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Triazoloacridin-6-ones as novel inhibitors of the quinone oxidoreductases NQO1 and NQO2

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ABSTRACT

A range of triazoloacridin-6-ones functionalized at C5 and C8 have been synthesized and evaluated for ability to inhibit NQO1 and NQO2. The compounds were computationally docked into the active site of NQO1 and NQO2, and calculated binding affinities were compared with IC_{50} values for enzyme inhibition. Excellent correlation coefficients were demonstrated suggesting a predictive QSAR model for this series of structurally similar analogues. From this we have identified some of these triazoloacridin-6-ones to be the most potent NQO2 inhibitors so far reported.

1. Introduction

The oxidoreductases, NAD(P)H:quinone oxidoreductase 1 (NQO1, DT-diaphorase, EC 1.6.99.2) and NRH:quinone oxidoreductase 2 (NQO2, EC 1.10.99.2), are homodimeric, cytosolic flavoproteins that catalyse the two electron reduction of quinones to hydroquinones.[1,2] Human NOQ1 and NQO2 share 54% and 49% cDNA and protein sequence identity, respectively.[3] Although both enzymes are expressed in many of the same tissues throughout the body, the highest expression of NQO1 is found in kidney, gut epithelium[4] and importantly, in many types of solid tumour including cancers of the colon, breast, lung and liver.[5] In contrast to NQO1, the NQO2 level in normal and malignant tissues is generally lower.[6] However, there are some cell types such as haemopoietic cells, where NQO2 is present and NQO1 is much reduced.[7,8]

Substrate specificity is different in the two enzymes, with NQO1 efficiently reducing 1,4-quinones,[9] whereas NQO2 can reduce *ortho*-quinones[10] and aromatic nitro compounds[11] as well as binding tyrosine kinase inhibitors such as imatinib.[12] The reductase properties of the enzymes have been exploited in cancer therapy with NQO1 being used to selectively activate quinone-based drugs such as mitomycin C, EO9 and RH1.[13] In contrast, NQO2 has been shown to activate the dinitrobenzene CB1954, which has led to the renewed evaluation of this drug in cancer chemotherapy.[14]

A common property of the two enzymes is their apparent ability to protect the tumour suppressor p53 against proteosomal degradation, which leads to stabilization and activation of p53.[15] Inhibition of NQO1 enzyme activity leads to destabilization of p53 and similar NQO1-mediated effects have been observed for other short-lived proteins including ornithine decarboxylase and p73α.[15–17] Additional effects of modulating NQO2 activity have been shown to alter signaling via the NF-κB pathway.[18] Thus, identification of novel, potent inhibitors of NQO1 and/or NQO2 could provide routes to gaining a better understanding of the cellular roles of these enzymes.

We recently applied a virtual screening strategy of the National Cancer database to identify novel inhibitors of NQO1.[19,20] This approach resulted in the discovery of the triazoleacridin-6-one, NSC645827, as a submicromolar inhibitor of NQO1.[19] We report here a study where we have synthesised a series of compounds structurally related to NSC645827 and evaluated their ability to inhibit NQO1. In addition, we have tested the ability of this series of compounds to inhibit the function of NQO2. We have further used computational molecular docking to identify structure/activity relationships (SAR) for inhibition of both NQO1 and NQO2 by these triazoloacridin-6-ones. We have then evaluated the compounds for their ability to interact with DNA and related this to their toxicity towards HCT116 colon cancer cells that show significant activity of both NQO1 and NQO2.

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2. Chemistry

Synthesis of a range of triazoloacridin-6-ones (Scheme 1), wherein there are two loci for diversification (positions 5 and 8), was carried out using an approach modelled on that described by Cholody et al.[23] The key intermediates in the syntheses were the 1-chloro-4-nitro acridones **2a–d** which were prepared using general methods detailed in the literature.[21,22] Reduction of the nitro-group of compounds **2a–d** was readily accomplished using stannous chloride in ethanolic HCl to furnish the corresponding 4-amino acridones **3a–d** in good yield. Exposure of the latter compounds to sodium nitrite in concd HCl at room temperature resulted in sequential diazotisation and cyclisation to give the triazoloacridin-6-ones **4a–d**. In the first instance, nucleophilic substitution of the chlorine atom at C5 of compounds **4a–d** was carried out, at slightly elevated temperature, using *N*,*N*-dimethylamino-ethylenediamine (**5**) in dimethylacetamide to give the 5-amino-triazoloacridin-6-ones **5a–d**. Subsequent oxidation of the terminal side-chain nitrogen was then accomplished using *m*CPBA in chloroform to give the *N*-oxides **10a–d**. Similarly, the remaining analogues (**6a–d, 7a–d, 8a–d, 9a–d**), bearing a variety of different amino substituents at C5, were prepared via exposure of compounds **4a–d** to the appropriate amine (**6–9**) at elevated temperature in dimethylacetamide.

3. Molecular modeling, biochemical and biological evaluation

Fifteen compounds from this series were assayed for their ability to inhibit purified recombinant human NQO1 and NQO2 (Table 1). The selection criterion for these compounds was based solely on their solubility.

The compounds were computationally docked into the active site of NQO1 and NQO2 using the X-ray crystal structures of human NQO1 with bound FAD (PDB code 1D4A, resolution at 1.7 Å)[25] and human NQO2 with bound FAD (PDB code 1QR2, resolution at 2 Å),[26] respectively. After docking, the calculated binding affinities (using the ChemScore[27] scoring function in GOLD 4.1) and experimental binding affinities, derived from IC_{50} values,[28] were compared for each enzyme (Table 1). Both enzymes demonstrate excellent correlation coefficients of $R^2 = 0.87$ for NQO1 and $R^2 = 0.93$ for NQO2 (Fig. 1A and B) suggesting a predictive QSAR model for this series of structurally similar analogues.

For NQO2, calculated binding affinities (ΔG_{calc}) and the experimental binding affinities derived from the observed IC_{50}s (ΔG_{exp}) span similar ranges, of 7 kJ/mol in both cases (Table 1). However, for NQO1, there is a more restricted range of calculated binding affinities (5 kJ/mol) compared to experiment (9 kJ/mol). This is reflected in the near twofold change in the slope of the regression lines for the two enzymes shown in Figure 1. This narrower calculated range of affinities for NQO1 may reflect the more limited steric fit of congeners modeled into the NQO1 active site compared to NQO2 (see below). Furthermore, compounds that are good inhibi-

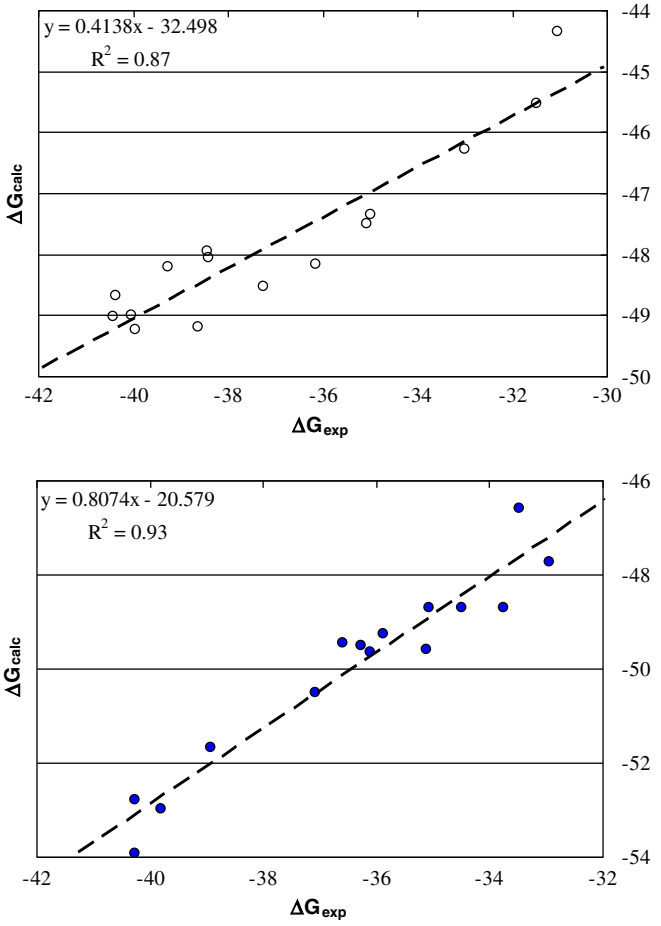


Figure 1. Calculated (ΔG_{calc}) and experimental (ΔG_{exp}) binding affinities (kJ/mol) are shown for (top) NQO1 as white circles and (bottom) for NQO2 as black circles. ΔG_{exp} is calculated from IC_{50} values using the Cheng–Prusoff equation.[28]

Table 1
Computationally-derived (ΔG_{calc}) and experimental (ΔG_{exp}) binding affinities (kJ/mol) and experimental IC_{50} values (nM) ± SD for the interaction of the triazoloacridin-6-ones with NQO1 and NQO2

ID	ΔG_{calc} NQO1	ΔG_{exp} NQO1	NQO1 (IC_{50})	ΔG_{calc} NQO2	ΔG_{exp} NQO2	NQO2 (IC_{50})	Ratio of IC_{50} NQO1/NQO2
5a	−49.2	−39.9	111 ± 25	−48.7	−33.8	1333 ± 577	0.08
5b	−49.2	−38.6	188 ± 10	−48.7	−35.1	783 ± 375	0.24
5c	−46.3	−33.0	1800 ± 10	−47.7	−33.0	1833 ± 289	0.98
5d	−47.5	−35.1	780 ± 53	−46.6	−33.5	1483 ± 525	0.53
6a	−48.1	−38.4	206 ± 5	−49.6	−36.1	517 ± 126	0.40
6c	−47.9	−38.4	203 ± 6	−49.5	−36.3	483 ± 104	0.42
6d	−48.2	−39.3	145 ± 59	−48.7	−34.5	987 ± 121	0.15
7c	−47.4	−35.0	808 ± 8	−52.8	−40.3	98 ± 13	8.24
8c	−49.0	−40.4	92 ± 3	−49.4	−36.6	427 ± 108	0.22
8d	−49.0	−40.0	107 ± 15	−49.6	−35.1	767 ± 153	0.14
9a	−44.3	−31.1	3933 ± 51	−50.5	−37.1	350 ± 132	11.24
9c	−45.5	−31.5	3283 ± 29	−49.3	−35.9	567 ± 208	5.79
10a	−48.5	−37.3	328 ± 3	−53.9	−40.3	98 ± 10	3.35
10c	−48.7	−40.4	94 ± 5	−51.7	−38.9	167 ± 42	0.56
10d	−48.2	−36.2	507 ± 6	−53.0	−39.8	117 ± 29	4.33

All experimental values are the mean of at least three independent experiments done in triplicate. Compounds are ordered in the table according firstly to substituent B and then substituent A.

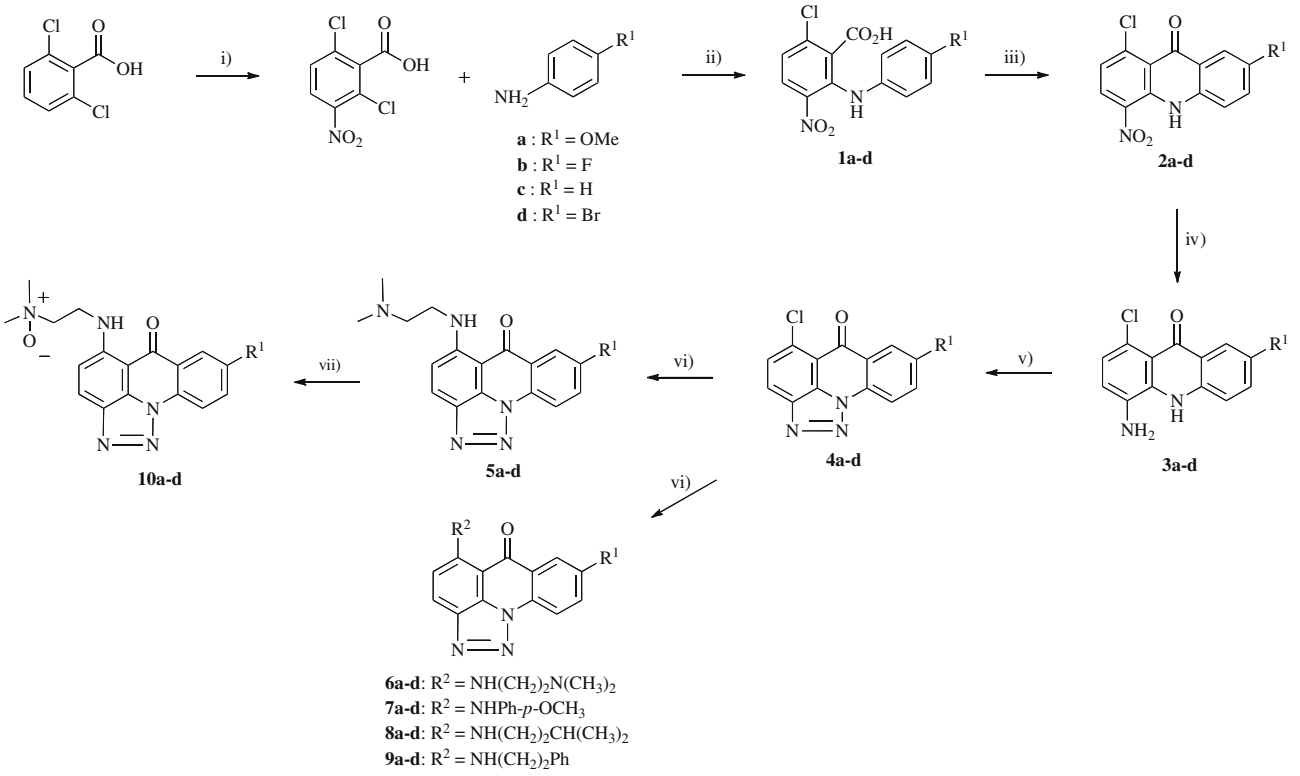
tors of NQO1 are not necessarily good inhibitors of NQO2. This is illustrated by comparing the ratio of IC_{50} for inhibition of NQO1 versus NQO2 for each compound (Table 1). For example, **5a** is one of the most potent inhibitors of NQO1 whereas it is a relatively weak inhibitor of NQO2. In contrast, **9a** is the poorest inhibitor of NQO1 while it shows reasonable activity towards NQO2.

The docking calculations predicted only one dominant binding mode for NQO1: the triazoloacridin-6-one scaffold is held in the active site of NQO1 by the formation of several strong hydrogen bonds (Fig. 2). Using **5a** as an example (Fig. 2A), the Oδ of Tyr126 forms a hydrogen bond with either N1 (2.6 Å) or N2 (2.4 Å) of the triazole ring and the OH of Tyr128 forms a strong hydrogen bond with the ketone group of C6 (1.5 Å). The hydrogen atom on the N5 side chain of the ligand also makes a hydrogen bond contact with the Oδ of Tyr128 (2.9 Å). This proton may also form an internal hydrogen bond with C6 if the N5 side chain is rotated. The ligand is positioned in the centre of the NQO1 binding pocket by π stacking interactions with the isoalloxazine ring of FAD, Trp105 and Phe178 (Fig. 2A). Thus, all ligands are able to make good hydrophobic contacts with active site residues and this is reflected in the lipophilic contribution to the docking score. However, the calculated binding affinity is compromised by clash penalties applied to the docking score as a consequence of steric hindrance with Phe106 forming part of internal wall of the NQO1 active site. Additional clashes are also observed with ligands which have a phenyl side chain (**7c**, **9a** and **9c**) forming unfavourable interactions with the glycerol moiety of FAD, Gly149 and Gly150. The N5 side chain has to project up and out of the active site and this is largely parallel to the FAD cofactor. This steric stress is reflected in both a lower calculated binding affinity and a reduced inhibitory capacity for these compounds (Table 1).

Compound **5a** is a potent inhibitor of NQO1 in with an IC_{50} of 111 nM and a calculated binding affinity of −49.2 kJ/mol. This compound has a methoxy substituent at C8 which is able to form an additional polar contact with His161 (3.1 Å, Fig. 2A). In addition to the lipophilic effect of the methoxy group with Phe 178, this may account for the 17-fold increased inhibitory potency over **5c** which has an IC_{50} of 1.8 μM and a H atom at C8. The methoxy substituent on **6a** is unable to make this hydrogen bond contact as a consequence of forming a polar contact with Gly149 (compare **6c**, Fig. 2B). Likewise, **10a** is predicted to form a polar contact with the FAD side chain (compare **10c,** Fig. 2C) resulting in a slight twist of the pharmacophore scaffold. Addition of fluorine (**5b**) and bromine (**5d**, **6d**, **8d** (Fig. 2D) and **10d**) substituents at C8 is well tolerated allowing favourable lipophilic active site interactions.

Despite the active sites of NQO1 and NQO2 being comparable (see Supplementary data), there are three notable variations: the Tyr126, Tyr128 and Met131 residues are replaced in NQO2 with Phe126, Ile128 and Phe131. This makes the NQO2 active site slightly larger and more hydrophobic than in NOQ1. These differences and the lack of a C-terminal domain may be part of the reason why NQO2 requires a different cofactor to NQO1.[11,29] Furthermore, they are proposed to account for the observed differences in substrate and inhibitor specificity between the two enzymes.

In contrast to NQO1, the docking studies suggest that triazoloacridin-6-ones are able to bind into the active site of NQO2 in several energetically favourable ways. Compounds **5a** (Fig. 3A), **5b** and **5c** do not form any polar contacts with active site residues and seem to be held in the binding pocket by lipophilic interactions with the isoalloxazine ring of FAD, Trp105, Phe106, Phe126, Ile128 and Phe178. These ligands form an internal hydrogen bond between the N5 proton and the ketone oxygen at C6 (2.0 Å, Fig. 3A); this limits rotation of the N5 side chain and holds the triazole scaffold in a planar conformation. The *N*-oxides, **10a** (Fig. 3B), **10c** and **10d** are the most potent inhibitors of NQO2 with IC_{50}



Scheme 1. General synthesis of the triazoloacrid-6-ones. Reagents and conditions: (i) H_2SO_4, HNO_3, 70 °C, 1 h[21]; (ii) *N*,*N*-dimethylaniline, 110 °C, 18 h, N_2[22]; (iii) dichloroethane, *N*,*N*-dimethylaniline, $POCl_3$, 70 °C, 1 h[22,23]; (iv) concd HCl, EtOH, $SnCl_2$, reflux, 6 h[23]; (v) $NaNO_2$, concd HCl, 4 h, rt[23]; (vi) dimethylacetamide, amine **5–9**, 60 °C, 4 h[23]; (vii) *m*CPBA, $CHCl_3$, 0 °C, 24 h.[24]

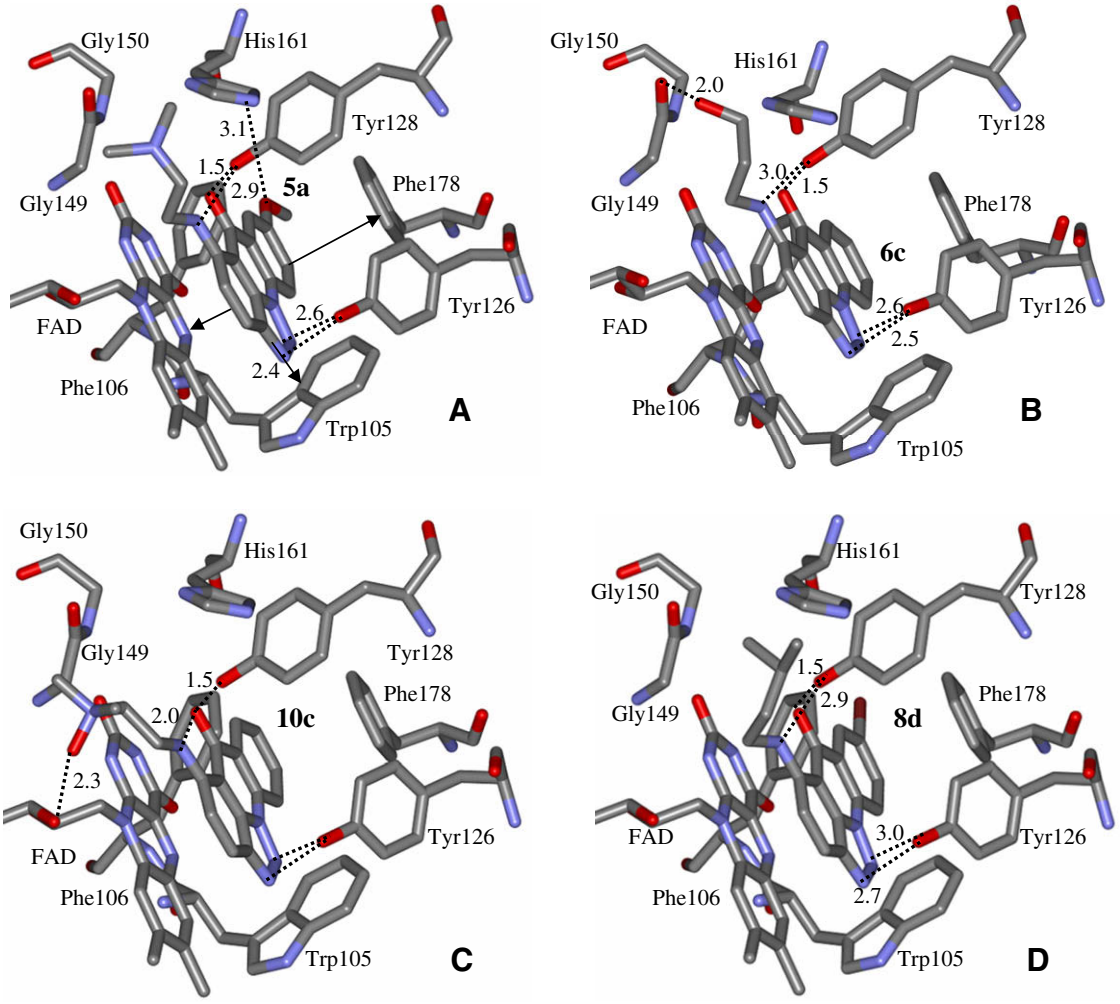


Figure 2. Compounds (A) **5a,** (B) **6c** (C) **10c**, (D) **8d** docked into the active site of NQO1. Polar contacts are shown as dashed lines and π–π stacking interactions are shown as solid arrows in A. Distances are given in Å.

values of 98, 167 and 117 nM, respectively. The N5 proton forms a hydrogen bond with Glu122 (2.5 Å) which causes the N5 side chain to rotate and the oxygen forms a hydrogen bond with a hydrogen on the FAD chain (2.7 Å, Fig. 3B). Compounds **7c** (Fig. 3C), and **9c** (Fig. 3D) fit into the binding pocket without any clash penalties arising from unfavourable interactions. The methoxy group on **7c** (Fig. 3C) forms a close hydrogen bond contact with Thr71 (2.4 Å) which causes the acridine scaffold to rotate slightly. The triazoloacridin-6-ones with fluorine and bromine substituents at C8 do not appear to play any part in affecting potency suggesting that electronic interactions are not important in binding to NQO2.

We have measured the toxicity of the triazoloacridin-6-ones in HCT116 colon cancer cells. Examples of dose response curves for toxicity are given in Figure 4 and from data like this we have derived values of IC_{50}, the concentration required to reduce cell survival by 50% relative to untreated controls, for each of the compounds. The values of IC_{50} obtained following either 24 or 96 h drug exposure are given in Table 2. These cells show significant activity of both NQO1 and NQO2 (1076 ± 70 and 493 ± 74 nmol DCPIP reduced/min/mg protein). At first sight there is no obvious relationship between inhibitory potency towards either, or both enzymes, and toxicity. Thus it is likely that there are other underlying mechanisms of toxicity.

A substantial number of triazoloacridin-6-ones have already been synthesized by Konopa and co-workers and some of these agents have shown significant anti-tumour activity in pre-clinical models.[23,30] The planar structure of the triazoloacridin-6-ones together with the known mechanism of action of related imidazoacridinones and anthracenediones, strongly suggests that interaction with DNA could be the underlying mechanism of toxicity of the triazoloacridin-6-ones in cells. Thus, we evaluated the ability of the compounds to bind to DNA by measuring changes in the melting temperature, T_m, of salmon sperm DNA (20 μg/ml) when treated with 10 μM of each of the triazoloacridin-6-ones. Values of T_m obtained at two different salt concentrations are given in Table 2. Consistent with recent studies by Koba and Kanopa[31] and Lemke et al.[32] we show that the triazoloacridin-6-ones cause a change in DNA melting temperature, T_m, but only at low salt concentrations. At physiologically relevant salt concentrations, little if any change in T_m is observed. Some examples of the DNA melting curves are given in Figure 5. In the figure, 10 μM adriamycin is used as a positive control and under these low-salt conditions adriamycin results in an 8 °C change in T_m. Nearly all the triazoloacridin-6-ones (except the **10** series) show changes in T_m, but the maximum change is no more than only 4 °C, suggesting they have only weak intercalating ability. Koba and Kanopa[31] went on to show, using DNA viscometry, that intercalation in DNA was unlikely to be the major mechanism of toxicity of these compounds. Some evidence for DNA cross-linking by the triazoloacridin-6-ones was demonstrated but this did not correlate with biological activ-

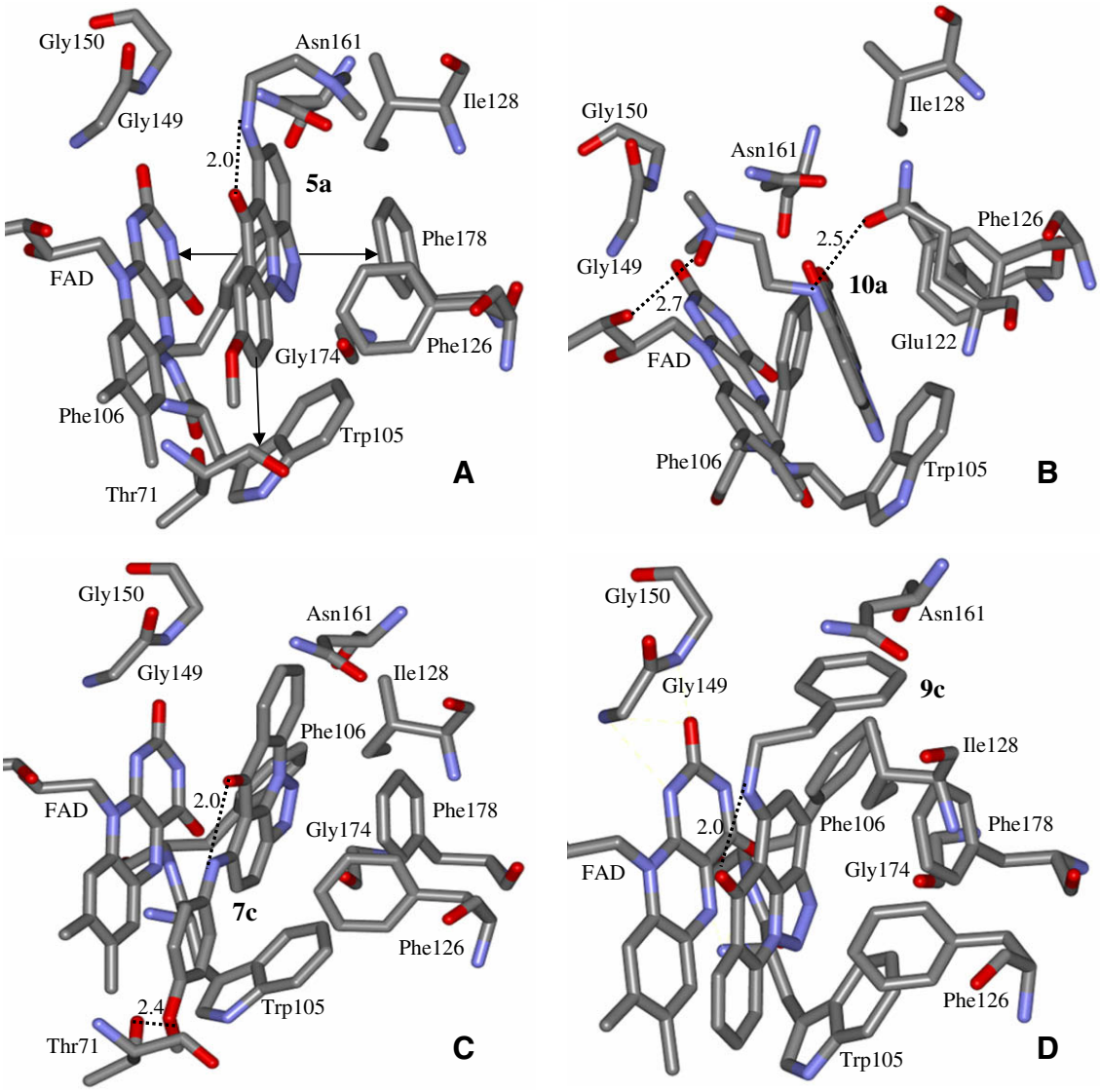


Figure 3. Compounds (A) **5a,** (B) **10a** (C) **7c**, (D) **9c** docked into the active site of NQO2. Polar contacts are shown as dashed lines and π–π stacking interactions are shown as solid arrows in A. Distances are shown in Å.

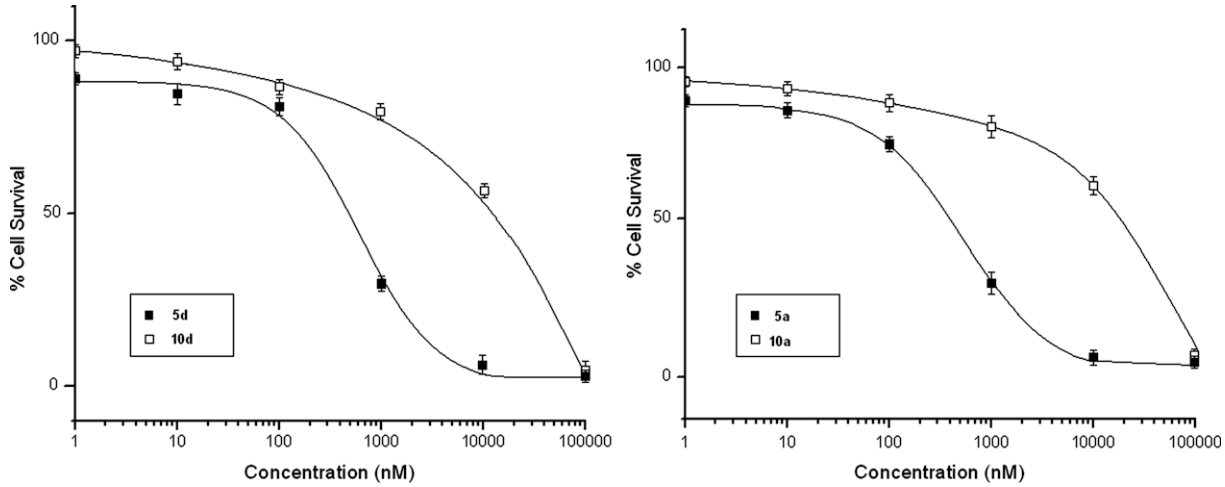


Figure 4. Survival of HCT116 cells when exposed to a variety of selected triazoloacridin-6-ones for 96 h. Data points are derived from at least three individual experiments.

Table 2
Toxicity of the triazoloacridin-6-ones towards HCT116 colon cancer cell line following 24 and 96 h exposure

ID	IC_{50} (μM) 24 h	IC_{50} (μM) 96 h	T_m DNA (high salt)	T_m DNA (low salt)
5a	1 ± 0.1	0.333 ± 0.148	0.08 ± 0.47	2.9 ± 0.73
5b	1.2 ± 0.8	0.465 ± 0.042	0.03 ± 0.31	2.6 ± 0.45
5c	20.5 ± 1.8	12.4 ± 3.2	0.33 ± 0.52	1.7 ± 0.34
5d	1.6 ± 0.09	0.495 ± 0.036	0.20 ± 0.64	2.6 ± 0.41
6a	1.5 ± 0.5	0.627 ± 0.136	0.19 ± 0.54	3.2 ± 0.44
6c	0.99 ± 0.3	0.630 ± 0.180	1.48 ± 1.56	3 ± 0.13
6d	2.8 ± 0.60	0.715 ± 0.111	0.42 ± 0.00	3 ± 0.12
7c	1.9 ± 0.22	0.630 ± 0.140	0.19 ± 0.00	0.2 ± 0.40
8c	3.2 ± 1	0.815 ± 0.262	0.23 ± 0.27	2 ± 0.31
8d	27 ± 2.2	7 ± 0.495	0.09 ± 0.47	1.9 ± 0.05
9a	12.5 ± 2.2	0.711 ± 0.278	0.08 ± 0.49	1.7 ± 0.39
9c	3.5 ± 1.1	0.803 ± 0.272	0.31 ± 0.16	1.7 ± 0.36
10a	57 ± 2.8	18.8 ± 0.138	0.30 ± 0.07	0.15 ± 0.41
10c	80 ± 4.4	23.8 ± 0.290	0.22 ± 0.04	0.5 ± 0.44
10d	48 ± 3.8	12 ± 0.126	0.45 ± 0.29	0.1 ± 0.12

Toxicity was measured using the MTT assay. Changes in DNA melting temperature, ΔT_m, in °C are given for salmon sperm DNA exposed to 10 μM of each compound in the presence of physiological (high) or low salt concentrations.[31,32] Each value ± SD is derived from three independent experiments.

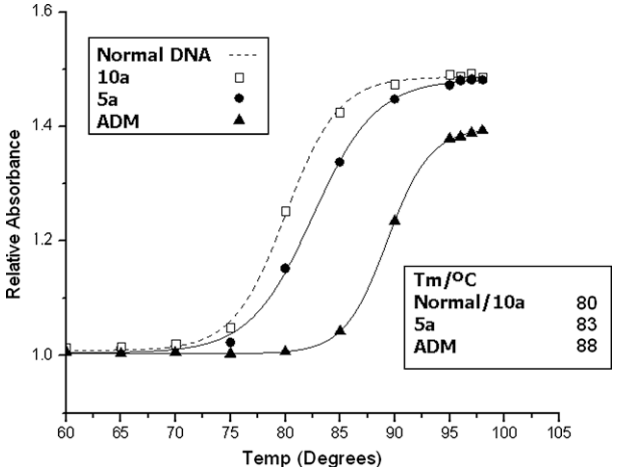


Figure 5. DNA melting curves for salmon sperm DNA in the presence of 10 μM adriamycin (Doxorubicin), **5a** or **10a**.

ity.[31] Nevertheless, from these observations it can be inferred that the triazoloacridin-6-ones have the potential for interacting with DNA.

The most striking observation from the toxicity results is the finding that the *N*-oxides (designated **10**) are consistently less toxic than the other triazoloacridin-6-ones. Each of the **10** compounds show a ~20-fold reduction in toxicity compared their parent, **5** compounds. This can be rationalized on the basis that the *N*-oxides have no effect on T_m whereas the parent compounds do interact with DNA. There are other examples in the literature which show planar, aromatic, *N*-oxides to be less toxic than their unsubstituted analogues. The best characterised example is the prodrug, banoxantrone (AQ4 N), which is substantially less toxic than its product of reductive activation, AQ4.[33–35] Interestingly, this has also been associated with decreased interaction of the *N*-oxide with DNA compared with the reduced analogue.[33,36] Inspection of the ability of the *N*-oxides to act as inhibitors of NQO1 and NQO2 shows that the *N*-oxides are consistently the most efficient inhibitors of NQO2, which is not the same for NQO1. The fact that these compounds are also the least toxic compounds in the series, suggests they might be useful pharmacological tools to study the biological role of NQO2 in cells.

One other compound in the series of triazoloacridin-6-ones shows a much reduced toxicity following both 24 and 96 h drug exposure and this is **5c**. It is a modest inhibitor of both NQO1 and NQO2 and is not an outlier in the binding affinity structure–activity relationships. It is not an *N*-oxide therefore this low toxicity would not be predicted. In a previously reported small series of triazoloacridin-6-ones[31], **5c** (designated C-1233) was shown to have the lowest toxicity both in vitro and in vivo and further it caused one of the smallest changes in T_m under low-salt conditions, which is consistent with the present findings.

4. Conclusions

We have shown that these triazoloacridin-6-ones are novel inhibitors of NQO1 and NQO2. In our previous study of inhibitors of NQO1[20] we demonstrated that the widely used inhibitor of NQO1, dicoumarol, gave an IC_{50} for enzyme inhibition of ~3 nM. Thus, although we have demonstrated a highly significant structure–activity relationship between binding affinity and inhibitory potency for this enzyme, it is unlikely that the compounds reported here will be useful as pharmacological inhibitors of NQO1. In contrast, this is not the case for NQO2. Not only have we demonstrated a clear structure–activity relationship, but we have also shown that those compounds in the **10** series are highly potent inhibitors (~100 nM). In the literature, resveratrol is used as standard inhibitor of NQO2. Using the enzyme assay reported here the IC_{50} for resveratrol is 450 ± 150 nM (unpublished results). Hence, some of the triazoloacridin-6-ones represent the most potent NQO2 inhibitors so far reported. Further, N-oxidation of the tertiary amines confers reduced toxicity and increased water solubility while maintaining inhibitory potency. This provides direction for future drug development as well as yielding compounds that may have useful pharmacological activity.

5. Experimental

5.1. Chemistry

Chemicals were purchased from Sigma–Aldrich Co., and Alfa Aesar. Melting points were measured using a Sanyo Gallenkamp MPD350 heater. Elemental analyses was carried out by the micro-analytical Laboratory, at the University of Manchester and is within ±0.3% of predicted values. Infrared spectra were recorded in the solid state using a Perkin–Elmer FT-IR instrument. Absorption maxima (ν_{max}) are recorded in wavenumbers (cm^{-1}). ^1H and ^{13}C NMR spectra were aquired using Bruker Avance 500, Bruker Avance 400, and Bruker Avance 300 spectrometers. ^1H and ^{13}C assignments were supported by ^1H COSY, DEPT and HMQC.

Chemical shifts are quoted in parts per million (ppm) to the nearest 0.01 ppm and referenced to the solvent peak. Mass spectroscopy was carried out by the Mass Spectrometry Laboratory, School of Chemistry, at the University of Manchester. Molecular ions, fractions from molecular ions and other major peaks are reported as mass/charge (m/z) ratios and are within ±5 ppm mass units for electrospray and ±10 ppm for HRMS. Solvents were evaporated on a Buchi RE111 rotaryevaporator equipped with a Buchi 461 water bath. Column chromatography was performed using silica gel (Sigma–Aldrich) 40–63 μm 60 Å. The eluents are specified in individual procedures. Distilled water was obtained from a Millipore Elix 5.

Synthesis of 2-(4-methoxyphenylamino)-6-chloro-3-nitrobenzoic acid (**1a**).[22] A solution of 4-methoxyaniline (0.700 g, 5.68 mmol) in *N,N*-dimethylaniline (2 mL) was added to a flask containing 2,6-dichloro-3-nitrobenzoic acid (1.00 g, 4.24 mmol) in *N,N*-dimethylaniline (3 mL). The mixture was heated to 120 °C and stirred for 18 h. Once cooled it was diluted with $CHCl_3$ (20 mL), extracted with 1 M NaOH (20 mL), and washed twice with 1 M NaOH (2 × 10 mL). The combined extracts were acidified with dilute HCl to pH 3 which gave a yellow precipitate. This was collected and dried to give **1a** (0.930 g, 68%): mp 211–213 °C (Lit.,[22] 210–212 °C); ν_{max}/cm^{-1} 3297w (N–H), 2848w (C–H), 1698s (C=O), 1589s, 1574s, 1510s; δ_H (300 MHz; DMSO-d_6) 8.69 (1H, s, NH), 8.04 (1H, d, *J* 8.9, C(4)H), 7.17 (1H, d, *J* 8.9, C(5)H), 6.91 (2H, d, *J* 8.9, C(10)H, C(12)H), 6.79 (2H, d, *J* 8.9, C(9)H, C(13)H), 3.70 (3H, s, C(14)H_3); δ_C (75 MHz; DMSO-d_6) 165.0 (q, C(7)), 155.6 (q), 153.3 (q), 138.4 (q), 136.5 (q), 134.7 (q), 134.0 (q), 127.6 (C(4)H), 122.6 (CH, Ph), 120.9 (C(5)H), 114.0 (CH, Ph), 55.2 (OCH_3); m/z (−ES) 323 (10, $[M-H]^-$, ^{37}Cl), 321 (30, $[M-H]^-$, ^{35}Cl); 321.0290 ($[M-H]^-$) found by −ES, required 321.0278, error 3.7 ppm.

Synthesis of 2-(4-fluorophenylamino)-6-chloro-3-nitrobenzoic acid (**1b**): A solution of 4-fluoroaniline (0.53 mL, 5.50 mmol) in *N,N*-dimethylaniline (1.5 mL) was added to a flask containing 2,6-dichloro-3-nitrobenzoic acid (1.18 g, 5.00 mmol) in *N,N*-dimethylaniline (3.0 mL). The mixture was heated to 110 °C with stirring under nitrogen for 18 h. Once cooled it was diluted with $CHCl_3$ (20 mL), extracted with 1 M NaOH (20 mL), and washed twice with 1 M NaOH (2 × 10 mL). The combined extracts were acidified with dilute HCl to pH 3 resulting in formation of a yellow precipitate. This was collected and dried to give **1b** (0.285 g, 18%): mp 176.5–177.0 °C; ν_{max}/cm^{-1} 3348w (N–H), 2980br (O–H), 2881w, (C–H), 1704s (C=O), 1588s, 1569s, 1530 m, 1504s; δ_H (300 MHz; DMSO-d_6) 10.73 (1H, s, C(7)O_2H), 8.57 (1H, s, NH), 8.05 (1H, d, *J* 8.9, C(4)H), 7.35 (1H, d, *J* 8.9, C(5)H), 7.02 (2H, app.t, *J* 8.8, C(10)H, C(12)H), 6.88 (2H, dd, *J* 8.7, 4.8, C(9)H, C(13)H); δ_C (75 MHz; DMSO-d_6) 165.0 (q), 164.0 (q), 140.9 (q), 139.3 (q), 136.5 (q), 135.8 (q), 131.1 (q), 129.8 (q), 127.5 (C(4)H), 123.1 (C(5)H), 120.6 (d, *J* 8.2, C(9)H, C(13)H), 115.5 (d, *J* 7.1, C(10)H, C(12)H); m/z (−ES) 311 (10, $[M-H]^-$, ^{37}Cl), 309 (30, $[M-H]^-$, ^{35}Cl). 309.0090 ($[M-H]^-$) found by −ES, required 309.0084, error 2.0 ppm.

Synthesis of 6-chloro-3-nitro-2-(phenylamino)benzoic acid (**1c**).[22] A similar procedure was adopted as above using aniline to give **1c** (0.552 g, 46%) as a fine, yellow powder: mp 203–205 °C (Lit.,[1] 199–204 °C); ν_{max}/cm^{-1} 3333w (N–H), 3040w (C–H), 1704s (C=O), 1583s, 1568s; δ_H (500 MHz; DMSO-d_6) 8.47 (1H, s, NH), 8.02 (1H, d, *J* 8.8, C(4)H), 7.41 (1H, d, *J* 8.8, C(5)H), 7.14 (2H, app.t, *J* 7.9 C(10)H, C(12)H), 6.85 (1H, app.t, *J* 7.4, C(9)H, C(11)H), 6.77 (2H, d, *J* 7.6, C(9)H, C(13)H); δ_C (125 MHz; DMSO-d_6) 165.1 (q, C(7)), 143.4 (q), 142.1 (q), 135.3 (C(4)H), 132.5 (q), 129.0 (q), 128.6 (C(10)H, C(12)H), 127.7 (C(11)H), 124.1 (q), 121.0 (C(9), C(13)H), 116.9 (C(5)H); m/z (−ES) 293 (8, $[M-H]^-$, ^{37}Cl), 291 (24, $[M-H]^-$, ^{35}Cl); 291.0178 ($[M-H]^-$) found by −ES, required 291.0178, error 0.0 ppm.

Synthesis of 2-(4-bromophenylamino)-6-chloro-3-nitrobenzoic acid (**1d**): 4-bromoaniline (7.29 g, 42.4 mmol, 5 equiv) was heated to 110 °C until completely melted. 2,6-Dichloro-3-nitrobenzoic acid (2.00 g, 8.47 mmol, 1 equiv) was then added in small portions over 20 min with stirring. Heating was continued and after 3 h a solid mass had formed which was kept at 110 °C for a further 4 h. Once cooled it was thoroughly crushed in 2 M NaOH (30 mL) and left to stir for 30 min. The unreacted aniline was filtered off and washed once with 2 M NaOH (5 mL) and twice with water (2 × 5 mL). The filtrate was then acidified with dilute HCl to pH 3 which gave a yellow precipitate. This was collected and dried to give **1d** (1.89 g, 60%) as a yellow powder. mp 198.8–202.0 °C; ν_{max}/cm^{-1} 3305w (N–H), 3090w, 2818 m (C–H), 1703s (C=O), 1596s, 1574s, 1528 m; δ_H (500 MHz; DMSO-d_6) 13.87 (1H, s, C(7)O_2H), 8.54 (1H, s, NH), 8.06 (1H, d, *J* 8.9, C(4)H), 7.51 (1H, d, *J* 8.9, C(5)H), 7.30 (2H, d, *J* 8.8, C(10)H, C(12)H), 6.71 (2H, d, *J* 8.8, C(9)H, C(13)H); δ_C (125 MHz; DMSO-d_6) 165.0 (q, C(7)), 143.4 (q), 143.0 (q), 135.2 (q), 134.5 (q), 133.5 (q), 131.5 (C(10)H, C(12)H), 127.3 (C(4)H), 125.2 (C(5)H), 118.7 (C(9)H, C(13)H), 112.0 (q, C(11)); m/z (−ES) 373 (25, $[M-H]^-$, $^{37}Cl^{81}Br$), 371 (100, $[M-H]^-$, $^{37}Cl^{79}Br$, $^{35}Cl^{81}Br$), 369 (80, $[M-H]^-$, $^{35}Cl^{79}Br$). 368.9282 ($[M-H]^-$) found by −ES, required 368.9283, error 0.3 ppm.

Synthesis of 1-chloro-7-methoxy-4-nitroacridin-9(10H)-one (**2a**).[22] A mixture of **1a** (1.00 g, 3.10 mmol), $POCl_3$ (1.88 mL, 20.2 mmol) and *N,N*-dimethylaniline (0.09 mL) in dichloroethane (10 mL) was heated under reflux for 2 h. The resulting red precipitate was filtered, washed with dichloroethane (2.0 mL), and dried to give **2a** (0.891 g, 87%) as a red needles. mp 263–264 °C (Lit.,[22] 262–263 °C); ν_{max}/cm^{-1} 3288 m (N–H), 3113w, 2839w (C–H), 1644s (C=O), 1606s, 1589s, 1565s; δ_H (300 MHz; DMSO-d_6) 11.64 (1H, s, NH), 8.55 (1H, d, *J* 8.9, C(3)H), 8.02 (1H, d, *J* 9.1, C(5)H), 7.59 (1H, d, *J* 2.9, C(8)H), 7.47 (1H, dd, *J* 9.1, 2.9, C(6)H), 7.38 (1H, d, *J* 8.9, C(2)H), 3.16 (3H, s, C(14)H_3); m/z (−ES) 305 (35, $[M-H]^-$, ^{37}Cl), 303 (100, $[M-H]^-$, ^{35}Cl); (+ES) 329 (20, $[M+Na]^+$, ^{37}Cl), 327 (60, $[M+Na]^+$, ^{35}Cl); 303.0180 ($[M-H]^-$) found by −ES, required 303.0173, error 2.4 ppm.

Synthesis of 1-chloro-7-fluoro-4-nitroacridin-9(10H)-one (**2b**): A mixture of **1b** (248 mg, 0.80 mmol), $POCl_3$ (0.50 mL, 5.25 mmol) and *N,N*-dimethylaniline (0.23 μL) in dichloroethane (25 mL) was heated under reflux for 2 h. The resulting red precipitate was filtered, washed with dichloroethane (2.0 mL), and dried to give **2b** (135 mg, 58%) as orange needles. mp 269.5–271.0 °C; ν_{max}/cm^{-1} 3305 m (N–H), 3078w (C–H), 1647s (C=O), 1624 m, 1589s, 1569s, 1525w, 1484s; δ_H (300 MHz; DMSO-d_6) 11.68 (1H, NH), 8.55 (1H, d, *J* 8.8, C(3)H), 8.12 (1H, dd, *J* 8.9, 4.6, C(8)H), 7.82 (1H, dd, *J* 8.9, 3.0, C(5)H), 7.73 (1H, app.dt, *J* 8.9, 3.0, C(6)H), 7.41 (1H, d, *J* 8.8, C(2)H); m/z (−ES) 293 (35, $[M-H]^-$, ^{37}Cl), 291 (100, $[M-H]^-$, ^{35}Cl); (+ES) 317 (35, $[M+Na]^+$, ^{37}Cl), 315 (100, $[M+Na]^+$, ^{35}Cl); 314.9960 ($[M+Na]^+$) found by +ES, required 314.9943, error 5.3 ppm.

Synthesis of 1-chloro-4-nitroacridin-9(10H)-one (**2c**).[22] A similar procedure was adopted as above using **1c** to give **2c** (1.23 g, 82%) as a bright red powder: mp 248–249 °C (Lit.,[22] 252–255 °C); ν_{max}/cm^{-1} 3307 m (N–H), 1644s (C=O), 1587, 1569 (N=O); δ_H (500 MHz; DMSO-d_6) 11.47 (1H, s, NH), 8.55 (1H, d, *J* 8.8, C(3)H), 8.18 (1H, d, *J* 8.0, C(5)H), 7.99 (1H , d, *J* 8.2, C(8)H), 7.89–7.82 (1H, m, C(6)H), 7.40 (1H, d, *J* 8.8, C(2)H), 7.38–7.41 (1H, m, C(7)H); δ_C (100 MHz, DMSO-d_6) 174.9 (q, C(9)), 141.8 (q), 138.4 (q), 137.0 (q), 133.9 (C(3)H), 130.1 (C(6)H), 125.6 (C(8)H), 123.2 (C(7)H), 122.5 (C(5)H), 121.8 (q), 118.6 (q), 118.3 (C(2)H), 99.2 (q); m/z (−ES) 275.1 (32, $[M-H]^-$, ^{37}Cl), 273.1 (100, $[M-H]^-$, ^{35}Cl). 297.0051 ($[M+Na]^+$) found by +ES, required 297.00543, error 2.7 ppm.

Synthesis of 7-bromo-1-chloro-4-nitroacridin-9(10H)-one (**2d**): A similar procedure was adopted as above using **1d** to give **2d** (1.61 g, 94%) as a bright red powder: mp 310.0–310.8 °C; ν_{max}/cm^{-1} 3291 m (N–H), 3074 m (C–H), 1658s (C=O), 1613, 1571s; δ_H

(500 MHz; DMSO-d_6) 8.50 (1H, d, J 8.8, C(3)H), 8.24 (1H, s, C(8)H), 7.98 (1H, d, J 8.6, C(5)H), 7.93 (1H, d, J 8.6, C(6)H), 7.39 (1H, d, J 8.8, C(2)H); m/z (−ES) 355 (25, $[M-H]^-$, $^{37}Cl^{81}Br$), 353 (100, $[M-H]^-$, $^{37}Cl^{79}Br$, $^{35}Cl^{81}Br$), 351 (80, $[M-H]^-$, $^{35}Cl^{79}Br$); (+ES) 378 (3, $[M+Na]^+$, $^{37}Cl^{81}Br$), 376 (12, $[M+Na]^+$, $^{37}Cl^{79}Br$, $^{35}Cl^{81}Br$), 374 (9, $[M+Na]^+$, $^{35}Cl^{79}Br$); 350.9178 ($[M-H]^+$) found by −ES, required 350.9178, error 0.1 ppm.

Synthesis of 4-amino-1-chloro-7-methoxyacridin-9(10H)-one (**3a**).[23] A solution of $SnCl_2$ (2.24 g, 11.8 mmol) in concd HCl (2.5 mL) was added to a suspension of finely powdered **2a** (0.800 g, 2.63 mmol) in ethanol–concd HCl (2 mL: 2 mL). The mixture was heated to reflux and stirred vigorously for 24 h and then allowed to cool. The HCl salt of the product was filtered from the solution as a peach coloured precipitate. This was added to water (100 mL), made basic with NaOH, and stirred for 30 min. The suspension was then filtered and washed with water to give **3a** (0.760 g, 86%) as a yellow powder: mp 215–218 °C (dec) (Lit.,[23] 215–217 °C (dec)); ν_{max}/cm^{-1} 3263 m (N–H), 3113w, 3004w, 2942w, 2840w (C–H), 1644s (C=O), 1605s, 1589s, 1566s; δ_H (300 MHz; DMSO-d_6) 10.58 (1H, s, NH), 7.67 (1H, d, J 9.0, C(5)H), 7.56 (1H, d, J 3.0, C(8)H), 7.37 (1H, dd, J 9.0, 3.0, C(6)H), 6.97 (1H, d, J 8.1, C(2)H), 6.90 (1H, d, J 8.1, C(3)H), 3.84 (3H, s, OMe); δ_C (75 MHz; DMSO-d_6) 175.6 (q, C(9)), 154.2 (q), 136.1 (q), 134.6 (q), 131.6 (q), 123.8 (C(6)H), 123.5 (C(2)H), 122.0 (q), 119.2 (C(5)H), 119.0 (q), 116.1 (q), 114.9 (C(3)H), 105.1 (C(8)H), 55.3 (OCH_3); m/z (−ES) 275 (30, $[M-H]^-$, ^{37}Cl), 273 (100, $[M-H]^-$, ^{35}Cl); (+ES) 299 (10, $[M+Na]^+$, ^{37}Cl), 297 (30, $[M+Na]^+$, ^{35}Cl), 277 (40, $[M+H]^+$, ^{37}Cl), 275 (100, $[M+H]^+$, ^{35}Cl); 275.0579 ($[M+H]^+$) found by +ES, required 275.0582, error 1.0 ppm.

Synthesis of 4-amino-1-chloro-7-fluoroacridin-9(10H)-one (**3b**): A solution of $SnCl_2$ (350 mg, 1.85 mmol) in concd HCl (1.0 mL) was added to a suspension of finely powdered **2b** (120 mg, 0.410 mmol) in ethanol–concd HCl (1 mL: 1 mL). The mixture was heated to reflux and stirred vigorously for 24 h and then allowed to cool. The HCl salt of the product was filtered from the solution as a peach coloured solid. This was added to water (15 mL), basified with NaOH, and stirred for 30 mins. The suspension was then filtered and washed with water to give **3b** (85 mg, 79%) as a yellow powder: mp 270–280 °C (dec); ν_{max}/cm^{-1} 3330 m, 3292 m, 3232w (N–H), 1570s (C=O), 1498s, 1461s; δ_H (300 MHz; DMSO-d_6) 7.74 (1H, dd, J 10.3, 3.2, C(8)H), 7.62–7.57 (1H, m, C(5)H), 7.37–7.33 (1H, m, C(6)H), 6.70 (1H, d, J 7.9, C(2)H), 6.61 (1H, d, J 7.9, C(3)H), 5.55 (2H, s br, NH_2); m/z (−ES) 263 (35, $[M-H]^-$, ^{37}Cl), 261 (100, $[M-H]^-$, ^{35}Cl); (+ES) 287 (30, $[M+Na]^+$, ^{37}Cl), 285 (100, $[M+Na]^+$, ^{35}Cl). 285.0197 ($[M+Na]^+$) found by +ES, 285.0201, error 1.5 ppm.

Synthesis of 4-amino-1-chloroacridin-9(10H)-one (**3c**).[23] A similar procedure was adopted as above using **2c** to give **3c** (1.07 g, 92%) as a yellow powder: mp 222–232 °C (dec) (Lit.,[23] 232–234 °C (dec)); ν_{max}/cm^{-1} 3364w, 3264w (N–H), 1621s (C=O), 1566s, 1526s; δ_H (500 MHz; DMSO-d_6) 10.49 (1H, s, NH), 8.15 (1H, dd, J 8.1, 2.2, C(8)H), 7.70 (1H, m, C(6)H), 7.65 (1H, d, J 7.9, C(5)H), 7.23–7.26 (1H, m, C(7)H), 7.00 (1H, d, J 8.2, C(2)H), 6.94 (1H, d, J 8.2, C(3)H), 5.65 (2H, s, NH_2); δ_C (125 MHz; DMSO-d_6) 176.4 (q, C(9)), 139.9 (q), 136.3 (q), 133.1 (q), 131.8 (C(6)H), 126.5 (C(8)H), 125.8 (q), 124.1 (q), 121.4 (C(3)H), 119.1 (q), 117.5 (C(2)H), 117.0 (C(5)H), 115.7 (C(7)H); m/z (−ES) 245 (32, $[M-H]^-$, ^{37}Cl), 243 (100, $[M-H]^-$, ^{35}Cl); (+ES) 269 (30, $[M+Na]^+$, ^{37}Cl), 267 (100, $[M+Na]^+$, ^{35}Cl), 147 (20, $[M+H]^+$, ^{37}Cl), 145 (60, $[M+H]^+$, ^{35}Cl); 245.0476 ($[M+H]^+$) found by +ES, required 245.0476, error 0.1 ppm.

Synthesis of 4-amino-7-bromo-1-chloroacridin-9(10H)-one (**3d**): A similar procedure was adopted as above using **2d** to give **3d** (0.825 g, 98%) as a yellow powder: mp 285–294 °C (dec); ν_{max}/cm^{-1} 3335 m, 3285 m (N–H), 1617s (C=O), 1597s, 1577s, 1519s; δ_H (500 MHz; DMSO-d_6) 10.88 (1H, s, NH), 8.27 (1H, d, J 2.3, C(8)H), 7.88 (1H, dd, J 8.9, 2.3, C(6)H), 7.74 (1H, d, J 8.9, C(5)H), 7.09 (1H, d, J 8.4, C(2)H), 7.03 (1H, d, J 8.4, C(3)H), 5.04 (2H, br s, NH_2); δ_C (75 MHz; DMSO-d_6) 175.8 (q, C(9)), 139.5 (q), 136.7 (q), 136.5 (C(6)H), 132.5 (q), 128.7 (C(8)H), 125.1 (q), 123.3 (C(2)H), 120.8 (q), 120.1 (C(5)H), 117.7 (C(3)H), 117.0 (q), 114.3 (q, C(7)); m/z (−ES) 325.0 (25, $[M-H]^-$, $^{37}Cl^{81}Br$), 323.0 (100, $[M-H]^-$, $^{37}Cl^{79}Br$, $^{35}Cl^{81}Br$), 321.0 (80, $[M-H]^-$, $^{35}Cl^{79}Br$); (+ES) 349.0 (12, $[M+Na]^+$, $^{37}Cl^{81}Br$), 347.0 (60, $[M+Na]^+$, $^{37}Cl^{79}Br$, $^{35}Cl^{81}Br$), 345.0 (45, $[M+Na]^+$, $^{35}Cl^{79}Br$), 327.0 (6, $[M+H]^+$, $^{37}Cl^{81}Br$), 325.0 (25, $[M+H]^+$, $^{37}Cl^{79}Br$, $^{35}Cl^{81}Br$), 323.0 (18, $[M+Na]^+$, $^{35}Cl^{79}Br$). 344.9397 ($[M+Na]^+$) found by +ES, required 344.9301, error −1.1 ppm.

Synthesis of 5-chloro-8-methoxy-6H-[1,2,3]triazolo[4,5,1-de]acridin-6-one (**4a**).[23] A suspension of **3a** (700 mg, 2.55 mmol) in concd HCl (8.5 mL) was stirred at room temperature for 30 min. A solution of $NaNO_2$ (235 mg, 3.40 mmol) in water (5.0 mL) was then added gradually and the mixture was stirred for 4 h. The grey precipitate was collected and washed with water to give **4a** (564 mg, 78%) as a grey power: mp 261–262 °C (Lit.,[23] 263–266 °C); ν_{max}/cm^{-1} 3070w, 1672s (C=O), 1594s , 1506s; δ_H (300 MHz; $CDCl_3$) 8.47 (1H, d, J 8.9, C(10)H), 8.37 (1H, d, J 8.5, C(3)H), 7.96 (1H, d, J 2.7, C(7)H), 7.72 (1H, d, J 8.5, C(4)H), 7.50 (1H, dd, J 8.9, 2.7, C(9)H), 4.00 (3H, s, OMe); m/z (+ES) 310 (25, [M+Na]+, ^{37}Cl), 208 (70, [M+Na]+, ^{35}Cl). 321.9960 ($[M+Cl]^-$) found by −ES, required 321.9964, error 0.4 ppm.

Synthesis of 5-chloro-8-fluoro-6H-[1,2,3]triazolo[4,5,1-de]acridin-6-one (**4b**): A suspension of **3b** (75 mg, 0.286 mmol) in concd HCl (1.0 mL) was stirred at room temperature for 30 min. A solution of $NaNO_2$ (25.6 mg, 0.371 mmol) in water (0.60 mL) was then added gradually and the mixture was stirred for 4 h. The precipitate was collected and washed with water to give **4b** (65 mg, 83%) as a grey power: mp 291.0–302.0 °C (dec); ν_{max}/cm^{-1} 3101 m (C–H), 1662s (C=O), 1638 m, 1595s, 1505s, 1478s; δ_H (300 MHz; DMSO-d_6) 8.68 (1H, d, J 8.5, C(3)H), 8.63–8.58 (1H, m, C(10)), 8.10–8.06 (1H, m, C(7)H), 7.96 (1H, app.dt, J 8.6, 3.4, C(9)H), 7.89 (1H, d, J 8.5, C(4)H); m/z (+ES) 298 (30, $[M+Na]^+$, ^{37}Cl), 296 (100, $[M+Na]^+$, ^{35}Cl).

Synthesis of 5-chloro-6H-[1,2,3]triazolo[4,5,1-de]acridin-6-one (**4c**).[23] A similar procedure was adopted as above using **3c** to give **4c** (0.732 g, 70%) as a grey power: mp 221–222 °C (Lit.,[23] 222–223 °C); ν_{max}/cm^{-1} 3076w (C–H), 1661s (C=O), 1634w, 1606s, 1594s, 1504s; δ_H (500 MHz; DMSO-d_6) 8.63 (1H, d, J 9.0, C(3)H), 8.49 (1H, d, J 7.7, C(7)H), 8.34 (1H, d, J 7.7, C(10)H), 8.04 (1H, app.t, J 7.7, C(9)H), 7.84 (1H, d, J 9.0, C(4)H), 7.71 (1H, app.t, J 7.7, C(8)H); δ_C (75 MHz, DMSO-d_6) 175.7 (q, C(6)), 143.5 (q), 135.4 (C(3)H), 134.7 (q), 129.5 (C(9)H), 128.4 (C(7)H), 127.7 (C(10)H), 127.0 (C(4)H), 124.5 (q), 115.3 (C(8)H); m/z (+ES) 280 (16, $[M+Na]^+$, ^{37}Cl), 278 (42, $[M+Na]^+$, ^{35}Cl), 258 (10, $[M+H]^+$, ^{37}Cl) 256 (30, $[M+H]^+$, ^{35}Cl); 256.0274 ($[M+H]^+$) found by +ES, required 256.0272, error 0.7 ppm.

Synthesis of 8-bromo-5-chloro-6H-[1,2,3]triazolo[4,5,1-de]acridin-6-one (**4d**): A similar procedure was adopted as above using **3d** to give **4d** (0.725 g, 95%) as a grey power. This powder is insoluble in all typical NMR solvents: mp 291.0–291.6 °C; ν_{max}/cm^{-1} 3087 m (C–H), 1654s (C=O), 1633 m, 1603s, 1591s, 1558s; δ_H (500 MHz; DMSO-d_6) 8.69 (1H, s), 8.45 (2H, m), 8.25 (1H, s), 7.90 (1H, s).

Synthesis of 5-(2-(dimethylamino)ethylamino)-8-methoxy-6H-[1,2,3]triazolo[4,5,1-de]acridin-6-one (**5a**).[23] *N,N*-dimethylethylendiamine (185 mg, 2.10 mmol) was added to a suspension of **4a** (200 mg, 0.701 mmol) in anhydrous dimethylacetamide (DMA) (3.0 mL) and the mixture was heated to 60 °C for 24 h. The reaction mixture was allowed to cool, then EtOH (2.0 mL) was added and the flask was left in a refrigerator for 2 days. The resulting yellow crystals were collected and washed twice with water (2 × 10 mL) and once with MeOH (10 mL) to give **5a** (175 mg, 74%): mp 173–175 °C (Lit.,[23] 173–174 °C); ν_{max}/cm^{-1} 3294w (N–H), 2990w, 2951w, 2823w, 2772w (C–H), 1662s (C=O), 1597s, 1548s, 1573s;

δ_H (300 MHz; DMSO-d_6) 9.44 (1H, s br, NH), 8.40 (1H, d, *J* 9.1, C(10)H), 8.30 (1H, d, *J* 9.3, C(3)H), 7.80 (1H, d, *J* 2.9, C(7)H), 7.57 (1H, dd, *J* 9.1, 2.9, C(9)H), 7.11 (1H, d, *J* 9.3, C(4)H), 3.94 (3H, s, OMe), 3.59 (2H, app.q, *J* 5.5, C(12)H_2), 2.61 (2H, t, *J* 5.9, C(13)H), 2.27 (6H, s, C(14)H_3, C(15)H_3); δ_C (75 MHz; DMSO-d_6) 176.2 (q, C(6)), 157.7 (q), 152.2 (q), 148.2 (q), 134.7 (q), 131.0 (q), 128.8 (C(3)H), 125.9 (q), 122.1 (C(9)H), 116.9 (C(10)H), 112.1 (C(4)H), 108.9 (C(7)H), 98.7 (q), 57.0 (C(13)H), 55.7 (OMe), 45.0 (C(14)H_3, C(15)H_3), 40.2 (C(12)H); *m/z* (+ES) 360.0 (40, $[M+Na]^+$), 338.1 (100, $[M+H]^+$); 338.1621 ($[M+H]^+$) found by +ES, required 338.1612, error 2.8 ppm.

Synthesis of 5-(3-hydroxypropylamino)-8-methoxy-6H-[1,2,3]triazolo[4,5,1-de]acridin-6-one (**6a**): A similar procedure was adopted as above using 3-amino-1-propanol to give **6a** (32.2 mg, 56%): mp 173–174 °C; ν_{max}/cm^{-1} 3339w (O–H), 3254 (N–H), 3046w, 2943w, 2866w (C–H), 1667s (C=O), 1601s, 1578s, 1556s, 1503s; δ_H (300 MHz; DMSO-d_6) 9.36 (1H, s br, NH), 8.38 (1H, d, *J* 8.9, C(10)H), 8.29 (1H, d, *J* 9.2, C(3)H), 7.75 (1H, s, C(7)H), 7.57 (1H, d, *J* 8.9, C(9)H), 7.12 (1H, d, *J* 9.2, C(4)H), 4.75 (1H, t, *J* 5.0, OH), 3.93 (3H, s, OMe), 3.56–3.64 (4H, m, C(12)H_2, C(14)H_2), 1.86 (2H, app.quin, *J* 6.3, C(13)H_2); δ_C (75 MHz; DMSO-d_6) 176.3 (q, C(6)), 157.7 (q), 152.5 (q), 139.6 (q), 134.6 (q), 128.9 (C(3)H), 125.9 (q), 121.9 (C(9)H), 116.9 (C(10)H), 111.7 (C(4)H), 108.97 (C(7)H), 106.5 (q), 98.7 (q), 58.1 (C(14)H_2), 55.7 (C(15)H_3), 40.0 (C(12)H_2), 31.7 (C(13)H_2); *m/z* (−ES) 323 (100, $[M-H]^-$); (+ES) 347 (100, $[M+Na]^+$), 325 (40, $[M+H]^+$); 325.1287 ($[M+H]^+$) found by +ES, required 325.1295, error 2.5 ppm.

Synthesis of 8-methoxy-5-(phenethylamino)-6H-[1,2,3]triazolo[4,5,1-de]acridin-6-one (**9a**): A similar procedure was adopted as above using 2-phenylethylamine to give **9a** (47.5 mg, 73%): mp 195–197 °C; ν_{max}/cm^{-1} 3280w (N–H), 2988w (C–H), 1657s (C=O), 1601s, 1579s, 1556s, 1503s; δ_H (300 MHz; $CDCl_3$) 9.42 (1H, s br, NH), 8.47 (1H, d, *J* 9.1, C(10)H), 8.14 (1H, d, *J* 9.2, C(3)H), 7.93 (1H, d *J* 2.9, C(7)H), 7.46 (1 h, dd, *J* 9.0, 2.9, C(9)H), 7.30–7.39 (5H, m, Ph), 6.88 (1H, d, *J* 9.2, C(4)H), 4.00 (3H, s, OMe), 3.77 (2H, app.q, *J* 6.9, C(12)H_2), 3.11 (2H, t, *J* 7.3, C(13)H_2) δ_C (75 MHz; $CDCl_3$) 178.0 (q, C(6)), 158.2 (q), 152.8 (q), 138.0 (q), 135.6 (q), 131.5 (q), 129.8 (q), 128.9 (C(3)H), 128.8 (2CH, Ph), 128.8 (2CH, Ph), 126.9 (CH, Ph), 126.4 (q), 122.7 (C(9)H), 117.2 (C(10)H), 110.6 (C(4)H), 108.8 (C(7)H), 99.9 (q), 55.9 (OMe), 44.9 (C(12)H_2), 35.8 (C(13)H_2); *m/z* (−ES) 369 (50, $[M-H]^-$); (+ES) 393 (100, $[M+Na]^+$), 371 (60, $[M+H]^+$); 371.1515 ($[M+H]^+$) found by +ES, required 371.1503, error 3.4 ppm.

Synthesis of 5-(2-(dimethylamino)ethylamino)-8-fluoro-6H-[1,2,3]triazolo[4,5,1-de]acridin-6-one (**5b**): A similar procedure was adopted as above using **4b** and *N,N*-dimethylethylendiamine to give **5b** (55 mg, 92%) as yellow crystals: mp 187.7–188.0 °C; ν_{max}/cm^{-1} 3283w (N–H), 2947w, 2774w (C–H), 1660s (C=O), 1629 m, 1609s, 1579s, 1548s, 1501s; δ_H (300 MHz; $CDCl_3$) 9.50 (1H, s br, NH), 8.55 (1H, dd, *J* 9.0, 4.5, C(10)H), 8.21 (1H, dd, *J* 9.0, 2.8, C(7)H), 8.19 (1H, d, *J* 9.2, C(3)H), 7.63 – 7.57 (1H, m, C(9)H), 6.93 (1H, d, *J* 9.2, C(4)H), 3.58 (2H, app.q, *J* 8.9, C(12)H_2), 2.73 (2H, t, *J* 6.3, C(13)H_2), 2.39 (6H, s, C(14)H_3, C(15)H_3); δ_C (75 MHz; $CDCl_3$) 177.1, 162.5, 159.3, 152.8, 135.5, 132.1, 131.8, 129.2, 127.2, 127.1, 121.7, 121.3, 117.8, 117.7, 113.9, 113.5, 111.1, 99.9, 57.6, 45.4, 41.1; *m/z* (+ES) 326 (100, $[M+H]^+$); 348.1241 ($[M+Na]^+$) found by +ES, required 348.1231 error 2.8 ppm.

Synthesis of 5-(2-(dimethylamino)ethylamino)-6H-[1,2,3]triazolo[4,5,1-de]acridin-6-one (**5c**).[23] A similar procedure was adopted as above using **4c** and *N,N*-dimethylethylendiamine to give **5c** (0.0473 g, 39.3%) as yellow crystals: mp 129–130 °C (Lit.,[23] 128–129 °C); ν_{max}/cm^{-1} 3293w (N–H), 2943w, 2767w, 1662s (C=O), 1600s, 1567s, 1542s, 1502s; δ_H (500 MHz; DMSO-d_6) 9.45 (1H, t, *J* 4.8, NH), 8.50 (1H, d, *J* 7.9, C(10)H), 8.42 (1H, dd, *J* 7.9, 1.3, C(7)H), 8.33 (1H, d, *J* 9.2, C(3)H), 8.01 (1H, app.dt, *J* 7.7, 1.3, C(9)H), 7.71 (1H, app.t, *J* 7.6, C(8)H), 7.15 (1H, d, *J* 9.2, C(4)H), 3.61 (2H, app.q, *J* 5.7, C(12)H_2), 2.61 (2H, t, *J* 6.0, C(13)H_2), 2.27 (6H, s, NMe_2); $\delta_{C\ (75\ MHz;\ DMSO\text{-}d6)}$ 176.6 (q, C=O), 152.1 (q), 134.7 (q), 134.6 (q), 134.0 (C(9)H), 131.7 (q), 128.7 (C(3)H), 127.4 (C(8)H), 126.6 (C(7)H), 124.6 (q), 115.2 (C(10)H), 112.1 (C(4)H), 99.0 (q), 57.1 (C(13)H_2), 45.1 (C(14)H_3, C(15)H_3), 40.3 (C(12)H_2); *m/z* (−ES) 306 (100, $[M-H]^-$); (+ES) 330 (100, $[M+Na]^+$), 308 (30, $[M+H]^+$); 330.1325 ($[M+Na]^+$) found by +ES, required 330.1325, error 0.1 ppm.

Synthesis of 5-(3-dydroxypropylamino)-6H-[1,2,3]triazolo[4,5,1-de]acridin-6-one (**6c**): A similar procedure was adopted as above using **4c** and 3-amino-1-propanol to give **6c** (0.076 g, 65%) as green crystals: mp 184–186 °C; ν_{max}/cm^{-1} 3310br (O–H), 3278w (N–H), 2875w (C–H), 1662s (C=O), 1599s, 1567s, 1501s; δ_H (500 MHz; DMSO-d_6) 9.39 (1H, t, *J* 5.8, NH), 8.48 (1H, d, *J* 8.1, C(10)H), 8.39 (1H, dd, *J* 7.9, 1.5, C(7)H), 8.31 (1H, d, *J* 9.4, C(3)H), 8.00 (1H, app.dt, *J* 7.8, 1.5, C(9)H), 7.70 (1H, app.t, *J* 7.6, C(8)H), 7.16 (1H, d, *J* 9.5, C(4)H), 4.76 (1H, t, *J* 5.1, OH), 3.62 (2H, app.q, *J* 6.3, C(14)H_2), 3.58 (2H, app.t, *J* 5.9, C(12)H_2), 1.85 (2H, app.p, *J* 6.3, C(13)H_2); δ_C (75 MHz; DMSO-d_6) 176.38 (q, C(6)), 152.09 (q), 134.51 (q), 134.29 (q), 133.79 (C(8)H), 131.33 (q), 128.40 (CH, C(9)), 127.02 (C(10)H), 126.38 (C(7)H), 124.26 (q), 115.03 (C(3)H), 111.40 (C(4)H), 98.77 (q), 58.14 (C(15)H_2), 39.85 (C(13)H_2), 31.76 (C(14)H_2); *m/z* (−ES) 293 (100, $[M-H]^-$); (+ES) 317 (100, $[M+Na]^+$); 293.1042 ($[M-H]^-$) found by −ES, required 293.1044, error −0.7 ppm.

Synthesis of 5-(4-methoxyphenylamino)-6H-[1,2,3]triazolo[4,5,1-de]acridin-6-one (**7c**): A similar procedure was adopted as above using **4c** and *p*-anisidine to give **7c** (0.116 g, 87%) as green crystals: mp 234–235 °C; ν_{max}/cm^{-1} 3242w (N–H), 2836w (C–H), 1664s (C=O), 1627 m, 1603s, 1584s, 1543s; δ_H (500 MHz; DMSO-d_6) 10.72 (1H, s, NH), 8.55 (1H, d, *J* 8.2, C(10)H), 8.46 (1H, dd, *J* 7.8, 1.5, C(7)H), 8.31 (1H, d, *J* 9.3, C(3)H), 8.06 (1H, app.dt, *J* 7.9, 1.5, C(9)H), 7.75 (1H, app.t, *J* 7.8 C(8)H), 7.44 (2H, d, *J* 8.9, C(14)H, C(16)H), 7.14 (1H, d, *J* 9.3, C(4)H), 7.09 (2H, d, *J* 8.9, C(13)H, C(17)H), 3.82 (3H, s, CH_3); δ_C (100 MHz; DMSO-d_6) 178.1 (q, C(6)), 158.5 (q), 151.2 (q), 136.5 (q), 135.5 (q), 134.9 (q), 132.3 (C(9)H), 131.1 (q), 129.2 (q), 128.0 (C(3)H), 127.31 (C(10)H), 126.5 (C(8)H), 125.0 (C(14)H, C(16)H), 115.9 (C(7)H), 115.7 (C(13)H, C(17)H), 113.0 (C(4)H), 100.8 (q), 56.0 (C(18)H_3); *m/z* (−ES) 341 (100, $[M-H]^-$); (+ES) 365 (100, $[M+Na]^+$), 343 (20, $[M+H]^+$); 343.1194 ($[M+Na]^+$) found by +ES, required 343.1190, error 1.3 ppm.

Synthesis of 5-(isopentylamino)-6H-[1,2,3]triazolo[4,5,1-de]acridin-6-one (**8c**): A similar procedure was adopted as above using **4c** and 3-methylbutylamine to give **8c** (0.09 g, 76%) as green crystals: mp 144–145 °C; ν_{max}/cm^{-1} 3273w (N–H), 3066w, 2950w, 2864w (C–H), 1657s (C=O), 1623 m, 1599s, 1582s, 1502s; δ_H (500 MHz; DMSO-d_6) 9.30 (1H, t, *J* 5.7, NH), 8.50 (1H, d, *J* 7.6, C(10)H), 8.40 (1H, dd, *J* 8.2, 1.2, C(7)H), 8.33 (1H, d, *J* 9.2, C(3)H), 8.01 (1H, app.dt, *J* 7.8, 1.2, C(9)H), 7.71 (1H, app.t, *J* 7.8, C(8)H), 7.16 (1H, d, *J* 9.2, C(4)H), 3.57 (2H, app.q, *J* 6.8, C(12)H_2), 1.76 (1H, app.quin, *J* 6.7C(14)H), 1.61 (2H, app.t, *J* 7.2, C(13)H_2), 0.98 (6H, d, *J* 6.6, C(15)H_3, C(16)H_3); δ_C (100; MHz, $CDCl_3$) 178.2 (q, C(6)), 152.9 (q), 135.4 (q), 135.3 (q), 133.7 (C(9)H), 132.3 (q), 128.9 (C(3)H), 127.7 (C(7)H), 126.5 (C(8)H), 125.1 (q), 115.7 (C(10)H), 110.9 (C(4)H), 100.0 (q), 41.5 (C(12)H_2), 37.9 (C(13)H_2), 25.8 (C(14)H), 22.5 (C(15)H_3, C(16)H_3); *m/z* (−ES) 305 (100, $[M-H]^-$); (+ES) 329 (100, $[M+Na]^+$), 307 (10, $[M+H]^+$); 329.1380 ($[M+Na]^+$) found by +ES, required 329.1373, error 2.2 ppm.

Synthesis of 5-(phenethylamino)-6H-[1,2,3]triazolo[4,5,1-de]acridin-6-one (**9c**): A similar procedure was adopted as above using **4c** and 2-phenylethylamine to give **9c** (0.102 g, 77%) as dark, green crystals: mp 153–155 °C. $C_{21}H_{16}N_4O$ requires C, 74.10; H, 4.74; N, 16.46. Found: C, 73.85; H, 4.77; N, 16.36. ν_{max}/cm^{-1} 3280w (N–H), 2926w, 2863w (C–H), 1658s (C=O), 1623 m, 1599s, 1567s; δ_H (500 MHz; DMSO-d_6) 9.27 (1H, t, *J* 5.8, NH), 8.44 (1H, d, *J* 7.9, C(10)H), 8.34 (1H, d, *J* 8.0, C(7)H), 8.26 (1H, d, *J* 9.2, C(3)H), 7.98

(1H, app.t, *J* 7.7, C(9)H), 7.67 (1H, app.t, *J* 7.7, C(8)H), 7.37 (2H, d, *J* 7.3, C(15)H, C(19)H), 7.33 (2H, app.t, *J* 7.3, C(16)H, C(18)H), 7.23 (1H, app.t, *J* 7.3, C(17)H), 7.15 (1H, d, *J* 9.2, C(4)H), 3.79 (2H, app.q, *J* 6.7, C(12)H_2), 3.01 (2H, t, *J* 7.3, C(13)H_2); δ_C (75 MHz; DMSO-d_6) 176.8 (q, C(6)), 152.2 (q), 138.5 (q), 134.8 (q), 134.6 (q), 134.1 (C(8)H), 131.6 (q), 128.9 (C(17)H, C(19)H), 128.8 (C(9)H), 128.5 (C(16)H, C(20)H), 127.3 (C(10)H), 126.7 (C(7)H), 126.5 (C(18)H), 124.5 (q), 115.3 (C(3)H), 111.9 (C(4)H), 99.1 (q), 44.0 (C(13)H_2), 34.8 (C(14)H_2); *m/z* (−ES) 339 (80, $[M-H]^-$); (+ES) 363 (100, $[M+Na]^+$); 363.1214 ($[M+Na]^+$) found by +ES, required 363.1216, error 0.6 ppm.

Synthesis of 5-(2-(dimethylamino)ethylamino)-8-bromo-6H-[1,2,3]triazolo[4,5,1-de]acridin-6-one (**5d**): A similar procedure was adopted as above using **4d** and *N,N*-dimethylethylendiamine to give **5d** (94.9 mg, 82%) as yellow crystals: mp 200.8–201.2 °C; ν_{max}/cm^{-1} 3251w (N–H), 2942w, 2793w (C–H), 1661s (C=O), 1625s, 1599s, 1574s and 1562s; δ_H (500 MHz; DMSO-d_6) 9.39 (1H, s, NH), 8.59 (1H, d, *J* 2.0, C(7)H), 8.35 (1H, d, *J* 8.6, C(10)H), 8.12 (1H, d, *J* 9.0, C(3)H), 7.90 (1H, dd, *J* 8.6, 2.0, C(9)H), 6.92 (1H, d, *J* 9.0, C(4)H), 3.63 (2H, br s, C(12)H_2), 2.77 (2H, br s, C(13)H_2), 2.41 (6H, s, Me_2); δ_C (75 MHz; $CDCl_3$) 176.6 (q, C(6)), 152.7 (q), 136.5 (C(9)H), 135.5 (q), 134.1 (q), 132.3 (q), 130.8 (C(7)H), 129.2 (C(3)H), 126.6 (q), 120.3 (q), 117.4 (C(10)H), 111.2 (C(4)H), 100.2 (q), 57.5 (C(13)H_2), 45.4 (C(14)H_3, C(15)H_3), 41.1 (C(12)H_2); *m/z* (+ES) 388 (100, $[M+H]^+$, ^{81}Br), 386 (100, $[M+H]^+$, ^{79}Br); 386.0611 ($[M+H]^+$) found by +ES, required 386.0611 error 0.0 ppm.

Synthesis of 5-(3-hydroxypropylamino)-8-bromo-6H-[1,2,3]triazolo[4,5,1-de]acridin-6-one (**6d**): A similar procedure was adopted as above using **4d** and 3-amino-1-propanol to give **6d** (92.6 mg, 83%) as yellow crystals: mp 210.8–211.0 °C; ν_{max}/cm^{-1} 3438 m (O–H), 3254w (N–H), 3076w, 2929w, 2873w (C–H), 1665s (C=O), 1622s, 1598s, 1566s; δ_H (300 MHz; $CDCl_3$) 9.36 (1H, s, NH), 8.58 (1H, d, *J* 2.2, C(7)H), 8.37 (1H, d, *J* 8.7, C(10)H), 8.12 (1H, d, *J* 9.3, C(3)H), 7.91 (1H, dd, *J* 8.7, 2.2, C(9)H), 6.93 (1H, d, *J* 9.3, C(4)H), 3.84 (2H, t, *J* 5.8, C(14)H_2), 3.63 (2H, app.q, *J* 6.4, C(12)H_2), 2.00 (2H, app.p, *J* 6.3, C(13)H_2); δ_C (75 MHz; DMSO-d_6) 175.2 (q, C(6)), 152.5 (q), 136.5 (C(9)H), 134.6 (q), 133.5 (C(7)H), 131.7 (q), 129.4 (C(3)H), 129.1 (q), 126.1 (q), 119.3 (q), 117.7 (C(10)H), 111.8 (C(4)H), 99.1 (q), 58.1 (C(12)H), 40.1 (C14)H), 31.7 (C(13)H); *m/z* (−ES) 373 (100, $[M-H]^-$, ^{81}Br), 371 (100, $[M-H]^-$, ^{79}Br); (+ES) 397 (50, $[M+Na]^+$, ^{81}Br), 395 (50, $[M+Na]^+$, ^{79}Br); 371.0150 ($[M-H]^-$) found by −ES, required 371.0150 error 0.2 ppm.

Synthesis of 5-(4-methoxyphenylamino)-8-bromo-6H-[1,2,3]triazolo[4,5,1-de]acridin-6-one (**7d**): A similar procedure was adopted as above using **4d** and *p*-anisidine to give **7d** (104 mg, 83%) as an orange powder: mp 240–245 °C (dec); ν_{max}/cm^{-1} 3236w (N–H), 1658s (C=O), 1621s, 1599s, 1574s, 1559s, 1508s; δ_H (300 MHz; $CDCl_3$) 10.71 (1H, s br, NH), 8.68 (1H, d, *J* 2.2, C(7)H), 8.46 (1H, d, *J* 8.7, C(10)H), 8.10 (1H, d, *J* 9.2, C(3)H), 8.01 (1H, dd, *J* 8.7, 2.2, C(9)H), 7.34 (2H, d, *J* 8.8, C(14)H, C(16)H), 7.14 (1H, d, *J* 9.2, C(4)H), 7.03 (2H, d, *J* 8.8, C(13)H, C(17)H), 3.88 (3H, s, OMe, C(18)H_3), 1.58 (3H, s, OMe, C(19)H_3); δ_C (75 MHz; $CDCl_3$) 177.1 (q, C(6)), 158.5 (q), 151.7 (q), 136.9 (C(9)H), 136.4 (q), 134.3 (q), 132.2 (q), 130.8 (C(7)H), 130.4 (q), 129.0 (C(3)H), 126.5 (C(14)H, C(16)H), 126.4 (q), 120.5 (q), 117.5 (C(10)H), 115.1 (C(13)H, C(17)H), 112.5 (C(4)H), 100.7 (q), 55.6 (C(18)H_3); *m/z* (−ES) 421 (50, $[M-H]^-$, ^{81}Br), 419 (50, $[M-H]^-$, ^{79}Br); 419.0139 ($[M-H]^-$) found by −ES, required 419.0138 error 0.2 ppm.

Synthesis of 5-(2-(dimethylamino)ethylamino)-8-methoxy-6H-[1,2,3]triazolo[4,5,1-de]acridin-6-one N-oxide (**10a**): Compound **5a** (100 mg, 0.296 mmol) was stirred in $CHCl_3$ (5 mL) until dissolved. The mixture was cooled to below 5 °C before 50% *meta*-chloroperbenzoic acid (mCPBA) (113 mg, 0.326 mmol) was added. The solution was stirred for 24 h at room temperature. The reaction mixture was added straight to a column of alumina for purification by flash chromatography using MeOH/$CHCl_3$ (1:10). This gave crude **10a** (182 mg) which was then dissolved in $CHCl_3$ (5 mL) and filtered to remove alumina residues and reduced under vacuum to give pure **10a** (65 mg, 62%) as a yellow powder: mp 147–148 °C; ν_{max}/cm^{-1} 3320 m (N–H), 1663 (C=O), 1602s, 1580s, 1565s; δ_H (500 MHz; D_2O) 7.17 (1H, s), 6.66 (1H, d, *J* 6.6), 6.42 (1H, d, *J* 7.2), 6.23 (1H, d, *J* 8.8), 5.78 (1H, s), 3.52 (2H, s, C(13)H_2), 3.42 (2H, s, C(14)H_2), 3.23 (3H, s, OMe), 3.19 (6H, s, Me_2); *m/z* (−ES) 352 (60, $[M-H]^-$); (+ES) 376 (100, $[M+Na]^+$), 354 (15, $[M+H]^+$); 354.1563 ($[M+H]^+$) found by +ES, required 354.1561 error 0.7 ppm.

Synthesis of 5-(2-(dimethylamino)ethylamino)-8-fluoro-6H-[1,2,3]triazolo[4,5,1-de]acridin-6-one N-oxide (**10b**): A similar procedure was adopted as above using **5b** to give **10b** (27 mg, 52%). as a yellow powder: mp 130–140 °C (dec); ν_{max}/cm^{-1} 3248br (N–H), 1663s (C=O), 1630s, 1578s, 1500s; δ_H (300 MHz; D_2O) 7.34 (1H, d, *J* 9.5, C(3)H), 7.20–7.08 (2H, m, C(9)H, C(10)H), 6.75 (1H, dd, *J* 9.4, 2.2, C(7)H), 6.38 (1H, d, *J* 9.5, C(4)H), 3.63 (2H, t, *J* 6.1, C(12)H_2), 3.49 (2H, t, *J* 6.1, C(13)H_2), 3.23 (6H, s, Me_2); *m/z* (−ES) 340 (100, $[M-H]^-$); (+ES) 364 (100, $[M+Na]^+$); 340.1216 ($[M-H]^-$) found by −ES, required 340.1215 error 0.2 ppm.

Synthesis of 5-(2-(dimethylamino)ethylamino)-6H-[1,2,3]triazolo[4,5,1-de]acridin-6-one N-oxide (**10c**): A similar procedure was adopted as above using **5c** to give **10c** (28 mg, 46%) as a yellow powder: mp 145–146 °C; ν_{max}/cm^{-1} 3271w (N–H), 2925w, 2863w (C–H), 1661s (C=O), 1624s, 1599s, 1601s, 1570s; δ_H (300 MHz; $CDCl_3$) 9.90 (1H, br s, NH), 8.51–8.57 (2H, m, C(7)H, C(10)H), 8.25 (1H, d, *J* 9.1, C(3)H), 7.90 (1H, app.t, *J* 7.7, C(9)H), 7.62 (1H, app.t, *J* 7.7, C(8)H), 7.15 (1H, d, *J* 9.1, C(4)H), 4.24 (2H, app.q, *J* 5.9, C(12)H_2), 3.70 (2H, t, *J* 5.9, C(13)H_2), 3.39 (6H, s, NMe_2); *m/z* (−ES) 322 (30, $[M-H]^-$); (+ES) 346 (100, $[M+Na]^+$); 324.1452 ($[M+H]^+$) found by +ES, required 324.1455 error −0.9 ppm.

Synthesis of 5-(2-(dimethylamino)ethylamino)-8-bromo-6H-[1,2,3]triazolo[4,5,1-de]acridin-6-one N-oxide (**10d**): A similar procedure was adopted as above using **5d** to give **10d** (50 mg, 50%). as a yellow powder: mp 143–144 °C; ν_{max}/cm^{-1} 3253br (N–H), 2956w (C–H), 1660s (C=O), 1622s, 1599s, 1565s; δ_H (300 MHz; $CDCl_3$) 10.06 (1H, s, NH), 8.64 (1H, d, *J* 2.2, C(7)H), 8.43 (1H, d, *J* 8.7, C(10)H), 8.24 (1H, d, *J* 9.2, C(3)H), 7.98 (1H, dd, *J* 8.7, 2.2, C(9)H), 7.15 (1H, d, *J* 9.2, C(4)H), 4.32 (2H, app.q, *J* 6.0, C(12)H_2), 3.68 (2H, t, *J* 6.1, C(13)H_2), 3.36 (6H, s, Me_2); *m/z* (−ES) 402 (100, $[M-H]^-$, ^{81}Br), 400 (100, $[M-H]^-$, ^{79}Br); (+ES) 426 (100, $[M+Na]^+$, ^{81}Br), 424 (100, $[M+Na]^+$, ^{79}Br); 402.0563 ($[M+H]^+$) found by +ES, required 402.0560 error 0.8 ppm.

5.2. Molecular modeling

For docking purposes, the crystallographic coordinates of human NQO1 (PDB code 1D4A; resolution 1.7 Å) and NQO2 (PDB code 1QR2; resolution 2 Å) were obtained from the Brookhaven Database. Hydrogen atoms were added to these structures which included the protein and FAD allowing for appropriate ionization at physiological pH. The protonated complexes were then minimized within SYBYL 7.3 whilst holding all heavy atoms stationary. The ligand was then removed leaving the enzyme active site available for subsequent docking studies. Inspection of the active sites of both enzymes showed a reasonable internal hydrogen bond network within the protein.

For preparation of ligand structures, fragments from SYBYL 7.3 were used to construct the compounds. Ligands were subject to 1000 iterations of energy minimization using the steepest descent algorithm using the Tripos force field. Flexible ligand docking was performed using the GOLD 4.1 software in combination with the ChemScore[27] scoring function. The FAD fragment was re-atom-typed to avoid underestimation by ChemScore of lipophilic/aromatic interactions. The active site was defined as being any volume

within 15 Å of N5 of the FAD cofactor. The top ten solutions for each ligand were retained and analyzed for favourable interactions within the active site of NQO1 and NQO2, including low protein–ligand clash, ligand distortion energies, lipophilic contacts and hydrogen bonding interactions.

5.3. Inhibition of NQO1 and NQO2

Recombinant human NQO1 and NQO2 were obtained from Sigma and diluted in 50 mM phosphate buffer to give an enzyme activity that would result in a change in optical absorbance of substrate of approximately 0.1 per minute. The enzyme reaction was started by adding 5 μL of this solution to 495 μL of 50 mM phosphate buffer at pH 7.4 containing either 200 μM NADH or NRH for NQO1 or NQO2, respectively, together with 40 μM DCPIP (dichlorophenolindophenol) and various concentrations of the potential inhibitor dissolved in DMSO (final concentration 1.0% v/v). Reactions were carried out at 37 °C and reduction of DCPIP was monitored at 600 nm in a Beckman DU 650 spectrophotometer. IC_{50} values were determined using nonlinear curve fitting as implemented in the program Excel for which a 50% reduction of the initial rate was attained. Each measurement was made in triplicate and the experiments carried out three times. Values of IC_{50} were converted to experimental binding affinities using the Cheng–Prusoff equation[28], where $\Delta G_{calc} = -RT \ln(IC_{50}/\{1 + ([S]/K_m)\}$ where $[S]$ is the substrate concentration and K_m is the Michaelis constant. Regression analyses were derived using Excel.

5.4. Toxicity assays

HCT116 colon cancer carcinoma cells were routinely maintained in monolayer culture in RPMI 1640, supplemented with 10% FCS (foetal calf serum) and 2 mM L-glutamine. Chemosensitivity was determined by MTT assay[37]. 2×10^3 cells were plated per well in a 96-well culture plate and incubated at 37° in an atmosphere of air plus 5% CO_2. Cells were than exposed continuously to drug for 4 days at 37 °C. Alternatively, 24 h drug exposure was used after which drug was removed and 0.2 ml of fresh medium was added and cells allowed to grow for a further 3 days. Subsequently, MTT was added and cells incubated for a further 4 h. After this time, culture medium and MTT solution were removed and formazan crystals were dissolved in DMSO. Optical density was measured at 550 nM on a BioTek—μQuant multiwell spectrophotometer using Gen5 software. Values of IC_{50} are the drug concentrations required to reduce optical density by 50% relative to untreated and/or vehicle only treated cells. All experiments were repeated at least three times.

5.5. Measurement of melting temperature

DNA stability was studied by establishing the melting temperature of SSDNA (Salmon Sperm DNA) in the presence of 10 μM of the triazoloacridin-6-ones in high and low salt phosphate buffers (High salt—150 mM NaCl, 15 mM sodium citrate, pH 7.0. Low salt—7.5 mM NaH_2PO_4, 1 mM EDTA, pH 7.0). Curettes were heated over the range 37–98 °C and absorbance at 260 nm was read every degree, using a Perkin–Elmer™ Precisely Lambda 25 UV/vis Spectrophotometer. Temperature was controlled by a Perkin-Elmer™ Peltier Temperature Programmer (PTP-6 Peltier System). Determination of the T_m was read from the mid-point of the hypochromic transition curves. Relative absorbance was plotted using Origin® 8.0 software. Curve fitting was done using the sigmoidal function which gave a Boltzmann distribution and regression analysis of the data. Values of T_m were derived from at least three independent 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.2009.11.059.

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