The treatment of *Acinetobacter baumannii* infections poses a significant clinical challenge, with isolates resistant to all commonly used agents increasingly being reported. With few new agents in the pipeline, clinicians are increasingly turning to combinations of antimicrobials in the hope that they may act synergistically together. In this study we assessed the activities of two glycopeptide-colistin combinations both *in vitro* and using a *Galleria mellonella* caterpillar model of *A. baumannii* infection. In checkerboard assays both vancomycin and teicoplanin were highly active against susceptible and multidrug-resistant strains of *A. baumannii* when combined with colistin (fractional inhibitory concentration [FIC] of <0.25). Treatment of *G. mellonella* caterpillars infected with lethal doses of *A. baumannii* resulted in significantly enhanced survival rates when either vancomycin or teicoplanin was given with colistin compared to colistin treatment alone \((P<0.05)\). This effect was most marked when vancomycin was the glycopeptide administered, although this agent was also highly effective as monotherapy, possibly through an immunomodulatory action on the *G. mellonella* response to *A. baumannii* infection. This work suggests that glycopeptide-colistin combinations are highly active against *A. baumannii* both *in vitro* and in a simple animal model of infection. They should be considered further as potential treatments for difficult-to-treat *A. baumannii* infections.

Over the last decade *Acinetobacter baumannii* has emerged as one of the most problematic nosocomial pathogens. Successful multidrug-resistant (MDR) clones have disseminated worldwide and often remain susceptible only to agents such as tigecycline and polymyxins, which are usually considered treatments of “last resort” \((17, 6)\). With resistance to these agents increasing being reported \((9, 16)\), the selection of effective therapies for the treatment of *A. baumannii* infections is particularly challenging.

Although some attempts to advance the development and introduction of new agents have been made, it is unlikely that any of these will be available for routine clinical use for a number of years. Clinicians are therefore forced toward using combinations of existing licensed drugs in the hope that they may act synergistically together. Although a number of antimicrobial combinations have been shown to be active against MDR *A. baumannii* (MDRAB) *in vitro*, there are only limited data on their efficacy in animal models, while those from humans are limited to individual case reports or retrospective cohort studies \((5)\).

Recently we observed a potent effect on the susceptibility of MDRAB to glycopeptides in the presence of low doses of colistin \((7)\). This occurred with both vancomycin and teicoplanin \((23)\) and in most cases reduced the concentration of the glycopeptide required to kill the organism to below pharmacodynamic breakpoints currently used to define susceptibility in Gram-positive bacteria. The effect is thought to be mediated via a permeabilizing effect of colistin on the *A. baumannii* outer membrane, facilitating the entry of glycopeptide molecules, which are usually excluded due to their size \((21)\). Although this combination appears to be a promising treatment option based on the *in vitro* data, further preclinical work is clearly needed before it can be considered for clinical use.

Assessment of the efficacy of antimicrobials using *in vivo* models is routinely undertaken prior to clinical trials. Although mammalian models are routinely employed for such studies, their use poses significant practical, financial, and ethical barriers. As a result, invertebrate models, such as the wax moth caterpillar *Galleria mellonella* (which can be easily sourced in most countries where larvae are sold commercially as reptile food), have been proposed as inexpensive and easy alternatives that are able to generate reliable and reproducible data on microbial virulence which mirror almost exactly those obtained using higher animals \((11, 19, 3)\). These insects have a well-developed innate immune system consisting of phagocytic cells, a complement-like system \((12)\), and an increasingly recognized arsenal of antimicrobial peptides \((2)\), making them highly attractive for the study of acute bacterial infections.

Recently, an assay using *G. mellonella* was developed to investigate the pathogenicity of *A. baumannii* by Peleg et al. \((18)\). As described, the model was also able to assess the effects of antimicrobial treatment on sensitive and resistant strains of *A. baumannii*. In this work we employed the *A. baumannii-G. mellonella* system to study the *in vivo* efficacies of glycopeptide-colistin combinations in an attempt to gain further insights into whether these therapies should be explored further for the treatment of MDRAB.
from Livefood UK Limited (Rooks Bridge, Somerset, United Kingdom) and suspensions containing 10^4, 10^5, and 10^6 CFU/larva of organisms in PBS, as buffered saline (PBS). In order to establish the inoculum required to kill AB210 were grown overnight in LB broth and washed twice in sterile phosphate-buffered saline (PBS) and prepared in sterile distilled water as 10,000-μg/ml stock solutions.

In vitro susceptibility and antimicrobial synergy studies. The susceptibilities of A. baumannii ATCC 19606 and AB210 to β-lactams, aminoglycosides, and quinolones were determined by the BSAC disc diffusion method (1). The MICs of colistin, teicoplanin, and vancomycin were determined by Etest (AB biotécnica, Solna, Sweden) on Iso-Sensitest agar. Synergy when colistin (0 to 16 μg/ml) was combined with teicoplanin or vancomycin (0 to 1,024 μg/ml) was assessed in standard checkerboard assays in microtiter plates with wells containing doubling dilutions of each agent in Iso-Sensitest broth. Plates were incubated at 37°C for 24 h and the absence of growth in nonturbid wells confirmed by the addition of Alamar blue reagent (Invitrogen, Paisley, United Kingdom). Synergy was assessed by the calculation of fractional inhibitory concentrations (FICs) and susceptible breakpoint indices (SBPIs) as previously described (7).

G. mellonella killing assay. G. mellonella caterpillars were obtained in bulk from LiveFood UK Limited (Roos Bridge, Somerset, United Kingdom) and stored at 15°C in wood shavings prior to use. A. baumannii ATCC 19606 and AB210 were grown overnight in LB broth and washed twice in sterile phosphate-buffered saline (PBS). In order to establish the inoculum required to kill G. mellonella over 48 to 96 h, 10 caterpillars were inoculated with 10 μl of bacterial suspensions containing 10^5, 10^6, and 10^7 CFU/larva of organisms in PBS, as determined by viable bacterial counts on Iso-Sensitest agar. Bacteria were injected into the hemocoel of the caterpillar via a left proleg using 25-μl Hamilton syringes (Cole-Parmer, London, United Kingdom). Caterpillars were incubated in petri dishes lined with filter paper at 37°C for 96 h and scored for survival by 2 independent observers daily. Insects were considered dead if they failed to respond to touch.

Antimicrobial treatment assays. The effects of treatment with colistin, gentamicin, teicoplanin, and vancomycin on A. baumannii-infected caterpillars were assessed as follows. Sixteen G. mellonella caterpillars were inoculated with a lethal dose of ATCC 19606 or AB210 via injection into a left proleg as described above. Antibiotics were administered via 10-μl injections into a left proleg within 30 min of inoculation. Doses were chosen to mimic those used to treat human infections and consisted of colistin at 2.5 mg/kg, vancomycin and teicoplanin at 10 mg/kg, and gentamicin at 5 mg/kg. Only caterpillars weighing 250 mg ± 25 mg were used in these experiments. Each drug was assessed individually, and vancomycin and teicoplanin were also assessed when combined with colistin. Sixteen uninjected caterpillars and 16 caterpillars injected with 10 μl of sterile PBS in place of bacteria were used as controls. To allow for the trauma associated with double injections, uninjected caterpillars inoculated twice with 10 μl of PBS were also used. Following infection and treatment, G. mellonella deaths were scored over 96 h of incubation at 37°C as described above. Experiments were performed three times on separate occasions.

Statistical analysis. Treatment assays were repeated three times on separate occasions with different batches of G. mellonella. Survival curves were analyzed using the log rank test, with a P value of ≤0.05 indicating statistical significance. The percent survival (and standard deviation) of antibiotic-treated caterpillars at the 96-h assay endpoint was calculated using pooled data across the replicate experiments. Mean values were compared using one-tailed, unpaired t tests. Assays using batches of caterpillars in which >2 uninfected and uninoculated animals died were not considered valid.

### RESULTS AND DISCUSSION

**Antimicrobial activity in vitro.** ATCC 19606 was susceptible to β-lactams, aminoglycosides, quinolones, and colistin. AB210 was resistant to all agents tested (including carbapenems) but susceptible to colistin (MIC, 1 μg/ml) and tigecycline. Both strains were highly resistant to vancomycin and teicoplanin (MIC, >256 μg/ml). Synergy between colistin and both glycopeptides was repeatedly observed in checkerboard assays, with FICs of <0.5 and SBPIs of >2 recorded for each combination (Table 1).

**A. baumannii-G. mellonella kill kinetics.** Both ATCC 19606 and AB210 were pathogenic to G. mellonella at >10^4 CFU/larva. Optimal killing for use in the antimicrobial treatment assays was observed using 10^5 CFU/larva of ATCC 19606 and 10^4 CFU/larva of AB210, as this led to staggered killing of >50% of the insects over the 96 h of the assay (Fig. 1).

**In vivo efficacies of antimicrobial monotherapies.** Administration of colistin and gentamicin protected G. mellonella from ATCC 19606-mediated killing, as >89% of the caterpillars were still alive at 96 h postinoculation. When teicoplanin was used to treat ATCC 19606 infections, survival rates were equivalent to those for the untreated controls (Table 1; Fig. 2A).

Both gentamicin and teicoplanin were ineffective versus the

<table>
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<td>93.75 ± 10.83</td>
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<td>97.92 ± 3.61</td>
<td>93.75 ± 10.83</td>
<td>60.42 ± 20.09</td>
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* a VAN, vancomycin; COL, colistin; TEIC, teicoplanin.

* b Calculated using a breakpoint of ≤2 μg/ml for susceptibility of Gram-positive bacteria to glycopeptides.

* c Mean from three replicate experiments ± standard deviation percentage points (pp).

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**TABLE 1. Summary of in vitro synergy testing and treatment efficacy in G. mellonella**

**FIG. 1. Kill kinetics of A. baumannii ATCC 19606 (A) and AB210 (B) in G. mellonella larvae over 96 h. Curves represent a single experiment performed using 10 insects.**
MDR isolate AB210, as would be predicted from the in vitro susceptibility data. Following administration of colistin to AB210-infected larvae, the survival rate was 47.92% (±9.53 standard deviation percentage points [pp]), compared with 89.58% (±9.58 pp) when colistin was used to treat ATCC 19606-infected animals (t test, P < 0.01) (Table 1). This relatively weak effect of colistin treatment is consistent with observations that polymyxins may not possess adequate bactericidal properties against MDR strains of *A. baumannii*. In time-kill assays, clinical isolates have been shown to readily regrow following exposure to colistin concentrations well in excess of the MIC (15), and colistin “heteroresistance” in isolates recovered from patients treated with standard doses of colistimethate sodium has been described (8). Treatment failures and unfavorable clinical outcomes have also been observed in patients administered colistin preparations for infections with strains of *A. baumannii* deemed to be susceptible (22, 14).

**In vivo efficacies of glycopeptide-colistin combination therapies.** The combination of colistin with either vancomycin or teicoplanin was highly effective in protecting larvae from ATCC 19606 or AB210 lethal infections. (Fig. 3). The combination of vancomycin with colistin appeared to be the most active combination, with survival of >90% of the animals in both cases (Table 1). The combination of teicoplanin and colistin was less active versus AB210 (66.67% [±7.22 pp] survival, compared with 95.83% [±9.55 pp] for ATCC 19606) but was still more active than colistin treatment alone (47.29 [±9.53 pp]) (t test, P < 0.05). Although in this work we have not assessed the dose of colistin required to enhance the activity of either of these glycopeptides in the *G. mellonella* model, previous in vitro studies have shown that only small amounts of colistin (0.5 µg/ml) may be required (7). Further studies to assess the pharmacokinetics of colistin-glycopeptide combinations will be important in determining optimal dosing regimens so as to minimize potential side effects.

**In vivo activity of vancomycin.** The results of experiments performed using vancomycin for the treatment of *G. mellonella* infected with *A. baumannii* were unexpected. Despite vancomycin having little in vitro activity against either ATCC 19606 or AB210 (MIC, >256 µg/ml), treatment resulted in survival of 93.75% (±10.83 pp) and 85.00% (±7.6 pp) of the larvae, respectively (Table 1 and Fig. 4). As this was not seen with teicoplanin, it suggests an intrinsic ability of vancomycin to modulate the response of *G. mellonella* to *A. baumannii* infection. The ability of glycopeptides to induce immunological reactions when administered to humans has been well documented. Vancomycin in particular has been implicated in a...
number of immune reactions, including anaphylaxis, which are reported to be far less common with teicoplanin. One such phenomenon is the so-called “red man syndrome,” which may occur in up to 90% of patients given vancomycin, depending on the rate of infusion (20). The severity can vary from a mild pruritus to an extensive rash with angioedema and is mediated by histamine release from mast cells following exposure to vancomycin (13). This is an anaphylactoid reaction independent of IgE and presumably represents an innate immune response. It is possible that in G. mellonella, vancomycin is able to “prime” the immune system in a similar way, enhancing the response to A. baumannii infection, which promotes the survival of vancomycin-treated larvae independent of any antimicrobial activity. The reason for a G. mellonella-specific immune response to vancomycin may relate to exposure to this naturally derived compound throughout its evolutionary history, a relationship which does not exist with semisynthetic glycopeptides such as teicoplanin.

In summary, we believe that these data support the notion that glycopeptide-colistin combinations may be a useful therapeutic option for the treatment of MDRAB infections. The findings in the G. mellonella model should now be assessed in mammalian models prior to clinical trials and pharmacokinetic-pharmacodynamic studies in A. baumannii-infected patients.

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