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Towards more specific O^6-methylguanine-DNA methyltransferase (MGMT) inactivators

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ABSTRACT

Searching for a novel family of inactivators of the human DNA repair protein O^6-methylguanine-DNA methyltransferase (MGMT) which is known to bind to the DNA minor groove, we have computationally modelled and synthesised two series of 2-amino-6-aryloxy-5-nitropyrimidines with morpholino or aminodiaryl substituents (potential minor groove binders) at the 4-position. Synthesis of these compounds was achieved by successive substitution of each of the two Cl atoms of 2-amino-4,6-dichloro-5-nitropyrimidine by the corresponding amino and aryloxy derivatives. Biochemical evaluation of these compounds as MGMT inactivators showed poor activities, but in general the 4-bromothenyloxy derivatives showed better inactivation than the benzyloxy versions. DNA binding assessment was not possible due to insolubility problems.

1. Introduction

It is well known that resistance to the cytotoxic effects of antitumor alkylating agents such as the nitrosoureas (e.g., bischloroethylnitrosourea, BCNU) or methylating agents (e.g., temozolomide, TMZ) can be mediated by the human DNA repair protein O^6-methylguanine-DNA methyltransferase (MGMT). MGMT removes alkyl groups from guanine residues that have been alkylated at the O^6-position by these drugs. The alkyl group is transferred to a cysteine residue in its active site in an auto-inactivating stoichiometric suicidal process.[1,2] MGMT can be expressed at high levels in certain tumour cells,[2,3] and hence it has become an important target for the development of drugs designed to improve the effectiveness of the treatment of tumours with this type of alkylating agent.[3–5]

MGMT binds to the minor groove of DNA in a rapid on–off process and alkyl transfer occurs when MGMT encounters a recognised substrate O^6-alkylguanine lesion.[6] More recently it has been shown that at least in vitro, minor groove binding can be cooperative.[7] The minor groove of DNA is also the interaction site for many enzymes and transcription control proteins. Consequently, compounds that target the minor groove have been investigated for their potential for treating cancer via inhibition of the action of DNA-dependent enzymes or by direct inhibition of transcription.[8–10] MGMT inactivators [such as O^6-(4-bromothenyl)guanine, (originally named PaTrin-2 and now Lomeguatrib, Fig. 1) and O^6-benzylguanine] that have been in clinical trials, have so far been shown not to improve patient outcomes.[11] Hence, new approaches need to be considered to enhance the inactivation of this protein in order to overcome the resistance to alkylating agents which are still some of the preferred drugs in cancer chemotherapy.

Current inactivators are based on free guanine and thus do not directly exploit the DNA minor groove binding characteristics of MGMT. Therefore, a novel approach to MGMT inactivation was considered: by attaching a minor groove binding moiety to an MGMT inactivator, we reasoned that it might better present the inactivating moiety to the active site of the protein. The possibility that the minor groove binding characteristics of the compound might result in an attenuation of the cooperative binding of MGMT[7] and reduce the overall rate of repair of cytotoxic DNA damage, was also considered.

Here we describe the modelling, synthesis and biochemical assessment of 4-nitropyrimidine derivatives that were intended to act as MGMT inactivators coupled to di-aromatic amino derivatives which could interact with the DNA minor groove (Fig. 1). The results obtained for the corresponding morpholine derivatives (**1** and **2** in Fig. 1) are also presented and it has to be noted that the ring numbering of these compounds is different that the rest of the pyrimidines here presented. Henceforth, and for the sake of clarity, we will use the ring numbers shown for compounds **3** and **4** in Figure 1 when referring to ring substitution for all compounds.

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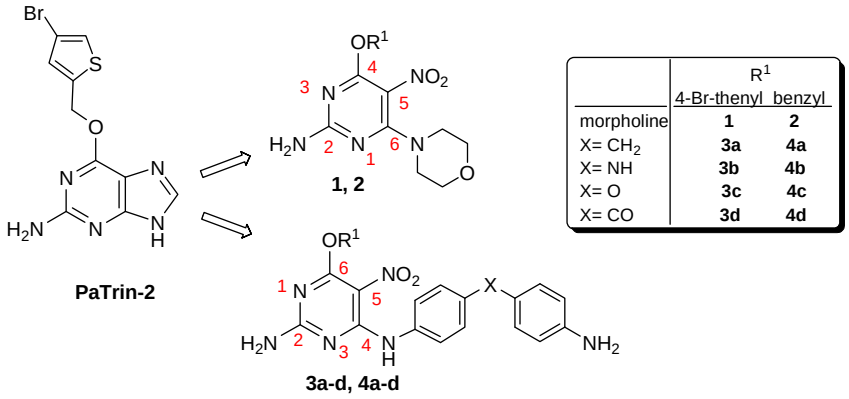


Figure 1. Structure of PaTrin-2 and the nitro derivatives **1**–**4** proposed in this study.

2. Results

2.1. Molecular modelling

To predict the affinity of the proposed compounds for MGMT and to gain insight into the forces operating in the MGMT–ligand complexes, a molecular modelling study was undertaken. PaTrin-2 was included to allow a better understanding of the corresponding MGMT activity. Thus, docking studies were carried out based on the crystal structure of MGMT[12] as a template and using the AUTODOCK4.2 package.[13] Fully optimized structures of the ligands (using B3LYP/6-31G[14]) were used for the docking experiments with the rigid structure of MGMT in a ligand-flexible approach. A detailed interaction analysis for each MGMT–ligand complex was then performed, and the binding energies were evaluated using a scoring function, based on the AMBER force-field that considers van der Waals' and electrostatic interactions.[15]

The repair mechanism of MGMT involves the transfer of an alkyl residue from guanine to the Cys145, and Pegg and co-workers[16] proposed that this Cys145 is highly reactive and exists as a thiolate anion at neutral pH. Thus, the interaction of the S atom in the Cys145 with the ether linkage of PaTrin-2 is crucial for the inactivation process to occur. Figure 2 shows the results obtained for the docking experiment of PaTrin-2 into the MGMT protein indicating how the ether O atom and the CH_2 group of the thenyl moiety are close to this Cys145.

LIGPLOT software (v. 4.4.2) was used to analyse the docking results, helping to determine the interactions formed between ligand and residues in the active site. This program takes into account hydrogen bonding (by means of distance cut-offs) and hydrophobic interactions and, despite its simplicity, provides a good insight of the interactions established within the complexes: the result obtained for the MGMT:PaTrin-2 complex is shown in Figure 3a. Several hydrophobic contacts are established between the 4-bromothenyl or the purine group and various MGMT residues (Met134, Val148, Asn157, Gly160 Tyr114 and Gly156). In general, the PaTrin-2 molecule is totally surrounded by hydrogen bond contacts, which indicates very good packing in the binding site of the protein, conferring substantial stability to the complex.

Details of the interactions observed in the docking of all the compounds studied and the active site of MGMT are presented in the Supplementary data section and here only the general trends will be discussed. In Figure 3, the plots obtained for compounds **1**, **2**, **3b** and **4b** are shown, as examples of the docking experiments performed and the interactions established.

Docking studies also provide information about the role of the amino acid residues in the active site of the binding process and the type of interactions involved. We found that some amino acids in the MGMT binding pocket are always involved in the interactions established with the ligands to form the complexes. For example, Pro140 was found to form hydrophobic interactions with all of the compounds except PaTrin-2. In all of the complexes,

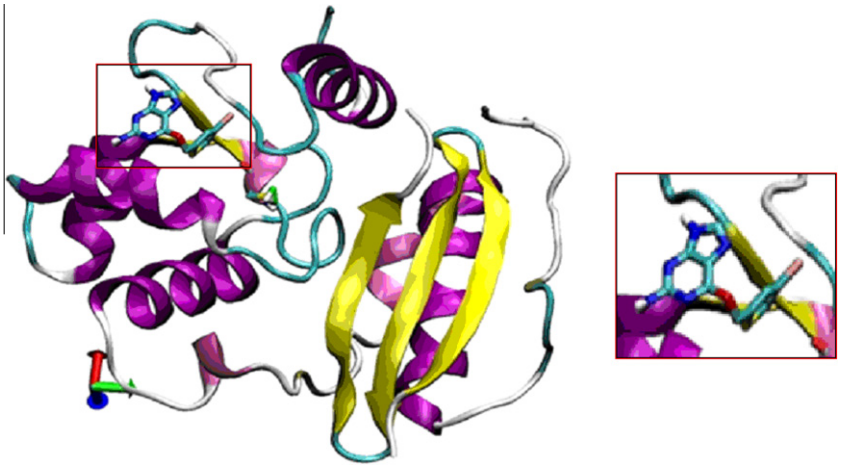


Figure 2. 3D-Representation using Rasmol (v.2.6) of the MGMT:PaTrin-2 complex obtained from molecular docking. The ether O atom of PaTrin-2 (red) can be found near the Cys145 (yellow).

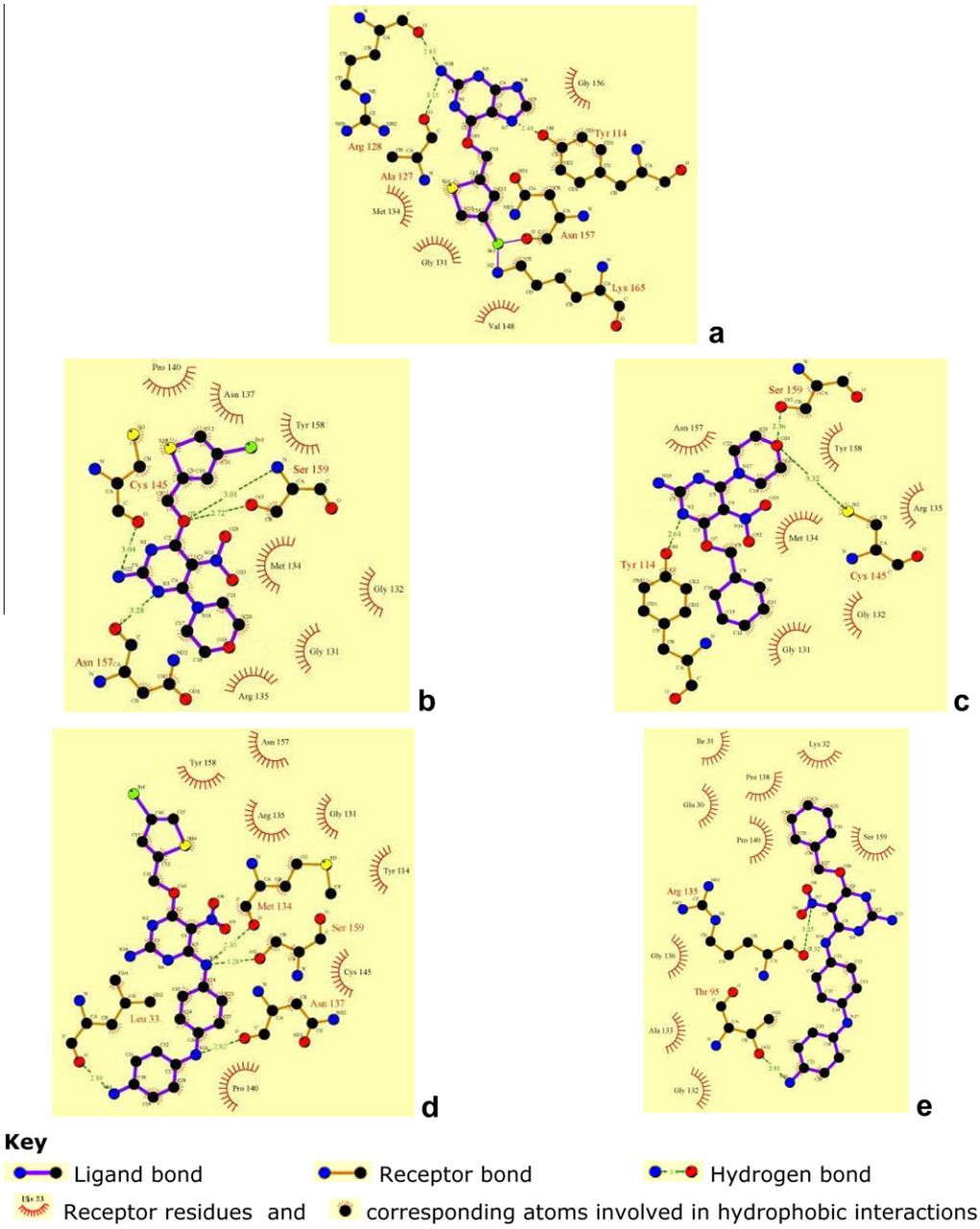


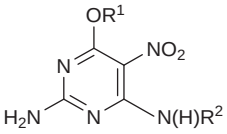
Figure 3. LIGPLOT representation. of the docking of (a) PaTrin-2, morpholine derivatives (b) **1** and (c) **2**, and diaromatic derivatives with NH linker (d) **3b** and (e) **4b**, into the MGMT binding pocket.

residues Arg135 and Ser159 were found to form hydrophobic or hydrogen bond interactions depending on the ligand. Interestingly, Tyr158 is involved in hydrophobic interactions only in complexes with 4-bromothenyl derivatives. The key residue, Cys145 appears to be involved in the formation of all the complexes studied but not always establishing interactions near the 4-bromothenyl or benzyl groups. We can consider the interaction of these residues ligand-specific because the involvement of the rest of the amino acid residues cannot be rationalised.

The binding energies obtained with the scoring function, which should be considered only as an indication of the potential of these compounds as MGMT inactivators, are shown in Table 1. An important conclusion is the strong interaction observed for the lead compound, PaTrin-2, followed by compounds **1** and **2** (morpholine substituted nitropirymidines) which show half of this binding strength. Thus, a better MGMT inhibitory activity would be expected for those compounds in comparison with the rest, which demonstrated much weaker binding. Additionally, larger interaction energy values are obtained for the 4-bromothenyl derivatives compare to the corresponding benzyl derivatives, in agreement with the best inactivating activity previously observed experimentally with PaTrin-2 and its 6-benzyloxy analogue.[17] The diaromatic compounds (**3a–d** and **4a–d**) are significantly poorer ligands, in agreement with the poor fit of the aromatic rings into the MGMT binding pocket due to steric hindrance. The significant improvement in interaction energy of the morpholino derivatives compared to the amino di-aromatic compounds could be explained because the attached group (morpholine) at the 4 position of the 5-nitropyrimidine is less bulky, more flexible and fits better into the active site of the protein.

In general, positive results were obtained from the docking experiments with PaTrin-2 and all the compounds proposed,

Table 1
IC_{50} values for inactivation of MGMT by compounds **1**, **2**, **3a–d** and **4c–d**, the 4-unsubtituted compound (**11**) and PaTrin-2 and docking binding energies calculated with AUTODOCK 4.2 for compounds **1**, **2**, **3a–d**, **4a–d** and PaTrin-2



Compound	R_1[a]	R_2	IC_{50} (μM)	Binding E (kcal mol^{-1})
1	BrTn	Morpholine–	1.0	−4.44
2	Bn	Morpholine–	19.0	−4.49
3a	BrTn	H_2N–Ph–CH_2–Ph–	20.0	−4.16
3b	BrTn	H_2N–Ph–NH–Ph–	4.7	−3.50
3c	BrTn	H_2N–Ph–O–Ph–	13.0	−1.52
3d	BrTn	H_2N–Ph–CO–Ph–	5.7	−1.98
4a	Bn	H_2N–Ph–CH_2–Ph–	—[b]	−2.67
4b	Bn	H_2N–Ph–NH–Ph–	—[b]	−1.80
4c	Bn	H_2N–Ph–O–Ph–	>600[c]	−0.77
4d	Bn	H_2N–Ph–CO–Ph–	>600[c]	−2.83
11	BrTn	H–	0.32	—
PaTrin-2			0.003	−8.95

[a] Bn = benzyl; BrTn = 4-bromothenyl.
[b] These compounds could not be tested because lack of material due to their difficult preparation.
[c] These compounds showed no inactivation at the highest concentration used in the assay (600 μM).

indicating stabilising interactions with the MGMT active site and for that reason we proceeded to prepare the new nitropyrimidines **1–4**.

2.2. Chemistry

The preparation of nitropyrimidines as analogues of O^6-benzylguanine has previously been reported.[18,19] In this work we present the preparation of the corresponding O^6-4-bromothenyl and O^6-benzyl nitropyrimidines introducing moieties at the amino group at the 4-position that we considered would interact with the DNA minor groove putatively presenting the MGMT inactivator moiety more effectively to the active site of the protein. To prepare these new derivatives, the synthesis of compound **5** is absolutely essential and we have already reported an efficient pathway for its synthesis.[20]

Starting from compound **5**, two possible routes could be considered to obtain the final nitropyrimidines that are conveniently functionalised at the 4-position. That is the subsequent substitution of each of the Cl atoms by amines and alkoxyde groups, or inversely.

For the first route, compound **5** was treated with sodium methoxide, as a model alkoxide for the more expensive 4-bromothenyl alcohol. Even at room temperature, the displacement of both of the chlorines of pyrimidine **5** by methoxide was observed. Since the selectivity of the reaction could not be controlled and the desired product was obtained in a very poor yield, the alternative route was investigated (Scheme 1).

The morpholino pyrimidine **6** was prepared using excess of morpholine in acetone. We found that this compound was obtained in higher yield at reflux temperature, whereby the disubstituted derivative was not detected in any of the experiments. Nevertheless, this was expected due to the lower nucleophilicity of secondary amines over alkoxyde groups. Further incorporation of 4-bromothenyloxy and benzyloxy groups into the morpholino derivative **6** was achieved using an improved version of the method previously reported by Pegg et al.[10] These authors reported the displacement of chlorine in 2-amino-4-chloro-5-nitropyrimidine to afford 2-amino-4-benzyloxy-5-nitropyrimidine at high temperatures (160 °C) with sodium in excess benzyl alcohol, also used as solvent. However, this approach would not be desirable for the expensive 4-bromothenyl alcohol and, hence, we used an equimolar amount of benzyl or 4-bromothenyl alcohol and sodium hydride in DMSO as solvent. Applying this protocol, compounds **1** and **2** were finally prepared (Scheme 1).

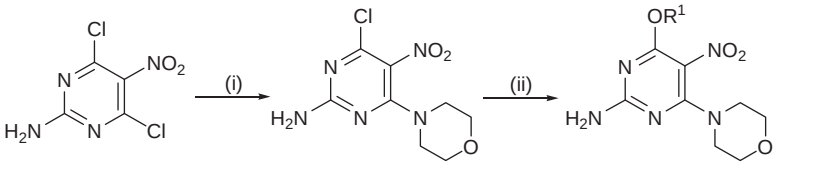
Having optimised the introduction of both the substituent in the 4-position and, then, the alkoxy moieties in the 6-position, we focused on the incorporation of aryl diamines, such as **7a–d** (commercially available), to the 4 position of the 5-nitropyrimidine **5** (Scheme 2). These amines could be positively charged at physiological pH and possess a concave shape, two important factors involved in DNA minor-groove recognition,[21] as has been investigated in our laboratory.[22] However, when compound **5** was treated with one equivalent of bis-(4-aminophenyl)methylene (**7a**) and triethylamine, a yellow precipitate was obtained that proved by NMR analysis to be the symmetric di-pyrimidine **8** (Scheme 2). Lower reaction temperatures or higher dilution of the reagents also led to the formation of this insoluble precipitate, and the desired monopyrimidine derivative was not detected. Thus, the selective introduction of one alkoxy group into **5** prior to the coupling reaction with these aryl amines was investigated.

The mono alkoxylation of **5** was performed using an equivalent amount of the corresponding alcohol and sodium hydride under the reactions conditions herein reported for the synthesis of **1** and **2**. However, the reaction was carried out in THF, due to the problems encountered in the purification of the products when DMSO was used. Optimization of the displacement of only one chlorine atom was required as substantial amounts of the disubstituted product were also obtained as previously reported. The reaction temperature was lowered to 0 °C to control the reactivity of the alkoxide and subsequent isolation of the compounds **9** and **10** (Scheme 2) was accomplished by successive column chromatographies on silica gel as the products and unreacted starting material have similar R_f values.

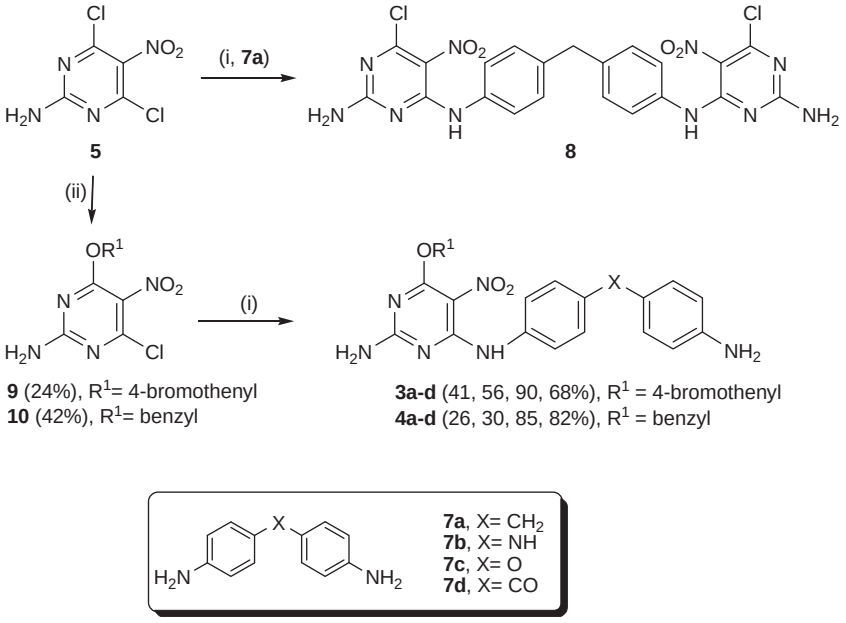
The final target compounds **3a–d** and **4a–d** (Scheme 2) were obtained in moderate to high yield (41–90%) from the addition reaction of compounds **9** and **10**, respectively to the corresponding commercial diaryl amines, **7a–d**.

2.3. Biochemistry

The 5-nitropyrimidines **1**, **2**, **3a–d**, and **4c–d** were tested for their ability to inactivate purified recombinant MGMT in vitro.



Scheme 1. Reagents and conditions: (i) morpholine in acetone at reflux, 1.5 h; (ii) R^1OH, NaH in DMSO, 80 °C, 2 h.



Scheme 2. Reagents and conditions: (i) **7a–d**, TEA in refluxing THF; (ii) R^1OH, NaH in THF, 0 °C.

PaTrin-2 was used as a positive control in all assays and the result obtained for the 4-unsubstituted O^6-(4-bromothenyl) derivative (**11**) was considered for comparison.[19] The resulting IC_{50} values are presented in Table 1. Compounds **4c** and **4d** were unable to inactivate MGMT at the highest concentrations used and only can be concluded that the IC_{50} for these compounds is >600 μM.

All other compounds showed much greater inactivation with IC_{50} values ranging from 1 to 20 μM (Table 1). The 2-amino-4-(4-bromothenyloxy)-6-morpholino-5-nitropyrimidine **1** was the most active compound synthesised, but substitution by polyaromatic amines in the benzyloxy derivatives (**4c** and **4d**), as just mentioned, clearly obliterated activity. This demonstrates that bulky groups at the 4-position of 5-nitropyrimidines negatively affect MGMT-inactivation capacity. The difficult preparation of the less accessible benzyloxy analogues **4a** and **4b** (X = CH_2, NH) did not provide enough material for their biochemical evaluation; however, it seems likely that they would follow the same pattern.

In the case of the morpholino derivatives, the benzyloxy compound **2** exhibited an activity approximately 20 times lower than the corresponding 4-bromothenyloxy derivative **1**. This trend was previously observed in our laboratory, since PaTrin-2 was found to be ten times more potent an inactivator than the corresponding O^6-benzylguanine (IC_{50} 0.04 μM).[16,23]

These results also indicate that, in general, the substitution at the 4-position of the pyrimidine ring is not well tolerated, since the introduction of morpholine resulted in a poorer biological activity. The 4-bromothenyloxy derivative unsubstituted in 4-position (**11**) previously prepared by our laboratory,[17,24] was more potent than any of the substituted versions (Table 1), suggesting as well that the 4-position is not best suited to derivatisation.

Thus far, no clear pattern has emerged by the comparison of the IC_{50} values of the amino diaryl compounds (**3**). However, it seems that those compounds with diaryl moieties containing groups that can form strong hydrogen bonds (X = NH, CO) were better MGMT inactivators and this may be a consequence of interactions with the amino acid residues involved in the MGMT binding process. However, it must be noted that compared to the IC_{50} value for PaTrin-2 (0.003 μM), all of the present compounds showed a very poor activity.

To assess the ability of the conjugated MGMT-inactivator/minor-groove binder derivatives (compounds **3** and **4**) to bind to DNA, we tried to perform thermal denaturation experiments in salmon sperm DNA (68% Adenine–Thymine base pair content). Unfortunately, these families of molecules were not soluble in the buffers tested (phosphate, MES, Tris) or in mixtures of DMSO or ethanol and the buffers. Hence, it was not possible to measure the ability of these compounds to bind to DNA.

3. Conclusions

Searching for novel MGMT inactivators that can also bind to the DNA minor groove, we have performed docking experiments with PaTrin-2 and two series of 2-amino-6-aryloxy-5-nitropyrimidines with (a) morpholino or (b) aminodiaryl (known minor-groove binder skeletons) substituents at the 4-position. Based on the computed stabilising interactions of these compounds with the MGMT active site, we synthesised both series of nitropyrimidines.

Thus, starting from 2-amino-4,6-dichloro-5-nitropyrimidine it was possible to prepare the morpholino derivatives by first substituting one of the Cl groups by morpholine and then introducing the aryloxy group by substitution of the second Cl group. In the case of the diaryloxy derivatives, the prerequisite was the introduction of the aryloxy group followed by the substitution of the second Cl group by the corresponding diaryldiamino compound.

Biochemical evaluation of these compounds as MGMT inhibitors demonstrated poor activities. The IC_{50} for the benzyloxy diaromatic compounds, **4c** and **4d**, is greater than 600 μM. All of the other derivatives, morpholino (**1** and **2**) and 4-bromothenyloxy amino diaryl (**3a–d**), showed better inactivation (IC_{50}s between 1 and 20 μM).

All of the compounds prepared had substantially higher IC_{50}s than PaTrin-2 and while the lack of the guanine core skeleton in these dual agents might explain this, previous nitro derivatives (both benzyloxy- and 4-bromothenyloxy-) have shown reasonably potent MGMT inactivation.[18,24] This might suggest that, even if binding of our compounds to DNA was weak, it could have the effect of reducing the compound's ability to inactivate MGMT: for alkyl transfer from alkyguanines in DNA, MGMT rotates the damaged base around the phosphodiester linkages and into the MGMT active site pocket, while an arginine residue occupies the vacated site in the helix. This may not be possible if the base is part of a structure that is bound to DNA, possibly in an

inappropriate topolgy, rather than being an integral part of the DNA molecule. On the other hand, if DNA binding is involved, for the present compounds, this may be too weak and hence much stronger anchoring to DNA might be necessary to facilitate MGMT inactivation.

In this context the actual nature of the minor-groove binding moieties may be important since these structures have been shown to better interact with the minor groove when cationic amidine-like groups are incorporated at both sides of the diaromatic system.[21,25] Finally, poor activity could be due to a non-optimal distance between the two functionalities (minor-groove binding and MGMT inactivation), incorporated in these molecules: linkers between these functions might therefore be incorporated in future dual agents.

4. Experimental

4.1. Modelling

The crystal structure of MGMT (PDB entry: 1QNT, resolution 1.90 Å) was used to derive a model for ligand binding in the active site. To reduce the required computational time for docking, we described the active site around the Cys145 residue, which is located within the residues 83–176 of the C-terminal domain. Hydrogen atoms were added to the crystal structure assuming a pH of 7, using the SYBYL program (v.7.2). Point charges were assigned to the MGMT protein according to the TRIPOS force-field. SYBYL (v.7.2) was used for the representation of the ligands, using the Gasteiger-Hückel algorithm for the calculation of the charges. The AUTODOCK program (v. 4.2)[12] was then used to perform the docking study. The residues Tyr114, Gly131, Met134, Arg135, Asn137, Pro140, Cys145, Val148, Tyr158, Ser159 and Gly160 were considered essential for the binding process, as previously reported by Wiessler and co-workers[26] we selected the centre of these residues as the position of the active site and centre of this grid (coordinates XYZ: 1.440, 41.180, 37.690). The grid point spacing was defined as 0.2 Å. Finally, the LIGPLOT program (v.4.4.2) was used for determining and plotting a 2D representation of the ligand–protein interaction.

4.2. Chemistry

All the commercial chemicals were obtained from Sigma–Aldrich or Fluka and were used without further purification. Deuterated solvents for NMR use were purchased from Apollo. Dry solvents were prepared using standard procedures, according to Vogel,[27] with distillation prior to use. Chromatographic columns used Silica Gel 60 (230–400 mesh ASTM). Solvents for synthesis purposes were used at GPR grade. Analytical TLC was performed using Merck Kieselgel 60 F254 silica gel plates. Visualisation was by UV light (254 nm). NMR spectra were recorded in a Bruker DPX-400 Avance spectrometer, operating at 400.13 MHz and 600.1 MHz for ^{1}H NMR; and at 100.6 MHz and 150.9 MHz for ^{13}C NMR. Shifts are referenced to the internal solvent signals and *J* values recorded in Hertz. NMR data were processed using Bruker Win-NMR 5.0 software. HRMS spectra were measured on a Micromass LCT electrospray TOF instrument with a WATERS 2690 autosampler with methanol, water or ethanol as carrier solvents. Melting points were determined using a Stuart Scientific Melting Point SMP1 apparatus and are uncorrected. Infrared spectra were recorded on a Mattson Genesis II FTIR spectrometer equipped with a Gateway 2000 4DX2-66 workstation and on a Perkin Elmer Spectrum One FT-IR Spectrometer equipped with Universal ATR sampling accessory. Samples analysis was carried out in Nujol using NaCl plates. Elemental analyses were carried out at the Microanalysis Laboratory, School of Chemistry and Chemical Biology, University College Dublin and at the Instituto de Química Médica (CSIC) in Madrid, Spain.

4.2.1. 2-Amino-4-chloro-6-morpholino-5-nitropyrimidine (6)

A solution of morpholine (479 mg, 5.5 mmol) in acetone (10 mL) was added dropwise to a stirred solution of 2-amino-4, 6-dichloro-5-nitropyrimidine (418 mg, 2 mmol) in acetone (10 mL), and the reaction mixture was refluxed for 1.5 h. The solvent was removed in vacuum and the residue was treated with water (10 mL). The suspension was filtered off and the yellow solid obtained was washed with water, suction-dried and collected (459 mg, 88%). mp 210–212 °C. ^{1}H NMR (DMSO-d_6) δ 3.37 (t, 4H, *J* 4.9, NCH_2CH_2O); 3.63 (t, 4H, *J* 4.9, NCH_2CH_2O); 7.49 (s, 1H, NH); 7.60 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ 46.6 (NCH_2CH_2O); 65.8 (NCH_2CH_2O); 122.0 (C5); 153.5, 156.8, 159.9 (C2, C4, C6). IR ν_{max} 3470, 3326, 3211 (NH_2); 1549 (NO_2) cm^{-1}. HRMS (ES) $[M+H]^+$ calcd for $C_8H_{11}ClN_5O_3$ 260.0550; found 260.0548. Anal. Calcd for $C_8H_{10}ClN_5O_3$: C, 37.0; H, 3.9; N, 27.0. Found: C, 37.0; H, 4.0; N, 26.7.

4.2.2. General procedure for the preparation of 4-alkoxy-6-morpholino derivatives

Sodium hydride (25 mg, 1 mmol) was added to a stirred solution of the corresponding alcohol (1.2 mmol) in dry DMSO (1 mL) under an argon atmosphere. Effervescence was observed due to the formation of hydrogen gas. After 30 min, **6** (260 mg, 1 mmol) was added in small portions and the reaction mixture was heated to 80 °C for 2–3 h. DMSO was removed in high vacuum and the residue was treated with water (4 mL), giving a viscous product.

4.2.2.1. 2-Amino-4-(4-bromothenyloxy)-6-morpholino-5-nitropyrimidine (1). The product was recrystallised from a mixture methanol/water to afford a yellow solid (191 mg, 46%). mp 152–154 °C. ^{1}H NMR (DMSO-d_6) δ 3.35–3.45 (m, 4H, NCH_2CH_2O); 3.55–3.75 (m, 4H, NCH_2CH_2O); 5.52 (s, 2H, OCH_2ThBr); 7.29 (br s, 3H, H3′, NH_2); 7.70 (s, 1H, H5′). ^{13}C NMR (DMSO-d_6) δ 46.9 (NCH_2CH_2O); 62.2, 66.0 (OCH_2Th, NCH_2CH_2O); 108.2, 111.5 (C4′, C5); 125.6 (C5′); 131.0 (C3′); 140.2 (C2′); 158.34, 160.0, 162.9 (C2, C4, C6). IR ν_{max} 3475, 3337, 3225 (NH_2); 1545 (NO_2) cm^{-1}. HRMS (ES) $[M+Na]^+$ calcd for $C_{13}H_{14}BrN_5O_4SNa$ 437.9848; found 437.9848. Anal. Calcd for $C_{13}H_{14}BrN_5O_4S$: C, 37.5; H, 3.4; N, 16.8. Found: C, 37.3; H, 3.4; N, 16.4.

4.2.2.2. 2-Amino-4-benzyloxy-6-morpholino-5-nitropyrimidine (2). The product was purified by column chromatography on silica gel (3:1 EtOAc/hexane) to give a bright yellow solid (137 mg, 42%). Mp 170–172 °C. ^{1}H NMR (DMSO-d_6) δ 3.36 (t, 4H, *J* 4.4, NCH_2CH_2O); 3.62 (t, 4H, *J* 4.4, NCH_2CH_2O); 5.39 (s, 2H, $OCH_2C_6H_5$); 7.23 (br s, 2H, NH_2); 7.32–7.42 (m, 5H, $OCH_2C_6H_5$). ^{13}C NMR (DMSO-d_6) δ 48.9 (NCH_2CH_2O); 66.0, 68.0 ($OCH_2C_6H_5$, NCH_2CH_2O); 111.7 (C5); 127.9, 128.1, 128.6, 136.4 ($OCH_2C_6H_5$); 158.3, 160.3, 163.5 (C2, C4, C6). IR ν_{max} 3469, 3336, 3223 (NH_2); 1548 (NO_2) cm^{-1}. HRMS (ES) $[M+Na]^+$ calcd for $C_{15}H_{17}N_5O_4Na$ 354.1178; found 354.1178. Anal. Calcd for $C_{15}H_{17}N_5O_4$: C, 54.4; H, 5.2; N, 21.1. Found: C, 54.5; H, 5.2; N, 20.7.

4.2.3. 2-Amino-4-(4-bromothenyloxy)-6-chloro-5-nitropyrimidine (9)

Sodium hydride (96 mg, 4 mmol) was added to a stirred solution of 4-bromothenyl alcohol (772 mg, 4 mmol) in dry THF (30 mL) under an argon atmosphere. Effervescence was observed. After 30 min, the mixture was cooled to 0 °C, followed by the addition of **5** (1.24 g, 6 mmol). The mixture was stirred overnight (the temperature rose from 0 °C to 13 °C during that period). Aqueous THF (10 mL) was added dropwise and the solvent was removed leaving a sticky oily residue. Hexane (30 mL) was added to form

a yellow solid, which was filtered and washed with hexane. The solid formed was purified by column chromatography on silica gel (2:3 EtOAc/hexane) to give a bright yellow solid (342 mg, 24%). mp 146–148 °C. ^{1}H NMR (DMSO-d_6) δ 5.61 (s, 2H, OCH_2ThBr); 7.34 (s, 1H, H3′); 7.75 (s, 1H, H5′); 8.12 (s, 1H, NH); 8.21 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ 63.3 (OCH_2ThBr); 108.4 (C4′); 123.0, 126.0, 131.7, 139.0 (C5–NO_2, C3′, C2′, C5′); 152.8, 160.5, 161.7 (C2, C4, C6). IR ν_{max} 3466, 3422, 3305 (NH_2); 1543 (NO_2) cm^{-1}. HRMS (ES) [M+H]$^+$ calcd for $C_9H_7BrClN_4O_3S$ 364.9111; found 364.9120.

4.2.4. 2-Amino-4-benzyloxy-6-chloro-5-nitropyrimidine (10)

Sodium hydride (96 mg, 4 mmol) was added to a stirred solution of benzyl alcohol (432 mg, 4 mmol) in dry THF (30 mL) under an argon atmosphere. Effervescence was observed. After 1 h, the mixture was cooled to 0 °C, followed by the addition of **5** (1.09 g, 5.2 mmol). The mixture was stirred overnight (the temperature rose from 0 °C to 13 °C during that period). Water (5 mL) was added dropwise to quench the excess of base. THF was removed in vacuum and the residue was treated with EtOAc, washed with water and dried over Na_2SO_4. Filtration and evaporation of the solvent left a brown residue which was purified by column chromatography on silica gel (1:2 EtOAc/hexane) to afford a bright yellow solid (476 mg, 42%). Mp 168–170 °C. ^{1}H NMR (DMSO-d_6) δ 5.46 (s, 2H, $OCH_2C_6H_5$); 7.27–7.50 (m, 5H, $OCH_2C_6H_5$); 8.06 (s, 1H, NH); 8.13 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ 69.3 ($OCH_2C_6H_5$); 123.1 (C5), 128.1, 128.4, 128.5, 135.4 ($OCH_2C_6H_5$); 152.6 160.7, 162.2 (C2, C4, C6). IR ν_{max} 3478, 3318, 3204 (NH_2); 1544 (NO_2) cm^{-1}. HRMS (ES) [M+H]$^+$ calcd for $C_{11}H_{10}ClN_4O_3$ 281.0441; found 281.0440.

4.2.5. General procedure for the preparation of 4-(4-bromothenyloxy)-6-diaromatic derivatives, 3

A solution of the corresponding diamine (0.8 mmol) in THF (6 mL) was added to a stirred solution of **9** (73 mg, 0.2 mmol) and triethylamine (112 μL, 0.8 mmol) in THF (6 mL). The mixture was refluxed for 2.5–48 h, depending of the starting diamine. THF was removed, water (20 mL) was added and the product was extracted with EtOAc (2 × 15 mL), washed with water and dried over Na_2SO_4.

4.2.5.1. 2-Amino-4-(4-bromothenyloxy)-6-[N-(4-(4-aminobenzyl)anilino)]-5-nitropyrimidine (3a). Filtration and evaporation of the solvent left a yellow residue, which was purified by column chromatography on silica gel (2:1 EtOAc/hexane) to obtain a yellow solid (43 mg, 41%). Mp 172–174 °C. ^{1}H NMR (DMSO-d_6) δ 3.73 (s, 2H, $C_6H_4CH_2C_6H_4$); 4.93 (br s, 2H, $C_6H_4NH_2$); 5.60 (s, 2H, ThBrCH_2O); 6.50 (d, 2H, *J* 8.5, H3″); 6.89 (d, 2H, *J* 8.5, H2″); 7.15 (d, 2H, *J* 8.5, H3′); 7.34 (s, 1H, H3‴); 7.53 (d, 2H, *J* 8.5, H2′); 7.65 (s, 1H, NH); 7.67 (s, 1H, NH); 7.71 (s, 1H, H5‴); 10.4 (s, 1H, NHC_6H_4). ^{13}C NMR (DMSO-d_6) δ 40.0 ($C_6H_4CH_2C_6H_4$); 62.44 (ThBrCH_2O); 108.2, 109.8 (C$_5$–NO_2), 114.2, 123.4, 125.6, 128.5, 128.7, 129.4, 131.0, 135.7, 139.1, 140.1, 146.8 (C5–NO_2, ThBr, C_6H_4); 156.5, 160.4, 164.2 (C2, C4, C6). IR ν_{max} 3450, 3409 (NH_2); 3336, 3296 (NH_2); 1554 (NO_2) cm^{-1}. HRMS (ES) [M+H]$^+$ calcd for $C_{22}H_{20}BrN_6O_3S$ 527.0423; found 527.0432. Anal. Calcd for $C_{22}H_{19}BrN_6O_3S$·0.2EtOAc: C, 50.3; H, 3.8; N, 15.4. Found: C, 50.4; H, 3.7; N, 15.45.

4.2.5.2. 2-Amino-4-(4-bromothenyloxy)-6-[N-(4-(4-aminophenylamino)anilino)]-5-nitropyrimidine (3b). Filtration and evaporation of the solvent gave a brown residue, which was purified by column chromatography on silica gel (2:1 EtOAc/hexane) to afford a brown solid (59 mg, 56%). Mp 200–202 °C. ^{1}H NMR (DMSO-d_6) δ 4.79 (br s, 2H, $C_6H_4NH_2$); 5.59 (s, 2H, ThBrCH_2O); 6.54 (d, 2H, *J* 8.5, H2″); 6.76 (d, 2H, *J* 8.5, H3′); 6.83 (d, 2H, *J* 8.5, H3″); 7.34 (s, 1H, H3‴); 7.37 (d, 2H, H2′); 7.54 (s, 1H, $C_6H_4NHC_6H_4$); 7.58 (br s, 2H, NH_2); 7.72 (s, 1H, H5‴); 10.4 (s, 1H, pyrimidine-NHC_6H_4). ^{13}C NMR (DMSO-d_6) δ 62.5 (ThBrCH_2O); 108.2, 109.6 (C$_5$–NO_2), 113.9, 115.1, 122.6, 124.9, 125.6, 128.0, 131.0, 132.0, 140.3, 144.2, 156.5, 158.8, 160.5, 164.4. IR ν_{max} 3451, 3411 (NH_2); 3331, 3218 (NH_2); 1552 (NO_2) cm^{-1}. HRMS (ES) [M+H]$^+$ calcd for $C_{21}H_{19}BrN_7O_3S$ 528.0375; found 528.0384. Anal. Calcd for $C_{21}H_{18}BrN_7O_3S$·0.5EtOAc: C, 48.3; H, 3.9; N, 17.1. Found: C, 48.25; H, 3.7; N, 16.9.

4.2.5.3. 2-Amino-4-(4-bromothenyloxy)-6-[N-(4-(4-aminophenoxy)anilino)]-5-nitropyrimidine (3c). The residue was purified by column chromatography on silica gel (2:1 EtOAc/hexane) to afford a bright yellow solid (95 mg, 90%). mp 202–204 °C. ^{1}H NMR (DMSO-d_6) δ 5.00 (br s, 2H, $C_6H_4NH_2$); 5.60 (s, 2H, ThBrCH_2O); 6.59 (d, 2H, *J* 8.5, H2″); 6.78 (d, 2H, *J* 8.5, H3″); 6.83 (d, 2H, *J* 9.0, H2′); 7.33 (s, 1H, H3‴); 7.57 (d, 2H, *J* 9.0, H2′); 7.64 (s, 2H, NH_2); 7.71 (s, 1H, H5‴); 10.4 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ 62.4 (ThBrCH_2O); 108.2, 109.7 (C$_5$–NO_2), 115.0, 116.6, 121.1, 125.0, 125.6, 131.0, 131.1, 140.1, 145.7, 145.8, 156.1, 156.5, 160.4, 164.2. IR ν_{max} 3430, 3416, 3337 (NH_2); 1559 (NO_2) cm^{-1}. HRMS (ES) [M+H]$^+$ calcd for $C_{21}H_{18}BrN_6O_4S$ 529.0215; found 529.0224 Anal. Calcd for $C_{21}H_{17}BrN_6O_4S$·0.2EtOAc: C, 47.9; H, 3.4; N, 15.4. Found: C, 47.9; H, 3.45; N, 15.0.

4.2.5.4. 2-Amino-4-(4-bromothenyloxy)-6-[N-(4,4′-aminobenzophenone)]-5-nitropyrimidine (3d). Filtration and evaporation of the solvent gave a yellow residue, which was purified by column chromatography on silica gel (2:1 EtOAc/hexane) to afford a bright yellow solid (74 mg, 68%). mp 196–198 °C. ^{1}H NMR (DMSO-d_6) δ 5.61 (s, 2H, ThBrCH_2O); 6.13 (br s, 2H, $C_6H_4NH_2$); 6.61 (d, 2H, *J* 8.5, H3″); 7.34 (s, 1H, H3‴); 7.54 (d, 2H, *J* 8.5, H2″); 7.61 (d, 2H, *J* 8.5, H3′); 7.72 (s, 1H, H5‴); 7.79 (s(br), 1H, NH); 7.82 (br s, 1H, NH); 7.89 (d, 2H, *J* 8.5, H2′); 10.6 (s, 1H, C_6H_4NH). ^{13}C NMR (DMSO-d_6) δ 62.6 (ThBrCH_2O); 108.2, 110.3 (C$_5$–NO_2), 112.7, 121.8, 124.1, 125.6, 130.0, 131.1, 132.6, 134.5, 140.0, 141.0, 153.8, 156.2, 160.4, 164.1; 192.6 (CO). IR ν_{max} 3483, 3415 (NH_2); 3367, 3321 (NH_2); 1653 (CO); 1531 (NO_2) cm^{-1}. HRMS (ES) [M+H]$^+$ calcd for $C_{22}H_{18}BrN_6O_4S$ 541.0215; found 541.0224. Anal. Calcd for $C_{22}H_{17}BrN_6O_4S$·0.2EtOAc: C, 49.0; H, 3.35; N, 15.0. Found: C, 49.0; H, 3.25; N, 15.0.

4.2.6. General procedure for the preparation of 4-benzyloxy-6-diaromatic derivatives, 4

A solution of the corresponding diamine (1 mmol) in THF (15 mL) or THF (10 mL)/DMF (2 mL) was added to a stirred solution of **10** (56 mg, 0.2 mmol) and triethylamine (139 μL, 1 mmol) in THF (5 mL) and the mixture was refluxed overnight or 48 h. THF, along with the excess of triethylamine, were removed on the rotary-evaporator and DMF was removed in high vacuum.

4.2.6.1. 2-Amino-4-benzyloxy-6-[N-(4-(4-aminobenzyl)anilino)]-5-nitropyrimidine (4a). The residue was treated with water and extracted with EtOAc (4 × 25 mL). The organic layer was washed with water, brine and dried over Na_2SO_4. Filtration and evaporation of the solvent gave a residue, which was purified by column chromatography on silica gel (2:1 EtOAc/hexane) to afford a yellow solid (23 mg, 26%). mp 210–212 °C. ^{1}H NMR (DMSO-d_6) δ 3.74 (s, 2H, $C_6H_5CH_2C_6H_5$); 4.86 (br s, 2H, $C_6H_4NH_2$); 5.47 (s, 2H, $C_6H_5CH_2O$); 6.48 (d, 2H, *J* 8.2, H3″); 6.87 (d, 2H, *J* 8.2, H2″); 7.16 (d, 2H, *J* 8.2, H3′); 7.33 (m, 1H, H4‴); 7.41 (t, 2H, *J* 7.0, H3‴); 7.50 (d, 2H, *J* 7.0, H2‴); 7.54 (d, 2H, *J* 8.2, H2′); 10.46 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ 40.3 ($C_6H_5CH_2C_6H_5$); 68.6 ($C_6H_5CH_2O$); 110.2 (C$_5$–NO_2); 114.4, 114.5, 123.6, 128.2, 128.4, 128.7, 128.9,

129.0, 129.6, 136.0, 136.5, 139.3, 147.2, 156.8, 161.0, 165.0. IR ν_{max} 3398, 3352 (NH_2); 3158 (NH_2); 1555 (NO_2) cm^{-1}. HRMS (ES) $[M+H]^+$ calcd for $C_{24}H_{23}N_6O_3$ 444.1753; found 444.1757. Anal. Calcd for $C_{24}H_{22}N_6O_3 \cdot 0.7EtOAc$: C, 63.9; H, 5.5; N, 16.7. Found: C, 63.6; H, 5.9; N, 16.9.

4.2.6.2. 2-Amino-4-benzyloxy-6-[N-(4-(4-aminophenylamino)anilino)]-5-nitropyrimidine (4b). The residue was purified by column chromatography on silica gel (2:1 EtOAc/hexane) to afford a brown solid (27 mg, 30%). mp 198–200 °C. ^1H NMR (DMSO-d_6) δ 4.30 (br s, 2H, $C_6H_4NH_2$); 5.59 (s, 2H, $C_6H_5CH_2O$); 6.50 (d, 2H, *J* 8.4, H2″); 6.75 (d, 2H, *J* 8.4, H3′); 6.80 (d, 2H, *J* 8.4, H3″); 7.48 (m, 5H, H4‴, H3‴, H2′); 7.51 (m, 5H, H2‴, $C_6H_5NHC_6H_5$, NH_2); 10.49 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ 68.5 ($C_6H_5CH_2O$); 110.0 (C5–NO_2); 114.0, 115.2, 122.7, 124.9, 128.2, 128.4, 128.9, 132.1, 136.6, 144.3, 144.5, 156.6, 160.9, 165.1. IR ν_{max} 3451 (NH_2); 3312, 3215 (NH_2); 1555 (NO_2) cm^{-1}. HRMS (ES) $[M+H]^+$ calcd for $C_{23}H_{22}N_7O_3$ 445.1706; found 445.1710. Anal. Calcd for $C_{23}H_{21}N_7O_3 \cdot 1.1EtOAc$: C, 60.9; H, 5.6; N, 18.1. Found: C, 61.4; H, 6.1; N, 18.5.

4.2.6.3. 2-Amino-4-benzyloxy-6-[N-(4-(4-aminophenoxy)anilino)]-5-nitropyrimidine (4c). The residue was purified by column chromatography on silica gel (2:1 EtOAc/hexane) to afford a bright yellow solid (76 mg, 85%). Mp 198–200 °C. ^1H NMR (DMSO-d_6) δ 4.91 (br s, 2H, $C_6H_4NH_2$); 5.37 (s, 2H, $C_6H_5CH_2O$); 6.51 (d, 2H, *J* 8.5, H2″); 6.79 (d, 2H, *J* 8.5, H3″); 6.74 (d, 2H, *J* 8.5, H2′); 6.74 (d, 2H, *J* 8.5, H3′); 7.26 (t, 1H, *J* 7.5, H4‴); 7.32 (t, 2H, *J* 7.5, H3‴); 7.38–7.44 (m, 2H, H2‴); 7.49 (m, 4H, H2′, NH_2); 10.3 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ 68.3 ($C_6H_5CH_2O$); 109.9 (C5–NO_2); 115.0, 116.6, 121.1, 125.0, 127.9, 128.1, 128.6, 132.2, 136.3, 145.7, 145.8, 156.0, 156.5, 160.7, 164.7. IR ν_{max} 3450, 3409 (NH_2); 3326, 3218 (NH_2); 1555 (NO_2) cm^{-1}. HRMS (ES) $[M+H]^+$ calcd for $C_{23}H_{21}N_6O_4$ 445.1624; found 445.1635. Anal. Calcd for $C_{23}H_{20}N_6O_4 \cdot 0.4EtOAc$: C, 61.6; H, 4.9; N, 17.5. Found: C, 61.5; H, 4.7; N, 17.8.

4.2.6.4. 2-Amino-4-benzyloxy-6-[N-(4,4′-diaminobenzophenone)]-5-nitropyrimidine (4d). The residue was treated with water and extracted with EtOAc (4 × 25 mL). The organic layer was washed with water, brine and dried over Na_2SO_4. Filtration and evaporation of the solvent gave a yellow residue, which was purified by column chromatography on silica gel (2:1 EtOAc/hexane) to afford a bright yellow solid (78 mg, 82%). mp 222–224 °C. ^1H NMR (DMSO-d_6) δ 5.48 (s, 2H, $C_6H_5CH_2O$); 6.15 (br s, 2H, $C_6H_4NH_2$); 6.61 (d, 2H, *J* 8.5, H3″); 7.36 (t, 1H, *J* 7.5, H4‴); 7.42 (t, 2H, *J* 7.5, H3‴); 7.49–7.58 (m, 4H, H2″, H2‴); 7.62 (d, 2H, *J* 8.5, H3′); 7.76 (s(br), 1H, NH); 7.78 (br s, 1H, NH); 7.91 (d, 2H, *J* 8.5, H2′); 10.7 (s, 1H, C_6H_4NH). ^{13}C NMR (DMSO-d_6) δ 68.4 ($C_6H_5CH_2O$); 110.5 (C_5–NO_2), 112.7, 121.7, 124.1, 127.9, 128.2, 128.6, 130.0, 132.6, 134.4, 136.2, 141.1, 153.8, 156.2, 160.7, 164.7; 192.6 (CO). IR ν_{max} 3484, 3414 (NH_2); 3376, 3316 (NH_2); 1632 (CO); 1538 (NO_2) cm^{-1}. HRMS (ES) $[M+Na]^+$ calcd for $C_{24}H_{20}N_6O_4Na$ 479.1444; found 479.1436. Anal. Calcd for $C_{24}H_{20}N_6O_4 \cdot 0.9EtOAc$: C, 61.9; H, 5.1; N, 15.7. Found: C, 61.9; H, 5.05; N, 15.9.

4.3. Biochemistry

Determination of the IC_{50} values for inactivation of MGMT by the study compounds was by a method previously described.[28] In brief, the compounds were dissolved at 10 mM in dry DMSO and serial dilutions prepared in DMSO. In each assay, fixed amounts of MGMT (50–60 fmol) were incubated with aliquots of the dilution series of test compound in a total volume of 200 μL of buffer containing 10 μg of calf thymus DNA at 37 °C for 1 h. Excess $[^3H]$-methylated DNA substrate (100 μL containing 4 μg of DNA and 100 fmol of O^6-$[^3H]$methylguanine) was added and incubation continued at 37 °C until the reaction was complete (1 h). For each assay, serial dilutions of PaTrin-2 were used as a positive control. Following the incubations, DNA was hydrolysed to acid solubility using 1 M perchloric acid and removed and washed once by centrifugation. The acid-insoluble $[^3H]$-methylated MGMT protein was quantitated by liquid scintillation counting. IC_{50} values were determined from the slope of the concentration-dependence plot and essentially represent the mean of 5 determinations.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.01.038.

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