Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 87–92

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

ANTIBIOTIC INHIBITORS OF THE PEPTIDYL TRANSFERASE CENTER. 1. CLINDAMYCIN AS A COMPOSITE ANALOGUE OF THE TRANSFER RNA FRAGMENTS L-PRO-MET AND THE D-RIBOSYL RING OF ADENOSINE

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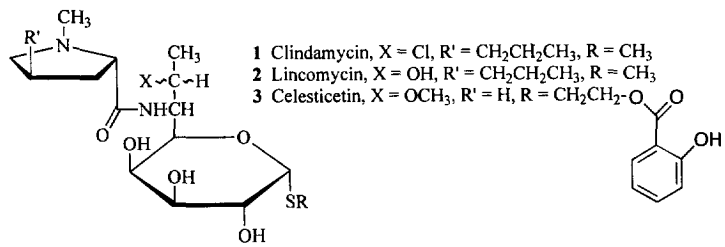
Received 11 August 1997; accepted 19 November 1997

Abstract: Clindamycin's three-dimensional structure is shown via a computer rendered stereomodel to be strikingly similar to L-Pro-Met and the D-ribosyl ring of adenosine. This discovery has important implications for the rational design of new lincosamides and for efforts to understand how this and related classes of agents selectively inhibit protein biosynthesis on the prokaryotic ribosome.

Clindamycin (**1**) is a 7-chloro-7-deoxy semisynthetic derivative of the naturally occurring antibiotic lincomycin (**2**). It possesses superior antibacterial activity to its parent.[1] Frequently used clinically in the treatment of infections caused by anaerobic bacteria, clindamycin has also become a preferred agent in the treatment of opportunistic infections caused by *Toxoplasma gondii* and *Pneumocystis carinii* in individuals with AIDS, and in the treatment of malaria resulting from 4-aminoquinoline- or sulfonamide/diaminopyrimidine-resistant strains of *Plasmodium falciparum*.[2]



1 and the lincosamide family of antibiotics inhibit ribosomal protein biosynthesis in prokaryotic microorganisms[3] by a molecular mechanism which has heretofore been unclear. Presented herein is a stereomodel of **1** (Fig. 1) which reveals that the antibiotic's three-dimensional structure closely resembles L-Pro-Met and the D-ribosyl ring of adenosine, biomolecules which occur proximate to one another at the 3'-ends of L-Pro-Met-tRNA and deacylated-tRNA for a brief interval following the formation of a peptide bond between L-Pro-tRNA and L-Met-tRNA. This finding strongly suggests that **1** and other lincosamides act as mimics of the 3'-ends of L-Pro-Met-tRNA and deacylated-tRNA as they are positioned during an initial phase of pretranslocation in the peptide elongation cycle. As such, **1** constitutes a structural analogue of an intermediate state in protein biosynthesis. This conclusion comports with: (*i*) lincosamide structure-activity relationships,[4] (*ii*) chemical footprinting data,[5,6] and (*iii*) tRNA and fragment binding studies.[7,8]

The peptidyl transferase center is a domain on the surface of the 50S ribosomal subunit which catalyzes

0960-894X/98/$19.00
PII: S0960-894X(97)10196-2

peptide bond formation. It contains at least three high affinity sites, the A', P',[6b] and E sites[9] to which residues at the 3'-termini of various tRNAs bind. **1** and **2** bind with moderately high affinity ($K_D \sim 10^{-5}$ to 10^{-6} M) and in a reversible, one-to-one, manner with a region of the peptidyl transferase center that encompasses portions of the center's A' and P' sites.[5] As a consequence, **1** competes for access to these sites with the 3'-terminal residues of tRNAs.[7] When **1** prevails, peptidyl-tRNAs (and possibly deacylated-tRNAs) prematurely dissociate from the ribosome.[8]

These findings have led a number of researchers to propose that this class of antibiotics acts through mimicry of one or more residues found at the 3'-end(s) of tRNA(s).[10] The substantiation of these hypotheses by x-ray crystallography[11] or NMR spectroscopy have been precluded by the immense size (~ 2,700 kdaltons) and complexity of the prokaryotic ribosome. However, a molecular modeling approach has been used in at least two previous attempts to establish a structural relationship between **2** and residues at the 3'-termini of aminoacyl- and/or peptidyl-tRNAs.[10a,b] One study presented a model comparing **2** to the 3'-terminus of L-Pro-tRNA,[10a] an aminoacyl-tRNA which binds to the A/A' site of the peptidyl transferase center. This hypothesis was later contradicted by experimental evidence showing that lincosamides block the attachment of 3'-tRNA fragments to both the center's A' and P' sites.[5,7] In another early study, computer-aided modeling was used to compare the three-dimensional structure of **2** with a hypothetical intermediate consisting of the 3'-termini of Gly-adenosine-5'-phosphate (A' site) and Gly-Gly-adenosine-5'-phosphate (P' site) poised at the instant *before* the peptide bond is formed.[10b] A subsequent effort to verify this model was unsuccessful,[12] casting doubt on its validity as well. These proposals appear to be the only reported attempts to reconcile the structure of a lincosamide antibiotic with residues at the 3'-ends of tRNAs using a modeling approach. While the studies erred in their assignments, the premise that lincosamide antibiotics are mimics of these residues remains widely accepted.

Close examination of clindamycin's structure reveals both the tRNA fragments it mimics, namely L-Pro-Met and the D-ribosyl ring of adenosine, and the specific molecular feature that causes it to interrupt translocation on the ribosome. This information is presented in a stereomodel which was derived in the following manner. The structures were generated using Chem3D Ltd™ molecular modeling software (Version 3.1, CambridgeSoft Corp., Cambridge, MA 02139). Structure optimization was achieved via the following sequence of commands: standard measurements, rectify, closures, structural errors. Energy minimization was conducted with the program's MM2 force field (rms errors and gradients were set at 0.1 for all of the aforementioned calculations). All of the structures were locally minimized and then imported into the molecular modeling program Sculpt™ (Version 2.1, Interactive Simulations, Inc., San Diego, CA 92121). L-Pro-Met and adenosine were individually overlaid on **1** by tethering selected atoms to one another and initiating Sculpt's auto minimization command. Once the energy values ceased changing, the molecules were conformationally fixed and **1** was separated from L-Pro-Met/adenosine. Initally, a number of unfavorable steric contacts were observed between atoms in the side chain of L-Met and the D-ribosyl ring of adenosine. This problem was overcome by separating L-Pro-Met/adenosine to a point just beyond their van der Waal radii. The resulting structurally correlated molecules were downloaded into Chem3D Ltd™ and are shown in stereoview format in Fig. 1. The

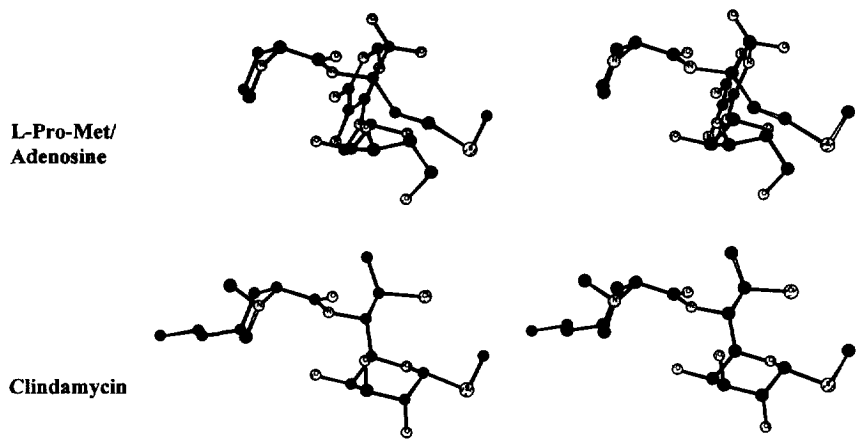


Figure 1. Stereoviews of Clindamycin (lower) and L-Pro-Met/Adenosine (upper)

model shows that *clindamycin's β-thiomethane-D-galactopyranosyl ring is a composite analogue of the L-Met side chain of L-Pro-Met-tRNA fused with the D-ribosyl ring of the 3'-terminal adenosyl moiety of deacylated-tRNA*. This conjoined feature of **1** constrains its conformational flexibility relative to the separate naturally occurring residues at the 3'-ends of peptidyl- and deacylated-tRNAs. Once bound to the A' and P' sites, clindamycin's rigidity prevents it from moving to the center's P' and E sites where presumably a different conformation is required. Consequently, newly formed peptidyl-tRNAs that have entered the pretranslocational state, or are stalled there,[8] are particularly vulnerable to premature displacement by the antibiotic from the peptidyl transferase center. **1** therefore interrupts ribosomal protein biosynthesis through an abortive translocation mechanism.

Support for **1** as a representation of L-Pro-Met and the D-ribosyl ring of adenosine is found in the experimental results from several areas of inquiry. For example, the stereomodel shows excellent correlation with existing lincosamide structure-activity relationships. When one or more of the three secondary hydroxyl groups on lincomycin's β-thiomethane-D-galactopyranosyl ring are inverted or replaced by a hydrogen atom, antimicrobial activity declines by almost 100-fold. Likewise, the oxidation of the β-thiomethane group into a sulfoxide or its replacement with a hydrogen atom also results in significant losses of activity.[4] These outcomes are expected based on the stereomodel because of the diminished resemblance of such derivatives to the D-ribosyl ring of adenosine or L-Met. In contrast, the *in vitro* antibacterial activity of lincosamides is frequently retained when the L-hygeric acid moiety (the modified L-Pro substituent) is replaced with other natural[4a] or unnatural[4b] L-amino acids. Such a change is similar to the center's P' site accommodating a different amino acid, an unremarkable event that occurs frequently during elongation.

1 and **2** are also widely used in the form of 2-phosphate derivatives.[3a,4a] These compounds possess improved aqueous solubility, muscle tolerance, and taste. Microbiological assays conducted *in vitro* indicate that 2-phosphate derivatives exhibit less than 1 to 2% of the activity of the parent compounds. In the

proposed stereomodel, the 2-phosphate group occupies a position on the β-thiomethane-D-galactopyranosyl ring that is identical to the 5'-phosphoryl group of the 3'-terminal adenosyl moiety of deacylated-tRNA. Based on this positioning, these compounds should possess enhanced antimicrobial activity. However, two factors apparently interfere: (*i*) organic compounds that are highly charged generally penetrate the microbial cell membrane poorly or not at all,[13] and (*ii*) the 2-phosphate group is on a *secondary* rather than a primary hydroxyl group, which restricts its conformational flexibility relative to the 5'-phosphoryl group of the 3'-terminal adenosyl moiety of deacylated-tRNA. The therapeutic role of these derivatives is therefore limited to functioning as prodrugs, as the 2-phosphate group is rapidly hydrolyzed *in vivo* to yield free **1** and **2**.[4a]

In addition to being consistent with the structure-activity data, the stereomodel assists our understanding of the elongation stage of protein biosynthesis in prokaryotes. Given the large number of possible L-amino acid combinations at the 3'-end of dipeptidyl-tRNA ($20^2 = 400$), it is unlikely that the ribosome accommodates each pair in a conformationally unique manner. Thus, we can infer that immediately following peptide bond formation, the 3'-terminal residues of all peptidyl/deacylated-tRNAs probably adopt a conformation similar to the one shown in the stereomodel. Accordingly, for a brief instant the newly linked amino acid of peptidyl-tRNA resides in the A' site while other amino acids in the peptide chain occupy the P' site simultaneously with the 3'-end of deacylated-tRNA (Fig. 2, A/A'-P':P/P'). This transient intermediate state, which is reminiscent of the classical model of protein biosynthesis,[14] is followed by the instantaneous movement of the 3'-terminal residues of deacylated- and peptidyl-tRNA into the center's P' and E sites, respectively. This movement, which occurs after the transition state, is reflected in the amended hybrid site model shown below.

Fig. 2 reconciles the hybrid site model of protein biosynthesis,[6a] which posits that the 3'-peptidyl ends of tRNAs remain anchored to the P' site throughout the elongation cycle, with chemical footprinting data for

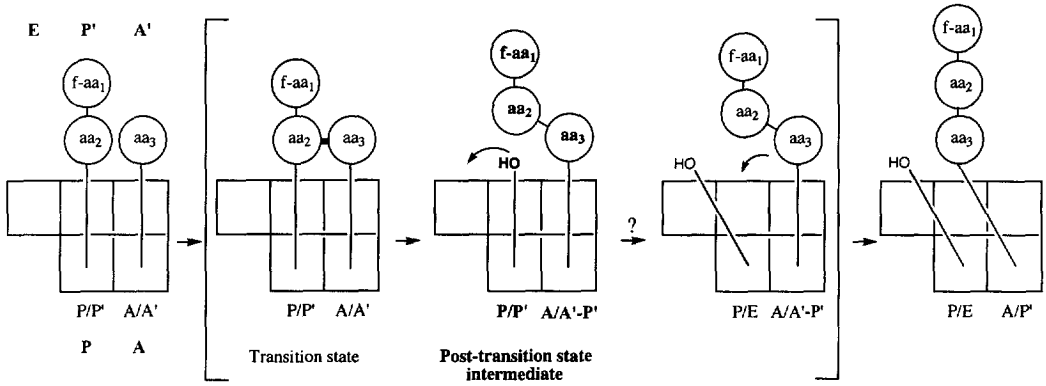


Figure 2. Amended hybrid site model depicting an early post-transition state intermediate (A/A'-P':P/P') that is mimicked by a number of antibiotic inhibitors of the peptidyl transferase center. Amino acids are represented by circles labeled aa_n, wherein n is 1, 2, or 3.

certain antibiotic inhibitors of the peptidyl transferase center which contain a peptidyl component to their

structures (*i.e.*, clindamycin, lincomycin, chloramphenicol, *etc.*).[5,6b] Reasoning from the hybrid site model, these inhibitors should bind exclusively to the P' site. However, the footprinting data for many of these inhibitors reveal protection of bases associated with both the A' and P' sites. Moreover, despite their peptidyl structure, the strongest protection exerted by these antibiotic inhibitors frequently involves bases that are associated with the A' site.[5,6b] By inserting a transition state as well as postulating the existence of short-lived intermediate states, the amended hybrid site model shown in Fig. 2 provides a more complete description of both inhibitor and tRNA binding to the peptidyl transferase center. Others have also recently interpreted the conflict between the antibiotic data and the hybrid site model by emphasizing the importance of the transition state.[6b]

Also consistent with the stereomodel are tRNA fragment binding studies conducted on clindamycin's parent, **2**, with CACCA-Leu and CACCA-Leu-Ac. It has been shown that the 3'-end of deacylated-tRNA binds to the P' site with significantly less affinity than to the E site.[6b,15] Interpreting the stereomodel in light of this finding, the portion of lincomycin's β-thiomethane-D-galactopyranosyl ring which resembles the 3'-adenosyl moiety of deacylated-tRNA should exert a weak inhibitory effect on P' site binding. By contrast, the stereomodel is predictive of a strong inhibitory effect on A' site binding since that portion of the ring which resembles the L-Met side chain presumably occupies the region of the A' site reserved for the newly linked amino acid of peptidyl-tRNA (see Fig. 2), or for an incoming amino acid of aminoacyl-tRNA. By providing substantiation that **2** indeed interferes with binding in these two ways, strongly at the A' site and weakly at the P' site, the fragment binding studies[7] provide additional support for the stereomodel.

One finding that appears to be at odds with the proposed stereomodel is the weak stimulation, rather than inhibition, of binding of tRNA fragments to the A' and P' sites by celesticetin (**3**),[7] a less active naturally occurring lincosamide. Several factors may account for this. The most probable is that **3** has a much slower onset of inhibition. This property has only recently been detected for **2**[16] and, if applicable to celesticetin, would have been accentuated by the low assay temperatures (0° and 4° C) employed in the governing studies.[7] Alternatively, **3** could have a lower affinity, as compared to **1** or **2**, for the peptidyl transferase center. Finally, it has been suggested that lincosamides act through an allosteric mechanism.[7] However, the failure of chemical footprinting studies to identify protected bases other than those associated with the A' and P' sites supports a direct rather than allosteric mechanism of inhibition for lincosamides.[5]

In conclusion, the stereomodel presented in this communication is generally consistent with the body of experimental evidence on **1** and **2**. Consequently, it provides a rational basis for the design and synthesis of a new generation of lincosamide and related antibiotics, arguing that modifications of this antibiotic class which enhance their similarity to the 3'-ends of peptidyl- and deacylated-tRNAs will result in compounds with greatly increased affinity for the ribosome. Additionally, and of potentially greater import, the stereomodel appears to disclose a three-dimensional "snapshot" of an intermediate state during prokaryotic protein biosynthesis, a key process in nature that is believed to have remained largely unchanged since the first protobacterium emerged from the primordial "soup" 3.8 to 4.0 billion years ago.[17]

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