Anthracycline biosynthesis in Streptomyces: engineering, resistance and antimicrobial activity

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General discussion

Anthracyclines are important anticancer drugs, despite severe dose-limiting side effects. Since their discovery in the 1950s[28], many efforts have been directed toward developing detoxified anthracyclines with better efficacy. More than 500 naturally occurring anthracyclines have been isolated from Actinobacteria[172,173,266], yet only daunorubicin (Dauno, Figure 1), doxorubicin (Doxo, Figure 1) and aclarubicin (Acla, Figure 1) are used in the clinic for cancer treatment[13]. Metabolic engineering strategies have yielded numerous derivatives mostly via combinatorial biosynthesis[216] and glycodiversification[105]. Moreover, comprehensive structural libraries of anthracyclines have been prepared through organic synthesis[17–19,378]. Despite these efforts, only semi-synthetic epirubicin, idarubicin, pirarubicin and valrubicin have been successfully introduced into the clinic[87].

Recent insights into the mode-of-action of anthracyclines have prompted renewed interest into this important class of anticancer compounds (**Chapter 2**). One of the most promising compounds is *N,N*-dimethyldoxorubicin (DMdoxo, Figure 1), a hybrid structure that combines the aglycone of Doxo with the amino sugar moiety of Acla, generated through semi-synthesis[16]. DMdoxo has potent cytotoxic activity but does not induce cardiotoxicity, the most acute and treatment-limiting side effect of Doxo[16]. Evaluation of anthracycline structural libraries indicated that the presence of a tertiary amine on the first sugar moiety of anthracyclines generally corresponds to the loss of DNA-damaging activity combined with improved cytotoxicity[17–19]. These results are promising for the development of detoxified anthracyclines.

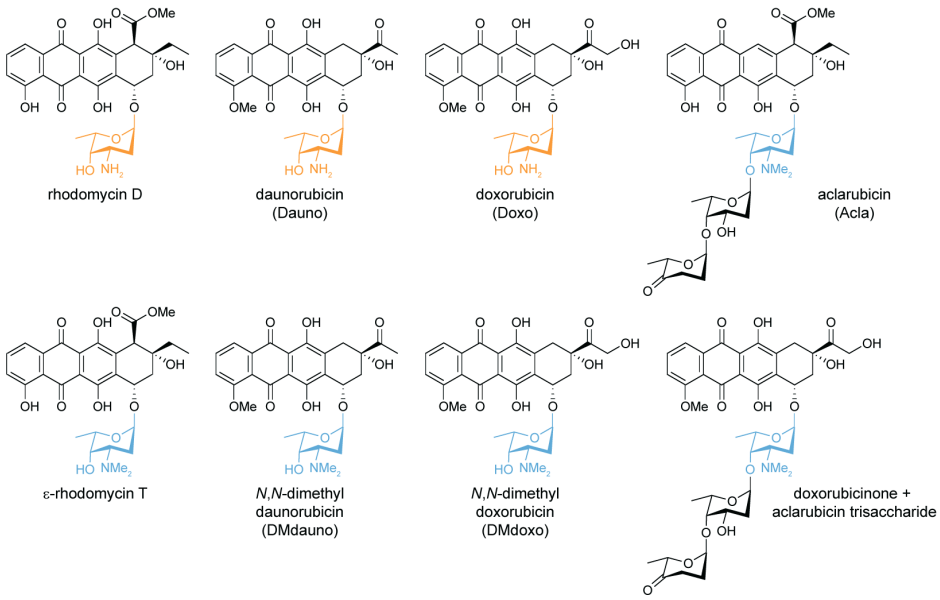

Figure 1. Chemical structures of relevant anthracyclines. The amino sugar L-daunosamine is represented in orange and the *N,N*-dimethylated derivative L-rhodosamine is represented in blue.

For clinical applications, Doxo is currently produced via a combination of fermentation and organic chemistry. In the first step, Dauno is obtained biosynthetically via fermentation of an industrial *Streptomyces peucetius* strain. Subsequently, biosynthetic Dauno is converted to Doxo via chemical 14-hydroxylation. Doxo can be obtained fully biosynthetically, but the final tailoring enzymatic reaction catalysed by the cytochrome P450 monooxygenase DoxA is notoriously inefficient[153], resulting in low yields. To date, DMdoxo has not been isolated from natural sources, but biosynthesis would be an attractive option for scaling up production of this promising compound.

For this reason, we aimed to engineer the Doxo biosynthetic pathway in *S. peucetius* for the production of biosynthetic *N,N*-dimethylated anthracyclines (**Chapter 3**). The challenges that we encountered instigated the study of anthracycline resistance mechanisms in *Streptomyces* (**Chapter 4**), and potential applications of detoxified anthracyclines as antibiotics (**Chapter 5**). Finally, to facilitate future screening and strain engineering efforts of *Streptomyces* producer strains, we developed a workflow for integrated quantitative proteomics and metabolomics from small-scale *Streptomyces* cultures (**Chapter 6**).

Challenges in engineering anthracycline biosynthesis

Combinatorial biosynthesis of anthracyclines has been widely applied for the generation of anthracycline hybrids in *Streptomyces*[216,266]. Especially the Acla-producer *Streptomyces galilaeus* has been focus of many studies, mainly via the introduction of tailoring enzymes from other anthracycline biosynthetic pathways[115,217–222,224]. Alternatively, anthracycline biosynthetic genes have been expressed in heterologous hosts, such as *Streptomyces venezuelae*[231,232], *Streptomyces coelicolor*[109] and *Streptomyces lividans*[109,117], that do not naturally produce anthracyclines.

Our approach to achieve biosynthesis of DMdauno and DMdoxo involved modifying the Doxo biosynthetic pathway in the industrial *S. peucetius* strain G001 (**Chapter 3**). We introduced the methyltransferase genes essential for TDP-L-rhodosamine biosynthesis, along with the corresponding glycosyltransferase genes from *S. galilaeus*, in G001. To abolish the production of non-dimethylated anthracyclines, we deleted *dnrS* encoding the Doxo glycosyltransferase. Subsequently, expression of the rhodomycin methyltransferase gene *rdmC* resulted in the successful production of DMdauno, although with limited productivity.

Our data showed that the presence of the rhodosamine moiety inhibits the three consecutive hydroxylation reactions required to form the Doxo aglycone structure. These reactions are catalysed by the cytochrome P450 monooxygenase DoxA, an enzyme unique to the Doxo pathway[266]. It has long been known that the final reaction in the biosynthetic pathway from Dauno to Doxo is extremely inefficient[153,254]. In fact, this is the reason why Doxo is currently produced semi-synthetically from daunorubicin in industry. Enzymatic assays of DoxA with *N,N*-dimethylated compounds showed significantly lower activity than with its natural substrates, indicating that this enzyme is the bottleneck for biosynthetic production of

DMdoxo. One potential solution could involve engineering of DoxA for improved activity. However, attempts to determine the structure of DoxA have thus far been unsuccessful, complicating rational engineering approaches. Alternatively, high-throughput methods using random or model-guided mutagenesis may prove successful in identifying DoxA variants with improved activity[379]. Another potentially limiting factor for DMdoxo biosynthesis is the significantly stronger toxicity of *N,N*-dimethylated anthracyclines to *S. peucetius* compared to the non-dimethylated variants. This finding motivated us to delve into anthracycline resistance in *Streptomyces*.

Cryptic anthracycline resistance genes in *Streptomyces*

Self-resistance mechanisms against toxic natural products are often encoded within biosynthetic gene clusters (BGCs)[57,323]. Anthracycline BGCs typically harbour ATP-binding cassette (ABC) transporter genes to support the efflux of the toxic metabolites[266]. Besides that, the presence of antibiotics in the environment resulted in the evolution or acquisition of highly specific resistance mechanisms against exogenous antibiotics[316,317]. In **Chapter 4**, we explored resistance against anthracyclines in actinomycetes via screening of our in-house strain collection and adaptive laboratory evolution experiments.

Screening of our in-house MBT collection of Actinobacteria revealed that while Doxo resistance was relatively common, all strains were highly sensitive to DMdoxo. Selected streptomycetes were challenged with DMdoxo in an adaptive laboratory evolution experiment, which resulted in strongly increased anthracycline resistance within five generations. Transcriptomics analysis of a DMdoxo-resistant *S. lividans* TK24 isolate revealed upregulation of two ATP-binding cassette (ABC) transporter gene pairs, *cdtAB* and *sclAB*. CdtAB and SclAB are homologous to the DrrAB transporter in the Dauno BGC that facilitates the efflux of Dauno and Doxo[64]. We detected SNPs in the promotor regions of *cdtAB* and *sclAB*, and within the coding regions of the corresponding regulatory genes *cdtR* and *sclR*. SclAB has been previously identified in a similar evolution experiment where *S. lividans* was challenged with the macrolide antibiotic spiramycin[305]. Deletion of the downstream TetR-family regulatory gene, *sclR*, or overexpression of *sclAB* provided mild resistance to various antibiotics, including daunorubicin[305,313]. Our work indicates that both CdtAB and SclAB provide particularly strong resistance to anthracyclines. Introducing expression cassettes containing *cdtAB* or *sclAB*, or disrupting *cdtR* or *sclR* in the parental strain, resulted in more than eight-fold increase in resistance to both Doxo and the stronger antibiotic DMdoxo. Homologs of the CdtAB and SclAB transporters occur frequently within the family of Streptomycetaceae, and their presence is not correlated to the occurrence of putative anthracycline BGCs.

Interestingly, in *S. lividans* the expression of the transporters was not directly activated during challenge with anthracyclines, but only via the accumulation of spontaneous mutations after prolonged exposure to the toxic compound DMdoxo. These findings show that apart from cryptic BGCs encoding undiscovered natural products, *Streptomyces* genomes also contain cryptic resistance mechanisms.

Potential antimicrobial applications of detoxified anthracyclines

Although anthracyclines were originally discovered through their antimicrobial activity, severe side effects prevented their development as antibiotics[10,13,30]. However, recent insights into the development of detoxified anthracyclines with limited side effects may open up a potential therapeutic window for their application as antibiotics. Interestingly, actinomycetes were highly sensitive to DMdoxo (Chapter 4), a promising detoxified anthracycline[15,16]. This further evoked our interest in exploring the potential antimicrobial applications of detoxified anthracyclines.

In **Chapter 5**, we evaluated the antimicrobial activity of a structurally diverse library of anthracyclines[16–19] against ESKAPE pathogens. *N,N*-dimethylated anthracyclines exhibited robust bactericidal activity against Gram-positive bacteria as well as Gram-negative *A. baumannii*. In particular, DMdoxo and an *N,N*-dimethylated Doxo trisaccharide derivative (Figure 1, compound **6** in Chapter 5) demonstrated the strongest antimicrobial activity. Importantly, although streptomycetes activated anthracycline resistance within a few generations upon exposure to subinhibitory concentrations (Chapter 4), we did not observe the development of resistance against Doxo and DMdoxo in methicillin-resistant *Staphylococcus aureus* (MRSA).

The presence of a tertiary amine on the first sugar moiety of anthracycline typically leads to the loss of DNA-damaging activity, which is associated with cardiotoxicity, the most treatment-limiting side effect of anthracyclines[17–19]. Our findings indicate that this is also correlated with improved antibiotic activity. The antimicrobial mode-of-action of anthracyclines remains poorly understood, although recently idarubicin was shown to target the cell membrane and bacterial topoisomerase II[345]. It would be of interest to further elucidate the antimicrobial mode-of-action of anthracyclines, and in particular the factors contributing to the improved activity of dimethylated anthracyclines. Although dimethylated anthracyclines have reduced toxicity, their limited side effects will still prevent their application as first-line antibiotics. Instead, we envision their potential application as in life-threatening situations, such as combating sepsis[346].

Toward high-throughput characterisation of *Streptomyces*

Streptomycetes remain an important source of new bioactive natural products[2–4]. To accelerate the discovery of these metabolites, high-throughput methods to characterise large strain collections under many experimental conditions are needed[52,356,357,359]. The mycelial morphology of streptomycetes in submerged cultures can cause significant biological variability[363]. Nonetheless, achieving reproducible small-scale cultivation of *Streptomyces* is feasible with tightly controlled inoculation strategies and well-characterised cultivation methods[361,362].

In **Chapter 6**, we present a workflow for integrated quantitative proteomics and metabolomics from microbioreactor (MBR) *Streptomyces* cultures. Our results indicate that reproducible and sensitive data can be obtained from small-culture volumes. However, further optimisation of the cultivation method is required to reduce biological variability. This methodology could complement existing approaches for natural product discovery, such as co-culture experiments, screenings with small-molecule elicitors, or computational methods[49,284].

Outlook

Anthracyclines have been cornerstones in oncology treatment for decades. Further understanding of biosynthesis and bioactivities of anthracyclines is important for anticancer and antimicrobial applications of these compounds.

Combinatorial biosynthesis is an effective approach for generating hybrid anthracyclines. In this work, expression of aclarubicin and rhodomycin biosynthetic genes in *S. peucetius* led to the biosynthesis of *N,N*-dimethylated anthracyclines. A similar approach could be applied for the biosynthesis of other detoxified anthracyclines, such as the *N,N*-dimethylated Doxo trisaccharide derivative, which demonstrated potent antimicrobial activity toward ESKAPE pathogens. Additionally, it would be of interest to explore the putative anthracycline BGCs that were detected in Streptomycetaceae to identify their cognate products and potentially discover new tailoring enzymes.

N,N-dimethylated anthracyclines demonstrated strong toxicity to Gram-positive bacteria, including actinomycetes. Although this finding presents promising prospects for their application as antibiotics, an important question that remains is the antimicrobial mode-of-action of anthracyclines. Uptake experiments utilising the fluorescent nature of anthracyclines via microscopy could offer valuable insights. Additionally, structure-activity relationship experiments may provide a better understanding of the factors contributing to the stronger antimicrobial activity of dimethylated anthracyclines. Finally, these insights may guide the engineering of anthracyclines for effective anticancer and antimicrobial applications.