY). Fetal calf serum was obtained from Armour Pharmaceutical (Kankakee, IL).

**Toxins.** T-2 and tetrool were purchased from Calbiochem (La Jolla, CA); the other trichothecene toxins were obtained from Sigma Chemical Co. (St. Louis, MO). T-2 was tritium-labeled by New England Nuclear (Boston, MA) or Amersham/Searle (Arlington Heights, IL) using a previously published procedure (Wallace et al., 1977). The toxin preparations had specific activities from 9.0 to 14.0 Ci/mmole and were equipotent to unlabeled T-2 in a protein synthesis inhibition assay. Radiolabeled tetrool was prepared from [³H]T-2 by a published procedure (Wei and Chu, 1985) and was the generous gift of Dr. R. Wannemacher, Jr. (U.S. Army Medical Research Institute of Infectious Diseases, Frederick, MD). In many cases, it was necessary to cut the radiolabeled tetrool to achieve desired molar concentrations so the specific activity varied. All toxins were dissolved in methanol and diluted so that the maximal alcohol concentration exposure to cells was 0.1%. This concentration of alcohol had no detectable effect on toxin-cell association.

**Toxin-cell association assay.** Cells were seeded in 24-well tissue culture plates. On the day of experimentation (1 × 10⁶ cells/well), the growth medium was replaced with 0.5 ml of Hanks’ 199 supplemented with 10% fetal calf serum, 50 μg/ml of gentamicin and 25 mM HEPES, pH 7.4 (complete H-199). Further incubations and manipulations at 37°C were carried out on top of a microscope slide warmer in a warmbox (both equilibrated to 37°C). This arrangement allowed much better temperature control than did a standard CO₂ incubator. Radiolabeled T-2 was added to the cells in 50-μl volumes and incubation carried out under the conditions and for the times stipulated. To determine cell-associated toxins, cells were rinsed three times with HBSS and solubilized in 1.0 ml of 0.1 M NaOH. A 0.5-ml aliquot was added to a scintillation vial with 0.1 ml of 0.1 M HCl and 5.0 ml of Aquasol 2 (New England Nuclear). The sample was then counted in a Beckman 5801 liquid scintillation spectrophotometer.

**Protein synthesis assay.** Cells in complete H-199 were incubated with toxin for the times and under the conditions indicated. Protein synthesis was measured by the addition of 1 μCi/well of [³H]leucine (New England Nuclear, 110-150 Ci/mmol) and incubation at 37°C, usually for 30 min. The pulse was terminated by rinsing the cells twice with HBSS and adding 0.10 ml of 0.1 M NaOH. After 5 to 10 min at 37°C, a prenumbered 11-mm disc (Schleicher and Schuell, 740E) was added to each well to absorb the solubilized cells. Each disc was then transferred to a bottle of 10% trichloroacetic acid and the samples were processed in mass as follows: two rinses with 5% trichloroacetic acid, two rinses with 50:50 ethanol:aceticone and one rinse with acetone. After drying, each disc was assayed for radioactivity in 2.0 ml of Liqua-scint (New England Nuclear).

**Purification of ribosomes.** Ribosomes from CHO were purified as described by Gupta and Siminovitch (1976).

**Toxin-ribosome binding assay.** A previously described filter assay was used (Middlebrook and Leatherman, 1989b). Usually, 10 to 20 μl of toxin(s) were added to 100 μl of a 10 OD₅₅₀/ml ribosomal suspension in buffer D (20 mM HEPES, pH 7.5; 120 mM KCl; 1.5 mM Mg acetate; 6 mM 2-mercaptoethanol) and incubation was carried out under the conditions stipulated. Binding was terminated by addition of the entire sample to a Whatman GF/F glass filter fiber, followed by four 4-ml washes. Each filter was then counted with Aquasol 2 in a liquid scintillation spectrophotometer.

**Sucrose density gradient analysis of polyribosome profiles.** A modification of the procedure described by Cundliffe et al. (1974). CHO in T-75 culture flasks were incubated to equilibrium at 37°C with site-saturating concentrations of labeled toxins (0.1 μg/ml of T-2 toxin or 10 μg/ml of tetrool). After washing four times in ice-cold HBSS, the cells were scraped into TMNa buffer (10 mM Tris-Cl, pH 7.4; 15 mM MgCl₂; 140 mM NaCl) and pelleted at 1000 × g for 10 min at 2°C. Cell pellets were lysed in TMNa buffer (on ice) containing 0.5% Nonidet P-40 (Sigma) and 200 μl of lyase was layered onto 4.5-ml, 10 to 30% continuous sucrose gradients prepared in TMNa buffer. The gradients were centrifuged at 42,000 rpm in an SW 50.1 rotor (Beckman) for 2.25 hr at 2°C. Under these conditions, the 40S and 60S subunits, and the 80S free ribosomes were simultaneously resolved and most of the polyribosomal material was pelleted. After centrifugation, the gradients were fractionated from the top with a Buchler Auto-Densi-Flow II gradient collector (Searle) pumping at a rate of 0.6 ml/min with a chart speed of 30 mm/min. Absorbance at 254 nm was monitored continuously through an LKB 2238 Uvicord S II UV spectrophotometer. The polyribosome pellet was resuspended in TMNa buffer and a portion of this pellet was used to determine polyosome absorbance. The remainder of the pellet and the gradient fractions were counted in a liquid scintillation counter using Aquasol 2 to determine polyribosome-bound toxin.

**Results**

Comparative dose/response curves for the inhibition of CHO protein synthesis by T-2 and tetrool are shown in figure 1. After 3 hr incubation with cells, protein synthesis was inhibited 50% by 0.003 μg/ml (6.2 nM) T-2. Tetrool also inhibited protein synthesis in CHO, but the curve was shifted to the right almost three orders of magnitude from that for T-2. It required 1 μg/ml (3.4 μM) to obtain a 50% inhibitory effect. Other than their positions, the two curves were similar. Both toxins induced a complete inhibition of protein synthesis and therefore appear to be full agonists with different potencies.

Binding isotherms for T-2 and tetrool at physiological temperature are depicted in figure 2. We observed binding of T-2 to CHO at much lower concentrations than for tetrool. In agreement with our previous work (Middlebrook and Leatherman, 1989a), we obtained an S-shaped curve with the midpoint at about 0.008 μg/ml (17 nM). Saturation was seen at a con-
that at concentrations examined in these studies, the binding of the two toxins was not substantially different. At a given concentration of toxin, there was a somewhat higher level of radiolabeled T-2 than tetraol association with CHO ribosomes. However, the ratio of T-2:tetraol ribosome binding decreased as the concentrations increased and an analysis of the data by the method of Scatchard (1949) indicated a slightly lower affinity of tetraol for the ribosome with the same apparent number of sites (data not shown). In marked contrast to the association of tetraol with cells, excess unlabeled tetraol competed completely for the binding of radiolabeled tetraol to ribosomes (fig. 3), demonstrating a specific interaction.

As another measure of toxin-ribosome binding, we examined the ability of T-2 and tetraol to compete with one another. Competition by the homologous and heterologous toxins was essentially the same, suggesting that the ribosomal binding affinities of the two toxins are close (data not shown). These results probably indicate that T-2 and tetraol bind to the same sites on the ribosome, although allosteric effects of one toxin on binding of the other cannot be ruled out.

Cross competition experiments performed with intact CHO produced quite different results (fig. 4). In each case, we used radiolabeled toxin concentrations which were just saturating (fig. 2B). Although it was low, we calculated the level of specific tetraol-CHO association and compared the data with specific T-2-CHO association (fig. 1). When the resulting curves were plotted with the toxins' inhibition of protein synthesis, the specific binding and the pharmacological effects correlated closely (fig. 1).

Because the protein synthesis inhibitory effects of T-2 are due to toxin-ribosome binding (Barbacid and Vasquez, 1974; Wei et al., 1974) we compared the binding of T-2 and tetraol to CHO ribosomes (fig. 3). Surprisingly, the binding of the two toxins was not substantially different. At a given concentration of toxin, there was a somewhat higher level of radiolabeled T-2 than tetraol association with CHO ribosomes. However, the ratio of T-2:tetraol ribosome binding decreased as the concentrations increased and an analysis of the data by the method of Scatchard (1949) indicated a slightly lower affinity of tetraol for the ribosome with the same apparent number of sites (data not shown). In marked contrast to the association of tetraol with cells, excess unlabeled tetraol competed completely for the binding of radiolabeled tetraol to ribosomes (fig. 3), demonstrating a specific interaction.

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the association of T-2 with cells was much more rapid than proportionately the same final specific activity in the medium. It is evident that with cells at tetraol were compared with the cellular uptake of tritiated water cells was much higher than the specific association, a pattern of cross competition, which was not increased by concentrations producing cross competition. In contrast, neither T-2 nor tetraol competed effectively served when compared to the data for T-2. First, many more concentrations producing cross competition, were used to determine the data for T-2. Second, and the data for T-2. Second, and the data for T-2. Third, the total association of tetraol with cells at 0°C or 4°C compared to 37°C was much lower proportionately than the ratios seen with T-2. That is, with T-

Fig. 4. T-2 and tetraol cross competition in CHO. A: T-2 toxin and tetraol competition for [3H]T-2 association with CHO; CHO were incubated at 37°C with 0.1 μg/ml of [3H]T-2 toxin and the indicated concentrations of unlabeled T-2 toxin (C) or unlabeled tetraol (Θ). After 3 hr, the cells were processed for cell-associated toxin as described under “Methods.” T-2 toxin and tetraol competition for [3H]tetraol association with CHO; CHO were incubated at 37°C with 10 μg/ml of [3H]tetraol and the indicated concentrations of unlabeled tetraol (A) or unlabeled T-2 toxin (Δ). After 3 hr of incubation with toxins, the cells were processed for cell-associated toxin as described under “Methods.” Data points represent the means of triplicate determinations with S.E.M.

Fig. 5. Comparative kinetics for the association of T-2 toxin, tetraol, and H2O with CHO. CHO were incubated at 37°C with 0.35 μg/ml of [3H] tetraol (Θ), 0.35 μg/ml of [3H]T-2 toxin (A) or [3H]H2O (C), each at 10 μCi/ml. After the indicated incubation times at 37°C, the cells were processed for cell-associated radioactivity as described under “Methods.” Data points are the averages of duplicate determinations.

that of either tetraol or water. The zero time point with T-2, which consisted of adding toxin to the medium and immediately washing the cells, produced more specifically cell-bound counts than a 90-min incubation with a like concentration of tetraol. A maximal, steady-state binding of T-2 was achieved in approximately 10 min, and was maintained throughout the course of the experiment (fig. 5). On the other hand, tetraol-cell binding reached a steady state in about 1 hr, but it was only 2.2% of the T-2 binding plateau. Furthermore, the cell binding kinetic curve for tetraol was not significantly different from that obtained with tritiated water, which we used as a measure of fluid phase pinocytosis.

If association of T-2 and tetraol with CHO was studied when toxins were added at concentrations which produce 90 to 95% inhibition of protein synthesis, additional and important information was brought to light. Total and specific binding of T-2 to CHO at physiological temperature rapidly attained plateau levels which were quantitatively close to one another, 3.0 vs. 2.5 million toxin molecules/cell (fig. 6). If association was carried out at either 4°C or 0°C, a longer time was required to reach the apparent plateau for both total and specific binding (fig. 6). Furthermore, the plateau level was about half that attained at 37°C (fig. 6), although extending the incubation at 4°C (but not 0°C) to 24 hr resulted in an increase in the binding to 2.5 million molecules bound per cell (data not shown).

Association of pharmacologically equipotent (much higher molar) concentrations of tetraol with CHO is demonstrated by the data in figure 7. Several important differences were observed when compared to the data for T-2. First, many more molecules of tetraol bound to (or were taken up by) CHO than with T-2, viz., 20 vs. 3 million (compare fig. 7A and 6A). Second, at physiological temperature, it required 2 to 3 hr to reach maximal total and specific binding of tetraol compared to 0.5 hr for T-2. In addition, the total association of tetraol with cells was much higher than the specific association, a pattern opposite that for T-2. Third, the total association of tetraol with cells at 0°C or 4°C compared to 37°C was much lower proportionately than the ratios seen with T-2. That is, with T-

Fig. 6. Comparative kinetics for the association of T-2 toxin, tetraol, and H2O with CHO. CHO were incubated at 37°C with 0.35 μg/ml of [3H] tetraol (Θ), 0.35 μg/ml of [3H]T-2 toxin (A) or [3H]H2O (C), each at 10 μCi/ml. After the indicated incubation times at 37°C, the cells were processed for cell-associated radioactivity as described under “Methods.” Data points are the averages of duplicate determinations.

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Fig. 8. Comparative kinetics for the association of T-2 toxin, tetraol, and H2O with CHO. CHO were incubated at 37°C with 0.35 μg/ml of [3H] tetraol (Θ), 0.35 μg/ml of [3H]T-2 toxin (A) or [3H]H2O (C), each at 10 μCi/ml. After the indicated incubation times at 37°C, the cells were processed for cell-associated radioactivity as described under “Methods.” Data points are the averages of duplicate determinations.

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Fig. 9. Comparative kinetics for the association of T-2 toxin, tetraol, and H2O with CHO. CHO were incubated at 37°C with 0.35 μg/ml of [3H] tetraol (Θ), 0.35 μg/ml of [3H]T-2 toxin (A) or [3H]H2O (C), each at 10 μCi/ml. After the indicated incubation times at 37°C, the cells were processed for cell-associated radioactivity as described under “Methods.” Data points are the averages of duplicate determinations.

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Fig. 10. Comparative kinetics for the association of T-2 toxin, tetraol, and H2O with CHO. CHO were incubated at 37°C with 0.35 μg/ml of [3H] tetraol (Θ), 0.35 μg/ml of [3H]T-2 toxin (A) or [3H]H2O (C), each at 10 μCi/ml. After the indicated incubation times at 37°C, the cells were processed for cell-associated radioactivity as described under “Methods.” Data points are the averages of duplicate determinations.

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Fig. 11. Comparative kinetics for the association of T-2 toxin, tetraol, and H2O with CHO. CHO were incubated at 37°C with 0.35 μg/ml of [3H] tetraol (Θ), 0.35 μg/ml of [3H]T-2 toxin (A) or [3H]H2O (C), each at 10 μCi/ml. After the indicated incubation times at 37°C, the cells were processed for cell-associated radioactivity as described under “Methods.” Data points are the averages of duplicate determinations.
Fig. 6. Effect of temperature on the total and specific association of T-2 toxin with CHO. A: Total association: [\(^{3}H\)]T-2 toxin (0.1 \(\mu\)g/ml) was incubated with CHO for the indicated times at 0\(^{\circ}\)C (\(\bigtriangleup\)), 4\(^{\circ}\)C (\(\bigtriangleup\)) or 37\(^{\circ}\)C (\(\bigtriangleup\)). The cells were then processed for cell-associated toxin as described under *Methods.* B: Specific association: the specific association of T-2 toxin with CHO at 0\(^{\circ}\)C (\(\bigtriangleup\)), 4\(^{\circ}\)C (\(\bigtriangleup\)) and 37\(^{\circ}\)C (\(\bigtriangleup\)) has been determined from the data in A by subtraction of the nonspecific binding measured in the presence of a 75-fold molar excess of unlabeled toxin. Data points for total association are the means of triplicate determinations with S.E.M.

In an attempt to compare the subcellular locations of T-2 and tetraol, we bound cells with radiolabeled toxins, washed and lysed the cells and centrifuged the samples on sucrose gradients. The profiles of radioactivity seen with T-2-bound cells coincided with the absorbance peaks defining the location of ribosomes and polysomes (fig. 8A). The ribosome-associated radioactivity measured as a function of time. Clearly, the rates of T-2- and tetraol-CHO dissociation were similar (fig. 8). When analyzed by a semilogarithmic plot (fig. 8, inset), both curves in figure 8 fit straight lines (correlation coefficients of 0.93 and 0.98 for T-2 and tetraol, respectively), whose equations gave calculated half-times of 3.3 and 3.7 hr for T-2 and tetraol, respectively.

2 at both lower temperatures, there was about 50% of the total association seen at 37\(^{\circ}\)C (fig. 6A). In contrast, with tetraol there was only 6 to 9% (4\(^{\circ}\)C) or 2 to 3% (0\(^{\circ}\)C) of the association at physiological temperature (fig. 7A). This temperature differential with T-2 and tetraol was even more pronounced when the specific association was considered. Once again, with T-2, the specific association at either 0\(^{\circ}\) or 4\(^{\circ}\)C (4–8 hr) was approximately 50% of the specific association at 37\(^{\circ}\)C (fig. 6B). Statistically, there was no specific association of tetraol with CHO at 0\(^{\circ}\)C, whereas we did observe specific association at 4\(^{\circ}\)C, approximately 60 to 70% that at 37\(^{\circ}\)C (fig. 7B).

The dissociation kinetics for cell-associated toxins are shown in figure 8. Cells were preincubated with concentrations of each toxin that produced similar pharmacological effects, i.e., 0.1 \(\mu\)g/ml of T-2 and 10 \(\mu\)g/ml of tetraol. Cells were then washed and
activity profile for tetraol-bound cells (total 15,000 ± 160 dpm) showed that most of the tetraol was on the top of the gradient with only a small fraction (2600 ± 250 dpm or 17%) in the ribosome/polysome region (fig. 9B). Thus it appears that virtually all the T-2 associated with cells was ribosome-bound, whereas a majority of cell-associated tetraol was free in the cytoplasm or bound to a very small organelle, vesicle or protein.

As a general rule, metabolizing systems of animals produce metabolites that are more polar than the parent compound. As a simple measure of the polarities of T-2 and tetraol, we measured their distribution coefficients between an aqueous buffer and benzene. The results indicated that T-2 and tetraol have diametrically opposed distribution coefficients. Although T-2 showed a preference for a nonpolar solvent by a ratio of 54:1, tetraol had a similar ratio of 1:270. If the distribution coefficients for that solvent system can be used as an estimate of lipophilicity, T-2 should be 14,000 times more lipophilic.

Discussion

Trichothecene toxins are among the most potent nonprotein inhibitors of protein synthesis in eukaryotic cell systems. A good deal of evidence points toward toxin binding to ribosomes as the molecular event which brings about this inhibition. There is a large range of inhibitory potencies in the trichothecene family of toxins. Delineating the bases for this variation in potencies should provide insights into the mechanism of action of these toxins with cells and possibly animals.
There was almost three orders of magnitude difference between T-2 and its metabolite, tetraol, at inducing protein synthesis inhibition in CHO (fig. 1). The molecular basis for this potency difference does not appear to exist at the level of toxin-ribosome binding because: 1) there was only a 2- to 5-fold difference in cell-free ribosomal binding of the two toxins (fig. 3) and 2) competition experiments strongly suggest that the two toxins bind to a common ribosomal site (data not shown). Therefore, we focused on interactions with the cell as the probable cause for the large potency difference.

The data presented in figures 1, 4, 6 and 7 established five important features of tetraol-cell vs. T-2-cell association. First, there was no appreciable binding of tetraol to CHO at concentrations of toxin that produced maximal cell binding with T-2 (fig. 1). Second, whatever the extracellular (exposure) concentration of toxin, similar levels of specifically bound T-2 or tetraol produced the same pharmacological response (fig. 1). Third, both T-2 and tetraol competed completely for radiolabeled T-2 binding to cells, the latter being about 200-fold less effective (fig. 4A), whereas neither T-2 or tetraol competed for more than about 15% of radiolabeled tetraol cell binding (with a reversal in competitive potencies) (fig. 4B). Fourth, at maximally effective pharmacological concentrations, the kinetics of total T-2 cell binding quickly reached a plateau (fig. 6A), whereas the kinetics for total tetraol cell binding were much slower (relatively speaking) and did not appear to plateau (fig. 7A). Finally, the kinetics of release or out transport of T-2 and tetraol were, within experimental error, identical.

Based on the above features, we propose the following explanation or model to account for the differences in toxicity between T-2 and tetraol: T-2 diffuses across the cell membrane readily and, once inside the cell, binds rapidly to ribosomes. Thus, cell-associated T-2 is really a measure of ribosomally bound T-2 inside a limiting sack, e.g., the plasma membrane. This model is supported by our previous studies with T-2 and cells (Middlebrook and Leatherman, 1989a,b) and by the data in figure 9 showing the ribosome profiles. Tetraol, on the other hand, is unable to cross the cell membrane by diffusion, possibly because of its hydrophilic nature. Thus, tetraol is taken inside the cell by a much slower, nonsaturable process similar, if not identical, to fluid-phase pinocytosis. Once inside the cell, tetraol escapes the pinocytic vesicles or lysosomes, binds to ribosomes and brings about inhibition of protein synthesis.

With such a model in mind, one can make certain predictions which appear to be borne out by our data. First, one would expect the rate of tetraol uptake to be the same as the uptake rate of other pinocytotically accumulated substances such as water or inulin. Indeed, as the data in figure 5 show, the kinetics of water and tetraol uptake were essentially the same. Second, the rate of uptake for tetraol should be much more temperature-dependent than that for T-2. Both the total uptake and specific binding of T-2 at 0° and 4°C were about 50% of those seen at physio-logical temperature (fig. 6). In contrast, the total uptake of tetraol at the lower temperatures was 10 to 15% of the 37°C uptake, whereas the specific uptake at 0° was statistically zero (fig. 7). There was a substantial specific uptake of tetraol at 4°C compared to physiological temperature, although it seemed to take much longer to attain that level (fig. 7). Finally, fluid-phase pinocytosis is a nonsaturable process so one would anticipate that the uptake of tetraol would be much more nonspecific than that for T-2, a prediction reflected by the data in figures 2, 4, 6 and 7.

It is clear that trichothecene toxins can differ markedly in their affinities for ribosomes (Wei et al., 1974) and that factor alone can account for some differences in their potencies, even if cellular uptake were similar. Sato and Ueno (1977) presented data which suggested that another factor determining toxicity for cells is the lipophilicity of individual trichothecenes. Without radiolabeled toxins, those workers were unable to define the molecular events underlying the observation that lipophilicity and toxicity are positively related. Although carried out with only two toxins, our experiments may indicate that the basis of the lipophilicity-toxicity correlation observed by Sato and Ueno (1977) is toxin entry into cells. It may be that differential uptake by target cells is an important, toxicity-determining property of other trichothecene toxins, but experiments with more trichothecenes would be required to state this as a general rule. However, it is probable that either ribosome affinity or lipophilicity could be the major potency determining factor for any given trichothecene or, in certain cases, both properties could contribute. Furthermore, the relationship between toxin-induced protein synthesis in cultured cells and toxin-induced lethality in animals is not clearly evident, so extrapolation to animal toxicity is not appropriate at this time.

References


