In vitro properties of antimicrobial bromotyrosine alkaloids

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A bromotyrosine alkaloid family of antimicrobial agents was synthesized using the known structure of a natural inhibitor of the mycobacterial mycothiol *S*-conjugate amidase (MCA) as a template. This series of compounds represents a novel class of anti-infective agents against Gram-positive pathogens, including mycobacteria and meticillin- and vancomycin-resistant *Staphylococcus aureus*. The fact that these compounds are active against mycobacterial strains in which the MCA gene is deleted and against Gram-positive bacteria lacking mycothiol suggests the existence of an alternative target for these compounds. One member of this family, EXEG1706, was identified as the lead compound possessing low MICs (2·5–25 μg ml^{-1}) for several clinical isolates, whilst having low toxicity for THP-1 monocytes and macrophages.

INTRODUCTION

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), and *Staphylococcus aureus* are two of several Gram-positive organisms causing significant morbidity and mortality in humans. *M. tuberculosis* is the second leading cause of death due to pathogenic agents worldwide (Dye *et al.*, 1999; World Health Organization, 2002). TB has again become a potential public health concern, partly because of its prevalence among the immunocompromised AIDS population and the homeless population, and partly because of an increase in drug-resistant *M. tuberculosis* strains worldwide (Negi *et al.*, 2003). *S. aureus* synthesizes several virulence factors, such as the capsule and protein A, that allow it to evade host immune defences and survive in hostile environments (for a review, see Lowy, 1998). Due to the large number of virulence factors, *S. aureus* is able to cause a variety of diseases, including major skin, soft tissue, respiratory, bone, joint and endovascular disorders. Drug resistance is also a major cause for concern, as the numbers of meticillin-resistant *S. aureus* (MRSA) infections in intensive care units steadily increased from 1987 to 1997 (Lowy, 1998), and MRSA resistant to intermediate concentrations of glycopeptides, including vancomycin, have been identified (Cassone *et al.*, 2004; Mallaval *et al.*, 2004). Considerable emphasis is now being placed on identifying new drug targets and on developing new antimicrobials to treat infections caused by this pathogen.

One potential drug target in mycobacteria is mycothiol (MSH) and MSH-dependent enzymes such as mycothiol *S*-conjugate amidase (MCA). MSH is the major low molecular mass thiol in *M. tuberculosis* and MCA functions in the MSH-dependent detoxification of oxidizing agents, electrophiles and several antibiotics. MSH binds to an alkylating agent (toxin), forming an *S*-conjugate. MCA then cleaves the MSH–toxin conjugate to release a mercapturic acid, which is excreted from the cell, and 1-D-*myo*-inositol-2-amino-2-deoxy-α-D-glucopyranoside, which is recycled into MSH biosynthesis (Newton *et al.*, 2000). MCA represents an important new antimicrobial target, as eukaryotes do not have MSH or MCA and instead rely on glutathione-dependent pathways for their detoxification processes. MSH and MCA homologues are commonly found in other genera within the *Actinomycetales*, including *Streptomyces*, *Nocardia* and *Corynebacterium*. Inhibition of MSH-dependent detoxification at the level of MSH–toxin conjugate binding to the MCA active site may represent a potent way of attacking mycobacterial infections.

Nicholas *et al.* (2001) screened a variety of marine extracts and identified novel alkaloid compounds with the ability to inhibit the mycobacterial MCA; two of these were novel bromotyrosine alkaloids (Nicholas *et al.*, 2001). The second alkaloid, known as compound 4, served as a template for our

Abbreviations: BCG, Bacille Calmette–Guérin; FACS, fluorescence-activated cell sorting; MCA, mycothiol *S*-conjugate amidase; MRSA, meticillin-resistant *Staphylococcus aureus*; MSH, mycothiol; MSSA, meticillin-sensitive *Staphylococcus aureus*; PI, propidium iodide; PMA, phorbol myristate acetate; TB, tuberculosis; VRSA, vancomycin-resistant *Staphylococcus aureus*; VRSH, vancomycin-resistant *Staphylococcus haemolyticus*.

design and synthesis of a series of compounds to be evaluated for antimicrobial activity (Arad & Av-Gay, 2003). An independent synthesis and evaluation of bromotyrosine alkaloids was also undertaken by Nicholas *et al.* (2002) in which the synthetic compounds were evaluated for their ability to inhibit MCA activity, but were not tested against other organisms. As none of the above compounds was tested for its biological activity against microbial pathogens, we tested them against a variety of micro-organisms. Here, we describe the synthesis and evaluation of these novel bromotyrosine alkaloids, some of which demonstrated high antimicrobial activity against a number of Gram-positive organisms. We further characterized EXEG1706, a promising compound showing high activity against *S.* spp. and *Mycobacteria* spp. and low toxicity to mammalian cells.

METHODS

Bacterial strains. The strains used in this study are shown in Table 1. Organisms were maintained in stock culture at −70 °C and freshly streaked for use before each assay. All organisms were grown in brain heart infusion (BHI) medium [solid medium contained 1·5 % (w/v) agar; Becton Dickinson] with the exception of *Escherichia coli* (Luria–Bertani broth), *Mycobacterium* spp. (Middlebrook 7H9 liquid medium and Middlebrook 7H10 solid medium; Difco Laboratories) and *Saccharomyces cerevisiae* (yeast nitrogen base without amino acids or ammonium sulphate; Becton Dickinson). *Streptococcus pneumoniae* strains were grown first on sheep's blood agar (PML Microbiologicals) before transfer to BHI.

Synthesis of bromotyrosine compounds. A series of novel bromotyrosine alkaloids was synthesized (Arad & Av-Gay, 2003) based on the structure of an alkaloid produced by an Australian nonverongid sponge, *Oceanapia* sp. (Nicholas *et al.*, 2001). The structures of the five compounds that had the best antimicrobial activity are shown in Fig. 1. Synthesis of three of these compounds has been described previously (Kende *et al.*, 2004; Nicholas *et al.*, 2002).

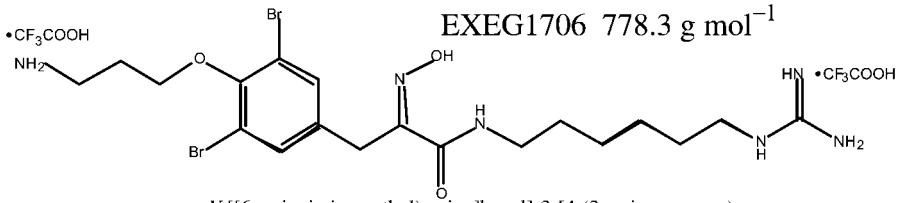
Fig. 2 summarizes the synthesis of EXEG3022, with a starting substrate of 4-hydroxyphenylpyruvic acid, which resulted in EXEG3022 in four synthetic operations. Pyruvic acid was reacted with *O*-(tetrahydro-2H-pyran-2-yl)hydroxylamine in methanol to give a crude *O*-(tetrahydro-2H-pyran-2-yl)oxime. This was directly coupled in dichloromethane with tert-butyl-*N*-(6-aminohexyl)guanidinyl bicarbamate in the presence of 1-hydroxybenzotriazole and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride to produce an 80 % yield of the guanidine amide derivative, labelled compound 2 (Wagner *et al.*, 1998). This intermediate was dissolved in dry tetrahydrofuran and stirred with *N*-bromosuccinimide to give a 76 % yield of the dibromophenol, labelled compound 3. Mitsunobu reaction of the phenol group of compound 3 with tert-butyl-*N*-(4-hydroxybutyl)guanidinyl bicarbamate, diethyl azodicarboxylate and triphenylphosphine in tetrahydrofuran produced the protected bisguanidine at a 37 % yield after recrystallization (compound 4). Finally, all five protecting groups were cleaved using 30 % trifluoroacetic acid in dichloromethane to give the bis(trifluoroacetate) salt of EXEG3022 at an ∼70 % yield. EXEG1707, which has a similar structure, was prepared in a parallel manner.

Drug dilution. For use in toxicity assays, unless otherwise specified, compounds were prepared as a 10 mg ml^{-1} solution in methanol and diluted in RPMI to 1 mg ml^{-1}. Further serial dilutions in RPMI containing 5 % fetal calf serum and 1 % glutamine (modified RPMI) gave the appropriate concentrations for individual assays.

Table 1. List of organisms used in this study

Organism	Strain*	Source or reference*
Bacillus subtilis	W168	ATCC
Enterococcus faecalis	ATCC 376	ATCC
Enterococcus faecium	1 CDC 2	CDC
Escherichia coli	JM109	Yanisch-Perron *et al.* (1985)
Mycobacterium smegmatis	MC^2-155	W. R. Jacobs, AECOM
Mycobacterium bovis BCG	ATCC 35734	ATCC
Saccharomyces cerevisiae	SD4191	ATCC
Staphylococcus aureus	ATCC 27217	ATCC
VRSA	VGH clinical isolate C4/11	This study
MRSA	VGH clinical isolate B-106BB8	This study
MSSA	VGH clinical isolate B-107BB8	This study
MRSA, quinolone resistant	VGH clinical isolate B-96BC10	This study
MRSA, quinolone sensitive	VGH clinical isolate A-105DD3	This study
MRSA, heteroresistant	VGH clinical isolate A-105DD2	This study
Staphylococcus epidermidis	Laboratory strain	ATCC
VRSH	VGH clinical isolate 1BA2 1994	This study
Staphylococcus saprophyticus		This study
Streptococcus pneumoniae	ATCC 49619	ATCC
Streptococcus pneumoniae	FC579	D. P. Speert, UBC

*AECOM, Albert Einstein College of Medicine, New York, USA; ATCC, American Type Culture Collection; CDC, Centers for Disease Control and Prevention; VGH, Vancouver General Hospital, 899 West 12 Ave, Vancouver, BC V5Z 1M9, Canada; UBC, University of British Columbia.



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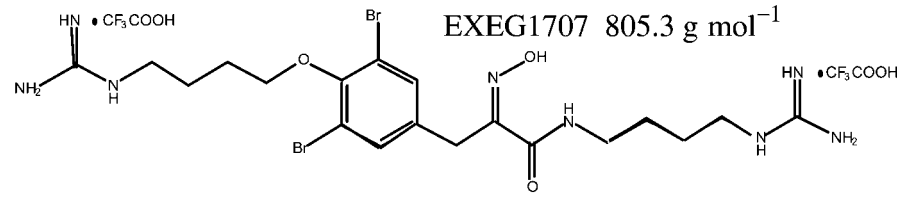
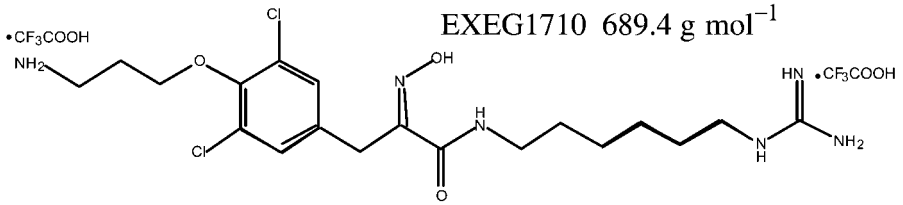
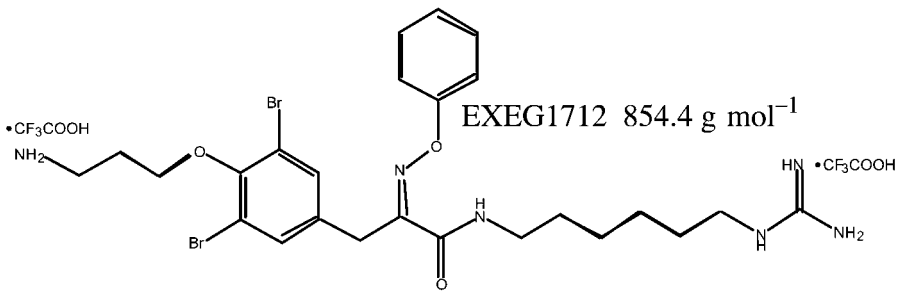


N-[[4-aminoiminomethyl)amino]butyl]-3-[4-[[4-(aminoiminomethyl)amino]butoxy]-3,5-dibromophenyl]-2-hydroxyiminopropionamide, bis(trifluoroacetic acid) salt



N-[[6-aminoiminomethyl)amino]hexyl]-3-[4-(3-aminopropoxy)-3,5-dichlorophenyl]-2-hydroxyiminopropionamide, bis(trifluoroacetic acid) salt



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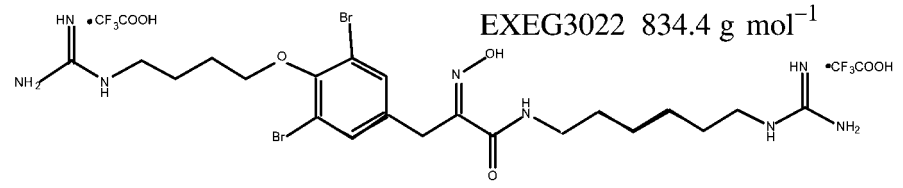

N-[[6-aminoiminomethyl)amino]hexyl]-3-[4-[[4-(aminoiminomethyl)amino]butoxy]-3,5-dibromophenyl]-2-hydroxyiminopropionamide, bis(trifluoroacetic acid) salt

Fig. 1. Chemical structures and molecular masses of synthetic bromotyrosine alkaloids. Bromotyrosine alkaloids were synthesized as described in Methods. The 5 compounds shown here were the most active out of 30 compounds tested. All molecular masses were calculated for the trifluoroacetic acid salts.

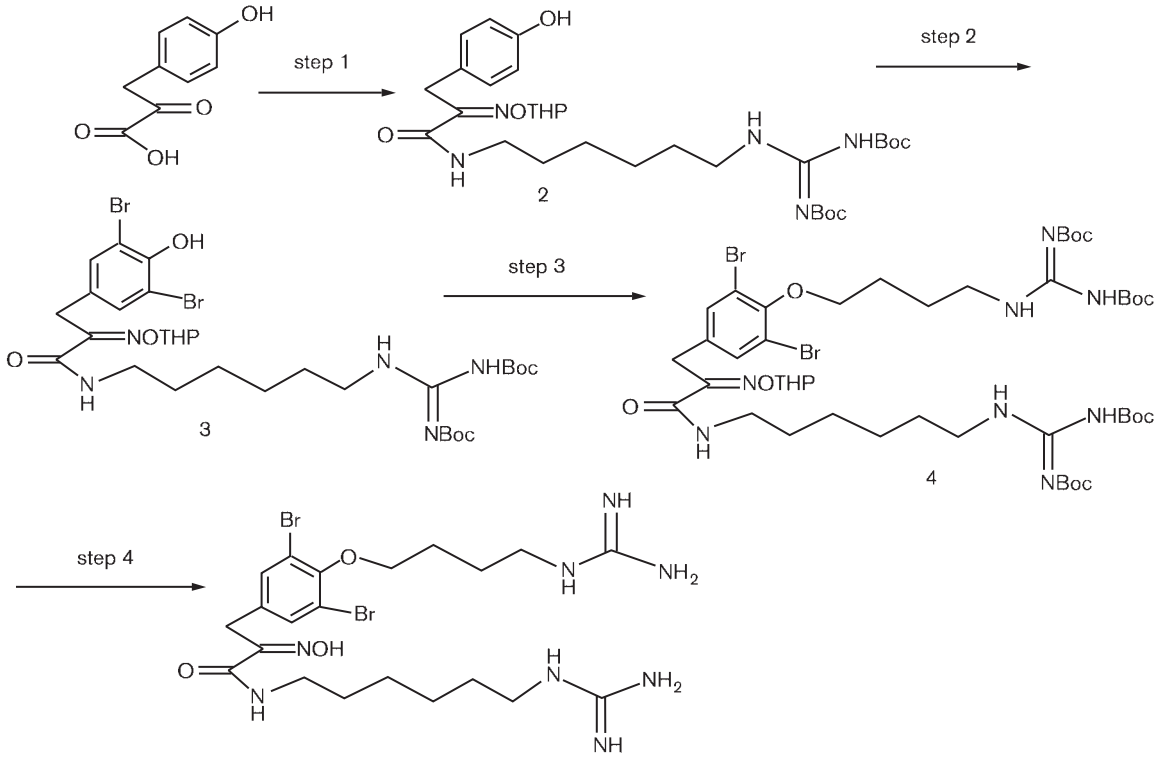

Fig. 2. Description of EXEG3022 synthesis from 4-hydroxyphenylpyruvic acid in four operations; see Methods for details. EXEG1707 was prepared in a parallel manner.

Disk diffusion assay. Test compounds were dissolved at 10 μg ml^{-1} in HPLC-grade methanol (Fisher Scientific). Sterile 59 mm filter paper disks (Difco Laboratories) were impregnated with 5–10 μl of a compound in solution and allowed to air dry. Test organisms were grown to mid-exponential phase and streaked evenly on to the surface of solid agar using a sterile swab. Disks containing test compounds or solvent alone were applied to the dry agar surface (maximum of six disks per 110 mm plate) and the plates were incubated at 37 °C until sufficient bacterial growth had occurred to observe growth inhibition zones, if present (up to 3 days).

For *Mycobacterium smegmatis*, disk assays were performed on 7H10 medium/1 % glucose plates. Medium for the *mshB* mutant strain was supplemented with 25 μg kanamycin ml^{-1} (Rawat *et al.*, 2003). Liquid medium for the control strain, $Mc^{2}155$-gent, with a gentamicin-resistance gene, and the *mca* mutant strain was supplemented with 10 μg gentamicin ml^{-1}, whilst solid medium was supplemented with 15 μg gentamicin ml^{-1} (Rawat *et al.*, 2004).

Tube and microdilution assays. Tube and microdilution assays were performed as described in the NCCLS guidelines (National Committee for Clinical Laboratory Standards, 1993).

Drug-toxicity assays. Drug toxicity was assessed by trypan blue exclusion staining of THP-1 monocytes and by fluorescence-activated cell sorting (FACS) analysis of propidium iodide (PI)-stained THP-1 cells exposed to increasing concentrations of the test drug. Growth of THP-1 cells, PI staining and FACS analysis were carried out as described previously (Pick *et al.*, 2004).

THP-1 monocytes were grown as described previously (Pick *et al.*, 2004). Different concentrations of drug were added to the medium and cells were exposed for 24 h at 37 °C. Cells were stained by adding 0·4 % trypan blue and washed twice with PBS (pH 7·4). Methanol was added to solubilize the stain and the absorbance of the methanol solution was measured at 605 nm.

Mammalian cell-toxicity assays. Phorbol myristate acetate (PMA)-differentiated THP-1 macrophages were grown in modified RPMI in 24-well tissue culture plates to approximately 3×10^{5} cells per well. Serial dilutions of EXEG1706 were added to test wells, whilst positive-control wells received 5 μg cycloheximide and negative-control wells received 1 % methanol. Plates were incubated for 24 h at 37 °C in the presence of 5 % CO_2. After incubation, the medium was removed from the wells and 150 μl 0·4 % trypan blue in PBS was added to stain the remaining macrophages. Viability was determined by counting dead cells (cells retaining the stain) and live cells (unstained cells, which actively pump the stain out of the cell). The trypan blue was then removed, the wells were washed twice with PBS and 300 μl methanol was added to each well. Aliquots (200 μl) were transferred to an ELISA plate and the absorbance was measured at 605 nm.

RESULTS

Synthesis and antimicrobial activity of bromotyrosine alkaloids

Thirty bromotyrosine alkaloid compounds were synthesized and evaluated for their ability to inhibit the growth of a number of micro-organisms (Arad & Av-Gay, 2003). Five compounds (Fig. 1) were found to have good activity against the organisms tested, and two, EXEG1706 and EXEG

Table 2. Diameters (mm) of disk assay growth inhibition zones for various organisms after treatment with synthetic bromotyrosine alkaloids

Each disk contained 100 μg test compound. ND, Not determined.

Compound	Growth inhibition zone (mm)									
	Saccharomyces cerevisiae	*B. subtilis*	*E. coli*	*S. aureus*	MRSA	MSSA	VRSA	*S. epidermidis*	*M. smegmatis*	BCG
EXEG1706	0	15	0	24	20	24	11	10	11	24
EXEG1707	0	20	7	19	17	19	9	9	10	16
EXEG1710	0	15	6	24	18	20	19	22	10	ND
EXEG1712	0	17	9	7	15	7	12	11	12	17
EXEG3022	15	ND	10	ND	18	16	ND	ND	16	ND

1710, were able to inhibit mycobacterial MCA *in vitro* (data not shown), confirming the results obtained by Nicholas *et al.* (2001).

Table 2 shows the results of disk sensitivity assays for these five compounds against several Gram-positive organisms, a Gram-negative organism and yeast. The best activity was observed against Gram-positive organisms, *Staphylococci* in particular. The compounds were generally less effective against Gram-negative organisms, including *E. coli* (Table 2), *Enterococcus faecalis* and *Enterococcus faecium* (see Table 4). One exception was EXEG3022, which had moderate activity against *E. coli* and was the only compound to have activity against the yeast, *Saccharomyces cerevisiae*. Of these five compounds of interest, EXEG1706 and EXEG1710 demonstrated the most potent activity against MRSA, meticillin-sensitive *S. aureus* (MSSA) and vancomycin-resistant *S. aureus* (VRSA). EXEG1706 and EXEG1710 also had moderate activity against *M. smegmatis* and EXEG1706 was very effective against the mycobacterial vaccine strain, *Mycobacterium bovis* Bacille Calmette–Guérin (BCG; Table 2) and a clinical *Streptococcus pneumoniae* isolate (not shown in Table 2, 22 mm diameter zone of inhibition for 100 μg compound applied to disk). Interestingly, EXEG1710 is a chlorotyrosine derivative with an otherwise identical chemical structure to EXEG1706.

The five bromotyrosine and chlorotyrosine alkaloids were tested further against MSSA, MRSA and VRSA. The MIC for each of the five alkaloids was obtained from a tube dilution assay carried out according to NCCLS guidelines (National Committee for Clinical Laboratory Standards, 1993). Table 3 shows that, while all compounds demonstrated effectiveness against these *S. aureus* strains, the compound with the lowest MICs was EXEG1706. Since EXEG1706 was the most promising antimicrobial from the group of novel bromotyrosine alkaloids tested, further studies were carried out with this compound.

Table 3. Growth inhibition by novel bromotyrosine antimicrobial compounds

Compound	MIC (μg ml^{-1})		
	MSSA	MRSA	VRSA
EXEG1706	2·5–25	2·5–25	2·5–25
EXEG1707	125	125	125
EXEG1710	100	100	100
EXEG1712	40	40	80
EXEG3022	50	50	70

Growth inhibition of clinical pathogens by EXEG1706

EXEG1706 antimicrobial activity was tested against an expanded panel of micro-organisms. Table 4 shows the MICs for EXEG1706 against a number of clinically important pathogens and *M. smegmatis*, which was grown in Middlebrook 7H9 medium rather than in BHI (see Methods). EXEG1706 MICs were between 2·5 and 25 μg ml^{-1} for most organisms tested. As observed previously, this compound was not effective against the Gram-negative bacteria tested nor *Enterococcus faecalis* and *Enterococcus faecium*. It was

Table 4. Growth inhibition of expanded strain panel by EXEG1706

Micro-organism	MIC (μg ml^{-1})
Bacillus subtilis	Resistant
Enterococcus faecalis	Resistant
Enterococcus faecium	Resistant
Mycobacterium smegmatis MC^{2}155	1·5–15·5
VRSA	2·5–25
MSSA	8–16
MRSA, quinolone resistant	4–16
MRSA, quinolone susceptible	4–8
MRSA, heteroresistant	8
Staphylococcus epidermidis	2·5–25
VRSH	2·5–25
Streptococcus pneumoniae ATCC 49619	Resistant
Streptococcus pneumoniae FC 579	2·5–25
Staphylococcus saprophyticus	2

also ineffective against one strain of *Streptococcus pneumoniae* tested. Low MICs were observed for pathogenic *S. aureus* and for *M. smegmatis* (MIC of between 1·5 and 15·5 μg ml^{-1} in Middlebrook 7H9 medium), encouraging further characterization of EXEG1706 antimicrobial activity.

Sensitivity of staphylococci to EXEG1706

Since most of the *Staphylococcus* spp. tested were quite sensitive to EXEG1706, the growth of three staphylococci was tested over a range of drug concentrations. Bacterial growth was monitored during a standard tube dilution assay by measuring the optical density of the culture at 605 nm. Fig. 3 represents the growth of VRSA, *Staphylococcus epidermidis* and vancomycin-resistant *Staphylococcus haemolyticus* (VRSH) in the presence of EXEG1706 after 24 h at 37 °C. The growth of all three organisms was completely inhibited at 25 μg EXEG1706 ml^{-1}. For *S. epidermidis*, growth inhibition occurred down to a concentration of 0·25 μg EXEG1706 ml^{-1}. For VRSH and VRSA, growth inhibition occurred down to the drug limits tested, i.e. 0·025 and 0·0025 μg ml^{-1}, respectively. This data indicated that VRSH and VRSA were the most sensitive to EXEG1706, as their growth was affected by very low concentrations of the drug.

EXEG1706 activity against mycobacterial mutants in MSH-related genes

Since the bromotyrosine compounds were based on compounds that inhibited MCA activity, we tested the activity of EXEG1706 on *M. smegmatis* strains in which we had inactivated the *mca* gene (Rawat *et al.*, 2004) or interfered with MSH biosynthesis by inactivating the *mca* homologue, *mshB* (Rawat *et al.*, 2003). In disk assays, EXEG1706 demonstrated significant activity against the wild-type strain at 50 μg ml^{-1}, and higher activity against *mca* or *mshB* mutants, as seen in Table 5.

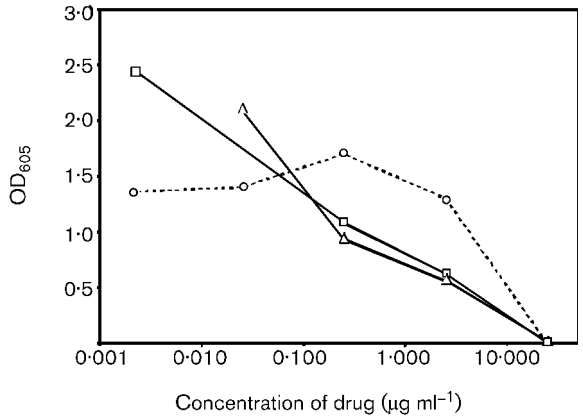

Fig. 3. Growth inhibition of staphylococci by EXEG1706. VRSA (□), VRSH (△) and *S. epidermidis* (○), were grown for 24 h in the presence of decreasing concentrations of EXEG1706. Bacterial growth was measured as OD_{605}.

EXEG1706 toxicity to macrophages and monocytes

EXEG1706 toxicity to mammalian cells was determined by exposing adherent THP-1 human macrophages to 12·5 μg EXEG1706 ml^{-1}. Compared with the solvent control, no decrease in adherent THP-1 cells was observed in the presence of EXEG1706 (data not shown), indicating that the drug was not toxic to the macrophages under these conditions.

This result was confirmed by the mammalian cell-toxicity assay, as described in Methods. Fig. 4(a) shows that below 100 μg EXEG1706 ml^{-1}, no significant macrophage toxicity was observed. Above 100 μg ml^{-1}, A_{605} increased with increasing drug concentration, indicating an increase in the number of dead macrophages stained with trypan blue. The insolubility of EXEG1706 in RPMI prevented testing of drug concentrations above 1000 μg ml^{-1}. Under the conditions used, the LD_{50} of EXEG1706 was calculated to be 400 μg ml^{-1}. EXEG1706 was more toxic to undifferentiated THP-1 monocytes when tested in the same manner, yielding an LD_{50} of 100 μg ml^{-1} (data not shown).

To determine the time required for EXEG1706 at its LD_{50} to have maximum toxic effect on PMA-differentiated THP-1 macrophages, the previous toxicity assay was carried out in the presence of 400 μg EXEG1706 ml^{-1}. Macrophage viability was assessed at 1, 2, 3, 4 and 24 h. As shown in Fig. 4(b), the maximum toxic effect of EXEG1706 at its LD_{50} occurred after 4 h of incubation.

EXEG1706 and the other novel bromotyrosine compounds were also tested for toxicity to THP-1 monocytes using a novel flow cytometry assay (Pick *et al.*, 2004). Undifferentiated THP-1 monocytes were exposed to different concentrations of EXEG1706, EXEG1710, EXEG1712 or EXEG3022 at 37 °C for 24 h. The percentage of dead cells was monitored by PI staining and analysed by FACS, and the LD_{50} was calculated. EXEG1710 was least toxic with an LD_{50} of 300 μg ml^{-1}. EXEG1706 was the next least toxic with an LD_{50} of 100 μg ml^{-1}. Both EXEG1712 and EXEG3022 were more toxic to monocytes, with LD_{50} values of 40 and 70 μg ml^{-1}, respectively. These two compounds had LD_{50}

Table 5. Diameters (mm) of disk assay growth inhibition zones for *M. smegmatis* parental wild-type strain, and *mshB* and *mca* mutant strains after treatment with EXEG1706

Data are presented as mean±range (mm) of duplicate samples of exponential cells cultured in Middlebrook 7H9 medium. NA, Not available.

Inhibitor (μg)	Mc^{2}155	*mshB*	Mc^{2}155-gent	*mca*
10·0	5·9±0·0	7·3±1·2	21·0±1·0	28·0±1·5
50·0	15·0±0·0	20·0±0·0	31·5±0·5	37·3±1·8
100·0	18·5±0·5	26·5±0·5	NA	NA

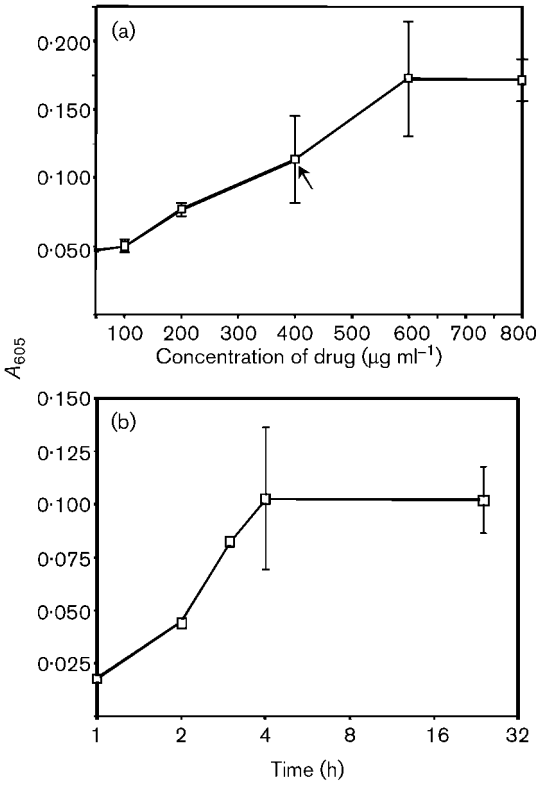

Fig. 4. EXEG1706 toxicity for differentiated THP-1 macrophages. (a) THP-1 macrophages were exposed to increasing concentrations of EXEG1706 for 24 h, stained with trypan blue and washed. The arrow denotes the LD_{50} value. (b) Time taken to achieve maximum toxicity for EXEG1706 at the LD_{50}. THP-1 macrophages were exposed to EXEG1706 at its LD_{50} (400 μg ml^{-1}). Samples were taken at various intervals, stained with trypan blue and washed. The number of dead (stained) cells was measured by determining A_{605}. Results are shown as the mean ± SEM for triplicate measurements.

values very close to their MIC values (Table 3), whereas EXEG1706 and EXEG1710 had MIC values considerably below their LD_{50} values. As EXEG1706 had the lowest MIC values, it was considered the most promising antimicrobial drug from this group.

DISCUSSION

Bacterial resistance to antibiotics is a serious public health concern because of nosocomial pathogens and organisms infecting those with immune systems weakened by disease or genetic disposition. *M. tuberculosis* causes significant disease among the AIDS and homeless populations. The worldwide incidence of TB is 8 million people a year, its prevalence is 1·86 billion people and the case fatality rate is 23 % (Dye *et al.*, 1999). Because of side effects and the length of time required for current TB treatment, patient non-compliance is common and contributes to the spread of multi-drug-resistant TB. Similarly, drug-resistant *S. aureus* strains are a major health concern. *S. aureus* is a problematic nosocomial pathogen and this is complicated further by the fact that humans are a natural reservoir for this organism (Lowy, 1998). Both MSSA and MRSA are persistent colonizers that can be carried asymptomatically, increasing the risk of subsequent infection for those colonized (Casewell & Hill, 1986; Sanford *et al.*, 1994; Wenzel & Perl, 1995). As with *M. tuberculosis*, individuals colonized with *S. aureus* often have immune deficiencies or other complicating health factors such as type 1 diabetes, intravenous drug usage, surgical interventions, haemodialysis and AIDS (Kluytmans *et al.*, 1995; Tuazon & Sheagren, 1974; Tuazon *et al.*, 1975; Weinke *et al.*, 1992; Yu *et al.*, 1986). Moreover, the emergence of VRSA is a near reality, as *S. aureus* isolates with intermediate sensitivity to vancomycin (Bartley, 2002; Mallaval *et al.*, 2004) and vancomycin-resistant coagulase-negative staphylococci have already been reported (Schwalbe *et al.*, 1987).

For the reasons outlined above, it has become even more important to identify new bacterial drug targets and develop new antimicrobial agents. A bromotyrosine alkaloid found in an extract from a non-verongid sponge inhibited the activity of mycobacterial MCA (Nicholas *et al.*, 2001). A series of synthetic bromotyrosine alkaloids was synthesized, modelled on the compound from the sponge extract, and their antimicrobial activities were evaluated (Arad & Av-Gay, 2003; Kende *et al.*, 2004). Five compounds from this series (Table 1) had good activity against a range of Gram-positive organisms, as demonstrated in disk diffusion assays (Table 2). None of the compounds was particularly effective against the Gram-negative organisms tested, suggesting either that their molecular target is absent in Gram-negative bacteria or that the compounds lack the ability to cross the Gram-negative cell wall.

M. smegmatis mutants defective in MSH biosynthesis have increased sensitivity to xenobiotics and alkylating agents (Rawat *et al.*, 2002). In our studies, a mutant disrupted in *mshB*, a gene paralogous to *mca* and involved in MSH biosynthesis, demonstrated increased sensitivity to EXEG1706 compared with the control, suggesting that MSH and MCA are involved in the protection against the bromotyrosine compounds. Furthermore, a mutant disrupted in *mca* also showed increased sensitivity. This suggests that the bromotyrosine compounds act on another, as yet unidentified, cellular target in mycobacteria and are not specific inhibitors of MCA. Since staphylococci do not produce MSH (Newton *et al.*, 1996) and there is no complete *S. aureus* MCA homologue, it is possible that another enzyme or set of enzymes serves as the target for these broad-range antimicrobial agents.

We took a bioinformatics approach to identify possible drug targets for the bromotyrosine compounds in Gram-positive bacteria. The identified *in vitro* target (MCA) and the MSH biosynthesis enzyme MshB have identical active sites situated around a zinc ion (Maynes *et al.*, 2003). Scrutiny of the MshB crystal structure has revealed a large family of related zinc hydrolases with a novel fold existing

among the Gram-positive bacteria (Maynes *et al.*, 2003; McCarthy *et al.*, 2003). A BLAST search using *M. tuberculosis* MCA revealed several homologues within staphylococci containing two active site motifs, HPDDE and HPDH (Maynes *et al.*, 2003), essential for the amide hydrolase reaction (data not shown). Since the synthetic bromotyrosine compound EXEG1706 is centred on an oxime moiety (hydroxamate) that binds metals, it is likely to be able to inhibit zinc metalloenzymes in other Gram-positive bacteria structurally related to *M. tuberculosis* MCA.

Interestingly, EXEG1706 and EXEG1710 gave rise to some of the largest zones of inhibition for the staphylococci and mycobacteria tested. These two compounds have an identical structure except for the presence of two bromines (EXEG1706) or chlorines (EXEG1710) substituted on the tyrosine moiety of each molecule. This suggests that this alkaloid structure is exceptionally active against these organisms. When MICs were calculated for each compound against MSSA, MRSA and VRSA, EXEG1706, EXEG1712 and EXEG3022 had the lowest MICs (Table 3). EXEG1706 demonstrated the lowest MICs of all, 2·5 μg ml^{-1} for all three organisms, making it the most potent of the five compounds, and thus became the lead antimicrobial compound and was characterized further for range of effectiveness and mammalian-cell toxicity.

EXEG1706 MICs were determined by standard tube assay (National Committee for Clinical Laboratory Standards, 1993). The results in Table 4 confirmed that EXEG1706 did not appear to be effective against Gram-negative organisms. It was also ineffective against *Bacillus subtilis* and one strain of *Streptococcus pneumoniae* (ATCC 49619); however, against all other organisms tested, EXEG1706 had a MIC of less than 25 μg ml^{-1}. This group included *M. smegmatis*, MSSA, quinolone-susceptible and -resistant MRSA, heteroresistant MRSA, VRSA and VRSH. In addition to the effectiveness of EXEG1706 against mycobacteria, its effectiveness against multi-drug-resistant staphylococci is of great interest. The low MIC of EXEG1706 against these organisms means that the dose of drug used in humans potentially could be kept low, minimizing mammalian-cell toxicity complications. To test the ability of EXEG1706 to inhibit growth, VRSA, VRSH and *S. epidermidis* were grown in the presence of decreasing concentrations of this bromotyrosine alkaloid. Only 25 μg EXEG1706 ml^{-1} completely inhibited growth of all three organisms (Fig. 3). The growth of *S. epidermidis* was inhibited down to a concentration of 0·25 μg EXEG1706 ml^{-1}, whereas the growth of VRSA and VRSH was inhibited down to 0·0025 and 0·025 μg ml^{-1}, respectively. This indicated that VRSA and VRSH were the most sensitive to this compound.

The sensitivity of mammalian cells to EXEG1706 was determined by exposing PMA-differentiated THP-1 macrophages to increasing concentrations of the compound (Fig. 4a). Toxicity was observed only above 100 μg EXEG1706 ml^{-1} and the LD_{50} was calculated to be 400 μg ml^{-1}. This LD_{50} value is at least 16-fold greater than effective concentrations of EXEG1706, as demonstrated in growth-inhibition assays with VRSA, VRSH and *S. epidermidis* and compared with the calculated MICs for staphylococci and mycobacteria from standard tube dilution assays. In addition, adherent THP-1 macrophages were not affected by 12·5 μg EXEG1706 ml^{-1} (data not shown). After 4 h in the presence of EXEG1706 at its LD_{50}, THP-1 macrophages exhibited maximum toxicity to this drug (Fig. 4b). Flow cytometry data demonstrating the toxicity of EXEG1706, EXEG1710, EXEG1712 and EXEG3022 on undifferentiated THP-1 monocytes has been reported previously (Pick *et al.*, 2004). With the exception of EXEG1710, EXEG1706 was the least toxic compound. For unknown reasons, EXEG1706 was also approximately four times more toxic to THP-1 monocytes than to macrophages, with an LD_{50} of 100 μg ml^{-1} for THP-1 monocytes. In the process of differentiating into macrophages, monocytes undergo changes in cell-surface marker expression, and macrophages have elevated endocytic and phagocytic capacity (Basta *et al.*, 1999; Coccia *et al.*, 1999; Gessani *et al.*, 1993). These changes may contribute to cell-specific differences in EXEG1706 sensitivity due to drug uptake, drug stability in the cells or availability of a toxicity target. That the EXEG1706 MICs are considerably below its LD_{50} indicates that this compound potentially could be used against antibiotic-resistant staphylococci and mycobacteria.

In this study, we have reported the synthesis and characterization of a series of bromotyrosine alkaloids based on a naturally occurring alkaloid. This novel class of antimicrobials shows promise against Gram-positive organisms, including mycobacteria and staphylococci, including MRSA, VRSA and VRSH. EXEG1706 emerged as the lead compound due to its low MICs against almost all Gram-positive organisms tested and its low toxicity for THP-1 cells. The lack of options available to treat infections caused by antibiotic-resistant, Gram-positive organisms makes it important to identify new classes of antimicrobials. EXEG1706 was crystallized in its trifluoroacetate salt and obviously a less dangerous salt form of this family of compounds needs to be designed. Nevertheless, the bromotyrosine alkaloid class, as demonstrated by the lead compound EXEG1706, was identified *in vitro* as an antimicrobial that provides new options for the treatment of multi-drug-resistant, Gram-positive infections. Animal studies are the next step in the evaluation of this family of compounds.

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