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Auranofin efficacy against MDR *Streptococcus pneumoniae* and *Staphylococcus aureus* infections

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Background: Auranofin is an FDA-approved, gold-containing compound in clinical use for the oral treatment of rheumatoid arthritis and has been recently granted by the regulatory authorities due to its antiprotozoal properties.

Methods: A reprofiling strategy was performed with a *Streptococcus pneumoniae* phenotypic screen and a proprietary library of compounds, consisting of both FDA-approved and unapproved bioactive compounds. Two different multiresistant *S. pneumoniae* strains were employed in a sepsis mouse model of infection. In addition, an MRSA strain was tested using both the thigh model and a mesh-associated biofilm infection in mice.

Results: The repurposing approach showed the high potency of auranofin against multiresistant clinical isolates of *S. pneumoniae* and *Staphylococcus aureus in vitro* and *in vivo*. Efficacy in the *S. pneumoniae* sepsis model was obtained using auranofin by the oral route in the dose ranges used for the treatment of rheumatoid arthritis. Thioglucose replacement by alkyl chains showed that this moiety was not essential for the antibacterial activity and led to the discovery of a new gold derivative (MH05) with remarkable activity *in vitro* and *in vivo*.

Conclusions: Auranofin and the new gold derivative MH05 showed encouraging *in vivo* activity against multiresistant clinical isolates of *S. pneumoniae* and *S. aureus*. The clinical management of auranofin, alone or in combination with other antibiotics, deserves further exploration before use in patients presenting therapeutic failure caused by infections with multiresistant Gram-positive pathogens. Decades of clinical use mean that this compound is safe to use and may accelerate its evaluation in humans.

Introduction

The widespread overuse of antibiotics during the past 50 years has led to the emergence of numerous antibiotic-resistant bacteria, which represent a global public health threat.[1] Antimicrobial drug resistance contributes to treatment failure, high medical bills and substantially higher rates of morbidity and mortality.[2] Although there have been recent developments in antibiotic research, antimicrobial resistance is a serious problem because of the scarcity of new antibiotics in the current drug development pipeline that are effective against pathogens such as MRSA, MDR *Streptococcus pneumoniae*, β-lactamase-producing Gram-negative bacteria or MDR and XDR strains of *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa* or *Enterococcus faecium*.[3] This situation is fostering prospective research into new antimicrobial active substances. However, there are controversies about the cost-effectiveness of such research, which focuses on the description of new substances with novel cellular interactions or of new useful activities for 'old' clinically used drugs by screening them against relevant disease targets to overcome resistance. As the development of new drugs becomes increasingly expensive, the latter strategy, which is known as drug repurposing or drug reprofiling,[4] has seen unexpected success and has been adopted by the NIH.[5] The screening of existing drugs for new purposes has two main advantages.

First, because these drugs have an established safety record there may be a significant saving in the time needed for the development process. Second, the combination of an off-patent drug, known clinical safety and possibly low production costs may bring down drug prices and so make reprofiled drugs affordable throughout the world.

We have screened a large compound library that includes drugs already approved for other uses by the FDA and found that auranofin, an oral gold-containing compound that was approved in 1985 for the treatment of rheumatoid arthritis (RA) in adults and is off-patent, shows encouraging *in vitro* and *in vivo* activity against Gram-positive organisms, including MDR clinical isolates of *S. pneumoniae* and *Staphylococcus aureus*.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. Pneumococcal strains were routinely grown in C medium[6] supplemented (or not) with 0.08% yeast extract (C+Y), at 37°C without shaking, or on reconstituted tryptose blood agar base (TSA) plates (Difco Laboratories) supplemented with 5% defibrinated sheep blood (Thermo Scientific, Hampshire, UK). To prepare frozen stocks, bacteria were grown in Todd–Hewitt broth supplemented with 0.5% yeast extract to an OD_{550} of 0.4 ($\sim 10^8$ cfu/mL) and stored at −70°C in 10% glycerol as single-use aliquots. *S. pneumoniae* clinical isolates used for the *in vivo* study were strain 48 (serotype 23F; ST 321; amoxicillin MIC=16 mg/L, erythromycin MIC=1024 mg/L, levofloxacin MIC=1 mg/L, chloramphenicol MIC=4 mg/L, tetracycline MIC 128 mg/L) and strain 3498 (serotype 8; ST 63; penicillin MIC=0.02 mg/L, erythromycin MIC >128 mg/L, ciprofloxacin MIC=64 mg/L, levofloxacin MIC=16 mg/L, chloramphenicol MIC=4 mg/L, tetracycline MIC >64 mg/L). The clinical isolate *S. aureus* 132 (ST 8; oxacillin MIC ≥4 mg/L; penicillin MIC=2 mg/L; ampicillin MIC=4 mg/L; cefoxitin MIC ≥8 mg/L; ciprofloxacin MIC >2 mg/L; levofloxacin MIC >4 mg/L; tobramycin >8 mg/L) was grown on tryptic soy agar or broth at 37°C supplemented with glucose (0.25%).

Susceptibility testing

Antibiotics were purchased from Sigma-Aldrich Chemical Co., St Louis, MO, USA. Susceptibility was determined by the agar dilution technique[7] or by the microdilution method, always following the recommendations of the CLSI guidelines.[8,9] The susceptibility studies were performed twice. The MIC was defined as the lowest concentration of the drug that prevented visible growth after 24 h of culture at 37°C. The proprietary library (Prestwick Chemical Library, Prestwick Chemical, Illkirch, France) containing *knowns* (i.e. off-patent) and novel structures has a wide selection of chemical space and pharmacophores.

Bactericidal assay

Pneumococci (1 mL) were grown in C medium to early exponential phase (OD_{550} ≈0.15) and then auranofin was added. Auranofin was dissolved in DMSO to provide a final concentration of 1–10 μM and resulting in 1% (v/v) of solvent in the broth. Controls were always run in parallel, replacing auranofin with DMSO. Samples were incubated at 37°C for up to 6 h, with the OD_{550} measured at intervals during the primary screening to monitor growth inhibition. Viable pneumococci were measured in blood agar plates. For each sample, a 10-fold dilution series was prepared in PBS

Table 1. Bacterial strains used in this study and antibacterial spectrum of auranofin

Strain	Description	MIC (mg/L)	Source/reference
Gram-positive bacteria			
Enterococcus faecalis	clinical isolate	2	HUB
Enterococcus faecium	clinical isolate CC17; MDR	1	35
Enterococcus casseliflavus	MDR clinical isolate	0.5	HUB
Nocardia otitidiscaviarum	clinical isolate	0.5	HUB
Streptococcus agalactiae	clinical isolate	0.25	HUB
Staphylococcus aureus			
ATCC 25923	quality control	0.5	ATCC
132	MRSA	2	36
Streptococcus pneumoniae			
R6	unencapsulated	0.12	37
D39_IU[a]	serotype 2	0.12	38
48	serotype 23F	0.12	17
3498	serotype 8	0.12	33
Streptococcus pyogenes	clinical isolate	0.12	HUB
Gram-negative bacteria			
Acinetobacter baumannii	XDR clinical isolate	>16	HUB
Escherichia coli ATCC 25922	serotype O6	>16	ATCC
Pseudomonas aeruginosa			
ATCC 27853	control strain for susceptibility testing	>16	ATCC
	XDR clinical isolate	>16	39
Klebsiella pneumoniae	ESBL-producing clinical isolate	>16	40

HUB, Hospital Universitario de Bellvitge, Barcelona, Spain.
[a]Strain IU1680 (Lilly).

and 10 μL of each dilution was plated. In some cases, 1 mL of culture was concentrated by centrifugation and plated. Colonies were counted after overnight incubation at 37°C.

Synthesis and analysis of auranofin analogues

Melting points (uncorrected) were determined on a Stuart Scientific electrothermal apparatus. NMR spectra were recorded on a Bruker Avance 300-AM (^{1}H, 300 MHz; ^{13}C, 75 MHz; ^{31}P, 121 MHz) at the Universidad Complutense de Madrid's NMR facilities. Chemical shifts (δ) are expressed in ppm relative to internal tetramethylsilane; coupling constants (*J*) are in hertz (Hz). The following abbreviations are used to describe peak patterns when appropriate: s (singlet); d (doublet); t (triplet), q (quartet); and m (multiplet). 2D NMR experiments (Heteronuclear Multiple Quantum Correlation and Heteronuclear Multiple Bond Correlation) of representative compounds were carried out to assign protons and carbons of the new structures. Element analyses (C, H, N, S) were obtained on a LECO CHNS-932 apparatus at the Universidad Autónoma de Madrid analysis services and were within 0.4% of the theoretical values, confirming a purity of at least 95% for all tested compounds. Analytical thin-layer chromatography was run on Merck silica gel plates (Kieselgel 60 F-254) with detection by UV light (254 nm), ninhydrin solution or 10% phosphomolybdic acid solution in ethanol. Flash chromatography was performed with a Varian 971-FP flash purification system using silica gel cartridges (Varian, particle size 50 mm). Unless stated otherwise, starting materials, reagents and solvents were purchased as high-grade commercial products from Sigma-Aldrich, Acros or Scharlab and used without further purification.

Sugars **1–3** and chloro(triethylphosphine)gold (**7**) were purchased as high-grade commercial products from Sigma-Aldrich. Ethylthio (triethylphosphine)gold (**4**), (2-diethylamino)ethylthio(triethylphosphine)gold (**5**), (2,3-dihydroxy)propylthio(triethylphosphine)gold (**6**) and 1*H*-isoindole-1,3(2*H*)-dione(triethylphosphine)gold (**8**) were synthesized according to described procedures.[10–13]

Synthesis of tetrahydro-1H-pyrrolo[1,2-c]imidazole-1,3(2H)-dione(triethylphosphine)gold (MH05)

Tetrahydro-1*H*-pyrrolo[1,2-*c*]imidazole-1,3(2*H*)-dione (60 mg, 0.43 mmol) was added to a solution of aqueous 1 M NaOH (0.5 mL, 0.5 mmol) in ethanol (5 mL) at 0°C under an argon atmosphere. Then, a solution of chloro(triethylphosphine)gold (100 mg, 0.28 mmol) in ethanol (10 mL) was added and the reaction mixture was stirred at 0°C for 75 min at room temperature. The mixture was evaporated under reduced pressure and the residue was resuspended in water (20 mL) and extracted twice with dichloromethane (30 mL each). The combined organic extracts were dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The residue was purified by column chromatography (hexane/ethyl acetate; 7:3) to yield compound **9** (called MH05 hereafter) (122 mg, 63%) as a white solid (melting point 135–136°C). ^{1}H NMR ($CDCl_3$) δ 1.24 (dt, *J*=18.6, 7.7, 9H, $3CH_3$), 1.75–1.84 (m, 1H, $1/2CH_2$cyc), 1.88 (dq, *J*=10.1, 7.6, 6H, $3CH_2CH_3$), 1.92–2.09 (m, 2H, CH_2cyc), 2.17 (dtd, *J*=11.1, 7.8, 5.4, 1H, $1/2CH_2$cyc), 3.15 (ddd, *J*=11.1, 7.8, 5.4, 1H, $1/2CH_2$cyc), 3.72 (dt, *J*=11.1, 7.1, 1H, $1/2CH_2$cyc), 4.04 (t, *J*=8.1, 1H, CH). ^{13}C NMR ($CDCl_3$) δ 9.16 ($3CH_3$), 17.78 (d, *J*=22.0 $2CH_2$), 27.4, 28.1, 46.5 ($3CH_2$), 66.6 (CH), 171.9, 184.7 (2C). ^{31}P NMR ($CDCl_3$) δ 34.1.; Anal. calculated for $C_{12}H_{22}AuN_2O_2P$: C 31.73, H 4.88, N 6.17; found: C 32.00, H 4.90, N 5.97.

Sepsis mouse model for S. pneumoniae

Experimental procedures were performed at the Instituto de Salud Carlos III (ISCIII), complying with Spanish government legislation (RD 53/2013) and European Community regulations (2010/63/EU). The Animal Care and Use Committee of the ISCIII approved experiments involving animals in this study (CBA PA 52_2011-v2). The peritonitis–sepsis infection model was based on methods described elsewhere,[14] using 4- to 6-week-old female BALB/c mice (weight range, 15–20 g) obtained from the ISCIII animal facilities. Briefly, groups of five mice were infected by the intraperitoneal route with 10^8 cfu/mouse for the serotype 23F strain (strain 48) or with 10^2 cfu/mouse for the serotype 8 strain (strain 3498), which were the MLDs producing 100% mortality for each strain over a 7 day follow-up period. Bacterial inoculation titres were calculated by serial dilution and plating onto blood agar plates for each experiment. Antibiotic treatment (or placebo) was administered every 24 h during the first 96 h by the oral route using a gavage device and starting 1 h after the pneumococcal challenge. The doses of auranofin and MH05 administered were 15, 10, 5 and 1 mg/kg for strain 48 and 15, 10 and 5 mg/kg for strain 3498. Bacterial counts were determined from blood collected from the tail vein of the mice at 24 h post-infection. The bacterial counts and the survival experiments were pooled as both experiments produced similar results.

Staphylococcus aureus infections: thigh model and mesh-associated biofilm infection

All animal studies were reviewed and approved by the Comité de Ética, Experimentación Animal y Bioseguridad of the Universidad Pública de Navarra (approved protocol PI-019/12). Work was carried out at the Instituto de Agrobiotecnología building under the principles and guidelines described in European Directive 2010/63/EU for the protection of animals used for experimental purposes. The intramuscular infection model was based on methods described elsewhere,[14,15] using 4- to 6-week-old female CD1 mice (weight range, 15–20 g) obtained from Harlan Laboratories, Spain. Briefly, groups of five mice were rendered neutropenic by intraperitoneal injections of cyclophosphamide at 150 mg/kg (4 days before inoculation) and 100 mg/kg (1 day before inoculation). Thigh infection was produced by injecting 0.1 mL of inoculum containing 10^5 cfu. Two and six hours post-infection, 0.1 mL (5 mg/kg) of auranofin or MH05 solution was injected subcutaneously into mice. For each experiment, one control group of mice received drug-free solution in the same volume and schedule as the active drug regimen. Twenty-four hours after infection, mice were euthanized and the muscle was removed and homogenized in 1 mL of PBS for bacterial recovery. Samples were serially diluted and plated onto TSA plates, in order to count the viable staphylococci.

For the *in vivo* implant infection model, a model of mesh-associated biofilm infection was performed as described elsewhere,[16] with some modifications. Prior to the surgical procedure, 0.5×0.5 cm polypropylene meshes (Prolene) were incubated with 0.5 mL of a 1:100 overnight dilution of a culture of the biofilm-forming strain *S. aureus* 132 for 75 min at 37°C with shaking. To calculate the initial inoculum, duplicate meshes were placed in 1 mL of PBS and vigorously vortexed. Samples were serially diluted and plated onto TSA plates in order to count viable staphylococci. CD1 mice were anaesthetized by intraperitoneal injection of a ketamine/xylazine mixture. After abdominal epilation and antisepsis of the operative field, the animals were operated on. An incision of 1.5 cm in the skin was made, with displacement of the subcutaneous space and opening of the peritoneal cavity. Then, a mesh coated with *S. aureus* strain 132 was fixed to the abdominal wall with one anchor point. Finally, the peritoneal cavity was closed by suturing with 6/0 Monosyn. The animals were put into a warm environment and, when awake, put back in their cages. Mice were treated with either auranofin (5 mg/kg, intraperitoneally) or MH05 (5 mg/kg, intraperitoneally) 2 h after implantation and at days 1, 2, 3, 4 and 5. For each experiment, one control group of mice received drug-free solution in the same volume and schedule as the active drug regimen. Six days after infection, mice were euthanized and mesh and surrounding tissue was extracted, placed in 1 mL of PBS and vigorously vortexed in order to count the viable staphylococci.

Statistical analysis

All *in vivo* results are representative of data obtained from repeated independent experiments, and each value represents the mean and standard

deviation for three or four replicates. Statistical analysis was performed by the two-tailed Student's *t*-test (for two groups), whereas analysis of variance (ANOVA) was chosen for multiple comparisons. Survival experiments were analysed by the long-rank test (Mantel–Cox), whereas bacterial clearance results for treated mice were compared with results obtained for the lethal group by using a two-tailed Student's *t*-test. GraphPad InStat version 3.0 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. Differences were considered statistically significant when $P<0.05$ and highly significant when $P<0.01$ or $P<0.001$.

Results

Antibacterial assays

The chemical library used in this study contained 1280 small molecules, consisting mostly of known off-patent drugs (85%) and a few other bioactive molecules. The primary screening against this collection of compounds tested the susceptibility of the *S. pneumoniae* D39 strain in liquid cultures. Thus, bacteria were cultivated in 96-well plates in the presence of 10 μM of each compound from the library. Screening selection criteria were based on the potency for growth inhibition in the bioassay together with either the novelty of the structures and/or their potential as antibiotics when reprofiling well-known drugs. We obtained ≈10% of primary positives, including an overall hit rate of 0.32% of knowns with non-described antibacterial properties. Of these known drugs for which no antibacterial activities were reported, auranofin (Figure 1a) was the most potent against pneumococcus, showing MICs of ∼0.1 mg/L for *S. pneumoniae* strains, irrespective of the capsule and antibiotic susceptibility (Table 1). The bactericidal effect of auranofin became evident at 0.34–0.68 mg/L (corresponding to 0.5–1 μM) as the viability of strains R6 and 48 substantially decreased after 4 h of treatment, i.e. ∼2 log in relation to the untreated control. The lethal effect was even more pronounced after 6 h, since reductions of 4 log for R6 and 3 log for strain 48, at 0.68 mg/L concentration, were observed (Figure 1b). It should be noted that at 6.78 mg/L (50× MIC) auranofin practically sterilized the cultures after 6 h.

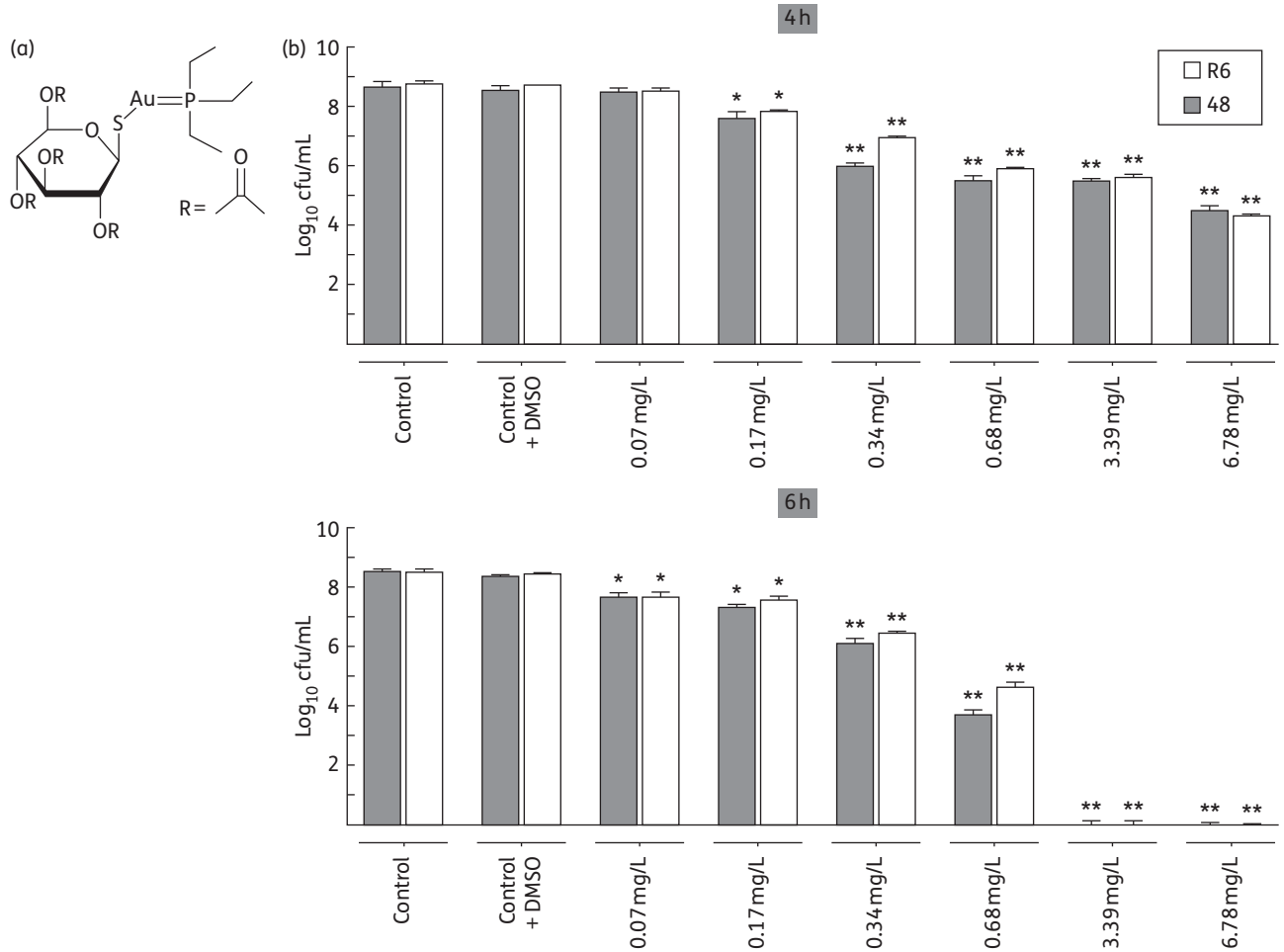
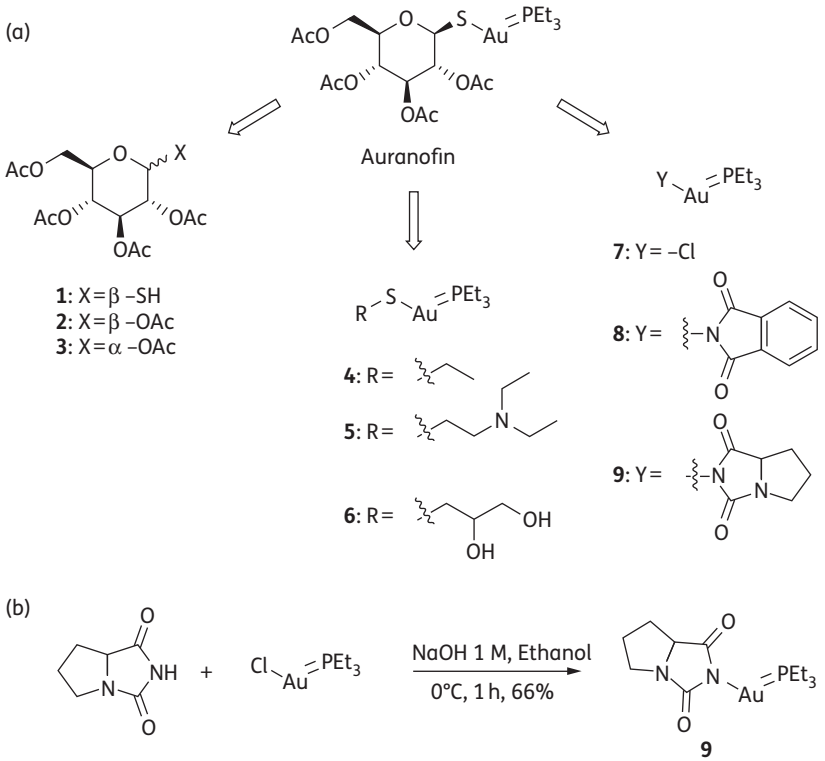


Figure 1. (a) Structure of auranofin. (b) Bactericidal effects of auranofin against *S. pneumoniae* strains R6 and 48. Exponentially growing bacterial cultures were incubated in the absence or presence of auranofin (0.07–6.78 mg/L) at 37°C. Viable cells were determined on blood agar plates after 4 or 6 h of treatment with auranofin. Controls reflect viable cells obtained in the absence of the drug or containing only the vehicle (DMSO) in which auranofin was dissolved. The lower limit of detection was 1 cfu/mL. Data are means from four independent experiments. Error bars represent standard deviations. Asterisks indicate that results are statistically significant compared with the control in the absence of auranofin (one-way ANOVA with a *post hoc* Dunnett's test; *$P<0.01$; **$P<0.001$).



(c)

Compound	MIC (mg/L)
Auranofin	0.12
4	0.42
5	0.12
6	0.25
7	0.21
8	0.42
9 (MH05)	0.21

Figure 2. (a) Design of auranofin analogues. (b) Synthesis of the new compound **9**, MH05. (c) Antibacterial activity of auranofin analogues **4**–**9** against *S. pneumoniae* D39.

The antibacterial spectrum of auranofin was defined by determining MICs for a panel of pathogenic clinical isolates (Table 1). A relevant feature of these values was the remarkable potency of auranofin against all Gram-positive clinical isolates tested, with inhibitory activities in the range 0.12–2 mg/L. Auranofin showed a restrictive antibacterial profile against Gram-positive bacteria when compared with data for Gram-negative organisms (MICs >16 mg/L), suggesting that the outer membrane may act as a permeability barrier for auranofin. Another important feature is that auranofin MIC values for *S. pneumoniae* and *S. aureus* were independent of the susceptibility pattern of these microorganisms.

Structural analogues of auranofin as antibiotics against S. pneumoniae

After testing the antibacterial activity of auranofin, we decided to explore some structural analogues obtained by structural modifications of the different features of the molecule (Figure 2a). Thus, sugars without gold and the phosphine moiety, such as 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose (compound **1**), 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose (compound **2**) and 1,2,3,4,6-penta-*O*-acetyl-α-D-glucopyranose (compound **3**), were selected to explore the role of the metal in antibacterial activity. Moreover, the sugar scaffold of auranofin was replaced by alkyl chains, keeping the sulphur atom as linker in compounds **4**–**6**. In addition, the influence of the organic moiety was explored in compounds **7**–**9**, where the sugar derivative was replaced by a chlorine atom or by nitrogen-containing heterocycles. Figure 2(c) shows the antibacterial activity of target compounds **4**–**9**, and auranofin as comparative control, against *S. pneumoniae* D39. Compounds **1**–**3** did not show any antibacterial activity under the experimental conditions used in the assay with living cells (data not shown). However, the previously described derivative compounds **4**–**8** showed potent activity in the bioassay and the new chemical entity—compound MH05, hereafter—showed antibacterial activity similar to that of auranofin.

Auranofin is active against infections caused by MDR pneumococci

To validate *in vivo* the bactericidal activity of auranofin and the synthetic compound MH05 against several pneumococcal strains, a mouse bacteraemia model of infection was used. Groups of five animals were infected with a lethal dose of the pneumococcal strains and antimicrobial treatment was started 1 h after bacterial challenge. Strain 48 (serotype 23F) is an MDR clinical isolate displaying a high level of antibiotic resistance, especially to β-lactam antibiotics and macrolides.[17] Infection with a high dose of this strain caused 100% mortality within the first 96 h (placebo; Figure 3a and b). However, mice treated with auranofin at doses of 1, 5 and 10 mg/kg by the oral route survived at rates of 50%, 30% and 20%, respectively. Treatments with 1 mg/kg showed statistically significant protective levels until the end of the experiment at day 7, which indicates that this drug reduces mortality against *S. pneumoniae* (Figure 3a). In addition, treatments with MH05 conferred protection at doses of 5 and 10 mg/kg (Figure 3b and c). Survival with the 10 mg/kg dose was 50%, whereas the 5 and 1 mg/kg doses induced 40% and 20% survival,

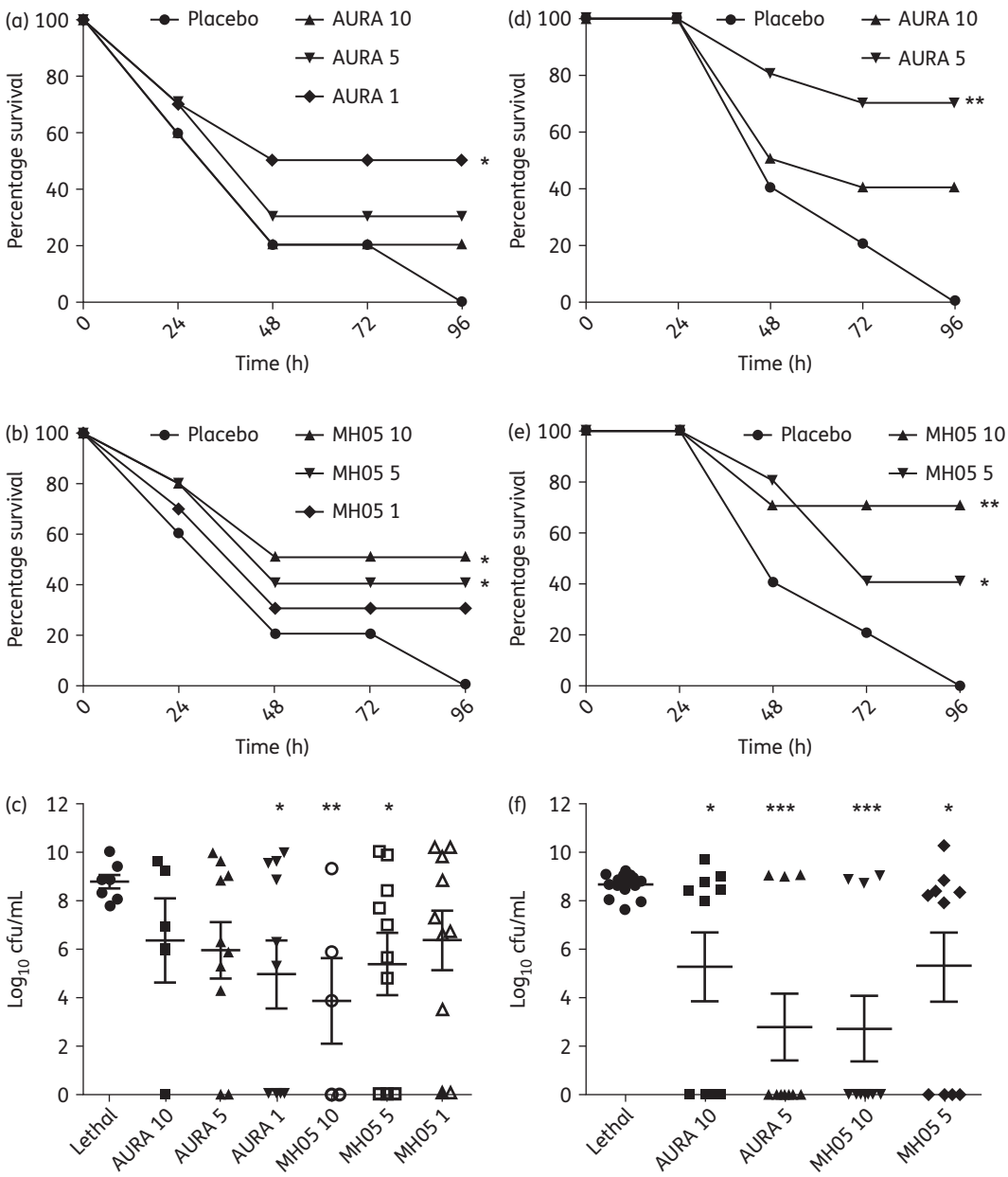


Figure 3. Percentage survival (a, b, d and e) and bacterial counts in blood (c and f) of groups of at least five mice intraperitoneally infected with the MLD of *S. pneumoniae* isolate serotype 23F (10^8 cfu/mouse) (a–c) or of *S. pneumoniae* isolate serotype 8 (10^2 cfu/mouse) (d–f). Experiments were repeated twice and animals were followed over a 7 day period. Antibiotic treatment or placebo was administered orally every 24 h during the first 96 h. AURA stands for auranofin and 1, 5 and 10 correspond to the dose of the drug (in mg/kg). To measure the level of bacteria in the bloodstream, blood samples were collected from the tails of the mice of each group 24 h after infection. The long-rank test was used for survival comparison and Student's *t*-test was used to estimate the difference between the treated groups and the untreated (lethal) group. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

respectively, at 96 h (Figure 3b). The levels of protection with MH05 were maintained unchanged from 96 h to the end of the survival period at day 7. To explore the level of bacteria in systemic circulation, blood samples were obtained daily from the tail veins of the mice. Overall, there was a reduction in the bacterial counts within the first 24 h of infection for all the doses investigated of auranofin and MH05; the results were statistically significant for 1 mg/kg auranofin and 10 and 5 mg/kg doses of MH05 (Figure 3c). A direct correlation between survival and colony counts was observed, as doses of 1 mg/kg for auranofin and 10 and 5 mg/kg for MH05 showed an increased number of animals with no bacteria in their blood within the first 24 h of infection (Figure 3a–c).

To extend our results to another serotype with a different antibiotic-resistance profile, mice were infected with strain 3498 (serotype 8). Animals in the lethal control (placebo) group succumbed at 96 h of infection after inoculation of a lethal dose of this strain (Figure 3d and e). In a similar way to that reported

above for strain 48, a significant increase in animal survival was only observed at the lowest concentration tested (5 mg/kg) (Figure 3d). The results, showing that auranofin, at a dose of 1 mg/kg (strain 48) or 5 mg/kg (strain 3498), has greater antimicrobial activity *in vivo* than at higher doses, are reminiscent of the paradoxical effect of certain antimicrobials—also known as the Eagle effect—wherein an antimicrobial is less effective at higher than at lower concentrations,[18] although a definite explanation will require further research.

Treatment with MH05 significantly increased the levels of protection for all the doses administered, with survival rates at 96 h post-infection (when all the mice in the lethal group had succumbed) of 70% and 40% for the 10 and 5 mg/kg doses, respectively (Figure 3e). These protective levels remained constant until the end of the experiment. Overall, our results indicate that auranofin and MH05 provide antimicrobial activity against MDR strains of *S. pneumoniae*, increasing survival rates. In terms of bacterial clearance, oral administration of the different compounds decreased pneumococcal levels in blood within the first 24 h of infection, when all animals were still alive (Figure 3f). Reduction of *S. pneumoniae* counts in the systemic circulation was statistically significant for the doses of 10 and 5 mg/kg for auranofin and of 10 and 5 mg/kg for MH05 (Figure 3f). The bacterial counts and survival experiments were pooled as both experiments produced similar results. Our findings confirm that auranofin and MH05 provided similar ranges of protection against pneumococcal sepsis caused by MDR strains by reducing bacterial levels in the bloodstream.

Auranofin is active against infections produced by MRSA

To evaluate the efficacy of auranofin and MH05 against *S. aureus* infections, we selected two relevant animal models, namely the intramuscular infection model (abscess) and the implant infection model (biofilm). For intramuscular infection, 10^5 cfu of the MRSA strain 132 were injected directly into the thigh of mice. Two and six hours after infection, mice were treated with two doses of 5 mg/kg auranofin or MH05 injected subcutaneously. This dose was based on the results obtained during the treatment of streptococcal infections. After 24 h, mice were killed and the infected tissue was removed to count the bacteria. The results revealed that two doses of auranofin or MH05 were sufficient to cause significant decreases in bacterial burden after 24 h, compared with results from the untreated control animals (Figure 4a). Interestingly, the efficacy of both compounds was very similar.

We next tested the efficacy of auranofin and MH05 used to treat *S. aureus* biofilm infections. For this purpose, polypropylene meshes coated with 10^5 cfu of the biofilm-positive *S. aureus* 132 were implanted into the intraperitoneal cavity of mice. Antimicrobial treatment was started 2 h after implantation and repeated daily, with 5 mg/kg/day of auranofin or MH05. At day 6, all animals were killed and meshes were extracted for bacterial counting. When the number of bacteria on meshes was determined, results showed that treatment with auranofin or MH05 significantly reduced the number of bacteria attached to the polypropylene meshes ($P<0.05$) (Figure 4b). Finally, we investigated whether auranofin and MH05 were able to protect against the bacterial population that propagates by detachment from the biofilm. To do so, mesh-surrounding tissue was extracted and

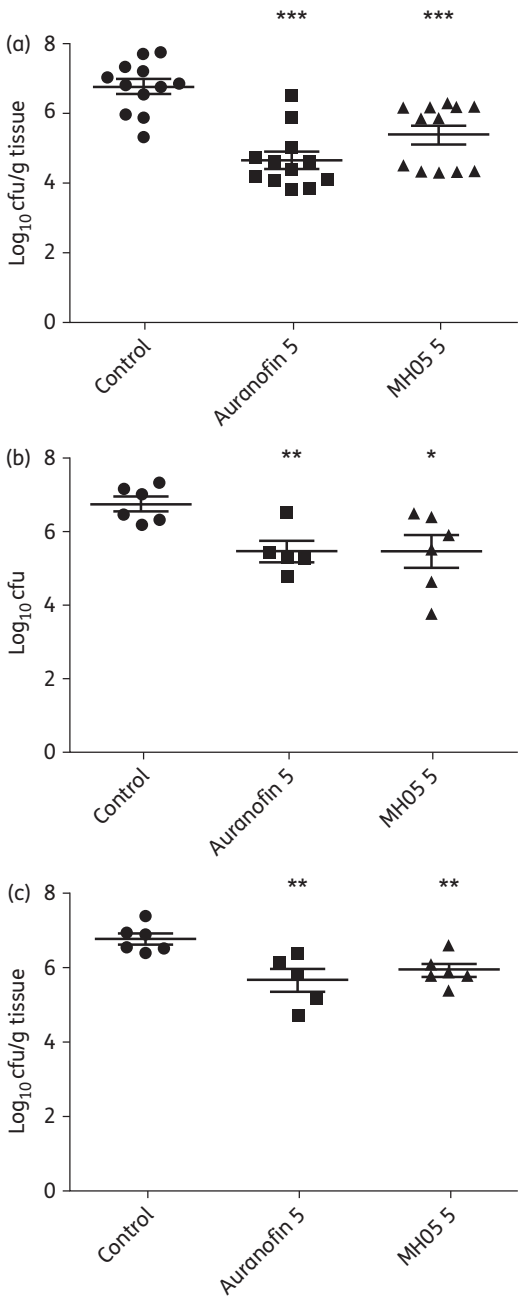


Figure 4. Efficacy of auranofin and MH05 in *S. aureus* murine infection models. (a) Efficacy of auranofin in the murine thigh infection model. Groups of mice ($n=12$) were rendered neutropenic and 10^5 cfu of the *S. aureus* 132 strain was injected into their thighs followed by subcutaneous administration of two 5 mg/kg doses of auranofin and MH05 at 2 and 6 h post-infection. The cfu recovered from the infected thighs after 24 h are shown. (b and c) Efficacy of auranofin in the biofilm infection model. (b) Mesh. Polypropylene meshes coated with *S. aureus* strain 132 were fixed to the abdominal wall of groups of mice ($n=6$), followed by intraperitoneal administration of a 5 mg/kg dose of auranofin or MH05 2 h post-infection and repeated at days 1, 2, 3, 4 and 5. Animals were killed, and meshes were extracted and placed in 1 mL of PBS at day 6 in order to count viable staphylococci. (c) Surrounding tissue. Error bars represent standard errors in the cfu measurements. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

bacterial colonization was determined. In contrast to the non-treated group (control), mice treated with auranofin or MH05 had significantly fewer bacteria colonizing the mesh-surrounding tissue ($P<0.01$) (Figure 4c). Taken together, these data demonstrated the *in vivo* efficacy of auranofin and its derivative MH05 for the treatment of implant-associated biofilm-forming MDR *S. aureus* strains.

Discussion

MDR and XDR Gram-positive bacteria are known to be the leading cause of healthcare-related and community-acquired infections.[19] Given the rapid spread of antimicrobial resistance, novel therapeutic strategies are needed to fight the increasing prevalence of MDR bacterial infections. A recent approach based on reprofiling efforts with off-patent compound libraries has the advantage of making use of well-characterized sets of compounds with known pharmacological properties.[20] A good example of the success of this strategy was the selection of auranofin (Ridaura®), an antiarthritic metallodrug, as an antimicrobial agent against pathogenic protozoa, such as *Plasmodium falciparum*, *Entamoeba histolytica* and *Giardia lamblia*.[21–24] Our phenotypic screening using the encapsulated *S. pneumoniae* D39 strain led to the selection of auranofin with potency in the range presented by the current antibiotics in clinical use for this pathogen. These results increase the clinical relevance of auranofin as an antibiotic, since *S. pneumoniae* has been reported to be responsible for 1.6 million deaths per year and the fourth cause of death in the world.[25]

The antibacterial properties of auranofin against *S. pneumoniae* D39 led us to explore some structural analogues obtained by modifications of the different features of the molecule. The comparative analysis of the activity of target compounds **1**–**9** and auranofin against *S. pneumoniae* clearly shows that removal of the gold and triethylphosphine in auranofin leads to a complete loss of activity, indicating that thioglucose is not the active part of the molecule responsible for antibiosis. In contrast, when this scaffold was replaced by alkyl chains (e.g. compounds **4**–**6**), antibacterial activity was restored. As for the influence of the atom bound to the metal, replacement of sulphur by chlorine did not produce any effect on antibacterial activity, since the chloro derivative **7** had the same MIC as auranofin, and nitrogen derivatives **8** and **9** (MH05) also showed striking activities. Therefore, replacement of the tetraacetylthioglucose scaffold of auranofin by alkyl sulphides or nitrogen-containing bicycles, such as phthalimide or bicyclohidantoins, maintains the antibacterial activity of the analogues against pneumococci. This work provided a new chemical entity (MH05), demonstrating the feasibility of the generation of novel structural families of antibiotics with an unexplored mechanism of action and deserving further preclinical research.

The spectrum of the antibacterial activity of auranofin was first explored *in vitro* against a panel of clinical isolates of Gram-positive pathogens by other authors,[26–28] but here we have included MDR strains of *S. pneumoniae* (with resistance to erythromycin and penicillin) and MRSA. The inhibition results showed no significant differences in potency and selectivity between the two gold derivatives, suggesting that the structural modifications that endow MH05 with the status of a new chemical entity do not hinder its antibacterial activity. Both auranofin and MH05 showed activities in the dose ranges used in current clinical practice. It is worth noting the high degree of selectivity of these gold compounds against the Gram-positive pathogens of the panel compared with activities against Gram-negative ones. This differential behaviour could be based on permeability variations caused by structural features, such as the outer membrane of Gram-negative bacteria. In addition, it is noticeable that auranofin and MH05 were equally effective against susceptible or MDR pneumococci and MRSA, which may suggest a new mechanism(s) of action for these metallodrugs, different from those of clinically prescribed antibiotics. Auranofin seems to exert powerful inhibition of thioredoxin reductases (TrxRs) in *E. histolytica*, preventing the reduction of thioredoxin and enhancing the susceptibility of trophozoites to reactive oxygen-mediated killing. The activity of auranofin in human amoebiasis—10-fold more potent against *E. histolytica* than metronidazole—has led the FDA to give it orphan-drug status.[23] TrxRs have been described as promising new targets also in *S. aureus*, a bacterium lacking the glutaredoxin (Grx) system.[29] However, *S. pneumoniae* possesses more complex redox regulation to cope with oxidative damage. Redox regulation in pneumococcus includes the Grx system and a well-known surface-located thioredoxin system common to all serotypes (Etrx1/Etrx2), which might be a clue to success against this human pathogen.[30] Redox systems are conserved through evolution and considered to be targets that deserve drug discovery efforts, in order to offer an alternative to existing therapies, including vaccines. Therefore, further research is needed to clarify the mechanism(s) of action of these metallodrugs in Gram-positive pathogens.

Auranofin was approved in 1985 for the treatment of RA. Recent *in vitro* studies described the high bactericidal activity of auranofin against MRSA.[27,28] Auranofin has a safe toxicity profile, ascertained during decades of use in humans, and well-known pharmacokinetic/pharmacodynamic characteristics,[31] warranting the rapid evaluation of this drug for Gram-positive infections *in vivo*. Our results have shown the capacity of auranofin to protect against infections with *S. pneumoniae* and *S. aureus*. Oral administration of auranofin and MH05 was chosen to treat pneumococcal sepsis in mice, because this is the appropriate route for auranofin treatment of RA in humans.[31] Here we demonstrate that oral administration of auranofin or MH05, within the dose range used for RA in the clinic, significantly reduced the burden of infection by MDR *S. pneumoniae* strains in mice. These results are very relevant in antimicrobial chemotherapy terms, as the pneumococcal strains investigated had high levels of resistance to β-lactam antibiotics, macrolides and/or fluoroquinolones. In the case of strain 48 (serotype 23F), the MIC of amoxicillin for this isolate is 16 mg/L, which might be of concern for the outcome of the infection.[17] Previous studies, using the same strain of mice and a pneumococcal strain with an MIC of penicillin 8-fold lower than that for strain 48 in our study, demonstrated that a cefotaxime dose of 50 mg/kg was needed to obtain 60% protection against sepsis.[32] However, our study demonstrates that administration of lower doses (5–10 mg/kg) of either auranofin or MH05 provided similar levels of protection against a pneumococcal strain for which the MIC of penicillin is much higher, suggesting that these two compounds might be promising alternatives against pneumococcal sepsis caused by strains harbouring high levels of β-lactam resistance. In the case of allergy to β-lactams,

macrolides and fluoroquinolones are antimicrobial alternatives against pneumococcal infections.[2] As the two strains used in this study were macrolide resistant, we investigated protection by auranofin and derivatives using a serotype 8 strain with marked resistance to fluoroquinolones. This clinical isolate belongs to a new recombinant MDR clone (serotype 8, ST 63), which recently emerged and spread in Spain as a cause of invasive diseases.[33] Levels of protection with auranofin and MH05 were higher against sepsis produced by this strain than those obtained against the serotype 23F strain. Treatment with levofloxacin at 50 mg/kg did not protect any mice after 72 h post-infection, resulting in bacterial levels in blood similar to those obtained with the lethal control at 24 h. This confirmed that administration of levofloxacin was not protective against the sepsis produced by the fluoroquinolone-resistant serotype 8 strain (data not shown). Along these lines, other authors have also shown that treatment of mice with levofloxacin and moxifloxacin against sepsis caused by a pneumococcal strain that was similar to the strain used in our study with respect to mutations at *gyrA* and *parC* and susceptibility to fluoroquinolones was poorly effective.[34] These results suggest that auranofin and MH05 might constitute novel antimicrobial alternatives against sepsis caused by pneumococcal strains with high levels of resistance to fluoroquinolones. Variation in bacterial levels in blood between the two pneumococcal strains investigated might be related to differences in the clinical isolates used for this study. Strain 48 is an MDR pneumococcal strain of serotype 23F that is virulent in mice only when high levels of bacteria are inoculated (LD_{100} $>5\times10^7$ cfu/mouse). However, strain 3498 (serotype 8) is highly virulent in mice (LD_{100} $<2\times10^2$ cfu/mouse). The marked differences in virulence between the two strains might be responsible for the increased clearance of strain 48 from the systemic circulation after drug treatment.

A high level of protection was also obtained with two *in vivo* models of infection with *S. aureus*. Furthermore, for both gold derivatives, protection levels in the infected animals were similar to or higher than those given by the antibiotics currently in use as research standards. In this respect, survival rates were significantly improved by treatment with either of the two metallodrugs. Despite the significant reduction in the implant-associated *S. aureus* biofilm load resulting from auranofin treatment, this reduction may not be sufficient to cure the infection, especially in patients with an impaired immune system. Thus, combinations of auranofin with other antibiotics that are currently used for the treatment of these infections require further exploration before use in clinical practice.

Use of a repurposing strategy with known drugs and a simple phenotypic assay have proved that auranofin behaves as an efficient antibiotic *in vivo* against two major pathogenic Gram-positive bacteria, *S. pneumoniae* and *S. aureus*, with a unique pharmacological profile, which is very probably due to its different mechanism(s) of action. Our medicinal chemistry efforts led to a novel chemical entity (MH05) that behaved *in vivo* as safely as the control standards, providing efficient protection against infections caused by the two main Gram-positive pathogens, which prompted us to develop new safe and efficient derivatives under the strictest regulatory rules. The *in vivo* bactericidal results shown here may lead to auranofin, alone or in combination with other antibiotics, being considered an alternative therapy to be further explored, under the pharmacological strategy for compassionate use against MRSA and MDR pneumococci, which are responsible for a wide variety of invasive infections.

Note added in proof

While this article was under consideration, Harbur *et al*. reported that auranofin inhibits the flavoenzyme thioredoxin reductase (TrxR) of *M. tuberculosis* and *S. aureus* (Harbut MB, Vilchèze C, Luo X *et al*. Auranofin exerts broad-spectrum bactericidal activities by targeting thiol-redox homeostasis. *Proc Natl Acad Sci USA* **112**: 4453–8).

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Transparency declarations

None to declare.

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