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COMMUNICATION

Harnessing the Dual Antimicrobial Mode of Action with a Lipophilic Mn(II) Complex Using the Principle of the Irving-Williams Series to Completely Eradicate *Staphylococcus aureus*

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The judicious selection of 5,7-dibromo-2-methy-8-quinolinol (BQ) as a Mn(II) ionophore results in the synthesis of $Mn(BQ)_2(DMSO)_2$·DMSO (1), a potent metalloantibiotic with a dual antimicrobial mode of action against four different strains of *Staphylococcus aureus* (SA) bacteria (MIC = 0.625 μg/mL). Additionally, 1 can overcome ciprofloxacin-resistance in methicillin-resistant SA bacteria.

The rising antimicrobial resistance (AMR), coupled with the dwindling rate of return in the discovery of antibiotics with novel modes of action, is an urgent global public health threat.[1-3] Although there are approximately 200 conserved essential proteins in bacteria that may become potential antimicrobial targets, only *ca*. 40 of them are accessed by the current commercial antibiotics.[4] As the result, the new antibiotics in the drug development pipeline that aim at these known targets will be prone to quick resistance development.[5,6] It is highly desirable to develop new antimicrobial agents with completely different targets than those of the current antibiotics.[7]

Recently, metal chelation to disrupt bacterial metal homeostasis has emerged as a new alternative antimicrobial mode of action to those of conventional antibiotics.[8-11] In this regard, 8-hydroxyquinoline (8-Hq) and its many derivatives constitute a large class of metal ionophores that have been explored as a novel class of antimicrobial agents.[12,13] Metal ionophores differ from ordinary chelating agents in their ability to penetrate the cell membrane, although the relationship between the molecular structure and cell membrane penetration in metal chelators is thus far poorly delineated. On the other hand, use of coordination compounds containing antimicrobial metals such as Cu(II), Ag(I), Ga(III), Zn(II), Mn(II), etc. is proven to be another effective approach to accessing certain unexploited antimicrobial modes of action.[14-17] In this communication, we describe our synthesis, structural characterization and antimicrobial activity of $Mn(BQ)_2(DMSO)_2$·DMSO (BQ = 5,7-dibromo-2-methy-8-quinolinol) that harnesses a dual antimicrobial mode of action using the principle of the Irving-Williams Series.[18] Specifically, for a given ligand, the relative stability exhibited by homologous divalent 3d metal complexes follows the trend $Cr^{2+} < Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} \geq Zn^{2+}$. Hence, $Mn((BQ)_2(DMSO)_2$·DMSO exhibits potent antimicrobial activity against four different strains of the gram-positive *Staphylococcus aureus* (SA) bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-intermediate *Staphylococcus aureus* with a measured MIC of 0.625 μg/mL owing to its ability to simultaneously deliver the antimicrobial Mn(II) into the bacterial cell and chelate to the other bio-essential metals including Fe(II), Zn(II) and Cu(II) *via* metal-exchange. As a result of its dual antimicrobial mode of action, $Mn((BQ)_2(DMSO)_2$·DMSO can completely eradicate methicillin-susceptible *Staphylococcus aureus* (MSSA) with the low concentration of 2.50 μg/mL as well as readily overcome the resistance in the phenotype of ciprofloxacin-resistant MRSA bacteria.

We began our studies with the use of the commercially available clioquinol (CQ; i.e., 5-chloro-7-iodo-8-quinolinol) as the ligand to form the bis(clioquinolato)manganese(II) for such investigation. CQ is the prototypical ionophore of Zn(II) and Cu(II) whose antimicrobial, antifungal, antiprotozoal and anticancer activity is attributable to its ability to disrupt cellular Zn(II)/Cu(II) homeosis.[19-22] However, the complexation of CQ with Mn(II) gave a product with limited solubility in water or DMSO, making it impossible to investigate the antimicrobial

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†Electronic Supplementary Information (ESI) available: [Experimental details including the synthesis, characterization, and antimicrobial studies of BQ and **1** CCDC-2267020 contains the supplementary crystallography data for this paper. The data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif]

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activity of the complex. We then synthesized the closest dibromo analogue of CQ, i.e., 5,7-dibromo-2-methy-8-quinolinol (broquinaldol; abbreviated as BQ hereafter) for this purpose in the hope that its Mn(II) complex would have better solubility in DMSO-H_2O mixture to allow for *in vitro* evaluation of its antimicrobial activity, while the cell membrane penetration of BQ could remain comparable to CQ. ~~Since Br is the middle congener between Cl and I, the bromo-analogue of a given organic compound can often be modulated to possess the intermediate physical and chemical properties of the chloro- and iodo-counterpart~~.

BQ was obtained *via* direct bromination of 2-methyl-8-quinolinol (i.e., 8-hydroxyquinaldine) with N-bromosuccinimide in dry toluene in quantitative yield as shown in **Scheme 1** (See **Figures S1-S5** in ESI for details of its spectroscopic characterization). The reaction of BQ with $MnCl_2{\cdot}4H_2O$ in an ethanol/water solution with Na_2CO_3 as a base gave a light-brown crystalline precipitate. The product was purified by recrystallization in DMSO to give dark red plate-shaped single crystals as shown in **Scheme 2** (see **Tables S1-S3** and **Figures S1-S2** in ESI for the syntheses, activity measurements, and spectroscopic characterization details). Single-crystal X-ray structure analysis showed that this Mn(II) complex crystallizes in the monoclinic space group Cc, consisting of one molecule in the asymmetric unit with a = 21.7849(4) Å, b = 15.7021(4) Å, c = 10.5287(3) Å, β = 112.1318(11)° and V = 3336.17(15) Å^3. Besides two DMSO molecules coordinating to the Mn(II) center, there is another DMSO molecule found in the lattice, establishing its molecular formula as $Mn(BQ)_2(DMSO)_2{\cdot}DMSO$ (**1**). ~~The final refinement converged with crystallographic agreement factors of R1=2.37% and wR2=4.88% for 7412 reflections with I>2σ(I) (R1=2.94%, wR2=5.00% for all data) and 379 variable parameters~~ (See Tables S1 and S2 for details of crystal data and structure refinement results). The Mn(II) center is found in a distorted octahedral coordination environment bound to two ligand and two *cis*-DMSO molecules. As shown by the space-filling model, the Mn(II) center is completely encased in the octahedral cavity formed by two lipophilic ligands and two DMSO molecules, effectively concealing its ionic character for better cell membrane penetration (*vide infra*) as illustrated in **Figure 1**.

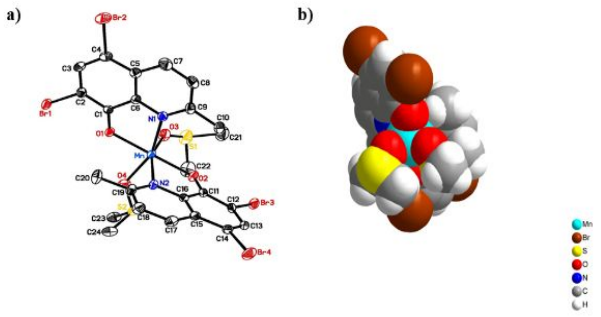

Figure 1. Molecular structure of **1** depicted by the ORTEP model with 50% probability ellipsoids (**a**; H-atoms and DMSO solvent are omitted for clarity), and by the space-filling model (**b**).

Because Mn(II) is located near the beginning of the Irving-Williams Series, any one of the bio-essential transition metal ions after it in this series including Fe(II), Zn(II) and Cu(II) should trigger the release of Mn(II) from the complex. We studied the metal-exchange reaction between **1** and $FeCl_2$, $ZnCl_2$ or $CuCl_2$, respectively, in an ethanol-DMSO solution. The results showed that a rapid exchange reaction occurred and completed within 30 min., causing Mn(II) to be released from **1** with the concomitant conversion of **1** into $M(BQ)_2$ (M = Fe(II), Zn(II) and Cu(II)) in solution as indicated by the presence of isosbestic points in their UV-VIS spectra as shown in **Figure 2** (**Figures S6 - S7** and refer to the SI for the metal-exchange details with $CuCl_2$ and $ZnCl_2$)

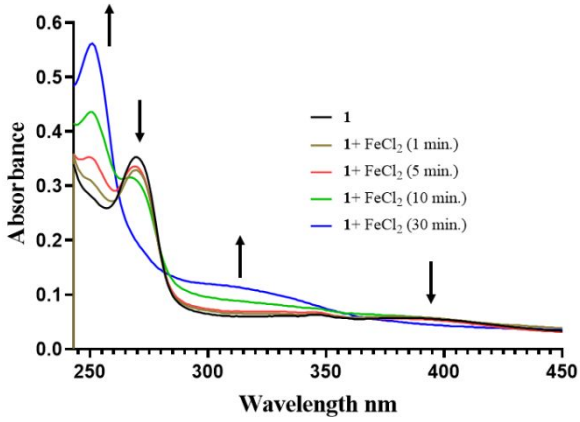

Figure 2. Metal-exchange reaction between **1** and $FeCl_2$ as followed by UV-VIS spectroscopy, showing the release of Mn(II) and concomitant conversion of **1** into $Fe(BQ)_2$ in solution.

We first measured the minimum inhibitory concentrations (MICs) of BQ in comparison with those of CQ against four different strains of the gram-positive *Staphylococcus aureus* (SA) to observe if antimicrobial activity could be retained in the dibromo analogue of CQ. These include a methicillin-susceptible strain of SA (MSSA; ATCC 6538), two methicillin-resistant strains of SA (MRSA$^{\alpha}$; ATCC BAA-44 and MRSA$^{\beta}$; USA 300, i.e., ATCC BAA-1717) and a vancomycin-intermediate strain of SA (VISA; ATCC 700699). To our surprise, the MIC of BQ was found to be 16 times lower than that of CQ against MSSA, and 32 times lower than CQ against MRSA$^{\alpha}$, MRSA$^{\alpha}$ and VISA, placing BQ among the most effective antimicrobial ionophores against SA bacteria in the family of 8-Hq derivatives (**Table 1**).[13]

Table 1. MIC values of **1** against four different strains of gram-positive SA bacteria in comparison with CQ, BQ and ciprofloxacin in μg/mL (in μM).

Bacteria Strains	CQ	BQ	Ciprofloxacin	1
MSSA (ATCC 6538)	10 (33)	0.625 (1.97)	0.250 (0.755)	0.625 (0.678)
MRSA$^{\alpha}$ (ATCC BAA-44)	20 (66)	0.625 (1.97)	16 (48.3)	0.625 (0.678)
MRSA$^{\beta}$ (ATCC BAA-1717)	20 (66)	0.625 (1.97)	16 (48.3)	0.625 (0.678)
VISA (ATCC 700699)	20 (66)	0.625 (1.97)	16-32 (48.3-96.6)	0.625 (0.678)

To put these findings in perspective, the MICs of ciprofloxacin – one of the most frequently prescribed antibiotics against these four strains of bacteria are also given in **Table 1**. Since BQ has virtually the same 3D structure as CQ, it is reasonable to conjecture that the improved antimicrobial activity of BQ is attributable to its more favourable bacterial cell penetration as both ionophores have the same antimicrobial mode of action. We then evaluated if similar bacterial cell penetration of BQ is retained in **1** by measuring the MICs of its Mn(II) complex against the same four strains of SA bacteria. The results showed that MICs of **1** are almost identical to those of BQ when expressed in the unit of μg/mL. This seemingly unchanged antimicrobial activity of **1** as compared to BQ is the result of the much higher molar mass of **1** (MM = 921.28 g/mole) *vs*. that of BQ (MM = 302.95 g/mole). Nevertheless, the measured MIC of **1** qualifies this Mn(II) complex as one of the most potent metal-based antimicrobial agents reported in the literature thus far.[15-17] To investigate whether the complexation of BQ to Mn(II) actually enhances antimicrobial activity, we used the colony forming unit (CFU) enumeration technique to examine the dose-dependent response of MSSA (ATCC 6538) toward **1** *vs*. BQ. The results clearly showed that **1** had truly enhanced antimicrobial activity when compared with BQ itself (**Figure 3**).

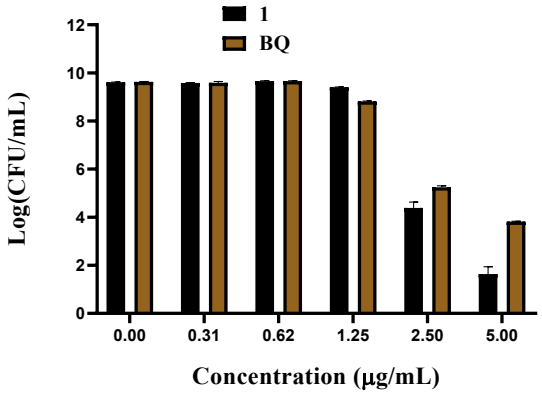

Figure 3. Growth-inhibitory effect of **1** against MSSA in comparison with BQ.

For example, at the concentration of 2.50 μg/mL, **1** achieved a 5-log reduction of bacterial colonies (i.e., complete eradication) *vs*. a 4-log reduction of bacterial colonies by BQ at the same concentration (i.e., **1** is ~10 times higher in antimicrobial potency than BQ). Furthermore, at the concentration of 5.00 μg/mL, **1** achieved a 7-log reduction of bacterial colonies *vs*. a 5-log reduction of bacterial colonies by BQ at the same concentration (i.e., **1** is ~100 times higher in antimicrobial potency than BQ). To ascertain that the enhanced antimicrobial activity of **1** is derived from an additional mode of action contributed by Mn(II) besides the chelation effect imparted by BQ itself, we measured the intracellular production of reactive oxygen species (ROS) in MSSA cells using the 2′,7′-dichlorofluorescin diacetate (DCFH-DA) cellular ROS assay technique. The results showed that the treatment with two different concentrations of **1** caused a dose-dependent increase of intracellular ROS production and a simultaneous decrease in the cell population of live bacteria in MSSA. Please note that BQ itself does not cause any intracellular ROS production. It is known that Mn can produce ROS in bacteria through two main pathways.[23-25] The first involves redox cycling, which transfers electrons between different oxidation states of Mn, leading to superoxide and hydrogen peroxide generation. The second pathway involves the disruption of metal homeostasis in bacteria due to excess Mn, which can compete with Fe for binding to proteins and enzymes, causing Fe depletion and ROS production. Overall, we conclude that **1** does possess a dual antimicrobial mode of action, albeit the additional mode of action contributed by Mn(II) plays a minor role as compared to the primary antimicrobial mode of action imparted by metal chelation of BQ.

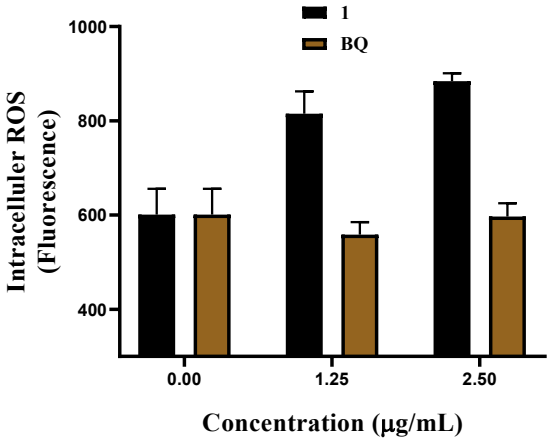

Figure 4. The ROS production in MSSA bacterial cells that are treated with two different concentrations of **1** or BQ after incubated for 2 hours.

The unique dual mode of action of **1**, coupled with the nonoverlapping nature of its antimicrobial mode of action with those of conventional antibiotics suggested that **1** might have the ability to overcome drug resistance in phenotypes of bacteria treated with conventional antibiotics. We first produced a ciprofloxacin-resistant phenotype of MSSA bacteria by treating the wild-type bacteria with a sub-lethal concentration of ciprofloxacin for 24 successive days. The MIC value of ciprofloxacin against this strain of bacteria had a 128-fold increase after 19 passages and remained unchanged until

passage 24. These mutant bacteria were then treated with **1** on passage 24 and lasted for 7 more passages. The results clearly showed that **1** can readily overcome the drug resistance in this phenotype of MSSA bacteria, indicating that the cellular target of **1** is completely different and nonoverlapping with that of ciprofloxacin (**Figure 5**).

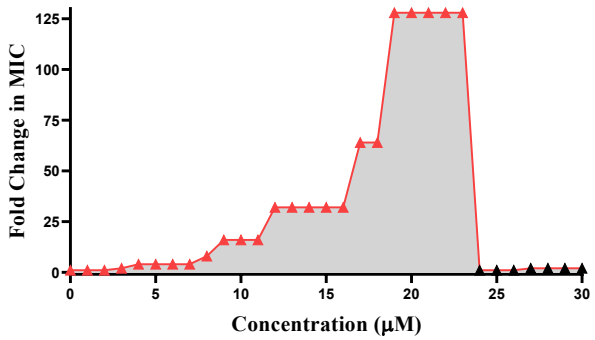

Figure 5. Results of resistance overcome assays of **1** in the phenotype of ciprofloxacin resistant MSSA bacteria.

Preliminary cytotoxicity studies of **1** in human embryonic kidney cells (HEK 293) using the MTT assay showed that IC_{50} of **1** was 7.23 ± 0.5 (see ESI for experimental details of the assay). Hence, the selectivity index (SI), defined as SI = $\frac{IC_{50} of\ mammalian\ cells}{MIC\ of\ bacterial\ cells}$, is estimated to be 11, suggesting that an acceptable therapeutic window (TW) may be found through *in vivo* toxicity studies, if **1** is to be developed as an alternative therapy in place of conventional antibiotics to treat drug-resistant MRSA.

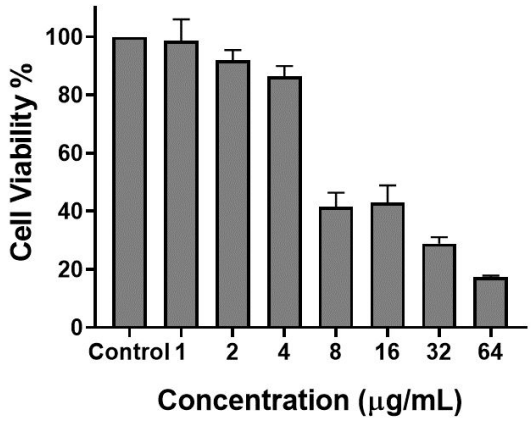

Figure 6. Cell viability curve of **1** in HEK 293 cells.

In conclusion, as a bio-essential element, Mn acts as a cofactor for many enzymes including manganese superoxide dismutase, arginase, and pyruvate carboxylase. It can be obtained directly from the diet or through a dietary supplement, suggesting that introducing Mn into the human body is unlikely to cause any serious toxic side effects. On the other hand, Mn(II) forms the second least stable complex with a given ligand in the Irving-Williams Series, making it the most suitable antimicrobial metal ion to be used to harness the dual antimicrobial mechanism with a judiciously selected metal ionophore. Such a push-and-pull approach may offer a unique opportunity to develop translatable Mn-based antimicrobial agents to treat bacterial infections by MRSA.

Author Contributions

S.D.H. and J.M. were primarily responsible for the conception of the project and the design of all experiments; K.M. contributed to the synthesis and characterization of the complex. B.B. contributed to the synthesis and characterization of BQ. J.A.K. contributed to the crystal structure analysis. K.M. and G.C. contributed to the *in vitro* antimicrobial studies. S.D.H. produced the manuscript with contributions and feedback from all authors. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

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