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PII: S0223-5234(17)30802-4

DOI: 10.1016/j.ejmech.2017.10.010

Reference: EJMECH 9802

To appear in: *European Journal of Medicinal Chemistry*

Received Date: 8 June 2017

Revised Date: 7 September 2017

Accepted Date: 4 October 2017

Please cite this article as: N. Nguyen, D.W. Wilson, G. Nagalingam, J.A. Triccas, E.K. Schneider, J. Li, T. Velkov, J. Baell, Broad activity of diphenyleneiodonium analogues against *Mycobacterium tuberculosis*, malaria parasites and bacterial pathogens, *European Journal of Medicinal Chemistry* (2017), doi: 10.1016/j.ejmech.2017.10.010.

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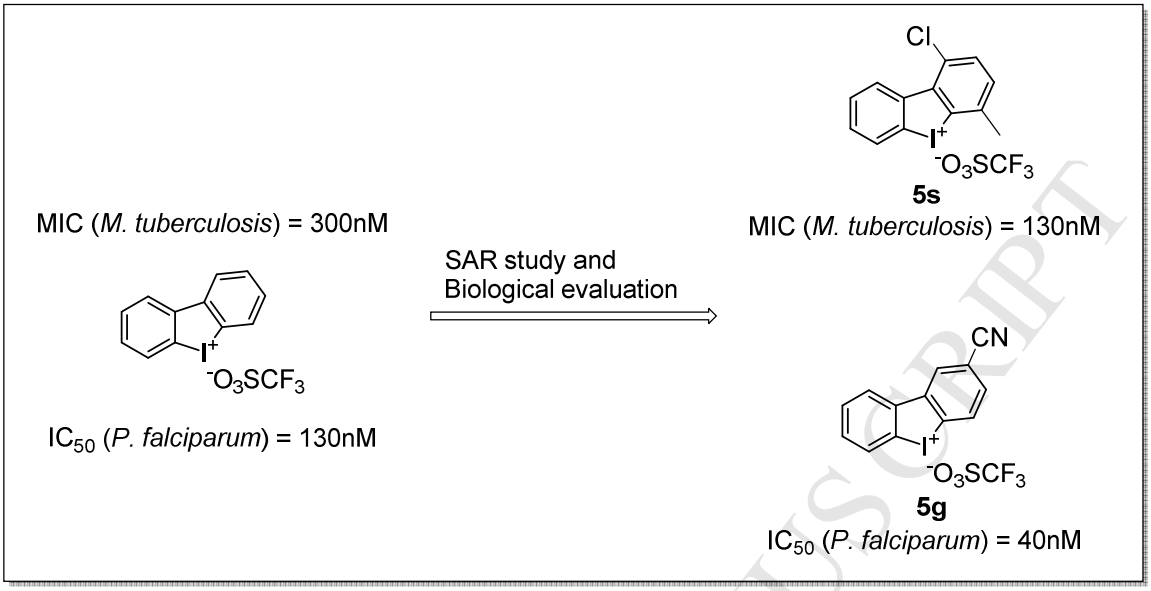
Graphical abstract



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Broad activity of diphenyleneiodonium analogues against *Mycobacterium tuberculosis*, malaria parasites and bacterial pathogens

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Key words: diphenyleneiodonium, type II NADH-quinone oxidoreductase, Gram-negative, *Plasmodium*, malaria.

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Abbreviations: DPI, diphenyleneiodonium; NADH, nicotinamide adenine dinucleotide reduced; NDH-2, type II NADH-quinone oxidoreductases

Abstract

In this study, a structure-activity relationship (SAR) compound series based on the NDH-2 inhibitor diphenyleneiodonium (DPI) was synthesised. Compounds were evaluated primarily for *in vitro* efficacy against Gram-positive and Gram-negative bacteria, commonly responsible for nosocomial and community acquired infections. In addition, we also assessed the activity of these compounds against *Mycobacterium tuberculosis* (Tuberculosis) and *Plasmodium* spp. (Malaria). This led to the discovery of highly potent compounds active against bacterial pathogens and malaria parasites in the low nanomolar range, several of which had favourable toxicity profiles against mammalian cells.

1. Introduction

Antibiotic resistance has evolved into a serious global health concern [1, 2]. In the United States over 23,000 people die each year due to infections with antibiotic-resistant bacteria. Notwithstanding the human cost, antibiotic resistance is also a massive economic burden which has been estimated to cost as much as $20 billion USD in excess healthcare expenses, with associated lost productivity estimated to be as high as $35 billion USD/year [2]. Sadly, the ‘magic bullet’ antimicrobial therapies we have gratuitously used over the past decades are rapidly losing their calibre. Modern healthcare over the last century has been founded on the basis that bacterial infections can be effectively treated using antimicrobial drugs. In a world without effective antibiotics, modern medical procedures that we take for granted, such as chemotherapy or simple surgery, will have increasing risk due to the threat of untreatable bacterial infections. Once again common bacterial infections will more than often result in death. Medicine is clearly entering a critical period, if bacteria continue developing resistance to multiple antibiotics at the present rate, and at the same time the pipeline continues to dry up, there could be catastrophic costs to healthcare and society [3].

In the developing world, infectious diseases caused by microbial pathogens remain a major disease burden with malaria (*Plasmodium* spp.; 429,000 deaths in 2015)[4] and TB (*Mycobacterium tuberculosis*; 1,800,000 deaths in 2015)[5] contributing to >2 million deaths every year globally [6]. Multi-drug resistant malaria parasites, particularly *P. falciparum* which causes the greatest burden of mortality, and *M. tuberculosis* are spreading in the Asia-Pacific region [7, 8], reducing the effectiveness of current front-line drugs and increasing potentially deadly treatment failures.

There is an urgent unmet medical need to discover new scaffolds with superior activity against these problematic human pathogens. Enzymes involved in energy metabolism are emerging as very important novel drug targets for anti-infective drug development [9-15]. Encouragingly, respiratory chain inhibitors appear to be the Achilles' heels of dormant non-replicating cells ('persisters'), that are often refractory to antibiotics and difficult to treat [16]. Instead of the multi-subunit complex I respiratory enzyme found in mammalian cells, protozoa, bacteria and plants possess a single sub-unit non-proton pumping, rotenone insensitive alternative complex I [10, 14, 17, 18]. This type II NADH-menaquinone oxidoreductase (NDH-2) contains a single non-covalently bound flavin adenine dinucleotide (FAD) cofactor and catalyzes the oxidation of NADH with menaquinone [19]. The absence of NDH-2 in the respiratory chain of mammalian mitochondria makes it a very attractive target for antibiotic drug development.

Diphenyleneiodonium (DPI) is known to be a potent, yet undeveloped, inhibitor of NDH-2 [15, 20]. A few very early reports investigated the potential of iodonium compounds, including DPI, as skin antiseptics against Gram-negative bacteria [21-23]. In the present study we have synthesized a series of novel DPI analogues and evaluated their *in vitro* effectiveness against infectious human pathogens that rely on NDH-2 for their energy needs, namely Gram-positive and Gram-negative bacteria commonly responsible for nosocomial

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and community acquired infections and *Mycobacterium tuberculosis*. In addition, we explored the sensitivity of *Plasmodium* spp. malaria parasites, which have a type II NADH-menaquinone oxidoreductase of unclear function, to the DPI analogues. The potential of these compounds as antimicrobial leads with a novel mode of action are discussed.

2. Results

2.1 Chemistry

The synthetic route to access the diphenyleneiodonium core typically involves 3 steps as described in the literature (Scheme 1)[24]. The biphenyl amine **3** is classically formed in good to excellent yield using a Suzuki-coupling reaction between an appropriately substituted 2-iodoaniline **1** and an appropriately substituted phenylboronic acid **2**. The biphenyl amine 3 was subsequently transformed into the biphenyl iodide **4** through diazotisation in Sandmeyer reaction, followed by oxidation of the biphenyl iodide **4** utilising *m*-CPBA under acidic conditions to afford the corresponding cyclic diphenyleneiodonium compound **5** (Scheme 1). The desired final products were obtained as precipitates from the reaction mixture and were easily isolated in high purity and acceptable yields. This procedure was successfully adopted for the synthesis of all novel DPI analogues reported herein as shown in Table 1.

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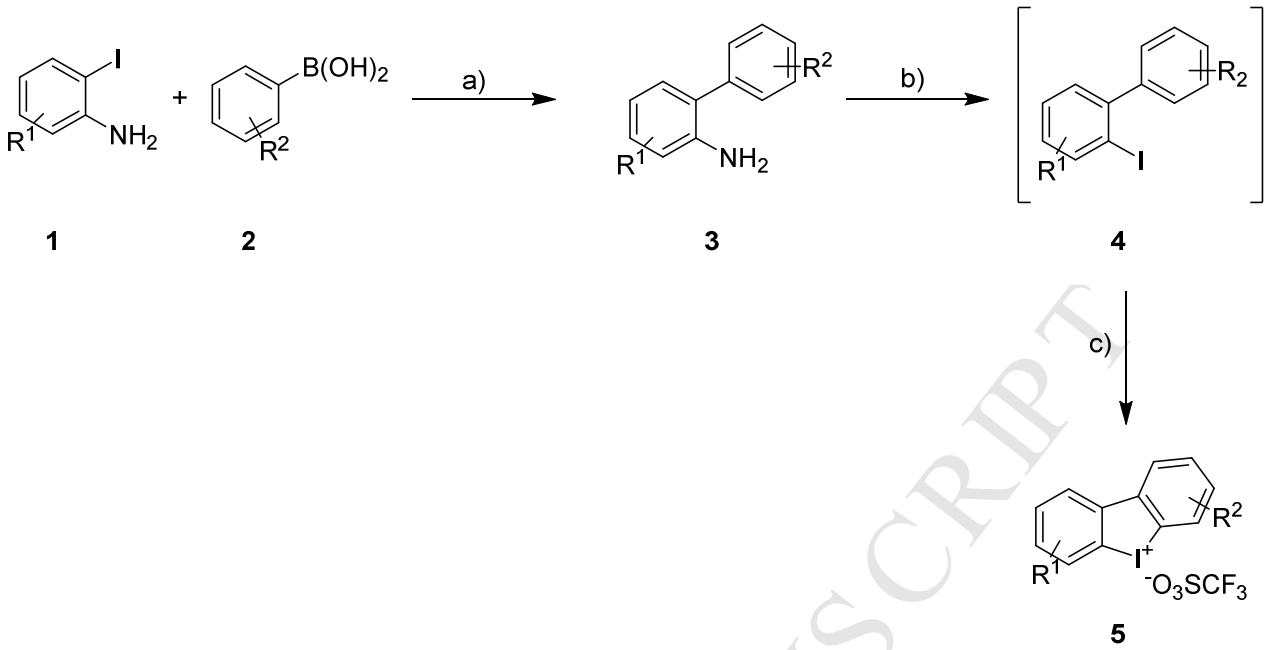
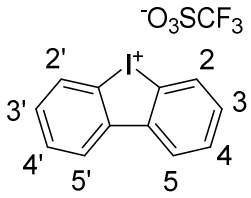


Scheme 1. Synthesis of Cyclic Diphenyleniodonium Trifluoromethanesulfonate **5**[a]. [a]Reagents and conditions: a) Dioxane/H_2O (9:1), K_2CO_3, TBAB, $Pd(dppf)Cl_2$, 130 ^oC, 1 h; b) 1. THF, 4 M HCl, 0 ^oC, aq. $NaNO_2$, 20 min. 2. aq. KI, 0 ^oC 10 min, r.t, 1 h; c) CH_2Cl_2, *m*-CPBA, TfOH, r.t, 1 h.

Table 1

Structures of DPI analogues synthesised and numbering system adopted



Compound	R	Compound	R	Compound	R	Compound	R
5o	H					**5aa**	5-F, 5'-F
5n	3-F	**5i**	4-F	**5d**	5-F	**5z**	5-F, 5'-Cl
5m	3-Cl	**5h**	4-Cl	**5c**	5-Cl	**5y**	5-Cl, 2'-Cl
5l	3-CN	**5g**	4-CN			**5x**	5-F, 2'Cl
5k	3-OMe	**5f**	4-OMe	**5b**	5-OMe	**5w**	5-Me, 2'-Cl
5j	3-Me	**5e**	4-Me	**5a**	5-Me	**5v**	5-Cl, 2-Cl
		5p	4,5-benzo			**5u**	5-Cl, 2-F
				5q	5-CF3	**5t**	5-Cl, 2-OMe

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5r	5-OCF3	**5s**	5-Cl, 2-Me

Note: The ring numbering system used herein is only for readers' convenience (not IUPAC nomenclature).

The compounds were made iteratively in two series. The first series comprises **5o**, **5a**, **5b**, **5c**, **5d**, **5e**, **5f**, **5g**, **5h**, **5i**, **5j**, **5k**, **5l**, **5m**, **5n**, **5p** and will be referred to hereon as DPI series 1, while DPI series 2 comprises **5q**, **5r**, **5s**, **5t**, **5u**, **5v**, **5w**, **5y**, **5x**, **5z**, **5aa**.

2.2 *Gram-positive and -negative bacteria MIC determination*

MIC determinations for DPI series 1 against several problematic Gram-positive and -negative bacterial species revealed very potent activity for several analogues. In support of our recent report [25], DPI itself (**5o**) displayed good activity against *Pseudomonas aeuruginosa* (MICs generally 2.3-75 µM) and *Acinetobacter baumannii* (MICs 0.58-19 µM). Activity was slightly weaker against *Klebsiella pneumoniae* (MICs 37 and 75 µM), vancomycin-resistant *Enterococcus faecium* (MICs 37 µM) but better against drug resistant strains of *Staphylococcus aureus* (MICs 4.7 and 19 µM) (Table 2).

Several analogues had very similar profiles to DPI, these being **5a**, **5h**, **5i**, **5l**, **5n** and **5p**, indicating that activity was not greatly influence by installation of a 5-Me, 4-Cl, 4-F, 3-CN, 3-F or a fused phenyl ring. One analogue lost significant activity in a broad spectrum sense, this being **5b**, suggesting that the electron donating methoxy group in the 5-position is particularly disfavoured. There were six other analogues (**5e**, **5f**, **5g**, **5j** and **5k**) that were broadly similar in profile to DPI with the exception of selectively weaker activity against certain strains, an example being **5f** (4-OMe) and **5k** (3-OMe) with strikingly weaker activity against Pa QLD PSA (70 µM), Pa M146201 and Ab07AC-366 (Table 2). In contrast, **5c** displayed exquisitely potent activity across a broad spectrum of strains, with potency greater than the detectable limit for 11 bacterial strains.

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The stability of the DPI analogues were investigated by incubating of analogue (**5j**) in bacterial culture media under standard assay conditions (37 °C, 200 µL Cation-Adjusted Mueller-Hinton Broth, 96-well polypropylene microtitre plates) and tested the resulting chemical species of the compound by LCMS in the next day. The LCMS analysis showed that the compound was stable under this condition.

Further, the impact of the counter anion towards the biological activity was investigated. A small selected set of chloride counter ion analogues were synthesised, where the chloride counter ion replaces the triflate counter ion. These chloride containing compounds were tested against a selected Gram-negative bacteria (Pa ATCC 27853). The results indicate that there is no notable anion linked trend in these results, hence there appears to be no strong link between the biological activity and the presence/absence of triflate anion.

Table 2

Gram-positive and -negative bacteria MIC determination

	MIC Activity µM (SI)								
	5o	**5a**	**5b**	**5c**	**5d**	**5e**	**5f**	**5g**	**PMB**
Pa ATCC 27853	75 (0.39)	18 (0.14)	>70 (>0.55)	<0.27 (<2.4)	2.2 (2.1)	>73 (>0.52)	>70 (>0.29)	71 (0.32)	0.77
Pa QLD PSA 1	9.3 (3.1)	4.5 (0.58)	>70 (>0.55)	0.54 (1.2)	4.5 (1.0)	36 (1.0)	70 (0.29)	18 (0.13)	0.77
Pa #912	4.7 (6.3)	1.1 (2.3)	70 (0.55)	<0.27 (<2.4)	4.5 (1.0)	9.0 (4.2)	8.7 2.3)	8.8 (2.5)	1.5
Pa 19147nm	2.3 (13)	0.57 (4.6)	70 (0.55)	<0.27 (<2.4)	<0.28 (<16)	9.0 (4.2)	18 (1.2)	4.4 (5.1)	25
Pa 18878B klon 1	4.7 (6.3)	1.1 (2.3)	70 (0.55)	<0.27 (<2.4)	<0.28 (<16)	9.0 (4.2)	35 (0.58)	8.8 (2.5)	>25
Pa M146201	9.3 (3.1)	2.3 (1.2)	>70 (>0.55)	0.54 (1.2)	1.1 (4.1)	36 (1.0)	70 (0.29)	8.8 (2.5)	3.1
Ab ATCC 19606	9.3 (3.1)	2.3 (1.2)	35 (1.1)	<0.27 (<2.4)	0.56 (8.2)	18 (2.2)	35 (0.58)	18 (0.13)	0.77
Ab 246-01-C	4.7 (6.3)	2.3 (1.2)	70 (0.55)	<0.27 (<2.4)	<0.28 (<16)	9.0 (4.2)	35 (0.58)	18 (0.13)	0.19
Ab ATCC 17978	2.3 (13)	1.1 (2.3)	35 (1.1)	<0.27 (<2.4)	0.56 (8.2)	9.0 (4.2)	18 (1.2)	4.4 (5.1)	0.38
Ab ATCC 19606 col10	4.7 (6.3)	2.3 (1.2)	17 (2.2)	<0.27 (<2.4)	0.56 (8.2)	9.0 (4.2)	8.7 (2.3)	2.2 (10)	98
Ab 07AC-336	19 (1.6)	2.2 (1.2)	70 (0.55)	<0.27 (<2.4)	1.1 (4.1)	18 (2.2)	70 (0.29)	71 (0.32)	6.2
Ab ATCC 17978 col10	0.58 (50)	0.57 (4.6)	35 (1.1)	<0.27 (<2.4)	<0.28 (<16)	1.1 (33)	4.4 (4.6)	2.2 (10)	12
Kp ATCC 13883	37 (0.78)	18 (0.14)	>70 (>0.55)	4.3 (0.15)	4.5 (1.0)	72 (0.52)	70 (0.29)	35 (0.63)	12
Kp M320445	37 (0.78)	36 (0.07)	70 (0.55)	8.6 (0.08)	9.0 (0.51)	72 (0.52)	70 (0.29)	71 (0.32)	0.38

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Kp #1	75 (0.78)	72 (0.04)	>70 (>0.55)	8.6 (0.08)	9.0 (0.51)	>72 (>0.52)	>70 (>0.29)	>71 (>0.32)	98
Kp 224-11-C	75 (0.78)	>72 (>0.04)	>70 (>0.55)	8.6 (0.08)	9.0 (0.51)	>72 (>0.52)	>70 (>0.29)	>71 (>0.32)	>25
Kp 248-33-D	37 (0.78)	>72 (>0.04)	>70 (>0.55)	8.6 (0.08)	9.0 (0.51)	72 (0.52)	>70 (>0.29)	71 (0.32)	12
Ec N2381	37 (0.78)	18 (0.14)	70 (0.55)	0.54 (1.2)	1.1 (4.2)	72 (0.52)	>70 (>0.29)	71 (0.32)	1.5
Ec N4149	37 (0.78)	>72 (>0.04)	>70 (>0.55)	0.54 (1.2)	1.1 (4.2)	>72 (>0.52)	>70 (>0.29)	>71 (>0.32)	1.5
Ec N11281	37 (0.78)	18 (0.14)	>70 (>0.55)	2.2 (0.31)	4.5 (1.0)	72 (0.52)	>70 (>0.29)	71 (0.32)	1.5
VRE ATCC 700221	4.7 (6.3)	2.3 (1.2)	8.7 (4.4)	<0.27 (<2.4)	0.56 (8.2)	4.5 (8.3)	4.4 (4.6)	1.1 (20)	>25
MRSA ATCC 43300	4.7 (6.3)	36 (0.07)	35 (1.1)	1.1 (0.61)	2.2 (2.1)	9.0 (4.2)	18 (1.5)	1.1 (20)	>25
VISA ATCC 700698	19 (1.6)	36 (0.07)	35 (1.1)	2.3 (0.29)	2.2 (2.1)	18 (2.2)	35 (0.58)	8.8 (2.5)	>25
VRSA ATCC 700699	19 (1.6)	36 (0.07)	35 (1.1)	1.1 (0.61)	2.2 (2.1)	18 (2.2)	35 (0.58)	8.8 (2.5)	>25

142 **Table 2**. (continued)

	MIC Activity µM (SI)								
	5h	**5i**	**5j**	**5k**	**5l**	**5m**	**5n**	**5p**	**PMB**
Pa ATCC 27853	8.7 (1.9)	18 (0.67)	72 (0.05)	>70 (>0.11)	35 (0.09)	8.7 (0.13)	8.9 (0.66)	8.4 (0.09)	0.77
Pa QLD PSA 1	8.7 (1.9)	18 (0.67)	>72 (>0.05)	70 (0.11)	8.8 (0.37)	2.2 (0.51)	8.9 (0.66)	4.2 (0.17)	0.77
Pa #912	4.3 (3.7)	2.2 (5.3)	4.5 (0.81)	>70 (>0.11)	4.4 (0.75)	1.1 (1.0)	1.1 (5.3)	2.1 (0.34)	1.5
Pa 19147nm	4.3 (3.7)	1.1 (11)	4.5 (0.81)	8.7 (0.81)	2.2 (1.5)	0.54 (2.0)	0.56 (11)	2.1 (0.34)	25
Pa 18878B klon 1	2.2 (7.5)	2.2 (5.3)	4.5 (0.81)	18 (0.39)	4.4 (0.75)	0.54 (2.0)	1.1 (5.3)	2.1 (0.34)	>25
Pa M146201	4.3 (3.7)	4.5 (2.7)	36 (0.11)	70 (0.11)	18 (0.19)	2.2 (0.51)	2.2 (2.6)	8.4 (0.09)	3.1
Ab ATCC 19606	4.3 (3.7)	4.5 (2.7)	9.1 (0.41)	18 (0.39)	18 (0.19)	2.2 (0.51)	4.5 (2.6)	4.2 (0.17)	0.77
Ab 246-01-C	4.3 (3.7)	4.5 (2.7)	18 (0.21)	35 (0.21)	18 (0.19)	2.2 (0.51)	4.5 (2.6)	4.2 (0.17)	0.19
Ab ATCC 17978	2.2 (7.5)	2.2 (5.3)	4.5 (0.81)	18 (0.39)	4.4 (0.75)	0.54 (2.0)	1.1 (5.3)	1.1 (0.69)	0.38
Ab ATCC 19606 col10	2.2 (7.5)	2.2 (5.3)	4.5 (0.81)	2.2 (3.1)	2.2 (1.5)	1.1 (1.0)	2.2 (2.6)	1.1 (0.69)	98
Ab 07AC-336	8.7 (1.9)	9.1 (1.3)	18 (0.21)	>70 (>0.11)	>71 (>0.05)	2.2 (0.51)	8.9 (0.66)	8.4 (0.09)	6.2
Ab ATCC 17978 col10	0.5 (30)	0.56 (2.0)	1.1 (3.2)	2.2 (3.1)	2.2 (1.5)	<0.27 (<4.1)	0.56 (11)	0.52 (1.4)	12
Kp ATCC 13883	8.7 (1.9)	18 (0.67)	72 (0.05)	>70 (>0.11)	18 (0.19)	8.7 (0.13)	36 (0.16)	8.4 (0.09)	12
Kp M320445	35 (0.47)	18 (0.67)	36 (0.11)	70 (0.11)	35 (0.09)	8.7 (0.13)	36 (0.16)	17 (0.04)	0.38
Kp #1	35 (0.47)	36 (0.33)	72 (0.05)	>70 (>0.11)	>71 (>0.05)	17 (0.06)	72 (0.08)	34 (0.02)	98
Kp 224-11-C	35 (0.47)	36 (0.33)	72 (0.05)	>70 (>0.11)	>71 (>0.05)	17 (0.06)	72 (0.08)	34 (0.02)	>25
Kp 248-33-D	17 (0.93)	18 (0.67)	72 (0.05)	>70 (>0.11)	>71 (>0.05)	17 (0.06)	36 (0.16)	34 (0.02)	12
Ec N2381	17 (0.93)	18 (0.67)	72 (0.05)	35 (0.21)	71 (0.05)	8.7 (0.13)	18 (0.08)	8.4 (0.09)	1.5
Ec N4149	17 (0.93)	36 (0.33)	72 (0.05)	>70 (>0.11)	71 (0.05)	8.7 (0.13)	18 (0.08)	34 (0.02)	1.5
Ec N11281	17 (0.93)	18 (0.67)	36 (0.11)	>70 (>0.11)	71 (0.05)	4.3 (0.26)	18 (0.08)	17 (0.04)	1.5
VRE ATCC 700221	8.7 (1.9)	4.5 (2.7)	4.5 (0.81)	4.4 (1.6)	2.2 (1.5)	1.1 (1.0)	2.2 (2.6)	1.1 (0.69)	>25
MRSA ATCC 43300	2.2 (7.5)	4.5 (2.7)	4.5 (0.81)	4.4 (1.6)	4.4 (0.75)	2.2 (0.51)	2.2 (2.6)	2.1 (0.34)	>25
VISA ATCC 700698	4.3 (3.7)	18 (0.67)	9.1 (0.41)	8.7 (0.78)	8.8 (0.37)	2.2 (0.51)	8.9 (0.66)	4.2 (0.17)	>25

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VRSA ATCC 700699	4.3 (3.7)	9.1 (1.3)	9.05 (0.398)	8.7 (0.78)	8.8 (0.37)	2.2 (0.51)	8.9 (0.66)	4.2 (0.17)	>25

(SI) = selectivity index relative to HEPG2 cells and was calculated by CC_{50} (1%FBS)/MIC.

2.3 Inhibition of Mycobacterium tuberculosis *growth*

Shown in Table 3 are the results of testing DPI series 1 compounds and DPI series 2 compounds against *M. tuberculosis*. It can be seen in Table 3 that all compounds displayed anti-mycobacterial activity and that the weakest compound, **5b**, nicely matched the observation that this compound was also the weakest in the bacterial panel. Further, **5e**, **5f**, **5j** and **5k** are also common to the groupings of compounds that are slightly less potent than DPI.

The observation that **5c** was very potent in the bacterial panel but also amongst the most potent compounds against *M. tuberculosis* led us to synthesise a set of second generation analogues based around the structure of **5c**. This compound contains a 5-chloro substituent and so our DPI series 2 focuses on analogues with a halogen in a 5-position. Gratifyingly, as show in Table 3, these compounds were uniformly extremely potent. In particular, **5s**, with 5-Cl, 2-Me substitution, was extremely potent, and 3-fold more potent than **5o** (DPI itself), with an MIC of 0.13 µM.

Table 3

Inhibition of *Mycobacterium tuberculosis* growth

DPI Series 1	MIC µM (SI)	DPI Series 2	MIC µM (SI) (SI)[a]
5o	0.30 (96)	**5q**	0.26 (0.09) (0.36)[a]
(5a	0.14 (19)	**5r**	0.25 (0.21) (0.99)[a]
5b	4.4 (8.8)	**5s**	0.13 (0.74) (2.8)[a]
5c	0.28 (2.4)	**5t**	0.51 (0.31) (0.44)[a]
5d	0.29 (16)	**5u**	0.27 (3.4) (10)[a]
5e	0.57 (66)	**5v**	0.50 (1.8) (3.7)[a]
5f	1.1 (19)	**5w**	0.27 (0.22) (1.8)[a]

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5g	0.29 (78)	**5x**	0.27 (0.19) (1.4)[a]
5h	0.54 (30)	**5y**	0.26 (0.32) (2.1)[a]
5i	0.29 (41)	**5z**	1.0 (0.38) (0.80)[a]
5j	0.57 (6.4)	**5aa**	0.28 (0.32) (0.58)[a]
5k	0.28 (24)		
5l	0.29 (12)		
5m	0.28 (3.9)		
5n	0.29 (20)		
5p	0.52 (2.4)		
Rifampicin	0.30		0.61

(SI) = selectivity index relative to HEPG2 cells and was calculated by CC_{50} (1%FBS)/MIC.

(SI)[a] = selectivity index relative to HEPG2 cells and was calculated by CC_{50} (10%FBS)/MIC.

2.4 In vitro activity of DPI against Plasmodium *spp.*

DPI has been shown to inhibit *P. falciparum* growth *in vitro*, however, the reported IC_{50} values and the proposed mechanism of action vary [26, 27] . In this study, the growth inhibitory IC_{50} for D10 (chloroquine sensitive; 0.13 µM) parasites after 90 hours of treatment (early ring stage to late trophozoite stage next cycle) was 3.3-fold higher than that achieved for the CS2 chloroquine resistant line (0.04 µM; Table 4 and Suppl. Fig. 1A), indicating that DPI is inhibitory to both chloroquine resistant and sensitive strains *in vitro*. The zoonotic human pathogen *P. knowlesi* YH1[28], an emerging pathogen in Southeast Asia and a laboratory adapted model for the major human pathogen *P. vivax*, had an IC_{50} 5.6 fold higher than that of the chloroquine sensitive D10 line at 0.74 µM (Table 4 and Suppl. Fig. 1A). Whether this represents a difference in parasite sensitivities, or is due to the shorter lifecycle of *P. knowlesi* (around 32 hours in these assays) and therefore reduced exposure time is not clear.

The *in vitro* growth inhibitory activity after 90 hours of treatment of the 26 DPI analogues was tested against the D10 chloroquine sensitive line (Table 5, Suppl. Fig. 1B and

1C). The activity of the analogues ranged from >3-fold decrease in growth inhibitory IC_{50} compared to DPI (to below 0.045 µM), to a loss of growth inhibition up to a concentration of 1 µM. Six of the 26 analogues had a greater than 2-fold increase in growth inhibitory activity. *P. knowlesi* growth inhibition was also tested against three analogues which displayed increased potency against *P. falciparum*. Analogues **5c** (*P.k.* YH1 0.08 µM versus D10 0.05 µM, p>0.054, Table 5), **5d** (*P.k.* YH1 0.08 µM versus D10 0.05 µM, p>0.032) and **5g** (*P.k.* YH1 0.11 µM versus D10 0.04 µM, p>0.0016), showed significantly improved potency over DPI and inhibited *P. knowlesi* growth to a similar extent to *P. falciparum*.

Next we attempted to make the D10 chloroquine sensitive line resistant to DPI. After 6 days of treatment with 0.2 µM DPI, the DPI concentration was raised to 0.4 µM for a further 6 days. The DPI concentration was then reduced back to 0.2 µM and parasites grown continuously under drug pressure. After a further 18 days of culture, viable parasites were visible and maintained under drug pressure until a stable population could be obtained (called D10-DPI^{R}). A sub culture of DPI selected parasites was made and the drug pressure was removed to test whether a tolerant, rather than resistant, parasite population had been selected for (line with DPI drug pressure removed for between 2-5 weeks is called D10-DPI^{off}). There was no statistical difference between the growth inhibitory IC_{50} for the D10-DPI^{r} line (0.36 µM) and the D10-DPI^{off} line (0.46 µM; p=0.264). In contrast, there was a ~2-fold difference between both the D10-DPI^{r} (p=0.011) and D10-DPI^{off} (p=0.005) lines compared to D10 (0.19 µM, Fig. 2) in parallel experiments. Sequence comparison between the D10-PfPHG parental, D10-DPI^{r} and D10-DPI^{off} lines showed no mutations in PfNDH2, suggesting it is unlikely that mutations in PfNDH2 are causing the reduced sensitivity to DPI. The sequence of Type II *Pf* dihydroorotate dehydrogenase, reported as an alternative target of PfNDH2 inhibitors [27], was also found to have no mutations associated with reduced sensitivity to DPI. It remains to be determined the mechanism by which resistance to DPI occurs in these

parasites. Attempts to select drug resistant parasites at a DPI concentration of 0.8 µM between days 6 and 12 were unsuccessful.

Changes in sensitivity of the D10-DPI^R line to more potent DPI analogues **5q**, **5c**, **5d** and **5g** were assessed by comparing the growth inhibitory IC_{50} between the resistant and parental lines (Table 5). The D10-DPI^R line was significantly more tolerant of analogue **5g** (D10-DPI^R 0.09 µM versus D10 0.04 µM, p=0.003), but not of **5c** (D10-DPI^R 0.07 µM versus D10 0.05 µM, p=0.165), **5d** (D10-DPI^R 0.08 µM versus D10 0.05 µM, p=0.0567) and **5q** (D10-DPI^R 0.14 µM versus D10 0.13 µM, p=0.091). These data indicate that selection for a DPI resistant line did not necessarily confer resistance to analogues of DPI.

In general, the potencies of DPI analogues in series 1 for bacterial species and *M. tuberculosis* (e.g increased potency for **5c**; reduced potency for **5b**) were reflected in the relative potency for *P. falciparum*. However, some discrepancies were observed with **5g** and **5l**, both potent inhibitors of *P. falciparum* that were very poor inhibitors of bacteria. This result suggests that the hydrophilic electron withdrawing groups at the 3- and 4-position are particularly disfavoured against bacterial isolates. In terms of series 2 compounds, there were broad similarities between the IC_{50} for *P. falciparum* and *M. tuberculosis* (R^2 0.27, p=0.101), with the exception of **5u** and **5v** which were inhibitory to *M. tuberculosis* but not *P. falciparum* growth, suggesting that 2 electron withdrawing substituents at the 2- and 5-position on the same phenyl ring are not tolerated for *P. falciparum* growth inhibition. When **5u** and **5v** were removed from the comparison, the correlation between growth inhibitory IC_{50}s for *P. falciparum* and *M. tuberculosis* improved significantly (R^2 0.455, p=0.046), confirming the broad similarity of drug potency for series 2 compounds against these important human pathogens.

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Table 4

In vitro activity of DPI against *P. falciparum* and *P. knowlesi* malaria

Parasite line	IC_{50}-90 hour (μM)
Pf D10 (chloroquine sensitive)	0.13
Pf CS2 (chloroquine resistant)	0.04
P. knowlesi YH1	0.74

Table 5

In vitro activity of DPI analogues against *P. falciparum* and *P. knowlesi* malaria

DPI Series 1	IC_{50} μM (SI) *Pf* D10	IC_{50} μM (SI) *Pf* $D10^r$	IC_{50} μM (SI) *Pk* YH1	DPI Series 2	IC_{50} μM (SI) (SI)[a] *Pf* D10	IC_{50} μM (SI) (SI)[a] *Pf* $D10^r$
5o	0.13 (221)			**5q**	0.13 (0.18) (0.72)[a]	0.14 (0.18) (0.70)[a]
5a	0.07 (37)			**5r**	0.13 (0.39) (1.9)[a]	
5b	0.17 (225)			**5s**	0.11 (0.82) (3.1)[a]	
5c	0.05 (13)	0.07 (8.9)	0.08 (8.5)	**5t**	0.13 (1.2) (1.75)[a]	
5d	0.05 (96)	0.08 (61)	0.08 (59)	**5u**	>1 (>0.93) (>2.7)[a]	
5e	0.08 (500)			**5v**	>1 (>0.89) (>1.9)[a]	
5f	0.10 (213)			**5w**	0.11 (0.63) (5.1)[a]	
5g	0.04 (531)	0.09 (256)	0.11 (232)	**5x**	0.19 (0.28) (2.1)[a]	
5h	0.08 (194)			**5y**	0.36 (0.23) (1.5)[a]	
5i	0.10 (125)			**5z**	>1 (>0.39) (0.83)[a]	
5j	0.23 (15.8)			**5aa**	0.84 (0.11) (0.19)[a]	
5l	0.05 (62)					
5m	0.07 (15)					

5n	0.06 (105)
5p	0.12 (5.8)
Chloroquine	0.03

(SI) = selectivity index relative to HEPG2 cells and was calculated by CC_{50} (1% FBS)/IC_{50}.

(SI)[a] = selectivity index relative to HEPG2 cells and was calculated by CC_{50} (10% FBS)/MIC.

Fig. 1A

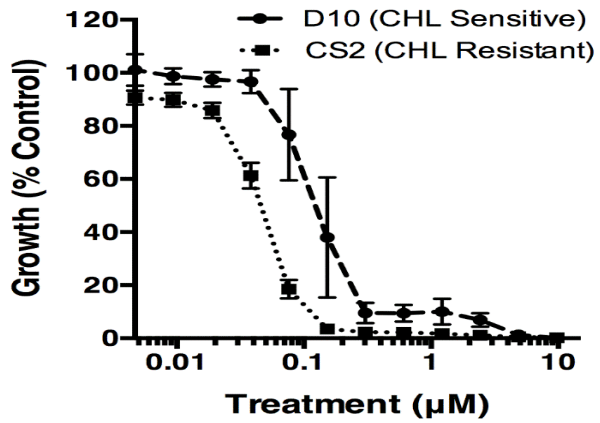


Fig. 1B

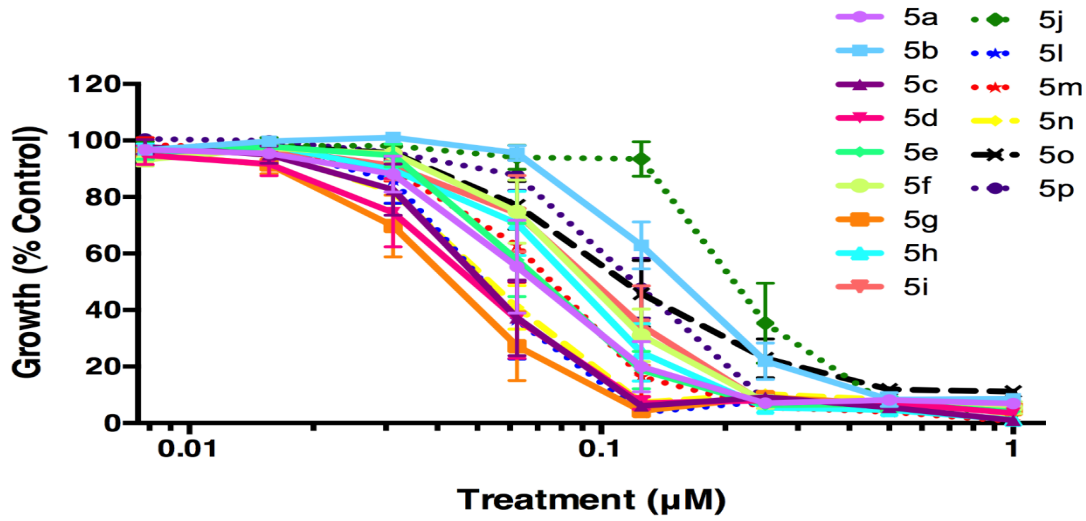
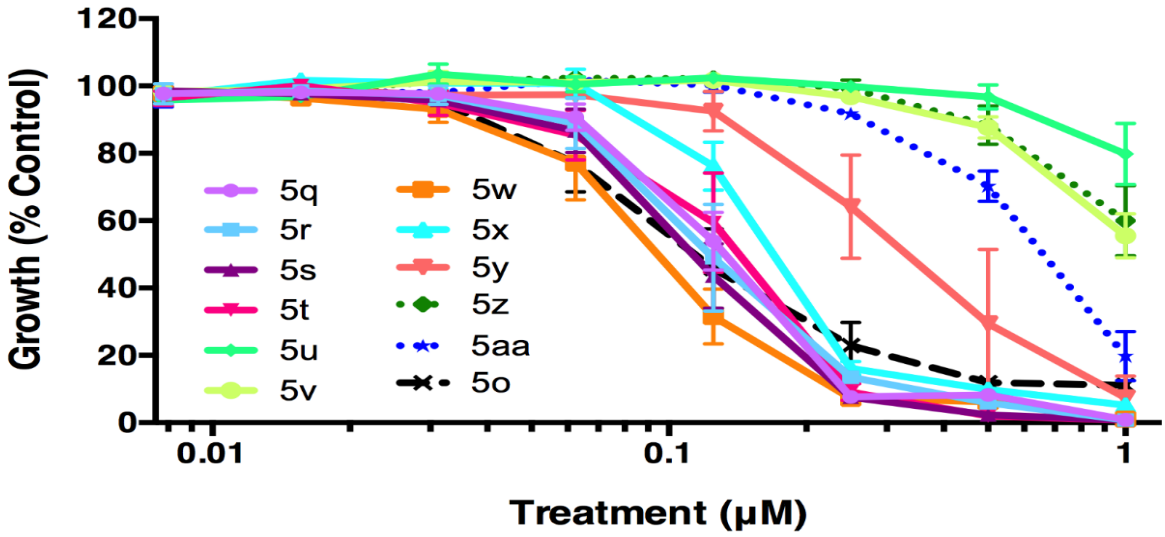


Fig. 1C



Supplementary Fig 1: *P. falciparum* growth inhibitory activity of DPI and analogues *in vitro*. **(A)** Dose response curves demonstrate that DPI is inhibitory to *P. falciparum* growth *in vitro*, with an IC_{50} <0.2 µM after 90 hours of parasite treatment for both chloroquine sensitive (D10) and chloroquine resistant (CS2) lines. Analogues of DPI in many cases demonstrated improved growth inhibitory activity against D10 parasites with **(B)** 11 out of 15 analogues for series 1, and **(C)** 1 out of 11 analogues for series 2 demonstrating >2 fold reduction in growth inhibitory IC_{50}.

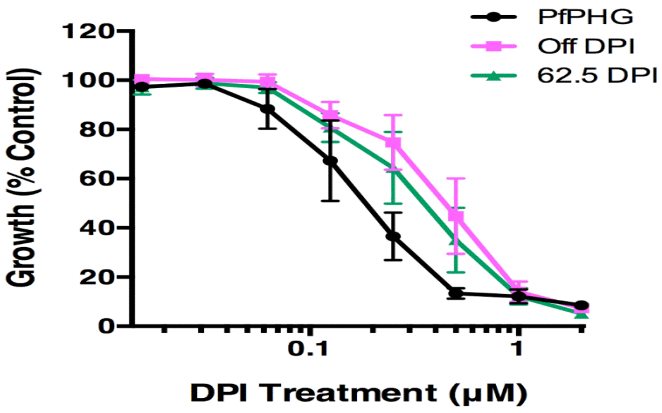


Fig 2. Parasite line selected for resistance to DPI shows reduced drug sensitivity. D10 parasites selected for resistance to DPI during continuous culture (D10-DPI^{R}) exhibited ~2 fold reduction in sensitivity to DPI, even after extended removal of drug pressure (D10-DPI^{off}, 2-5 weeks without drug pressure) when compared to D10 parental parasites.

2.5 Cytotoxicity assay

In parallel with the above assays, we assessed both series of DPI analogues for cytotoxicity against HEPG2 cells. As shown in Table 6, DPI series 1 displayed a wide range of cytotoxicity in 1% FBS, from as low as 0.66 µM for **5c** to as high as 38 µM for **5b**. Ten of the 15 series 1 compounds had improved antimalarial activity in the low nM range (Table. 5; **5a**, **5d**, **5e**, **5f**, **5g**, **5h**, **5i**, **5k**, **5l**, **5m**) whilst maintaining a CC_{50} above 1 µM. For *M. tuberculosis* (Table. 4), 8 out of 15 compounds met this criteria (**5a**, **5d**, **5g**, **5i**, **5k**, **5l**, **5m**, **5n**), confirming that improved DPI analogue activity against broad pathogens is not absolutely at the expense of mammalian cell toxicity. As seen for compound **5c** from which series 2 was derived, analogues with a halogen in a 5-position were significantly more cytotoxic (Table 6). However, this is at conditions artificially low in serum concentration (1%). When retested in the presence of 10% FBS, cytotoxicity decreased up to 8-fold and toxicity would decrease further *in vivo* where serum concentration is 100%. Nevertheless, cytotoxicity, particularly for the series 2 compounds, is a clear potential liability and will need to be addressed in future DPI analogue optimisation studies.

Table 6

Cytotoxicity Assay

	CC_{50}[µM]			CC_{50}[µM]	
DPI Series 1	**1%FBS**	**10%FBS**	**DPI Series 2**	**1%FBS**	**10%FBS**
5o	29.2		**5q**	0.02361	0.0947
5a	2.6		**5r**	0.05026	0.2508
5b	38.3		**5s**	0.09295	0.3488
5c	0.66		**5t**	0.1504	0.2242
5d	4.6		**5u**	0.9303	2.724
5e	37.5		**5v**	0.8938	1.848
5f	20.2		**5w**	0.0601	0.4863
5g	22.3		**5x**	0.05238	0.3864

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5h	16.1	**5y**	0.08457	0.5436
5i	11.9	**5z**	0.3915	0.8331
5j	3.6	**5aa**	0.08862	0.161
5k	6.8			
5l	3.3			
5m	1.1			
5n	5.9			
5p	0.72			

3. Discussion

There has been a steady decline in the number of FDA approved antibiotics, with only 10 antibiotics considered "New Molecular Entities" being approved by the FDA from 2004 to 2012 [29, 30]. This is dwarfed in comparison to the 20 new classes of antibiotics developed between 1930 and 1962, and the 30 new antibiotics approved between 1983 and 1992 [29]. There were no new classes of antimicrobials discovered between 1968 and 2000, and the two novel classes discovered in 2000 and 2003 (daptomycin and linezolid) are only effective against Gram-positive bacteria [29]. The lack of new antibiotics renders physicians impotent to treat emerging resistance to existing antibiotics. Globally, spreading resistance to antimalarial and anti-tubercular drugs is of urgent concern [7, 8], with increasing treatment failures and the potential for increased mortality in the years ahead unless alternative treatments become available. The present report helps address this urgent need for new anti-infectives by evaluating the *in vitro* antibacterial and antimalarial activity of a novel series of DPI compounds.

In view of the increasing incidence of MDR pathogens there is an urgent need for new antibiotics with novel modes of action. Agents that selectively target respiratory enzymes that are unique to the pathogen such as NDH-2 in the electron transport chain of bacteria and

certain parasites will offer a superior approach for treating persistent infections, greatly reduced treatment periods and provide a high selectivity for the pathogen versus the host. DPI has been show to inactivate flavin enzymes via a radical reaction mechanism which leads to the covalent modification of the reduced flavin cofactor [31-33]. The reduced flavin transfers an electron to DPI, generating semiquinone and a diphenyliodyl radical. The free radical fragments to give iodobenzene and a phenyl radical, the latter undergoes recombination with the flavin semiquinone to form various phenyl adducts [32]. We have previously shown that DPI inhibits NDH-2 activity in isolated *Escherichia coli* membranes [34]. Coincidently, we also demonstrated that a secondary mode of action of the polymyxin lipopeptide antibiotics against Gram-negative bacteria involves the inhibition of NDH-2 activity [34].

The unique structure of the Gram-negative cell wall provides an often impermeable barrier to antibiotics, particularly hydrophobic compounds [35]. A major advantage of diaryl-iodonium compounds is their amphipathic character which allows them to easily cross even the most formidable membrane barrier such as the Gram-negative cell wall. Despite their apparent attractiveness as anti-infective agents, iodonium compounds have undergone limited drug development.

A few very early reports investigated the potential of iodonium compounds including DPI as skin antiseptics against Gram-negative bacteria [21-23]. The compounds *per se* or as emulsions with pine oils showed bactericidal activity. The MIC values reported against both Gram-positive and Gram-negative bacteria obtained in the present study were much lower (~10-fold) compared to those of early reports, suggesting DPI is much more active than previously thought [36-38]. This discrepancy may have arisen due to the fact these authors employed DPI dissolved in aqueous solutions without any co-solvent and then attempted to estimate the concentration using the dipicrylamine chemical reactivity technique [36-38].

Acute toxicity studies with dogs (received an intraperitonal dose of 70 mg/kg) and monkeys (received intraperitonal doses of doses of 30 and 50 mg/kg) indicated DPI has some toxic effects on the central nervous system and skeletal muscle at high concentrations [39, 40]. DPI has also been shown to act as a primary eye irritant and a moderate irritant via dermal exposure in humans [41, 42]. In oral exposure studies with dogs at levels of 100 mg/kg, principle toxicity included frequent vomiting, reduced cardiac efficiency and electrolyte imbalance [43, 44]. DPI has also been reported to induce hypoglycaemia in rats at high concentrations, which can be minimized by fortifying the drinking water with glucose [45]. The LD50 of DPI administered orally to rats is 60 mg/kg. Based on these animal toxicity studies it appears that DPI related toxicity occurs at very high concentrations well beyond the very low μM levels required to kill the tested bacterial species *in vitro.* This offers some assurance of safety and a high degree of pathogen versus host selectivity, however, this will also be dependent upon the pharmacokinetics of DPI. Encouragingly, tissue distribution studies in rats that were administered [^{125}I]-DPI revealed a predominant localization of radioactivity in the liver, kidneys, heart and adipose tissue [46].

From a medicinal chemistry perspective, an iodonium chemotype would usually be viewed with great caution. However, data achieved in this study which shows analogues can be synthesised with improved pathogen killing activity that maintain desirably low mammalian cell toxicity suggests such a compound could be considered worthy of further elaboration to investigate structure-activity relationships. For the first time, we have reported here a detailed assessment of the SAR across a range of microorganisms for a novel set of DPI analogues. We show that some of our novel DPI analogues are much more potent than DPI itself with IC_{50} values in the low nanomolar range. However, some compounds, particularly those with a halogen in a 5-position in series 2, are relatively cytotoxic to

mammalian cells and need to be further modified to determine if cytotoxicity can be reduced while potency against bacterial and parasite pathogens is maintained.

While DPI is one of the less cytotoxic compounds against HEPG2 cells, it is one of the most potently cytotoxic compounds against THP1 cells. Therefore there seems to be something of a disconnect between our observation of *in vitro* cytotoxicity and the historical use of DPI *in vivo* with an apparently acceptable, albeit moderate, toxicity profile. For this reason, while cytotoxicity-associated liability would necessarily require monitoring, it is possible that the extreme potency of some of our DPI analogues could find an application in certain antibacterial settings after further improvements in the cytotoxicity profile. Whether sufficiently divergent SAR can be established to build on the antibacterial potency we have achieved and minimise mammalian cytotoxicity for further development of this class for *in vivo* use will be the focus of future research.

4. Conclusion

A series of DPI analogues were synthesised and subsequently assessed for their biological activity. Several of these compounds exhibited high potency, with low nanomolar activity against problematic Gram-negative and Gram-positive bacteria, *Mycobacterium tuberculosis* and *Plasmodium* spp., protozoan parasites.

5. Experimental

5.1 Chemistry

All non-aqueous reactions were performed under an atmosphere of nitrogen, unless otherwise specified. Commercially available reagents were used without further purification. Thin-layer chromatography (TLC) was performed on silica gel $60F_{254}$ pre-coated aluminum sheets (0.25 mm, Merck). Automated flash column chromatography was performed with a Biotage Isolera

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One instrument using Biotage SNAP cartridges packed with silica (KP-SilTM). Nuclear Magnetic Resonance (NMR) spectra were recorded at 400.13 Hz on an Avance III Nanobay 400 MHz Bruker spectrometer coupled to the BACS 60 automatic sample changer. Proton resonances are annotated as: chemical shift (δ), multiplicity (s, singlet; d, doublet; m, multiplet), coupling constant (*J*, Hz), and the number of protons. Mass spectrometry was performed with an Agilent 6224 TOF LC/MS coupled to an Agilent 1290 Infinity (Agilent, Palo Alto, CA). All data were acquired and reference mass corrected via a dual-spray electrospray ionisation (ESI) source. Analytical HPLC was acquired on an Agilent 1260 Infinity analytical HPLC coupled with a G1322A degasser, G1312B binary pump, G1367E high-performance autosampler, and G4212B diode array detector. Conditions were as follows: Zorbax Eclipse Plus C18 rapid resolution column (4.6 × 100 mm) with UV detection at 254 and 214 nm, 30 °C; the sample was eluted using a gradient of 5−100% solvent B in solvent A, where solvent A was 0.1% aq. TFA and solvent B was 0.1% TFA in CH_3CN (5−100% B [9 min], 100% B [1min]; 0.5 mL/min).

*5.1.1 General Method A: Preparation of Biphenylamine Derivatives **3a-3aa**.*

Substituted 2-iodoaniline **1** (1.0 eq.), substituted phenylboronic acid **2** (1.2 eq.), K_2CO_3 (3.0 eq.), tetrabutylammonium bromide (0.1 eq.), $PdCl_2$(dppf) (0.1 eq.) and dioxane/H_2O (9:1) (0.5 M) were added to a 10 mL microwave-vial. The vial was sealed with a cap and placed in a Cem Discover-microwave cavity. After irradiation at 130 °C for 1 h and subsequent cooling, the solvent was removed *in vacuo*. The residue was taken up into EtOAc (30 mL) and washed once with water and brine. The organic layer was dried over $MgSO_4$, filtered, and concentrated. The crude product was purified by flash column chromatography using 0-10% EtOAc/petroleum benzine to give the biphenylamine product **3a-3aa**.

5.1.2 The following compounds have all been reported previously and their ^{1}H spectra data showed good agreement with the literature data:

2'-Methyl-[1,1'-biphenyl]-2-amine (**3a**)[47], 2'-Methoxy-[1,1'-biphenyl]-2-amine (**3b**)[48], 2' Fluoro-[1,1'-biphenyl]-2-amine (**3c**)[49], 2'-Chloro-[1,1'-biphenyl]-2-amine (**3d**)[50], 3'-Methyl-[1,1'-biphenyl]-2-amine (**3e**)[51], 3'-Methoxy-[1,1'-biphenyl]-2-amine (**3f**)[51], 3'-Fluoro-[1,1'-biphenyl]-2-amine (**3h**)[51], 3'-Chloro-[1,1'-biphenyl]-2-amine (**3i**)[51], 4'-Methyl-[1,1'-biphenyl]-2-amine (**3j**)[51], 4'-Methoxy-[1,1'-biphenyl]-2-amine (**3k**)[51], 4'-Fluoro-[1,1'-biphenyl]-2-amine (**3m**)[51], 4'-Chloro-[1,1'-biphenyl]-2-amine (**3n**)[51], [1,1'-Biphenyl]-2-amine (**3o**)[52], *2-(Naphthalen-2-yl)aniline (**3p**)[53]*, (2'-Amino-[1,1'-biphenyl]-2-yl)-2,2,2-trifluoroethan-1-one (**3r**)[54].

*5.1.3 2'-Amino-[1,1'-biphenyl]-3-carbonitrile (**3g**).* Light yellow solid (0.29 g, 65% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81 (ddd, *J* = 2.2, 1.7, 0.8 Hz, 1H), 7.78 – 7.72 (m, 1H), 7.68 – 7.63 (m, 1H), 7.57 (td, *J* = 7.7, 0.5 Hz, 1H), 7.24 (ddd, *J* = 8.0, 7.4, 1.6 Hz, 1H), 7.12 (dd, *J* = 7.6, 1.4 Hz, 1H), 6.90 (td, *J* = 7.5, 1.1 Hz, 1H), 6.85 (dd, *J* = 8.0, 0.8 Hz, 1H), 4.19 (br s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 143.42, 140.94, 133.64, 132.64, 130.72, 130.31, 129.68, 129.48, 125.02, 118.95, 118.72, 116.04, 112.95. LCMS Rt 3.21 min, *m/z* 195.1 $[M + H]^+$.

*5.1.4 2'-Amino-[1,1'-biphenyl]-4-carbonitrile (**3l**).* Brown oil (0.27 g, 61% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (dq, *J* = 5.4, 1.7 Hz, 2H), 7.65 – 7.61 (m, 2H), 7.26 (d, *J* = 7.7 Hz, 1H), 7.15 (d, *J* = 7.6 Hz, 1H), 6.93 (t, *J* = 7.5 Hz, 1H), 6.87 (d, *J* = 8.0 Hz, 1H), 4.41 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 144.68, 143.40, 132.71, 130.33, 129.93, 129.72, 125.55, 119.10, 118.94, 116.19, 110.96. LCMS Rt 3.22 min, *m/z* 195.1 $[M + H]^+$.

*5.1.5 2'-(Trifluoromethyl)-[1,1'-biphenyl]-2-amine (**3q**).* Dark brown oil (0.39 g, 74% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47 – 7.34 (m, 4H), 7.25 (td, *J* = 7.8, 1.6 Hz, 1H), 7.13 (dd, *J* = 7.5, 1.4 Hz, 1H), 6.92 (t, *J* = 8.8 Hz, 2H), 4.42 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 147.05, 144.04, 132.84, 132.52, 130.97, 129.25, 127.39, 122.54, 121.83, 121.53, 119.26, 118.50, 115.80. LCMS Rt 3.39 min, *m/z* 239.3 $[M + H]^+$.

5.1.6 *2'-Chloro-5'-methyl-[1,1'-biphenyl]-2-amine (**3s**).* Brown solid (0.39 g, 79% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 (d, *J* = 8.1 Hz, 1H), 7.24 (ddd, *J* = 8.0, 7.4, 1.6 Hz, 1H), 7.20 – 7.11 (m, 2H), 7.08 (dd, *J* = 7.5, 1.5 Hz, 1H), 6.91 – 6.82 (m, 2H), 3.72 (s, 2H), 2.37 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 143.87, 137.70, 137.24, 132.61, 130.81, 130.49, 129.93, 129.69, 129.15, 125.66, 118.46, 115.63, 20.94. LCMS Rt 3.41 min, *m/z* 218.1 $[M + H]^+$.

*5.1.7 2'-Chloro-5'-methoxy-[1,1'-biphenyl]-2-amine (**3t**).* Brown oil (0.46 g, 87% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.46 – 7.35 (m, 1H), 7.26 – 7.19 (m, 1H), 7.09 (dd, J = 7.5, 1.5 Hz, 1H), 6.95 – 6.78 (m, 4H), 3.83 (s, 3H), 3.62 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.70, 143.81, 138.86, 130.69, 130.41, 129.30, 125.55, 125.25, 118.45, 116.77, 115.69, 115.36, 55.73. LCMS Rt 3.35 min, *m/z* 234.1 $[M + H]^+$.

*5.1.8 2',5'-Dichloro-[1,1'-biphenyl]-2-amine (**3u**).* Colourless oil (0.43 g, 80% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.46 (d, *J* = 8.6 Hz, 1H), 7.38 (s, 1H), 7.32 (dd, *J* = 8.5, 2.6 Hz, 1H), 7.29 – 7.24 (m, 1H), 7.07 (dd, *J* = 7.6, 1.5 Hz, 1H), 6.89 (ddd, *J* = 10.7, 8.5, 4.6 Hz, 2H), 4.11 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 143.72, 139.70, 133.05, 132.46, 131.93, 131.12, 130.36, 129.70, 129.22, 124.17, 118.58, 115.83. LCMS Rt 3.49 min, *m/z* 238.0 $[M + H]^+$.

*5.1.9 2'-Chloro-5'-fluoro-[1,1'-biphenyl]-2-amine (**3v**).* Yellow oil (0.38 g, 76% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.46 – 7.35 (m, 3H), 7.34 – 7.28 (m, 1H), 7.16 (td, *J* = 8.2, 6.3 Hz, 1H), 6.63 (dd, *J* = 11.1, 8.5 Hz, 2H), 5.07 – 3.98 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.72, 160.26, 143.69, 139.91, 139.83, 131.31, 131.23, 130.32, 129.64, 129.02, 124.38, 118.98, 118.76, 118.53, 116.36, 116.13, 115.82. LCMS Rt 3.88 min, *m/z* 222.1 $[M + H]^+$.

*5.1.10 2'-Chloro-3-methyl-[1,1'-biphenyl]-2-amine (**3w**).* Brown oil (0.32 g, 79% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.59 – 7.48 (m, 1H), 7.36 (q, *J* = 2.9 Hz, 3H), 7.16 (dd, *J* = 7.4, 0.6 Hz, 1H), 6.98 (dd, *J* = 7.5, 1.3 Hz, 1H), 6.85 (t, *J* = 7.5 Hz, 1H), 4.03 (s, 2H), 2.30 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 142.05, 138.37, 134.14, 132.11, 130.36, 130.06, 129.13, 128.28, 127.33, 125.13, 122.59, 117.96, 17.95. LCMS Rt 3.44 min, *m/z* 218.1 $[M + H]^+$.

*5.1.11 2'-Chloro-3-fluoro-[1,1'-biphenyl]-2-amine (**3x**).* Brown oil (0.31 g, 69% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.57 – 7.50 (m, 1H), 7.38 – 7.32 (m, 3H), 7.15 (dd, *J* = 7.4, 0.8 Hz, 1H), 6.97 (dd, *J* = 7.6, 1.1 Hz, 1H), 6.84 (t, *J* = 7.5 Hz, 1H), 3.87 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 161.82, 159.40, 145.77, 145.72, 134.89, 134.44, 130.50, 130.32, 129.74, 129.63, 128.64, 128.24, 114.18, 110.99, 110.97, 105.30, 105.07. LCMS Rt 3.40 min, *m/z* 222.1 $[M + H]^+$.

*5.1.12 2',6-Difluoro-[1,1'-biphenyl]-2-amine (**3y**).* Brown solid (0.36 g, 85% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.46 – 7.37 (m, 2H), 7.27 – 7.14 (m, 3H), 6.66 – 6.59 (m, 2H), 4.12 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.23, 161.67, 159.80, 159.20, 146.27, 146.22, 132.47, 132.44, 130.38, 130.30, 130.06, 129.95, 124.65, 124.61, 120.03, 119.86, 116.47, 116.25, 110.99, 110.97, 109.29, 109.09, 105.16, 104.93. LCMS Rt 3.28 min, *m/z* 206.1 $[M + H]^+$.

*5.1.13 2'-Chloro-6-fluoro-[1,1'-biphenyl]-2-amine (**3z**).*Brown oil (0.27 g, 77% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.58 – 7.50 (m, 1H), 7.37 (t, *J* = 4.0 Hz, 3H), 7.07 (ddd, *J* = 10.8, 8.1, 1.3 Hz, 1H), 6.89 (d, *J* = 7.3 Hz, 1H), 6.80 (td, *J* = 7.9, 5.2 Hz, 1H), 3.56 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 153.01, 150.63, 136.86, 136.83, 133.89, 132.84, 132.72, 131.89, 130.16, 129.53, 127.41, 127.31, 127.27, 125.81, 125.78, 117.67, 117.59, 114.83, 114.64. LCMS Rt 3.41 min, *m/z* 222.1 $[M + H]^+$.

*5.1.14 2',3-Dichloro-[1,1'-biphenyl]-2-amine (**3aa**).* Brown solid (0.28 g, 75% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45 (d, *J* = 8.6 Hz, 1H), 7.39 (d, *J* = 2.5 Hz, 1H), 7.34 – 7.23 (m, 2H), 7.08 (dd, *J* = 7.6, 1.5 Hz, 1H), 6.91 (dd, *J* = 13.8, 7.5 Hz, 2H), 4.48 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 143.73, 139.69, 133.02, 132.43, 131.91, 131.10, 130.34, 129.68, 129.19, 124.12, 118.53, 115.80. LCMS Rt 3.47 min, *m/z* 238.0 $[M + H]^+$.

*5.2.1 General Method B: Synthesis of Cyclic Diphenyleniodonium Trifluoromethanesulfonate Derivatives **5a-5aa**.*

The preparation was performed according to the literature procedure.[24] To a stirred solution of biphenylamine **3a-3aa** (1.0 eq.) in THF (0.1 M) was added 4 M HCl (3 mL), and the solution was cooled in an ice water bath. A solution of $NaNO_2$ (1.2 eq.) in H_2O (3 mL) was added dropwise. After 20 min, a solution of KI (2.5 eq.) in H_2O (3 mL) was added, and stirred for 10 min in an ice water bath. Then the solution was slowly warmed to room temperature and stirred for 1 h before an aqueous solution of 20% $Na_2S_2O_3$ was added until the colour of the mixture didn't change. The phases were separated, and the aqueous phase extracted with EtOAc (15 mL x 3). Then the combined organic layers were washed with H_2O and brine, dried over $MgSO_4$, and concentrated. The residue was purified by flash column

chromatography using 0-5% EtOAc/petroleum benzine to give the biphenyliodide **4**. These compounds were used directly in the next step.

To a stirred solution of biphenyliodide **4** (1.0 eq.) in anhydrous CH_2Cl_2 (0.2 M) was added *m*-CPBA (75%, 1.5 eq.), TfOH (0.003 eq.). The solution was stirred for 1 h at room temperature. The solvent was removed by rotary evaporation and Et_2O (2 mL) was added to the remained solid. The mixture was stirred for 20 min, and then filtered. The obtained solid was washed with Et_2O (3x), dried in a vacuum oven to afford the desired cyclic diphenyleniodonium trifluoromethanesulfonate **5a**-**5aa**.

5.2.2 The following compounds have all been reported previously and their ^{1}H spectra data showed good agreement with the literature data:

1-Methyldibenzo[*b*,*d*]iodol-5-ium trifluoromethanesulfonate (**5a**)[55], 2-Methoxydibenzo[*b*,*d*]iodol-5-ium trifluoromethanesulfonate (**5k**)[24] 3-Methoxydibenzo[*b*,*d*]iodol-5-ium trifluoromethanesulfonate (**5k**)[24], 3-Cyanodibenzo[*b*,*d*]iodol-5-ium trifluoromethanesulfonate (**5l**)[24], 3-Chlorodibenzo[*b*,*d*]iodol-5-ium trifluoromethanesulfonate (**5m**)[55], 3-Fluorodibenzo[*b*,*d*]iodol-5-ium trifluoromethanesulfonate (**5n**)[55], Dibenzo[*b*,*d*]iodol-5-ium trifluoromethanesulfonate (**5o**)[24], Benzo[*b*]naphtho[1,2-*d*]iodol-11-ium trifluoromethanesulfonate (**5p**)[24]

*5.2.3 1-Methoxydibenzo[b,d]iodol-5-ium trifluoromethanesulfonate (**5b**).* Off-white solid (0.05 g, 22% yield). ^{1}H NMR (400 MHz, DMSO) δ 8.83 (dd, *J* = 8.1, 1.4 Hz, 1H), 8.28 (dd, *J* = 8.2, 1.1 Hz, 1H), 7.91 – 7.88 (m, 1H), 7.86 – 7.82 (m, 1H), 7.74 – 7.60 (m, 2H), 7.58 – 7.50 (m, 1H), 4.11 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 160.26, 141.91, 131.48, 131.30, 130.96, 130.76, 130.34, 130.07, 122.81, 122.49, 120.56, 114.16, 57.10. LCMS Rt 2.89 min,

519 *m/z* 309.0 [M - OTf]$^+$. HRMS (ESI) calcd for $C_{13}H_{10}IO$ [M- OTf]$^+$, 308.9771, found
520 308.9771. HPLC purity >95%, Rt 4.59 min.
521
522 *5.2.4 1-Chlorodibenzo[b,d]iodol-5-ium trifluoromethanesulfonate (**5c**).* Colourless solid (0.3
523 g, 87% yield). ^{1}H NMR (400 MHz, DMSO) δ 9.14 (d, *J* = 7.6 Hz, 1H), 8.30 (t, *J* = 7.6 Hz,
524 2H), 7.98 (d, *J* = 7.8 Hz, 1H), 7.91 (t, *J* = 7.4 Hz, 1H), 7.80 (t, *J* = 7.3 Hz, 1H), 7.66 (t, *J* =
525 8.0 Hz, 1H). ^{13}C NMR (101 MHz, DMSO) δ 140.74, 137.85, 134.34, 133.35, 131.92, 131.21,
526 131.09, 131.01, 130.92, 130.38, 123.50, 121.66. LCMS Rt 2.99 min, *m/z* 312.9 [M - OTf]$^+$.
527 HRMS (ESI) calcd for $C_{12}H_7ClI$ [M- OTf]$^+$, 312.9275, found 312.9276. HPLC purity >95%,
528 Rt 4.86 min.
529
530 *5.2.5 1-Fluorodibenzo[b,d]iodol-5-ium trifluoromethanesulfonate (**5d**).* Colourless solid (0.2
531 g, 66% yield). ^{1}H NMR (400 MHz, DMSO) δ 8.42 (d, *J* = 7.9 Hz, 1H), 8.25 (dd, *J* = 8.2, 0.7
532 Hz, 1H), 8.10 (dd, *J* = 8.0, 0.7 Hz, 1H), 7.89 (t, *J* = 7.6 Hz, 1H), 7.83 – 7.68 (m, 3H). ^{13}C
533 NMR (101 MHz, DMSO) δ 160.05, 139.50, 139.45, 132.09, 132.00, 131.59, 131.54, 131.08,
534 130.51, 130.29, 130.12, 127.41, 122.03, 121.28, 118.89, 118.68. LCMS rt 2.81 min, *m/z*
535 297.0 [M - OTf]$^+$. HRMS (ESI) calcd for $C_{12}H_7FI$ [M- OTf]$^+$, 296.9571, found 296.9570.
536 HPLC purity >95%, Rt 4.53 min.
537
538 *5.2.6 2-Methyldibenzo[b,d]iodol-5-ium trifluromethanesulfonate (**5e**).* Off-white solid (0.2 g,
539 71% yield). ^{1}H NMR (400 MHz, DMSO) δ 8.46 (d, *J* = 7.5 Hz, 1H), 8.34 (s, 1H), 8.21 (d, *J* =
540 7.9 Hz, 1H), 8.07 (d, *J* = 8.2 Hz, 1H), 7.86 (t, *J* = 7.0 Hz, 1H), 7.71 (t, *J* = 7.4 Hz, 1H), 7.55
541 (d, *J* = 8.2 Hz, 1H), 2.52 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 142.19, 142.15, 141.43,
542 132.53, 131.48, 131.17, 131.08, 130.62, 127.82, 127.37, 122.16, 118.42, 21.22. LCMS rt 2.88

min, *m/z* 293.0 [M - OTf]$^+$. HRMS (ESI) calcd for $C_{13}H_{10}I$ [M- OTf]$^+$, 292.9822, found 292.9821. HPLC purity >95%, Rt 4.61 min.

*5.2.7 2-Cyanodibenzo[b,d]iodol-5-ium trifluoromethanesulfonate (**5g**).* Off-white solid (0.04 g, 39% yield). ^{1}H NMR (400 MHz, DMSO) δ 9.10 (s, 1H), 8.63 (s, 1H), 8.41 (s, 1H), 8.19 (d, *J* = 46.2 Hz, 2H), 7.86 (d, *J* = 51.6 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 143.53, 140.76, 133.69, 132.51, 132.25, 131.37, 131.01, 128.10, 127.31, 123.01, 118.26, 114.26. LCMS rt 2.70 min, *m/z* 303.9 [M - OTf]$^+$. HRMS (ESI) calcd for $C_{13}H_7IN$ [M- OTf]$^+$, 303.9618, found 303.9618. HPLC purity >95%, Rt 4.14 min.

*5.2.8 2-Chlorodibenzo[b,d]iodol-5-ium trifluoromethanesulfonate (**5h**).* Off-white solid (0.13 g, 69% yield). ^{1}H NMR (400 MHz, DMSO) δ 8.68 (d, *J* = 2.2 Hz, 1H), 8.61 – 8.57 (m, 1H), 8.21 (dd, *J* = 15.0, 8.3 Hz, 2H), 7.88 (t, *J* = 7.2 Hz, 1H), 7.81 – 7.72 (m, 2H). ^{13}C NMR (101 MHz, DMSO) δ 144.32, 141.02, 136.87, 132.53, 132.18, 131.26, 131.12, 131.03, 128.03, 127.17, 122.70, 119.99. LCMS rt 2.91 min, *m/z* 312.9 [M - OTf]$^+$. HRMS (ESI) calcd for $C_{12}H_7ClI$ [M- OTf]$^+$, 312.9275, found 312.9276. HPLC purity >95%, Rt 4.82 min.

*5.2.9 2-Fluorodibenzo[b,d]iodol-5-ium trifluoromethanesulfonate (**5i**).* Off-white solid (0.07 g, 56% yield). ^{1}H NMR (400 MHz, DMSO) δ 8.52 (ddd, *J* = 12.7, 8.9, 2.0 Hz, 2H), 8.27 – 8.19 (m, 2H), 7.92 – 7.84 (m, 1H), 7.78 – 7.71 (m, 1H), 7.62 (td, *J* = 8.8, 2.8 Hz, 1H). ^{13}C NMR (101 MHz, DMSO) δ 165.72, 163.79, 163.26, 144.88, 144.79, 141.28, 141.25 (J_{CF} = 3.1 Hz), 133.02, 132.93, 132.13, 131.26, 131.08, 127.98, 122.70, 119.10, 118.86, 116.05, 116.03, 114.59, 114.34. LCMS rt 2.81 min, *m/z* 297.0 [M - OTf]$^+$. HRMS (ESI) calcd for $C_{12}H_7FI$ [M-OTf]$^+$, 296.9571, found 296.9570. HPLC purity >95%, Rt 4.39 min.

*5.2.10 3-Methyldibenzo[b,d]iodol-5-ium trifluoromethanesulfonate (**5j**).* Off-white solid (0.09 g, 67% yield). ^{1}H NMR (400 MHz, DMSO) δ 8.43 (dd, *J* = 7.9, 1.2 Hz, 1H), 8.36 (d, *J* = 8.1 Hz, 1H), 8.19 (dd, *J* = 8.2, 0.8 Hz, 1H), 7.99 (s, 1H), 7.87 – 7.81 (m, 1H), 7.69 (dd, *J* = 11.1, 4.3 Hz, 2H), 2.50 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 142.21, 142.03, 139.63, 132.17, 131.16, 131.13, 131.02, 130.74, 127.16, 127.10, 122.14, 121.79, 21.68. LCMS Rt 2.89 min, *m/z* 293.0 [M - OTf]$^{+}$. HRMS (ESI) calcd for $C_{13}H_{10}I$ [M- OTf]$^{+}$, 292.9822, found 292.9821. HPLC purity >95%, Rt 4.70 min.

5.2.1.1 DPI Series 2 analogues

*5.2.1.2 1-(Trifluoromethyl)dibenzo[b,d]iodol-5-ium trifluoromethanesulfonate (**5q**).* Colourless solid (0.11 g, 77% yield). ^{1}H NMR (400 MHz, DMSO) δ 8.63 (d, *J* = 8.2 Hz, 1H), 8.50 (d, *J* = 8.3 Hz, 1H), 8.42 – 8.34 (m, 2H), 7.99 (ddd, *J* = 8.5, 7.3, 1.3 Hz, 1H), 7.92 (t, *J* = 8.0 Hz, 1H), 7.86 – 7.79 (m, 1H). ^{13}C NMR (101 MHz, DMSO) δ 139.44, 139.19, 135.83, 132.10, 131.50, 130.83, 130.26, 130.20, 130.13, 124.90, 121.99. LCMS Rt 2.89 min, *m/z* 347.0 [M - OTf]$^{+}$. HRMS (ESI) calcd for $C_{13}H_{7}F_{3}I$ [M- OTf]$^{+}$, 346.9539, found 346.9541. HPLC purity >95%, Rt 5.11 min.

*5.2.1.3 1-(2,2,2-Trifluoroacetyl)dibenzo[b,d]iodol-5-ium trifluoromethanesulfonate (**5r**).* Colourless solid (0.12 g, 69% yield). ^{1}H NMR (400 MHz, DMSO) δ 8.52 (d, *J* = 8.1 Hz, 1H), 8.33 (d, *J* = 8.2 Hz, 2H), 7.97 (dd, *J* = 12.0, 4.6 Hz, 2H), 7.82 (ddd, *J* = 8.5, 3.6, 2.0 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 147.67, 139.48, 134.51, 131.96, 131.76, 131.58, 131.31, 130.44, 130.16, 125.94, 124.48, 124.08, 123.04, 122.73, 121.89, 121.84, 119.53, 119.31, 116.73, 116.33, 79.75, 79.42, 79.09, 31.13. LCMS Rt 2.92 min, *m/z* 362.9 [M - OTf]$^{+}$.

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HRMS (ESI) calcd for $C_{13}H_7F_3IO$ $[M - OTf]^+$, 362.9488, found 362.9490. HPLC purity >95%, Rt 5.31 min.

*5.2.1.4 1-Chloro-4-methyldibenzo[b,d]iodol-5-ium trifluoromethanesulfonate (**5s**).* Colourless solid (0.14 g, 78% yield). ^{1}H NMR (400 MHz, DMSO) δ 9.15 (dd, *J* = 8.2, 1.4 Hz, 1H), 8.45 (dd, *J* = 8.3, 1.0 Hz, 1H), 7.97 – 7.89 (m, 2H), 7.87 – 7.76 (m, 1H), 7.53 (d, *J* = 8.2 Hz, 1H), 2.72 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 141.78, 139.12, 137.52, 134.42, 131.98, 131.81, 131.31, 131.28, 131.21, 130.47, 128.25, 121.35, 25.70. LCMS Rt 2.97 min, *m/z* 326.9 $[M - OTf]^+$. HRMS (ESI) calcd for $C_{13}H_9ClI$ $[M- OTf]^+$, 326.9432, found 326.9433. HPLC purity >95%, Rt 5.04 min.

*5.2.1.5 1-Chloro-4-methoxydibenzo[b,d]iodol-5-ium trifluoromethanesulfonate (**5t**).* Colourless solid (0.18 g, 67% yield). ^{1}H NMR (400 MHz, DMSO) δ 9.08 (d, *J* = 8.1 Hz, 1H), 8.42 (d, *J* = 8.3 Hz, 1H), 7.93 (dd, *J* = 8.1, 4.6 Hz, 2H), 7.80 (t, *J* = 7.8 Hz, 1H), 7.36 (d, *J* = 8.8 Hz, 1H), 4.09 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 161.18, 146.20, 143.05, 140.51, 136.81, 136.69, 135.91, 135.60, 128.92, 127.50, 126.54, 124.30, 117.41, 63.08. LCMS Rt 2.87 min, *m/z* 342.9 $[M - OTf]^+$. HRMS (ESI) calcd for $C_{13}H_9ClIO$ $[M- OTf]^+$, 342.9381, found 342.9382. HPLC purity >95%, Rt 5.07 min.

*5.2.1.6 1-Chloro-4-fluorodibenzo[b,d]iodol-5-ium trifluoromethanesulfonate (**5u**).* Colourless solid (0.2 g, 72% yield). ^{1}H NMR (400 MHz, DMSO) δ 9.16 (dd, *J* = 8.2, 1.4 Hz, 1H), 8.41 (dd, *J* = 8.3, 1.0 Hz, 1H), 8.04 (dd, *J* = 8.9, 5.1 Hz, 1H), 7.99 – 7.94 (m, 1H), 7.90 – 7.81 (m, 1H), 7.66 (dd, *J* = 8.9, 7.2 Hz, 1H). ^{13}C NMR (101 MHz, DMSO) δ 160.83, 158.37, 140.48, 139.97, 139.93, 136.41, 136.34, 132.52, 131.80, 131.32, 131.11, 128.36, 128.33, 125.95, 122.74, 122.55, 119.54, 117.32, 117.10, 111.26, 110.97. LCMS Rt 2.87 min, *m/z* 330.9 [M -

OTf]$^+$. HRMS (ESI) calcd for $C_{12}H_6ClFI$ [M- OTf]$^+$, 330.9181, found 330.9181. HPLC purity >95%, Rt 4.60 min.

*5.2.1.7 1,4-Dichlorodibenzo[b,d]iodol-5-ium trifluoromethanesulfonate (**5v**).* Colourless solid (0.11 g, 71% yield). ^{1}H NMR (400 MHz, DMSO) δ 9.14 (dd, *J* = 8.2, 1.4 Hz, 1H), 8.46 (dd, *J* = 8.4, 1.1 Hz, 1H), 7.98 (ddd, *J* = 11.2, 9.2, 4.9 Hz, 2H), 7.91 – 7.76 (m, 2H). ^{13}C NMR (101 MHz, DMSO) δ 141.60, 139.14, 135.90, 132.52, 132.48, 131.89, 131.71, 131.60, 131.46, 129.73, 128.01, 122.64. LCMS Rt 2.76 min, *m/z* 346.9 [M - OTf]$^+$. HRMS (ESI) calcd for $C_{12}H_6Cl_2I$ [M- OTf]$^+$, 346.8886, found 346.8889. HPLC purity >95%, Rt 4.87 min.

*5.2.1.8 6-Chloro-1-methyldibenzo[b,d]iodol-5-ium trifluoromethanesulfonate (**5w**).* Colourless solid (0.25 g, 69% yield). ^{1}H NMR (400 MHz, DMSO) δ 8.90 (d, *J* = 7.6 Hz, 1H), 8.43 (d, *J* = 8.0 Hz, 1H), 7.99 (d, *J* = 7.6 Hz, 1H), 7.80 (t, *J* = 7.5 Hz, 1H), 7.66 (d, *J* = 6.2 Hz, 2H), 2.72 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 140.98, 139.78, 138.64, 134.66, 133.53, 132.18, 131.36, 131.05, 130.79, 128.55, 126.22, 122.92, 26.14. LCMS Rt 2.78 min, *m/z* 327.0 [M - OTf]$^+$. HRMS (ESI) calcd for $C_{13}H_9ClI$ [M- OTf]$^+$, 326.9432, found 326.9433. HPLC purity >95%, Rt 5.02 min.

*5.2.1.9 6-Chloro-1-fluorodibenzo[b,d]iodol-5-ium trifluoromethanesulfonate (**5x**).* Colourless solid (0.12 g, 69% yield). ^{1}H NMR (400 MHz, DMSO) δ 8.38 (t, *J* = 2.1 Hz, 1H), 8.26 (d, *J* = 8.8 Hz, 1H), 8.13 (dd, *J* = 8.0, 1.0 Hz, 1H), 7.89 – 7.72 (m, 3H). ^{13}C NMR (101 MHz, DMSO) δ 160.23, 141.35, 136.71, 132.87, 132.78, 132.60, 131.16, 129.37, 129.19, 127.44, 122.76, 119.48, 118.99, 118.78. LCMS Rt 2.93 min, *m/z* 330.9 [M - OTf]$^+$. HRMS (ESI) calcd for $C_{12}H_6ClFI^+$ [M- OTf]$^+$, 330.9181, found 330.9182. HPLC purity >95%, Rt 5.15 min.

*5.2.1.10 1,6-Dichlorodibenzo[b,d]iodol-5-ium trifluoromethanesulfonate (**5y**).* Colourless solid (0.09 g, 65% yield). ^{1}H NMR (400 MHz, DMSO) δ 9.17 – 9.05 (m, 1H), 8.48 (dd, *J* = 8.4, 1.0 Hz, 1H), 8.07 (dt, *J* = 24.6, 12.3 Hz, 1H), 8.02 – 7.91 (m, 2H), 7.73 (t, *J* = 8.2 Hz, 1H). ^{13}C NMR (101 MHz, DMSO) δ 142.60, 138.43, 134.68, 134.16, 133.36, 133.11, 131.33, 131.11, 130.57, 129.31, 125.55, 124.31, 122.75, 119.54. LCMS Rt 2.95 min, *m/z* 346.9 [M - OTf]$^{+}$. HRMS (ESI) calcd for $C_{12}H_6Cl_2I$ [M- OTf]$^{+}$, 346.8886, found 346.8887. HPLC purity >95%, Rt 4.87 min.

*5.2.1.11 1-Chloro-9-fluorodibenzo[b,d]iodol-5-ium trifluoromethanesulfonate (**5z**).* Colourless solid (0.1 g, 70% yield). ^{1}H NMR (400 MHz, DMSO) δ 8.25 (dd, *J* = 8.1, 0.9 Hz, 1H), 8.16 (dd, *J* = 7.7, 1.1 Hz, 1H), 8.01 (dd, *J* = 8.0, 0.9 Hz, 1H), 7.91 – 7.68 (m, 3H). ^{13}C NMR (101 MHz, DMSO) δ 161.52, 158.91, 137.37, 137.31, 134.20, 134.01, 133.28, 133.20, 132.18, 129.78, 128.71, 128.55, 127.31, 122.77, 121.78, 121.74, 119.79, 119.54. LCMS Rt 2.93 min, *m/z* 330.9 [M - OTf]$^{+}$. HRMS (ESI) calcd for $C_{12}H_6ClFI$ [M- OTf]$^{+}$, 330.9181, found 330.9182. HPLC purity >95%, Rt 4.96 min.

*5.2.1.12 1,9-Difluorodibenzo[b,d]iodol-5-ium trifluoromethanesulfonate (**5aa**).* Off-white solid (0.12 g, 66% yield). ^{1}H NMR (400 MHz, DMSO) δ 8.15 (dd, *J* = 7.6, 1.4 Hz, 2H), 7.89 – 7.71 (m, 4H). ^{13}C NMR (101 MHz, DMSO) δ 161.26, 158.67, 133.07, 127.36, 122.74, 121.66, 119.80, 119.67, 119.54. LCMS Rt 2.89 min, *m/z* 314.9 [M - OTf]$^{+}$. HRMS (ESI) calcd for $C_{12}H_6F_2I$ [M- OTf]$^{+}$, 314.9477, found 314.9477. HPLC purity >95%, Rt 4.61 min.

5.3 Biological Asssay

5.3.1 Organisms

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Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli, Enterococcus faecalis, Enterococcus faecium, Staphylococcus aureus, Acinetobacter baumannii and *Mycobacterium tuberculosis H37Rv* strains employed in this study were clinical isolates or obtained from the American Type Culture Collection (Rockville, MD, USA). Bacterial isolates (excluding *M. tuberculosis*) were stored in tryptone soy broth (Oxoid) with 20% glycerol (Ajax Finechem, Seven Hills, NSW, Australia) at -80 °C. *M. tuberculosis* H37Rv (ATCC 25618) was propagated in 37 °C in 7H9 media (BD Diagnostic Systems, Sparks, MD, USA) supplemented with 10% albumin-dextrose-catalase (ADC), 0.5% glycerol and 0.02% tyloxapol. Isolates were stored at -80 °C in the same media with 30% glycerol.

P. falciparum (D10-PfPHG[56] , CS2-PHG[57]) and *P. knowlesi* (YH1[28]) parasites were cultured in human O^+ erythrocytes according to the method of Trager and Jensen.[58] Briefly, parasites were grown in RPMI-HEPES culture medium (pH 7.4) supplemented with 50 μM hypoxanthine, 25 mM $NaHCO_3$, 20 μM gentamicin and 0.5% Albumax II (Gibco, Melbourne, VIC, Australia). Cultures were maintained in airtight boxes in a 37 ºC incubator in an atmosphere of 1% O_2, 4% CO_2 and 95% N_2.

5.3.2 Gram-positive and Gram-negative bacterial panel assay

The MICs of test compounds against *P. aeuruginosa, K. pneumoniae, E. coli, E. faecalis, E. faecium, S. aureus* and *A. baumannii* strains were determined by the broth microdilution method according to the guidelines of the Clinical and Laboratory Standards Institute [25]. Experiments were performed with Cation-Adjusted Mueller-Hinton Broth (CaMHB) in 96-well polypropylene microtitre plates. Wells were inoculated with 100 μL of bacterial suspension prepared in CaMHB (containing ~10^6 colony forming units (cfu) per mL) and 100 μL of CaMHB containing increasing concentrations of DPI (0 to 128 μM). The MICs were defined as the lowest concentration at which visible growth was inhibited

following 18 h incubation at 37 °C. The IC_{50} of the antibiotic polymixin B (Sigma) was assayed as a growth inhibitory positive control.

5.3.3 Mycobacterium tuberculosis growth inhibition

For MIC determination compounds were serially diluted in 96 well tissue culture plates in 10 µL of purified H_2O in triplicate (0 to 10 µM). *M. tuberculosis* grown in supplemented 7H9 media (90 µL) was adjusted to an OD_{600nm} of 0.001, added to wells and incubated for 7 days at 37 °C. Resazurin (10 µL; 0.05% w/v; Sigma-Aldrich, Australia) was then added, incubated for 4-24 h at 37 ^{o}C and fluorescence measured at 590 nm using a FLUOstar Omega microplate reader (BMG Labtech, Germany). The MICs were defined as the lowest concentration at which bacterial growth was completely inhibited compared to non-treated bacteria. The IC_{50} of the antibiotic rifampicin (Sigma) was assayed as a growth inhibitory positive control.

5.3.4 Plasmodium *spp. growth inhibition assays*

Parasites were synchronized to early ring stages using a combination of heparin synchronization [59] and sorbitol lysis. Malaria growth inhibition assays using ring stage parasites were setup in a 96-well round bottom plate as described previously [56] at 1% parasitaemia and 1% haematocrit in a final volume of 45 µL. A 10x final concentration of DPI and controls were added to make the final volume to 50 µL. *P. falciparum* assays were cultured for 90 h, through the next cycle of replication, until the parasites were mainly mature trophozoites (36-42 h post-invasion). *P. knowlesi* assays were cultured for 50 h, again until the parasites were late trophozoites of the next growth cycle. Assays were stained with 10 µg/mL ethidium bromide (EtBr, Bio-Rad, Melbourne, VIC, Australia) for 1 h and then washed prior to flow cytometry (Becton Dickinson LSR) assessment of parasitaemia. GFP

and non-GFP fluorescent parasites were gated and counted according to established protocols [60]. Flow cytometry data was analysed using FlowJo software (Tree Star, St, Ashland, OR, USA). The IC_{50} of parasite growth inhibition was determined using Graphpad PRISM (Graphpad Software, La Jolla, CA, USA) following the recommended protocol for non-linear regression of a log(inhibitor) *vs*. response curve [60]. The IC_{50} of the antimalarial chloroquine (Sigma) was assayed as a growth inhibitory positive control.

5.3.5 P. falciparum resistance selection and sequencing

The central 1567bp (18bp-1585bp) of the *Pf*NDH2 sequence was PCR amplified from the D10-*Pf*PHG parental, D10-DPI^{r} and D10-DPI^{off} lines using the primers *Pf*NDH2 F GGTTAATATATAATGTTAGTAAAGTTCAGG and *Pf*NDH2 R CATTTTTTTATCATTTGATGAAAGGAC. The sequence of Type II *Pf* dihydroorotate dehydrogenase was amplified from the target lines using the primers *Pf*DHOD F GTGTGATAGATAGCTCCAGTCG and *Pf*DHOD R GCACTTATGTGTCGCCCG. Sanger sequencing of the resulting PCR products was conducted at the Australian Genome Research Facility, with alignments compared using Geneious (Biomatters, New Zealand).

5.3.6 Cytotoxicity Assay

HepG2 cells (ATCC HB-8065) were seeded as 4000 cells per well in a 384-well plate in DMEM medium (GIBCO-Invitrogen #11995-073), with 1% FBS or 10% FBS as specified. Cells were incubated for 24 h at 37 ^{o}C, 5% CO_2 to allow cells to attach to the plates. Compounds were added into each well with a series of concentrations from 300 µM to 0.14 µM in 3-fold dilution, the cells were then incubated for 24 h at 37 ^{o}C, 5% CO_2. After the incubation, 10 µM resazurin (dissolved in PBS) was added to each well. The plates were then incubated for 2 h at 37 ^{o}C, 5% CO_2. The fluorescence intensity was read using a Polarstar

Omega plate reader with excitation/emission 560/590. The data was analysed using Prism software. Results are presented as the average percentage of control ± SD for each set of duplicate wells using the following equation: Percentage Viability = ($FI_{Compound}$ – $FI_{Negative}$/$FI_{Untreated}$ –$FI_{Negative}$) x 100.

Conflict of interest

The authors declare they have no conflict of interest.

Acknowledgements

T.V, J.B and J.L are supported by the Australian National Health and Medical Research Council (NHMRC) and National Institute of Health (USA). T.V. is an Australian NHMRC Industry Career Development. DW was supported by a NHMRC Peter Doherty Australian Biomedical Fellowship (APP1035715).

Appendix A. Supplementary data

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Highlights

- A series of compounds based on the NDH-2 inhibitor diphenyleneiodonium (DPI) were synthesised.
- Compound **5s** and **5g** exhibited low nanomolar activity against *M. tuberculosis and P. falciparum* respectively.

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