Silver(I) Complexes of 9-Anthracenecarboxylic Acid and Imidazoles: Synthesis, Structure and Antimicrobial Activity

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SUPPLEMENTARY DATA

BIOLOGICAL SCREENING

In vitro antimicrobial screening

Minimal growth media (MM) was composed of 2% (w/v) glucose, 0.5% (w/v) ammonium sulphate and 0.17% (w/v) yeast nigtogen base (without amino acids or ammonium sulphate). To solidify the media 2% (w/v) bacteriological agar was added when required. Yeast extract peptone dextrose (YEPD) media was composed of 2% (w/v) glucose, 2% (w/v) bacteriological peptone and 1% (w/v) yeast extract. To solidify the media 2% (w/v) bacteriological agar was added when required. Nutrient Broth was obtained from Scharlau Microbiology and made up according to the manufacturer's instructions (13 g in 1 litre of deionised water). Phosphate buffered saline (PBS) was obtained from Aldrich and made up according to the manufacturer's instructions (1 tablet in 200 cm^3 of deionised water).

In vitro bacterial susceptibility testing

E. coli and methicillin-resistant *S. aureus* (MRSA) were grown on nutrient broth agar plates at 310 K and maintained at 277 K for short-term storage. Cultures were routinely sub-cultured every 4-6 weeks. All assays were run in triplicate and on three independent occasions. Fresh solutions of complexes were prepared immediately prior to testing. Complexes (0.020 g) were added to DMSO (1 cm^3) then water (9 cm^3) was added to give a stock solution (concentration 2000 μg cm^{-3}). Complexes with low solubility were tested as fine suspensions. The stock solution (1 cm^3) was added to water (9 cm^3) to yield a solution/suspension with a concentration of 200 μg cm^{-3}.

Nutrient broth (100 μl) was added to each well of a 96-well, flat-bottomed microtitre plate. Water (100 μl) was added to column 1 of the plate (negative control with no bacterial cells). Column 2 was the positive control (medial with bacterial cells). 100 μl of the above complex solution (200 μg cm^{-3}) was added to every well in column 3. Serial dilutions (1:1) were made from column 3-12 to produce a test concentration range of 100–0.2 μg cm^{-3}.

E. coli and MRSA were grown overnight to the stationary phase in nutrient broth at 310 K and 200 rpm. The cells were diluted to give an optical density of 0.1 at λ = 600 nm. The cell suspension (100 μl) was added to every well in columns 2–12. The completed plates were incubated at 310 K in a static incubator and the final optical density recorded. MIC_{50} values (minimum concentration required to inhibit 50% of cell growth) were then determined and expressed in terms of μM concentration.

In vitro fungal susceptibility testing

C. albicans (ATCC 10231) was grown on YEPD agar plates at 310 K and maintained at 277 K for short-term storage. Cultures were routinely sub-cultured every 4-6 weeks. All assays were run in triplicate and on three independent occasions. Fresh solutions of complexes were prepared immediately prior to testing. Complexes (0.020 g) were dissolved in DMSO (1 cm^3) and added to water (9 cm^3) to give a stock solution (concentration 2000 μg cm^{-3}). Complexes with low solubility were tested as fine suspensions. The stock solution (or suspension) (0.5 cm^3) was added to water (9.5 cm^3) to give a solution with a concentration of 100 μg cm^{-3}. MM (100 μl) was added to each well of a 96-well, round-bottomed microtitre plate. Water (100 μl) was added to column 1 of the plate (negative control, media with no fungal cells). Column 2 was the positive control (media and fungal cells only). 100 μl of the above complex solution (100 μg cm^{-3}) was added to every well in column 3. Serial dilutions (1:1) were made from columns 3-12 to produce a test concentration range of 50–0.1 μg cm^{-3}.

C. albicans was grown to the stationary phase overnight at 310 K on YEPD media. The cells were washed with PBS solution and resuspended in MM at a density of 5×10^5 cells cm^{-3}. The cell suspension (100 μl) was added to every well in columns 2–12. The completed plate was then covered with acetate foil (Sarstedt) to prevent dehydration. The plate was incubated at 310 K with continuous shaking for 24 h. The optical density (λ = 540 nm) of each well was recorded at 1 h intervals. MIC_{100} values were then determined and expressed in terms of μM concentration.

In vivo cytotoxicity studies using *Galleria mellonella*

Since the immune system of insects bears a number of strong structural and functional similarities to the innate immune system of mammals,[38,39] insects can be used to screen the *in vivo* effect of drugs without the necessity of mammalian testing. The larvae of the greater wax moth, *G. mellonella*, have been utilised successfully since 1982 in *in vivo* studies of the pathogenicity of bacteria and fungi.[40-44] With specific reference to *C. albicans*, the *G. mellonella* model has provided invaluable information from fungal virulence studies.[45-49] Furthermore, the curative effects of antifungal prescription drugs[50] and some silver(I) compounds[51] on *C. albicans*-infected *G. mellonella* have already been determined.

G. mellonella larvae in the sixth developmental stage were used to determine the *in vivo* cytotoxicity of ketoconazole, $AgNO_3$ and complexes **1**-**7**. Ten healthy larvae, between 0.20-0.40 g in weight and with no cuticle discolouration, were used in each test. Fresh solutions of the test complexes were prepared immediately prior to testing. Test compounds (0.020 g) were dissolved in DMSO (1 cm^3) and added to water (9 cm^3) to give a stock solution (concentration 2000 μg cm^{-3}). Test solutions were made from this stock solution. In cases of limited solubility the resulting suspensions were used for the tests. Each compound was tested at two concentrations; 100 μg cm^{-3} and twice their MIC_{100} value (in μg cm^{-3}). The test solution (20 μl) was administered to the larvae by injection directly into the haemocoel through the last pro-leg. The base of the pro-leg can be opened by applying gentle pressure to the sides of the larvae and this aperture will re-seal after removal of the syringe without leaving a scar. Larvae were placed in sterile petri dishes and incubated at 303 K for 72 h. Larvae survival was monitored after this 72 h period. Death was assessed by the lack of movement in response to stimulus together with discolouration of the cuticle. Three controls were employed in all assays. The first consisted of untouched larvae maintained at the same temperature as the test larvae. The second was larvae with the pro-leg pierced with an inoculation needle but no substance injected into them. The third control was larvae that were inoculated with 20 μl of the same sterile water used to make the test solutions.

In vivo antifungal screening using *G. mellonella*

G. mellonella larvae in the sixth developmental stage were used to determine the *in vivo* antimicrobial activity of ketoconazole, $AgNO_3$ and complexes **1**-**7**. As with the cytoxicity studies (see above) ten larvae were injected with a fresh solution (or suspension) of the complex. Test concentrations were again 100 μg cm^{-3} and twice their MIC_{100} value (in μg cm^{-3}). *C. albicans* was grown to the stationary phase in YEPD media at 310 K for 24 h. The cell concentration was assessed using a haemocytometer following dilution of the culture with PBS. The cells were washed three times with PBS and re-suspended in sterile PBS following harvesting by centrifugation (4000 x g for 10 min) to yield a suspension of 1×10^8 cell cm^{-3}.

Two treatments (prophylactic and treatment of infection) were employed in the screening of the test solutions. Five controls were used in each assay. The first control consisted of untouched larvae maintained at the same temperature as the test larvae (negative

control). The second was larvae with the pro-leg pierced with an inoculation needle but no substance injected into them. The third control was larvae that were inoculated with the same sterile water (20 µl) used to make the test solutions, and the fourth was larvae inoculated with the same sterile PBS solution used to wash and make the *C. albicans* suspension. The positive control consisted of larvae inoculated with *C. albicans* and no test solution administered. The latter control is the one given in the tabulated results (Table 3). Significance was determined using the log rank (Mantel-Cox) method. Positive results were placed into one of three categories: * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$.

Prophylactic treatment. The test solution (20 µl) was administered to the larvae by injection directly into the haemocoel through the last pro-leg. Larvae were then placed in sterile petri dishes and incubated at 303 K for 1 h, after which time they were inoculated with 20 µl of the *C. albicans* cell suspension (2×10^6 cells). This number represents a lethal dose of fungal cells. The larvae were incubated at 303 K for 72 h and larvae survival was monitored immediately after this period.

Treatment of infection. Larvae were inoculated with 20 µl of the *C. albicans* suspension (2×10^6 cells) by injection directly into the haemocoel through the last pro-leg. The larvae were then placed in sterile petri dishes and incubated at 303 K for 1 h, after which time the test solution (20 µl) was administered by injection. The larvae were incubated at 303 K for 72 h and larvae survival was monitored immediately after this period.

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