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Discovery of a Cryptic Intermediate in Late Steps of Mithramycin Biosynthesis

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Abstract

MtmOIV and MtmW catalyze the final two reactions in the mithramycin (MTM) biosynthetic pathway, the Baeyer-Villiger opening of the fourth ring of premithramycin B (PMB), creating the C3 pentyl side chain, strictly followed by reduction of the distal keto group on the new side chain. Unexpectedly this results in a C2 stereoisomer of mithramycin, iso-mithramycin (iso-MTM). Iso-MTM undergoes a non-enzymatic isomerization to MTM catalyzed by Mg^{2+} ions. Crystal structures of MtmW and its complexes with co-substrate NADPH and PEG, suggest a catalytic mechanism of MtmW. The structures also show that a tetrameric assembly of this enzyme strikingly resembles of the ring-shaped β subunit of a vertebrate ion channel. We show that MtmW and MtmOIV form a complex in the presence of PMB and NADPH, presumably to hand over the unstable MtmOIV product to MtmW, yielding iso-MTM, as a potential self-resistance mechanism against MTM toxicity.

Graphical Abstract

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Keywords

Biosynthesis; Protein structures; Isomerization; Biocatalysis; Natural products

Introduction

Small molecule natural products, which have evolved structurally to exert specific biological functions over millions of years, provide the producer organisms with evolutionary advantages. As chemical warfare agents, these molecules usually bind (or react with) house-keeping elements of the competing organisms and inhibit their normal life cycles. As a double-edged sword, such natural products may create similar threats to the producing organisms themselves. To avoid self-destruction, many natural product biosynthetic gene clusters encode not only enzymes for their production, but also additional regulatory factors to control the expression of the genes, transporters to ensure efficient efflux of the toxic molecule or dedicated resistance mechanisms, such as enzymes that degrade or tightly bind the product molecules that linger in the cytoplasm.^[1]

Mithramycin (MTM, **1**) is an aureolic acid-type anticancer antibiotic produced by *Streptomyces argillaceus*. MTM is a potent transcriptional inhibitor targeting G/C-rich promoters.^[2] In accordance with this mechanism of action, MTM was recently found to potently inhibit growth of cancer cells that depend on oncogenic transcription. Specifically, MTM is a highly potent antagonist of the bone and soft tissue cancer Ewing sarcoma,^[3] which is driven by transcription factor EWS-FLI1 and prostate cancers driven by TMPRSS2-ERG.^[4] MTM binds DNA at X(G/C)(G/C)X sequences, explaining its preference for G/C-rich promoters.^[5] Structurally, MTM is a polyketide drug, composed of a tricyclic aglycone, C2- and C6-linked tri- and disaccharide chains, respectively, and a multifunctional C3-pentyl side chain (Scheme 1).^[6] The specific structure of the aglycone is

critical for DNA binding, because MTM binds DNA as a dimer, in which the two molecules of MTM are bound to each other by a divalent metal ion coordination to aglycone oxygens.^[7] Another key structural feature, the stereochemistry at C2, results in the right-handed twist of the dimeric core scaffold of MTM, which lies in the minor groove of the DNA duplex.^[8]

The biosynthesis of MTM begins with the synthesis of the decaketide backbone assembly by a type-II polyketide synthase (PKS), which includes cyclizing reactions, oxidation/hydroxylation, ketoreduction and a methyl transfer step, to produce the first isolable pathway products, the tetracyclic intermediates 4-O-dimethyl-premithramycinone and premithramycinone (**2**).^[9] In a series of post-PKS tailoring steps, **2** is glycosylated and methylated resulting in premithramycin B (PMB, **3**), presumably the last non-bioactive intermediate of this pathway.^[5] The fourth ring of **3** is oxidized by the Baeyer-Villiger monooxygenase MtmOIV to form premithramycin B-lactone (**5**), which upon opening provides the C3-pentyl side chain in mithramycin-DKA (**6**).^[10] A decarboxylation reaction, which produces MTM DK (**4**), is followed by the reduction of the 4'-keto group of the pentyl side chain catalyzed by MtmW, which finalizes the biosynthesis of MTM.^[11] In absence of MtmW, **4** undergoes spontaneous degradation to form shunt products mithramycins SK (**7**), SDK (**8**), and SA (**9**).^[12] The activity of MtmOIV and MtmW in the above-mentioned process is critical, because these reactions yield key structural features that properly shape MTM for its biological activity.^[8] Only MTM and its analogues with the tricyclic core appear to be biologically active.

To gain a better understanding of the final biosynthetic steps for development of potential drugs for the treatment of Ewing sarcoma and prostate cancers caused by aberrant ETS-fused transcription factors, we focused on the enzymes responsible for the generation of the C3-side chain, Baeyer-Villiger monooxygenase MtmOIV and ketoreductase MtmW, whose activity is likely coupled to channeling MTM out through the efflux channel MtrAB.^[13] Herein, we report the evidence for enzymatic cooperation of MtmOIV and MtmW essential for MTM formation, through formation of a transient complex, as well as crystallographic analysis of MtmW.

Results and Discussion

The ketoreductase MtmW, encoded by gene *mtmW*, was proposed to reduce the keto group at the 4' position of the C3-side chain in MTM DK (**4**), the latest intermediate on the pathway to MTM.^[10] MtmW shares sequence homology with other bacterial ketoreductases of aldo-keto reductase (AKR) superfamily (Supplemental Figure 1), which, intriguingly, include the cytosolic β -subunit (Kv β) of the Shaker family of voltage-dependent potassium channels (Kv1) in vertebrates.^[14]

We tested the activity of the purified recombinant MtmW protein, by incubating MtmW, MtmOIV and their cofactors (NADPH and FAD) with PMB in a Tris-HCl buffer at pH 8.25. This pH was chosen to maximize the activity of MtmOIV in generating the proposed intermediate MTM DK (Scheme 1).^[10] Formation of MTM was observed in this assay (Figure 1C), while in the absence of MtmOIV or MtmW no MTM was observed (Figure

1D). These results demonstrated that the recombinant, MtmW protein was active and fully functional.

Surprisingly, in the presence of both enzymes, only very small amounts of MTM, intermediates and shunt products of the conversion reaction were observed, while a new peak (Figure 1C, retention time: 10.6 min; Supplemental Figure 2) emerged as major product in this assay. The production of this new compound clearly required MtmW, since this product was not observed in the control assays carried out in the absence of MtmW or when MtmW was heat-denatured (Figure 1, traces D-F). HR-MS showed that this new compound has an identical molecular weight to that of MTM, hence we named this molecule iso-MTM (**10**, Supplemental Figures 3-5). Purification of iso-MTM was performed, and the compound was analyzed by NMR spectroscopy. The NMR spectra (Supplemental Figures 6-10, Supplemental Tables 1-2) revealed that iso-MTM is a C2 epimer of MTM. The NMR data for iso-MTM (**10**), when compared with those for MTM^[15], revealed a distinct difference in the coupling constant of C2-H in the ¹H NMR spectrum. In MTM the C2 proton resonates at 4.78 ppm with a coupling constant of 11.5 Hz (trans-coupling),^[16] whereas in **10**, a coupling constant of 6.7 Hz (cis-coupling) and a chemical shift of 4.40 ppm were found, indicating that **10** was a C2 epimer of MTM (Figure 2). These data also confirmed that MtmW reduced the C4' keto group, as seen in MTM.

Because MtmW on its own does not convert PMB (Figure 1, trace F), one concludes that the reaction starting with PMB is carried out sequentially by MtmOIV followed by MtmW. We hypothesized that one of the species formed in the presence of MtmOIV alone (Figure 1, panels D and E) is a substrate of MtmW. In the previous studies, **4** was proposed to be the natural substrate of MtmW since **4** differs from MTM only by C-4' keto group (Scheme 1).^[10, 12, 17] For this reason, all of the major products of PMB by MtmOIV (pH 8.25, shown in Figure 1D) were isolated by HPLC and tested as substrates of MtmW. None of these compounds were further converted by MtmW. This result suggested two non-mutually exclusive possibilities: 1) the product of MtmOIV that was a pathway intermediate was short-lived and degraded and 2) the proper sequence of reactions could only be carried out by a complex of MtmOIV and MtmW, where a true pathway intermediate forms transiently, whereas in the absence of MtmW PMB is converted to shunt products. To exclude the possibility of degradation of this intermediate under HPLC conditions, we carried out sequential reactions with MtmOIV followed by MtmW (Figure 3). No new product was observed when we directly added MtmW to a one-hour MtmOIV reaction (Figure 3B). Similarly, no new product was detected when we removed MtmOIV by ultra-filtration after a one-hour MtmOIV reaction and then added MtmW (Figure 3C).

The conditions for the MtmOIV-MtmW assays were further optimized by pH adjustment, to pH 7.5. Under these conditions, PMB was fully converted, mainly into iso-MTM, and into small amounts of MTM after 15 min incubation, while no MtmOIV shunt products were accumulated (Figure 4A). These results indicate that if the substrate of MtmW is formed by MtmOIV, it is short-lived and/or that it can only form by an MtmOIV-MtmW complex. In both cases, the data strongly argue that the intermediate would be handed over by MtmOIV to MtmW upon their interaction with each other.

We tested whether MtmOIV and MtmW (each at 20 μ M) form a complex by a electrophoretic mobility shift assay^[18] (EMSA; Figure 5A) in the absence and in the presence of PMB and NADPH (a co-substrate for both enzymes). (MtmOIV is strongly bound to FAD; therefore, addition of FAD was not necessary.) Besides the bands corresponding to MtmOIV and MtmW, no new band was observed at any conditions, indicating that the two proteins did not form a long-lived complex. However, a distinct and reproducible smear of the band corresponding to MtmW was observed in the presence of the MtmOIV (lanes 3-6), which migrated much more slowly. This effect was not observed in the absence of MtmOIV (lane 1), indicating that MtmW and MtmOIV formed a complex that dissociated during the initial stages of the electrophoresis. This smear was enhanced in the presence of either NADPH or PMB or both ligands. The formation of MtmOIV-MtmW complex in a substrate-dependent fashion is consistent with the above mechanistic models of the proper sequence of reaction occurring only when the product of MtmOIV can be handed over directly to MtmW. We also performed this assay as a titration, where the concentration of MtmOIV increased while the MtmW concentration was kept constant at 10 μ M (Figure 5B). In agreement with the previous experiment, we observed gradual weakening of the intensity of the band corresponding to the free MtmW upon titrating MtmOIV, indicating weak MtmOIV-MtmW binding.

In our earlier studies of MtmOIV, **4** was proposed to be the initial major product of the MtmOIV reaction, which would then undergo Favorskii-like rearrangements producing MTM SK (**7**) and MTM SDK (**8**) or a retro-Aldol reaction producing MTM SA (**9**).^[10, 19] The actual substrate of MtmW could be MTM-DKA (**6**), which could never be isolated due to its instability, but was observed by LC-MS during the MtmOIV reaction.^[10, 19a]

The bioactivity of iso-MTM was initially investigated by the disk diffusion assay, where we observed a comparable zone of inhibition of the Gram-positive *Staphylococcus aureus* to that for MTM, 3.5 cm and 3.7 cm in diameter, respectively. The MIC values for iso-MTM and MTM against *S. aureus* were found to be 230 nM and 115 nM, respectively. Neither iso-MTM nor MTM were active against the Gram-negative *Salmonella enterica*. Bioactivity against the EWS-FLI1 containing Ewing sarcoma cancer cell line TC-32^[4] was also assayed: iso-MTM and MTM had an IC₅₀ of 209 nM and 45 nM, respectively. From these observations it appeared that iso-MTM was not as cytotoxic as MTM. However, when butanol extractions of the culture supernatant samples containing iso-MTM were analyzed by LC/MS, they were found to contain ~ 50% MTM, which was likely generated by spontaneous isomerization from iso-MTM. Overnight incubation in the supernatant from an *S. aureus* LB culture resulted in a mean isomerization of 70.5% of the iso-MTM into MTM, indicating that iso-MTM was largely biologically inactive and consistent with our hypothesis that extracellular factors such as pH, ions, secreted enzymes, or *S. aureus* metabolites were responsible for the isomerization.

The ratio of iso-MTM to MTM varied over time. We observed that iso-MTM slowly and non-enzymatically converted into the less sterically hindered MTM in the pH range 4-8, with the conversion somewhat more favorable at higher pH. Addition of Mg²⁺ ions rapidly promoted this conversion (Supplemental Figures 11-13). Since the tricyclic core of MTM analogues is known to chelate Mg²⁺, we propose a mechanism of this conversion based on

Mg²⁺-dependent keto-enol tautomerism (Figure 6), via *si*-protonation at the C2-position in its last step. This was verified by incubating iso-MTM with and without MgCl₂ and observing a MgCl₂-dependent isomerization of iso-MTM into MTM. Since Mg²⁺ binds the deprotonated species, the proposed mechanism is consistent with the observed pH dependence.

The last step in MTM biosynthesis is catalyzed by ketoreductase MtmW. As judged by S-200 size-exclusion chromatography, the assembly state of MtmW was most consistent with a 290 kDa octamer (Supplemental Figure 14). However, we could not rule out a tetrameric assembly, because of a limited resolving power of this method for proteins in this size range. Analytical ultracentrifugation of MtmW resolved this ambiguity and yielded a sedimentation coefficient of 10.8S (Supplemental Figures 15 and 16), in excellent agreement with the value of 11.8S for an octameric state of MtmW modeled as an oblate spheroid. Structurally and biochemically characterized AKR superfamily proteins that are most similar in sequence to MtmW include an octameric 3-hydroxybutanal reductase STM2406 from *Salmonella enterica* serovar Typhimurium (36% sequence identity; Supplemental Figure 1)^[20] and a tetrameric cytosolic β subunit (Kv β) of the Shaker family of voltage-dependent potassium channels (Kv1) in vertebrates (34% sequence identity).^[21]

We crystallized MtmW (N3) in two crystal forms: the apo-MtmW crystallized in one form (diffracting to 1.8 Å resolution) and its complex with NADP⁺ in the other (2.1 Å resolution), with a similar crystal packing (Supplemental Table 3). We determined the crystal structures of these two states of MtmW. The structure of an MtmW monomer (Figure 7) consists of a common TIM barrel fold, which makes up most of the protein, a small N-terminal two-stranded β -sheet capping the TIM barrel and two extra helices outside of the barrel. Other short helices were also present, but their degree of disorder varied among monomers of the same crystal form and between the two forms. The apo-MtmW crystals contained two MtmW monomers per asymmetric unit, with each monomer generating its own tetramer by crystal symmetry operations. In one monomer, two loops spanning residues 26-30 and 224-238 were not observed in the electron density map due to disorder. In the other monomer, in addition to two analogous loops (residues 24-30 and 218-247), a short loop spanning residues 55-56 was also disordered. The disordered loops are located on the same face of the protein (in the back of the structure in Figure 7).

The structure shows that the monomers of MtmW form a well packed homotetramer (colored in Figure 8A), in which both the monomer fold and the tetrameric assembly are highly conserved in STM2406 and similar aldo-keto reductases as well as in Kv β proteins. The tetramers in both crystal forms are loosely packed against each other (Figure 8B) so that their molecule, with the 4-fold rotational symmetry axis running along the middle of the channel (Figure 8A). The two helices lying outside of the TIM barrels are located on the periphery of the tetrameric scaffold. The crystal structure of MtmW in complex with NADP⁺ showed that the cofactor was bound on the outside of the channel to each monomer (Figure 8), where the access to the nicotinamide moiety of NADPH would occur between the tetramer subunits. The nicotinamide moiety of the bound NADP⁺ exits into a large cleft (Figures 8 and 9), where substrate binding and catalysis must take place. The NADP⁺ binding interface is highly conserved among homologous proteins of known structure. As

predicted from the sequence alignment, the catalytic tetrad universally conserved in the AKR is also present in MtmW at proper positions (Supplemental Figure 1; conserved catalytic Tyr57 is shown in Supplemental Figure 17).

In addition to these crystals, extensive crystallization trials were carried out to obtain crystals of MtmW in complex with MTM and iso-MTM. While we did not observe either of these ligands in the active site of MtmW, the crystals that were grown with MtmW preincubated with iso-MTM and NADP⁺ yielded strong tubular electron density in the putative substrate binding cleft that was best modelled by a terminal region of a PEG molecule, in addition to a well resolved NADP⁺ molecule (Figure 9, Supplemental Figures 17A, 18). The PEG is bound in the substrate binding pocket because of its proximity to the nicotinamide ring of NADP⁺ and Tyr57. The structurally analogous substrate binding pocket STM2406 (Supplemental Figure 17, panels B and C) was functionally validated by mutagenesis.^[20] The loops that were disordered in the apo- and NADP⁺-bound crystal structures were all ordered in the MtmW-NADP⁺-PEG crystal structure. These conformational changes are likely coupled to MtmOIV binding, which we observed to be enhanced in the presence of NADPH and PMB. The PEG is bound in a hydrophobic pocket, which is large enough to accommodate the tricyclic mithramycin core and the 3-side chain. Some of the residues lining the pocket are located on the flexible loops: Leu24, Tyr26, Pro27, Val56, Tyr57, others include Met86, and Pro87. The cleft defined by the nicotinamide ring of NADP⁺ contains charged residues Glu308, Arg125, Glu155, consistent with the polar nature of the 3-side chain of MTM.

In vertebrate Kv1 channels, each Kv β subunit forms a complex with a T1 domain of the integral membrane β subunit responsible for the functional tetrameric assembly of the ion channel.^[21-22] T1 is bound to Kv β on the face of the tetramer analogous to the solvent exposed face of the MtmW octamer (Supplemental Figure 19, top tetramer in Figure 8B). Kv β is also an active NADPH-dependent reductase whose dominant endogenous substrate is yet unknown, but the change in the redox state, even when induced by surrogate substrates, has been shown to regulate the channel.^[23] By the same logic, we propose that MtmW, being the ultimate mithramycin biosynthetic enzyme, may be associated with a transmembrane channel to regulate the export of mithramycin.

Conclusion

In this study, we have demonstrated that the MTM producer *Streptomyces argillaceus* employs an unanticipated strategy, where the enzyme-driven biosynthesis ends in production of the inert iso-MTM, rather than MTM, which then non-enzymatically converts to the toxic MTM species. Mechanistically, reductase MtmW must interact with the Baeyer-Villiger monooxygenase MtmOIV to form a complex, to efficiently convert biologically inactive, non-toxic PMB into the still inert iso-MTM. Our enzymatic assays and the EMSA yielded strong evidence for a physical functional complex of MtmOIV and MtmW. Because MtmW resembles a cytoplasmic subunit of a potassium channel, we propose that it may be directly coupled to the iso-MTM export through the heterodimeric efflux pump MtrA-MtrB. The formation and export of an inert precursor, rather than the toxic MTM, would explain self-resistance of the producing organism to the toxic MTM. Once secreted, iso-MTM can be

converted to bioactive MTM in the presence of environmental Mg^{2+} , as we demonstrated. This self-resistance mechanism appears to be an unusual strategy, facilitated by the formation of a complex of biosynthetic enzymes potentially interacting with a membrane transport channel to avoid damage by the active natural product to the host cells. Analogy for this model can be found in the actinorhodin biosynthesis in *Streptomyces coelicolor*.^[24] The diffusible, gamma form of actinorhodin is only found after passage through an RND transporter and in conjunction with a basic extracellular environment. A similar transporter-mediated catalysis may also be present in the MTM pathway, as iso-MTM has not been found in *S. argillaceus* cultures. Further studies of MtrA-MtrB and its interaction with MtmW will be necessary to test this hypothesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

HPLC chromatograms of reactions catalysed by MtmW and MtmOIV at pH 8.25 (50 mM Tris-HCl pH 8.25, 500 μ M NADPH, 250 μ M FAD, 5 μ M MtmOIV, 15 μ M MtmW, 300 μ M PMB (**3**) or MTM (**1**), as indicated). A) Pure PMB as standard; B) pure MTM as standard; C) PMB + MtmOIV + MtmW + FAD + NADPH; D) PMB + MtmOIV + FAD + NADPH; E) PMB + MtmOIV + boiled MtmW + FAD + NADPH; F) PMB + MtmW + FAD + NADPH.

iso-mithramycin (**10**)

C2-H: δ 4.49 (d, J = 6.7 Hz)

Figure 2.
Determination of the C2 stereochemistry of **10**.

Figure 3.

HPLC chromatograms of reactions catalysed by MtmW and MtmOIV. The conditions were the same as in Figure 1C with the exception that MtmW was added to the MtmOIV reaction in different ways. A) MtmW was added together with MtmOIV (this trace is the same as in Figure 1C); B) MtmW was added after 1 h of the MtmOIV reaction; C) MtmW was added after 1 h of the MtmOIV reaction and removal of MtmOIV by filtration.

Figure 4.

HPLC chromatogram of reactions catalysed by MtmW and MtmOIV at pH 7.5 (the other conditions are described in Figure 1). A) PMB + MtmOIV + MtmW + FAD + NADPH; B) PMB + MtmOIV + FAD + NADPH; C) PMB + MtmOIV + FAD + NADPH incubated for 15 min, then MtmOIV was removed and MtmW was added; D) PMB + MtmW + FAD + NADPH.

Figure 5.

A) A Coomassie blue stained 1.7% agarose gel showing the EMSA with MtmOIV (20 μ M in monomers, 10 μ M in dimers) and MtmW (20 μ M in monomers, 2.5 μ M in octamers). The smear above the MtmW band in lanes 3-6 indicated that MtmW was retarded by binding MtmOIV. B) A similar gel showing a titration of MtmW (10 μ M) with MtmOIV. The fraction of free MtmW is shown below each lane.

Figure 6.
Mechanism of the conversion of iso-MTM to MTM.

Figure 7.

A monomer of MtmW. A view into the TIM barrel is shown, with the short N-terminal β -sheet capping the barrel. The N- and C-termini are labeled as "N" and "C", respectively.

Figure 8.

Two orthogonal views of the MtmW octamer of the MtmW-NADP⁺ complex. A) The view into the inter-subunit channel. B) The view where the channel is oriented along the vertical axis. The bound NADP⁺ is shown by purple sticks.

Figure 9.

The crystal structure of MtmW-NADP⁺-PEG complex. NADP⁺ is shown by purple sticks, PEG (orange sticks) is well defined by the polder omit electron density map, contoured at 5.5 σ (shown as a mesh). The residues in the putative substrate binding site are shown as sticks with the labels. The orientation of this MtmW monomer is the same as that of the similarly colored monomer in Figure 8A.

Scheme 1.

Biosynthesis of mithramycin, with involved intermediates and shunt products.