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Brief Communication

Biosynthetic Studies and Genetic Engineering of Pactamycin Analogs with Improved Selectivity toward Malarial Parasites

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SUMMARY

Pactamycin, one of the most densely functionalized aminocyclitol antibiotics, has pronounced antibacterial, antitumor, antiviral, and antiplasmodial activities, but its development as a clinical drug was hampered by its broad cytotoxicity. Efforts to modulate the biological activity by structural modifications using synthetic organic chemistry have been difficult because of the complexity of its chemical structure. However, through extensive biosynthetic studies and genetic engineering, we were able to produce analogs of pactamycin that show potent antimalarial activity, but lack significant antibacterial activity, and are about 10–30 times less toxic than pactamycin toward mammalian cells. The results suggest that distinct ribosomal binding selectivity or new mechanism(s) of action may be involved in their plasmodial growth inhibition, which may lead to the discovery of new antimalarial drugs and identification of new molecular targets within malarial parasites.

INTRODUCTION

Pactamycin is a structurally unique antitumor antibiotic isolated from the soil bacterium *Streptomyces pactum* (Bhuyan, 1962). It shows potent antimicrobial (Bhuyan, 1962), antitumor (White, 1962), antiviral (Taber et al., 1971), and antiprotozoal (Otoguro et al., 2010) activities, and literally affects cell growth of all three phylogenetic domains, eukarya, bacteria, and archaea. Its broad-spectrum growth inhibitory activity is mainly due to its ability to bind a conserved region within the 30S ribosomal subunit of most organisms, inhibiting the translocation of certain mRNA-tRNA complexes and blocking protein synthesis (Brodersen et al., 2000; Dinos et al., 2004). Although pactamycin was first reported in the 1960s and its various biological activities have been extensively investigated, further development of this compound was hampered by its wide-ranging cytotoxicity. Moreover, its complex chemical structure has rendered its structural modification via synthetic organic chemistry difficult.

The biosynthetic origin of pactamycin had been studied by feeding experiments with isotopically labeled precursors (Rinehart et al., 1981; Weller and Rinehart, 1978). It has been suggested that the five-member ring aminocyclitol moiety of pactamycin is derived from glucose, whereas 6-methyl salicylic acid (6MSA) is derived from acetic acid. The 3-aminoacetophenone moiety is derived from an unknown branch of the shikimate pathway (Rinehart et al., 1981). More insights into the biosynthesis of pactamycin have emerged from the recent identification of the biosynthetic gene cluster of pactamycin in *S. pactum* (Ito et al., 2009; Kudo et al., 2007). On the basis of the latter study, it was postulated that PtmC (a radical SAM enzyme), PtmJ (a glycosyltransferase), and PtmG (a putative deacetylase) are involved in the formation of the cyclopentitol core (**I**) (Figure 1A), which is then converted to compound **II** through isomerization and aminotransferation. However, little is known about the mode of formation of this interesting aminocyclopentitol core structure.

No less intriguing an aspect of pactamycin biosynthesis is its high degree of tailoring modifications (e.g., *N*-carbamoylation, *N*-methylation, *C*-methylation, hydroxylation, and 6MSA attachment), which are all confined within the highly compacted core structure (Figure 1A). Feeding experiments using ^{13}C-methionine revealed that five methylation events are involved in pactamycin biosynthesis, three of which are *C*-methylations and two are *N*-methylations (Weller and Rinehart, 1978). Most remarkably, the C-7/C-8 hydroxyethyl moiety is derived from two units of methionine through "tandem" *C*-methylations. *C*-Methylation also occurs at the neighboring C-5 position of the core cyclopentitol unit. In silico analysis of the pactamycin cluster suggests that these *C*-methylations may be catalyzed by radical SAM-dependent enzymes. Similar enzymes have been proposed to play a role in the biosynthesis of clorobiocin (Westrich et al., 2003), fortimicin KL1 (Kuzuyama et al., 1995), fosfomycin (Higgins et al., 2005), mitomycin (Mao et al., 1999), and many other important primary and secondary metabolites (Sofia et al., 2001). Such tailoring enzymes are certainly important from a pharmaceutical point of view, because inactivation of these enzymes may result in pactamycin analogs that have been deemed difficult to obtain by synthetic organic chemistry. Herein, we describe the functional analysis of the putative tailoring genes in *S. pactum* ATCC 27456 and postulate their roles in pactamycin biosynthesis. In addition, the study resulted in a number of pactamycin analogs that showed potent

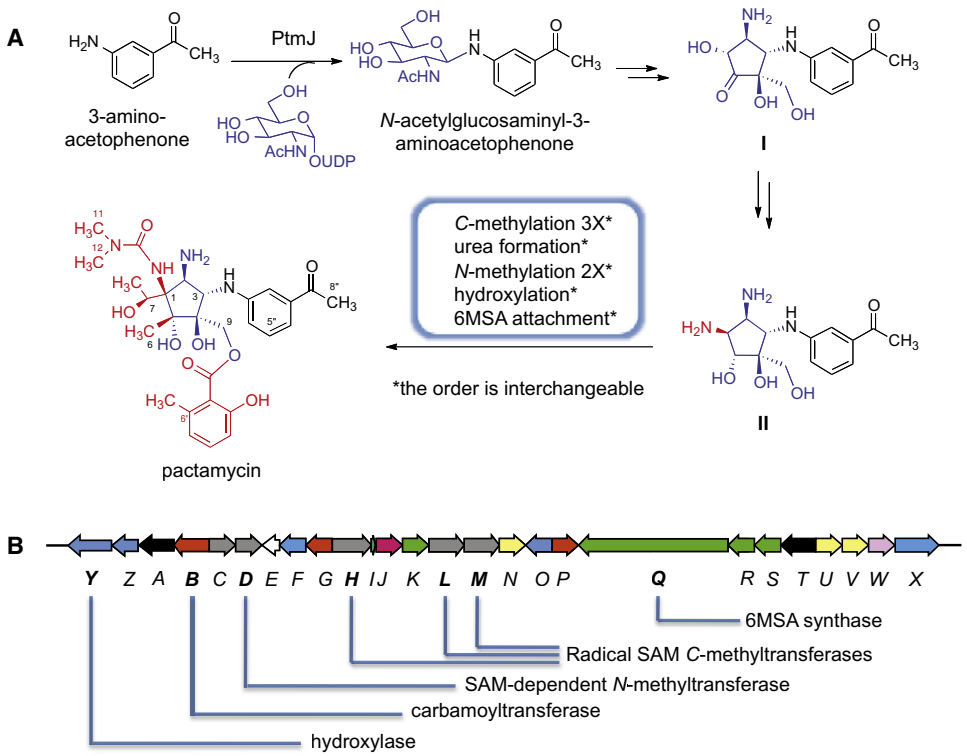

Figure 1. Biosynthesis of Pactamycin

(A) A synopsis of the proposed biosynthetic pathway to pactamycin is shown.

(B) The core biosynthetic gene cluster of pactamycin and the putative tailoring genes are shown.

For additional details supporting data from this figure, see Figure S1.

antimalarial activity, but in contrast to pactamycin, have no significant antibacterial activity and reduced cytotoxicity against mammalian cells.

RESULTS AND DISCUSSION

Inactivation of the Carbamoyltransferase PtmB

Structurally, pactamycin consists of an aminocyclopentitol unit, two aromatic rings, and a 1,1-dimethylurea. The urea moiety may be derived from *N*-carbamoylation of the C-1 amino group. A gene encoding a protein with high identity to carbamoyltransferases has been identified within the pactamycin cluster and is predicted to mediate this reaction (Ito et al., 2009). Interestingly, the protein (PtmB) is more similar to carbamoyltransferases that catalyze *O*-carbamoylation than those that catalyze *N*-carbamoylation. To investigate whether PtmB is involved in pactamycin biosynthesis, the gene was inactivated by targeted in-frame deletion. Analysis of the culture broth of the Δ*ptmB* strain revealed that the mutant was not able to produce pactamycin, suggesting a direct involvement of *ptmB* in pactamycin biosynthesis. However, no other pactamycin-related products could be detected in the samples. Complementation experiments by introducing into the Δ*ptmB* strain an integrative plasmid harboring the intact *ptmB* gene resulted in conjugants that were able to produce pactamycin, albeit in low yields, eliminating the possibility of polar effects in the Δ*ptmB* strain.

Formation of the *N,N*-Dimethylurea Unit

Further modification of the urea functionality to *N,N*-dimethylurea may be catalyzed by SAM-dependent *N*-methyltransferases. However, because *ptmD* is the only *N*-methyltransferase gene present in the cluster, it is predicted to play a role in both *N*-methylation reactions. To test this hypothesis, we inactivated *ptmD* in *S. pactum* and analyzed the antibiotic production by LC-MS. As expected, the Δ*ptmD* strain was not able to produce pactamycin or 7-deoxypactamycin, but instead produced two new metabolites with *m/z* 531 $(M+H)^+$ and *m/z* 515 $(M+H)^+$ (Figure 2B). MS/MS analysis of the products gave fragmentation patterns that are consistent with those predicted for *N,N*-didemethylpactamycin and *N,N*-didemethyl-7-deoxypactamycin, respectively (see Figure S2 available online), indicating that PtmD is responsible for the *N*-methylations of the urea moiety.

The Roles of the Fe-S Radical SAM *C*-Methyltransferases

In addition to the carbamoyltransferase PtmB and the *N*-methyltransferase PtmD, the radical SAM-dependent enzymes PtmH, PtmL, and PtmM are believed to be involved in the decoration of the aminocyclopentitol unit. The involvement of these enzymes in pactamycin biosynthesis is particularly fascinating, because they may catalyze methylation of nonreactive carbons within a fairly crowded five-member ring structure. Moreover, one of the enzymes was predicted to catalyze methylation of

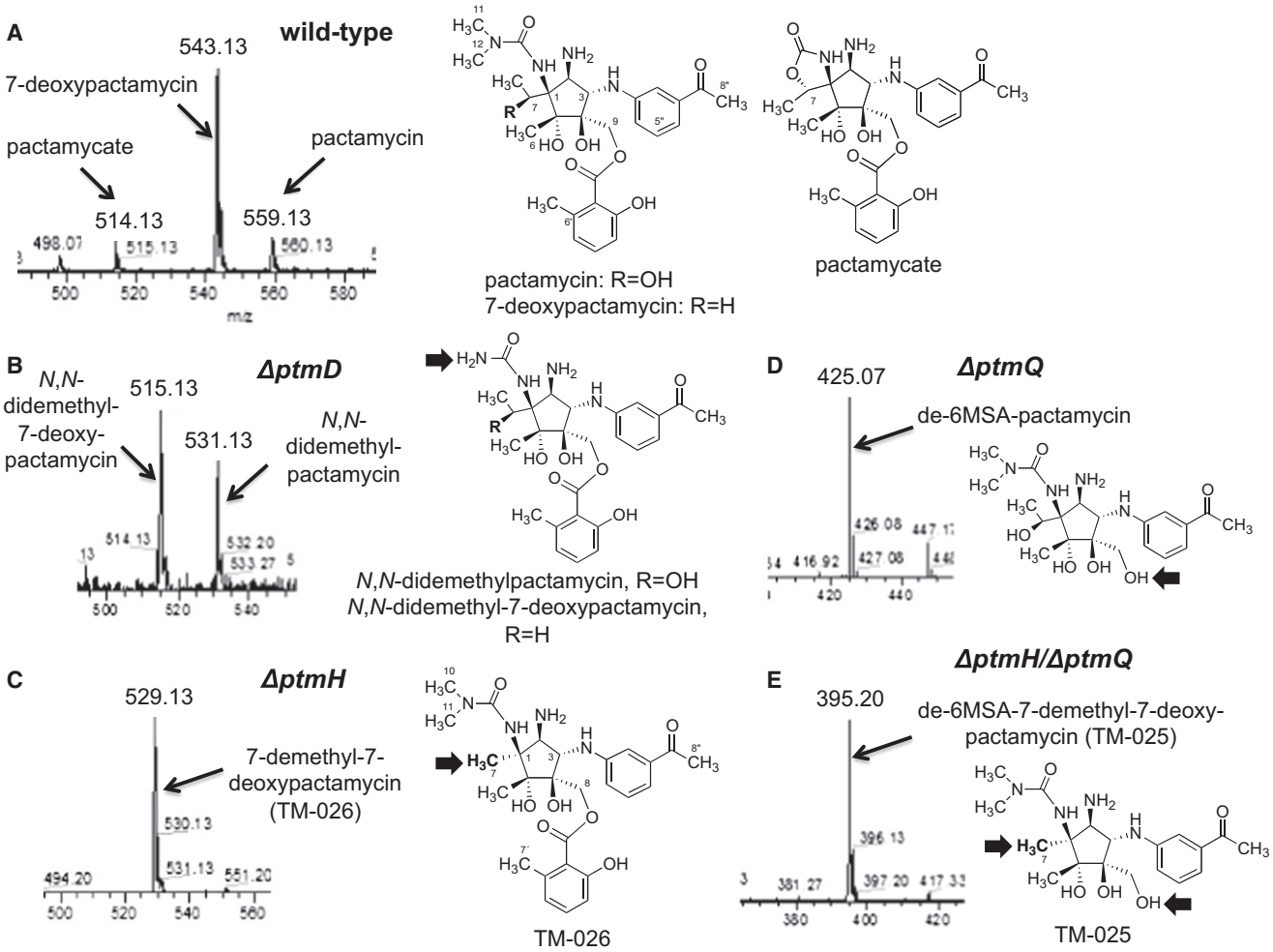

Figure 2. Partial Mass Spectra of the Ethyl Acetate Extracts Obtained from the Culture Broths of the Wild-Type and the Mutant Strains of *S. pactum*

(A–E) Wild-type (A), Δ*ptmD* (B), Δ*ptmH* (C), Δ*ptmQ* (D), and Δ*ptmH*/Δ*ptmQ* (E) strains are shown. Thick arrows indicate positions at which modifications take place. For additional experiments supporting data from this figure, see Figures S2–S4.

a methyl group (C-7), which itself is derived from methionine involving another radical SAM *C*-methyltransferase enzyme. These tandem methylations result in an ethyl moiety, which is then hydroxylated to form the hydroxyethyl side chain.

To explore the roles of those enzymes in pactamycin biosynthesis, we inactivated the genes individually and examined their phenotypes. LC-MS analysis of the culture broths of the mutants revealed the lack of pactamycin production in those strains, suggesting the involvement of *ptmH*, *ptmL*, *and ptmM* in pactamycin biosynthesis. Although Δ*ptmL* and Δ*ptmM* did not give any detectable amount of pactamycin or its analogs, Δ*ptmH* produced a new compound with *m/z* 529 $[M+H]^+$ (Figure 2C). On the basis of 1D and 2D NMR and MS/MS analyses (Figures S2 and S3), the compound was determined to be 7-demethyl-7-deoxypactamycin (TM-026). The results suggest that PtmH is involved in the formation of the hydroxyethyl unit and that hydroxylation occurs after the second methylation. PtmY, which shares high identity with cytochrome P450 monooxygenases, is proposed to play a role in the hydroxylation reaction. However, inactivation of *ptmY* in *S. pactum* resulted in mutant strains that not only produced 7-deoxypactamycin but also a small amount of pactamycin (data not shown). The result thus, unfortunately, did not provide solid evidence for the catalytic function of PtmY, because both compounds are routinely found in different ratios in the culture broths of the wild-type strain. Therefore, either PtmY is not directly involved in the hydroxylation of C-7 or its function in Δ*ptmY* strain was complemented by another monooxygenase. On the other hand, PtmL and PtmM may catalyze the attachment of C-6 and C-7. Inactivation of these genes may have blocked the methylation reactions but, as in the case of Δ*ptmB*, no intermediate compounds were accumulated in the culture broths or in the cells at a detectable level. Complementation experiments by introducing expression plasmids harboring intact *ptmH*, *ptmL*, or *ptmM* into the corresponding mutant strains of *S. pactum* showed that all conjugants were able to produce pactamycin, suggesting that there was no polar effect that obstructed pactamycin biosynthesis in the mutants.

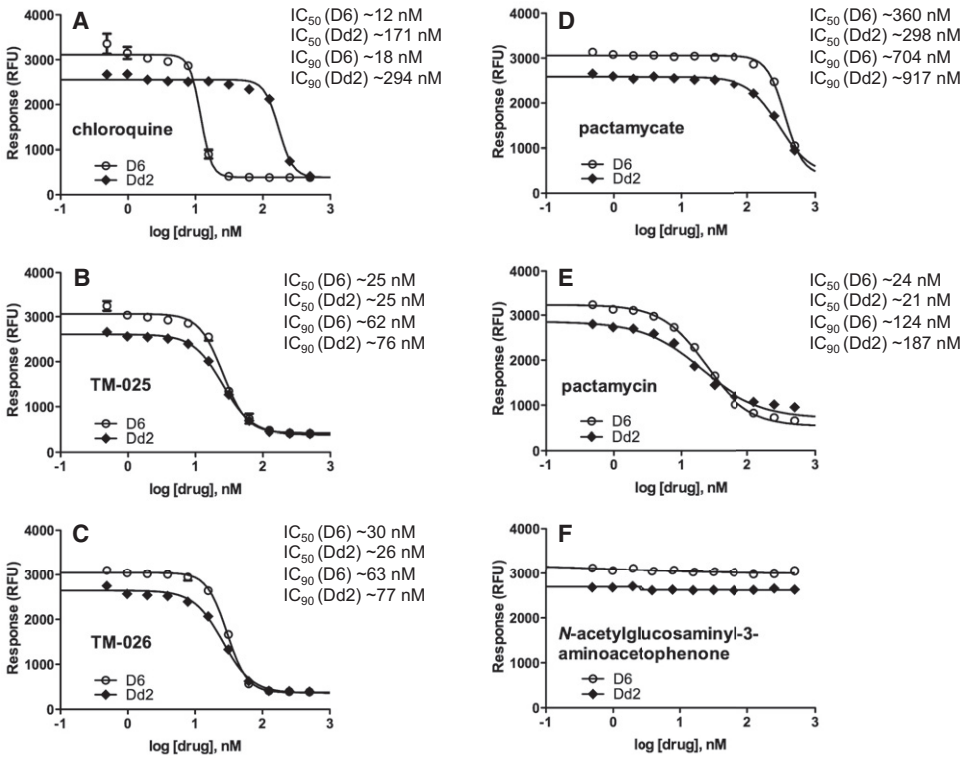

Figure 3. Antimalarial Activity of Pactamycin Analogs against the Chloroquine-Sensitive (D6) and Chloroquine-Resistant (Dd2) Strains of *Plasmodium falciparum*

(A–F) Chloroquine (A), TM-025 (B), TM-026 (C), pactamycate (D), pactamycin (E), and *N*-acetylglucosaminyl-3-aminoacetophenone (F) are shown. All values are presented as relative fluorescence units (RFU). Each value is mean ± SEM of quadruplicate values from a representative experiment. The 50% and 90% inhibitory concentrations (IC_{50} and IC_{90}) were determined by nonlinear regression analysis of logistic dose-response curves (GraphPad Prism software).

Double Knockout of *ptmH* and *ptmQ*

Although the production of TM-026 by the Δ*ptmH* strain revealed the function of *ptmH* in pactamycin biosynthesis, the result was somewhat unexpected. Previously, we had demonstrated that inactivation of the iterative type I polyketide synthase gene *ptmQ* within the cluster effectively halted the formation of 6MSA, and the mutant strain produced de-6MSA-pactamycin and de-6MSA-pactamycate (pactamycin analogs without 6MSA) (Ito et al., 2009). The result had led us to conclude that the attachment of 6MSA occurs last in the pathway. However, the formation in the Δ*ptmH* strain of TM-026 that lacks a methyl and a hydroxy group yet retains the 6MSA moiety suggests otherwise. Moreover, it is also quite puzzling that the product of the Δ*ptmD* strain contains, except for the two *N*-methyl groups, all other functionalities. These results suggest that some tailoring enzymes involved in pactamycin biosynthesis have relaxed substrate specificity. The promiscuity of some of these enzymes not only makes the sequence or the timing of the tailoring processes unclear, but also hinders the production of smaller analogs of pactamycin. To generate less-decorated pactamycin analogs, we constructed a double gene knockout mutant, in which both *ptmH* and *ptmQ* are inactivated. As expected, mutant strains resulting from this double gene knockout produced another new analog of pactamycin with *m/z* 395 $[M+H]^+$, which, on the basis of the careful structural characterization using 1D and 2D NMR and MS/MS analyses, was identified as de-6MSA-7-demethyl-7-deoxypactamycin (TM-025) (Figure 2E; Figures S2 and S4).

Antimalarial Activity of TM-025 and TM-026

During our initial screening for new antimalarial natural products, we found that de-6MSA-pactamycin demonstrated a potent activity against both chloroquine-sensitive and -resistant strains of *Plasmodium falciparum* at low nanomolar concentrations (data not shown). However, similar to pactamycin, de-6MSA-pactamycin also showed significant antimicrobial activity and high cytotoxicity against mammalian cells (Ito et al., 2009). More recently, Otoguro and co-workers reported a similar finding, in which pactamycin and 7-deoxypactamycin showed high activities against both the drug-resistant K1 and drug-susceptible FCR3 strains of *P. falciparum* (Otoguro et al., 2010). They noted that 7-deoxypactamycin, which lacks a hydroxy group at C-7, was 40 times more active than pactamycin. However, its cytotoxicity was also much higher than that of pactamycin. The new compounds TM-025 and TM-026, which were subjected to testing against *P. falciparum* D6 (chloroquine sensitive) and Dd2 (chloroquine resistant) strains, showed pronounced antimalarial activity with IC_{50} values between 25 and 30 nM against both strains, on a par with pactamycin (Figure 3). However, the IC_{90} values for TM-025 and TM-026 (62–77 nM) were significantly lower than those for pactamycin (124 and 187 nM), particularly against the Dd2 strain, indicating

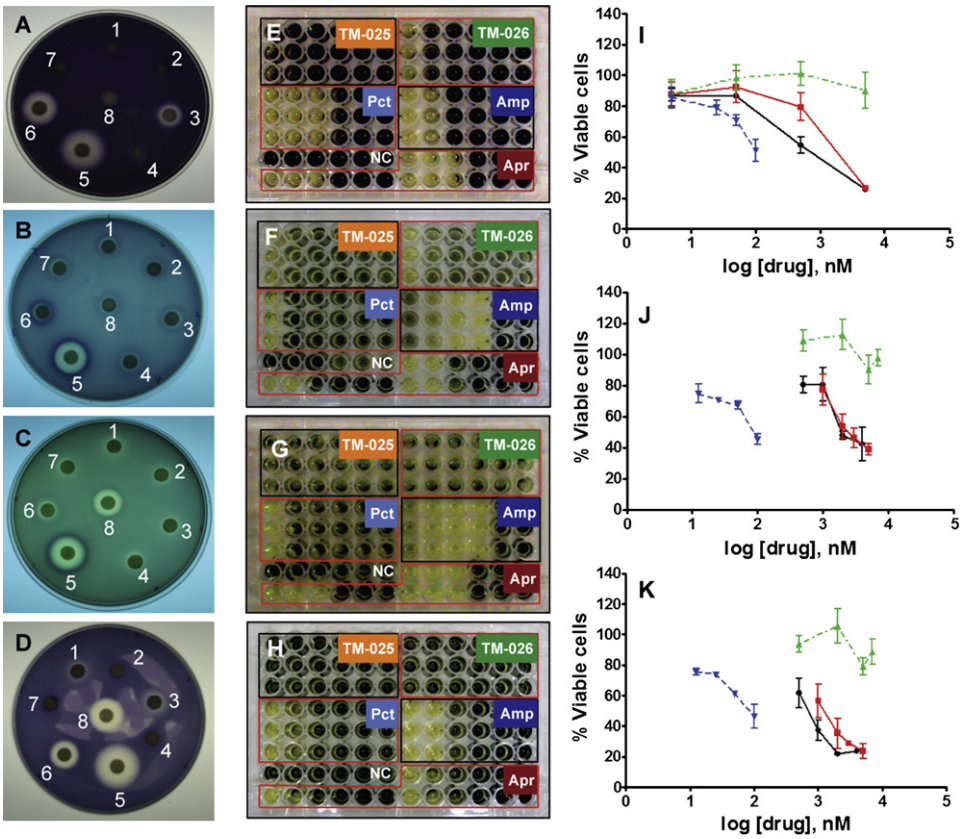

Figure 4. Antibacterial and Cytotoxicity Assays of Pactamycin Analogs

(A–D) Agar-diffusion assay of pactamycin analogs against *S. aureus* (A), *B. subtilis* (B), *P. aeruginosa* (C), and *E. coli* (D) are shown. **1**, 20 μl 10 mM TM-025; **2**, 20 μl 1 mM TM-025; **3**, 20 μl 10 mM TM-026; **4**, 20 μl 1 mM TM-026; **5**, 20 μl 10 mM pactamycin; **6**, 20 μL 1 mM pactamycin; **7**, 20 μl 10 mM *N*-acetyl-glucosaminyl-3-aminoacetophenone; **8**. 5 μl 1 mg/ml ampicillin.

(E–H) Microdilution assay of pactamycin analogs against *S. aureus* (E), *B. subtilis* (F), *P. aeruginosa* (G), and *E. coli* (H) are shown. **TM-025**: 1 mM–10 nM; **TM-026**: 1 mM–10 nM; **Pct** (pactamycin): 0.5 mM–10 nM; **Amp** (ampicillin): 10 mg/ml–0.1 μg/ml; **Apra** (apramycin)**:** 5 mg/ml–50 ng/ml; **NC**, negative control. Molar concentrations were used for pactamycin analogs to reflect more accurate comparisons between those compounds.

(I–J) Cytotoxicity assay of pactamycin analogs against HCT116 cells using broad-range concentrations at 48 hr (I), narrow-range concentrations at 24 hr (J), and narrow-range concentrations at 48 hr (K). Blue triangles represent pactamycin, black circles represent TM-025, red squares represent TM-026, and green triangles represent *N*-acetyl-glucosaminyl-3-aminoacetophenone. All values are presented as percentage of viable cells, which was calculated relative to the no treatment and solvent only wells. Each value is mean ± SEM of triplicate values from a representative experiment.

some superiority of the new analogs in their antimalarial activity over the parent compound. The results also suggest a possible acquired resistance within the parasites against pactamycin (Figure 3E). As a comparison, the less active analog pactamycate showed IC_{50} and IC_{90} values at around 300 nM and 800 nM, respectively, against both strains. Moreover, the inhibitory patterns of TM-025 and TM-026 appeared to be somewhat different from that of pactamycin, which suggests that the new analogs may inhibit plasmodial growth via a distinct mechanism of action. The putative pactamycin biosynthetic precursor *N*-acetylglucosaminyl-3-aminoacetophenone was also tested, and its lack of activity suggests that the antimalarial activity is not due to the aminoacetophenone moiety.

Antibacterial Activity of TM-025 and TM-026

Most surprising results emerged from the antibacterial assays. Although pactamycin demonstrates a strong antibacterial activity against both Gram-positive and -negative bacteria, neither TM-025 nor TM-026 showed any significant activity in the agar diffusion and microdilution assays at the concentrations used (Figures 4A–4H). The IC_{50} values of pactamycin were about 10 μM against *Staphylococcus aureus*, 500 μM against *Pseudomonas aeruginosa* and *Bacillus subtilis*, and 100 μM against *Escherichia coli*, whereas the IC_{50} values of TM-025 and TM-026 were consistently higher (mostly >1 mM) against all tested bacterial strains. The results suggest that the new analogs have less affinity to the bacterial ribosome or interact with it in a less damaging fashion than pactamycin.

Cytotoxic Activity of TM-025 and TM-026

To determine the effect of TM-025 and TM-026 on mammalian cells, their cytotoxic activity was evaluated and compared to that of pactamycin in a WST-8–based assay using HCT116 human colorectal cancer cells. The results revealed that the

new analogs are significantly less toxic than pactamycin, with estimated IC_{50} values between 1000 and 3000 nM, or about 10–30 times higher than that of pactamycin (IC_{50} ~100 nM) (Figures 4I–4K). The results support the notion that the new analogs have less affinity toward the ribosome of mammalian cells, or their antimalarial activity is the result of a different mechanism of action. Further work is currently under way to determine the structure-activity relationship of this untapped class of natural products and to unveil their mechanism(s) of action against plasmodium parasites.

SIGNIFICANCE

Pactamycin is one of the most densely functionalized aminocyclitol antibiotics, with pronounced biological activities. Literally, it affects cell growth of all three phylogenetic domains; therefore, despite its potent biological activity, it was not considered to be a viable drug lead. However, the identification of the pactamycin cluster and the development of gene inactivation methodologies in *Streptomyces pactum* in our laboratory have made it possible for us to investigate the biosynthesis of pactamycin at the molecular level and to employ genetic engineering technologies to produce analogs with improved biological properties. The data presented here highlight the importance of biosynthetically based structure modification methodologies in generating pactamycin analogs that have been deemed inaccessible by synthetic organic chemistry. The analogs are structurally less complex and more stable than pactamycin and show potent antimalarial activity, but, in contrast to pactamycin, have significantly reduced antibacterial activity and cytotoxicity against mammalian cells. The results suggest that the compounds either have higher selectivity toward the plasmodial ribosome, or new mechanism(s) of action in plasmodial growth inhibition are involved, which could lead to a new direction in the discovery and development of drugs against malaria and other life-threatening protozoal infections.

EXPERIMENTAL PROCEDURES

Bacterial strains (Table S1), plasmids (Table S1), primers (Table S2), general DNA manipulations, complementation experiments, compound purification, structure elucidations, and biological assays are described in Supplemental Experimental Procedures.

Construction of the ptmB, ptmD, ptmH, ptmL, ptmM, ptmY, and ptmH/ptmQ Knockout Mutants

Two ~1 kb PCR fragments upstream and downstream of the genes were fused and cloned into the HindIII/XbaI site of pBluescript II SK(-) vector. For *ptmB*, the products were subsequently excised with HindIII and XbaI, treated with Klenow enzyme, and ligated into pTMW018 (predigested with BamHI and treated with Klenow enzyme) to create pTMW025 (Table S1). For *ptmD*, *ptmH*, *ptmL*, *ptmM*, and *ptmY*, the products were excised and cloned into the HindIII/XbaI site of pTMN002 to create pTMN003, pTMW026, pTMW027, pTMN004, and pTMN005, respectively. All plasmids were then individually introduced into *S. pactum* ATCC 27456 by conjugation using the *E. coli* donor strain ET12567/pUZ8002. For *ptmH/ptmQ* mutant, the plasmid pTMN001 (Ito et al., 2009) was transferred into *E. coli* ET12567/pUZ8002 and subsequently was introduced into the Δ*ptmH* strain by conjugation. Screening and analysis of the mutants were performed as described elsewhere (Ito et al., 2009).

Analysis of Metabolites from the ptmB, ptmD, ptmH, ptmL, ptmM, ptmY, and ptmH/ptmQ mutants

The mutant strains of *S. pactum* were cultured in modified Bennet medium and the products were extracted and analyzed as described in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chembiol.2011.01.016.

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