An Evaluation of Minor Groove Binders as Anti-Lung Cancer Therapeutics

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Abstract

A series of 47 structurally diverse MGBs, derived from the natural product distamycin, was evaluated for anti-lung cancer activity by screening against the melanoma cancer cell line B16-F10. Five compounds have been found to possess significant activity, more so than a standard therapy, Gemcitabine. Moreover, one compound has been found to have an activity around 70-fold that of Gemcitabine and has a favourable selectivity index of greater than 125. Furthermore, initial studies have revealed this compound to be metabolically stable and thus it represents a lead for further optimisation towards a novel treatment for lung cancer.

Keywords: Minor Groove Binders; Anti-cancer; Lung Cancer

Cancer is a major health problem responsible for approximately 13 % of the deaths worldwide. The incidence is estimated to be around 13 million cases a year, according to WHO.[1] In particular, lung cancer is one of the most frequent cancers causing around 1.4 million deaths per annum. The treatment of lung cancer is based on chemotherapy and/or surgery. However, surgery is limited to those tumours with peripheral location. Other types of tumours with a more difficult access, such as small-cell lung cancer, are generally treated with chemotherapeutic agents. A first-line chemotherapy treatment is a platinum based combination of cisplatin or carboplatin with etoposide or irinotecan.[2] The mechanism of action of platinum based compounds is localised at the major groove of the DNA where the drug interferes with the genetic transcription. These modifications result in variations in the product from crucial genes involved in cell-cycle replications and finally induce cell apoptosis. Nonetheless, treatment with many traditional anticancer drugs, such as cisplatin, is related to drug resistances and unsuccessful therapeutic outcome. Therefore, the need of new anticancer compounds that could overcome those resistances by binding to the smaller groove of the DNA.[3]

Minor Groove Binders (MGBs) are a class of compound that specifically, and reversibly, bind to the minor groove of DNA. They recognise specific base sequences of DNA with high selectivity and achieve efficacy by interfering with transcription factors and altering gene expression.[4]
Our approach at the University of Strathclyde has been to diversify the structure of the first discovered MGB, distamycin, and to generate a portfolio of significantly active Strathclyde Minor Groove Binders (S-MGBs). We can now design novel S-MGBs with tailored activities through a detailed understanding of DNA binding, sequence selectivity, and physicochemical characteristics (figure 1).

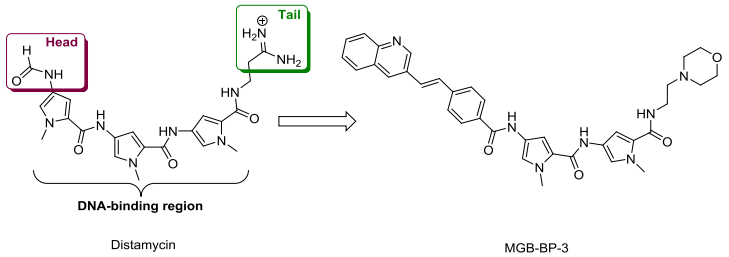


Figure 1. *Distamycin and our lead Gram-positive antibacterial compound, MGB-BP-3.*

Deviations from the distamycin structure have included tuning the basicity (pK_a) of the tail group amidine at the C-terminus, the addition of larger alkyl side chains and the introduction of thiazole rings. Aromatic rings replaced the distamycin formyl group and, importantly, the *N*-terminal amide was replaced by its isosteric alkene.[5] These structural changes systematically modified the physicochemical properties to increase hydrophobic contacts with the target DNA resulting in a library of highly potent compounds displaying distinct biological activities. The physicochemical aspects of this design hypothesis were investigated in detail and shown to lead to strong DNA binding driven largely by enthalpic interactions.[6] Furthermore, studies suggest that the physicochemical properties of MGBs substantially determine their net uptake into target cells. Our knowledge in this area has led to the discovery of a family of compounds with significant anti-Gram-positive bacterial activity, the most active of which, MGB-BP-3, has successfully completed Phase I clinical trials for the treatment of *Clostridium difficile* infections. Furthermore, other S-MGBs have been shown to be active against *Trypanosoma* both in cell-based studies and in mouse models of disease and leishmaniasis.[7]

Distamycin itself does not possess significant cytotoxicity; however, other classes of MGB have displayed significant potential as anti-cancer agents, most notably those which are directly derived from the distamycin framework. Tallimustine (figure 2) in which the formyl head group of distamycin has been replaced with a benzoic acid mustard (BAM) moiety displays high activity against various murine tumors and human xenografts. Most other BAM compounds alkylate and cross-link within DNA's major groove; however, with the minor groove binding affinity conferred by the distamycin framework, tallimustine has been shown to preferentially monoalkylate the 3'-adenine-N3 atom of 5'-TTTTGA-3' sequences in the minor groove.[8] In distamycin type molecules, increasing the number of pyrroles is linked to an increase in DNA binding affinity and this is true of tallimustine where an increase to four pyrroles significantly increases the cytotoxicity.[9,10] Development of tallimustine was promising as phase I and II clinical trials demonstrating significant antitumor, but notably myelotoxicity led to the phase II clinical development being halted.[11, 12, 13]

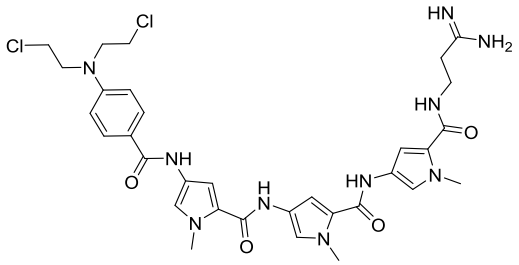


Figure 2. *Tallimustine.*

As a class of compounds, S-MGBs are not inherently cytotoxic: structural alterations away from that of distamycin led to anti-infective compounds with favourable selectivity indices.[14] Despite the lack of class specific toxicity, some S-MGBs inhibit the growth of lung cancer cells. This paper describes the evaluation of a panel S-MGBs as potential lung cancer therapeutics.

The compounds synthesised for this investigation are typical of S-MGBs in general. They include compounds with amide, amidine, or alkene linked head groups with a range of polar and non-polar

features, *N*-alkyl pyrroles, a standard feature of minor groove binders, *C*-alkyl thiazoles, a specific feature of S-MGBs, and tertiary amine tail groups with a variety of basicities (figure 3). These structural variations give rise to a set of compounds with a wide range of physicochemical properties and DNA-binding specificity.

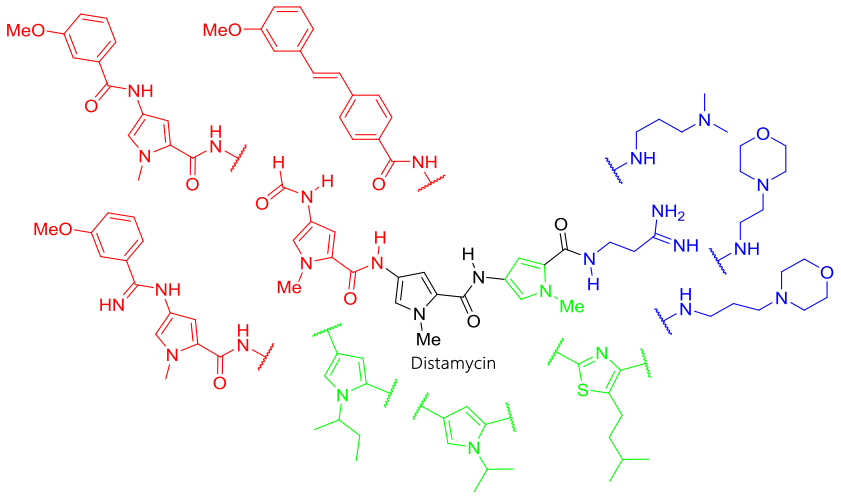
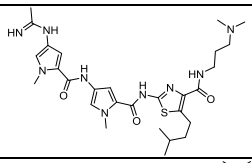
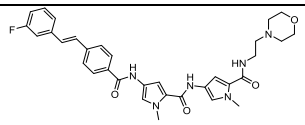
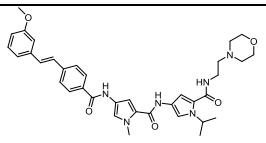
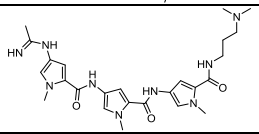
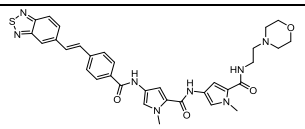
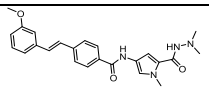
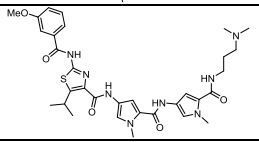
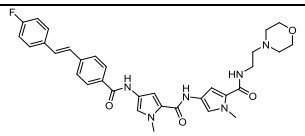
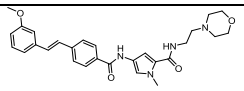
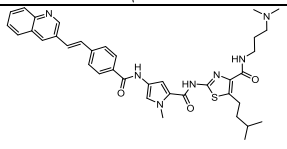
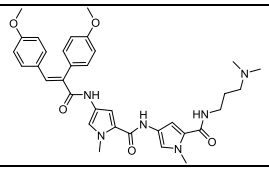
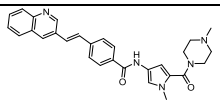
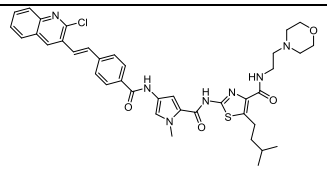
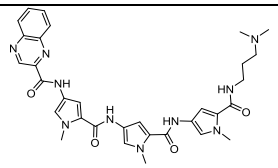
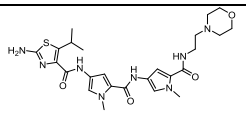
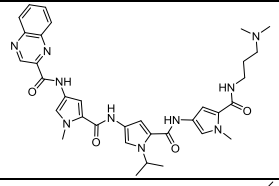
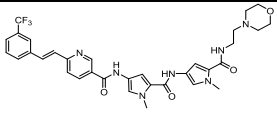
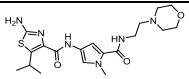
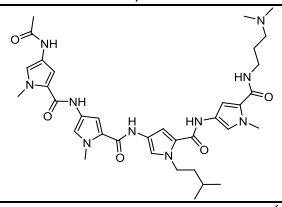
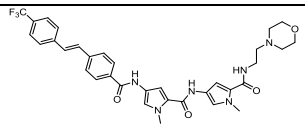
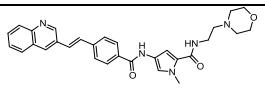
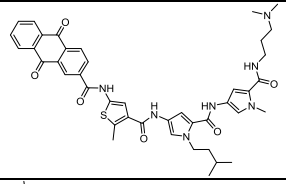
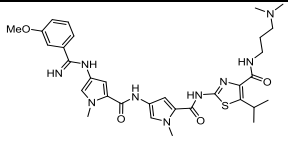
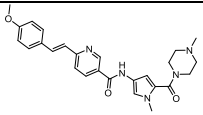
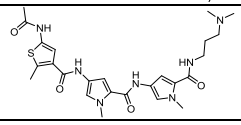
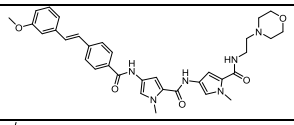
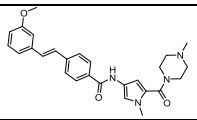
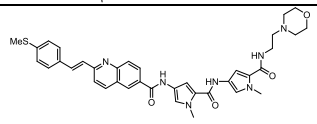
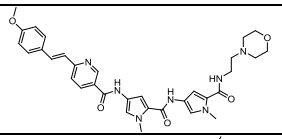
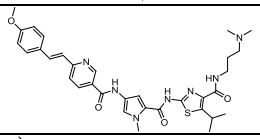
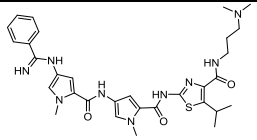
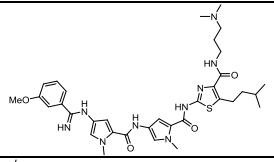
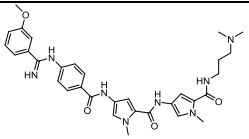
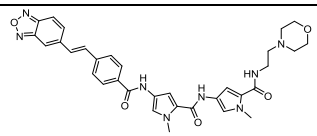
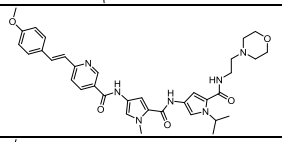
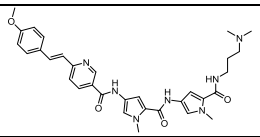
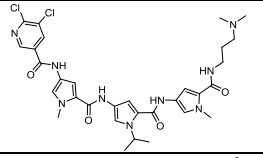
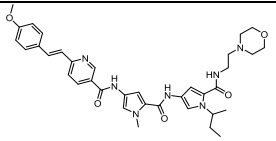
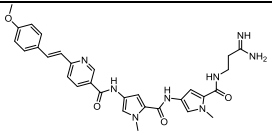
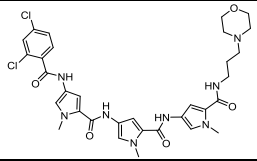
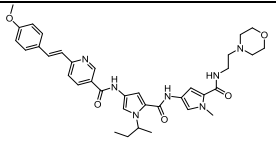
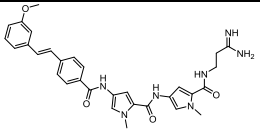
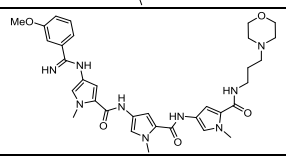
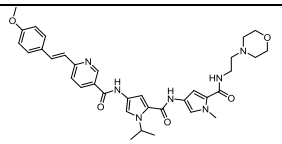
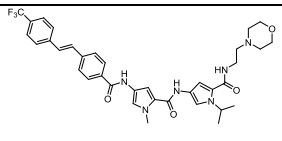


Figure 3. *Exemplars of the structural variations in the S- MGB set investigated in this study.*

Synthesis of these MGBs was achieved in a modular fashion, initially building from the C-terminus, tail group end, of the molecule and ending with a coupling reaction to attach the head group. The synthesis of many of the compounds under study in this letter has been published previously[7] and that of the novel compounds can be found in the electronic supplementary information as the synthetic methods are similar.

All MGBs were purified by HPLC to give a final purity greater than 98%. Table 1 shows the structures of the MGBs that were investigated in this study.

S-MGB	Structure	S-MGB	Structure	S-MGB	
1		**17**		**33**	
2		**18**		**34**	
3		**19**		**35**	
4		**20**		**36**	

5		**21**		**37**	
6		**22**		**38**	
7		**23**		**39**	
8		**24**		**40**	
9		**25**		**41**	
10		**26**		**42**	
11		**27**		**43**	
12		**28**		**44**	
13		**29**		**45**	
14		**30**		**46**	
15		**31**		47	

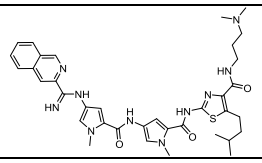
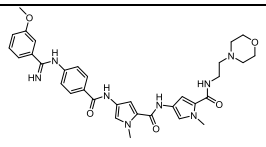
16		**32**			

***Table 1**. S-MGBs evaluated in this study*

In this study the melanoma cancer cell line B16-F10, which quickly metastases to the lungs, was used for initial screening. This cell line was derived from pulmonary metastatic cells isolated from the lungs of mice after ten successive passages of B16 sub-lines.[16] The original B16-F10 cell line was transfected with firefly luciferase gene to develop the B16-F10-luc murine melanoma cell line (Bioware® B16-F10-luc-G5, Caliper Life Sciences; Hopkinton, USA). The integration of this gene allows disease progression to be monitored by luciferin-luciferase bioluminescence imaging.[17] 46 compounds from the S-MGB library were tested at a single concentration of 15 µg/mL for their ability to inhibit the growth of B16-F10-luc cells. The bioluminescence emitted from the cells treated with MGBs was compared to the bioluminescence emitted for the solvent control cells to establish a baseline (Table 2).

S-MGBs have been extensively investigated as anti-infective agents, initially as antibacterial and more recently as antiparasitic agents.[7] Two key properties of S-MGBs characterize their biological activity, namely DNA binding and access to cells. Most S-MGBs are based on the distamycin structure and thus possess an affinity towards DNA binding; however, it is access to cells that has been found to be the most important factor explaining activity, and selective toxicity. For example, S-MGBs possess very strong anti-Gram-positive activity but little anti-Gram-negative activity and this appears principally to be due to efflux from Gram-negative cells. Since S-MGBs show anti-Gram-negative activity in the presence of efflux pump inhibitors it is possible that efflux is the chief cause of the lack of activity in this class of bacteria. The action of efflux pumps on S-MGBs appears to be related to their physicochemical properties and it is thus likely that these will be important in explaining anti-lung cancer activity. To this end, $logD_{7.4}$ was predicted using the software MarvinSketch (Version 15.6.29.0, ChemAxon, http://www.chemaxon.com), in an attempt to gain some insight into the differences in activity. These results, alongside the activity data, are presented in table 2.

MGB	Inhibition (% ± SD)	$LogD_{7.4}$	MGB	Inhibition (% ± SD)	$LogD_{7.4}$	MGB	Inhibition (% ± SD)	$LogD_{7.4}$
1	NA	0.18	**17**	46 ± 0.43	4.11	**33**	88 ± 3.1 ***	4.50
2	NA	-2.42	**18**	37 ± 17	4.11	**34**	55 ± 26	3.92
3	NA	2.77	**19**	NA	4.11	**35**	55 ± 24	4.06
4	53 ± 7.3	6.51	**20**	NA	2.59	**36**	69 ± 14**	4.56
5	NA	7.56	**21**	NA	1.08	**37**	57 ± 2.5 *	0.93
6	NA	1.90	**22**	NA	3.86	**38**	2.2 ± 6.9	1.27
7	NA	0.80	**23**	77 ± 5.7 *	4.85	**39**	72 ± 4.4 **	4.37
8	40 ± 26	4.97	**24**	34 ± 4.7	2.29	**40**	59 ± 13 *	3.26
9	NA	0.56	**25**	NA	3.81	**41**	76 ± 5.1 **	4.24
10	19 ± 47	4.99	**26**	44 ± 10	2.83	**42**	74 ± 12**	2.95
11	57.12 ± 11.48 *	2.22	**27**	34 ± 5.4	3.41	**43**	NA	-1.74
12	30.92 ± 28.62	3.15	**28**	83 ± 3.5 ***	3.51	**44**	NA	-1.13
13	16.13 ± 8.45	2.39	**29**	84 ± 3.4 ***	4.04	**45**	NA	-0.48
14	NA	3.15	**30**	84 ± 3.8 ***	4.04	**46**	67 ± 12	0.50
15	NA	1.48	**31**	85 ± 2.9 ***	3.51			
16	54.10 ± 9.16	3.81	**32**	77 ± 12**	2.12			

***Table 2.** The effect of treatment with different MGBs compounds on the proliferation of B16-F10-luc cells. Cells (3.7 x 10^5 cells/well) were incubated for 24 hours in the presence of medium containing DMSO (solvent control, 2.44 % v/v DMSO) or*

different MGBs compounds (15.36 µg/ml with 2.44 % v/v DMSO). The effect of treatment on cell proliferation is shown as the mean percentage inhibition of n=3 experiments compared to the mean solvent control where NA is not active ($p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ comparing bioluminescence of the cells treated with MGBs with the cells with solvent control). $LogD_{7.4}$ predicted using the software MarvinSketch (Version 15.6.29.0, ChemAxon, http://www.chemaxon.com).*

The data shown in table 2 indicate that this selection of S-MGBs contains both significantly active and inactive compounds with respect to B16-F10 cells. It is significant that in this set of S-MGBs none of those bearing an amide linked head group has any appreciable activity against the B16-F10 cell line. Many of the inactive compounds in this set have shown appreciable activity against other disease targets: many have moderate antibacterial properties with MICs around 10 µM; and, **45** has been shown to be significantly active in a mouse model of *Trypanosoma congolense* (manuscript submitted). This illustrates that the structure and physicochemical properties of S-MGBs influence their activity profiles against different organisms and cells. Compounds **34**-**41** represent a subset of short length S-MGBs having one pyrrole ring less than the others. A greater number of pyrrole rings usually correlates to greater DNA binding affinity and in many contexts greater activity; therefore, it is interesting to note that these shorter compounds still are active against B16-F10 cells. Moreover, this subset of compounds has been shown to in general to lack antibacterial activity, further evidence of differential activity profiles for S-MGBs (manuscript submitted).

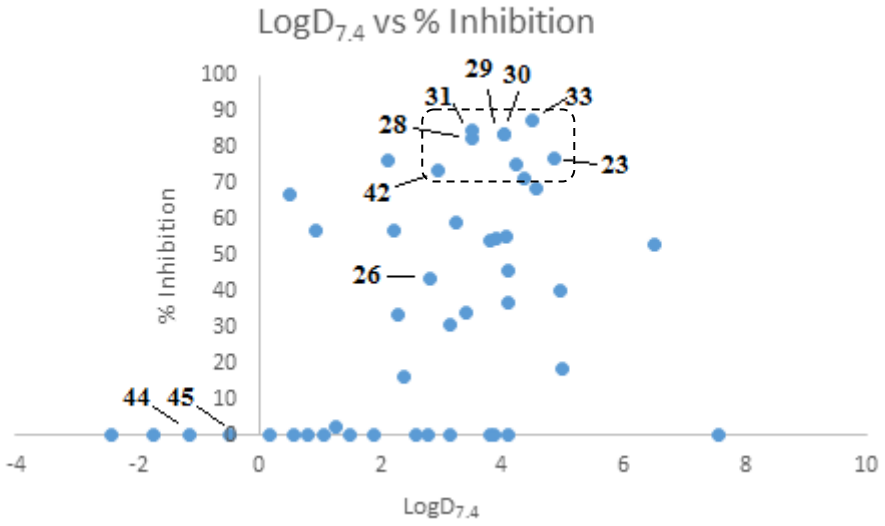


Figure 4. *$LogD_{7.4}$ vs % inhibition of B16-F10-luc cells. $LogD_{7.4}$ predicted using the software MarvinSketch (Version 15.6.29.0, ChemAxon, http://www.chemaxon.com). Dotted box shows clustering of active compound around $LogD_{7.4}$ range of 3-5.*

In an effort to explain the observed activities by considering the physicochemical properties of S-MGBs, $logD_{7.4}$, as a measure of lipophilicity, was plotted against the % inhibition of B16-F10-luc cells (figure 4). It is clear that lipophilicity, measured by $logD_{7.4}$, alone cannot explain the activities of this set of S-MGBs; however, the most active compounds are clustered within a $logD_{7.4}$ range of 3-5. The importance of lipophilicity is neatly explained through compounds **45**, **44**, **26** and **28**-**31**, which all contain the same 4-methoxyphenyl-2-pyridylethenyl head group. **45** and **44** have basic amidine and dimethylamino tail groups, giving them very low $logD_{7.4}$ values (-0.48 and -1.13, respectively) and no activity. The morpholino tail group in **26**, with a $logD_{7.4}$ of 2.83, gives a moderate activity of 44% inhibition and further increasing the lipophilicity by inclusion of a branched alkyl side chain (**28**-**31**, $logD_{7.4}$ 3.51 and 4.04) affords an excellent inhibition of around 84%.

The % inhibition data was used to select a number of active compounds with a variety of structural features. For example, compound **28** was chosen to represent compounds **28** to **33** as these all

possess the same core structure but differ in their alkyl side chain. The mean IC_{50} was determined for compounds **23**, **28**, **33** and **42** (table 3). In these studies, gemcitabine, a drug commonly used to treat cancer, was used as the positive control. **23**, **28**, and **33** had a smaller IC_{50} than gemcitabine ($p \leq 0.05$; **Error! Reference source not found.**) whereas **42** had similar activity to gemcitabine.

MGB	IC_{50} (μM ± SD)	$LogD_{7.4}$
Gemcitabine	11.0 ± 1.7	ND
23	0.16 ± 0.01	4.85
28	0.81 ± 0.08	3.51
33	1.1 ± 0.51	4.50
42	10.4 ± 0.50	2.95
47	2.2 ± 0.22	5.45

Table 3. *The mean half maximal inhibitory concentration (IC_{50}) values for different MGBs compounds tested against B16-F10-luc cells. The values shown are from three experiments and three replicates were used for each concentration tested in each experiment.*

Given the significant activity of compound **23**, which contains an unusual trifluoromethyl substituent, the initial screening data were scrutinised for information that could prompt the design of a compound with greater activity. Compound **25** had no activity in the initial screen; however, compound **33**, with an isopropyl instead of a methyl side chain, had an IC_{50} of 1.1 μM, highlighting the importance of this simple alteration. The isopropyl side chain also appears in compound **35**, (IC_{50} of 0.81 μM), and although its methyl side chain analogue, **26**, was active (44% growth inhibition), there is still a substantial increase associated with the presence of the isopropyl group. This prompted the design of compound **47** which is an analogue of the most active compound, **23**, but with the inclusion of the isopropyl side chain. However, **47** (IC_{50} of 2.16 μM) was 13-fold less active than **23**. With the inclusion of the isopropyl group, the lipophilicity of compound **47** ($logD_{7.4}$ = 5.45) is notably higher than that of **23** ($logD_{7.4}$ = 4.85), suggesting that a $logD_{7.4}$ less than 5 is required for optimal cellular activity. Nonetheless, with an activity approximately 70-fold greater than Gemcitabine, compound **23** is a significant find. Compound **23** has also been shown to have no appreciable activity in any of our routine screens against fungi, bacteria or parasites. Moreover, we have previously reported compound **23** to have no measureable toxicity against HEK cells at 20 μM thus giving a satisfactory selectivity index of >125.[7] This discovery encouraged the further investigation of the biological action of S-MGBs in the context of anti-lung cancer therapeutics, in particular compound **23**'s potential for further development.

Minor groove binders that have seen use previously as anti-cancer agents are often modified with an DNA-alkylating moiety, such as in tallimustine. DNA binding is thus a plausible mechanism of action for this class of minor groove binder. In the case of other classes of minor groove binders, principally anti-infective agents including S-MGBs, an alternative mechanism of action has been suggested that involves cell membrane permeabilisation.[18] S-MGBS that have strong affinities for DNA tend to also have strong antibacterial properties and more recently, we have found that significantly active S-MGBs gain access to both bacterial cells and parasites, where nuclear localisation is observed.[7] However, penetration into mammalian cells has not yet been observed for any significantly active and non-toxic S-MGB that has undergone extensive investigation as anti-infective agents. To understand better the behaviour of S-MGBs in mammalian cells the strongly fluorescent probe S-MGB (**48**) was used in fluorescence microscopy studies.

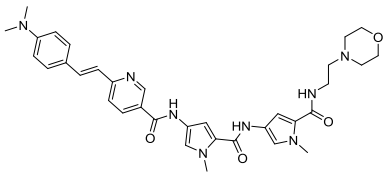


Figure 5. *Compound **48**, A fluorescent probe S-MGB.*

To assess intracellular localisation in mammalian cells, B16-FO Luc cells were treated with 3 μg/ml and 30 μg/ml of compound **48**, using 250 μM Hoechst 33342 as a control, and were monitored for fluorescence using the UV and the DAPI filter sets.

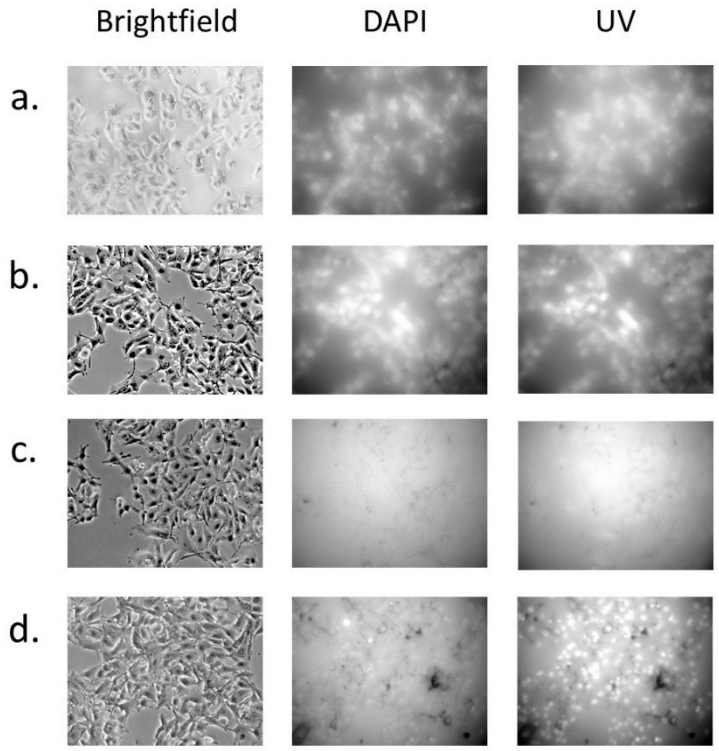


Figure 6*. Fluorescence microscopy of B16/FO/Luc cells treated with S-MGB compound **48**. Cells were exposed to various concentrations of the S-MGB for 1 hr and examined under an inverted fluorescent microscope using the X20 objective with brightfield and UV or DAPI filter sets. Row (a), 3 μg/ml compound **48**; (b), 30 μg/ml compound **48**; (c) DMSO only; (d), 250 μM Hoechst 33342. Similar results were obtained in two experiments with separately prepared cells.*

There was clear evidence that compound **48** was internalised within the cells showing an increase in fluorescence with increasing concentration of the S-MGB. Comparison with the bright field image showed that fluorescence was concentrated in the nucleus, as observed for the positive control, Hoechst 33342. This assessment of a model, fluorescent S-MGB supports DNA binding as a plausible mechanism of action in mammalian cells.

Knowledge of potential *in vivo* stability is a crucial indicator of whether or not a compound is suitable for further development in a drug discovery programme. The majority of drug metabolism takes place in the liver, so we carried out an assessment of rat hepatocyte uptake, and metabolism of compound **23** over a period of 24 h in primary monolayer cultures, and monitored cell viability over this time. There was no evidence of uptake of the drug into cells over the 24 h period when using red, green or blue filters for detecting fluorescence. Although cell morphology showed no signs of overt toxicity (figure 7), fewer cells were consistently observed in drug treated dishes than in controls post-treatment.

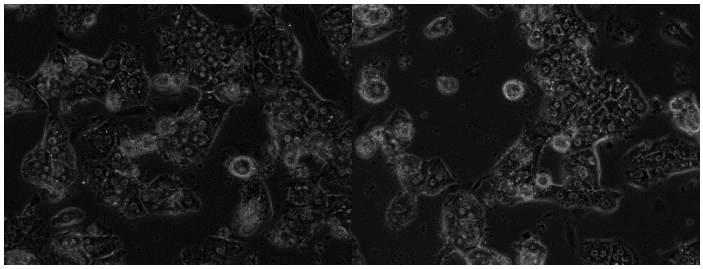


Figure 7. Phase contrast images of hepatocyte monolayers exposed to DMSO control (Left) and 7.5µM compound 23 for 60 min (Right). Zeiss Axiovision Fluorescence microscope, x 20 wet lens (N/A 0.5). There was no evidence of drug uptake during 24 h exposure using either green, red or blue filter settings.

Aliqots of the medium of the hepatocyte incubations were extracted at 15 minutes, 60 minutes and 24 hours and analysed by LC-MS in order to further assess the stability of these compounds and to identify any metabolites. Figure 8 shows the extracted ion trace for the 15 minute aliqot.

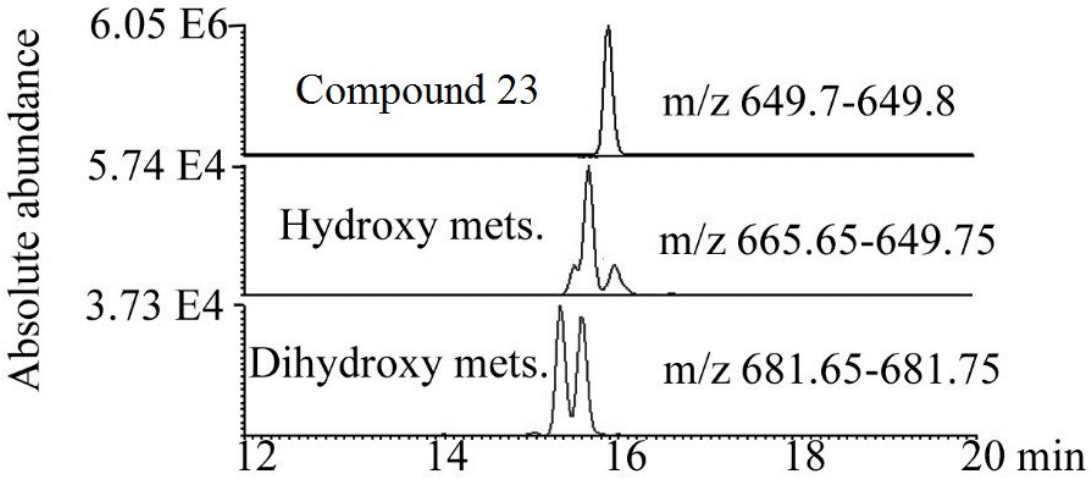


Figure 8. *Extracted ion traces for compound **23** and its hydroxy and dihydroxy metabolites (mets).*

Compound **23** showed only around 1% metabolism, estimated from the peak heights of the metabolites relative to the parent compound, over the 24-hour monitoring period of the experiment, the vast majority of this occurring within the first 15 minutes. The m/z of the metabolites are 665.2703 and 681.2653 and thus likely correspond to a mono and di-hydroxylated analogue of compound **23,** which has a m/z of 649.2749. From these data, compound **23** can be concluded to be particularly stable over a sufficiently long time period to be deemed appropriate for further development.

This study has indicated the importance of the activity profile of S-MGBs and serves to provide further evidence that subtle structural variations can influence the activity profiles of compounds within this class. A sub-set of S-MGBs has been identified which have significant activity against a lung cancer cell line, more so than a current treatment option, Gemcitabine. As a class, we have also demonstrated that a plausible mechanism of action for S-MGBs on mammalian cells is through DNA binding.

These general findings have allowed us to select one compound, which is around 70-fold more active than Gemcitabine in our *in vitro* assay, and possesses a favourable selectivity index of >125 against the HEK cell line. Moreover, we have demonstrated its stability in a hepatocyte model and thus identified this compound as worthy for further development as an anti-lung cancer therapeutic.

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