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New Auranofin Analogs with Antibacterial Properties against *Burkholderia* Clinical Isolates

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Abstract: Bacteria of the genus *Burkholderia* include pathogenic *Burkholderia mallei*, *Burkholderia pseudomallei* and the *Burkholderia cepacia* complex (Bcc). These Gram-negative pathogens have intrinsic drug resistance, which makes treatment of infections difficult. Bcc affects individuals with cystic fibrosis (CF) and the species *B. cenocepacia* is associated with one of the worst clinical outcomes. Following the repurposing of auranofin as an antibacterial against Gram-positive bacteria, we previously synthetized auranofin analogs with activity against Gram-negatives. In this work, we show that two auranofin analogs, MS-40S and MS-40, have antibiotic activity against *Burkholderia* clinical isolates. The compounds are bactericidal against *B. cenocepacia* and kill stationary-phase cells and persisters without selecting for multistep resistance. *Caenorhabditis elegans* and *Galleria mellonella* tolerated high concentrations of MS-40S and MS-40, demonstrating that these compounds have low toxicity in these model organisms. In summary, we show that MS-40 and MS-40S have antimicrobial properties that warrant further investigations to determine their therapeutic potential against *Burkholderia* infections.

Keywords: *Burkholderia*; *Burkholderia cepacia* complex; auranofin; antimicrobials; antibiotic resistance; cystic fibrosis

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1. Introduction

Antibiotics are one of the greatest medical advances of the 20th century, with their widespread discovery starting in the early 1900's [1,2]. However, antibiotic resistance is now a global crisis, responsible for approximately 700,000 deaths annually [2], with that number projecting to increase each year [3]. The "golden era" of antibiotic discovery, which lasted approximately 20 years, led to the identification of vancomycin, methicillin, cephalosporins, and many other antibiotics [1,2]. No new antibiotic from a new class has reached the clinic for many decades [4]. When a new antibiotic is developed that does not have a novel mechanism of action, resistance mechanisms are already present. Therefore, antimicrobials with novel mechanisms of action are needed to prevent this quickly generated resistance.

Bacteria of the genus *Burkholderia* [5] includes difficult-to-treat human pathogens such as the *Burkholderia cepacia* complex (Bcc), *Burkholderia mallei* and *Burkholderia pseudomallei* [6]. Bcc is a group of more than 20 species that cause life-threatening bacterial infections in cystic fibrosis (CF) patients [7]. *Burkholderia cenocepacia* infections, in particular, have one of the worst clinical outcomes [5,8], causing decreased lung function and *cepacia* syndrome, a sepsis with necrotizing pneumonia [8,9]. Additionally, CF patients infected with *B.*

cenocepacia are often ineligible for lung transplants [10], a common life-saving procedure [8], because *B. cenocepacia* infections have a high risk of reoccurrence [11].

New antibiotics are urgently needed to combat *Burkholderia* infections. Promising emerging therapeutics are those with heavy metals [12], especially gold [13–17], which have been used in medicine since 2500 B.C.E. Recently, the gold-containing, anti-arthritis drug auranofin [18,19] was found to be active against *Mycobacterium tuberculosis* and Gram-positive bacteria [20]. Auranofin inhibited the function of the enzyme thioredoxin reductase, interrupting thiol-redox homeostasis [20,21]. However, auranofin lacked significant activity against Gram-negative bacteria with minimum inhibitory concentrations (MICs) > 16 mg/L [20].

We previously found that auranofin is active against *Helicobacter pylori* and synthesized sugar-modified analogs have improved antibiotic activity and reduced toxicity to mammalian cells [21]. By varying the structures of the thiol and phosphine ligands on auranofin, we expanded the antibacterial activity of the auranofin analogs to the Gram-negative pathogens including *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Escherichia coli* [22]. In this work, we describe the characterization of two auranofin analogs, WB-19-HL4170 (MS-40S) and WB-19-HL4118 (MS-40), which show potential as antimicrobials against Bcc strains, *B. pseudomallei* and *B. mallei*. MS-40S and MS-40 are bactericidal against *B. cenocepacia*, kill persister cells, and do not select for multistep resistant mutations while maintaining low toxicity to eukaryotic systems.

2. Results

2.1. MIC of Auranofin Derivatives against a Panel of Burkholderia Cepacia Complex Species

We tested auranofin and ten auranofin analogs (Figure 1) against a panel of Bcc bacteria, comprising of clinical isolates from CF patients and strains from environmental sources. Auranofin (Figure 1, top left) and the analogues belonging to group one (Figure 1, top right) were largely inactive against members of the Bcc (Table S1), with most of the MICs being 128 μg/mL or higher. Group one analogs have modifications of the thioglucose ligands, with or without additional replacing of trimethylphosphine (-PMe_3) to triethylphosphine (-PEt_3) coordinated bonding to the gold atom. Auranofin, however, showed high activity against *B. mallei* with MICs ranging from 0.25–1 μg/mL. In *B. pseudomallei*, auranofin had an MIC of 64 μg/mL. Auranofin and the group one derivatives in this study were shown to have diverse activity in other Gram-negative and Gram-positive bacteria, but they were inactive or had low activity in *P. aeruginosa*, *Enterobacter cloacae*, and *K. pneumoniae* [22].

Group two (Figure 1, bottom) includes the analog WB-19-HL4118 (MS-40) which had shown a low MIC in Gram-negative and Gram-positive bacteria, such as in *A. baumannii*, *E. cloacae*, *E. coli*, and *S. aureus* [22]. Therefore, we synthetized additional analogs with similar structures to MS-40. Group two analogs showed lower MICs against members of the Bcc (Table 1) than group one (Table S1). Remarkably, MS-40 and WB-19-HL4170 (MS-40S) showed the strongest activity (Table 1). These two compounds also have high activity in *B. mallei* and *B. pseudomallei* strains as well. The synthesis of MS-40S and MS-40 is shown in Scheme 1. The remaining derivatives from this group have moderate MICs, ranging from 8 to 64 μg/mL, with only a few being 128 μg/mL or higher. The structures of the group two derivatives have substitution of thioglucose ligands with mercaptoethanol ($HOCH_2CH_2SH$) or mercaptoethanol modification, suggesting that the thioglucose was unable to permeate into most of the Bcc bacterial cell.

Next, we compared the MICs of MS-40S and MS-40 to common antibiotics used to treat CF patients infected with *Burkholderia* spp. Those include ceftazidime [23,24], meropenem [23–26], doxycycline [27], and tobramycin [24–26]. The combination therapy ceftazidime–avibactam is considered the last resort treatment for those infected with *Burkholderia* species [9]; therefore, we determined the MIC of ceftazidime–avibactam and these four antibiotics against the Bcc panel (Table 2). The MICs of MS-40 and MS-40S are much lower than the antibiotic tobramycin and are similar to the other antibiotics, including the last resort combination treatment ceftazidime–avibactam. Additionally, doxycycline,

against some isolates from the Bcc, had MIC values as low as 1 and 2 μg/mL. Taken together, the initial MIC testing shows MS-40S and MS-40 are comparable to antibiotics used currently in the clinic, having MICs lower than most and even have similar values to the combination therapy ceftazidime–avibactam.

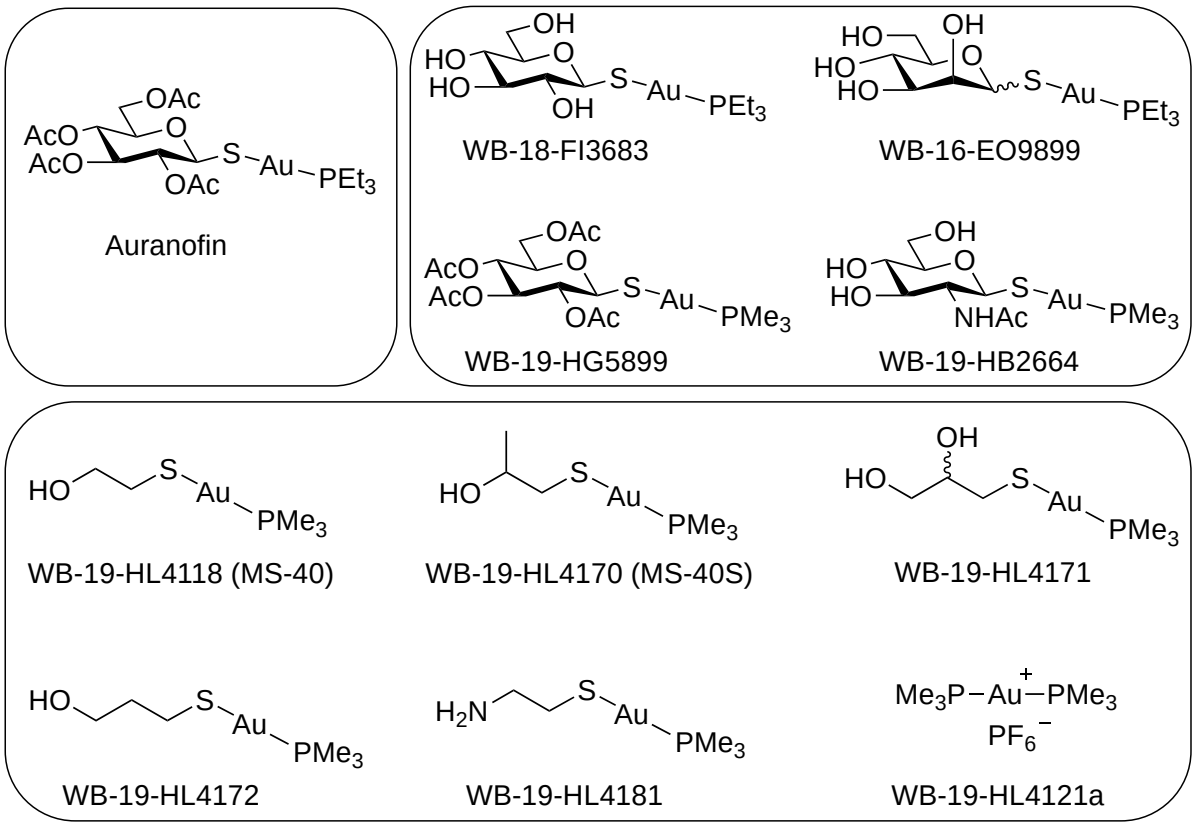
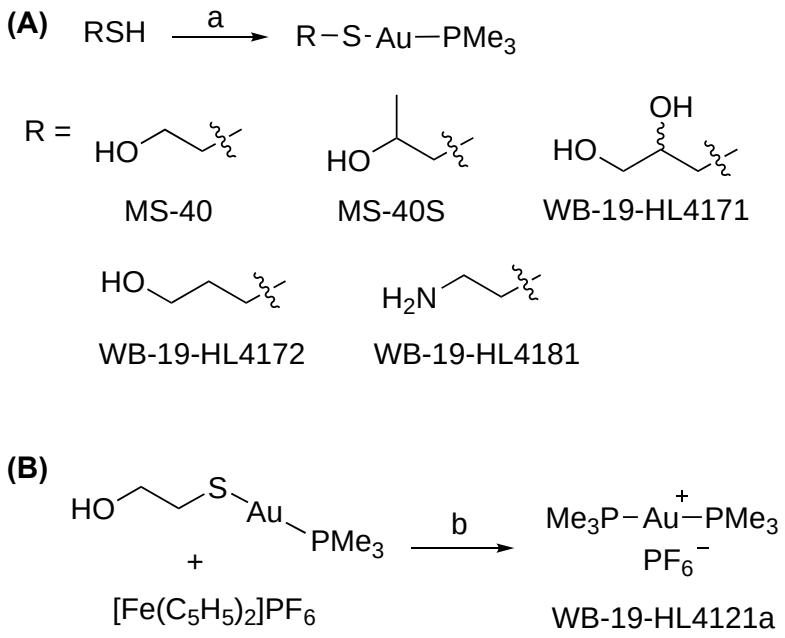


Figure 1. Chemical structure of auranofin and auranofin analogs. (**Top left**) Auranofin. (**Top right**) Group one auranofin analogs containing modifications of the thioglucose and replacement of triethylphosphine ($P(CH_2CH_3)_3$; PEt_3) with trimethylphosphine ($P(CH_3)_3$; PMe_3). (**Bottom**) Group two auranofin analogs, contains mercaptoethanol ($OHCH_2CH_2SH$) replacing the thioglucose, then further modifications of mercaptoethanol.

Table 1. Minimum inhibitory concentrations (MICs) of group two auranofin derivatives against *Burkholderia cepacia* complex (Bcc) bacteria, *B. mallei* and *B. pseudomallei*.

Organism	MIC (μg/mL)						
	Auranofin	WB-19-HL4118	WB-19-HL4170	WB-19-HL4171	WB-19-HL4172	WB-19-HL4181	WB-19-HL4121a
B. cenocepacia K56-2	>128	4	8	32	32	32	128
B. lata BCC6	128	8	8	32	16	16	32
B. contaminans MF16	128	8	8	64	32	64	32
B. contaminans FFH-2050MA	128	4	16	32	16	32	32
B. dolosa CEP021	>128	16	32	64	32	64	128
B. multivorans ATCC17616	128	8	16	32	32	32	64
B. cenocepacia 140485	128	8	16	16	32	32	32
B. ubonensis LMG 20358	>128	16	16	>128	32	32	128
B. contaminans FFH-4004	>128	8	16	32	32	32	64
B. mallei China 5 (NBL 4)	0.25	0.12	0.12	ND	ND	ND	ND
B. mallei Ivan (NCTC 10230)	0.5	0.12	0.12	ND	ND	ND	ND
B. mallei China 7 (NBL 7)	1	0.25	0.25	ND	ND	ND	ND
B. pseudomallei 1710b	64	4	4	ND	ND	ND	ND
B. pseudomallei MSHR465a	64	4	4	ND	ND	ND	ND
B. pseudomallei HBPUB10134a	64	4	2	ND	ND	ND	ND
B. pseudomallei MSHR305	ND	2	ND	ND	ND	ND	ND

The reported MIC values are from three biological replicates. In the case where a 2-fold difference occurred, the higher value is reported. ND; not determined.



Scheme 1. (**A**) Synthesis of MS-40S. (**B**) Synthesis of MS-40. Reagents and conditions: a: $NaOCH_3$, CH_3OH, room temperature, 2 h; b: dichloromethane, 0 °C, 23 h.

Table 2. Minimum inhibitory concentrations (MICs) of MS-40S compared to clinical antibiotics used to treat CF patients.

Organism	MIC (μg/mL)							
	Auranofin	MS-40	MS-40S	MEM	TOB	CAZ	DOX	CZA [a]
B. cenocepacia K56-2	>128	4	8	16	512	32	4	4
B. lata BCC6	128	8	8	4	128	64	4	16
B. contaminans MF16	128	8	8	8	32	32	16	8
B. contaminans FFH-2050MA	128	4	16	4	128	8	2	4
B. dolosa CEP021	>128	16	32	8	128	16	16	8
B. multivorans ATCC17616	128	8	16	4	64	16	2	8
B. cenocepacia 140485	128	8	16	16	128	8	1	8
B. ubonensis LMG 20358	>128	16	16	16	128	16	4	4
B. contaminans FFH-4004	>128	8	16	8	64	8	8	4

[a] 8 μg/mL avibactam. The reported MIC values are from three biological replicates. In the case where a 2-fold difference occurred, the higher value is reported. MEM, meropenem; TOB, tobramycin; CAZ, ceftazidime; DOX, doxycycline; CZA, ceftazidime–avibactam.

2.2. MS-40S Has Broad Bactericidal Activity

Individuals with CF are commonly infected by multiple bacteria, causing polymicrobial infections [28,29]. Therefore, for MS-40S and MS-40 to be effective antimicrobials, it is imperative for these compounds to kill additional CF pathogens. Common bacteria that cause CF lung infections, besides *Burkholderia* spp., are *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Stenotrophomonas maltophilia*, and *Achromobacter xylosoxidans* [28,30]. Other pathogenic Gram-negative bacteria that were shown to, although rarely, cause CF pulmonary infections include *Escherichia coli* [31], *Escherichia vulneris* [32], *Klebsiella pneumoniae* [30], and *Acinetobacter* species [32].

We thus tested MS-40S against CF pathogens to determine their MIC and MBC (minimum bactericidal concentration), and the same was done with MS-40 (Table 3). MS-40S have low MICs for the Gram-positive bacterium *S. aureus*, one of which is an MRSA strain. MS-40S is also bactericidal against the other Gram-negative bacteria tested with MICs in the range of 1–16 μg/mL and most of the MBCs were between 1- and 4-fold of their respective MICs. *P. aeruginosa* is a common multi-drug resistant bacterium [33], with some

strains/clinical isolates being extensively-drug resistant [34], and has moderate MIC values between 16 and 64 µg/mL and its MBCs between 2- and 16-fold higher than its MICs. MS-40 shows similar results to MS-40S. Overall, the MICs/MBCs against CF pathogens show MS-40S and MS-40 have broad-spectrum bactericidal activity, indicating their potential as a therapeutic option for CF patients.

Table 3. MS-40S is bactericidal against other bacteria that infect CF patients.

Organism	MIC (µg/mL)		MBC (µg/mL)	
	MS-40	MS-40S	MS-40	MS-40S
Burkholderia cenocepacia K56-2	4	8	8 *	>32 *
Stenotrophomonas maltophilia DH57	1	2	4	8
Stenotrophomonas maltophilia K279a	2	2	2	8
Pseudomonas aeruginosa PA01	16	32	128	256
Pseudomonas aeruginosa PA7	32	64	64	128
Escherichia coli 120955	4	4	32	64
Escherichia vulneris CEP511	2	4	2	4
Klebsiella pneumoniae 120310	2	4	4	8
Acinetobacter baumannii ATCC 17978	2	2	4	8
Staphylococcus aureus ATCC 27700	0.25	0.5	2	2
Staphylococcus aureus 107094	0.25	0.25	1	1
Achromobacter xylosoxidans ACH03	8	16	8	32

The reported MIC values are from three biological replicates. In the case where a 2-fold difference occurred, the higher value was taken. * Indicates MBC was determined from exponential time kill curve.

2.3. MS-40S Does Not Select for Multistep Resistant Mutants

New antimicrobials are urgently needed because resistance to current antibiotics has arisen and spread to many bacteria [3,35,36]. Ideally, resistance will occur slowly for new antimicrobials, or not at all. We therefore characterized the occurrence of resistance to MS-40S due to repeated exposure and continuous growth [37]. Bacteria grown in the presence of subinhibitory concentrations of each compound (0.5× MIC) were subcultured and grown overnight in Luria–Bertani (LB) broth. These cultures were then used for the next MIC test, and this process was repeated for a total of 24 days (Figure 2, left). We performed this procedure for MS-40S, MS-40 and the antibiotics meropenem and doxycycline. These antibiotics were chosen because they are commonly used to treat cystic fibrosis patients infected with *Burkholderia* species [23–27], and they have different mechanisms of action and resistance [3,38].

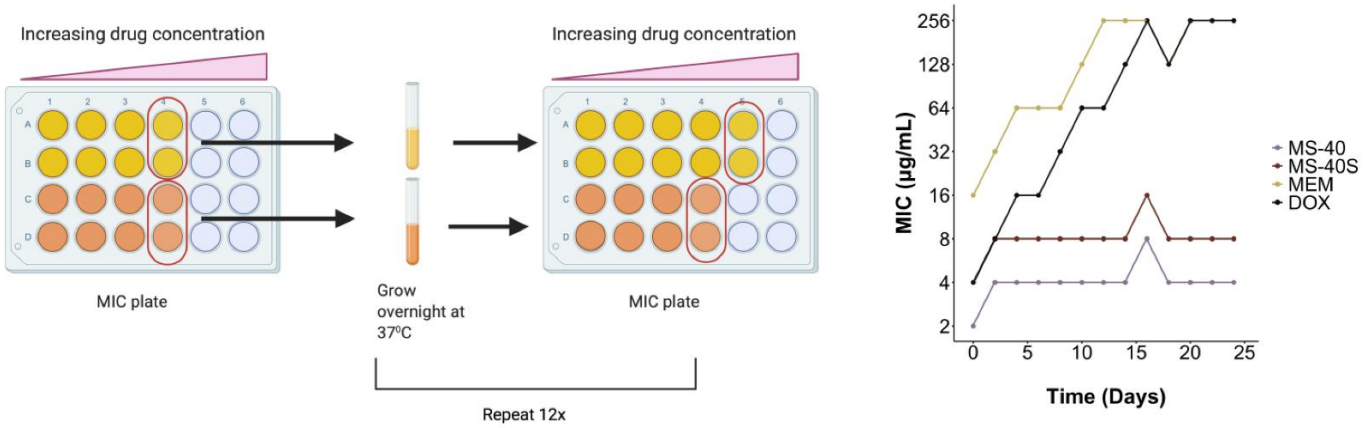


Figure 2. Resistance is not generated to MS-40S. Repeated exposure of a continuously grown culture to sub-lethal concentrations of the antimicrobials were achieved by determining the MIC of the compound. Then, for each compound, 30 µL of bacteria from the well with growth with the highest concentration of compound was grown overnight and used in the next MIC test. This was repeated over 24 days, with the MIC tested every second day. MEM, meropenem; DOX, doxycycline.

Figure 2 (right) shows that resistance against meropenem and doxycycline arose quickly. While their starting MICs values were 16 and 4 μg/mL, respectively, both MICs reached 256 μg/mL after 12–16 days. Remarkably, no apparent increase in their MICs was observed for MS-40S and MS-40, demonstrating a desired property as a potential therapeutic agent.

2.4. MS-40S Is Bactericidal against Both Replicating and Non-Replicating Cells

Antibiotics are classified either as bactericidal, if they kill cells and reduce the population by 99.99%, or bacteriostatic, if they prevent cell growth/division, but do not kill more than 99.99% of the population [38,39]. It is common for antibiotics to only target actively dividing cells because their targets are involved in replication or other energy-dependent processes [38], rendering them less effective when cells are not replicating or respiring [40]. In time kill experiments, we found MS-40S to be bactericidal to both exponential (replicating) and stationary (non-replicating) phase cells, and the same was found for MS-40 (Figure 3; top 4 panels). Interestingly, MS-40S is more effective at killing stationary phase (Figure 3; middle right) than exponential phase cells (Figure 3; middle left), reducing the culture by approximately three $\log_{10}$ units and nine $\log_{10}$ units at 4× MIC in the exponential and stationary phase, respectively. For comparison purposes, we show that doxycycline and ceftazidime–avibactam are both unable to kill cells in stationary phase (Figure 3; bottom right) and ceftazidime–avibactam is slow at killing exponential phase cells, regardless of the concentration (Figure 3; bottom left).

The finding that MS-40S and MS-40 are able to kill stationary phase cells highlights their potential as future therapeutics. Stationary phase cells contain a higher amount of persister cells that could be a common cause of relapses in infections [10,41].

2.5. MS-40S Kill and Inhibit the Formation of Persister Cells

Persister cells, a subpopulation that is not killed by an antimicrobial, are thought to be a common cause of relapses in infections and persistent infections [41,42]. Persisters are thought to form via randomly overexpressing a resistance factor, decreased growth rate, decreased cellular energy, and/or a slower lag phase [42,43]. Once the antibiotic is removed, they will begin to grow normally, without inherited resistance, termed "persister awakening" [43]. A stationary phase population has increased amounts of persisters because it is slower growing and is metabolically dormant [41]. MS-40S and MS-40 can kill stationary phase cells effectively, so we reasoned that the compounds might kill persister cells.

We exposed an exponentially growing *B. cenocepacia* K56-2 population to 5× MIC of ciprofloxacin (MIC, 2 μg/mL) for 3 h, to enrich the surviving population in persister cells. After the treatment, surviving cells were washed and collected in phosphate buffered saline (PBS) to prevent persister awakening [44], then exposed to the MS-40S and MS-40. Figure 4 show persister cells, in the presence of MS-40S and MS-40 (Figure 4, top panels), are killed to a concentration below/close to the limit of detection, whereas the persister cells re-exposed to ciprofloxacin or those without antibiotics are not killed. This demonstrates that MS-40S and MS-40 can indeed kill persister cells created by other antibiotics.

To determine the amount of persisters remaining after 24 h of exposure, we performed the persister frequency assay [45]. We exposed a culture with a CFU/mL of 1×10^8, to one of the antimicrobials for 24 h. Compounds tested included MS-40S, meropenem, ceftazidime–avibactam, and MS-40. The remaining cells, enriched in persisters, were plated on LB to determine CFU/mL and percent survival was calculated by the $\log_{10}$ CFU/mL values. After 24 h, no persisters were formed after exposure to 10× MIC MS-40S, and a very low persister frequency with 5× MIC MS-40S (Figure 4, bottom). No persisters were formed exposed to 5× and 10× MIC of MS-40 and meropenem also produced a low amount of persisters at both concentrations tested. The last resort combination therapy ceftazidime–avibactam (CZA) produced the most persisters in this assay, with approximately 30% of the culture enriched in persisters surviving after treatment.

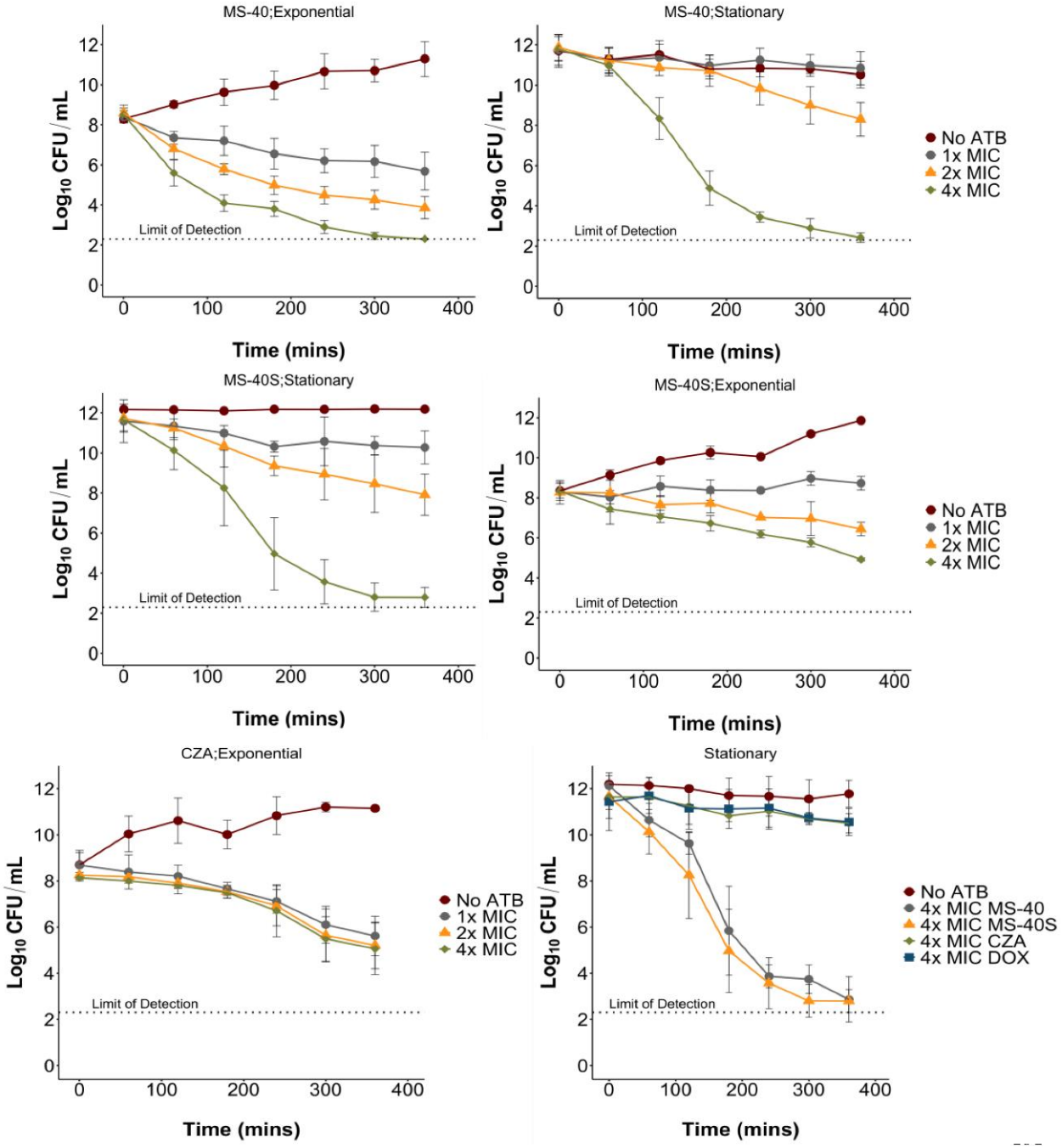


Figure 3. Exponential and stationary time kills of *Burkholderia cenocepacia* K56-2. The compounds used were MS-40, MS-40S, and ceftazidime–avibactam (CZA) at 1×, 2×, and 4× MIC, as well as doxycycline (DOX) at 4× MIC. The cultures were grown overnight for stationary phase cells or grown overnight, subcultured, and grown to early exponential phase. Samples were taken every hour for six hours to determine CFU/mL. No ATB; no antibiotic.

Taken together, these results show MS-40S, as well as MS-40, can kill persister cells created by other antibiotics and can inhibit persister cell formation. This suggests that MS-40S, as well as MS-40, have the potential to effectively eradicate an infection, reducing the risk of a relapse in infection after the treatment regime.

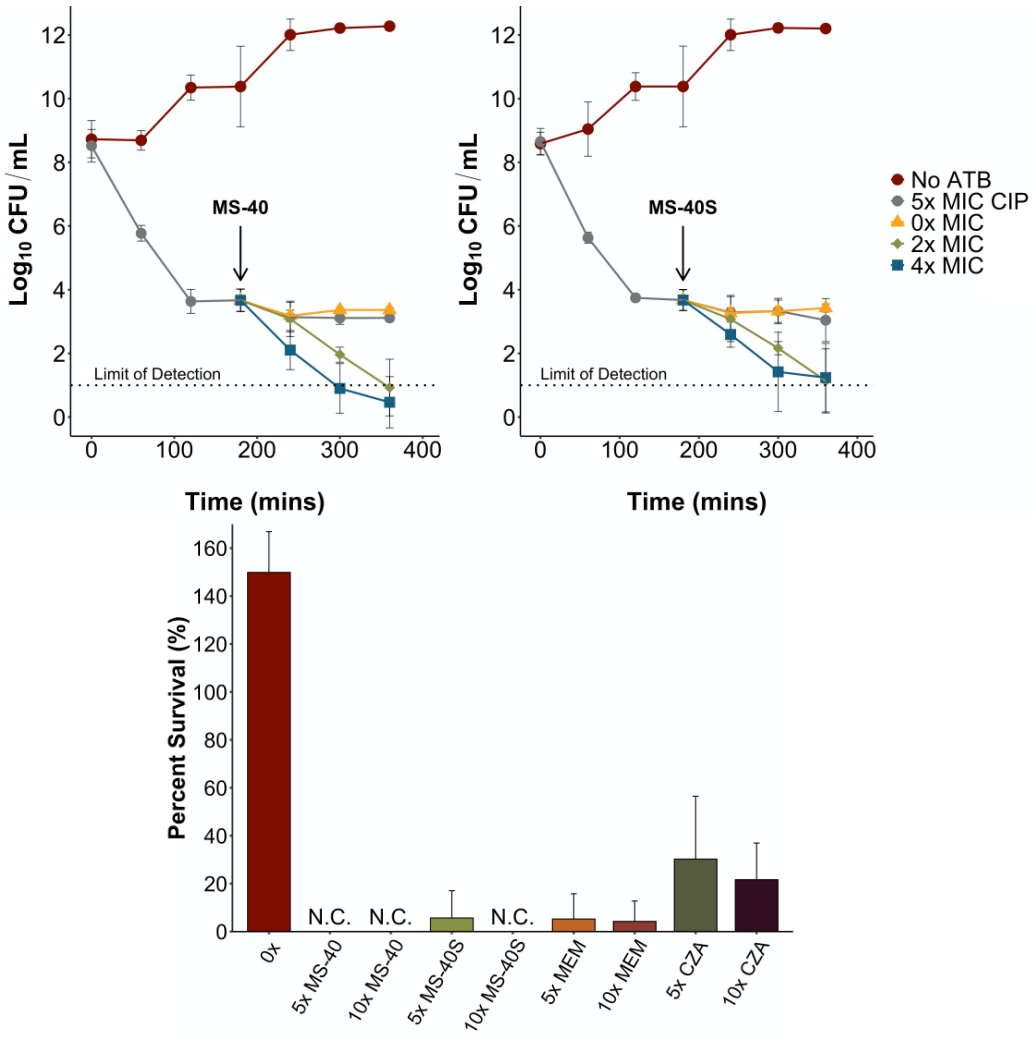


Figure 4. MS-40S kills and inhibits the formation of persister cells. (**Top**) An exponential phase culture with approximately 1×10^8 CFU/mL was exposed to 5× MIC ciprofloxacin (CIP) to generate persister cells. At three hours post-exposure, persister cells were washed, resuspended in PBS and exposed to 0×, 2×, or 4× MIC of MS-40 (**top left**) and MS-40S (**top right**) for an additional three hours. No ATB; no antibiotic. (**Bottom**) An overnight culture of *B. cenocepacia* K56-2 was incubated with the corresponding antimicrobial, MS-40, MS-40S, meropenem (MEM), or ceftazidime–avibactam (CZA). Percent survival was calculated by the $\log_{10}$ CFU/mL of the surviving population/$\log_{10}$ CFU/mL of the initial population. N.C., no colonies.

2.6. C. elegans and G. mellonella Toxicity

In preliminary cytotoxicity tests, MS-40 was shown to have lower toxicity in human A549 cells than auranofin [21,22]; however, the novel MS-40S has not been tested. To show that these compounds are safe for eukaryotic cells, we first used *C. elegans* as a model organism.

We performed a survival assay with *Caenorhabditis elegans* exposed to MS-40S, and three clinical antibiotics: the combination ceftazidime–avibactam, meropenem, and doxycycline, as well as MS-40 (Table 4). We calculated the Survival_{100}/MIC value, which is a ratio of the highest concentration with 100% survival to the compound's MIC [46]. This is a preliminary view to a compound's therapeutic index. MS-40S has similar Survival_{100}/MIC values as clinical antibiotics, with MS-40S, doxycycline, and ceftazidime–avibactam having values of 8, 16, and 32, respectively, and MS-40 and meropenem having a value of four. Similar to *C. elegans*, *Galleria* larvae were also well tolerated to MS-40S (Table 5). This was compared to MS-40 and a clinical antibiotic, doxycycline, which has similar MIC values

to MS-40S and MS-40. Concentrations used ranged from 10 to 1 mg/kg. MS-40S and MS-40 were safe for the larvae at concentrations of 10 mg/kg with a percent survival of approximately 80% and higher. This was similar as the clinical antibiotic, doxycycline. Taken together, *C. elegans* and *Galleria* toxicity models show that MS-40S, as well as MS-40, have low toxicity in these eukaryotic organisms.

Table 4. Percent survival of *C. elegans* exposed to MS-40, MS-40S, and clinical antibiotics.

Antibiotic	Concentrations (μg/mL)								$Survival_{100}$/MIC Ratio *
	256	**128**	**64**	**32**	**16**	**8**	**4**	**DMSO Control**	
MS-40	74.4	81.8	94.7	92.9	100	100	100	100	4
MS-40S	85.7	97.6	100	100	100	100	100	100	8
MEM	93.2	97.4	100	100	100	100	100	100	4
DOX	79.5	91.5	100	98.2	100	97.4	100	100	16
CZA [a]	88.2	100	100	100	100	100	100	100	32

The values in bold indicate percent survival of the compound at its MIC value. * The $Survival_{100}$/MIC ratio is calculated as the highest concentration in which there is 100 percent survival divided by the compound's MIC. [a] Ceftazidime with 8 μg/mL avibactam. Values reported are representative of three experimental replicates.

Table 5. Percent survival of *Galleria* exposed to doxycycline (DOX), MS-40, and MS-40S.

		Galleria Survival		
Compound	**Dose Concentration (mg/kg)**	**24 h**	**48 h**	**72 h**
DOX	10.0	93.3	93.3	93.3
	5.0	93.3	90.0	90.0
	2.0	93.3	93.3	93.3
	1.0	96.7	96.7	96.7
MS-40	10.0	90.0	83.3	80.0
	5.0	96.7	93.3	90.0
	2.0	90.0	80.0	80.0
	1.0	100	96.7	96.7
MS-40S	10.0	90.0	76.7	76.7
	5.0	93.3	86.7	86.7
	2.0	83.3	83.3	83.3
	1.0	96.7	86.7	86.7
DMSO	10.0	93.3	86.7	86.7

Data reported are representative of three survival curves (n = 30) for each concentration. The survival differences between the DMSO control and treatments were not statistically different (log-rank test).

3. Discussion

Here, we show initial antibiotic properties of two auranofin analogs, MS-40 and the novel compound MS-40S, against the cystic fibrosis pathogen *B. cenocepacia* K56-2. The antibiotic properties were explored in parallel with commonly used antibiotics, namely doxycycline, meropenem, and ceftazidime–avibactam. This comparison shows MS-40S and MS-40 to have potential to be developed as antibiotics. One difference between MS-40S and MS-40 and the antibiotics used in this study is the ability of MS-40S and MS-40 to eliminate non-replicating cells. It is common for antibiotics to act on essential targets, such as those involved in cell wall synthesis, DNA replication, and translation [38]. In stationary phase, most of these processes are decreased, preventing the antibiotics from acting upon the cell. We confirmed this with two antibiotics with different mechanisms of action (MOA): doxycycline, a tetracycline that binds to the 30 s subunit of the ribosome, preventing translation elongation [47], and ceftazidime–avibactam, a cephalosporin–β-lactamase inhibitor combination that inhibits cell wall synthesis [48]. These two antibiotics

did not kill cells in stationary phase. Our data show that MS-40S and MS-40 are bactericidal against both replicating and non-replicating cells. Interestingly, MS-40S was shown to kill a greater amount of stationary phase cells than exponential phase, unlike MS-40. This suggests that the MOA of these two compounds may be slightly different.

B. cenocepacia strains are inherently resistant to many available antibiotics [6,10,49], leaving only a few available for treatment. As shown in the resistance studies, resistance is not easy to achieve for MS-40S and MS-40. Mutational resistance can be achieved by altering the antibiotic gene targets, decreasing the binding affinity of the antimicrobial to the gene product, decreasing the uptake/increase in efflux, or, lastly, by changing global responses such as changing a metabolic pathway [3]. Meropenem and doxycycline quickly generated multistep resistance, possibly by one of the mechanisms listed above; however, resistance did not emerge for MS-40S and MS-40. This might be due to MS-40S and MS-40 not being affected by the change of porins/efflux pumps that can cause resistance to other antimicrobials [3], especially in *Burkholderia* species [6,10,50]. Alternately, mutations in the gene targets of MS-40S and MS-40 could have resulted in a reduced fitness of the resistant mutant cell, preventing the mutant from outcompeting the sensitive cells [51,52].

Additionally, MS-40S and MS-40 can clear difficult-to-eradicate persister cells which commonly cause relapses in infections [42]. This suggests these compounds could eradicate the difficult-to-treat persistent infections in the CF lung, helping CF patients infected with *Burkholderia* species become eligible for lung transplants by [11]. MS-40S and MS-40 were shown to eliminate persister cells in two ways. The first way was by killing an enriched population of ciprofloxacin-generated persisters, reducing the population by a further 1–2 $\log_{10}$ CFU/mL. The second way was from a stationary-phase population of bacteria exposed solely to MS-40S and MS-40, with MS-40S only having a small amount of persisters at 5× MIC and MS-40 producing no persisters. Alternatively, the persister frequency was low for meropenem (5–7%), and approximately 30% for ceftazidime–avibactam, similar to the amount produced by *Burkholderia pseudomallei* exposed to ceftazidime [45]. Therefore, MS-40S and MS-40 could be used on their own to eliminate infections, or in tandem with current antibiotics to help eradicate infections [53].

We have also showed MS-40S and MS-40 have low toxicity in *C. elegans* and *G. mellonella*. One limitation of these compounds is that we did not observe *in vivo* antibiotic activity in *C. elegans* and *G. mellonella* infected with *B. cenocepacia* K56-2 (data not shown). To help explain why we were not seeing a protective effect, we tested the MIC of MS-40S and MS-40 in 50% human serum. In the presence of 50% human serum, the MICs of both compounds increased to 128 µg/mL, suggesting these compounds bind non-specifically to proteins, which could decrease their antimicrobial activity. To increase the efficacy of these potent antimicrobials, a drug delivery system must be developed. One possible route is the creation of an MS-40S/MS-40-loaded liposome, as has been shown with rifampicin in the treatment of pulmonary *Mycobacterium abscessus* infections [54]. Creating an aerosolized antimicrobial therapy would also allow us to achieve higher concentrations of the drug, and increase lung penetration, which is especially important for CF pulmonary infections [55].

Auranofin, and previously published auranofin analogues, inhibits thioredoxin reductase, an enzyme that plays a role in thiol-homeostasis in the cell [20,21]. However, it is unclear whether MS-40S and MS-40 share the same target with auranofin. It is assumed that the active component of auranofin is the gold atom, which binds the sulfur in the active site of thioredoxin reductase, inhibiting the formation of the critical disulfide bond, disrupting the function of the enzyme [18,21]. Auranofin was shown to have other effects on the bacterial cell, such as inhibiting DNA, protein, and cell wall synthesis. Thus, thioredoxin reductase may not be the sole antimicrobial target [17]. It is assumed that MS-40 and MS-40S will have similar effects inside the cell as auranofin, possibly having a multi-target mechanism of action. The lack of multi-step resistance may support this multi-target mechanism of action because multiple mutations would be needed to generate resistance. The other potential targets of auranofin and auranofin derivatives are not known. Other factors in the mechanism of action of the antimicrobials, which can explain differences in activity

among compounds with slightly different structures, can be associated efflux pumps and transporters. Future research avenues could include determining the mechanism of action of MS-40S and MS-40, including the targets of the compounds, permeability factors, such as transporters and efflux pumps, and how these compounds kill persister cells and stationary phase cells.

4. Materials and Methods

4.1. Materials

All reagents and solvents were used as received from Sigma-Aldrich or Fisher Scientific unless noted. Reactions were monitored by thin layer chromatography (TLC) using TLC plates pre-coated with silica gel 60 F_{254} (SiliCycle, Québec, QC, Canada), visualized with a handheld ultraviolet device either directly or after staining with 5% H_2SO_4 in ethanol. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance Spectrospin DRX500 spectrometer, referenced either to the non-deuterated residual solvent peaks or tetramethyl silane peak (TMS, δ 0.00 ppm). ^{31}P NMR spectra were recorded on a Bruker Avance Spectrospin DPX200 spectrometer, using freshly prepared triphenylphosphine solution (0.1 M in $CDCl_3$, δ −6.00 ppm) as the external standard.

4.2. Bacterial Strains and Growth Conditions

Strains used are shown in Table S2. All strains were grown in LB at 37 °C with shaking at 230 rpm. *B. ubonensis* was grown at 30 °C with shaking at 230 rpm. New Brunswick Innova40 shaking incubator was used for liquid cultures. A Barnstead Lab-Line Standing Incubator was used for LB-agar plates and 96-well plates.

4.3. Synthesis of Auranofin and Auranofin Derivatives

Auranofin, WB-19-HL4118 (MS-40), WB-18-FI3683, WB-16-EO9899, WB-19-HG5899 and WB-19-HB2664 were synthesized following our previously published protocol (22).

WB-19-HL4170 (MS-40S). To a solution of Me_3PAuCl (150 mg, 0.486 mmol) and 1-mercapto-2-propanol (43 µL, 0.486 mmol) in MeOH (10 mL), $NaOCH_3$ was added (25 wt% in methanol, 125 µL). The solution was stirred at room temperature for 2 h. The reaction mixture was then concentrated on a rotary evaporator, diluted with dichloromethane, and poured into water followed by 3 times extraction by dichloromethane. The combined organic phase was dried over Na_2SO_4, concentrated, passed through a PTFE syringe filter (0.2 µm), and dried under vacuum to afford the product as light beige crystals (172 mg, 97%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.70 (dqd, *J* = 9.4, 6.1, 3.4 Hz, 1H), 3.47 (s, 1H), 3.09 (dd, *J* = 12.7, 3.3 Hz, 1H), 2.82 (dd, *J* = 12.7, 9.0 Hz, 1H), 1.60 (d, *J* = 10.4 Hz, 5H), 1.24 (d, *J* = 6.1 Hz, 2H), (Figure S1). ^{13}C NMR (126 MHz, $CDCl_3$) δ 70.41, 38.47, 21.57, 16.03 (d, *J* = 35.7 Hz), (Figure S2). ^{31}P NMR (162 MHz, $CDCl_3$) δ −0.31 (Figure S3).

WB-19-HL4171, WB-19-HL4172 and WB-19-HL4181 were synthesized following the same procedure as MS-40 above.

WB-19-HL4171. Light yellow crystals (183 mg, 99%) from Me_3PAuCl (150 mg, 0.486 mmol) and 1-thioglycerol (36 µL, 0.486 mmol). ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.83–3.60 (m, 4H), 3.10 (dd, *J* = 12.8, 4.4 Hz, 1H), 3.00 (dd, *J* = 12.8, 7.7 Hz, 1H), 2.93 (s, 1H, OH_α), 2.32 (s, 1H, OH_β), 1.60 (d, *J* = 10.4 Hz, 9H), (Figure S4). ^{13}C NMR (126 MHz, $CDCl_3$) δ 74.49, 65.58, 32.65, 16.13 (d, *J* = 35.8 Hz), (Figure S5). ^{31}P NMR (162 MHz, $CDCl_3$) δ −0.36, (Figure S6).

WB-19-HL4172. Light grey semi-solids (175 mg, 99%) from Me_3PAuCl (150 mg, 0.486 mmol) and 3-mercapto-1-propanol (42 µL, 0.486 mmol). ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.83 (t, *J* = 5.9 Hz, 2H), 3.19 (s, 1H), 3.04 (t, *J* = 6.8 Hz, 2H), 1.92 (p, *J* = 6.6 Hz, 2H), 1.60 (d, *J* = 10.4 Hz, 9H), (Figure S7). ^{13}C NMR (126 MHz, $CDCl_3$) δ 62.30, 39.20, 25.82, 16.07 (d, *J* = 35.5 Hz), (Figure S8). ^{31}P NMR (162 MHz, $CDCl_3$) δ −0.41, (Figure S9).

WB-19-HL4181. Colorless viscous solids (113 mg, quantitative) from Me_3PAuCl (100 mg, 0.324 mmol) and cysteamine hydrochloride (37 mg, 0.324 mmol). ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.01 (t, *J* = 6.3 Hz, 2H), 2.88 (t, *J* = 6.3 Hz, 2H), 1.99 (s, 2H), 1.59

(d, J = 10.4 Hz, 9H), (Figure S10). ^{13}C NMR (126 MHz, $CDCl_3$) δ 47.89, 32.94, 16.02 (d, J = 35.6 Hz), (Figure S11). ^{31}P NMR (162 MHz, $CDCl_3$) δ −0.15, (Figure S12).

WB-19-HL4121a. To a solution of 2-mercaptoethanolatotrimethylphosphine gold(I) (MS-40, 200 mg, 0.570 mmol) in 100 mL of dichloromethane, ferrocenium hexafluorophosphate (95 mg, 0.286 mmol) was added. The solution was stirred at 0 °C for 23 h. After filtration, the filtrate was concentrated and was then transferred to a 20-mL scintillation vial with a total volume of ~5 mL. The solution was placed in an ether vapor environment at 4 °C overnight. The yellow crystals formed were washed by diethyl ether and were further purified by preparative silica gel TLC (10:1 v/v dichloromethane/methanol) to give the product as a white solid (22 mg, 7.5%). ^{1}H NMR (400 MHz, CD_2Cl_2) δ 1.60 (t, J = 4.1 Hz, 1H), (Figure S13). ^{31}P NMR (162 MHz, CD_2Cl_2) δ 8.2, 143.8 (septet, J_{PF} = 710 Hz), (Figure S14).

4.4. Auranofin Derivatives and Antibiotic Stock Solutions

Stock solutions were prepared by dissolving the compounds in dimethyl sulfoxide (DMSO) at a concentration of 20 mg/mL. Antibiotics were suspended at the following concentrations: tobramycin (Alfa Aesar, Haverhill, MA, USA), 10 mg/mL in H_2O; chloramphenicol (Sigma, St. Louis, MO, USA), 20 mg/mL in ethanol; ceftazidime (Sigma), 10 mg/mL in 0.1 M NaOH; meropenem (Sigma), 10 mg/mL in DMSO; doxycycline (Sigma), 25 mg/mL in H_2O; ciprofloxacin (Sigma), 10 mg/mL in 0.1 M HCl; and avibactam (MedKoo Biosciences, Morrisville, NC, USA), 10 mg/mL in DMSO.

4.5. Antimicrobial Susceptibility Testing and Multistep Resistance to Active Derivatives

The compounds were diluted from their stock solutions to 256 μg/mL in Cation-Adjusted Mueller Hinton Broth (CAMHB) for use in the experiment. Determination of the MIC was followed by standards set by the Clinical Laboratory Standards Institute (CLSI) [56]. The 96-well plates were filled with 50 μL of CAMHB, combined with a concentration gradient of compound to be tested. Bacterial culture was diluted to a turbidity equal to MacFarland Standard 0.5, then diluted 100-fold in CAMHB. A total of 50 μL of culture was transferred into each well. After incubation at 37 °C with no shaking for 18 h, MIC was read visually as the lowest concentration of antibiotic that prevented growth.

To determine the rate of multistep resistance mutations from serial passaging, the assay was performed as described previously [37,57]. From the MIC plate, 30 μL from the well that had bacterial growth at the highest concentration of the antimicrobial (0.5× MIC) for each of the compounds tested, was inoculated into 2 mL of LB without compound and incubated overnight at 37 °C with shaking. These overnight cultures were then used as the culture for a second MIC test, and this was repeated 12 times for a total of 24 days of continuous growth.

4.6. Time Kill Assays

Bacterial cultures were grown overnight and either subcultured to an OD_{600} of 0.025 or left in stationary phase. If subcultured, the bacteria were grown to early exponential phase (OD_{600} of 0.13–0.18). The bacteria were exposed to the antibiotics at 1×, 2×, and 4× the MIC, as well as no antibiotic for a negative control. Each hour from time zero to six hours, a sample of each condition was serially diluted to a dilution factor of 10^{-8}, and 5 μL of each dilution was spotted onto LB agar. Plates were incubated for 24 h at 37 °C to determine CFU/mL.

4.7. Time Kill of Persister Cells

The generation and collection of persister cells was adapted from Bahar et al. [44]. Briefly, persister cells were generated by subculturing an overnight culture of *B. cenocepacia* K56-2 in LB and grown until it reached early exponential phase (OD_{600} of 0.13–0.18). The culture was then exposed to 5× MIC of ciprofloxacin (CIP; MIC = 2 μg/mL) with 0× MIC as

a control for three hours. For the initial time zero count, a sample was taken and diluted to a factor of 10^{-8} and 5 µL was spotted onto LB. After the initial count, ciprofloxacin was added to the corresponding culture. A sample was taken every hour for three hours for CFU/mL counts, as mentioned above. After the third hour, the remaining population, enriched in persister cells, was collected, washed, and resuspended in phosphate buffered saline (PBS), divided into five tubes, and again exposed to 5× MIC ciprofloxacin or different concentrations of MS-40S or MS-40 (2× and 4× the MIC), along with a no antibiotic condition as a control. Samples were taken every hour for an additional three hours. Plates were incubated at 37 °C for 24 h and counted for CFU/mL.

4.8. Persister Frequency Assay

The persister frequency assay was performed as described in Ross et al. [45]. An overnight culture of *B. cenocepacia* K56-2 was subcultured to a concentration of 1×10^8 CFU/mL in 2 mL LB. Antimicrobials tested, meropenem, ceftazidime–avibactam, MS-40S, and MS-40 were added to a final concentration of 5× and 10× MIC. The cultures were exposed to the antibiotics for 24 h at 37 °C with shaking. After 24 h the culture was plated on LB to determine CFU/mL. Plates were incubated at 37 °C for 24 h.

4.9. C. elegans Survival

Caenorhabditis elegans was used as a model organism to test the toxicity of the compounds. The survival was performed as described in Selin et al. [46]. *C. elegans* DH26 eggs were incubated at 26 °C until they reached the L4 stage, at approximately 48 h. L4 stage worms were collected and washed with M9 media. Worms were suspended in 100 µL of M9 and transferred to the NGMII plates containing *E. coli* OP50. Approximately ten non-infected *C. elegans* OP50-fed worms, in triplicate, were exposed to a serial dilution of antibiotics to be tested in liquid killing media (LKM; 80% M9 buffer 20% liquid NGMII) in a 96-well plate. The range of concentrations used was 4–128 µg/mL for the following antimicrobials: MS-40S, MS-40, meropenem, doxycycline, and ceftazidime–avibactam, along with a no antibiotic control. Worms were counted at day 0 and incubated at 25 °C. After 24 h, worms were counted for percent survival and the $Survival_{100}$/MIC ratio was calculated. Worms that appeared straight were considered dead, and those were moving and S-shaped were counted as alive. Three experimental replicates were performed.

4.10. Galleria Toxicity

Galleria mellonella was also used as a model organism to study the toxicity of MS-40S and MS-40. The experiments were performed as done in Naguib and Valvano 2018 [58] and Cruz et al., 2018 [59]. *Galleria* larvae were stored at 16 °C in wood shavings and used within 2 weeks of receiving them. Larvae, with an approximate weight of 250 mg, were injected with 10 µL in the last, left proleg using a Hamilton micro-syringe (Hamilton, Nevada, USA). For each compound, MS-40S, MS-40, and doxycycline were diluted in PBS, and 10, 5, 2, and 1 mg/kg were injected in 10 worms for each condition. A total of 10 worms were also injected with 10 µL of PBS for a negative control, and 10 worms were not injected. Survival was measured every 24 h for 72 h. Larvae were considered dead if non-motile and unresponsive to touch. Three experimental replicates were performed. Survival curves were made on GraphPad Prism 6.

5. Conclusions

To conclude, we have shown MS-40 and the novel compound MS-40S have potent bactericidal activity towards pathogenic *Burkholderia*, including the cystic fibrosis multi-drug resistant pathogens from the Bcc, *B. pseudomallei* and *B. mallei*. MS-40S and MS-40 kill both *B. cenocepacia* replicating and non-replicating cells, including persister cells, with little occurrence of resistance. MS-40S and MS-40 also have bactericidal activity against other pathogens involved in the CF lung microbiome. We also demonstrate in *C. elegans* and *Galleria* models, MS-40S and MS-40 were non-toxic. The novel compounds are

comparable to current clinical antibiotics used to help those infected with *B. cenocepacia* and other *Burkholderia* species. We propose that MS-40S and MS-40 have unique properties as antimicrobials and studying the mechanism of action of these will help in the development of novel antibiotics to treat multi-drug resistant CF lung infections.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/antibiotics10121443/s1, Table S1. Minimum inhibitory concentrations (MICs) of group one auranofin derivatives against a panel of *Burkholderia cepacia* complex (Bcc) bacteria. Table S2. Bacterial species and strains used in this study. Figure S1. ^{1}H NMR spectrum of compound WB-19-HL4170 (MS-40S) in $CDCl_3$. Figure S2. ^{13}C NMR spectrum of compound WB-19-HL4170 (MS-40S) in $CDCl_3$. Figure S3. ^{31}P NMR spectrum of compound WB-19-HL4170 (MS-40S) in $CDCl_3$. Figure S4. ^{1}H NMR spectrum of compound WB-19-HL4171 in $CDCl_3$. Figure S5. ^{13}C NMR spectrum of compound WB-19-HL4171 in $CDCl_3$. Figure S6. ^{31}P NMR spectrum of compound WB-19-HL4171 in $CDCl_3$. Figure S7. ^{1}H NMR spectrum of compound WB-19-HL4172 in $CDCl_3$. Figure S8. ^{13}C NMR spectrum of compound WB-19-HL4172 in $CDCl_3$. Figure S9. ^{31}P NMR spectrum of compound WB-19-HL4172 in $CDCl_3$. Figure S10. ^{1}H NMR spectrum of compound WB-19-HL4181 in $CDCl_3$. Figure S11. ^{13}C NMR spectrum of compound WB-19-HL4181 in $CDCl_3$. Figure S12. ^{31}P NMR spectrum of compound WB-19-HL4181 in $CDCl_3$. Figure S13. ^{1}H NMR spectrum of compound WB-19-HL4121a in CD_2Cl_2. Figure S14. ^{31}P NMR spectrum of compound WB-19-HL4121a in CD_2Cl_2.

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