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Bioreductively Activated Reactive Oxygen Species (ROS) Generators as MRSA Inhibitors

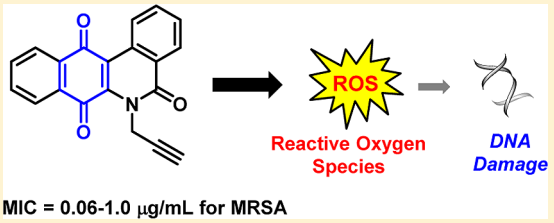
Vinayak S. Khodade,[†] Mallojjala Sharath Chandra,[†] Ankita Banerjee,[‡] Surobhi Lahiri,[‡] Mallikarjuna Pulipeta,[‡] Radha Rangarajan,[‡] and Harinath Chakrapani*,[†]

[†]Indian Institute of Science Education and Research Pune, Dr. Homi Bhabha Road, Pune, 411008 Maharashtra, India

[‡]Vitas Pharma Research Private Limited, Technology Business Incubator, University of Hyderabad, C. R. Rao Road, Gachibowli, Hyderabad 500046, India

Supporting Information

ABSTRACT: The number of cases of drug resistant *Staphylococcus aureus* infections is on the rise globally and new strategies to identify drug candidates with novel mechanisms of action are in urgent need. Here, we report the synthesis and evaluation of a series of benzo[b]phenanthridine-5,7,12(6*H*)-triones, which were designed based on redox-active natural products. We find that the *in vitro* inhibitory activity of 6-(prop-2-ynyl)benzo[b]phenanthridine-5,7,12(6*H*)-trione (**1f**) against methicillin-resistant *Staphylococcus aureus* (MRSA), including a panel of patient-derived strains, is comparable or better than vancomycin. We show that the lead compound generates reactive oxygen species (ROS) in the cell, contributing to its antibacterial activity.

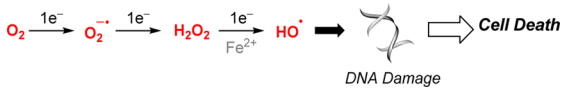


KEYWORDS: *Drug resistance, reactive oxygen species, MRSA, superoxide radical, RecA, DNA damage*

Because of the high levels of morbidity and mortality, drug-resistant infections have now become a major public health problem globally.[1] The pathogen *Staphylococcus aureus* (*S. aureus*), for example, has acquired resistance to several antibiotics including the methicillin-based drugs. Methicillin-resistant *S. aureus* (MRSA) strains are fast becoming resistant to other frontline antibiotics as well.[2] The global pipeline for new antibiotics is weak, and hence, the development of new strategies that specifically address drug resistance is necessary.

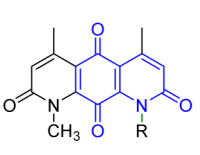
Redox-active natural products were long considered as innocuous byproducts of metabolism of microorganisms without any major function.[3,4] It is increasingly clear that these secondary metabolites are produced to mediate a number of cellular processes including gene expression, interspecies communication, and defense.[3,4] The ability of such small molecules to keep competitors in check through the generation of reactive oxygen species (ROS) has attracted attention for new drug development.[3,5] ROS including superoxide radical $O_2^{-\bullet}$, hydrogen peroxide H_2O_2, and hydroxyl radical $^{\bullet}OH$ are generated as a natural consequence of respiration but can cause cellular damage at elevated levels (Scheme 1).[6]

Scheme 1. Reactive Oxygen Species (ROS) and Their Possible Cellular Effects

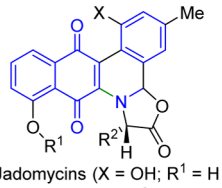


Potentiating these ROS in cancers using small molecules has been considered as a possible drug design strategy.[7] For example, deoxynyboquinone, a natural product derivative, has potent tumoristatic activity in animal models, and its efficacy is, in part, dependent on generation of ROS (Chart 1).[8,9] The potential for ROS generators to inhibit growth of certain mycobacteria is also reported.[10,11] The oxidative stress-inducing antimalarial drug artemisinin, when conjugated with mycobactin,[12] and the antileprosy drug Clofazimine[13] have potent inhibitory effects on multidrug-resistant strains of *Mycobacterium tuberculosis*. Here, we report results of design and synthesis of natural product-inspired ROS generators and their potential to inhibit multidrug-resistant *S. aureus*.

Chart 1



R = H, Deoxynyboquinone
R = Me, SCH538415



Jadomycins (X = OH; R^1 = H or carbasugar, OH; R^2 = alkyl)

We proposed to synthesize and study benzo[b]-phenanthridine-5,7,12(6*H*)-triones of general structure **1**

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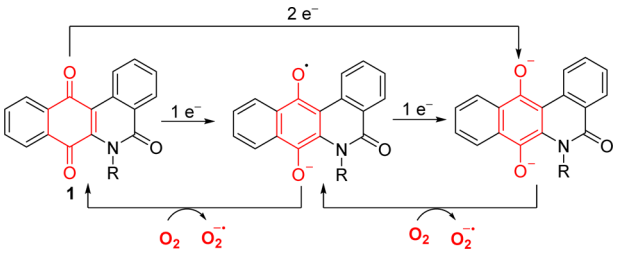
(Scheme 2). This scaffold contained the amide adjoining the quinone of deoxynyboquinone and the aryl ring-like

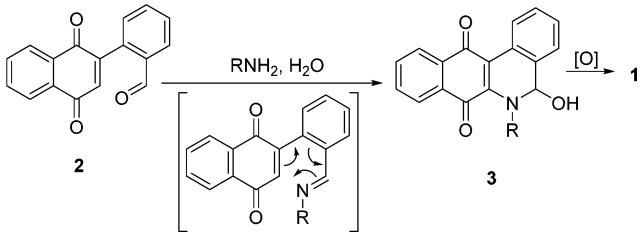
Scheme 2. Proposed Mechanism of $O_2^{-\bullet}$ generation from 1



jadomycins,[14] another class of natural products that are known to cleave DNA in the presence of metal ions such as Cu(II)[15] presumably through the generation of hydroxyl radical (Chart 1).[16] **1** is predicted to undergo bioreduction to its semiquinone,[17] which then is reoxidized by molecular oxygen, forming $O_2^{-\bullet}$ (Scheme 2). During this oxidation, **1** is regenerated and becomes available for further ROS generation via bioreduction (See Supporting Information, Scheme S3).

The synthesis of **1** can be achieved in two steps from 2-(2-formyl-phenyl)-1,4-naphthoquinone **2** (Table 1), which in turn

Table 1. One-Pot Synthesis of 1a−1n by the Reaction of 2 with a Primary Amine



entry	R	compd	% yield[a]
1	Me	**1a**	73
2	Et	**1b**	50
3	^{n}Pr	**1c**	45
4	cyclohexyl	**1d**	23
5	allyl	**1e**	21
6	propargyl	**1f**	20 (37)[b]
7	CH_2CO_2Me	**1g**	39
8	benzyl	**1h**	39
9	2-CF_3PhCH_2	**1i**	14
10	4-$ClPhCH_2$	**1j**	20
11	4-$OMePhCH_2$	**1k**	17
12	4-NO_2PhCH_2	**1l**	22
13	4-CF_3PhCH_2	**1m**	47
14	(3,4,5-triOMe)$PhCH_2$	**1n**	13

[a]Isolated yield. [b]Addition of H_2O_2 (10 equiv) to the reaction mixture.

can be synthesized from commercially available 2-bromo-1,4-naphthoquinone (Supporting Information).[14] The addition of an amine to **2** would result in the formation of an imine, which sets up the molecule for an intramolecular 6π-electrocyclic ring closure to afford the alcohol **3**; subsequent oxidation of **3** should give the desired lactams of structure **1** (Table 1).[14]

When **2** was reacted with methylamine in an open container, we found **1a** as the major product in 73% yield implying that the intermediate alcohol (**3a**, R = Me, Scheme S2, Supporting Information) was oxidized during the reaction (Table 1). Using this one-pot procedure, compounds **1b−1g** were prepared from **2** (Table 1, entries 2−7). The use of an aromatic amine such as aniline did not produce the desired product but instead gave an inseparable mixture of products. The one-pot methodology was, however, found to be compatible with benzylamines, and **1h−1n** were prepared in moderate yields (Table 1, entries 8−14).

To test if this library of compounds had an effect on the growth of bacterial cells, the minimum inhibitory concentration (MIC) was determined for the series against methicillin sensitive *S. aureus* (MSSA). Five of the analogues tested were found to inhibit MSSA at 32 μg/mL or less (Table 2). The best

Table 2. Calculated Partition Coefficients (ClogP) and Minimum Inhibitory Concentration (MIC) against Methicillin-Sensitive *S. aureus* (MSSA) of 1a−1n

entry	compd	ClogP[a]	MIC (μg/mL)[b]
1	**1a**	2.63	1.0
2	**1b**	3.16	>32
3	**1c**	3.69	>32
4	**1d**	4.66	>32
5	**1e**	3.40	>32
6	**1f**	2.73	0.5
7	**1g**	2.82	>32
8	**1h**	4.40	>32
9	**1i**	5.28	>32
10	**1j**	5.11	>32
11	**1k**	4.32	32
12	**1l**	4.14	8.0
13	**1m**	5.28	>32
14	**1n**	3.70	8.0
15	tobramycin		1
16	fosfomycin		8
17	vancomycin		0.5

[a]Calculated using Chembiodraw Ultra 13.0. [b]Minimum inhibitory concentration (MIC) is defined as the lowest concentration required to inhibit visible bacterial growth; MSSA = MSSA 29213.

inhibitor was **1f** with MIC of 0.5 μg/mL against MSSA and MRSA (Table 2, entry 6, and Table 3, entry 1). The activity was comparable to vancomycin, the drug of choice in the clinic for multidrug resistant MRSA infections. This compound was identified as the lead for further studies.

Table 3. MICs of 1f against Methicillin-Resistant *S. aureus* (MRSA) Strains

entry	strain	MIC (μg/mL)	MIC (μM)	MIC of vancomycin (μg/mL)
1	MRSA 33591	0.5	1.6	0.5−2
2	MRSA 7419	0.12	0.38	1.0
3	B19506	0.06	0.19	0.5−2
4	MRSA K-1	0.25	0.80	1.0
5	MRSA 7425	0.12	0.38	0.12
6	MRSA 7386	1.0	3.19	1.0
7	E9902	0.5	1.60	0.5
8	E151	0.5	1.60	0.5
9	E288	0.5	1.60	0.5
10	B21838	0.5	1.60	0.5
11	B853	1.0	3.19	1.0
12	MRSA B0085	0.5	1.60	1.0

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First, we attempted to improve the yield of **1f**. A mechanism for the electrocyclic ring closure followed by hydration and oxidation was proposed (Scheme S2, Supporting Information). Aerobic oxidation presumably involved hydrogen peroxide and when the reaction mixture was supplemented with H_2O_2, an increased yield of **1f** (37%, Table 1, entry 6) was observed. The overall yield of **1f** could thus be improved to 24% in two steps from commercially available 2-bromo-1,4-naphthoquinone. In contrast, jadomycins are synthesized in >20 steps[14] while deoxynyboquinone was prepared in seven linear steps.[9]

Cyclic voltammetry analysis revealed that $1e^-$ reduction potentials (E_{red}) of **1f** was −1.00 V (Table S1, Supporting Information), which appears appropriate for metabolism by bioreductive enzymes.[17] This compound was found to be unreactive with biological thiols such as glutathione (see Supporting Information, Figure S1), possibly due to steric hindrance created by the substituents on the quinone ring. A luminol-based chemiluminescence assay was used to detect $O_2^{-\bullet}$, and we found generation of $O_2^{-\bullet}$ only in the presence of NQO1-naphthoquinone oxidoreductase (DT-Diaphorase, DT-D),[18] a model bioreductive enzyme (Figure 1a).[19,20] A HPLC-based dihydroethidium (DHE) assay was used to independently assess $O_2^{-\bullet}$ production (Figure 1b).[21,22] Here, the conversion of DHE to 2-hydroxyethidium (2-OH-E^+) is indicative of $O_2^{-\bullet}$ generation.[23] During incubation of **1f** in the presence of DT-D, the formation of 2-OH-E^+ was observed, thus, confirming the intermediacy of $O_2^{-\bullet}$ (Figure 1b). In addition, H_2O_2, the product of $1e^-$ transfer to $O_2^{-\bullet}$, was measured using an Amplex Red-based fluorescence assay. Again, we found that **1f** was capable of generating H_2O_2 in the presence of DT-D.[24]

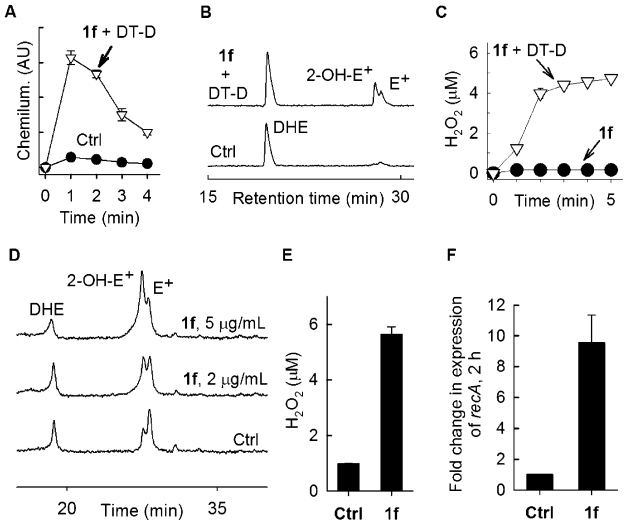

Figure 1. (a) Time course of $O_2^{-\bullet}$ generation was estimated by a luminol-based chemiluminescence assay in the presence of DT-diaphorase. Control (Ctrl) is **1f** incubated in pH 7.4 buffer. (b) Superoxide generated during incubation of **1f** with DT-D was estimated using a dihydroethidium (DHE) assay. Superoxide specifically react with DHE to produce 2-hydroxyethidium (2-OH-E^+); ethidium (E^+) is formed by nonspecific oxidation of DHE and is indicative of a general increase in oxidative species. Ctrl is **1f** in pH 7.4 buffer. (c) Hydrogen peroxide produced during incubation of **1f** (2 μg/mL) with DT-D for 5 min was quantified by an Amplex Red-based fluorescence assay. (d) $O_2^{-\bullet}$ generated during incubation of *S. aureus* with **1f** was estimated using a dihydroethidium (DHE) assay. $O_2^{-\bullet}$ specifically react with DHE to produce 2-hydroxyethidium (2-OH-E^+); ethidium (E^+) is formed by nonspecific oxidation of DHE and is indicative of a general increase in oxidative species. (e) Hydrogen peroxide generation during incubation of *S. aureus* with **1f** (2 μg/mL) for 60 min as measured using an Amplex Red-based fluorescence assay. Ctrl is untreated bacteria. (f) ROS accumulation in the cell can damage DNA, leading to the upregulation of the DNA repair enzyme, *RecA*. Quantitative real time PCR analysis of *recA* expression in MSSA cells incubated with 6.7 × MIC concentrations, i.e., 3.35 μg/mL for 120 min showed a significant increase in *recA* levels. Ctrl is untreated bacteria.

Next, the possibility of **1f** generating intracellular $O_2^{-\bullet}$ in bacteria was examined using a DHE assay.[21,22] The bacterial control showed unreacted DHE (Figure 1d), and in the presence of **1f**, the formation of 2-OH-E^+ in a concentration-dependent manner was observed, suggestive of $O_2^{-\bullet}$ production intracellularly (Figure 1d). Superoxide accumulation intracellularly leads to the production of H_2O_2 through dismutation.[6] H_2O_2 diffuses out of the cell and can be measured using Amplex Red. In this assay, extracellular H_2O_2 serves as a surrogate marker for intracellular ROS levels.[25] When *S. aureus* cells are exposed to **1f**, we find increased H_2O_2 confirming that **1f** was capable of enhancing ROS (Figure 1e). The yield of H_2O_2 produced by **1f** was nearly quantitative, a testament to efficient ROS generation by this compound (Figure 1e).

A possible consequence of increased $O_2^{-\bullet}$ and H_2O_2 is the generation of $^\bullet$OH. However, because of its extremely short half-life, detection of $^\bullet$OH is challenging. Instead, reported methods measure all elevated oxidative species including $^\bullet$OH. Using dichlorofluorescein-diacetate ($DCFH_2$-DA) fluorescence assay, the levels of oxidative species generated intracellularly in *S. aureus* was estimated. The results of this assay indicate that **1f** at 0.5 μg/mL was capable of generating oxidative species intracellularly (Figure S5, Supporting Information).

Lethality induced by elevated ROS is attributable to DNA damage by $^\bullet$OH leading to single and double strand breaks.[6] In response to DNA damage, DNA rescue pathways are activated.[6] Symptomatic of one such rescue pathway is the expression of *RecA*, a repair protein crucial to homologous recombination.[26] In the presence of **1f**, we found a significant upregulation of *RecA* expression suggesting that DNA damage repair response is activated in *S. aureus* upon treatment with **1f** (Figure 1f). We next examined the effect of **1f** on the viable colony count of MSSA in the presence of thiourea, a $^\bullet$OH quencher. The growth inhibition of **1f** is reduced in the presence of thiourea, suggesting that quenching the ROS in the cell reduces the antibacterial effect of the compound (Figure S6, Supporting Information). These data provide further support for a ROS based mechanism contributing to antibacterial activity.

To test for synergy between **1f** and other known antibiotics, we conducted synergy time kill assays with ciprofloxacin (Figure S7, Supporting Information) and tobramycin (Figure S8, Supporting Information) against MRSA 33591. We found no evidence for synergy suggesting that the mechanism of action of **1f** was distinct from these clinically-used antibiotics. The lack of synergy shows that mechanism of action of **1f** is independent of the antibiotics tested and could represent a novel way to inhibit bacterial growth. If the mechanism is novel, we hypothesized that it would be able to overcome existing resistance. Hence we tested the activity of the compound against a panel of 11 clinical isolates of MRSA. We found the activity to be well-conserved between the reference strain MRSA 33591 and the clinical isolates (Table 3, entries 2−12). Finally, we tested the growth inhibitory potential of **1f** against mammalian cells. When screened against A549 human lung

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carcinoma cells, the compound showed a 50% growth inhibitory concentration (GI_{50}) of 8.6 μM (Supporting Information). The selectivity index (SI) (GI_{50}/MIC) for MRSA 33951 is >5, which is favorable for further development.

Thus, taken together, our investigations reveal **1f** as a potent MRSA inhibitor with a unique mechanism of action that involves enhancement of ROS levels in cells. Several redox-active analogues of **1f** (Table S1, Supporting Information) were capable of undergoing bioreduction to generate superoxide in buffer (Figure S3, Supporting Information) and increase hydrogen peroxide levels in MRSA (Figure S4, Supporting Information) but were poor *S. aureus* inhibitors (Table 2).[3,4,25,27−29] Hence, generation of ROS by these redox-active small molecules appears necessary but not sufficient for inhibiting MRSA growth (Figure S4, Supporting Information).[3,4] It is noteworthy that even small structural modifications to Jadomycins resulted in significant differences in DNA damaging capability,[30] and SCH538415 (Chart 1), the structural analogue with an additional *N*-methyl group had a 10-fold lower potency in comparison with deoxynyboquinone.[9]

In summary, we report a natural product-inspired redox-active small molecule that is able to overcome drug resistance in MRSA. The in vitro potency of this compound is comparable or better than that of vancomycin, the drug of last resort for such infections.

■ ASSOCIATED CONTENT

Supporting Information

Preparative procedures, assay protocols, NMR spectra, and other experimental data. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*(H.C.) E-mail: harinath@iiserpune.ac.in.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

MIC, minimum inhibitory concentration; GI_{50}, 50% growth inhibitory concentration; ROS, reactive oxygen species; SI, selectivity index; DHE, dihydroethidium; DT-D, DT-diaphorase

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