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The Antibacterial Drug MGB-BP3: from discovery to clinical trial

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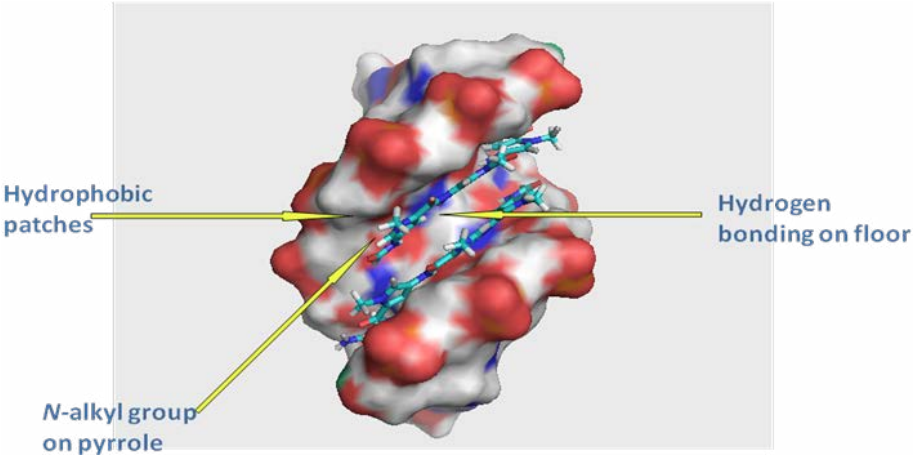
Introduction

It goes without saying now that there is a severe risk to health world wide because of the continued emergence of resistance of bacteria to many of the currently available antibacterial drugs. About 12 years ago at Strathclyde we began a project to see whether it would be possible to transform the oligoamide natural products, distamycin (**1**) and netropsin (**2**), into useful antibiotics by modifying their structures so that toxicity and unwanted biological activity was removed and selective, high antibiotic activity obtained. These natural products were well known to bind to the minor groove of DNA and details of the configuration of binding were known from X-ray crystallography [1,2]. A firm basis therefore existed for the design of new minor groove binding ligands. The research plan was to introduce additional hydrophobic components into the ligands so that binding to the non-polar regions of the minor groove could be obtained and so that the physicochemical properties of the new compounds be made more drug-like than those of distamycin and netropsin. These were new concepts at the time (Figure 1). However it was known that the sequence selectivity of binding of oligoamide natural products was tunable by including *N*-methylimidazole at positions where binding adjacent to a GC base pair was required because substitution of the pyrrole CH by N removed a steric clash between ligand and minor groove [3]. The ability of large synthetic ligands to bind with high selectivity to extended sequences of DNA has been exploited outstandingly by Dervan and his colleagues [4]. The Strathclyde approach in contrast was to concentrate on smaller compounds of up to 700 D and to vary structure by changes in head group, alkyl substituent, and tail group (Figure 2).



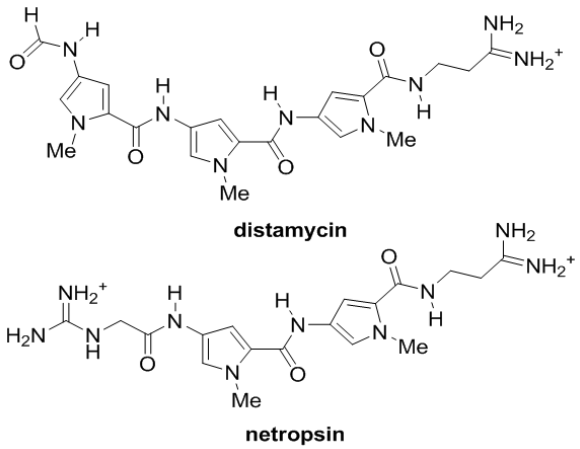


Figure 1

Getting to grips with drug-like minor groove binders

In the first phase of the work, studies were aimed at learning how different structural modifications would influence binding to DNA and biological activity. For the former, capillary electrophoresis and footprinting were primary techniques and for the latter, routine assays were run against a short panel of Gram-positive and Gram-negative bacteria and fungi. This led to a number of design principles that could be used to invent compounds with potentially useful properties as follows [5]:

- *N*- and *C*-alkyl substituents up to C5, including branched chains, were acceptable for DNA binding and led to antibacterial and antifungal activity provided that they were not attached to adjacent rings of the oligoamides;
- Bulky head groups such as *t*-butyl prevented binding and gave biologically inactive compounds;
- 1,5-dimethyl substitution in pyrrole oligomers prevented binding and gave biologically inactive compounds;
- Substantial variation was possible in the structure of the amino tail group;
- Whereas the inclusion of imidazoles (polar) led to toxic ligands, GC reading could be established using thiazoles (non-polar) in accordance with the initial design plan.

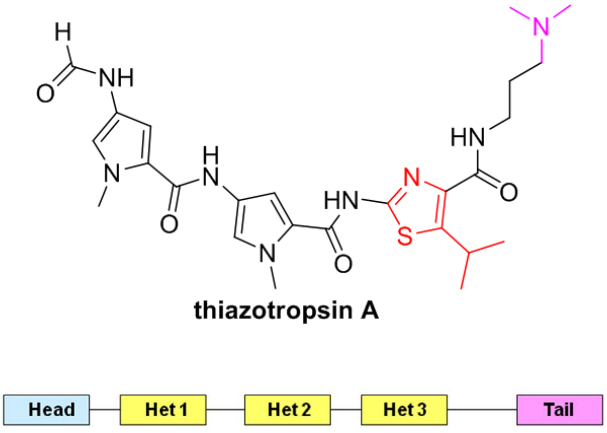


Figure 2

The most detailed work was carried out with a compound known as thiazotropsin A (**3**, Figure 2), which contained a *C*-isopropylthiazole and a dimethylaminoethyl tail group as modifications of the distamycin structure. Thiazotropsin A did show significant antibacterial activity (IC_{50} ~ 5μM against a range of Gram-positive bacteria) but its main value was as a pilot compound that taught how such small ligands with potentially useful biological activity bind to DNA. Professor Keith Fox (University of Southampton) used footprinting techniques to show that thiazotropsin A had a strongly preferred binding site (ACTAGT) [5] and this sequence was incorporated into decamers and dodecamers for further investigations of DNA binding [6]. The dissociation constant for binding to an oligonucleotide containing ACTAGT was determined by isothermal calorimetry as 50 nM and it was further shown that this affinity was 100-fold greater than to an oligonucleotide containing ATATAT. NMR studies using both chemical shift changes on ligand binding and NOE experiments provided clear evidence for the structure of the complex [7]: thiazotropsin A bound in a ratio of 2 molecules of ligand to the minor groove in an antiparallel configuration such that the thiazole ring was adjacent to G and a specific hydrogen bond was formed between the thiazole N and the guanosine NH_2. In order to accommodate two molecules of ligand, the minor groove broadened significantly; such a change would be expected to influence the conformation of DNA not just locally but allosterically at sites not directly interacting with the ligand. Interestingly, the coverage of thiazotropsin A of 6 base pairs is a similar length to sequences identified as sigma factor promoter binding in bacterial gene expression (see below).

Discovery leading to MGB-BP3

With this knowledge available, further modifications were introduced at the head group consistent with the design model (Figure

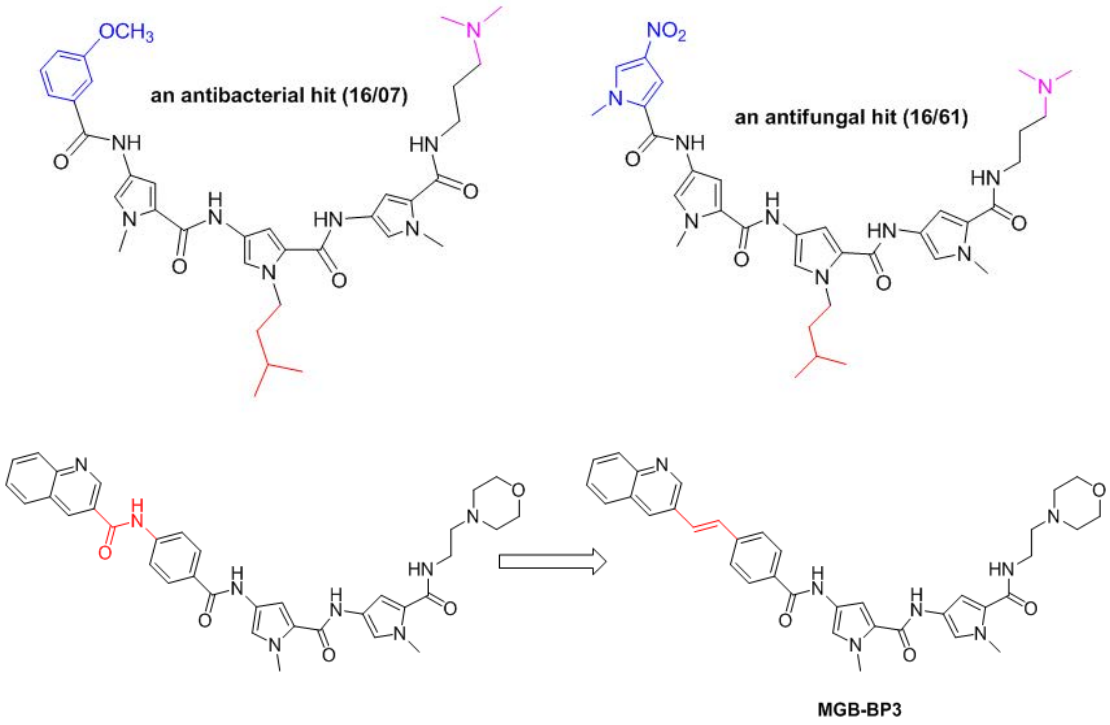


Figure 3 Early hits introducing major modifications to the distamycin structure and the structural change (isosteric replacement of amide by alkene) that led to the discovery of MGB-BP3.

1) and to create more sites for medicinal chemical modification. Principal amongst these was the use of aromatic head groups. Including such substructures, significant hits were found for both antibacterial and antifungal activity (Figure 3). At this stage it was decided to concentrate upon antibacterial activity and what turned out to be the key structural modification was introduced. In order to increase further the non-polar interactions, it would be worth investigating whether an alkene, the non-polar isostere of an amide, could be used as a link between the component rings of the minor groove binder. This change led to 'AIK 19/56/2', first prepared by Dr Abedawn Khalaf in 2006, and now known as MGB-BP3 (Figure 3) [8].

In making this change it was clear that a potential hydrogen bonding site would be lost but there could be compensation from non-polar interactions. Moreover, the alkene link would not be susceptible to hydrolysis and importantly for subsequent biological studies, a fluorophore was introduced. Confirmation of strong binding to DNA for the alkene class of compound was obtained by melting temperature measurements which showed no loss of affinity to the dodecamer GCGATATATGCG/CGCTATATACGC. Many compounds with alkene links to the head group showed substantial antibacterial activity with very little evidence for mammalian cell toxicity but MGB-BP3 was significantly the most active having an MIC less than 1 μg/ml against many Gram-positive bacteria.

Gram-negative activity, however, was not

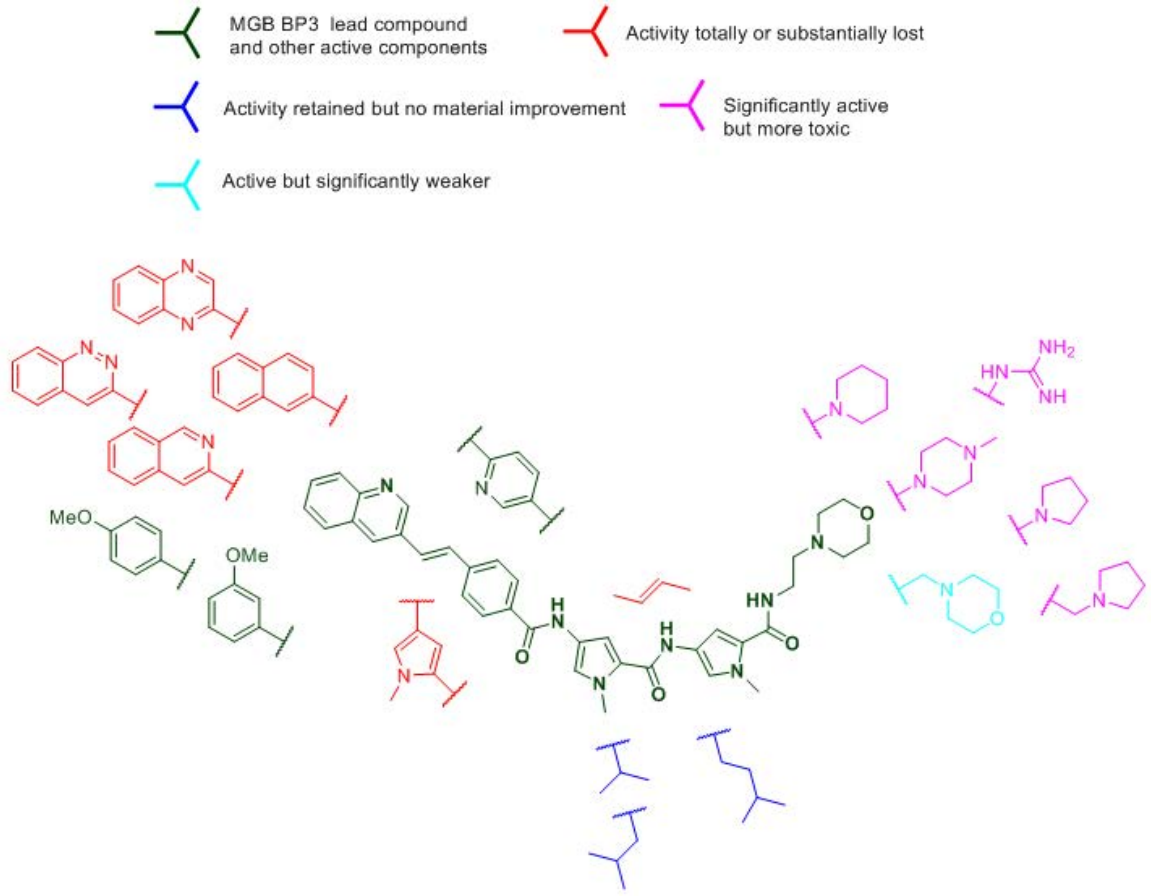


Figure 4 Summary of structure-activity for MGB-BP3-like compounds against Gram-positive bacteria.

observed. It could be argued that it might be possible to replace one of the other amide links by an alkene also. With some effort, examples of such compounds were synthesized with the alkene between Het 2 and Het 3 (using the labeling in Figure 2). Significantly, none of these compounds was found either to bind strongly to DNA or to have antibacterial activity. The use of an alternative isostere, the diazo group, did not give compounds with significant antibacterial activity or DNA binding [8a]. A further indication of the special characteristics of MGB-BP3 was found by removing the quinoline nitrogen atom to give a naphthalene head group, moving it to give an isoquinoline, or adding additional nitrogen atoms; none of these changes led to strongly antibacterial compounds. A summary of the structure-activity relationships established for antibacterial activity is shown in Figure 4. Patents were filed for all of the significant discoveries [9].

At this point in the project the University of Strathclyde put in a significant effort to find a commercial partner for the development of these discoveries. A deal was done in 2010 to license the two patents to a new Scottish company, MGB Biopharma. This company has subsequently raised money and managed the development of MGB-BP3 to reach Phase 1 clinical trials in 2015. Substantial scientific support has been provided by the University of Strathclyde, the vast majority of which has been funded by internal University funds and UK research council grants. The remainder of this paper concentrates upon the scientific results that have supported the development programme with reference to formulation, selectivity, and mechanism of action.

Selectivity of MGB-BP3 and related compounds

A key property of the MGB-BP3 class of antibacterial compound is their selectivity for Gram-positive bacteria and their lack of apparent activity against mammalian cells. This is well illustrated by data from another compound of the class, AIK 20/25/1, which is almost as active as MGB-BP3 and is the second compound in line for development. Figure 5 shows the effect on cellular viability for this compound comparing a mammalian cell line (HS27 murine fibroblast) with *Staphylococcus aureus*. The difference is striking. No evident toxicity was found with the HS27 cells but catastrophic death was found for the bacteria. Similar results have been obtained for many compounds in this series. The catastrophic bactericidal activity is not typical of a drug's acting at a single pharmacological

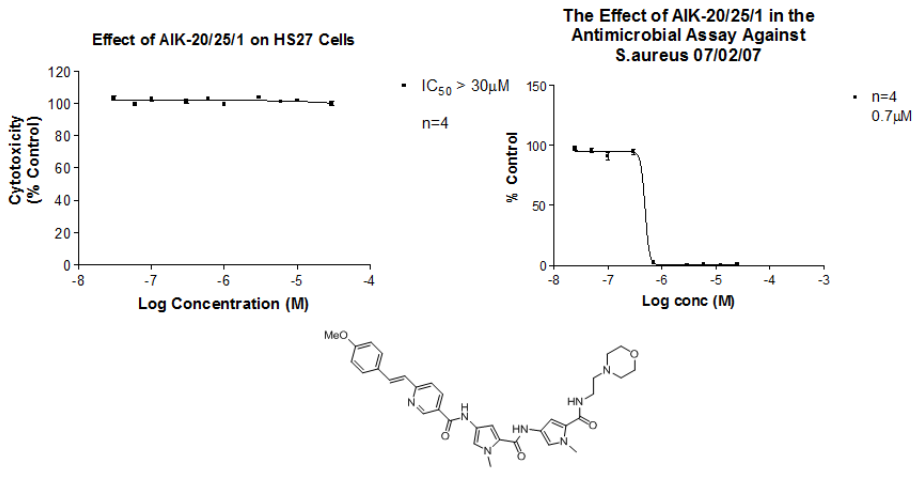


Figure 5 Selectivity for Gram-positive bacteria shown by a close relative of MGB-BP3

target but suggests that the minor groove binders exert their effect by interfering with a number of biochemical pathways that together lead to catastrophic death; this will be discussed further below.

As was noted above, the MGB-BP3 class of antibacterial compound did not show significant activity against Gram-negative bacteria. Nevertheless some significant hits were obtained in particular with compounds containing thiazoles and with amidine-linked head groups such as MGB-BP4 (Figure 6). It was important to establish the reasons for these differences. Taking advantage of the intrinsic fluorescence of the MGB-BP3 class of compounds, it was possible to show that MGB-BP3 readily entered the cells of Gram-positive bacteria but no uptake of fluorescence was seen in Gram-negative bacteria.

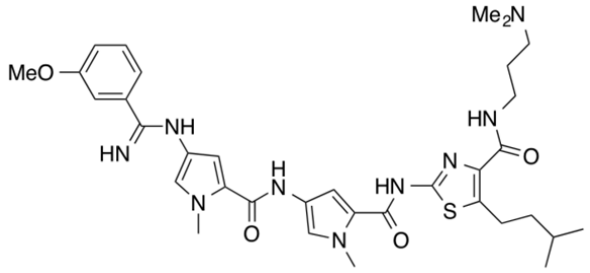


Figure 6 A hit, MGB-BP4, in a screen for anti-Gram-negative compounds

However when *E. coli* cells were treated with lysozyme and EDTA so that the outer cell membrane was removed and so-called spheroplasts were formed, intracellular fluorescence was observed [10]. The conclusion that penetration of the cells is necessary for activity is obvious. It was shown later that a major contribution to the failure of the MGB-BP3 family to attach Gram-negative bacteria was due to efflux pumps. When cells of *Pseudomonas aeruginosa* were treated with both an efflux pump inhibitor (Phe-Arg-β-naphthylamide) and a minor groove binder, some compounds were found to be significantly active. As this work was proceeding, the activity of the Strathclyde minor groove binders against other infectious organisms, particularly parasites, was also being investigated. Different compounds showed distinct activity profiles. In summary the following activities have been found: *in vitro* against *Candida albicans* and *Asperg illusniger*; *in vitro* against *Plasmodium falciparum* DD3, a drug resistant strain;*in vitro* against *Trypanosoma brucei* (human parasite) and *in vivo* against *Trypanosoma congolense* (animal parasite); *in vivo* against *Leishmania major* and *Leishmania donovani*. Now these parasites are all eucaryotes and again selectivity with respect to mammalian cells was found. This point is emphasised by the successful proof of concept experiments with animals in models of sleeping sickness and leishmaniasis in which effective minor groove binders did not show toxicity to the animals at therapeutically effective doses [11]. A general significant scientific point can be identified from these results, namely that the toxicity of a minor groove binder depends not only upon its ability to bind to DNA but also on the way in which the target cell handles the drug. To put it simply, if the drug does not enter the cell, it cannot reach DNA to exert its effect. What reaches the DNA is evidently both compound specific and cell specific.

These concepts can now be examined in terms of the antibacterial minor groove binders such as MGB-BP3. MGB-BP3 clearly is able to enter Gram-positive bacterial cells as shown by fluorescence microscopy. On the other hand, there is no evidence for the penetration of BP3 into a number of mammalian cell lines that have been studied, including B16FOluc (mouse lung carcinoma) and A549 (human lung carcinoma). Now these are all cell lines in which membrane components such as efflux pumps may not be as active as in primary cells. Both primary macrophages and primary hepatocytes have been examined and in neither case was MGB-

BP3 found to enter these cells. The experiments were shown to be competent because other minor groove binders such as Hoechst dyes were found to enter the cells and to concentrate in the nuclei [12]. It therefore seems that by chance MGB-BP3 has a favourable combination of properties to act as an antibacterial drug, namely selective penetration of the target bacterial cell and high affinity for DNA. Attention can now turn to the consequences of DNA binding in the bacterial cell.

Mechanism of action of MGB-BP3

In order to understand the effect of MGB-BP3 as an anti-Gram-positive compound, the modern technique of RNA-seq has been used. In these experiments, *Staphylococcus aureus* cells were treated with MGB-BP3 at a sub-lethal concentration so that its effect on cellular biochemistry could be determined by the differential expression of genes compared with an untreated sample as shown in the transcriptome [13]. Large quantities of data were generated and it is important to establish the significance of the data. Therefore two independent experiments have been carried out at increasing sequencing depth and the data analysed by four software methods. Several hundred genes have been found to be either up- or down-regulated, including about 70 essential genes, and the response of *S. aureus* has now been characterised. For example it was not surprising to find that the gene encoding the histone-like protein that protects the bacterial chromosome was substantially up-regulated on challenge by MGB-BP3. Other notable changes were the down-regulation of phosphomevalonate kinase, an enzyme that has been identified as a potential drug target. When all of the changes were transferred to a metabolic map for *S. aureus* the areas of greatest effect were nucleic acid and nucleic acid component metabolism (up and down), lipid metabolism (mostly up), and glycolysis and oxidative phosphorylation (up and down). In terms of the kill curves' profiles the implication that a catastrophic failure in energy production leads to cell death is attractive. In order to increase confidence in the significance of these observations, a number of enzymes was selected and the change in their expression investigated by qRT-PCR. In summary it was found that the qRT-PCR data set matches the RNA-seq data set with respect to relative gene expression levels between different genes, that the qRT-PCR matches the RNA-Seq data set with respect to the effect of MGB-BP3, and that some genes analysed by qRT-PCR are essential genes in *S. aureus*.

A further point arising from the RNA-seq experiments is that the target sites for binding MGB-BP3 to DNA were identified. Consensus sequences of AT rich sites were found consistent with the known binding of MGB-BP3 to DNA from footprinting experiments. MGB-BP3 showed especially strong footprints at AT rich sequences such as TTTAAACGTT and ATATATGTATA [14]. The binding sites identified by RNA-seq were TAAGAG and AAAGAAAA. Together these data point to a variety of AT rich sites that are competent for binding to MGB-BP3. It is interesting that a recent study also using RNA-seq drew attention to a number of AT rich sites as promoter binding sites in *S. aureus*,of which TATAGT, TATAAA, TAAAAT, TATTTT, TATAAT, and TATACT were highlighted [15]. The probability that MGB-BP3 exerts its gene expression modifying and hence antibacterial effects by binding to some of these promoter sequences is strong.

Formulation of MGB-BP3

Clinical trials of course refer to a medicine and not simply to an active pharmaceutical ingredient. MGB Biopharma has invested heavily in the development of MGB-BP3 into a medicine and has brought it to the stage of a Phase 1 clinical trial in an oral formulation for the treatment of *Clostridium difficile* infections. The probability is that MGB-BP3 exerts its antibacterial effects

by modifying gene expression through binding to some of these promoter sequences.

The underlying science for formulation has also been studied at the University of Strathclyde. Although MGB-BP3 is dibasic, its pK_as are low, 3.49 (quinoline) and 5.26 (morpholine) indicating that at physiological pH greater than 99% of the molecules will be neutral. In order to obtain aqueous formulations, it is necessary to maintain a pH of about 5. The low pK_a of the morpholine is especially striking being at least 2.5 below a typical *N*-alkyl morpholine. It was also observed that when a solution of MGB-BP3 and its close relatives was brought from pH 3 towards pH 7, turbidity appeared at about pH 5 and, if allowed to stand, the solution transformed into a gel. The gel returned to mobile solution on gentle warming. A possible explanation for these phenomena is that MGB-BP3 and its close relatives form dimers in aqueous solution similar to the configuration found in DNA binding (Figure 1). Direct evidence for this possibility came from an NMR study of the MGB-BP3 analogue with a 3-methoxyphenyl head group in place of the 3-quinolyl head group (Figure 7) [16].

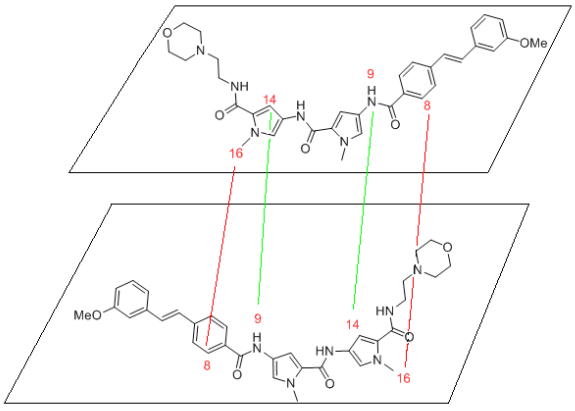


Figure 7 Self association of an MGB-BP3 relative as shown by NOE studies in aqueous solution.

At room temperature substantially broad signals were observed but at 80 °C it was possible to obtain well-resolved [^{1}H] spectra for this compound and also to measure NOE effects. As shown in Figure 7, NOEs were observed consistent with the existence of antiparallel dimers of exactly the same configuration that would bind to DNA according to the design paradigm for the Strathclyde compounds.

Conclusion

MGB-BP3 has been developed up to Phase I by MGB Biopharma. It is highly effective against Gram-positive bacteria but not Gram-negative bacteria. Nevertheless, a number of hits have been found in the Strathclyde MGB library with activity against Gram-negative bacteria and a hit to lead programme of research for Gram-negative activity is now under way. Further,proof of concept experiments with other Strathclyde MGBs in animal models for parasitic diseases have been successful supporting the case for Strathclyde MGBs as a discovery platform for anti-infective medicines of many types. With respect to the mechanism of action of the lead antibacterial drug, MGB-BP3, evidence has accrued that itcauses a substantial biochemical and physiological response in *S. aureus* consistent with DNA binding in particular at promoter sites. Other experiments suggest that the selectivity for bacterial compared with mammalian cells probably relates to differential access to cells. This is obviously of crucial importance for MGB-BP3 and other MGBs as drugs of the future and it isa problem under active investigation.

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