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Antimicrobial Activity of Tryptanthrins in *Escherichia coli*

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Tryptanthrins have potential therapeutic activity against a wide variety of pathogenic organisms, although little is known about their mechanism. Activity against *Escherichia coli*, however, has not been examined. The effects of tryptanthrin (indolo[2,1-*b*]quinazolin-6,12-dione) and nine derivatives on growth, survival, and mutagenesis in *E. coli* were examined. Analogues with a nitrogen atom at the 4-position of tryptanthrin stopped log phase growth of *E. coli* cultures at concentrations as low as 5 μM. Tryptanthrins decreased viability during incubation with cells in buffer by factors of 10^{-2} to $< 10^{-6}$ at 0.2−40 μM. Derivatives with an oxime group at the 6-position exhibited the greatest bactericidal activity. Most tryptanthrins were not mutagenic in several independent assays, although the 4-aza and 4 aza-8-fluoro derivatives increased frameshift mutations about 22- and 4-fold, respectively. Given the structure of trypanthrins, binding to DNA may occur by intercalation. From analysis using a sensitive linking number assay, several tryptanthrins, especially the 4-aza and 6-oximo derivatives, intercalate into DNA.

Introduction

Indolo[2,1-*b*]quinazolin-6,12-dione (tryptanthrin, Figure 1) and its analogues have generated interest as a class of potential therapeutic agents because of their ease of synthesis, stability, and broad spectrum of activity against pathogenic organisms. Historically, plant extracts containing this alkaloid have been used as a remedy for athlete's foot[1] and as anti-inflammatory and fever-reducing agents.[2] Tryptanthrins possess antibacterial activity against *Bacillus subtilis*[3] and *Mycobacterium tuberculosis*;[4,5] antifungal activity against various *Trichophyton*, *Microsporum*, and *Epidermophyron* species;[1] and activity against growth of *Leishmania donovani*,[6] *Trypanosoma brucei*,[7] and *Plasmodium falciparum*.[8,9] In mammalian cells, tryptanthrins are reported to possess cytotoxic activity against many cancer cell lines.[10] They can decrease the activity of cyclooxygenase-2 (COX-2)[11,12] and inhibit the expression of nitric oxide synthase and prostaglandin E(2) in cells.[13] Hepatocyte growth factor in human fibroblasts[14] and the multidrug resistance gene *MDR1* in breast cancer cells[15] are inhibited by tryptanthrins. In mice, interleukin-2 and interferon-γ have been reported to be inhibited by treatment with these compounds,[2,16] and in certain systems tryptanthrins may possess antitumor activity.[17,18]

Although IC_{50} values into the low ng/mL range have been demonstrated against a wide variety of pathogenic organisms, very little insight has been gained into the mechanism(s) of action of tryptanthrins at the cellular and molecular levels. To our knowledge, the only published hypothesis of any detail concerning tryptanthrins' mechanism of action involved hemin and hemozoin binding as a factor in antimalarial activity.[19−21] However, this would not account for the toxicity toward other organisms that cause tuberculosis,[4] trypanosomiasis,[7] and leishmania[6] or the observed antibacterial or antifungal activity.[1,22] In the case of inhibition of *MRD1*, tryptanthrin may act by down-regulating gene expression.[15] From the very diverse collection of target prokaryotic and eukaryotic organisms and the wide range of reported biological effects, tryptanthrins likely act via multiple biochemical pathways.

With regard to mechanisms of toxicity from a broad perspective, it is not currently known if tryptanthrins act as mutagens or inhibitors of key metabolic pathway(s) or if they act physically, such as by disrupting membranes. It is also unclear if tryptanthrins act as static agents, preventing growth and replication, or if they can act as direct lethal agents. A priori, the planar structures of tryptanthrins suggest that they could bind to DNA by intercalation, or they may form covalent adducts to nucleobases. Either action could lead to mutagenesis or the inhibition of DNA synthesis, RNA synthesis, or gene expression. To begin to address the broad issue of how tryptanthrins might affect survival and mutagenesis, we have examined their effects in *E. coli*, with which no prior reports are available. *E. coli* is a well-established model for the study of xenobiotics on genetic material that can be extrapolated to other organisms. The genetic tools available for *E. coli* should facilitate future mechanistic studies of tryptanthrin action. Presented herein is the first analysis of the effects of tryptanthrin and several analogues (Figure 1) on the growth, survival, and mutagenesis of *E. coli*. In addition, we demonstrate that certain tryptanthrins alter the twist of the DNA helix consistent with their binding to DNA by intercalation.

Results

Effect of Tryptanthrins on Growth of *E. coli* and Viability in LB Media. *E. coli* strain AS19 was grown in LB[a] media at 37 °C

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[a] Abbreviations: LB, Luria−Bertaini medum; AMP, ampicillin; CAP, chloramphenicol; RIF, rifampicin.

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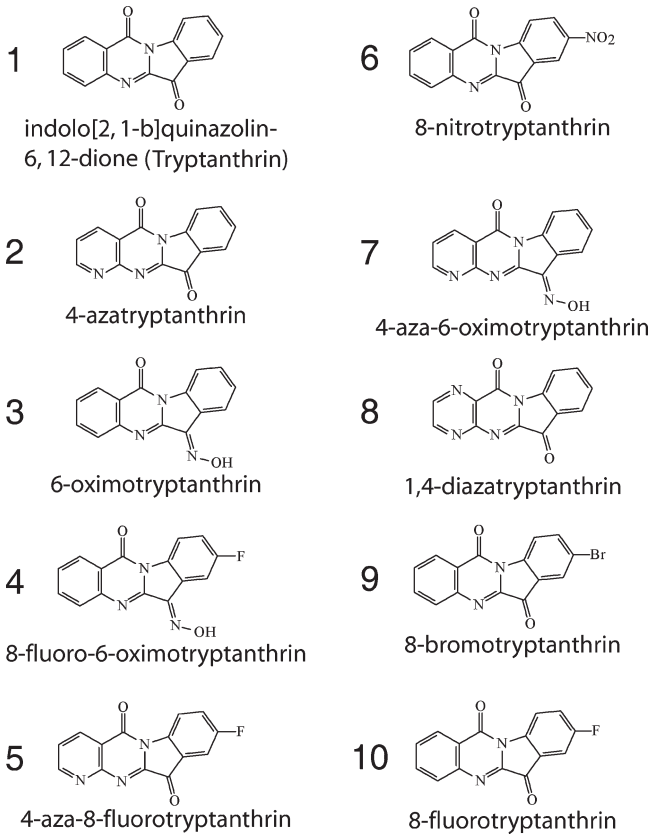

Figure 1. Tryptanthrin structure. The molecular structures of the 10 tryptanthrin derivatives are shown. The numbers indicate the notation used throughout.

in the presence of various concentrations of tryptanthrins (Figure 1) to assess their effect on growth. The effects of various concentrations of tryptanthrin **5** on culture growth are shown in Figure 2. A concentration of 0.01 μg/mL had no effect on growth, while 0.1 μg/mL had a small effect (Figure 2A). At 1 μg/mL, tryptanthrin **5** stopped growth within 4 h and little change in the OD_{650} occurred over the next 20 h of incubation (Figure 2A). Concentrations of 2.5−20 μg/mL still required about 4 h to stop growth (Figure 2, B). Following 20−24 h of incubation, cell viability was reduced to about 15% in cells treated with 10 μg/mL tryptanthrin **5** (data not shown).

Results of individual treatment of AS19 with seven other tryptanthrins at 10 μg/mL are shown in Figure 2C, D. A range of effectiveness in stopping growth was evident. Tryptanthrins **3**, **4**, and **7** had only a slight effect on growth. Tryptanthrins **1** and **6** slowed growth and reduced the OD_{650} end point by about 2-fold, while tryptanthrins **2**, **5**, and **8** caused cessation of growth several hours after addition. Results of Figure 2 are representative of multiple repetitions of these experiments. Tryptanthrins **9** and **10** showed weak growth inhibition (as **3**, **4**, and **7**) (data not shown).

From dose dependence experiments (similar to that shown in Figure 2), estimations of the concentration that reduced the rate of growth by half can be determined. For this, the OD_{650} 4 h after the tryptanthrin addition, at which point control cells were in late log phase growth, was plotted against the concentration of tryptanthrin added. Representative data are shown in Figure 3 for tryptanthrin **8**. For all tryptanthrins that resulted in a measurable reduction in OD_{650}, concentrations of 0.6−2 μg/mL were effective in reducing growth by half (Table 1).

Tryptanthrins **3**, **4**, and **7** failed to exhibit a significant effect on growth. As the 6-oxime derivatives are photolytically unstable, these compounds (**3**, **4**, **7**, and **8**) may initially bind to DNA by intercalation, and following subsequent photoactivation, they may react with DNA to form covalent adducts. This possibility was tested in two ways. First, following incubation of cells for several hours in the presence of the tryptanthrin (conditions that stop growth), samples were exposed to 360 nm light, and then growth was resumed. In these experiments, no inhibition of growth was observed following 360 nm light exposure for cells treated with tryptanthrins **3**, **4**, and **7** (data not shown). As a control in this experiment, cells were also treated with tryptanthrin **8**, which resulted in growth inhibition. Exposure of cells to 360 nm light, following addition of tryptanthrin **8**, resulted in similar growth inhibition kinetics without irradiation. In other experiments, tryptanthrins were exposed to light in DMSO, prior to addition to cells; however, no light-dependent effect was observed (data not shown).

Effect of Tryptanthrins on *E. coli* Viability during Incubation in M9 Buffer. To assess the bactericidal activity of tryptanthrins on *E. coli*, cells were grown overnight in LB, adjusted to 10^5-10^6 cells/mL in M9 buffer, and incubated in M9 buffer for 2−3 days in the presence of different concentrations of tryptanthrins. At roughly 12 h intervals, cells were sampled and plated to measure viability. Representative data from at least four to eight repetitions of each experiment are shown in Figure 4 for tryptanthrins **1**−**8**. Data are presented as surviving fraction of cells relative to untreated cells incubated in M9 buffer.

Several aspects of these experiments are significant. First, most tryptanthrins resulted in a significant decrease in viability of $10^{-4}-10^{-6}$ by 3 days of incubation in the presence of 10 μg/mL tryptanthrin. Tryptanthrins **2**, **5**, and **8**, which caused a cessation in growth in LB media, were effective bactericidal agents at concentrations lower than 10 μg/mL. Tryptanthrin **2** was equally effective at concentrations from 0.05 to 10 μg/mL, although the reduction in survival was less than for some other analogues. The activities of tryptanthrins **9** and **10** at 0.4 and 10 μg/mL were similar to those for tryptanthrin **2**. Tryptanthrin **4** was very effective at both inhibiting growth and killing. Significantly, tryptanthrins that failed to show substantial growth inhibition in LB media (tryptanthrins **3**, **4**, and **7**) were effective, at the higher concentrations, in killing cells during incubation in M9 buffer.

Mutagenic Potential of Tryptanthrins in *E. coli*. Several selection systems were utilized to test for the potential induction of mutations by tryptanthrin treatment. AS19 containing plasmid pBRF14C (with a 106 bp inverted repeat in the chloramphenicol acetyl transferase gene) was used as a system to test for induction of DNA secondary structure mutations (deletion of inverted repeats).[23,24] In addition, the frequency of rifampicin resistant mutations, which detects a more general array of mutations,[25] was measured. Cells were treated with 10 μg/mL tryptanthrins in M9 buffer to reduce survival by about 10^{-3}. Following this, about 10^3-10^4 cells were inoculated into 10 mL of LB, and the culture was grown overnight at 37 °C with shaking. Cells were plated to measure viability, CAP-resistant revertants, and RIF-resistant revertants. In seven independent repetitions of this experiment no consistent increases in mutation frequencies were observed for the deletion of the inverted repeat or reversion to rifampicin resistance. Thus, tryptanthrins do not possess, at least

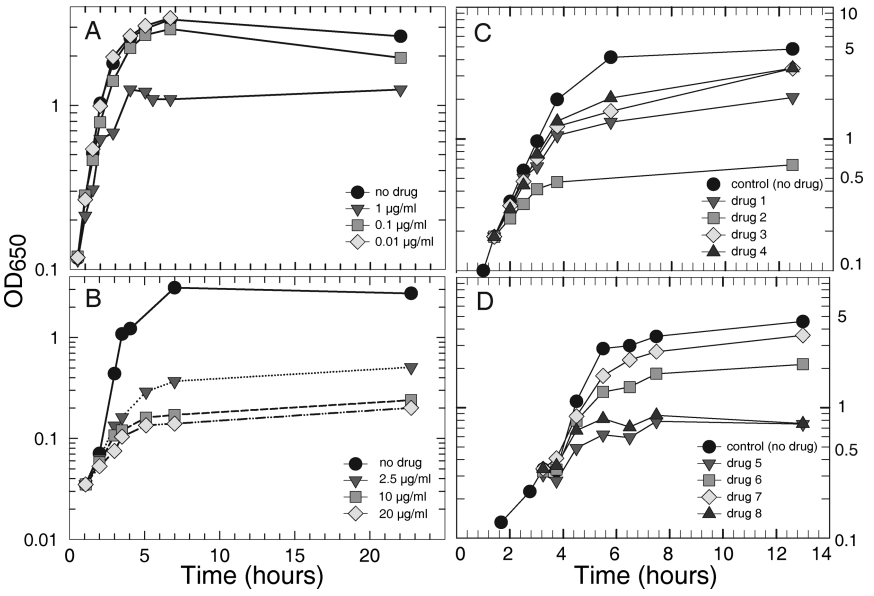

Figure 2. Effect of 4-aza-8-fluorotryptanthrin on growth of *E. coli* AS19. (A, B) The effect of tryptanthrin **5** (4-aza-8-fluorotryptanthrin) on growth of *E. coli* AS19 in LB is shown at concentrations indicated. A culture is started from an overnight, tryptanthrin is added, and the culture is incubated at 37 °C with shaking at 200 rpm. OD_{650} values were determined at various times of growth: (A) no drug (black circle), 0.01 μg/mL (gray diamond), 0.1 μg/mL (gray box), 1.0 μg/mL (black down-triangle); (B) no drug (black circle), 20 μg/mL (gray diamond), 10 μg/mL (gray box), 2.5 μg/mL (black down-triangle). Panels A and B are representative of more than six repetitions of this experiment. (C, D) The effects of tryptanthrins **1**−**8** on growth were compared at 10 μg/mL: (C) no drug (black circle), tryptanthrin **1** (black down-triangle), tryptanthrin **2** (gray box), tryptanthrin **3** (gray diamond), tryptanthrin **4** (black up-triangle); (D) no drug (black circle), tryptanthrin **5** (black down-triangle), tryptanthrin **6** (gray box), tryptanthrin **7** (gray diamond), tryptanthrin **8** (black up-triangle). The growth curves in panels C and D. are representative of data from three to six repetitions of each tryptanthrin analyzed.

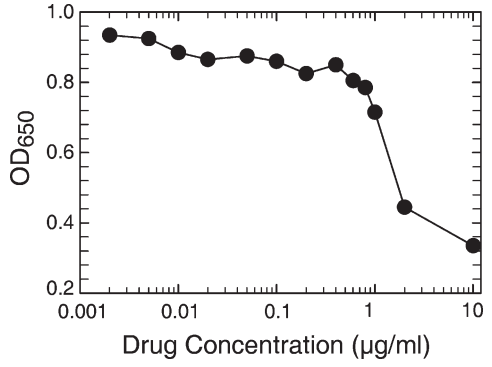

Figure 3. Dose dependence of growth inhibition. To determine a concentration where the rate of growth is reduced by 50%, the OD_{650} at 4 h after tryptanthrin **8** addition, when growth is in exponential phase, is plotted against tryptanthrin concentration. These values for all tryptanthrins that lead to growth inhibition are listed in Table 1.

Table 1. Tryptanthrin Concentrations That Reduce the Rate for Growth by 50%

tryptanthrin	concentration (μg/mL)[a]
1	2.25 ± 0.25
2	1.3 ± 0.2
3	NR[b]
4	NR[b]
5	0.6 ± 0.1
6	0.6 ± 0.1
7	NR[b]
8	1.5 ± 0.1
9	NR[b]
10	NR[b]

[a] The concentration shown is that required to reduce the OD_{650} to half that in untreated cells at 4 h after tryptanthrin addition. Concentrations represent the average of two to three independent experiments. [b] NR: no results due to minimal effect of these tryptanthrins on growth inhibition.

under the conditions of this experiment, a general mutagenic potential or an increase in the frequency of deletion of inverted repeats.

Given that certain intercalating agents are generally thought to induce frameshift mutations, a specific Lac^+ reversion mutagenesis assay that tests for frameshift mutations[26] was also applied for several of these tryptanthrins. Strains WAR176 and WAR177 that report +G and −G frameshifts, respectively, were tested. Cells were grown, from overnight cultures, for 10 generations in MM Gly media in the presence of 5 or 10 μg/mL tryptanthrins. Cell growth and numbers of Lac^+ revertants were measured to determine mutation frequencies. Growth in the presence of most tryptanthrins did not induce frameshift mutations in this system. However, the 4-aza and 4 aza-8-fluoro derivatives (tryptanthrins **2** and **5**, respectively) increased frameshift mutations about 22- and 4-fold, respectively, in both the +G and −G reporter strains (data not shown).

Tryptanthrins Alter DNA Supercoiling. Intercalating drugs bind to DNA and alter the helical twist, resulting in DNA unwinding by about 28° per drug bound.[27] The analysis of DNA topology, therefore, provides a very sensitive assay for intercalation. To assess intercalation or other binding that alters the twist of DNA, covalently closed circular DNA is treated with a topoisomerase in the presence of a suspected intercalating drug and analyzed by agarose gel electrophoresis. Negative supercoils remaining after relaxation indicate that drug binding altered the twist of DNA. DNA, with no drug present, was relaxed in the presence of Topo I (Figure 5A, lane 2, sample C). In the presence of tryptanthrin **6** at 100 μg/mL, DNA also appeared to be completely relaxed,

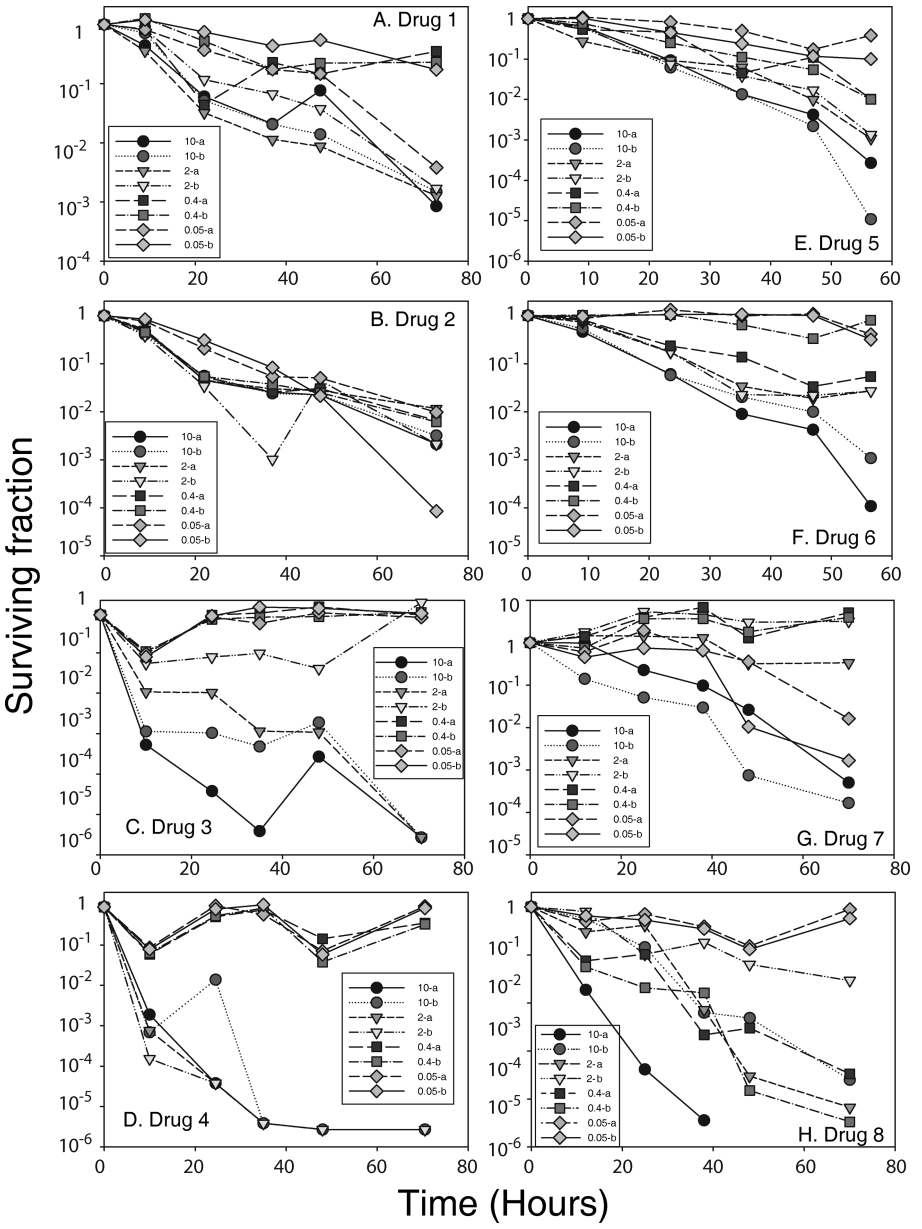

Figure 4. Survival during exposure to tryptanthrins in M9 buffer. Tryptanthrins were added to a suspension of AS19 at 10^5-10^6 cells/mL, and incubation at 37 °C was continued for up to 72 h. Samples were removed at various times, and viability was determined by plating on LB + AMP plates. Surviving fraction is that relative to cells incubated in M9 buffer without tryptanthrin addition. Data shown are for duplicate samples treated with tryptanthrins **1**−**8** in panels A−H, respectively. For all panels treatment was with 10 μg/mL (black circle, gray circle), 2 μg/mL (dark-gray triangle, light-gray triangle), 0.4 μg/mL (black box, gray box), 0.05 μg/mL (dark-gray diamond, light-gray diamond). Data are representative of four to eight independent experiments.

suggesting that minimal binding occurred. The sample containing tryptanthrin **2**, however, remained highly supercoiled, indicative of minimal supercoil relaxation presumably due to substantial tryptanthrin binding (lane 4). Tryptanthrins **1**, **3**, **4**, and **5** (lanes 3, 5, 6, 7) showed various levels of negative supercoiling indicative of different levels of binding. To demonstrate that the tryptanthrins were not inhibiting Topo I activity, the DNA was first relaxed with topoisomerase I, tryptanthrins were then added, and the reactions were allowed to continue. The DNA samples would remain relaxed if a drug inhibited the enzyme. As evident in Figure 5A, lanes 10−14, the distribution of topoisomers was similar to that seen in lanes 3−7. This shows that topoisomerase altered the linking number of relaxed DNA in the presence of tryptanthrins. These results demonstrate that tryptanthrins do not inhibit human DNA topoisomerase I, and the enzyme relaxed positive and negative supercoils in the presence of drug.

To better resolve the distribution of supercoils, samples were separated on an agarose gel containing chloroquine to unwind about six to eight supercoils (or introduce about six to eight positive supercoils in relaxed DNA). The multiple bands of the relaxed topoisomer samples are thereby separated, facilitating comparison and analysis (Figure 5B). Lanes 1, 6, and 13 contain control DNA relaxed in the absence of tryptanthrins. Tryptanthrins **6**−**10** showed only a small linking number deviation, evident as a series of bands 1−2 superhelical turns higher in the gel. Tryptanthrins **1**, **4**, and **5** showed a significant shift in the linking number pattern. Tryptanthrins **2** and **3** exhibited a very large change in linking number, indicative of a high level of drug intercalation (Figure 5B, lanes 3 and 4).

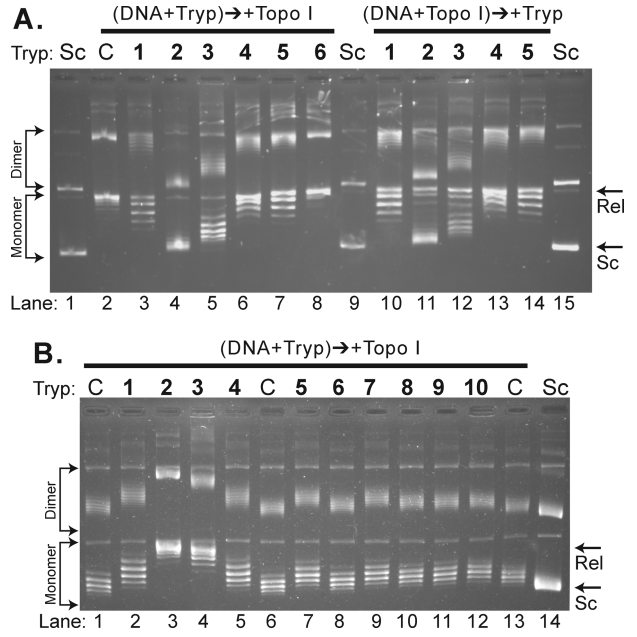

Figure 5. DNA unwinding by tryptanthrins. Plasmid pGEM DNA was incubated with human DNA topoisomerase I in the presence of tryptanthrins and the mobility of the reaction products resolved by agarose gel electrophoresis. (A) Standard agarose gel. Lanes 1, 9, and 15 contain purified supercoiled DNA. In lanes 2−8, DNA was relaxed with topoisomerase in the presence of 100 μg/mL of the tryptanthrins indicated above the lanes. In lanes 10−14, DNA was relaxed by topoisomerase treatment before addition of the tryptanthrin. This plasmid preparation contained both monomer and dimer plasmids, indicated to the left of the gel. The positions of migration of supercoiled (Sc) and relaxed (Rel) plasmid monomers are indicated at the right of the gel. (B) This agarose gel contained 2 μg/mL chloroquine in the gel and buffer, which relaxes about eight negative supercoils, facilitating analysis of the center of the topoisomer distributions.

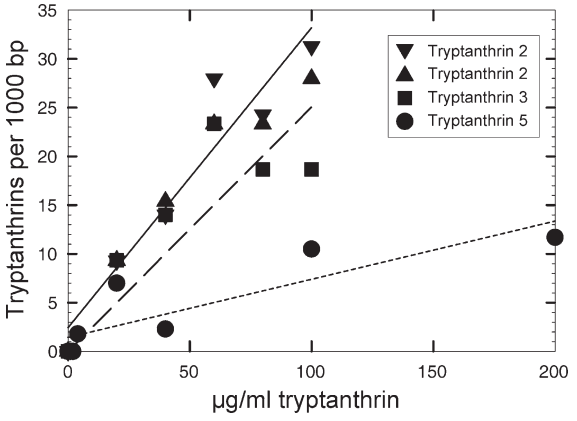

Figure 6. Dose dependence of DNA unwinding. The extent of DNA unwinding was determined from gels as shown in Figure 5 and as described under Materials and Methods. Data are presented as tryptanthrins bound per 1000 bp. Two independent experiments for tryptanthrin **2** are shown (black up-triangle, black down-triangle). Single analyses are shown for tryptanthrins **3** (black box) and **5** (black circle). Lines are least-squares fits to data for tryptanthrin **2** (solid line), tryptanthrin **3** (long dashed line), and tryptanthrin **5** (short dashed line).

Dose dependence unwinding analyses for tryptanthrins **2**, **3**, and **5** are shown in Figure 6. As the concentration of tryptanthrin increased, the number of negative supercoils relaxed by Topo I decreased. This is consistent with an increase in bound tryptanthrins with increasing concentration. The numbers of drugs bound per plasmid are calculated from the number of supercoils restrained and a value of 28° unwinding per drug, as described in Materials and Methods. Data were analyzed by linear regression to estimate the number of drugs bound at 100 μg/mL (0.4 mM). At 0.4 mM, 33 molecules of tryptanthrin **2** were bound per 1000 bp, or 1 drug per 30.3 bp. From all our analyses, the number of tryptanthrin molecules bound per 1000 bp at 100 μg/mL are shown in Table 2 for all analogues.

Discussion

Tryptanthrins have been evaluated as a class of therapeutic agents. Interest in these compounds stems from their stability, ease of synthesis, and reports of therapeutic application against pathogenic agents, including bacteria, fungi, and activity against cancer cells. However, the mechanism(s) of action of tryptanthrins remain unknown at the cellular or molecular level. To begin understanding the molecular mechanisms involved in the activity of tryptanthrins, we have initiated a study utilizing *E. coli* as a model system to evaluate the bacteriostatic, bactericidal, and potential mutagenic properties of a series of tryptanthrin analogues. Ten tryptanthrin analogues were utilized; three contained an oxime group in place of the 6-keto moiety. The reason for the oxime substitution was 2-fold. First, the oxime analogues are fluorescent and appear photolytically unstable, opening up the possibility of future fluorescent microscopy studies. Second, it is known that oximes in general tend to be toxic toward single cell organisms, resulting in the possible addition of a second pharmacophore into this class of molecules.[10]

Several features of this analysis are worth noting (Table 2). First, at 6−40 μM, some tryptanthrins showed bacteriostatic activity in that they stopped growth, with some decrease in viability, while other analogues did not. Second, several tryptanthrins exhibited significant bactericidal activity on prolonged exposure in M9 buffer. Third, most derivatives were not particularly mutagenic, even following treatment that decreased survival. However, two derivatives, including tryptanthrin **2** that was the strongest DNA intercalator, mildly elevated the frequency of frameshift mutations. From the lack of a general mutator effect, it is assumed that these compounds are not forming covalent adducts with DNA. Fourth, significantly, several of these derivatives bound to DNA, resulting in the unwinding of negative supercoils, a hallmark of DNA intercalating drugs.

The results presented indicate two general features with regard to a structure−activity correlation. In terms of bacteriostatic activity, most tryptanthrins containing the nitrogen heteroatom in the 4-position (tryptanthrins **2**, **5**, **7**, and **8**) increased their potency relative to those analogues that did not contain the heteroatom. The tryptanthrin analogues that exhibited the highest bactericidal activity (tryptanthrins **3** and **4**) contained the oxime functionality at the 6-position. Tryptanthrin **8**, the 1,4-diaza derivative, was also a very effective bactericidal agent. Tryptanthrin **7** contained both the 6-oxime and the 4-position nitrogen heteroatom. It exhibited poor bacteriostatic activity but reasonable bactericidal activity, suggesting that the oxime may have masked the activity of the heteroatom. By comparison of tryptanthrins **3** and **4** at 2 μg/mL, the 8-fluoro moiety appeared to have increased the bactericidal effectiveness, as compound **4** was more effective in killing than compound **3**. The 8-bromo

Table 2. Summary of Tryptanthrin Activities

derivative	growth inhibition[a]	bactericidal activity		DNA intercalation (drug/kbp at 100 μg/mL)[c]
		μg/mL	survival[b]	
1, tryptanthrin	+	2	$2 \times 10-3$	~10
2, 4-azatryptanthrin	++	0.05	5×10^{-3}	33
3, 6-oximotryptanthrin	±	2	$10^{-2}-10^{-4}$	25
		10	2×10^{-6}	
4, 8-fluoro-6-oximotryptanthrin	±	2	2×10^{-6}	~10
5, 4-aza-8-fluorotryptanthrin	++	0.4	2×10^{-2}	7.3
		2	1×10^{-3}	
		10	$10^{-3}-10^{-5}$	
6, 8-nitrotryptanthrin	+	2	3×10^{-2}	< 5
		10	$10^{-3}-10^{-4}$	
7, 4-aza-6-oximotryptanthrin	±	0.05	$10^{-2}-10^{-3}$	~7
		10	3×10^{-4}	
8, 1,4-diazatryptanthrin	++	0.4	5×10^{-6}	< 5
9, 8-bromotryptanthrin	±	0.4		< 5
		10	10^{-2}	
10, 8-fluorotryptanthrin	±	0.4		< 5
		10	10^{-2}	

[a] Growth inhibition at 10 μg/mL: ++, growth inhibition by 4 h after tryptanthrin addition; +, growth slowed by 4 h with a 2- to 4-fold reduction in OD_{650}; ±, mild effect of growth inhibition. [b] Survival is the surviving fraction of cells after 60−72 h of exposure to tryptanthrins at the concentration indicated. [c] DNA intercalation represents the tryptanthrins bound by intercalation per 1000 bp at 100 μg/mL. This value is calculated as described in Materials and Methods.

(tryptanthrin **9**) and 8-flouro (tryptanthrin **10**) derivatives were less effective bacteriostatic agents than the parent compound (Table 2), and the bactericidal activity was similar to the 4-aza derivative, tryptanthrin **2**. In the present study, compound **8**, the 1,4 diaza derivative, was overall the most effective antagonist to *E. coli* when taking into account the ability to inhibit growth as a static agent and to reduce survival of the organism. In summary, these results indicate that the tryptanthrins may have multiple mechanisms of action that depend upon modification of the parent tryptanthrin **1**.

Various theories have been suggested as mechanisms of action for tryptanthrins, but at present, little is known. The planar conformation of tryptanthrins in solution or as a solid may suggest action as DNA intercalators,[28,29] which we confirmed here. In light of recently published results that indicate that tryptanthrins have the potential to assume enantiomeric conformations upon binding to a substrate in vivo,[28,29] the conformational and electronic behavior of these molecules may be more complex than initially thought. These compounds are presumably not forming covalent adducts with DNA, as this would also likely lead to large increase in mutation rate. Our results show that tryptanthrins **2** and **3**, the 4-aza and 6-oximo derivatives, strongly unwind DNA supercoils, consistent with intercalation into the DNA double helix. Several other derivatives (tryptanthrins **1**, **4**, **5**, and **7**) show lower levels of unwinding, while the remaining analogues show a minimal effect. At present, no strong correlation exists between DNA binding and the measured biological effects.

Although tryptanthrins have a broad spectrum of activity against different organisms, no single analogue is extremely potent toward all the different types of organisms. For example, the most potent analogue against malaria may have very poor activity against tuberculosis.[5,8,9] This suggests different mechanisms of action at the molecular level and/or the involvement of different receptors or biochemical pathways among the different organisms. *E. coli* is certainly an excellent model for understanding many basics of DNA interaction; inhibition of DNA, RNA, or protein biosynthesis; or inhibition of basic metabolic pathways. However, it is conceivable that certain toxicological effects involving higher eukaryotic organisms may not be evident in this system. Experiments are in progress to increase the activity of tryptanthrins in *E. coli* and to understand the mechanisms of action in various biochemical pathways.

Materials and Methods

Bacterial Strains and Media. *E. coli* AS19, containing plasmid pBRF14C,[23] was used for the bacteriostatic and bactericidal analyses. AS19 was selected for permeability to actinomycin,[30] which makes it more susceptible to uptake of small molecules.[31] Strains WAR176 and WAR177, containing the +G and −G frameshift reporters (CC107 and CC108),[26] respectively, were used for the frameshift mutation assay. Cells were grown in Luria−Bertani medium (LB) (10.0 g of tryptone, 5.0 g of yeast extract, and 10.0 g of NaCl per liter of H_2O). LB plates contained 15.0 g of agar per liter. M9 buffer contained 1 g of NH_4Cl, 5.8 g of Na_2HPO_4, and 3 g of KH_2PO_4 per liter of H_2O. Ampicillin plates (LB + AMP) contained 30 μg/mL ampicillin. Rifampicin and chloramphenicol plates (LB + RIF, LB + CHL) contained 100 μg/mL rifampicin and 25 μg/mL chloramphenicol, respectively.

Tryptanthrin Derivatives. The tryptanthrin derivatives are shown in Figure 1. Reaction of commercially available isatins with the appropriate isatoic anhydride, according to the protocol of Mitscher,[32] afforded the series of tryptanthrin analogues as crystalline solids. The oximes (**3**, **4**, **7**, and **8**) were prepared by reaction of the corresponding tryptanthrin with hydroxylamine hydrochloride.[33] The oximes were synthesized and purified by recrystallization in the dark. The tryptanthrins utilized in this study were dissolved at 1−2 mg/mL (3−8 mM) in dimethyl sulfoxide (DMSO) and stored at −80 °C. Tryptanthrins **1**−**10** are known compounds. Structural identities were confirmed via high resolution DART mass spectroscopy and NMR, while purity of 95% or greater was attained via multiple recrystallizations (minimum of 3). Melting points were compared to those previously reported.

Effect of Tryptanthrins on Cell Growth and Survival. To determine the effect of tryptanthrins on growth, a colony of *E. coli* AS19 harboring pBRF14C was picked from an LB + AMP plate, inoculated into LB medium containing 30 μg/mL ampicillin, and grown overnight at 37 °C without shaking.

Ten millimeter aliquots of a culture in LB, adjusted to OD_{650} = 0.05−0.1, were placed in 125 mL flasks. Tryptanthrins were then added from stock solutions in DMSO (from 0.01 to 20 μg/mL), and the cultures were grown with shaking at 200 rpm at 37 °C. During growth, samples were taken at routine intervals, and the OD_{650} was measured. For viability measurement, after appropriate dilutions in M9 buffer, about 200−400 viable cells were plated on LB + AMP plates. Plates were incubated for 24 h at 37 °C before counting. Cell viability was determined as the number of cells/mL following tryptanthrin treatment divided by the number of cells/mL without treatment.

For measurement of survival in M9 buffer, an overnight culture was diluted to 10^5−10^6 cells/mL, tryptanthrins were added to a final concentration of 0.2−40 μM, and the cultures were incubated for up to 3 days at 37 °C. At various times, appropriate dilutions of cells in M9 buffer were plated (200−400 cells per plate) on LB + AMP plates. Viable cells were counted after 24 h.

Measurement of the Mutagenic Activity of Tryptanthrins in *E. coli*. The mutagenic potential of tryptanthrins on *E.coli* AS19 was assessed using two different mutational assays: rifampicin resistance[25] and the deletion of a 106-bp inverted repeat from the chloramphenicol acetyltransferase gene in pBRF14C,[22] restoring chloramphenicol resistance in *E. coli* strain AS19. For this, an overnight culture was diluted in M9 buffer and treated with tryptanthrins as described above for the measurements of survival in M9 buffer. The next day survival measurements were made and 0.5 mL of the cultures was inoculated into 10 mL of LB + AMP and grown with shaking at 100 rpm for 24 h. Cells from this culture were pelleted and resuspended in 1 mL of M9 buffer. Appropriate dilutions were plated on LB + AMP plates (200−400 cells per plate) to determine viable cell counts, and 0.1−0.2 mL of the resuspension was plated on multiple LB + CHL or LB + RIF plates to ascertain numbers of CHL or RIF revertants, respectively. CHL and RIF revertants were counted after 48 h of incubation at 37 °C.

A second assay for mutagenesis involved measurement of +G or G frameshift mutations resulting in Lac^+ revertants using the frameshift reporter strains of Cupples et al.[26] For this analysis, cells were grown for 10 generations in the presence of tryptanthrin analogues before plating for viable cells on glucose plates and for Lac^+ revertants on lactose plates, as described.[26]

Alteration of DNA Supercoiling by Tryptanthrin. Plasmid DNA (0.25 μg of pGEM-3Z, 2743 bp), purified by alkaline lysis followed by purification from a CsCl−ethidium bromide gradient,[24] was incubated with human topoisomerase I (TopoGen, Inc.), in 25 μL of Topo reaction buffer (10 mM Tris, pH 7.9, 150 mM NaCl, 0.1 mM spermidine, and 5% glycerol, plus 1 U of topoisomerase I) in the presence of tryptanthrins (10−100 μg/mL) for 2 h at 37 °C. In some cases DNA was relaxed by treatment with topoisomerase I 1 h prior to addition of the tryptanthrins, followed by an additional 1−2 h of incubation. The reaction was stopped by the addition of 5 μL of 5% sarkosyl, 25% glycerol with bromophenol blue dye (0.005%). Then 8−10 μL aliquots were loaded onto 1.5% agarose gels in Topo TAE buffer (40 mM Tris-acetate, 5 mM sodium acetate, 1 mM EDTA, pH 8.3) with or without 2 μg/mL chloroquine (Sigma). DNAs were separated by electrophoresis at 50 V for 3−4 h. Gels were then stained with ethidium bromide (0.5 μg/mL) for 15 min and photographed. The distribution of topoisomers were analyzed using Kodak 1D image analysis software.

On comparing the peak of the no-drug control topoisomer distribution (L_o) to the peak of the distribution (L_{drug}) of drug-treated samples, we calculated $L_{drug} - L_o$. The number of tryptanthrin bound to the DNA was calculated as $[(L_{drug} - L_o) \times 360°]/28°$. 28° is a general unwinding angle for intercalating drugs.[27]

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