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Determining the Antibacterial Activity and Mode of Action of Tirandamycin

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Determining the Antibacterial Activity and Mode of Action of Tirandamycin

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Abstract

Tirandamycin is a small molecule natural product that has been isolated from various species of marine and terrestrial *Streptomyces*. The natural product has shown antibacterial activity against an array of Gram-positive and Gram-negative bacteria, showing promise as a pharmaceutical drug. Tirandamycin has 14 known derivatives, many of which have been created synthetically. Some of its derivatives are particularly potent against the high-risk bacteria vancomycin-resistant *Enterococcus faecium*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus pneumoniae* and *Escherichia coli*. However, the antibacterial potency of these derivatives has not been tested systematically leading to the possibility of discovering more potent derivatives of the drug which could combat the rise of multi-drug resistant forms of these bacteria. Additionally, understanding tirandamycin's structural basis of inhibition would allow for future manipulation of tirandamycin's base structure to maximize antibacterial potency. While it is known that tirandamycin targets bacterial RNAp, there is still a need to determine where tirandamycin specifically binds to the enzyme, including the interactions between its amino acid residues and tirandamycin. A screening of the derivatives of tirandamycin against these high-risk bacteria is proposed to discover possibly potent but currently untested derivatives of tirandamycin. Additionally, x-ray crystallography of the tirandamycin-RNAp complex and site-directed mutagenesis of *E. coli* RNAp is proposed to determine the structural basis of inhibition by tirandamycin.

I. Introduction

a. Overview of natural products

The use of natural products produced by plants, animals, and microorganisms has been common throughout history, with some of the earliest known uses dating back to 1500 BCE Egypt.[1] In the past hundred years, the science behind these natural products has developed, and they can now be understood in a biological and chemical context. This allows for their antibacterial or antifungal potency to be dramatically increased as well as their structural basis of inhibition to be uncovered. This information can be used to chemically manipulate their structure to better bind and inhibit a target resulting in more effective and less toxic medicines.

Due to the wide range of organisms that produce these natural products, thousands have been categorized to date, all with unique structures that target different bacteria and fungi, and some even emerging as chemotherapeutic agents.[2] The 3-acyltetramic acid family of natural products contain important microbial derived antibiotics, including tirandamycin A and B, tirandalydigin, streptolydigin, nocamycin, and Bu-2313 A (Figure 1).[3]

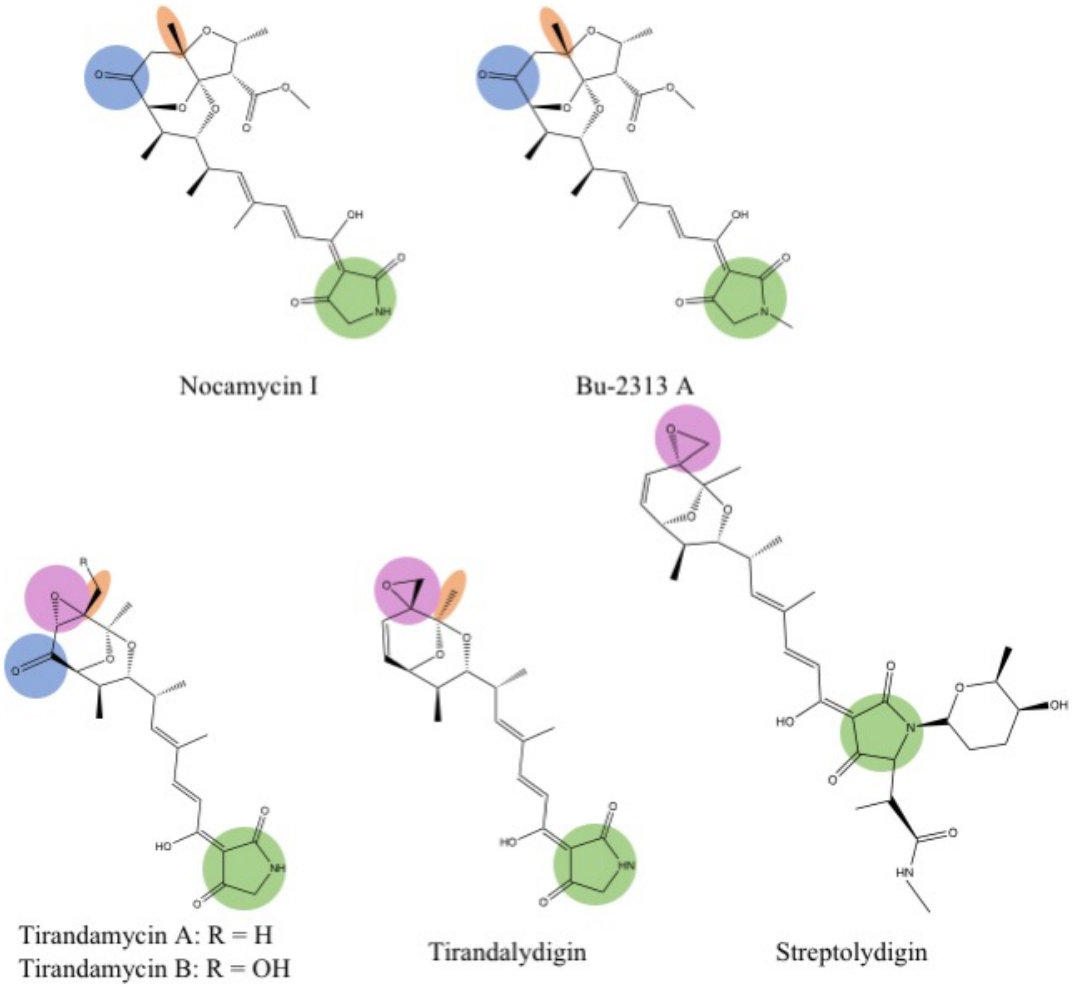

Figure 1. Structures of natural products in the tetramic acid family. The common structural features including the epoxide (pink), C-18 methyl (orange), ketone (blue), and pyrrolidine-2,4-dione (green) groups are highlighted.

The tetramic acid family of natural products are usually isolated from terrestrial and marine organisms, including bacteria, fungi, and sponges, and they exhibit antitumor, antibacterial, and antiviral activities.[4] Each differs in the degree of oxidation of their bicyclic ketal ring system as well as the pyrrolidine-2,4-dione substituents (Figure 2). By comparing their structures and biological activities, a description of how these chemicals work from a microbiological stance is beginning to emerge. Possible routes for chemical modifications of

these products that can lead to novel, more potent, antibacterial and chemotherapeutic drugs with lower levels of cell toxicity are also being developed.[5–8]

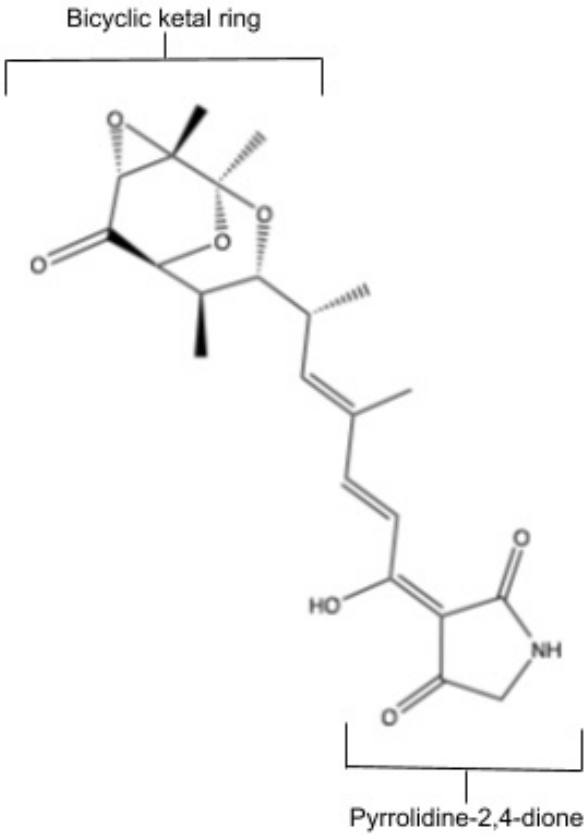

Figure 2. Pyrrolidine-2,4-dione and bicyclic ketal ring system structures of the tetramic acid family of natural products as seen on tirandamycin A.

b. Tirandamycin as a target for pharmaceutical research

Tirandamycin has been shown to be an antibacterial agent effective against an array of Gram-positive bacteria, including vancomycin-resistant *enterococcus* (VRE), and the Gram-negative *Escherichia coli* (Table 1).[9,10] Additionally, tirandamycin has been shown to have little to no cytotoxic activity in human cells.[5,11] It is important to recognize that tirandamycin is not the most potent antibacterial in the tetramic acid family of natural products as streptolydigin is a better antibacterial agent against *E. coli*.[10] However it offers promise for the

development of new medicines because the chemical synthesis of multiple derivatives of this compound has already been achieved.[12–14] Therefore, to develop tirandamycin into a useful pharmaceutical drug, its potency must first be optimized. This can be done by uncovering the mechanism behind tirandamycins function as a generic anti-bacterial.

		Minimum Inhibitory Concentration (μg/ml)						
Gram-Stain	Bacteria	A	B	C	D	K	L	Iso-B
(-)	*Escherichia coli*	100[19]		0.302[15]		39.53[15]		
(+)	*Bacillus halodurans*		1[17]					
(+)	*Bacillus thuringiensis*		32[16]				128[16]	
(+)	*Bacillus subtilis*	8[18]	100[17]				>128[16]	
(+)	Vancomycin-resistant *Enterococcus faecium*	0.94[9]	43.35[9]	4.84[9]	>3.61[9]	79.06[15]		
(+)	*Staphylococcus areus*	250[18]	>128[16]	4.84[15]		>158.11[15]	>128[16]	
(+)	Multi-drug-resistant *Staphylococcus aureus*			9.69[15]		>158.11[15]		
(+)	*Streptococcus agalactiae*	2.5[5]	2.5[5]					5.0[5]
(+)	*Streptococcus bovis*	50[19]						
(+)	*Streptococcus faecalis*	<0.5[18]						
(+)	*Streptococcus hemolyticus*	<0.5[18]						
(+)	*Streptococcus pneumoniae*	<0.5[18]						
(+)	*Streptococcus pyogenes*	12.5[19]						

Table 1. Minimum inhibitory concentration of tirandamycin derivatives against *Bacillus, Escherichia, Enterococcus, Staphylococcus,* and *Streptococcus* genus bacteria. Each derivative is represented by its letter, where Iso-B stands for isotirandamycin B.

Tirandamycin is a secondary metabolite that has been isolated from several species of marine and terrestrial *Streptomyces* and their mutant strains.[20] As most products in the tetramic acid family, it features a bicyclic ketal moiety at the end of a conjugated 5 carbon chain.[21] There are currently 14 known tirandamycin derivatives existing both naturally and as genetically engineered intermediates (Figure 3).[5,9,11,15,16,22] These derivatives all vary in their bicyclic ketone degree of oxidation, stereochemistry, and connectivity. Common structural

features include the tetramic acid, epoxide, ketone, and methyl groups, as well as a C-4 C-5 trans conformation.

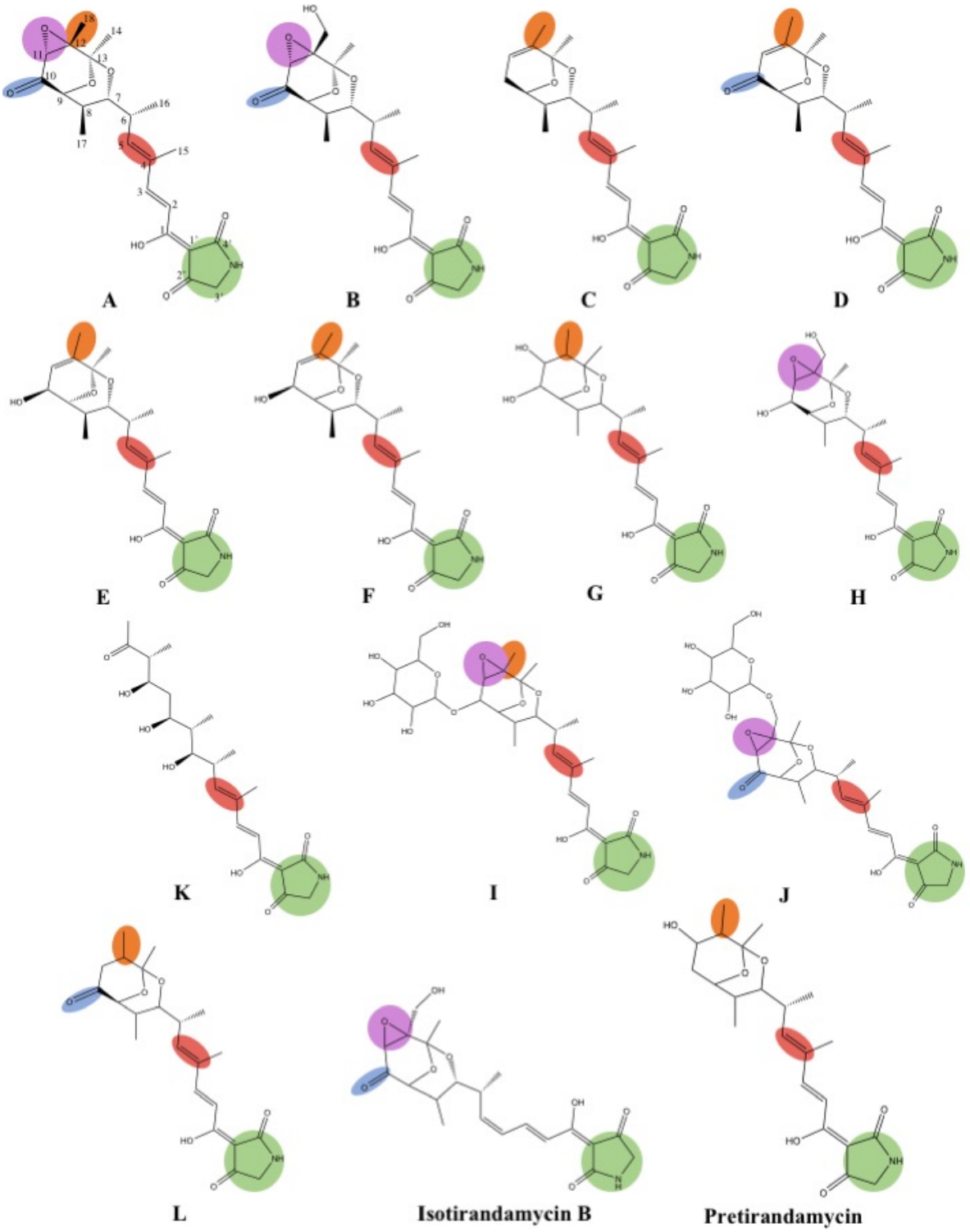

Figure 3. Structures of the 14 derivatives of tirandamycin with common structural features highlighted. The epoxide (pink), methyl (orange), ketone (blue), and pyrrolidine-2,4-dione (green) groups are highlighted. Additionally, the C-4 C-5 trans isomers are shown in red. The carbon numbering of tirandamycin is shown on structure A.

Structure-activity relationships (SARs) are a key aspect of optimizing drug potency and represent the relationship between a compounds structure and its biological activity.[23] SARs are particularly important to examine when comparing the potency of tirandamycin's derivatives, as the different substituents on the bicyclic ketal ring of each derivative will likely have an effect on how potent they are. According to SARs, the most potent structures should have substituents or functional groups that are interacting with the target enzyme and are important for tirandamycins biological activity. Alternatively, some derivatives may have unnecessary substituents or functional groups that would have no effect on the biological activity of tirandamycin. This would be represented as two derivatives with equal potency towards a specific bacterium with only one having an extra substituent or functional group attached. By measuring the antibacterial potency of tirandamycin's derivatives, scientists have been able to uncover the importance of structure-activity relationships and come closer to finding the mechanism behind some of tirandamycin's antibacterial properties.

c. The antibacterial activity of tirandamycin

Shortly after its discovery in 1971, tirandamycin A was tested for antibacterial activity against an array of Gram-positive bacteria of the *Bacillus*, *Staphylococcus* and *Streptococcus* genus.[18] The natural product showed potent antibacterial activity towards *B. subtilis*, *S.*

faecalis, *S. hemolyticus*, and *S. pneumoniae* with minimum inhibitory concentrations (MICs) of 8 μg/ml, < 0.5 μg/ml, < 0.5 μg/ml, and < 0.5 μg/ml, respectively. Tirandamycin A and its many derivatives have since been tested for antibacterial activity against a wider range of Bacillus, Streptococcus, Staphylococcus, Enterococcus, and Escherichia bacteria.

Tirandamycin is a known inhibitor of bacterial ribonucleic acid polymerase (RNAp), as shown in a study that tested tirandamycin A for inhibition of the RNAp of *E. coli* and the RNAp from rat liver nuclei.[10] Bacterial RNAp is an enzyme responsible for RNA initiation, elongation and termination.[24,25] The α, β, β', and ω subunits make up the core enzyme. The σ factor is a separate protein that binds to the core RNAp to form the holo-enzyme and allows it to locate specific DNA promoter sequences within the double-stranded DNA (Figure 4). After RNAp binds to DNA, the DNA template must move through the enzyme for RNA transcriptions, a process referred to as translocation.[26] During this process, the "F bridge helix" in the β' subunit alternates between a straight and bent structure, causing a conformational change in the enzyme that lets each amino acid of the DNA template into the active center to be transcribed into the corresponding RNA amino acid.[27] This process is vital for RNAp function. Whereas tirandamycin was able to effectively inhibit the chain initiation and elongation of bacterial RNAp, it had no effect on the function of animal polymerase. This provides evidence that tirandamycin could be safe for use on humans. Additionally, it shows tirandamycins likely bacterial target, and suggests that tirandamycins potency might vary for each species of bacteria due to differences in their RNAp amino acid sequence.

Although the specific molecular interactions between bacterial RNAp and tirandamycin are still unknown, the suggested binding interactions of RNAp and the natural product streptolydigin have been studied. Both tirandamycin and streptolydigin have a bicyclic ketal

group at the end of an unsaturated 6 carbon chain and have been compared as structurally analogous to each other, making data collected from one natural product presumed to be applicable to the other.[9]

The x-ray crystal structure of the streptolydigin-RNAp complex provides a picture of its general binding site on the enzyme. This technique was used to uncover streptolydigin as an allosteric inhibitor of *Thermus thermophilus* RNA polymerase, stabilizing the straight-bridge helix conformation and preventing the cycling into a bent-bridge helix conformation.[4] The x-ray crystal structure showed the natural product binding to an area on the β and β′ subunits of bacterial RNAp, interacting with the trigger loop region and bridge helix of the enzyme (Figure 4).

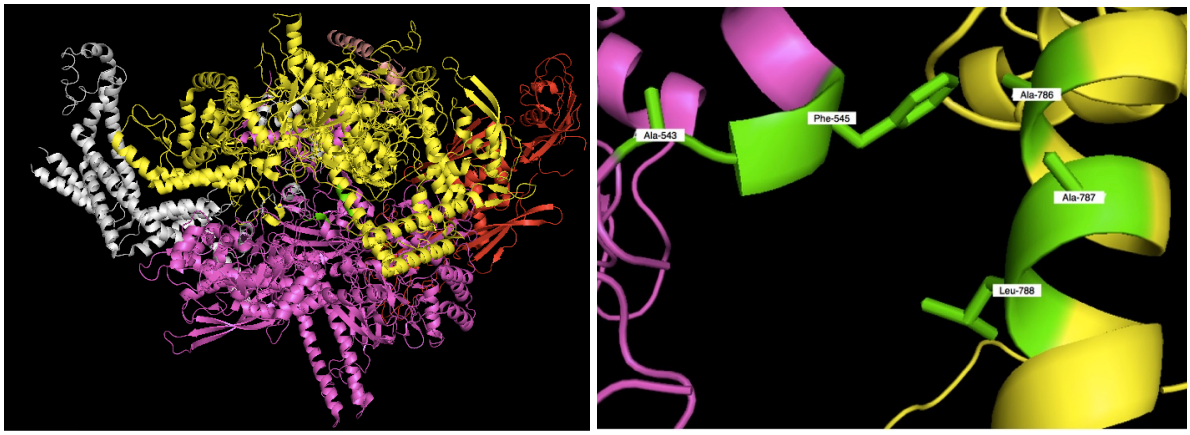

Figure 4. Structure of *E. coli* RNAp with five subunits (left) and potential tirandamycin binding site (right) pictured. The α subunit is shown in red, β subunit in pink, β' subunit in yellow, ω subunit in salmon, and the attached σ^{70} factor is shown in white. The proposed binding site of tirandamycin is shown in green with the amino acids that are presumed critical for streptolydigin binding labeled.

In addition to this, the study did saturation mutagenesis and analysis of *T. thermophilus* to find amino acid substitutions that lead to streptolydigin-resistant RNAp. Substitution of small, hydrophobic amino acid residues 543 Ala and 544 Gly in the β subunit to larger, more hydrophilic residues Val and Arg caused RNAp streptolydigin resistance. This suggests that there are favorable hydrophobic interactions at C-4, C-5, C-15, and C-16. Additionally, the authors suggest that the amino acid substitution of residue 788 Leu to Val introduces a branched side chain that likely results in steric hinderance at C-18, C-10 and C-11 for streptolydigin binding. These interactions are likely to contribute to tirandamycins biological activity, as both compounds are structurally similar in these specific areas having methyl groups at C-4 and C-6 on their linear six carbon chain and an epoxide group at C-12 on their bicyclic ketal moiety. Because of the high degree of RNAp sequence conservation between bacteria, the *T. thermophilus* RNAp structure is considered an accurate model representative of the transcription apparatus in all bacteria.[29] This allows the data collected with streptolydigin and *T. thermophilus* to be applicable to many different species of bacteria. The close relationship between the natural products streptolydigin and tirandamycin also suggests it is likely that tirandamycin binds to the same location on bacterial RNAp and uses the same structural basis of inhibition as streptolydigin. The results of other studies which have compared the antibacterial potency of different tirandamycin derivatives against other bacteria give further information about the structure-activity relationship presumably between tirandamycin and bacterial RNAp (Figure 5).

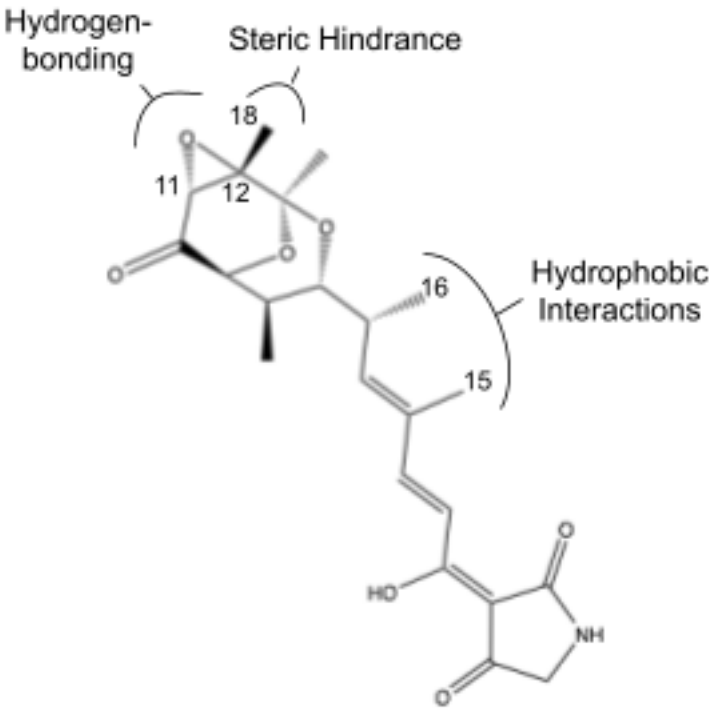

Figure 5. Annotated structure of tirandamycin with predicted structure-activity relationships shown.

There are some discrepancies in the data collected on the potency of tirandamycins derivatives, likely due to the fact that they have been evaluated in many different experiments but never all together. This can provide mixed reviews on whether certain functional groups are important for tirandamycins biological function. It is important, however, to take all the data into account to understand all the current theories about the SARs of tirandamycin.

Tirandamycins A and B were isolated and characterized from a marine-derived *Streptomyces sp*. along with intermediates C and D.[9] Analysis of these metabolites occurrence in the biosynthetic pathway showed that tirandamycin C was the earliest intermediate, followed by tirandamycin D, A, and B. Due to the increasing level of oxidation on the bicyclic ketal rings with each succeeding derivative, the authors came to two conclusions. First, it is likely

that the epoxide group on carbon-11 and carbon-12 of tirandamycins bicyclic ketal rings is necessary for tirandamycin's function. Second, both tirandamycin A and B are present as final products in which the only difference is the addition of a hydroxyl group on the carbon-18 methyl. The authors suggest that this step may not be necessary for tirandamycin's function, and therefore both derivatives are abundantly produced final products. This would suggest that it is the step of adding the epoxide group which is introduced after tirandamycin C in the biosynthetic pathway that gives these products their antibacterial properties.

In addition, the paper looked at the activity of tirandamycin A through D against VRE, and the results agreed with the importance placed on the C-11 C-12 epoxide group. Tirandamycin A (MIC = 2.25 μM) was drastically more potent than tirandamycin B (MIC = 100 μM), C (MIC = 110 μM), and D (MIC > 9 μM). From the data it was inferred that the increased potency of tirandamycin A could be attributed to the C-10 ketone and C-11 C-12 epoxide. The hydroxy group at C-18 on tirandamycin B was proposed to have an adverse effect on tirandamycin's antibacterial activity, as its potency was similar to that of tirandamycin C.[17] It can be extrapolated from this data that oxidation of carbons inside the bicyclic ketal ring system may help tirandamycin better inhibit VRE, while building oxygen containing substituents onto C-18 could cause steric hindrance between tirandamycin and its binding site.

In contrast to its poor anti-VRE activity, tirandamycin B was found to be about equally effective against *Streptococcus agalactiae* (MIC = 5.7 μg/mL) as tirandamycin A (MIC = 5.9 μg/mL). In addition to these derivatives, a new tirandamycin analogue, isotirandamycin B, was tested against *S. agalactiae* and had a significantly weaker effect on the bacteria, with an MIC of 11.5 μg/mL. These results suggest that the presence of a hydroxy group at carbon-18 increases rather than inhibits tirandamycins antibacterial potency against *S. agalactiae*. The

additional testing of isotirandamycin B suggests another interesting component of tirandamycins structure. Isotirandamycin B has a completely identical structure to tirandamycin B but has undergone E to Z isomerization of the carbon-4 carbon-5 double bond. This shows the trans conformation of tirandamycins carbon chain is likely necessary for tirandamycin's function.[5]

When tested against *Bacillus thuringiensis*, tirandamycin B has significantly more potent antibacterial activity compared to tirandamycin L, with MICs 32 μg/mL and 128 μg/mL, resepectively.[16] Derivative B and L are structurally identical compounds with two exceptions, tirandamycin L does not have the epoxide group connecting C-11 and C-12 or the hydroxyl group on C-18 that B has. This suggests that, similarly to *S. agalactiae*, the oxidative modification of the bicyclic ketone group on tirandamycin is important for its antibacterial activity against *B. thuringiensis*.

Tirandamycin K, the first linear tirandamycin derivative, was isolated along with tirandamycin C. Both derivatives were tested for antibacterial activity against *Escherichia coli*, VRE, *Staphyloccus aureus*, and multidrug resistant *S. aureus*. In all cases, tirandamycin C showed significantly more potent antibacterial activity than tirandamycin K, which had no effect on *S. aureus* at up to 400 μM. Therefore, this study concluded that the bicyclic ketal rings are likely necessary for antibacterial function.[15]

As bacteria continue to evolve and mutate, the need for new antibacterial drugs with different modes of action is of constant importance. The emergence of multi-drug resistant bacteria including VRE, *S. agalactiae*, group A and B *Streptococcus*, *S. aureus*, and *E. coli* have global health implications and these issues must be addressed by constantly searching for novel antibacterial chemicals that can combat this phenomenon.[30–33] In addition to this,

penicillin-resistant and multi-drug resistant *S. pneumoniae* has become a growing issue as one of the leading causes of pneumonia and meningitis.[34,35] It is important to capitalize on the discovery of tirandamycin, whose many derivatives offer another way to combat these bacteria while they are evolving to be immune to current medicines, which are slowly becoming less and less potent.[36] Aim 1 will address this need by conducting a larger screening of the current known tirandamycin derivatives A-L against the listed bacteria.

Additionally, the bacteria *Escherichia coli* seems to be a particularly good target for further research regarding the possible pharmaceutical implications of tirandamycin. *E. coli* is responsible for a wide range of infections including urinary tract infections and pneumonia.[37,38] In intensive care units, ventilator-associated pneumonia has a mortality rate of around 13% with higher rates for surgical patients.[39] *Escherichia coli* has been shown to be one of the most common causes of ventilator-assisted pneumonia, second only to *Pseudomonas aeruginosa*.[40] With the emergence of ampicillin, cefotaxime, and multi-drug resistant strains of *E. coli*, the World Health Organization named *Enterobacteriaceae*, including *E. coli*, critical priority pathogens.[41–43] While many of the compounds in the tetramic acid family of natural products have antibacterial properties against *E. coli*, only the mechanism of streptolydigin has been studied.[10,19,28] Due to the structural similarities between streptolydigin and tirandamycin, it is likely that these two natural products use the same mechanism of action.[9] However, further data are needed to confirm this relationship. Discovery of tirandamycin's interaction with *E. coli* RNAp would create a further understanding of how these natural products inhibit bacterial RNAp and provide the information needed for the rational design of more potent antibiotics based on tirandamycin's structure. Aim 2 will further the understanding of this interaction by using x-ray crystallography of *E. coli* RNAp interacting with tirandamycin to create an image

of where tirandamycin interacts with E. coli on bacterial RNAp and showing what amino acids may be present at that binding site.

Aim 3 proposes making single amino acid substitutions at the binding site of tirandamycin determined in aim 2 using site-directed mutagenesis on *E. coli* RNAp to determine the specific amino acid interactions that contribute to tirandamycins SARs. Amino acid substitutions that cause *E. coli* RNAp resistance to tirandamycin would suggest there are interactions happening between tirandamycin and the amino acid that are crucial for tirandamycins biological activity. By finding these critical amino acid residues, the structure of tirandamycin could be manipulated to form more favorable interactions between bacterial RNAp and the natural product, allowing for the creation of more potent forms of tirandamycin.

II. Research Proposal

a. Aim 1: Screening of tirandamycins A-L against VRE, group A and B *S. agalactiae*, *S. aureus*, *S. pneumoniae* and *E. coli*.

Tirandamycins A, B, C, and K have been the main focus of research regarding the bacteria *E. coli*, VRE, *S. agalactiae*, and *S. aureus*; however, these derivatives have mainly been tested individually and not in comparison to one another. In addition, tirandamycins E through L have rarely been tested for any kind of antibacterial activity at all. To move forward in the development of novel chemicals to combat the drug resistant bacteria VRE, group A and B *S. agalactiae*, *S. aureus*, *S. pneumoniae* and *E. coli*, all tirandamycin derivatives should be compared on the basis of their antibacterial activity against these pathogens and their structure to accurately deduce how to optimize their potency. These specific bacteria have been chosen because tirandamycin has already been shown to be a potent antibacterial of *Streptococcus, Staphylococcus, Enterococcus,* and *Enterobacteriaceae.* Additionally, vancomycin-resistant *Enterococcus faecium*, *Staphylococcus aureus*, *Streptococcus pneumoniae,* and *Escherichia coli* have been labeled as "global priority pathogens" by the World Health Organization, and Group A and B *S. agalactiae* have been listed as a "concerning threat" by the CDC due to their growing resistance to erythromycin and clindamycin.[43–46]

Aim 1 will determine the most potent structure of existing tirandamycin derivatives using drug screening of tirandamycins A through L against VRE, group A and B *S. agalactiae*, *S. aureus*, *S. pneumoniae,* and *E. coli*. Vancomycin-resistant *enterococcus* would be spread on m-Enterococcus Agar in 96-well plates and spotted with 100 µl serial dilutions of the derivatives of tirandamycin listed above, including isotirandamycin B in dimethyl sulfoxide (DMSO). Dilutions will start at 200 µg/ml, going down by a concentration of 10 µg/ml until

the minimum inhibitory concentration (MIC) of each derivative is reached (Figure 6).[42–45] MICs would be calculated by finding the smallest concentration of tirandamycin that still has a clear zone of inhibition. This process would be repeated for *S. agalactiae*, *S. pneumoniae*, and *S. aureus* on Blood Agar, and *E. coli* on Nutrient Agar.[9] All bacteria would be incubated for 24 hours at 35 °C to facilitate bacteria growth.[49,51–54]

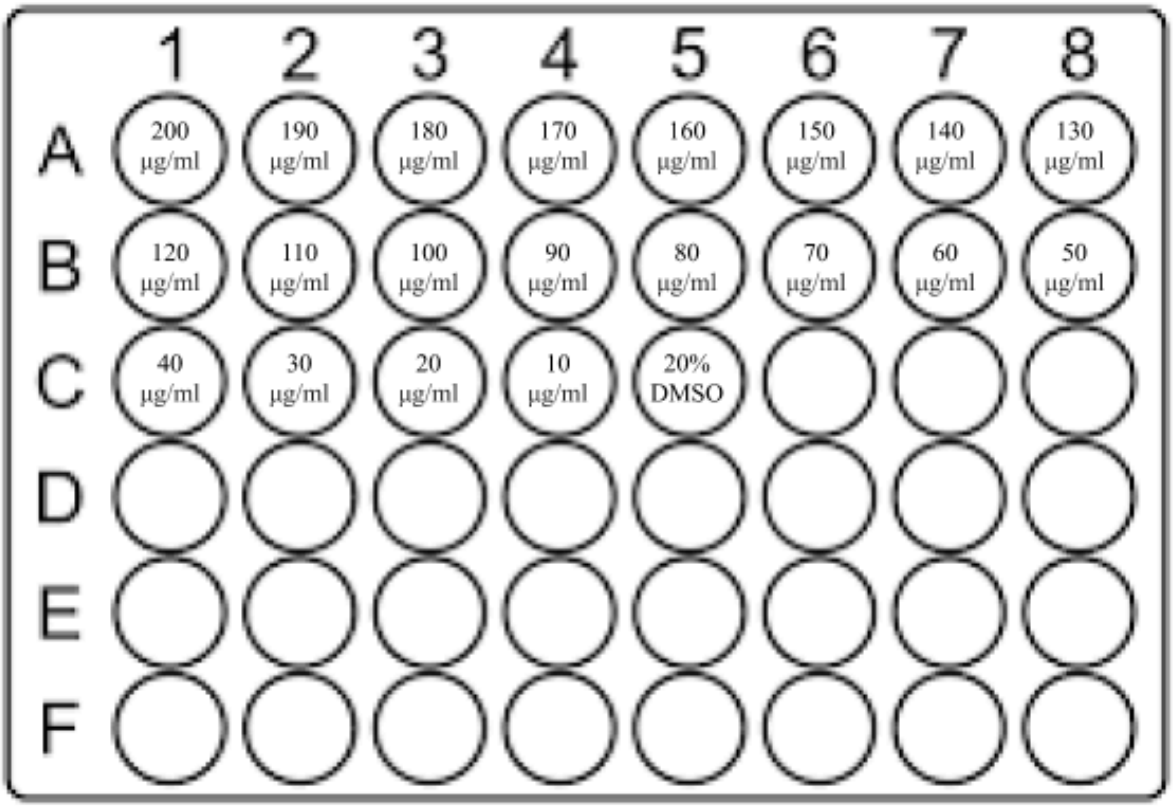

Figure 6. Serial dilutions shown in µg/ml of tirandamycin to 20% DMSO solution.

DMSO would be an optimal solvent to use, as it has been successfully used as a solvent in studies with *Streptococcus pneumoniae* and *Staphylococcus aureus* previously and has been tested against *Escherichia coli,* showing no inhibition of the bacteria when using up to 20% DMSO by volume to solid medium agar.[55–57] Additionally DMSO is known to be a strong organic solvent which can dissolve both hydrophobic and hydrophilic compounds making it

ideal for the derivatives of tirandamycin, a compound with hydrophilic bicyclic moiety and tetramic acid structure and a hydrophobic carbon chain.[58] While DMSO has been used as a solvent for the Gram-negative *E. coli* and three of the five selected Gram-positive bacteria, the toxicity effects of various organic solvents has been shown to vary across Gram-positive bacteria based on their specific species making the effect of DMSO on group A and B *S. agalactia*, and VRE unknown.[59] Due to this variation, DMSO could have an inhibitory effect on these Gram-positive bacteria. Therefore, the agar plates would also be spotted with 100 µl of 20% DMSO as a negative control to ensure the solvent is not contributing to inhibition of growth. If DMSO at 20% concentration by volume is shown to inhibit growth of the bacteria, the serial dilutions should be repeated using an alternative solvent such as methanol which has also been shown to have low levels of toxicity against various Gram-positive bacteria and can dissolve organic materials.[56] The concentration of DMSO and methanol to water can also be increased in the case of low tirandamycin solubility or decreased due to bacterial inhibition.

The previously studied tetramic acid streptolydigin would also be spotted on agar plates of *S. aureus* and *E. coli* using the same serial dilutions and solvent concentrations, as a positive control. This would allow the effect of experimental conditions such as the chosen solvent or solvent concentration on the antibacterial potency of streptolydigin and therefore tirandamycin to be evaluated. Streptolydigin would be a good positive control as it has been previously tested for antibacterial activity against *S. aureus* and *E. coli.*[19] Tirandalydigin, another tetramic acid analogue, would be used as a positive control for VRE because it's MIC for vancomycin-resistant *Enterococcus faecalis* is also known.[19] Besides tirandamycin, there are no products in the tetramic acid family that have been tested against *S. pneumoniae* and *S. agalactiae.* Therefore ceftriaxone, a known antibacterial agent for both, would be used as a positive control

for these bacteria.[60,61] The solvent type and concentration would be the same as used for the tirandamycin derivatives across all positive controls.

The inhibitory effects of tirandamycin would be measured using the MICs of each derivative for the 7 bacteria tested, providing a total of 91 MIC values. Due to potential differences in the RNAp amino acid sequences of each individual bacteria, it is likely that the most potent tirandamycin derivative will not be the same for all 7 bacteria. Organizing the derivatives of tirandamycin in order of potency for each individual bacterium would make it possible to analyze the affect certain functional groups and substituents have on tirandamycin potency. Because each derivative would be tested using the same experimental conditions, this data would provide a far clearer and more accurate picture of the structure-activity relationships of tirandamycin than the fragmented data currently available. Specifically, a comparison of tirandamycins A and H with the tirandamycin derivatives D and L could provide information as to whether the C-11 C-12 epoxide group is necessary for tirandamycins biological activity. A comparison of tirandamycin A and isotirandamycin B would also show how a trans to cis conformation change on the C-4 C-5 double bond affects tirandamycin potency. As previously mentioned, the hydroxyl group on C-18 of tirandamycin B has been predicted to have an adverse effect on tirandamycins antibacterial activity against vancomycin-resistant *Enterococcus* due to steric hindrance. This proposal could be tested by comparing the minimum inhibitory concentrations of tirandamycin A, B, and J. If added functional groups at C-18 cause steric hindrance between tirandamycin and bacterial RNAp, then derivatives A, B, and J should exhibit decreasing potency with functional groups of increasing size.

b. Aim 2: X-ray crystallography of tirandamycin in complex with *E. coli* RNA polymerase

Knowing the location of tirandamycin's binding site on bacterial RNAp is necessary to increase tirandamycin's potency, as it can identify key interactions between the functional groups of the antibiotic and the amino acid residues of the enzyme. Structural studies be done using x-ray crystallography of a *E. coli* RNAp in complex with tirandamycin. In order to determine where the most favorable interactions take place between tirandamycin and RNAp, the tirandamycin derivative that had the lowest MIC for *E. coli* RNAp in aim 1 should be used. X-ray crystallography would be ideal for *E. coli* RNAp because crystallization of the enzyme has been successfully achieved using the *E. coli* σ^{70} holoenzyme.[62] This not only proves that the crystallization of *E. coli* RNAp is possible but removes the need for a "crystal screen" as the most effective choice of precipitant, buffer, protein concentration, temperature, and crystallization technique are all known.[63–67]

While the x-ray crystal structure of the *E. coli* RNAp-tirandamycin complex has not yet been determined, an *E. coli* RNAp-benzoxazinorifamycin complex and *T. thermophilus* RNAp-streptolydigin complex has been crystallized.[28,68,69] From looking at the binding site of streptolydigin on *T. thermophilus* it can be assumed that tirandamycin binds at the same location between the β and β' subunits of *E. coli* RNAp.[28] Both studies regarding benzoxazinorifamycin and streptolydigin used the same technique to form the enzyme-compound complex crystal structures however, it is possible that small differences between the compounds or enzymes previously used could cause issues to arise when using the same procedure for *E. coli* and tirandamycin. Specifically, benzoxazinorifamycin binds to a different area on the β subunit of *E. coli* RNAp than where tirandamycin is presumed to bind.[69] While there is evidence that tirandamycin acts as an allosteric inhibitor of bacterial RNAp and forces

a conformational change in the enzyme, benzoxazinorifamycin is not an allosteric inhibitor and instead blocks the active site of RNAp.[70] Additionally, *T. thermophilus* and *E. coli* share many conserved residues; however, there are some differences between the two enzymes that could affect the ability for the RNAp-tirandamycin complexes to form.[28] To account for this, suggested changes to the procedure for RNAp-tirandamycin complex crystallization are given.

Aim 2 will locate the tirandamycin binding site on *E. coli* RNAp using x-ray crystallography of both the bacterial RNAp alone and tirandamycin interacting with bacterial RNAp. As described in previous studies, *E. coli* RNAp core and σ^{70} holoenzyme would be prepared and purified by creating a polycistronic plasmid, pGEMABC, for expressing the rpoB and rpoC genes responsible for encoding the β and β′ subunits.[64] The core RNAp subunits would be transformed with pGEMABC and pACYCDuet-1_Ec_rpoZ. Column chromatography would be used for purification of *E. coli* RNAp after adding excess σ^{70} to core RNAp. The RNAp crystals would be obtained using hanging drop vapor diffusion. A 50/50 mixture of protein solution and crystallization solution would be incubated at 22 °C over the reservoir.[69]

For crystallization of *E. coli* RNAp in complex with tirandamycin, RNAp crystals would be soaked in crystallization solution containing 20 mM MES, 13 mM magnesium formate, 2 mM spermine, 2 mM DTT, 5% PEG400, 10% MPD, and 0.1 mM of tirandamycin overnight at 22 °C and flash frozen by liquid nitrogen. This procedure has been used previously for the enzyme-crystal structures using rifamycin and streptolydigin. The two procedures used the same concentration of drug (0.1 mM) but varied in the period of time which the crystals were soaked. The *T. thermophilus* crystal was soaked in streptolydigin for only 15 minutes while the *E. coli* crystal was soaked benzoxazinorifamycin overnight. Previous studies have shown

that if the desired compound is not binding to the crystallized enzyme, increasing the period of time which crystallized enzymes are soaked in the desired compound as well as increasing the concentration of the compound in solution can help the complex crystals form.[71] Therefore, if the procedure mentioned above does not result in crystals with tirandamycin bound to *E. coli* RNAp, *E. coli* RNAp holoenzyme should be soaked in tirandamycin using higher concentrations of the compound for longer periods of time. As a negative control, *E. coli* RNAp crystals should also be soaked in a crystallization solution without the added tirandamycin to ensure the solution does not cause any conformational change of *E. coli* RNAp on its own.

The crystal structures of *E. coli* RNAp and the *E. coli* RNAp-tirandamycin complex will be compared first to observe any conformational change induced by tirandamycin. A straight-bridge helix conformation would be expected for the RNAp-tirandamycin complex, as was seen in the *T. thermophilus* RNAp-streptolydigin complex. The crystal structure will also show the location of tirandamycin's binding site, allowing for the visualization of all amino acids in contact with the structure of tirandamycin. Interacting hydrogen, oxygen, and nitrogen atoms between tirandamycin and the amino acid residues of *E. coli* RNAp would be examined for bond length, as an estimated 1.5-2.5 Å between a hydrogen atom and a nitrogen or oxygen atom would indicate possible hydrogen bonding.[72,73] Hydrophobic interactions and possible sources of steric hindrance could also be studied using the crystal structure. This information would provide a basis for the structure-activity relationships of tirandamycin.

c. Aim 3: Site-directed mutagenesis of *E. coli* RNAp

Site-directed mutagenesis is a valuable tool that can be used to determine structure-function relationships between enzymes and inhibitors.[74] This can be readily done to make mutants of

E. coli RNAp because the primary DNA sequence encoding each individual subunit of the wild type enzyme, which is required for site-directed mutagenesis, is already known.[75] Once the binding site for tirandamycin on *E. coli* RNAp is confirmed, site-directed mutagenesis can be used to verify the amino acids that are crucial to tirandamycins binding affinity and its function as an antibacterial. On the enzyme's five subunits, it is likely tirandamycin would bind on the β and β' subunits, interacting with the trigger-loop region and bridge helix.[28] By comparing the amino acids of *T. thermophilus* RNAp that streptolydigin binds to with the corresponding amino acid residues in *E. coli* RNAp, it is possible to predict what type of interactions tirandamycin has with *E. coli* RNAp (Figure 7). The effect each amino acid mutation has on tirandamycins antibacterial activity will be evaluated by testing the MIC of tirandamycin for each *E. coli* mutant RNAp, as described below.

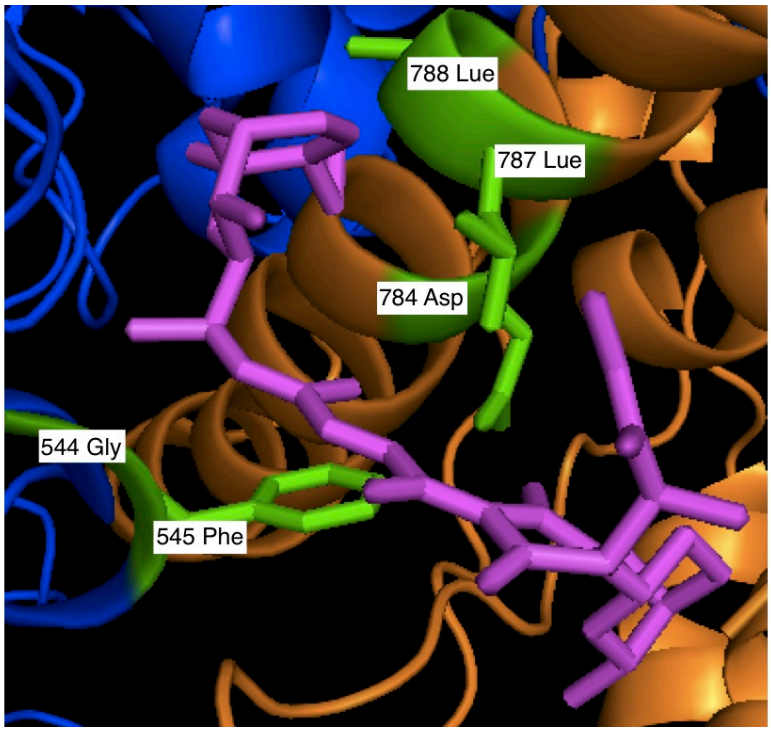

Figure 7. Streptolydigin-*thermophilus* crystal structure, with critical amino acids around streptolydigins bicyclic ketal ring and C-4 C-6 methyl groups labeled (green). Streptolydigin is shown in pink, interacting with the β (blue) and β' (orange) subunits of *T. thermophilus.*

Based on the known crystallography data, it is likely that amino acid residues 543 Ala, 544 Gly, and 545 Phe on the β subunit of *E. coli* RNAp have hydrophobic interactions with the C-4 through C-6 carbon chain and methyl groups on tirandamycin. To test this assumption, Asn should be substituted for residue 545 Phe, introducing a relatively large polar amino acid in the place of the wild type hydrophobic benzyl group at this position. Additionally, the much smaller hydrophobic amino acid Ala should be substituted at this position. If this substitution causes an increase in tirandamycin potency it could be possible that steric hindrance with the C-4 and C-6 methyl groups interferes with tirandamycin's ability to bind to bacterial RNAp, and removing one or more of these groups would result in a more potent form of the drug. Two mutations should also be made for both 543 Ala and 544 Gly to test for hydrophobic interactions and steric hindrance at this position by substituting each with the larger amino acid Leu and the polar amino acid Ser.

The conserved β' subunit residues 784 Asp, 787 Leu, and 788 Leu on *T. thermophilus* RNAp were also shown to be important for streptolydigin's binding affinity and should be examined for possible interactions between *E. coli* RNAp and tirandamycin. Specifically, substitution of Leu 788 to Val caused streptolydigin resistance in the bacterial RNAp, most likely due to steric hindrance at the β carbon. It is important to note that this residue is near the epoxide group on streptolydigin which is attached solely to C-12, while on tirandamycin this epoxide group is attached to both C-11 and C-12. It is possible that this attachment to both carbons changes the positioning of the epoxide resulting in decreased steric hindrance between tirandamycin and amino acid residue Leu 788. This mutation should therefore be done when testing *E. coli* to see if it leads to tirandamycin resistance. If hydrophobic interactions between

the 788 Leu and C-12 and C-18 are important for tirandamycins binding affinity, substituting Ala at this position should decrease tirandamycin potency.

The backbone of residues 784 and 787 are thought to interact with the C-12 epoxide group as well as C-11 and C-10 on streptolydigins bicyclic ketal moiety. It is possible that hydrogen bonding between the peptide backbone at this position and the oxygen groups on tirandamycin may be a crucial part of tirandamycins structural basis for inhibition, as increased oxygenation of this region of tirandamycin has previously shown to cause an increase in potency. Single amino acid mutations of each of these residues to Pro would change the structure of this backbone and could potentially remove the possibility of hydrogen bonding and lead to tirandamycin resistance.

Using this information, new and improved tirandamycin analogues could be created by examining which functional groups are interacting with these amino acids and how the structure could be manipulated to create more favorable interactions between the wild type enzyme and tirandamycin analog. The amino acid substitutions of tirandamycin resistant *E. coli* RNAp mutants would correspond to specific residues that effect tirandamycins ability to bind or inhibit the RNAp, further explaining tirandamycins structural basis for binding.

It is important to consider the effect which these single amino acid mutations would have on the conformation and enzymatic activity of *E. coli* RNAp in the absence of tirandamycin when evaluating the results of site-directed mutagenesis. It is possible that certain single amino acid residue changes affect the conformation of *E. coli* RNAp enough to cause the enzyme to lose its function in the absence of tirandamycin. In one study, a mutation which caused a single amino acid substitution of the β subunit on *E. coli* RNAp blocked *E. coli* cell growth at 42 °C, the temperature at which *E. coli* RNAp must be heated to for transformation to occur.[76]

Because there is evidence that tirandamycin binds to the β subunit of *E. coli* RNAp, it is likely that the β subunit would be targeted in site-directed mutagenesis. Therefore, a control experiment to ensure enzymatic activity after each single amino acid substitution would be needed. Additionally, a mutation in the enzyme could allosterically block the binding site of tirandamycin.[77] Crystallization and x-ray crystallography of each tirandamycin-resistant *E. coli* RNAp enzyme should be evaluated for conformational changes that might block the tirandamycin binding site. Additionally, it is possible that by artificially packing the enzyme in a crystal structure, its natural conformation is altered. These control experiments are further described in the procedure below.

Aim 3 would use site-directed mutagenesis to locate the amino acid residues that are crucial for tirandamycin binding affinity to *E. coli* RNAp. The appropriate oligonucleotide primers would be ordered for the amino acid substitutions described above. Site-directed mutagenesis would be performed using standard procedures.[78] To control for enzymatic function, each *E. coli* mutant would be spread in 96-well plates on Nutrient agar and spotted with 100 μl serial dilutions starting with a 200 μg/ml concentration of the tirandamycin-DMSO solution described in aim 1. Furthermore, 100 μl of 20% DMSO by volume would also be spotted as a control. Any *E. coli* mutants that cannot grow in the 20% DMSO solution would be assumed to have a mutation that renders the enzyme inactive. The procedure explained in aim 2 would be used to recrystallize each tirandamycin-RNAp complex for the *E. coli* mutants that become tirandamycin-resistant to ensure allosteric changes in the enzyme are not the cause of any change in the antibacterial activity of tirandamycin. By including these control experiments, the mutations that cause tirandamycin resistance can be assumed to contribute to necessary interactions for bacterial inhibition between the drug and RNAp amino acid residues.

III. Significance

As an RNAp inhibitor, tirandamycin has an advantage over currently used antibacterials. Rifamycin's are currently the only class of RNAp inhibitors that have been approved for clinical use.[79] Rifamycin-resistant strains of *S. aureus*, *S. pneumoniae,* and *E. coli* have also begun to evolve.[80–84] This leaves relatively few competitors for antibacterials that act as RNAp inhibitors. Additionally, because bacterial RNAp is an essential enzyme and highly conserved, it is likely that antibacterial agents targeting this region would be potent against a wide variety of bacteria.[85] Bacterial RNAp are also vastly different from eukaryotic RNAp, so drugs that target this enzyme have no effect on human RNAp, making this enzyme a key antibacterial target.[86] Tirandamycin also has shown little to no cell toxicity in human cells, making it a promising option for further pharmaceutical research.[5,11]

Tirandamycin has a complex structure, like many natural other products, and many of its derivatives have already been obtained by chemical synthesis.[12–14] Comparatively, structure-based drug design commonly used in pharmaceutical research often lacks the ability to create drugs that are as effective antibacterials as those produced through thousands of years of evolution.[87] It is therefore advantageous to utilize the naturally produced antibiotic tirandamycin by uncovering its mode of action and making synthetic improvements upon the compound to create the optimal molecule.

As bacteria continue to mutate and grow resistant to previously used antibacterial compounds the need for a novel drug is always of great importance. Currently, the bacteria vancomycin-resistant *Enterococcus faecium*, group A and B *S. agalactiae*, *S. aureus*, *S. pneumoniae* and *E. coli* are all considered high-risk bacteria because they have continued to evolve and become resistant to many of the commonly used antibacterials. While tirandamycin

has shown antibacterial activity against all of these bacteria, many of its derivatives have not yet been tested for antibacterial activity. This leaves a large gap in the knowledge of the impact the natural product could have as a novel antibacterial drug. By exploring the varying antibacterial activity of its derivatives against an array of high-risk bacteria, the full potential of this promising antibacterial could be uncovered and lead to finding more potent naturally occurring forms of the drug. The drug screening proposed in aim 1 would provide this information.

Understanding the mechanism of action of tirandamycin would allow for the elucidation of the structure-function relationship of tirandamycin and its many derivatives. Having this information could lead to improving on the structure of tirandamycin to become a more potent antibacterial. *E. coli* is a great target for this research as the need for a new, more potent antibacterial against this bacterium is critical due to the sudden rise in multidrug resistant *E. coli.* Aim 2 and 3 would allow for a deeper understanding of how tirandamycin and the other related natural products in the tetramic acid family function. Assuming tirandamycin is an allosteric inhibitor similar to streptolydigin, this could include increasing tirandamycins binding affinity to the β and β' subunits of *E. coli* RNAp. Due to the increased risk associated with *E. coli*, VRE, group A and B *S. agalactiae*, *S. aureus*, and *S. pneumoniae* and the high potential of tirandamycin as a pharmaceutical drug, this research is a necessary next step for antibacterial exploration.

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