Anthracycline biosynthesis in Streptomyces: engineering, resistance and antimicrobial activity

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N,N-dimethylated anthracyclines with improved antibiotic activity

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Abstract

The sharp increase in infections associated with multi-drug resistant pathogens calls for alternatives for existing clinical antibiotics. A major issue that prevents clinical application of many antibiotic candidates is cytotoxicity. One example are members of the anthracycline polyketides, which are DNA-targeting drugs that while originally discovered as antibiotics, now find widespread clinical use as anticancer agents. Here we show that *N,N*-dimethylated anthracyclines, which have strongly reduced side effects, show improved activities as antibiotics. Screening a collection of structurally diverse anthracyclines against a panel of ESKAPE pathogens showed potent activity against Gram-positive bacteria. *N,N*-dimethylated variants had significantly improved antibacterial activity and also demonstrated moderate activity against Gram-negative *Acinetobacter baumannii*. *N,N*-dimethyldoxorubicin (compound **2**) and an *N,N*-dimethylated doxorubicin trisaccharide derivative (compound **6**) demonstrated the highest antimicrobial activity. These compounds exhibited a two-fold higher activity against methicillin-resistant *Staphylococcus aureus* (MRSA) compared to doxorubicin in phosphate buffered saline, and even 16-fold higher activity in pooled human plasma ($LC_{99.9}$ of 2 μM). A resistance development assay indicated that MRSA failed to develop resistance to *N,N*-dimethyldoxorubicin over 25 passages, indicating robust antimicrobial activity. These findings suggest that *N,N*-dimethylated anthracyclines hold promise as potential last-resort antibiotics in the battle against multi-drug resistant pathogens.

Introduction

The current state of antimicrobial resistance presents a global crisis in public health. The widespread emergence of multi-drug resistant (MDR) pathogens calls for the development of novel antibiotics[336,337]. Ongoing efforts encompass a variety of approaches, including the screening of microbial collections for bioactive natural products and chemical synthesis. However, the discovery of novel antibiotic classes has declined dramatically in recent decades[338,339]. As an alternative strategy, there is a growing interest in revisiting compound classes not conventionally applied as antimicrobial drugs[340,341].

Anthracyclines represent a class of highly potent anticancer compounds[15,266]. They are aromatic type II polyketides featuring a linear tetracyclic 7,8,9,10-tetrahydro-5,12-naphthacenequinone scaffold and are decorated with one or more sugar moieties[30]. Many of these compounds were initially discovered due to their antimicrobial activity[10,30]. However, their potential as antibiotics was constrained by severe side effects, directing their exclusive application as chemotherapeutic agents[13]. The most prominent clinically used natural anthracyclines are daunorubicin (**32**, Figure 1) and doxorubicin (**1**, Figure 1), both isolated from *Streptomyces peucetius*[8–10], and aclarubicin (**12**, Figure 1), a natural product of *Streptomyces galilaeus*[33]. Furthermore, semi-synthetic anthracyclines such as epirubicin (**13**, Figure 1) and idarubicin have gained widespread use in clinical oncology[13].

Anthracyclines intercalate into DNA, which results in two distinct activities[14,15]. Firstly, they induce DNA damage by causing DNA double-strand breaks through the inhibition or poisoning of topoisomerase II, an essential enzyme involved in DNA replication and repair. Secondly, anthracyclines cause chromatin damage by evicting histones, the proteins involved in packaging DNA into a condensed structure. The major side effects associated with anthracycline treatment, including cardiotoxicity, the development of secondary tumours and infertility, result from the combination of these two activities, such as is the case for daunorubicin (**32**) and doxorubicin (**1**)[14,16]. In contrast, anthracyclines that primarily induce chromatin damage, such as aclarubicin (**12**) and *N,N*-dimethyldoxorubicin (**2**, Figure 1), are associated with reduced side effects. These "detoxified" anthracyclines lack cardiotoxicity and do not induce second tumours[14,16], and can be more safely administered to patients in a poor conditions. A comprehensive evaluation of a structurally diverse library of anthracyclines revealed that the presence of a tertiary amine on the first sugar moiety generally results in the loss of DNA-damaging activity while exhibiting improved cytotoxicity[17–19]. This finding offers a promising strategy for the development of improved anthracyclines.

Anthracyclines with limited side effects offer the potential for a therapeutic window as antimicrobial agents, opening up new possibilities in addressing antimicrobial resistance. In recent years, the antimicrobial activity of several anthracyclines against a range of pathogens has been explored in a limited number of studies, primarily through high-throughput screenings of compound libraries. For instance, in a screening of the National Cancer Institute compound

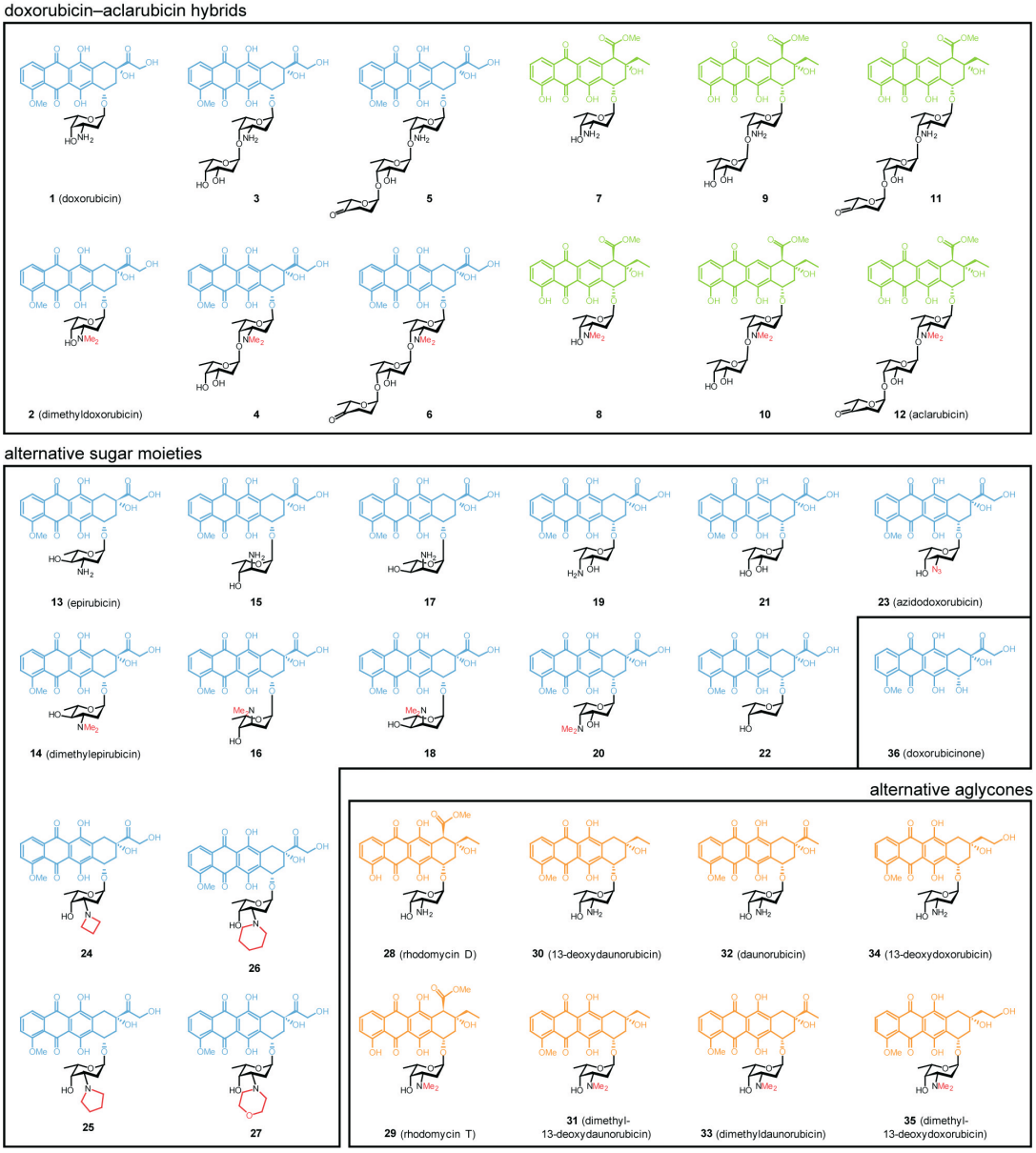

Figure 1. Chemical structures of compounds 1–36, evaluated in this study. Doxorubicin–aclarubicin hybrids (**1**–**12**), (*N,N*-dimethylated) doxorubicin epimers (**13**–**18**), (*N,N*-dimethylated) doxorubicin regio-isomers (**19** and **20**), doxorubicin with replacement of removal of the basic amine group (**21**–**23**), doxorubicin with alternative alkylation (**24**–**27**), (*N,N*-dimethylated) doxorubicin derivatives differing in the aglycone part (**28**–**35**), and doxorubicinone (**36**). The doxorubicin aglycone is depicted in blue, the aclarubicin aglycone in green, and alternative aglycones in yellow. Tertiary amines and the azide group are indicated in red.

library, six anthracyclines were identified as active against *Borrelia burgdorferi*, among which daunorubicin (**32**) and *N,N*-dimethyldaunorubicin (**33**, Figure 1)[342]. In another high-throughput screening of an FDA-approved drugs library, daunorubicin (**32**) exhibited antimicrobial activity against *Staphylococcus aureus* small-colony variants[343]. Additionally, daunorubicin (**32**) demonstrated potent antimicrobial activity against *Mycobacterium absceccus*[344]. Furthermore, idarubicin exhibited bactericidal activity against methicillin-resistant *S. aureus* (MRSA) and methicillin-resistant *Staphylococcus epidermis*[345].

The mechanism underlying the antimicrobial activity was attributed to cell membrane disruption activity[345]. Another potential application of anthracyclines lies in the treatment of sepsis. Epirubicin (**13**) conferred robust protection against sepsis in an experimental mouse model at a low dose regiment[346]. However, a comprehensive comparison of a diverse library of anthracyclines against a wide panel of pathogens has been lacking, underscoring the urgent need or a more in-depth investigation.

The goal of this study was to evaluate the antimicrobial activity of a library of 35 structurally diverse anthracyclines against ESKAPE pathogens. Compounds with potent cytotoxic activity against human cancer cell lines also exhibited enhanced antimicrobial activity, particularly against Gram-positive bacteria. Generally, the *N,N*-dimethylated compounds exhibited increased antimicrobial activity. Among these compounds, *N,N*-dimethyldoxorubicin (**2**) and compound **6**, composed of the doxorubicin aglycone combined with the aclarubicin trisaccharide (Figure 1), exhibited the strongest antimicrobial activity, especially for Gram-positive bacteria including MRSA.

Results

Minimal inhibitory concentration of a 35-compound anthracycline library

Recent advances in the development of less toxic anthracyclines present a promising opportunity to explore their potential as antibiotics. To search for anthracyclines with antibiotic activity, we screened a library consisting of 35 different compounds (Figure 1). The library is composed of a set of structurally diverse anthracyclines, each characterised by variations in the aglycone or the sugar moiety. The anthracyclines are divided into three main groups. The first group consists of doxorubicin and aclarubicin hybrids (**1**–**12**)[18]. The second group consists of (*N,N*-dimethylated) doxorubicin variants featuring alternative sugar moieties: diastereomers (**13**–**18**)[19], regio-isomers (**19**–**20**)[17], replacement or removal of the basic amine (**21**–**23**)[17], and variations in the amine alkylation (**24**–**27**)[17]. The last group consists of (*N,N*-dimethylated) doxorubicin variants with alternative aglycones (**28**–**35**)[17]. The anticancer activities of these anthracyclines have been previously investigated[17–19].

Since anthracyclines are known to exhibit in particular bioactivity against Gram-positive bacteria, while their activity against Gram-negative bacteria is generally low, the antimicrobial assays were initially focused on the Gram-positive *Bacillus subtilis* and methicillin-resistant

Staphylococcus aureus USA300 ($MRSA_{USA300}$). Minimum inhibitory concentrations (MICs) were determined using the antimicrobial broth microdilution susceptibility assay. In this assay, a mid-logarithmic growth-phase culture was exposed to anthracycline concentrations ranging from 0.5 to 64 µM (see Materials and Methods for details).

The anthracyclines had varying antibiotic activity (Table 1). Doxorubicin (**1**) exhibited an MIC of 8 µM against *B. subtilis* and of 16 µM against $MRSA_{USA300}$. Importantly, *N,N*-dimethyldoxorubicin (**2**) exhibited a four-fold lower MIC against both strains. Among the doxorubicin–aclarubicin hybrids, the dimethylated variants consistently demonstrated stronger antimicrobial activity. Notably, the doxorubicin–aclarubicin hybrid with the doxorubicin aglycone and aclarubicin trisaccharide (compound **6**) exhibited the highest activity of the entire library against *B. subtilis* (0.5 µM) and $MRSA_{USA300}$ (1 µM). Similarly, for the diastereomers and regio-isomers, the dimethylated variants consistently demonstrated stronger antimicrobial activity. The non-dimethylated variants generally did not exhibit any antimicrobial activity, with the exception of epirubicin (**13**) that exhibited intermediate bioactivity, with an MIC of 16 µM against both Gram-positive strains. Removal or replacement of the basic amine group had moderate effect on the antimicrobial activity.

Table 1. Minimal inhibitory concentration of anthracyclines 1–35 against Gram-positive bacteria.

#	Group	A*	S*	M*	*B. subtilis* (µM)	$MRSA_{USA300}$ (µM)
1	doxorubicin–aclarubicin hybrids	doxo	1	x	8	16
2	doxorubicin–aclarubicin hybrids	doxo	1	Me_2	2	4
3	doxorubicin–aclarubicin hybrids	doxo	2	x	›64	›64
4	doxorubicin–aclarubicin hybrids	doxo	2	Me_2	4	8
5	doxorubicin–aclarubicin hybrids	doxo	3	x	16	›64
6	doxorubicin–aclarubicin hybrids	doxo	3	Me_2	0.5	1
7	doxorubicin–aclarubicin hybrids	acla	1	x	64	›64
8	doxorubicin–aclarubicin hybrids	acla	1	Me_2	32	32
9	doxorubicin–aclarubicin hybrids	acla	2	x	32	64
10	doxorubicin–aclarubicin hybrids	acla	2	Me_2	2	8
11	doxorubicin–aclarubicin hybrids	acla	3	x	32	›64
12	doxorubicin–aclarubicin hybrids	acla	3	Me_2	2	4
13	diastereomers	doxo	1	x	16	16
14	diastereomers	doxo	1	Me_2	4	8
15	diastereomers	doxo	1	x	›64	›64
16	diastereomers	doxo	1	Me_2	32	16
17	diastereomers	doxo	1	x	›64	64
18	diastereomers	doxo	1	Me_2	16	16

[continued on next page]

Table 1. *[continued]*

#	Group	A*	S*	M*	*B. subtilis* (µM)	$MRSA_{USA300}$ (µM)
19	regio-isomers	doxo	1	x	64	64
20	regio-isomers	doxo	1	Me_2	4	4
21	replacement/removal of amine	doxo	1	OH	16	32
22	replacement/removal of amine	doxo	1	H	8	32
23	replacement/removal of amine	doxo	1	N_3	4	16
24	alternative amine alkylation	doxo	1	cyclic	2	4
25	alternative amine alkylation	doxo	1	cyclic	1	2
26	alternative amine alkylation	doxo	1	cyclic	1	4
27	alternative amine alkylation	doxo	1	cyclic	1	2
28	alternative aglycones	other	1	x	8	8
29	alternative aglycones	other	1	Me_2	8	8
30	alternative aglycones	other	1	x	16	32
31	alternative aglycones	other	1	Me_2	8	16
32	alternative aglycones	other	1	x	4	8
33	alternative aglycones	other	1	Me_2	2	4
34	alternative aglycones	other	1	x	›64	›64
35	alternative aglycones	other	1	Me_2	32	32

* Column A: the aglycone structure is indicated as: acla, aclarubicin aglycone; doxo, doxorubicin aglycone; other, alternative aglycone. Column S: number of sugar moieties. Column M: modification or replacement of the amine group: x, no modification; Me_2, dimethylation; OH, replacement of amine by hydroxyl group; H, removal of amine; N_3, replacement of amine by azide; cyclic, amine alkylation with cyclic group.

However, compounds **24**–**27** exhibited low micromolar MICs (1–4 µM) against both strains, indicating that tertiary alkylation of the amine group resulted in a significantly increased bioactivity. For the compounds with alternative aglycones, dimethylation resulted in a two-fold increase in antimicrobial activity, with the exception of rhodomycin D (**28**), which exhibited the same MICs as its dimethylated derivative rhodomycin T (**29**). Notably, both 13-deoxydoxorubicin (**34**) and its dimethylated derivative (**35**) exhibited poor antimicrobial activity.

Taken together, *N,N*-dimethylation or tertiary alkylation of the amine group resulted in increased antimicrobial activity against Gram-positive bacteria. A subset of the anthracyclines was chosen for further studies of their antimicrobial activity against a broader range of pathogens. For this we selected the following compounds: doxorubicin–aclarubicin hybrids with the doxorubicin aglycone (**1**–**6**), harbouring different numbers of sugar moieties, with or without dimethylation; aclarubicin (**12**), which features an alternative aglycone with a dimethylated trisaccharide; azidodoxorubicin (**23**), which contains an azide group; and the aglycone doxorubicinone (**36**).

Bactericidal activity of anthracyclines against ESKAPE pathogens

The bactericidal activity of compounds **1**–**6**, **12**, **23** and **36** was tested against different clinical isolates from the ESKAPE panel[347], including *Enterococcus faecium* LUH15122, *S. aureus* LUH14616 ($MRSA_{LUH14616}$), *Klebsiella pneumoniae* LUH15104, *Acinetobacter baumannii* RUH875, *Pseudomonas aeruginosa* LUH15103, and *Enterobacter cloacae* LUH15114. The Gram-positive *Staphylococcus epidermidis* LUH15163 and *Streptococcus pyogenes* LUH2762, were also included. Mid-logarithmic growth-phase cultures were exposed to compounds at concentrations ranging from 0.061 to 64 µM (See Materials and Methods for details).

Table 2. Bactericidal activity of anthracyclines against planktonic bacteria. Killing of *E. faecium* LUH15122, *S. aureus* LUH14616, *S. epidermidis* LUH15163, *S. pyogenes* LUH2762, *K. pneumoniae* LUH15104, *A. baumannii* RUH875, *P. aeruginosa* LUH15103, and *E. cloacae* LUH15114 after 24 h exposure to 0.061 to 64 µM of compound **1**–**6**, **12**, **23** or **36** in PBS with or without 50% pooled human plasma. The results are expressed as the 99.9% lethal concentration ($LC_{99.9}$).

			PBS								
Gram	**Species**	**Strain**	**1**	**3**	**5**	**2**	**4**	**6**	**12**	**23**	**36**
+	*E. faecium*	LUH15122	›64	›64	64	2	16	4	8	›64	›64
	S. aureus	LUH14616	8	›64	›64	4	8	4	16	›64	32
	S. epidermidis	LUH15163	8	›64	16	1	4	1	8	64	32
	S. pyogenes	LUH2762	4	16	4	1	2	1	8	8	16
-	*K. pneumoniae*	LUH15104	›64	›64	›64	›64	›64	›64	›64	›64	›64
	A. baumannii	RUH875	64	›64	›64	16	32	16	›64	›64	›64
	P. aeruginosa	LUH15103	›64	›64	›64	›64	›64	›64	›64	›64	›64
	E. cloacae	LUH15114	›64	›64	›64	›64	›64	›64	›64	›64	›64
			PBS with 50% pooled human plasma								
Gram	**Species**	**Strain**	**1**	**3**	**5**	**2**	**4**	**6**	**12**	**23**	**36**
+	*E. faecium*	LUH15122	›64	›64	›64	›64	›64	›64	›64	›64	›64
	S. aureus	LUH14616	32	›64	64	2	8	2	›64	64	›64
	S. epidermidis	LUH15163	›64	›64	64	2	4	1	›64	›64	›64
	S. pyogenes	LUH2762	4	64	16	1	2	1	32	›64	›64
-	*K. pneumoniae*	LUH15104	›64	›64	›64	64	›64	›64	›64	›64	›64
	A. baumannii	RUH875	64	›64	›64	8	32	32	›64	›64	›64
	P. aeruginosa	LUH15103	›64	›64	›64	›64	›64	›64	›64	›64	›64
	E. cloacae	LUH15114	›64	›64	›64	32	›64	›64	›64	›64	›64

The results are shown in Table 2 and Supplementary Figure S1. Doxorubicin (**1**) exhibited activity against $MRSA_{LUH14616}$, *S. epidermis* and *S. pyogenes*. Compound **3**, featuring a non-dimethylated disaccharide, only displayed activity against *S. pyogenes*. The non-dimethylated trisaccharide (compound **5**) was active against *S. epidermis* and *S. pyogenes*. The dimethylated doxorubicin variants (compounds **2**, **4** and **6**) effectively killed all Gram-positive strains at concentrations in the low micromolar range (1–16 µM). *N,N*-dimethyldoxorubicin (**2**) and compound **6**, which

features the doxorubicin aglycone with the aclarubicin trisaccharide, exhibited particularly strong activity against *S. epidermis* (1 µM) and *S. pyogenes* (1 µM). Aclarubicin (**12**) had moderate activity against all Gram-positive strains (8–16 µM). Azidodoxorubicin (**23**) was active only against *S. pyogenes*, while doxorubicinone (**36**) exhibited moderate activity overall. Interestingly, the dimethylated doxorubicin variants (compounds **2**, **4** and **6**) exhibited moderate bactericidal activity against *A. baumannii* (16–32 µM). Finally, none of the compounds inhibited growth of *K. pneumoniae*, *P. aeruginosa* or *E. cloacae*).

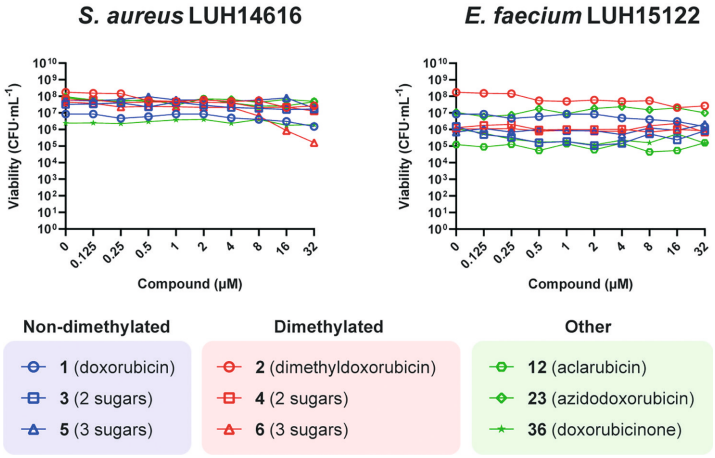

Figure 2. Bactericidal activity of anthracyclines against mature biofilms. Viability of 7-days-mature biofilms of *E. faecium* LUH15122 and *S. aureus* LUH14616 after 24 h exposure to 0.125 to 32 µM of compound **1**–**6**, **12**, **23** or **36**. The results are expressed as the number of viable bacteria in colony forming units (CFU) per mL for each compound concentration. Non-dimethylated doxorubicin variants are represented in blue, dimethylated doxorubicin variants are represented in red, and others in green.

To simulate physiologically relevant conditions, the bactericidal assays were repeated in PBS with 50% pooled human plasma. Under these conditions, the dimethylated doxorubicin derivatives retained their bactericidal activity against $MRSA_{LUH14616}$, *S. epidermis* and *S. pyogenes* (1–8 µM), whereas the non-dimethylated compounds and aclarubicin demonstrated reduced efficacy (Table 2, Supplementary Figure S1). *E. faecium* was resistant to all compounds in 50% plasma. Importantly, the dimethylated compounds also retained their activity against *A. baumannii* in 50% plasma.

We also investigated the effect of shorter treatment time on the bactericidal activity. Therefore, $MRSA_{LUH14616}$ and *A. baumannii* RUH875 were exposed for 1, 2, 3, 4 or 24 h to doxorubicin (**1**) or *N,N*-dimethyldoxorubicin (**2**) in PBS, PBS with 2% TSB and PBS with 50% pooled human plasma (Supplementary Table S2). Both compounds were active against both strains after 1 h, but more efficient killing was shown after 24 h exposure.

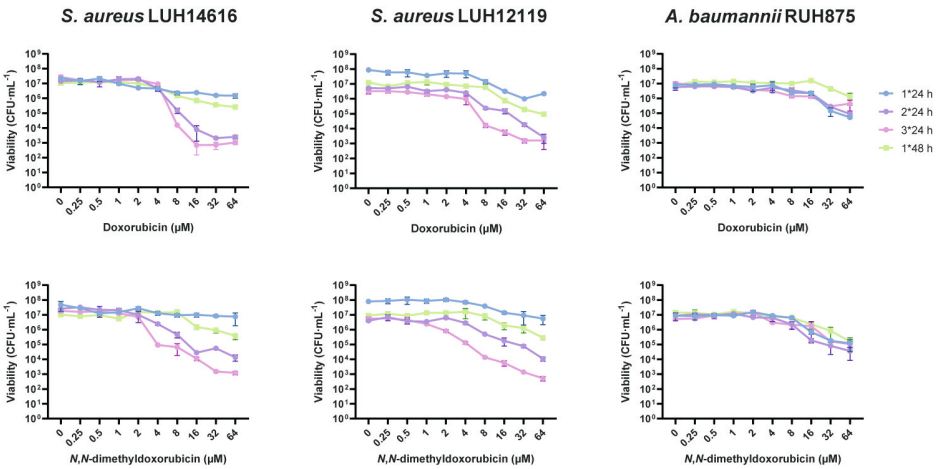

Figure 3. Bactericidal activity of anthracyclines against immature biofilms of MRSA. Viability of 24-h-immature biofilms of *S. aureus* LUH14616, *S. aureus* LUH12119 and *A. baumannii* RUH875 after one, two or three times 24 h or one time 48 h exposure to 0.125 to 32 μM of doxorubicin (**1**) or *N,N*-dimethyldoxorubicin (**2**). The results are expressed as the number of viable bacteria in colony forming units (CFU) per mL for each compound concentration.

Antibiofilm activity of anthracyclines

The efficacy of compounds **1**–**6**, **12**, **23** and **36** in eradicating mature bacterial biofilms of $MRSA_{LUH14616}$ and *E. faecium* LUH15122 after 24-h exposure was evaluated (see Materials and Methods for details). At the highest tested concentration of 32 μM, compounds **1**–**6**, **12**, **23** and **36** did not eradicate mature biofilms of $MRSA_{LUH14616}$ and *E. faecium* (Figure 2). The most notable antibiofilm activity was observed with compound **6**, which killed 99.6% of mature biofilm of $MRSA_{LUH14616}$ at 32 μM.

We further tested the antibiofilm activity of anthracyclines against less mature biofilms of *S. aureus* using a stronger treatment regiment. In this experiment, we included a second *S. aureus* strain ($MRSA_{LUH12119}$). We exposed immature (24-h-old) biofilms of $MRSA_{LUH14616}$ and $MRSA_{LUH12119}$ to doxorubicin (**1**) and *N,N*-dimethyldoxorubicin (**2**) with one, two or three times 24 h or one time 48 h incubation time (see Materials and Methods for details). Multiple treatment of immature biofilms of both MRSA strains improved the efficacy of the anthracyclines, whereas longer treatment time had only moderate effect (Figure 3). Three times 24 h treatment with 32 μM of doxorubicin (**1**) or *N,N*-dimethyldoxorubicin (**2**) killed 99.9% of both $MRSA_{LUH14616}$ and $MRSA_{LUH12119}$.

Resistance development of MRSA to anthracyclines

In light of the growing concern for resistance against antibiotics, we evaluated the potential $MRSA_{LUH14616}$ and $MRSA_{LUH12119}$ to develop resistance to anthracyclines. Serial passaging of the two MRSA strains in the presence of sub-inhibitory concentrations of doxorubicin (**1**) resulted in the selection of isolates with a maximum of 16-fold increase in MIC after 25 passages (Figure 4). Importantly, in the case of *N,N*-dimethyldoxorubicin (**2**), the MIC did not increase more than

two-fold despite of 25 passages. In contrast, exposure to the antibiotic rifampicin resulted in a rapid increase in resistance, with a 256-fold increase in MIC after just eight passages, and reaching an MIC of 2 mg·mL^{-1} after 11 passages (Figure 4).

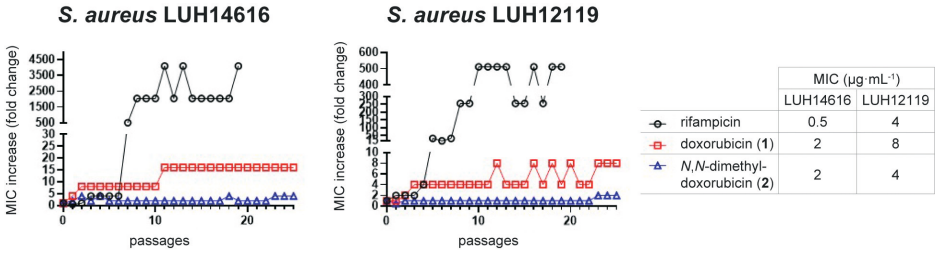

Figure 4. Resistance development of MRSA against doxorubicin and *N,N*-dimethyldoxorubicin. Resistance development of *S. aureus* LUH14616 and LUH12119 to doxorubicin (**1**), *N,N*-dimethyldoxorubicin (**2**) and rifampicin. Values are fold changes in minimal inhibitory concentration (MIC) relative to the MIC of the first passage.

Discussion

The escalating global crisis of antimicrobial resistance, coupled with a concerning stagnation in the discovery of novel antibiotics, underscores the urgent need for innovative strategies in addressing this challenge[337,338]. In this context, we explored the potential application of detoxified anthracyclines, well-known anticancer drugs, as antibiotics. Our findings revealed that dimethylated anthracyclines exhibit robust bactericidal activity against Gram-positive bacteria as well as Gram-negative *A. baumannii*. Considering that dimethylated anthracyclines lack DNA-damaging activity, which is associated with reduced side effects[16], this makes detoxified anthracyclines promising candidates as antibiotics.

Evaluation of a library of anthracyclines indicated that dimethylation consistently increased the activity against *B. subtilis* and MRSA$_{USA300}$. In particular compound **6**, featuring the doxorubicin aglycone with a dimethylated trisaccharide, exhibited a very low MIC of only 1 μM against MRSA$_{USA300}$. Furthermore, the doxorubicin derivatives with cyclic amines (compounds **24**–**27**) exhibited low MICs (2–4 μM) against MRSA$_{USA300}$. Subsequent evaluation of the bactericidal activity of a subset of the library against pathogens of the ESKAPE panel indicated that dimethylated anthracyclines effectively killed mainly Gram-positive bacteria. *N,N*-dimethyldoxorubicin (**2**) and compound **6** exhibited an $LC_{99.9}$ of 4 μM against MRSA$_{LUH14616}$ and of 1 μM against clinical isolates of *S. epidermis* and *S. pyogenes*. The compounds also demonstrated moderate activity against Gram-negative *A. baumannii*, and retained their activity in physiologically relevant conditions (50% human plasma). Importantly, MRSA$_{LUH14616}$ and MRSA$_{LUH12119}$ did not develop resistance to *N,N*-dimethyldoxorubicin (**2**) after 25 passages in sub-inhibitory conditions. However, the anthracyclines were ineffective in eradicating mature biofilms of MRSA$_{LUH14616}$ and *E. faecium*. Immature biofilms of MRSA$_{LUH14616}$ and MRSA$_{LUH12119}$ could be killed for 99.9% by multiple exposures to 32 μM of doxorubicin (**1**) or *N,N*-dimethyldoxorubicin (**2**).

N,N-dimethylation of anthracyclines generally results in the elimination of DNA double-strand break activity in human cancer cell lines, while cytotoxicity remains due to the release of nucleosomes from defined areas in the genome[17–19]. Obviously, histone release will not be a factor relevant for the antimicrobial activities. Still, our results indicate that *N,N*-dimethylation improves the antimicrobial activity of anthracyclines. Additionally, cyclic amines demonstrated low MIC values against *B. subtilis* and $MRSA_{USA300}$. This is in contrast to the anticancer activities, which were unaffected by alternative alkylation of the amine moiety of doxorubicin[17]. Furthermore, changing the position of the amine in the sugar moiety had minor effects on cytotoxicity[17], while this strongly reduced the antimicrobial activity (compounds **15**, **17** and **19**). The cytotoxicity of anthracyclines is associated with histone eviction activity[16], but the antimicrobial mode-of-action of anthracyclines has been poorly studied as the drugs usually have major toxic activities to experimental animals and patients. A recent study reported the antimicrobial activity of the cancer drug idarubicin, another doxorubicin derivative with a modified aglycone structure that is also cardiotoxic, and presented a model for its mode-of-action[345]. Idarubicin was found to target the cell membrane and bacterial topoisomerase II subunits GyrA and GyrB[345]. The increased hydrophobicity of dimethylated anthracyclines may result in better membrane solubility, which could offer an explanation for their improved antimicrobial activity. Additionally, it is possible that DNA gyrase inhibitory activity is improved in the dimethylated anthracyclines, adding another potential factor to their improved efficacy. We are currently investigating the mode-of-action of dimethylated anthracyclines via resistance assays and uptake experiments.

Repurposing of anticancer compounds as antimicrobials has gained increasing attention in recent years. For example, the toxic cancer drug mitomycin C, the antimicrobial properties of the DNA-targeting anticancer compound mitomycin C have been evaluated, revealing promising activity against bacterial persist cells[348] and efficient killing of Gram-negative *A. baumannii*[349]. Additionally, mitoxantrone, another toxic cancer drug that acts via DNA damage and free radical formation, has been proposed as potential antibiotic for the treatment of vancomycin-resistant *Enterococcus faecalis* (VRE)[350]. However, the application of anticancer compounds as antibiotics is limited by their inherent toxicity to human cells. Consequently, their application as antimicrobial agents must be approached strategically, such as through topical treatments or as a last resort in cases involving MDR pathogens. Alternatively, derivatives of anticancer compounds may be found that exhibit reduced toxicity, with detoxified anthracyclines serving as an example. In the context of doxorubicin treatment, cardiotoxicity is the dose limiting side effect, restricting administration to a cumulative dose of 450–550 $mg \cdot m^{-2}$ [351]. Anthracyclines with reduced cardiotoxicity like *N,N*-dimethyldoxorubicin (**2**) could be applied at higher accumulative dosages[15]. The feasibility of employing anthracyclines as antibiotics relies on the specific applications and pharmacodynamics. However, the low bactericidal concentrations observed for both *N,N*-dimethyldoxorubicin (**2**) and compound **6** are a basis for further optimisation of these drugs for *in vivo* application as antibiotics for especially Gram-positive bacteria.

In conclusion, our work provides a beautiful example of how compounds from the antibiotic class of anticancer drugs can be modified to reduce the side effects in patients while enhancing the antibiotic activities. The detoxification of anticancer compounds presents a promising alternative strategy to replenish the antibiotics discovery pipelines and combat antimicrobial resistance.

Materials and Methods

Chemical compounds

The anthracyclines used in this work are presented in Figure 1. The 35-compound anthracycline library consists of doxorubicin and aclarubicin derivatives with variations in the tetracyclic aglycone and the sugar moieties. Doxorubicin (**1**) was purchased from Pharmachemie (the Netherlands). Aclarubicin (**12**, sc-200160) and doxorubicinone (**36**, sc-218273) were purchased from Santa Cruz Biotechnology (USA). Epirubicin (**13**) was purchased from Accord Healthcare Limited (UK). Daunorubicin (**32**) was purchased from Sanofi-Aventis (the Netherlands). All other anthracyclines were synthesised as described previously[16–19].

Bacterial strains and cultivation conditions

The bacterial strains used in this work are listed in Supplementary Table S1. Clinical isolates of *Enterococcus faecium* (LUH15122), *Staphylococcus aureus* (LUH14616, LUH12119 and USA300), *Staphylococcus epidermidis* (LUH15163), *Streptococcus pyogenes* (LUH2762), *Klebsiella pneumoniae* (LUH15104), *Acinetobacter baumannii* (RUH875), *Pseudomonas aeruginosa* (LUH15103), and *Enterobacter cloacae* (LUH15114), and the model strain *Bacillus subtilis* 168 were used. Before each experiment, glycerol stocks were streaked onto blood agar plates, and incubated at 37 °C overnight. Subsequently, approximately five colonies were inoculated into tryptic soy broth (TSB). The bacteria were cultured at 37 °C at 200 rpm to mid-logarithmic growth phase, and diluted to the desired inoculum concentration, based on the optical density at 600 nm (OD_{600}).

Minimum inhibitory concentration

Minimum inhibitory concentrations (MICs) were determined using the antimicrobial broth microdilution susceptibility assay according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Briefly, a mid-logarithmic growth-phase culture (OD_{600} of 0.5) was exposed to anthracycline concentrations ranging from 0.5 to 64 µM. Cation-adjusted Mueller–Hinton Broth (CAMHB) was prepared by supplementing Mueller–Hinton Broth (MHB) with 0.5 mM $CaCl_2$ and 0.5 mM $MgCl_2$. Anthracyclines were dissolved in DMSO to a concentration of 6.4 mM and subsequently diluted to 128 µM in CAMHB. The compounds solutions were serially diluted two-fold in CAMHB into microtiter plates to a volume of 50 µL per well. The bacterial suspension was 100-fold diluted in CAMHB, and 50 µL was added to each well to achieve a final OD_{600} of 0.0025. Plates were sealed with an adhesive membrane, and incubated at 37 °C overnight. Biomass formation in each well was visually inspected. The MIC was defined as the

lowest anthracycline concentration at which no growth was observed. *N,N*-dimethyldoxorubicin (**2**) was tested at two-fold dilutions of 112 µM or 149 µM instead of 64 µM for *B. subtilis* and USA300, respectively. Compounds **4**–**7**, **11**, **21**, **22** and **26** were tested at two-fold dilutions of 85 µM instead of 64 µM for both strains. In these cases, the MIC values were rounded up to 0.5, 1, 2, 4, 8, 16, 32, 64 or ›64 µM.

Bactericidal activity

Bactericidal activity was determined according to the CLSI guidelines. Briefly, a mid-logarithmic growth-phase culture was centrifuged for 10 min at 3000 rpm and washed with 10 mL of phosphate buffered saline (PBS; pH 7.4, 140 mM NaCl). Anthracyclines were dissolved in PBS to a concentration of 426.7 µM, and subsequently serially diluted two-fold in PBS into microtiter plates to a volume of 30 µL per well to achieve a final concentration in the range of 0.061–64 µM for Gram-positive strains and 4-64 µM for Gram-negative strains. Subsequently, 50 µL of PBS with 4% TSB or 50 µL of pooled human plasma (Sanquin) was added to each well, resulting in a final concentration of 2% TSB or 50% (v/v) plasma. Except in case of *S. pyogenes* LUH2762, where a final concentration of 10% TSB was applied in both PBS and plasma experiments. Finally, the bacterial suspension was diluted in PBS to approximately $5{\cdot}10^6$ CFU·mL^{-1}, and 20 µL was added to each well to achieve a achieve final concentration of approximately $1{\cdot}10^6$ CFU·mL^{-1}. Plates were sealed with an adhesive membrane, and incubated at 37 °C for 24 h. Viability was determined via a colony forming units (CFU) assay. A dilution series of the bacterial suspensions was prepared in PBS and plated on Mueller–Hinton (MH) agar plates. Plates were incubated at 37 °C for 24 h. The bactericidal activity was reported as the 99.9% lethal concentration ($LC_{99.9}$), i.e., the lowest anthracycline concentration that killed ≥99.9% of bacteria. One replicate of *N,N*-dimethyldoxorubicin (**2**) with each strain was tested at two-fold dilutions of 112 µM instead of 64 µM. In this cases, the MIC values were rounded up to 0.5, 1, 2, 4, 8, 16, 32, 64 or ›64 µM. To study shorter treatment times of planktonic bacteria, the plates were incubated for 1, 2, 3 or 4 h instead of 24 h.

Treatment of immature and mature biofilms

A mid-logarithmic growth-phase culture was centrifuged for 10 min at 3400 rpm. The biomass was resuspended in 2 mL of PBS, and subsequently diluted in brain heart infusion (BHI) medium to approximately $1{\cdot}10^7$ CFU·mL^{-1}. Subsequently, 100 µL of the bacterial suspension was added to each well of a microtiter plate. Plates were sealed with an adhesive membrane, and incubated for 24 h (immature) or 7 days (mature) at 37 °C in a humidified atmosphere. For mature biofilm experiments, plates were sealed with a breathable membrane. After incubation, planktonic bacteria were carefully removed from each well. The biofilm was washed twice with 100 µL of PBS. Subsequently, 100 µL of anthracyclines diluted in PBS in the range of 0.125–32 µM was added to each well. As untreated control, bacteria were exposed to PBS without anthracyclines. For immature biofilms anthracyclines were diluted in PBS with 2% TSB. After 24 h incubation at 37 °C in a humidified atmosphere, the biofilm was washed twice with PBS and resuspended in 100 µL PSB by 10 min of sonication. Viability was determined via a CFU assay of MH agar and reported as the $LC_{99.9}$ value. For mature biofilm treatment with *N,N*-dimethyldoxorubicin,

the tested concentrations deviated from the intended range. In this case, the $LC_{99.9}$ value was rounded up to 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32 or ›32 µM. For multiple treatment of immature biofilms, after 24 h incubation with anthracyclines, the biofilm was washed twice with PBS. The biofilm was treated with 100 µL of fresh anthracycline solution of the same concentration, and incubated for another 24 h. For prolonged treatment of immature biofilms, the incubation time was extended to 48 h.

Resistance development

Development of resistance to anthracyclines was performed as described by Habets and Brockhurst[352]. For comparison, development of resistance to the clinically relevant antibiotic rifampicin (Sigma-Aldrich) was determined. Briefly, *S. aureus* LUH12119 and LUH14616 were cultured overnight at 37 °C at 200 rpm in modified RPMI-1640 medium (with 20 mM HEPES and L-glutamine, without sodium bicarbonate). Compounds were dissolved in PBS and serially diluted two-fold in modified RPMI-1640 medium into microtiter plates to a volume of 100 µL per well (with a final concentration of 0.5–64 µM for anthracyclines and 0.5–4.1 $mg \cdot mL^{-1}$ for rifampicin). Subsequently, 5 µL of overnight culture was added to each well. Plates were sealed with an adhesive membrane, and incubated at 37 °C at 200 rpm for 24 h. Biomass formation in each well was visually inspected. The MIC was defined as the lowest anthracycline concentration at which no growth was observed. Subsequently, 5 µL of the culture at 0.5-fold MIC was added to 100 µL of fresh medium containing a two-fold dilution range of the compounds. The plates were treated and analysed as described above. This process was repeated for 25 passages.

Supplementary Information

Table S1. Bacterial strains used in this work.

Gram	Species	Strain	Class
+	*Bacillus subtilis*	168	Model strain
	Enterococcus faecium	LUH15122	MDR
	Staphylococcus aureus	LUH14616	MRSA
	Staphylococcus aureus	LUH12119	MRSA
	Staphylococcus aureus	USA300	MRSA
	Staphylococcus epidermidis	LUH15163	MDR
	Streptococcus pyogenes	LUH2762	MDR
-	*Klebsiella pneumoniae*	LUH15104	MDR
	Acinetobacter baumannii	RUH875	MDR
	Pseudomonas aeruginosa	LUH15103	MDR
	Enterobacter cloacae	LUH15114	MDR

Table S2. Short treatment time of anthracyclines against planktonic bacteria. Killing of *S. aureus* LUH14616 and *A. baumannii* RUH875 after 1, 2, 3, 4 or 24 h exposure to 1 to 64 µM of doxorubicin (**1**) or *N,N*-dimethyldoxorubicin (**2**) in PBS, PBS with 2% TSB or PBS with 50% pooled human plasma. The results are expressed as the 99.9% lethal concentration ($LC_{99.9}$).

***S. aureus* LUH14616**										
	doxorubicin (**1**)					*N,N*-dimethyldoxorubicin (**2**)				
Medium	**1h**	**2h**	**3h**	**4h**	**24h**	**1h**	**2h**	**3h**	**4h**	**24h**
PBS	›64	›64	›64	64		›64	›64	32	32	
PBS + 2% TSB	›64	›64	64	64	32	›64	›64	32	16	4
PBS + 50% plasma	›64	›64	›64	64	32	›64	›64	32	16	1
***A. baumannii* RUH875**										
	doxorubicin (**1**)					*N,N*-dimethyldoxorubicin (2)				
Medium	**1h**	**2h**	**3h**	**4h**	**24h**	**1h**	**2h**	**3h**	**4h**	**24h**
PBS	64	64	64	64		›64	64	64	32	
PBS + 2% TSB	›64	›64	64	64	64	32	32	16	16	16
PBS + 50% plasma	›64	64	32	32	64	32	32	16	16	8

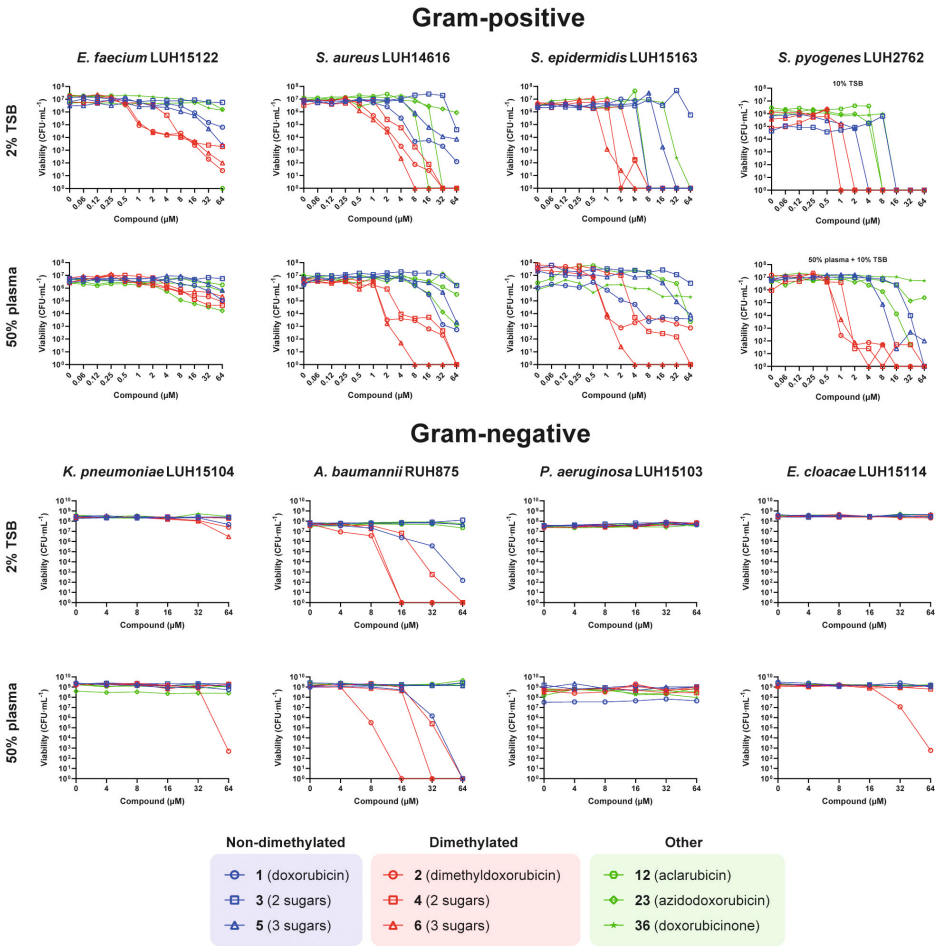

Figure S1. Bactericidal activity of anthracyclines against planktonic bacteria. Viability of *E. faecium* LUH15122, *S. aureus* LUH14616, *S. epidermidis* LUH15163, *S. pyogenes* LUH2762, *K. pneumoniae* LUH15104, *A. baumannii* RUH875, *P. aeruginosa* LUH15103, and *E. cloacae* LUH15114 after 24 h exposure to 0.061 to 64 μM of compound **1**–**6**, **12**, **23** or **36** in PBS with or without 50% pooled human plasma. The results are expressed as the number of viable bacteria in colony forming units (CFU) per mL for each compound concentration. Non-dimethylated doxorubicin variants are represented in blue, dimethylated doxorubicin variants are represented in red, and others in green.