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Synthesis, anti-microbial, toxicity and molecular docking studies of *N*-nitroso-*N*-phenylhydroxylamine (cupferron) and its derivatives

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ABSTRACT

Bacterial resistance to antimicrobial agents is increasing at an alarming rate globally and requires new lead compounds for antibiotics. In this study, *N*-phenyl-*N*-nitroso hydroxylamine (cupferron) and its derivatives have been synthesised using readily available starting materials. The compounds were obtained in high yield and purity. They show activity towards a range of Gram-positive and Gram-negative pathogenic bacteria, with minimum inhibitory concentration (MIC) values as low as 2 $\mu g.mL^{-1}$ against the tested organisms, especially for Gram-positive species. Toxicity studies on the lead compound **3b** indicate insignificant effects on healthy cell lines. Molecular docking studies on the lead compound identify possible binding modes of the compound, and the results obtained correlate with those of *in vitro* and MIC studies. The lead compound shows excellent drug-likeness properties.

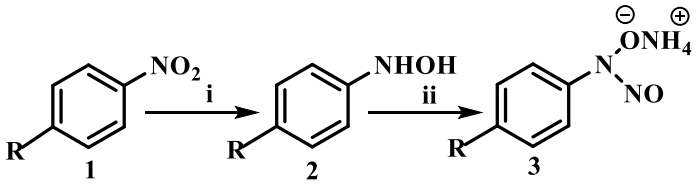
Key words:

Antibiotics, antimicrobial resistance, bacteria, cupferron

There is growing concern globally over antimicrobial resistance in a range of microorganisms of clinical relevance, including and especially hospital acquired infections (nosocomial infections).[1,2] This is a particular consideration in developing countries where resistant communicable diseases can spread rapidly due to limited resources for treatment and management, often with fatal results.[3,4] Natural selection processes favour resistant strains, leading to organisms that are increasingly tolerant of antibiotics.[5] Resistance can also emerge by mutations that alter the drug binding sites in molecular targets and by increased expression of the endogenous efflux pump.[5] Recently, the World Health Organisation (WHO) released a list of drug-resistant bacteria that pose a direct threat to human health, emphasising the urgent need for the development of new antibiotics.[6] The list particularly highlights the danger of Gram-negative bacteria that are already resistant to multiple antibiotics,[7] and includes *Klebsiella pneumonioe, Pseudomonas aeruginosa and Escherichia coli*, *Salmonella typhi, Staphylococcus aureus* and *Streptococcus pyogenes*. These organisms have the ability to develop new resistance pathways, and crucially, translate that resistance to genetic material that flows between species, resulting in drug resistance in wild-type bacteria as well.[8,9] As part of the ongoing search for potent antimicrobial agents to address this threat, we report a study on the evaluation of *N*-aryl-*N*-nitroso hydroxylamine derivatives (commonly referred to as cupferron) as possible antimicrobial agents.

Previous research on cupferron and its analogues like alanosine shows that they are good nitric oxide (NO) donors,[10] which have several applications in pharmacology, *e.g.* vasodilation, inhibition of blood clotting and as antineoplastic agents.[10] Nitric oxide is a key molecule in healthy brain function and is produced by neuronal nitric oxide synthase. This molecule is responsible for killing invading microorganisms.[11] Adding the ONNO-donor moiety to cyclooxygenase-2 (COX-2) inhibitors provides an alternative for designing drugs that can help in adverse cardiovascular

events.[11,12] Cupferron and its derivatives have an ONNO moiety attached directly to carbon, and have been shown to produce NO.[12] The advantage of this type of NO donor is that after release of the NO moiety, the by-product(s) can be regarded as non-toxic.[12] This spurred our interest in cupferron as a biologically active compound,[13] which is further encouraged because there is little known about the biological activity of cupferron compounds.[14] We herein report the synthesis of cupferron and some derivatives as well as their evaluation as potential lead compounds for antimicrobial studies.



Scheme 1: Synthesis of cupferron and derivatives; (i) Zn/NH_4Cl, aq/MeOH; 60 °C, (ii) NH_3/ *n*-C_4H_9-ONO, ether, 0 °C, for **1**–**3**, a–c: R = H, R = Cl and R = $ClCH_2$, respectively.

Reduction of nitroarenes **1a-c** with ammonium chloride and zinc in aqueous methanol led to the formation of aryl hydroxylamines **2a-c** (yield: 95%, 95% and 88%), respectively. Nitrosation of **2a-c** in diethyl ether with ammonia formed cupferron and its derivatives **3a-c** (yield: 61%, 57% and 57%), respectively (Scheme 1). Compound **3a** has been previously reported,[15] but incompletely characterised. In this paper we provide complete characterisation data (see Supporting Information Figures S1–12).

All of the synthesised compounds were tested against Gram-positive (*Staphylococcus aureus* ATCC-25923, *Streptococcus pyogenes* ATCC-19615, *Bacillus subtilis* ATCC-23857 and *Corynebacteria species* ATCC-49368) and Gram-negative (*Escherichia coli* ATCC-25922, *Salmonella typhi* ATCC-6539, *Klebsiella pneumoniae* ATCC-13883 *and Pseudomonas aeruginosa* ATCC-27853) species. The results are shown in Table 1 (see also Supporting Information Figures

S13–16). By and large, the cupferron derivatives show good activity against Gram-positive bacteria (MIC 2–20 μg.mL^{-1}), with lesser activity towards Gram-negative species. The antimicrobial effect is especially evident with chlorocupferron **3b**, which afforded the broadest antimicrobial activity and lowest MIC values.

Table: 1. Antimicrobial studies of cupferron and its derivatives[a]

Compound	**R**	**Microorganism (MIC in μg.mL^{-1})**							
		Sa	Sp	Bs	Cs	Ec	St	Kp	Pa
3a	**H**	20	20	16	20	NA	20	NA	NA
3b	**Cl**	4	4	2	8	20	20	20	20
3c	**CH_2Cl**	16	8	16	16	NA	NA	NA	NA
DMSO[b]	NA	NA	NA	NA	NA	NA	NA	NA	NA
Cip[c]				2	4				8
G[c]		8					16		
S[b]			8			16		16	

[a] Gram-positive bacteria: *S. aureus* (Sa), *S. pyogenes* (Sp), *B. subtilis* (Bs), *C. species* (Cs), Gram-negative bacteria: *E. coli* (Ec), *S. typhi* (St), *K. Pneumoniae* (Kp), *P. aeruginosa* (Pa). NA = no activity. Values are the average of three closely related experimental values. [b]DMSO included as a control due to its use as a solvent carrier for the test compounds. [c]Cip = ciprofloxacin, G = gentamicin, S = streptomycin

Zone of inhibition studies of all test materials were pursued at concentrations between 5–15 mg.mL^{-1} (Table 2). Globally, the results match the MIC studies. The two substituted cupferrons afforded larger zones of inhibition than the parent compound, with chlorocupferron giving the largest zones of inhibition. Highest activity was once again noted for Gram-positive bacteria and this is especially evident at the lowest concentration of 5 mg.mL^{-1}. For Gram-positive bacteria, chlorocupferron **3b** afforded zones of inhibition similar to (but slightly larger than) the best positive

control, ciprofloxacin. For Gram-negative bacteria, the experimental compounds generally showed weak activity compared to positive controls.

Table: 2. Zone of inhibition evaluation of the compounds and the control

		Organisms and zones of inhibition (mm)							
Entry	**Conc.** ($mg.mL^{-1}$)	**Sa**	**Sp**	**Bs**	**Cs**	**Ec**	**St**	**Kp**	**Pa**
3a	5	–[a]	8±0.1	10±0.1	–	–	–	–	–
	10	9±0.1	10±0.2	15±0.1	–	7±0.1	–	–	–
	15	12±0.1	20±0.2	17±0.5	10±0.1	10±0.2	–	–	–
3b	5	10±0.2	12±0.2	14±0.6	13±0.1	10±0.4	9±0.1	8±0.1	5±0.1
	10	12±0.2	15±0.6	19±0.3	16±0.5	14±0.1	13±0.5	11±0.4	10±0.2
	15	25±0.5	27±0.6	35±0.8	23±0.3	20±0.3	19±0.6	15±0.4	13±0.6
3c	5	8±0.1	10±0.9	10±0.1	13±0.4	–	–	–	–
	10	10±0.1	12±0.3	14±0.2	14±0.7	7±0.1	–	7±0.1	10±0.1
	15	20±0.8	18±0.2	18±0.1	17±0.8	10±0.1	–	10±0.2	15±0.1
Cip	5	7±0.3	10±0.7	12±0.4	10±0.5	–	–	–	15±0.4
	10	10±0.1	13±0.1	15±0.1	16±0.4	11±0.7	15±0.9	12±0.6	18±0.5
	15	22±0.9	26±0.9	27±0.6	19±0.6	23±0.4	32±0.4	25±0.4	25±0.5
G	5	10±0.2	3±0.1	5±0.6	–	–	10±0.1	–	–
	10	11±0.5	13±0.4	10±0.4	10±0.3	8±0.4	12±0.5	14±0.5	–
	15	22±0.4	25±0.4	24±0.3	23±0.7	12±0.3	26±0.2	18±0.3	–
S	5	2±0.1	2±0.1	–	–	–	–	–	–
	10	4±0.1	3±0.3	10±0.3	8±0.1	–	7±0.3	–	11±0.2
	15	8±0.1	10±0.6	20±0.6	20±0.8	–	14±0.2	–	15±0.3

[a](–) = No effect. Data with mean ± standard deviation from 3 replicates

The toxicity effect of the lead compound **3b** was evaluated at various concentrations (10, 25, 50 and 100 μM) on healthy human cell lines (kidney LLC-PK1-(ATCC-CL-101) epithelial cells, and liver cell line HepG2-(ATCC-HB-8065)) using the MTT assay, which measures the mitochondria-dependent metabolic activity of cells. Comparative analyses were carried out between the control and treated cell populations. The data obtained are presented in (Figures 1 and 2). The compound displayed low toxicity effects on the cells. Generally, the cells maintained viability of 80% and above. Statistical analyses (Student's t-test, 95% confidence interval) showed no significant differences between the treated cells and the control at the lower concentrations (Figure 2), confirming low toxicity toward these cell lines under the tested assay conditions.

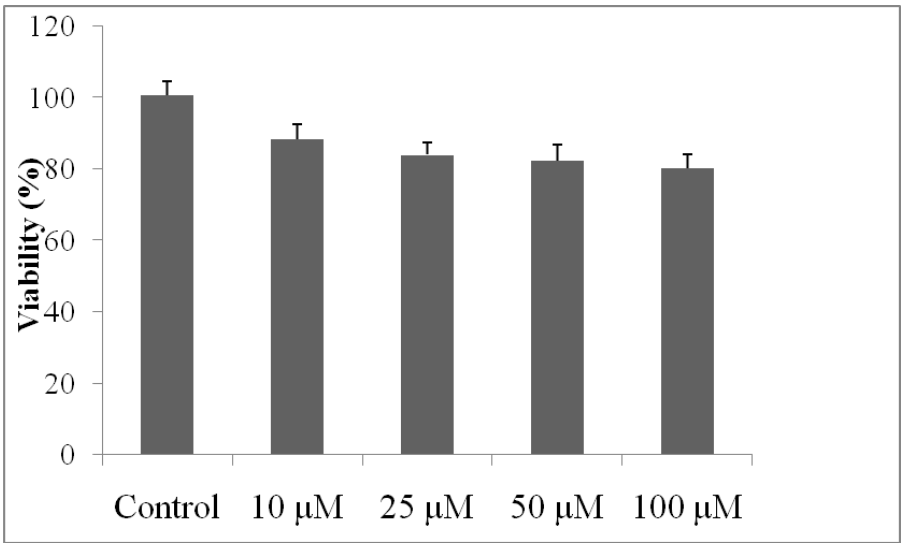


Figure 1: Viability of HepG2-(ATCC-HB-8065) cell lines (% in relation to control) after treatment with different concentrations of **3b**. Control = untreated cells in 10% DMSO

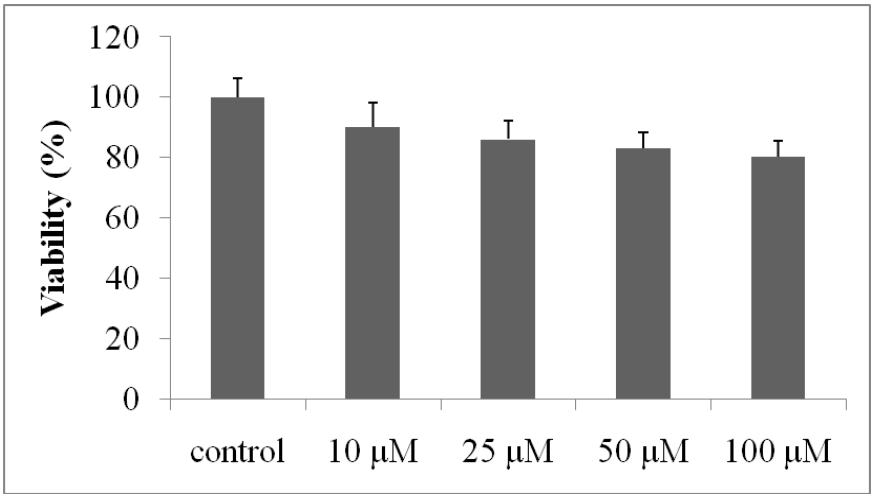


Figure 2: Viability of LLC-PK1-(ATCC-CL-101) cell lines (% in relation to control) after treatment with different concentrations of **3b**. Control = untreated cells in 10% DMSO

Molecular docking studies were carried out to understand the binding profile of the lead compound **3b** and to support the *in vitro* and the MIC results. One of the modes of action of antimicrobial agents is inhibition of the bacterial protein synthesis machinery by small molecules that bind to key structures, blocking their function.[16,17] The ability of the lead compound to inhibit protein synthesis via binding to some key proteins was investigated. This study was done using appropriate datasets and software packages against a selected set of relevant target proteins from the pathogenic organisms, including: *B. subtilis*, *E. coli*, *P. aeruginosa*, *S. aureus*, *K. pneumoniae*, *S. typhi*. The

modeling indicated that the compound was capable of forming bonds with 2-7 amino acid residues in the receptor active pocket.(supporting information Table S1). In general, the compound showed moderate enthalpy of binding ranging from –5.39 to –8.78 kcal/mol (supporting information, Table S1). The highest binding overall score was obtained on *Bsobg* from *B. subtilis* and the lowest on *FtsZ* from *S. pneumoniae*. These results support the *in vitro* and MIC studies. To understand the interaction in somewhat more detail, the compound was subjected to 2D protein-ligand interaction analysis (supporting information Figure S17). Figure S17 exemplifies the potential binding conformation of the docked compound. Furthermore, the compound represents at two H-bond and the commonly interacting residues (supporting information Figure S17) are encircled in red colour. The typical mode of this interaction between the lead compound (**3b**) with *Fabl* protein from *E. coli* is presented in both 2D and 3D conformation in Figures 3a and 3b.

The pharmacokinetics, pharmacodynamics and physicochemical properties were evaluated for **3b**, which is predicted to have good oral bioavailability and shows desirable drug-likeness (Table 4). The results are consistent with Lipinski's rule of five. Expected poor intestinal absorption accompanies molecules with a topological polar surface area value at or above 140 $Å^2$.[18,19] **3b** is calculated to have a topological polar surface area value of 32.7 $Å^2$, which is well within the suggested general upper limit of 140 $Å^2$, and is within the suggested upper limit of 90 $Å^2$ for penetrating the blood-brain-barrier, falling into the preferred range of <70 Å.[20] This indicates a good potential permeability through cellular plasma membranes. Also, according to Lipinski, the probability of being absorbed depends upon the molecular lipophilicity (miLogP) value and is best in the range –0.4 to 5.0. On the basis of this, the compound was found to have miLogP value of 2.5 and this is within the acceptable range (Table 4). The compound has a molecular weight of 187.59 which is compatible with the upper limits 500 as outlined by Lipinski. The results suggest that the

compound has high potential to be absorbed in the intestine (HIA^+) and potentially has the ability to cross the blood brain barriers (BBB^+). It also maintained zero (0) alert to pan assay interference compounds (PAINS) and did not receive an alert as a potential inhibitor of CYP2D6 (Table 4). Thus, the compound possesses desirable physicochemical and pharmacokinetics properties and can be considered as a drug-like molecule.

Table 4: Physicochemical and Pharmacokinetics Analysis

Entry	Properties	Results
1	Molecular weight (≤500)	187.59
2	H-bond acceptors (≤10)	5
3	H-bond donors (≤5)	1
4	miLogP (≤5)	2.5
5	Drug likeness	-0.73
6	Blood-Brain Barrier	Positive
7	CYP2D6 inhibitor	No
8	Human Intestinal Absorption	Positive
9	PAINS	0 Alert
10	Molar refractivity	46
11	Topological Polar Surface Area	$32.7Å^2$
12	Number of Rotatable Bonds	2
13	Number of Atoms	6

Cupferron and two derivatives were synthesised and tested against Gram-positive and Gram-negative bacterial strains. The *in vitro* evaluation and MIC studies show promising results,

especially against the Gram-positive organisms tested. In particular, chlorocupferron **3b** demonstrates excellent antimicrobial activity, in particular against Gram-positive organisms, with MIC values ranging from 2–8 $\mu g.mL^{-1}$ for these species of concern. The lead compound **3b** shows low toxicity effects on healthy human cell lines and no significant difference with the control at p-value < 0.05. Furthermore, molecular docking studies of **3b** identified a range of potential interactions with the principal binding protein-site combination. Of those identified the binding with Arg15, Thr9, and Thr16, from *B. subtilis* protein appeared strongest and correlated well with the experimental results (MIC and zone of inhibition). The compound was predicted by *in silico* ADMET studies to possess good drug-likeness properties. This provides a strong platform for a medicinal chemistry approach to fine tune the characteristics and activity of the lead compound.

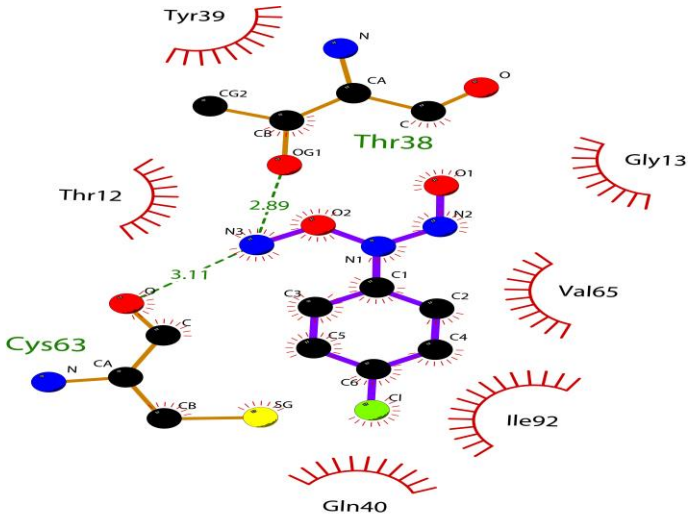


Figure 3a: 2D representation of the interaction of the lead compound **3b** with the *Fabl* protein from *E. coli*.

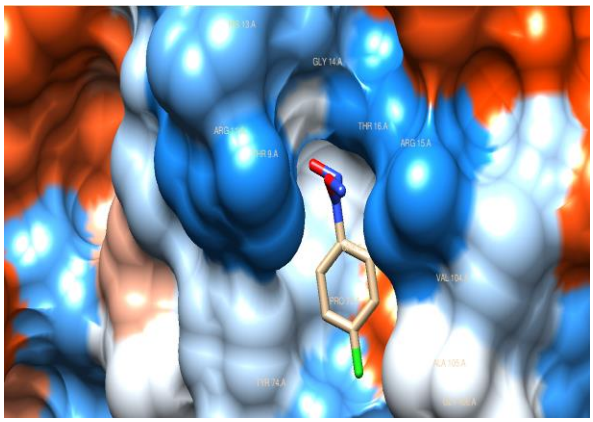


Figure 3b: 3D representation of the interaction of the lead compound **3b** in pocket of the *Fabl* protein from *E. coli*.

Author contribution statement

All authors approve the submission of this manuscript. All authors participated in the conceptualisation of the study, in the drafting of the manuscript and the interpretation of the data. IW conducted the experimental work.

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Declaration of competing interest

The authors declare no conflict of interest.

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Supporting information

The supporting information associated with this article can found, in the online version, at…

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