Synthesis and biological evaluation of novel diaziridinylquinone–acridine conjugates

Angela M. Di Francesco[a,b], Stephen P. Mayalarp[c], Susan Kim[d], John Butler[b] and Moses Lee[d]

The synthesis and biological evaluation of a homologous series of conjugates (9–13) of 2,5-diaziridinylbenzoquinone (DZQ) and 9-carbonylacridine, a DNA intercalating moiety, via a polymethylene unit ($n = 2$–6) are described. In addition, the non-acridine compound 14, analogous to compound 12, and the 5-methyl-DZQ derivatized conjugate 15, an analog of compound 10, were also prepared. Through a Comet assay, compounds 9–13 were shown to produce DNA interstrand cross-links at submicromolar concentrations, consistent with K562 leukemia cells accumulating in the G_2/M stage in the cell cycle. The cytotoxicity of compounds 9–15 was examined using a MTT assay on several human cancer cell lines, including chronic myeloid leukemia K562, the non-small cell lung cancers H596 and H460, and colon carcinoma cells BE and HT29. H460 and HT29 are rich in DT-diaphorase (DTD), and H596 and BE cells have negligible amounts of functional DTD. Under continuous exposure of drugs, except to the non-aziridine compound 19b, the IC_{50} values of all other compounds were determined to be in the range of 0.3–11.3 nM. Compound 10, which has a propyl linker group, was subjected to *in vivo* studies. When BDF1 mice with established mouse mammary carcinoma were treated with compound 10 (2 mg/kg at day 1 and 5 mg/kg at day 7), a significant delay (9–10 days) in cancer growth was recorded when compared to untreated controls. Furthermore, administration of compound 10 to *nu/nu* BDF1 mice bearing human lung cancer H460 xenograft (1.5 mg/kg for 10 for five consecutive days from day 13 and 17) also showed a significant reduction in tumor growth compared to untreated controls. The half-life of compound 10 in the presence of five different peptidases (porcine esterase, carboxypeptidase A, B and Y, and pepsin) was determined to be between 30 and 60 h. *Anti-Cancer Drugs* 14:601–615 © 2003 Lippincott Williams & Wilkins.

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[a]Division of Pediatric Oncology, Catholic University, Rome, Italy, [b]Biological Sciences, Salford University, Manchester, UK, [c]PE Biosystems, Warrington, UK and [d]Department of Chemistry, Furman University, Greenville, SC, USA.

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Correspondence to M. Lee, Department of Chemistry, Furman University, Greenville, SC 29613-1120, USA.
Tel: +1 864 294-3368; fax: +1 864 294-3559;
e-mail: Moses.Lee@furman.edu

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Introduction

One approach to the design of selective anticancer drugs is to exploit the presence of hypoxic tissues within the solid tumor [1,2]. Solid tumors contain regions of hypoxia that account for 5–30% of the total viable tumor cell population. This hypoxic condition, which develops because of inefficient vascularization in solid tumors larger than a few millimeters in diameter [3,4], places these cells in a unique environment, such as lower pH [4,5], which makes them an attractive target for selective chemotherapy. These cells tend to be resistant to both radio- and chemotherapy, and may be the limiting factor in much clinical cancer treatment [6–8]. Because solid tumors are known to have a highly reducing environment, compounds that can be activated by reduction reaction and in a weakly acidic environment, such as mitomycin C **1** [9], AZQ **2** [10], EO-9 **3** [11,12], tirapazamine **4** [13] and trenimon **5** [14], as depicted in Figure 1, have been developed as anticancer treatments [15].

Several aziridinylbenzoquinones, such as AZQ, have undergone clinical trials as potential anticancer agents. AZQ is a bioreductively activatable agent which has shown clinical activity in brain tumors [16,17] as well as acute non-lymphocytic leukemia [18,19]. Trenimon was widely used in Europe for the treatment of breast and ovarian carcinomas [20]. The indoloquinone EO-9 was also considered to be a potential anticancer agent [12]. Tirapazamine exhibits excellent activity against MV-522 human lung adenocarcinoma xenograft in nude mice when administered in combination with taxol and oxaliplatin [21]. RH1 **6**, which exhibits potent cytotoxicity, has recently been selected for clinical evaluation in the UK [22]. These types of compounds can be activated toward DNA alkylation as a result of bioreduction which is mainly brought about by NADPH:cytochrome P-450 reductase, a one-electron reducing enzyme which produces a semiquinone radical, or via NAD[P]H:oxidoreductase [DT-diaphorase (DTD)], a two-electron

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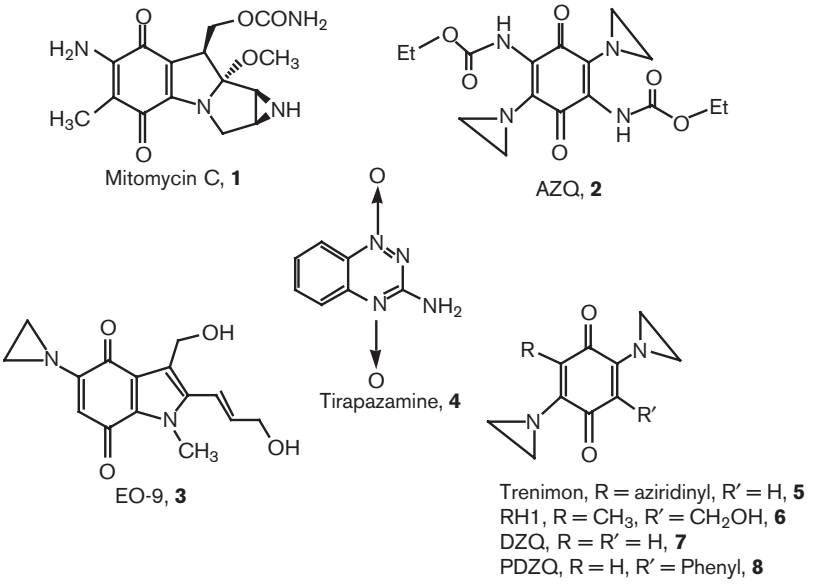
Fig. 1



Examples of bioreductively activatable anticancer agents.

reducing enzyme which results in the formation of an activated hydroquinone. High levels of DTD in tumor cells might enhance bioreductive DNA alkylation, since it has been shown that DTD reduces quinones to hydroquinones [23,24]. Upon reduction, the hydroquinones or semiquinones of AZQ, or DZQ, MeDZQ and RH1, or trenimon react with DNA and effectively produce interstrand cross-links, thereby exerting their biological activity.

DZQ **7**, the simplest analog of AZQ, MeDZQ (dimethylDZQ), trenimon and PDZQ (**8**) [25,26], also has potent anticancer properties, and upon reduction it intercalates into GC sites to produce a DNA interstrand cross-link at 5′-TGC sites where it displays cytotoxic activity [23,27–29]. Recently, a conjugate of DZQ with a triplex oligonucleotide was reported and it was shown to have improved sequence-directed cross-linking activity [30]. Several reports have demonstrated that attachment of DNA-modifying agents to sequence-specific binders can dramatically improve the anticancer activity of nitrogen mustards, such as the conjugates of a benzoic acid mustard group with distamycin analogs [31–33]. In this paper compounds **9–13** were designed in which the DZQ moiety is tethered to an intercalating agent, 9-carbonylacridine. Acridines that are substituted in the 9-position were chosen as the DNA-targeting vehicle because their conjugates to aniline *N*-mustards have enhanced DNA interaction and anticancer activity over the alkylating agent alone [34]. For **9–13**, the number of methylene groups in the tether was varied from two to six to provide the flexibility in optimizing the interactions of both the DZQ and the acridine groups with the DNA base pairs. In order to ascertain the effects of the acridine moiety on the biological activity of **9–13**, a non-acridine compound (**14**) was also designed and evaluated. Compound **15**, a 6-methylated analog of **10** and related to RH1, was also prepared with the hope of enhancing its selectivity for DTD [22]. This assumption was based on the observation that, as the methyl analog **15** cannot undergo Michael addition reactions, it would give better cytotoxic differentials than the non-methylated counterparts. Essentially, MeDZQ is a poorer substrate for DTD than DZQ, but is much more toxic and produces large cytotoxic differentials [38,41].

Methods

N-(*N*′-Butoxycarboxamidoethyl)acridine-9-carboxamide 16a

A suspension of acridine-9-carboxylic acid (0.5 g, 2.24 mmol), dicyclohexylcarbodiimide (0.93 g, 4.5 mmol) and hydroxybenzotriazole monohydrate (0.30 g, 2.2 mmol) in dry dichloromethane (15 ml), at room temperature under nitrogen was stirred for 30 min and then treated with *N*-BOC-ethylenediamine (0.39 ml, 2.47 mmol). The mixture was left stirring for 17.5 h at room temperature (22°C) after which it was subjected to 3 h of sonication. The mixture was filtered, the residue was washed with dichloromethane and the filtrate was evaporated *in vacuo*. The residue was chromatographed on silica gel using chloroform:methanol (19:1) as eluent to give compound **16a** as a yellow solid (681 mg, 83%). M.p.

189–191°C; R_f 0.29 [chloroform:methanol (9:1)]; IR (film) 3316 (broad), 2977, 2931, 1704, 1659 and 1519; UV-vis (EtOH) 359, 246 and 211 nm; ^{1}H-NMR ($CDCl_3$, 200 MHz) 8.15 (2 H, dd, 8.5 and 1.5), 7.96 (2 H, dd, 8.5 and 1.5), 7.74 (2 H, dd, 8.5 and 1.5), 7.49 (2 H, td, 8.5 and 1.5), 7.15 (1 H, bs), 5.16 (1 H, bs), 3.83 (2 H, q, 6.0), 3.51 (2 H, q, 6.0), 1.35 (9 H, s); EIMS (*m/z*) 365 (M +), 265, 248, 235, 222, 206 and 178; accurate mass for $C_{21}H_{23}N_3O_3$: calcd 365.1739; found 365.1739; elemental analysis for $C_{21}H_{23}N_3O_3.H_2O$: calcd C, 65.80; H, 6.53; N, 10.97; found C, 65.92; H, 6.78; N, 11.07.

Synthesis of compounds 16b–e

The procedure for preparing compounds **16b–e** were similar to that used for the synthesis of compound **16a** except the appropriate *N*-BOC-alkyldiamine (2.5 ml, 14.3 mmol) was used. *N*-(*N*′-butoxycarboxamidopropyl) acridine-9-carboxamide **16b** was isolated as a yellow solid (2.06 g, 38%). M.p. 123°C; R_f 0.53 [chloroform:methanol, (19:1)], IR (film) 3365, 2923, 1696, 1582 and 1525; UV-vis (EtOH) 360, 247 and 211; ^{1}H-NMR ($CDCl_3$, 300 MHz) 8.18 (2 H, d, 8.0), 7.92 (2 H, d, 8.0), 7.80 (2 H td, 8.0 and 1.5), 7.66 (2 H, td, 8.0 and 1.5), 6.30 (1 H, t, 5.0), 3.72 (2 H, q, 6.0), 3.31 (2 H, q, 6.0), 1.86 (2 H, quin, 6.0), 1.38 (9 H, s); FABMS (*m/z*) 380 (M + H); accurate mass for $C_{22}H_{25}N_3O_3$: calcd 380.1974; found 380.1973.

N-(*N*′-*t*-butoxycarboxamidobutyl)acridine-9-carboxamide **16c** was isolated as a yellow solid (507 mg, 58%). M.p. 147–150°C; R_f 0.24 [chloroform:methanol (19:1)]; IR (film) 3586, 3291, 2971, 2939, 2877, 1694, 1948 and 1523; UV-vis (EtOH) 360, 248 and 211; ^{1}H-NMR ($CDCl_3$, 200 MHz) 8.05 (2 H, d, 8.5), 7.89 (2 H, d, 8.5), 7.67 (2 H, td, 8.5 and 1.5), 7.45 (2 H, td, 8.5 and 1.5), 7.23–7.08 (1 H, bm), 4.80–4.68 (1 H, bm), 3.76–3.60 (2 H, m), 3.25–3.09 (2 H, m), 1.90–1.60 (4 H, m), 1.40 (H, s); EIMS (*m/z*) 393 (M +), 320, 235, 206 and 178; accurate mass for $C_{23}H_{27}N_3O_3$: calcd 393.2052; found 393.2059.

N-(*N*′-butoxycarboxamidopentyl)acridine-9-carboxamide **16d** was isolated as a yellow solid (0.51 g, 81%). M.p. 136–138°C; IR (film) 3265 (broad), 2942, 1691, 1643 and 1517; UV-vis (EtOH) 360, 245 and 211; ^{1}H-NMR ($CDCl_3$, 200 MHz) 8.18 (2 H, d, 8.5), 8.02 (2 H, d, 8.5), 7.76 (2 H, ddd, 8.5, 8.5 and 1.5), 7.55 (2 H, ddd, 8.5, 8.5 and 1.5), 6.59 (1 H, bs), 4.58 (1 H, bs), 3.72 (2 H, q, 7.0), 3.15 (2 H, q, 7.0), 1.89–1.74 and 1.64–1.50 (6 H, m), 1.32 (9 H, s); *m/z* (E1) 407 (M +), 350, 334, 306, 291, 277, 235, 221, 206 and 178; elemental analysis for $C_{24}H_{29}N_3O_3$: calcd C, 70.76; H, 7.13; N, 10.32%; found C, 70.83; H, 7.51; N, 10.41.

N-(*N*′-butoxycarboxamidohexyl)acridine-9-carboxamide **16e** was isolated as a yellow solid (157 mg, 83%). M.p. 129–131°C; R_f 0.66 [chloroform:methanol (19:1) run 2 times]; IR (film) 3280 (broad), 3066, 2973, 2933, 2854, 1691, 1639, 1540 and 1517; UV-vis (EtOH) 359, 250 and 211; ^{1}H-NMR ($CDCl_3$, 200 MHz) 8.19 (2 H, d, 8.5), 8.03 (2 H, d, 8.5), 7.77 (2 H, ddd, 8.5, 8.5 and 1.5), 7.55 (2 H, ddd, 8.5, 8.5 and 1.5), 6.60 (1 H, bs), 4.56 (1 H, bs), 3.70 (2 H, q, 6.0), 3.18–3.03 (2 H, m), 2.0–1.2 (8 H, m), 1.40 (9 H, s,); EIMS (M/z) 421 (M +), 364, 348, 320, 291, 277, 263, 235, 206 and 178; accurate mass for $C_{25}H_{31}N_3O_3$: calcd 421.2365; found 421.2362.

N-(2′-Aminoethyl)acridine-9-carboxamide 17a

To a cold (ice bath) suspension of *N*-(*N*′-butoxycarboxamidoethyl)acridine-9-carboxamide **16a** (0.642 g, 1.76 mmol) in THF (40 ml) was added 6 M HCl (40 ml) and the resulting yellow solution was left stirring for 15 h while warming to room temperature. The mixture was evaporated to dryness *in vacuo* and the resulting residue was dissolved in water (500 ml) and washed with chloroform (2 × 100 ml). The aqueous layer was basified to pH 12 with 4 M NaOH and the resulting solution extracted with chloroform (4 × 100 ml). The organic extracts were combined, washed with brine, dried (Na_2SO_4) and the solvent removed under reduced pressure to afford compound **17a** as a yellow solid (479 mg). R_f 0.07 [chloroform:methanol (9:1)]; IR (film) 3407, 3201, 3048, 2939, 2858, 1635 and 1581; UV-vis (EtOH) 360, 251 and 211; ^{1}H-NMR ($CDCl_3$, 200 MHZ) 8.20 (2 H, dd, 8.5 and 1.5), 8.06 (2 H, dd, 8.5 and 1.5), 7.77 (2 H, td, 8.5 and 1.5), 7.75 (2 H, td, 8.5 and 1.5), 6.80 (1 H, bs), 3.77 (2 H, q, 6.0), 3.10 (2 H, t, 6.0); EIMS (*m/z*) 265 (M +), 249, 235, 221, 206, 193 and 178; accurate mass for $C_{16}H_{15}N_3O$: calcd 265.1215; found 265.1225.

Synthesis of compounds 17b–e

N-(3′-Aminopropyl)acridine-9-carboxamide **17b** was prepared using a similar procedure to the synthesis of compound **17a**. Compound **17b** was obtained as a yellow solid (1.18 g, 87%). IR (film) 3262, 3059, 2931, 2856, 1643, 1547 and 1520; UV-vis (EtOH) 360, 244 and 210; ^{1}H-NMR ($CDCl_3$, 300 MHz) 8.05 (2 H, d, 9.0), 7.90 (2 H, d, 9.0), 7.65 (2 H, td, 9.0 and 1.5), 7.43 (2 H, td, 9.0 and 1.5), 3.72 (2 H, q, 6.0), 2.86 (2 H, t, 6.0), 1.80 (2 H, quin, 6.0), 1.70 (2 H, bs); FABMS (*m/z*) 280 (M + H); accurate mass for $C_{17}H_{17}N_3O$: calcd 280.1450; found 280.1465.

N-(4′-Aminoethyl)acridine-9-carboxamide **17c** was obtained as a yellow solid (317 mg, 84%). R_f 0.09 [chloroform:methanol (9:1)]; IR (film) 3237, 3077, 2935, 2867, 1650, 1619 and 1552; UV-vis (EtOH) 360, 245 and 211; ^{1}H-NMR ($CDCl_3$, 200 MHz) 8.20 (2 H, d, 8.5), 8.05 (2 H, d, 8.5), 7.77 (2 H, td, 8.5 and 1.5), 7.55 (2 H, td, 8.5 and 1.5), 7.46–7.37 (1 H, m), 3.73 (2 H, q, 6.0), 2.77 (2 H, t, 6.0), 1.89–1.55 (4 H, m); CIMS (*m/z*) 294 (M + H), 277; accurate mass for $C_{18}H_{20}N_3O$: calcd 294.1606; found 294. 1606.

N-(5′-Aminopentyl)acridine-9-carboxamide **17d** was obtained as a yellow solid (487 mg, 88%); R_f 0.09 [chloroform:methanol (9:1)]; IR (film) 3237, 3070, 2933, 2859, 1645, 1552; UV-vis (EtOH) 360, 243 and 211; ^{1}H-NMR ($CDCl_3$, 200 MHz) 8.21 (2 H, d, 8.5), 8.05 (2 H, d, 8.5), 7.78 (2 H, ddd, 8.5, 8.5 and 1.5), 7.58 (2 H, ddd, 8.5, 8.5 and 1.5), 6.45 (1 H, bs), 3.74 (2 H, q, 7.0), 2.75 (2 H, t, 7.0), 1.88–1.73 and 1.60–1.48 (6 H, m); EIMS (*m*/*z*) 307 (M +), 227, 263, 249, 235, 211, 206 and 178; accurate mass for $C_{19}H_{21}N_3O$: calcd 307.1685; found 307.1685.

N-(6′-Aminohexyl)acridine-9-carboxamide **17e** was obtained as a yellow solid (230 mg, 76%); R_f 0.09 [chloroform:methanol (9:1)]; IR (film) 3255 (broad), 3058, 2927, 2856, 2356, 2362, 2343, 1645, 1550, 1517; UV-vis (EtOH) 360, 250 and 211; ^{1}H-NMR ($CDCl_3$, 300 MHz) 8.09 (2 H, d, 8.5), 7.94 (2 H, d, 8.5), 7.69 (2 H, ddd, 8.5, 8.5 and 1.5), 7.46 (2 H ddd, 8.5, 8.5 and 1.5), 6.65 (1 H, J 6.0), 6.68 (2 H, q, 6.0), 2.65 (2 H, t, 6.0), 1.80–1.34 (8 H, m); CIMS (*m*/*z*) 322 (M + H); EIMS (*m*/*z*) 321 (M +), 291, 277, 263, 235, 221, 206 and 178; accurate mass for $C_{20}H_{23}N_3O$: calcd 321.1841; found 321.1844.

2-(4′-*N*-(2″-(9‴-acridinecarboxamido)ethyl)benzamido)-quinone 19a

A suspension of the 4-(2′-quinone)benzoic acid **18** (414 mg, 1.82 mmol), dicyclohexylcarbodiimide (749 mg, 3.63 mmol) and hydroxybenzotriazole monohydrate (245 mg, 1.82 mmol) in dry DMF (10 ml) at room temperature under nitrogen was stirred for 25 min and was then added a solution of the amine *N*-(2′-aminoethyl)acridine-9-carboxamide (481 mg, 1.82 mmol) in dry DMF (2 ml). The mixture was left stirring for 17 h after which it was diluted with dichloromethane, washed several times with water, then brine, dried (Na_2SO_4) and the solvent removed *in vacuo*. The resulting residue was chromatographed twice on silica gel using chloroform:methanol (19:1) to give 2-(4′-*N*-(2″-(9‴-acridinecarboxamido)ethyl)benzamido)quinone **19a** as a yellow solid (297 mg, 34%). M.p. 235–238°C; R_f 0.3 [chloroform:methanol (9:1)]; IR (film) 3322 (broad), 3064, 2946, 1652 and 1591; UV-vis (EtOH) 360, 251 and 202; ^{1}H-NMR ($CDCl_3$, 200 MHz)8.16 (2 H, bd, 8.5), 7.90 (2 H, bd. 8.5), 7.88 (2 H, dd, 8.0 and 1.5), 7.72 (2 H, ddd, 8.5, 8.5 and 1.5), 7.52 (1 H, t, 6.0), 7.50 (2 H, dd, 8.8 and 1.5), 7.40 (2 H, ddd, 8.5, 8.5 and 1.5), 7.14 (1 H, t, 6.0), 6.86–6.85 (3 H, m), 4.05–3.96 (2 H, m), 3.91–3.84 (2 H, m); FABMS (*m*/*z*) 476 (M + H), 393; accurate mass for $C_{29}H_{22}N_3O_4$: calcd 476.1610; found 467.1611.

Synthesis of compounds 19b–e

The procedure to prepare 2-(4′-(*N*-(3″-acridine-9‴-carboxamido)propyl)benzamido)quinone was similar to that used for the synthesis of compound **19a**. 2-(4′-(*N*-(3″-Acridine-9‴-carboxamido)propyl)benzamido)quinone **19b** was isolated as an orange/yellow solid (526 mg, 41%). M.p. 189–196°C; R_f 0.11 [dichloromethane:ethyl acetate (3:1)]; IR (film) 3322, 2922, 2844, 1628, 1572, 1525, 1433, 1238, 1089, 756, 639 ; ^{1}H-NMR ($CDC1_3$, 200 MHz) 8.28 (2 H, d, 8.5), 8.02 (2 H, d, 8.5), 7.91 (2 H, d, 8.0), 7.78–7.66 (4 H, m), 7.51 (2 H, d, 8.0), 6.90–6.82 (3 H, m), 3.98–3.81 (2 H, m), 3.19–3.09 (2 H, m), 1.84–1.75 (2 H, m; FABMS (*m*/*z*) 512 (M + Na), 490 (M + 1); accurate mass for $C_{30}H_{24}N_3O_4$: calcd 490.1776; found 490.1776.

2-(4′-(*N*-(4″-Acridine-9‴-carboxamido)butyl)benzamido) quinone **19c** was isolated as a yellow solid (225 mg, 42%). M.p. 189–191°C; R_f 0.38 [chloroform:methanol (9:1)]; IR (film) 3293 (broad), 3070, 2931, 2869, 1650 and 1544; UV-vis (EtOH) 360, 251 and 202; ^{1}H-NMR ($CDCl_3$, 300 MHz) 8.22 (2 H, d, 8.5), 8.04 (2 H, d, 8.5), 7.83 (2 H, d, 8.0), 7.78 (2 H, ddd, 8.5, 8.5 and 1.5), 7.59 (2 H, ddd, 8.5, 8.0 and 1.5), 7.52 (2 H, d, 8.0), 6.88–6.85 (3 H, m), 6.63–6.58 (1 H, m), 6.49–6.55 (1 H, m), 3.79–3.69 (2 H, m), 3.64–3.56 (2 H, m), 1.95–1.82 (4-H, m); EIMS (*m*/*z*) 503 (M +); accurate mass for $C_{31}H_{25}N_3O_4$: calcd 503.1845; found 503.1837; elemental analysis for $C_{31}H_{25}N_3O_4.0.15\ CH_2Cl_2$: calcd. C, 72.48; H, 4.92; N, 8.24%; found C, 72.40; H, 4.70; N, 8.45.

2-(4′-(*N*-(5″-Acridine-9‴-carboxamido)pentyl)benzamido) quinone **19d** was isolated as a yellow solid (250 mg, 34%). R_f 0.24 [chloroform:methanol (19:1) run 2 times]; IR (film) 3324 (broad), 3079, 2927, 2852, 2364, 2337, 1652, 1635 and 1540; UV-vis (EtOH) 360, 251 and 203; ^{1}H-NMR ($CDCl_3$, 200 MHz) 8.20 (2 H, d, 8.5), 8.03 (2 H, d, 8.0), 7.77 (2 H, ddd, 8.5, 8.5 and 1.5), 7.60–7.51 (4 H, m), 7.35 (2 H, d, J 8.5), 6.91–6.86 (3 H, m), 6.60 (1 H, bs), 6.34 (1 H, bs), 3.78 (2 H, q, 7.0), 3.54 (2 H, q, J 7.0), 1.98–1.85 and 1.80–1.55 (6 H, m); EIMS (*m*/*z*) 517 (M +); accurate mass for $C_{32}H_{27}N_3O_4$: calcd 517.2001; found 517.2003.

2-(4′-(*N*-(6″-acridine-9‴-carboxamido)hexyl)benzamido) quinone **19e** was isolated as a yellow solid (158 mg, 43%). R_f 0.5 [chloroform:methanol (9:1)]; IR (film) 3288 (broad), 3050, 2931, 2858, 2364, 2341, 1650, 1591 and 1548; UV-vis (EtOH) 360, 251 and 202; ^{1}H-NMR ($CDCl_3$, 200 MHz) 8.23 (2 H, d, 8.5), 8.06 (2 H, d, 8.5), 7.79 (2 H, ddd, 8.5, 8.0 and 1.5), 7.75 (2 H, 8.0), 7.58 (2 H, ddd, 8.5, 8.5 and 1.5), 7.49 (2 H, d, 8.0), 6.90–6.85 (3 H, m), 6.41 (1 H, t, 6.0), 6.30 (1 H, t, 6.0), 3.73 (2 H, q, 6.0), 3.49 (2 H, q, 6.0), 1.86–1.49 (8 H, m); FABMS (*m*/*z*) 532 (M + H), 462, 425; accurate mass for $C_{33}H_{29}N_3O_4$: calcd 531.2157; found 531.2163.

2-4′-benzamido-*N*′-[2″-(acridine-9‴-carboxamido)ethyl]-3,6-diaziridinyl-1,4-benzoquinone 9

To a cold (ice bath) suspension of 2-(4′-*N*-(2″-(9‴-acridinecarboxamido)ethyl)benzamido)quinone **19a**

(285 mg, 0.6 mmol). in dry ethanol (6 ml) under nitrogen was added aziridine (62 µl, 1.2 mmol). The mixture gradually turned orange, and after 2 h, it was taken off the cooling bath and evaporated to dryness under reduced pressure. The resulting mixture was chromatographed on silica gel using chloroform:methanol (19:1) as eluent to afford compound **9** as a red solid (90 mg, 27%). R_f 0.48 [chloroform:methanol (19:1) run 3 times]; IR (film) 3305 (broad), 3072, 3006, 2927, 2364, 2323, 1652, 1569 and 1540; UV-vis (EtOH) 342 and 257; ^{1}H-NMR ($CDCl_3$, 200 MHz) 8.17 (2 H, d, 8.5), 7.934 (2 H, d, 8.5), 7.85 (2 H, d, 8.0), 7.73 (2 H, td, 8.5 and 1.5), 7.46–7.34 (5 H, m), 7.26 (1 H, t, 7.0), 5.97 (1 H, s), 4.05–3.93 (2 H, m), 3.85–3.78 (2 H, m), 2.20 (4 H, s), 1.95 (4 H, s); FABMS (*m/z*) 558 (M + H), 530, 393; accurate mass for $C_{33}H_{28}N_5O_4$: calcd 557.2063; found 557.2049.

Synthesis of the target compounds 10–13

2-4′-Benzamido-*N*′-[3″-(acridine-9‴-carboxamido)propyl]-3,6-diaziridinyl-1,4-benzoquinone **10** was prepared in a similar way as compound **9**. 3-(4′-(*N*-(3″-Acridine-9‴-carboxamido)propyl)benzamido)-2,5-diaziridinylquinone **10** was isolated as a red solid (234 mg, 34%). M.p. 270°C (dec); R_f 0.31 [$CHCl_3$:MeOH, (19:1)]; IR (film) 3444, 3322, 2922, 2856, 1654, 1433, 1377, 1272, 1238, 1144, 758, 640; UV-vis (EtOH) 218; ^{1}H-NMR ($CDCl_3$, 300 MHz) 8.28 (2 H, d, 8.5), 8.10 (2 H, d, 8.5), 7.88 (2 H, d, 8.5), 7.84 (2 H, td, 8.5 and 1.0), 7.63 (2 H, td, 8.5 and 1.0), 7.45 (2 H, d, 8.5), 7.23 (1 H. t, 6.0), 7.11 (1 H, t, 6.0), 6.03 (1 H, s), 3.89–3.76 (4 H, m), 2.14–2.07 (2 H, m), 2,27 (4 H, s), 1.98 (4 H, s); FABMS (*m/z*) 594 (M + Na), 572 (M + H); accurate mass for $C_{34}H_{30}N_5O_4$: calcd 572.2298; found 572.2309.

2-{4′-Benzamido-*N*′-[4″-(acridine-9‴-carboxamido)butyl]}-3,6-diaziridinyl-1,4-benzoquinone **11** was isolated as a red solid (60 mg, 26%). R_f 0.48 [chloroform:methanol (9:1); IR (film) 3315 (broad), 3074, 3004, 2932, 2854, 2242, 1650, 1571 and 1552; UV-vis (EtOH) 344, 250 and 218; ^{1}H-NMR ($CDCl_3$, 200 MHz) 8.10 (2 H, d, 8.5), 7.94 (2 H, d, 8.5), 7.74 (2 H, d, 8.0), 7.70 (2 H, ddd, 8.5, 8.5 and 1.5), 7.48 (2 H, ddd, 8.5, 8.5 and 1.0), 7.31 (2 H, d, 8.0), 7.14 (1 H, t, 5.5), 6.79 (1 H, t, 5.5), 5.90 (1 H, s), 3.7–3.63 (2 H, m), 3.54–3.43 (2 H, m), 2.15 (4 H, s), 1.92 (4 H, s), 1.90–1.76 (4 H, m); FABMS (*m/z*) 586 (M + H), 557, 393; accurate mass for $C_{35}H_{32}N_5O_4$: calcd 586.2454; found 586.2468.

2-{4′-Benzamido-*N*′-[5″-(acridine-9‴-carboxamido)pentyl]}-3,6-diaziridinyl-1,4-benzoquinone **12** was isolated as a red solid (70 mg, 22%). R_f 0.49 [chloroform:methanol (19:1) run 3 times]; IR (film) 3320, 3070, 2993, 2927, 2850, 1650, 1625 and 1571; UV-vis (EtOH) 342, 250 and 218; ^{1}H-NMR ($CDCl_3$, 200 MHz) 8.10 (2 H, d, 8.5), 7.96 (2 H, d, 8.5), 7.71 (2 H, ddd, 8.5, 8.5 and 1.5), 7.62 (2 H, d, 8.0), 7.47 (2 H, ddd, 8.5, 8.5 and 1.5), 7.27 (2 H, d, 8.0), 7.10 (1 H, t, 6.0), 6.60 (1 H, t, 6.0), 5.93 (1 H, s), 3.66 (2 H, q, 6.0), 3.42 (2 H, q, 6.0), 2.19 (4 H, s), 1.93 (4 H, s), 2.00–1.49 (6 H, m); FABMS (*m/z*) 600 (M + H), 572, 462, 448, 393; accurate mass for $C_{36}H_{34}N_5O_4$: calcd 600.2611; found 600.2601.

2-{4′-Benzamido-*N*′-[6″-(acridine-9‴-carboxamido)hexyl]}-3,6-diaziridinyl-1,4-benzoquinone **13** was isolated as a red solid (42 mg, 24%). R_f 0.59 [chloroform:methanol (19:1) run 3 times]; IR(film) 3313 (broad), 3077, 2929, 2856, 1648, 1573 and 1537; UV-vis (EtOH) 342, 250 and 221; ^{1}H-NMR ($CDCl_3$, 200 MHz) dH 8.22 (2 H, d, J 8.5), 8.07 (2 H, d, J 8.5), 7.80 (2 H, ddd, J 8.5, 8.5 and 1.5), 7.74 (2 H, d, J 8.5), 7.59 (2 H, ddd, J 8.5, 8.5 and 1.5), 7.39 (2 H, d, J 8.5), 6.45 (1 H, t, J 6.0), 6.31 (1 H, t, J 6.0), 6.0 (1 H, s), 3.74 (2 H, q, J 6.0), 3.56–3.43 (2 H), 2.24 (4 H, s), 1.98 (4 H, s), 1.88–1.51 (8 H, m); FABMS (*m/z*) 614 (M + H); accurate mass for $C_{37}H_{36}N_5O_4$: calcd 614.2767; found 614.2768.

3-(4′-(*N*-(5″-*N*-butoxycarbonyl)pentyl)benzamido)-2,5-diaziridinylquinone 14

This compound, **14**, was synthesized by following the general aziridination procedure described for the synthesis of compound **9**. The reaction product was purified by chromatography on silica with $CHCl_3$:MeOH (19:1) as elution system, affording a red solid (8 mg, 17%). M.p. 190–5°C; ^{1}H-NMR ($CDCl_3$, 300 MHz) 7.90 (2 H, d, 8.5), 7.6 (2 H, d, 8.5), 6.0 (s, 1 H), 3.2–3.6 (m, 4 H), 1.9–1.6 (m, 6 H), 2.2 (s, 4 H), 2.0 (s, 4 H), 1.4 (s, 9 H); FAB-MS (+ KI): 533 ($M + K^+$, 13%), 376 (10), 338 (10), 192 (40), 136 (33), 39 (100); HRCIMS: found 495.2599, $C_{27}H_{35}N_4O_5$ requires 495.2607.

3-(4′-(*N*-(3″-Acridine-9‴-carboxamido)propyl)benzamido)-2,5-diaziridinyl-5-methylquinone 15

Compound **15** was synthesized by following the aziridination reaction described for the preparation of conjugate **9** (480 mg, 0.95 mmol) as starting reagent. The resulting residue was chromatographed twice on silica gel with CH_3Cl:MeOH (9:1) as elution system, to afford compound **15** as red solid (53 mg, 9.5%). M.p. 250–254°C; ^{1}H-NMR ($CDCl_3$, 300 MHz) 8.26 (d, 2 H, 9), 8.09 (d, 2 H, 9), 7.9–7.8 (m, 4 H), 7.6 (m, 2 H), 7.42 (d, 2 H, 9), 7.2 (m, 1 H, NH), 7.06 (m, 1 H, NH), 3.9–3.7 (m, 4 H, CH_2), 2.3 (s, 4 H,), 2.13–2.01 (m, 5 H, CH_3 and CH_2), 1.90 (s, 4 H); FAB-MS (*m/z*): 586 (M + + 1, 27%), 391 (9), 307 (7),154 (100), 136 (99), 77 (56), 39 (46); HRCIMS: found 586.2445, $C_{35}H_{32}N_5O_4$ requires 586.2454.

Stability studies on compounds 9–13

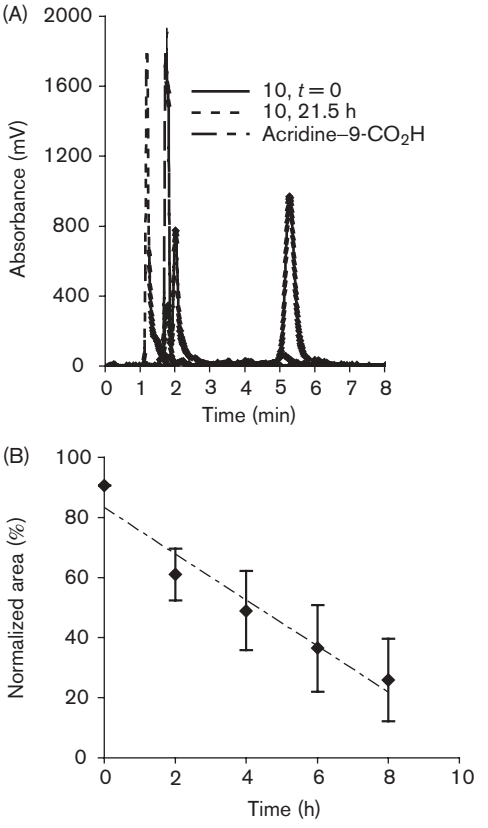
A stability study was carried out in the presence of the following different carboxypeptidases: porcine esterase (3120 U/ml), carboxypeptidase A (1122 U/ml), carboxypeptidase B (reconstituted in 100 µl of distilled water, 1760 U/ml), carboxypeptidase C (reconstituted in 100 µl of

distilled water, 870 U/ml) and pepsin (1.6 mg/ml in PBS, 5120 U/ml). To a solution of the compounds (5 µl of 10 mM stock solution in DMSO, final concentration = 62.5 µM) in PBS (785 µl) was added the enzyme (10 µl of stock) at room temperature. Then 150 µl of the resulting suspension was manually injected in the high-performance liquid chromatograph (HPLC) at different time points from time 0 up to 49 h later. The HPLC analysis was carried out using a Gilson 306 solvent delivery system, a Gilson 117 UV-vis detector and a ASPEC XL autoinjector. The system was controlled using a Gilson controller software which included data capture and processing function. A S5CN-2360 column was used. The analysis was carried out under isocratic conditions of methanol:phosphate buffer 0.05 M, pH 7, 40:60 or 60:40 v/v depending on the polarity of the compound investigated. A wavelength of 360 nm was used throughout the experiments. Under the conditions described, for example, compound **10** gave a RT of 4.52 min. The relative amounts of the compounds were quantified by comparing the percent area of the peak in the chromatograms at different time points to the starting compound at time 0. Plots of percent area versus times allowed the calculation of the half-life of the compound under the conditions described.

The same experiment was also carried out in RPMI 1640 medium with 10% (v/v) FCS at 37°C. In this case, 10 µl of the compounds (10 mM stock in DMSO; final concentration = 100 µM) was added to 990 µl of medium. The stability study was then carried out as above except that the temperature was maintained at 37°C throughout all the experiment. Under these conditions, the RT of **10**, for instance, was slightly higher than before, appearing at 4.95 min.

In order to identify one possible product from the breakdown of **10**, a solution of 9-acridinecarboxilic acid in medium (100 µM) was also injected into the column. This gave a single peak at 1.72 min (Fig. 2A). All of the experiments were repeated for a minimum of 3 times.

Fig. 2



(A) Comparative view of typical chromatograms from a stability study of **10** in medium. Compound **10** is shown at time 0 (continuous line) and after 21.5 h in medium (dashed line), and 9-acridinecarboxylic acid (dash/dotted line) is shown for comparison. (B) Stability study on **10** in medium with 10% (v/v) FCS at 37°C. Plot of the normalized areas of **10** peak versus time. The half-life under these condition was $t_{1/2} = 5.8 \pm 1.4$ h. The plot is representative of three different experiments.

Rates of reduction by human DTD

The reduction of the quinones by human DTD was followed by the corresponding reduction of cytochrome *c* [41]. The quinones (50 µM) were put into a 1-ml quartz cuvette containing 0.7 mg/ml BSA, 70 µM cytochrome *c*, 10 µl of recombinant human DTD (sp. act. 602 pmol/min/mg) and 25 mM Tris–HCl buffer, pH 7.4. The reaction was initiated by the addition of NADH (250 µM). The reduction of the cytochrome *c* was followed at 550 nm ($\varepsilon_{550nm} = 21 \times 10^3\ M^{-1}\ cm^{-1}$). Each quoted value is the average of three determinations (typical errors ± 15%).

Cytotoxicity studies

K562, H460, H596, HT29 and BE cells were obtained from the ATCC (Rockville, MD) or from the European Collection for Animal Cell Cultures (Porton Down, UK). T K562 is a human chronic myelogenous leukemia cell line, and was included as it was used in previous diaziridinylquinone cytotoxicity studies and is p53 deficient [28,45]. HT29 is a human colon cancer line and H460 is a human lung cancer cell line. These two cell lines express relatively high levels of DTD [38,41]. The corresponding matched cell lines, H596 (human lung) and BE (human colon), were chosen as they do not have detectable DTD activity [26,28,46]. Continuous challenge cytotoxicity studies were carried out on these cells (600/well) in 96-well plates using the MTT method [40]. After 5 days, the absorbances were read on a multiscan plate reader at 540 and 640 nm. Plots were then drawn for inhibition of cell growth as a function concentration. From these plots, IC_{50} values (concentration of drug necessary to inhibit 50% growth) were calculated. Each compound was tested in triplicate at each concentration on at least two occasions.

Cell cycle studies

Exponentially growing K562 cells were subcultured at a density of 8×10^4 cells/ml in RPMI 1640 medium containing 10% FCS and left growing for 24 h at 37°C in a humidified 5% CO_2 atmosphere. After this time, the cells were treated with the drugs at the appropriate concentrations (multiples of the MTT IC_{50} values, $10 \times IC_{50}$) and left growing overnight. The following day, the cells were harvested and transferred into plastic tubes. The tubes were then centrifuged for 5 min at 1500 r.p.m., and the resulting cell pellets were washed once with 500 µl of PBS, treated with 500 µl of 70% ice-cold ethanol and then left at 4°C for 5 min. After a further centrifugation (2000 r.p.m. for 5 min), the pellets were re-hydrated in 1 ml PBS. This washing was repeated twice. Following another centrifugation, the cell pellets were treated with 50 µl of DNase-free RNase solution (100 µg/ml) for 5 min at room temperature in order to remove the RNA. The cell pellets were then treated with 300 µl of propidium iodide solution in PBS (50 µg/ml) for 15 min, mixed and then processed by flow cytometry. The red fluorescence of propidium iodide was measured at > 620 nm using a Becton Dickinson FACScan. The data were acquired with CellQuest software and analyzed using a cell cycle fitting program, ModFit LT (Verity Software House).

Comet studies

The alkaline Comet assay for measuring DNA cross-links was adapted from previous methods [47]. Exponentially growing cells were subcultured in 24-well plates at 2×10^4cells/well and allowed to attach overnight. The following day, the cells were then treated with either equidose or equitoxic concentrations (IC_{50} multiple doses) of the quinones and left for 24 h before samples processing. Our previous Comet studies on diaziridinylquinones have shown that the quinone–DNA interactions and DNA repair occur within this time period [45]. The cells were then harvested and transferred into plastic tubes in an ice bucket. The samples were then divided into two—half were irradiated to 30 Gy in a ^{131}Cs source (4.2 Gy/min) for the cross-link studies, while the other half remained unirradiated in order to allow for evaluation of strand breakage efficacy of the acridine quinone compounds. Drug-untreated irradiated and unirradiated samples were run as positive and negative controls for DNA damage, respectively. The cross-linking efficiency was estimated by considering the decrease of the tail DNA compared to drug-untreated irradiated control [47].

All of the cells were then embedded in low-melting-point agarose on pre-coated microscope slides and left on ice for a few min while the gel set. All of the slides were then treated with a lysis buffer (100 mM EDTA, 10 mM Tris–HCl, 1% Triton X-100, 1% DMSO, 2.5 M NaCl) for 1 h. The slides were then gently soaked with four changes of distilled water at 15 min intervals. After this washing procedure, the slides were transferred in the dark to an electrophoretic tank filled with alkali unwinding buffer (50 mM NaOH, 1 mM EDTA, pH 12.5). After 45 min in the alkali buffer, the slides were electrophoresed for 25 min using a voltage of 21 V. The medium was then carefully removed and the slides were transferred to a plastic box where they were individually soaked twice for 5 min in 1 ml of neutralizing buffer (0.4 M Tris–HCl, pH 8.0) and twice with 1 ml of PBS. The slides were then allowed to dry overnight at room temperature. The following morning, the slides were re-hydrated in double-distilled water for 30 min and then individually soaked in 1 ml of SYBR Green I dye. This solution was made up by dissolving 10 µl of the commercial stock solution (Bio-Products, Rockland, ME) in 100 ml water. The slides were then rinsed twice with double-distilled water to remove excess dye and then dried at room temperature in the dark. A few drops of distilled water were then used to re-hydrate the cells, which were subsequently visualized using fluorescence microscopy. The statistical calculations of the tail moments were carried out on 50 cells/slide using Komet Assay Software Version 4 (Kinetic Imaging).

Modeling studies

A preliminary computer modeling of the energy-minimized structures on **9–11** was carried out in collaboration with Kate Hemming (Ridge Danyers, UK). In this modeling study, a Silicon Graphics Iris 4D/310GTX workstation and QUANTA 4.0 software (including CHARMm *22.2*) working under IRIS 4.0.5 was used.

In vivo studies

The *in vivo* study was done using a BDF1 strain of mice, all male and aged 15 weeks. Mean weight at time of injection was 35 ± 4 g and the mean tumor volume (mouse mammary carcinoma, derived at the Paterson Cancer Research Institute) at the time of first injection was 100 mm^3. The mice, in groups of eight, were injected at day 0 with a dose equivalent of 2 mg/kg body weight in arachis oil (volume around 0.3 ml/injection). A further injection of 5 mg/kg was done exactly 1 week later. The tumor volumes were measured by standard caliper technique. In another study with a different strain of mice and more animals, cyclophosphamide showed a 12-day delay in the growth of the same mouse carcinoma. The drug was used at 250 mg/kg (MTD of 400 mg/kg).

The *in vivo* xenografts investigations using H460 cancer in *nu/nu* BDF1 mice were conducted by Mr Gary Owen at the Paterson Laboratories (Manchester, UK). Briefly, the long-term toxicity studies were carried out on BDF1 adult mice (approximately 10–12 weeks old) of any gender. Two mice per group (treated and untreated ones) were i.p. injected with **9** (5 mg/kg) and **10** (10 mg/kg) on day 0. The samples were made just prior use in

DMA:arachis oil (5:95). The animals' weights were recorded prior treatment and then daily for the duration of the study.

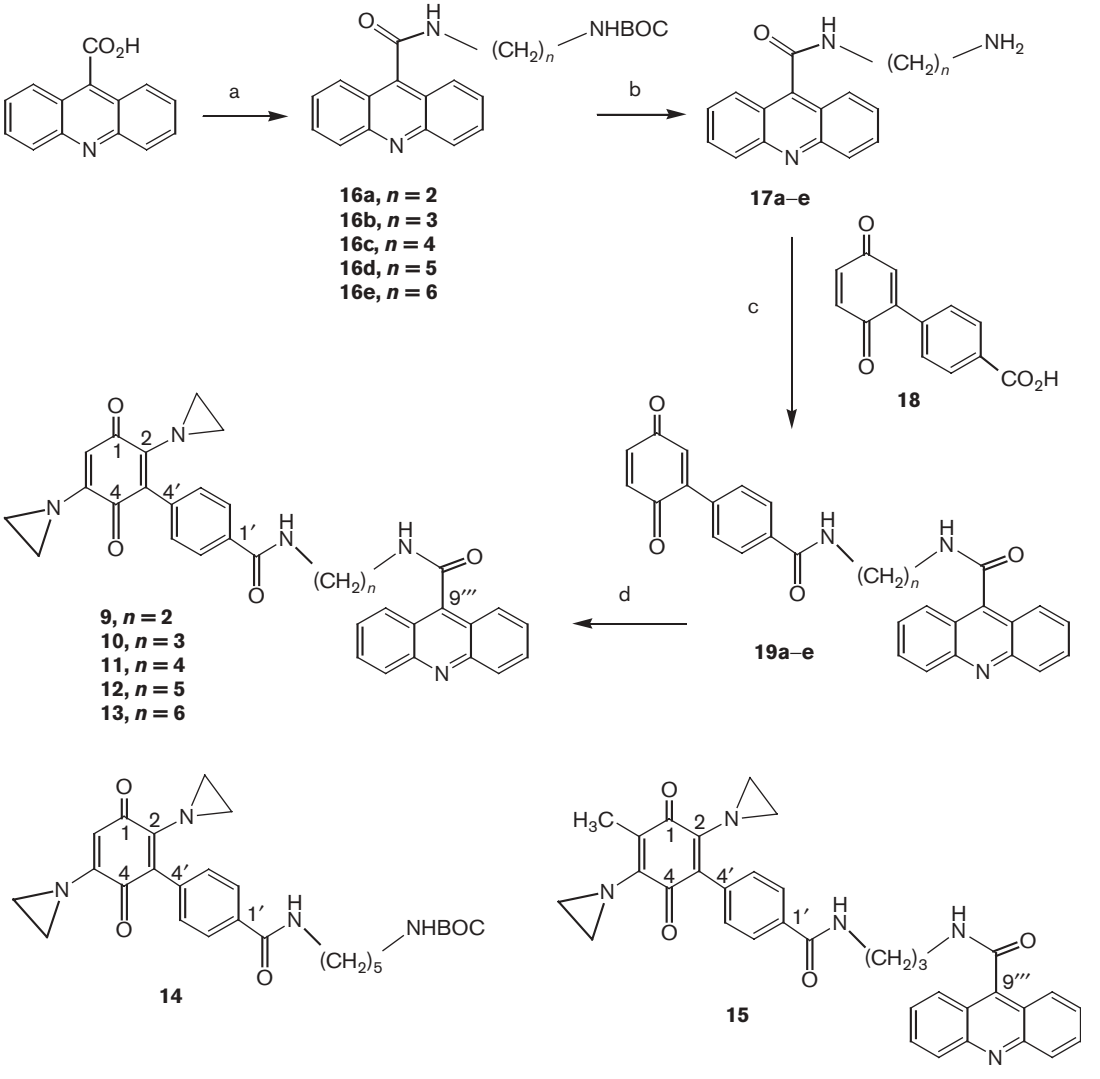
For the evaluation of the efficacy as antitumor agents, NSCLC cells (H460) were s.c. implanted in *nu/nu* BDF1 mice (five animals per experimental group; a total of 15 animals). After the tumors were well established, **9** and **10** (in DMA:arachis oil, 5:95) were i.p. injected for 5 days consecutively at doses of 1.0 and 1.5 mg/kg/day, respectively. The tumor volumes (in mm^3) and the mice weights were measured daily for a total of 33 days to assess tumor growth and toxicity.

Results and discussion

Synthesis

Compounds **9–13** were prepared according to the synthetic approach given in Scheme 1. Reaction of acridine-9-carboxylic acid with a suitable *N*-BOC protected diaminoalkane ($n = 2$–6 methylene units) in the presence of dicyclohexylcarbodiimide (DCC) and hydroxybenzotriazole monohydrate (HOBt) gave the desired amides **16a–e**, respectively, in good yields. It was found that the percent yields of these reactions were significantly improved, by around 20%, if the reaction mixtures were sonicated for several h prior to work-up. Removal of the *t*-butoxycarbonyl (BOC) protecting group of **16a–e**

Scheme 1



[a]BOCNH-$(CH_2)_n$-NH_2 (n=2–6), DCC, HOBt, dry DMF, rt. [b](i) HCl, THF, H_2O; (ii) NaOH. [c]Quinone benzoic acid **18**, DCC, HOBt, dry DMF. [d]Aziridine, dry ethanol, 0°C.

with hydrochloric acid, followed by base treatment, provided **17a–e** as their free bases. The amino group of **17a–e** was coupled with 4-(*2′*-quinone)benzoic acid **18** [35] in the presence of DCC and HOBt to give compounds **19a–e** in good yields. Reaction of the quinone moiety of **19a–e** with aziridine finally provided the target **9–13** as reddish solids. Compounds **14** and **15** were prepared in reasonable yields following a similar strategy. The 6-methyl analog of quinone acid **18**, used in the preparation of compound **15**, was obtained through the oxidation of 2,5-dimethoxy-4-(4′-carboxyphenyl)toluene. The latter intermediate was successfully afforded in high yield under the Suzuki reaction's conditions [36] by coupling the commercially available 4-bromo-benzoic acid (Sigma-Aldrich, Poole, UK) to 2,5-dimetoxy-4-methyl-boronic acid. The latter one was in turn synthesized in high yield from 4-bromo-2,5-dimethoxy toluene by following the standard procedure described by Manthey *et al.* [37]. Infrared, UV-vis, mass spectrometry, ^{1}H-NMR and elemental analyses were used to characterize the compounds described in this paper.

Stability studies on 10

The stability of **10** in the cell culture medium and in the presence of five different carboxypeptidases, such as porcine esterase, carboxypeptidase A, B and Y, and pepsin, was investigated using a HPLC technique. The apparent half-lives in the presence of the five different enzymes at room temperature are $33.1 \pm .1$, 62.5 ± 4.5, 63.1 ± 2.3, 38.0 ± 1.0 and 57.3 ± 0.1 h, respectively.

All of the chromatograms showed similar profiles in that several peaks were observed and they were all at much earlier retention times than **10**, suggesting that all the products are more polar than **10** itself. The chromatograms from the incubations in culture medium enriched with 10% FCS showed one species with a retention time of 1.72 min, which was the same retention time as pure 9-acridinecarboxylic acid. The results from these investigations (a representative chromatogram from experiments in culture medium is given in Fig. 2A) are intriguing, because it appears that whereas the model **10** is relatively stable in PBS in the presence or absence of the different enzymes, it is much less stable in the cell culture medium. This implies that there are other more effective peptidases in the cell culture medium than those used in the PBS experiments and/or the DZQ–acridine compounds (**9–13**) react with components which are present in the medium, but not in PBS. Some typical reactions that could occur in the medium include direct Michael additions of the quinones to proteins, reduction of the quinones, ring-opening reactions and hydrolysis. The potential peptidase product, 9-acridinecarboxylic acid, was detected in the cell culture medium, but this could have been formed simply from slow hydrolysis of the amide group at 37°C. A representative time-dependent disappearance of **10** in cell culture medium is depicted in Figure 2(B). As several products were detected in all of the solutions, it was not feasible doing a full kinetic study of the reactions. In either event, time course toxicity studies proved that the species produced in medium from **10** were not more toxic than the starting compound (data not shown). It could be seen from Figure 2(B) that the cytotoxicity profile did not change dramatically after 6 h (the half-life in cell culture medium) and, indeed, the toxicity values approached a plateau after this time at a concentration of 1.3 ± 0.5 nM.

Rates of reduction by human DTD

The relative rates of reduction of **9–15** and **19b** by human recombinant DTD are shown in Table 1. The rates with RH1 (**6**) and PDZQ (**8**) are also included as positive references. It is worthy to note that all of the compounds investigated were substrates for human recombinant DTD; almost comparable to RH1 or PDZQ despite the presence of the relatively bulky methylene spacers and the acridine group. These results are consistent with a previous proposal that certain quinones can have a relatively bulky group on one side of the molecule and yet still be good substrates for the enzyme [38]. All of the DZQ–acridine compounds have similar rates of reduction by the enzyme. This is consistent with them all having the same diaziridinylquinone group and shows that the nature of the bulky side groups is not very important. Interestingly, the methylated analog **15** was reduced less efficiently. This is probably due to the expected decrease in reduction potential of the quinone (methyl groups can donate electrons into the quinone ring) and, possibly, due to some increase in steric hindrance when the quinone locates in the active site of the enzyme. Consistent with this finding is the ability of DZQ to act as a better substrate for DTD than MeDZQ [38]. Additionally, **19b** appears to be a relatively poor substrate for the enzyme. This is surprising as the reduction potential of this quinone should be much more positive than that of the other quinones, due to the lack of an electron-donating effect of the nitrogen on the aziridine group, and there should be much less steric hindrance at the active site of the enzyme. One possibility that could explain the results

Table 1 The rates of reduction of the quinone compounds by human recombinant DTD (data are an average of two determinations)

Compound	*k* (μmol/min/mg)
RH1, **6**	74.1
PDZQ, **8**	71.0
9	42.7
10	57.7
11	42.4
12	46.9
13	38.2
14	31.1
15	15.8
19b	10.4

is that this quinone may be able to react directly with the enzyme via Michael additions and inactivate it. It is probable that reactions of this type also occur with simple benzoquinones, e.g. 1,4-benzoquinone appears to be a poorer substrate for the enzyme than 2,5-dimethyl-1,4-benzoquinone [39].

Cytotoxicity studies

The ability of **9–15** and **19b** to inhibit the growth of tumor cells in culture was evaluated using a MTT-based assay [40]. The IC_{50} values for a 5-day continuous exposure of the compounds are shown in Table 2. Five different types of human cancer cells were used in this study, and they included K562 (chronic myeloid leukemia), H460 and H596 (both non-small cell lung cancer), and HT29 and BE (both colon cancer) cells. Even though HT29 and H460 are rich in DTD, K562 has low levels of the enzyme and the other two types of cells have undetectable levels. The cytotoxicity of RH1 are also included in this study as a reference compound. It is worthy to note that the IC_{50} values for **10** taken after continuous exposure and 6-h exposure are very similar: 1.3 and 1.8 nM, respectively.

The *in vitro* cytotoxicity data given in Table 2 show that all of the compounds are almost as potent as RH1 and agent **9** is the most toxic. Several trends were observed from these studies. The compound with no aziridines, **19b**, was significantly less toxic than all of the other compounds. This is consistent with the requirement of alkylation as the mechanism for toxicity. Furthermore, the acridine moiety must contribute to the toxicities of these compounds because **14**, which did not have an acridine, was significantly less cytotoxic than any of the conjugates.

Further analysis of the cytotoxicity data revealed that **9–15** and **19b** were slightly more cytotoxic against cells rich in DTD than cells poor in DTD. However, the differentials were not large when compared to RH1, whose main mechanism of activation to a DNA cross-linking agent is proposed to involve reduction by DTD [22]. Therefore this data would imply that there might be other underlying mechanisms for activation and/or detoxification of these acridine compounds. Because methylated analog **15** could not undergo Michael addition reactions, it should give much better cytotoxic differentials than the non-methylated compounds. Although the methylated **15** was found to be slightly more toxic than its non-methylated parent, **10**, it gave poor differentials between high- and low-DTD cell lines (Table 2).

Table 2 Cytotoxicity data and DTD metabolism of DZQ–acridine conjugates 9–13, and their analogs 14, 15 and 19b

Compound	K562	H460	H596	HT29	BE
RH1, **6**	0.09	0.05	2.3 (46)[a]	11.3	580 (52.7)[b]
PDZQ, **8**	3.3	3.9	8.5 (2.2)	8.8	16.1 (1.8)
9	0.3	1.1	1.7 (1.5)	0.59	0.8 (1.3)
10	1.7	1.3	8.0 (6.2)	2.9	3.4 (1.2)
11	1.3	2.6	4.2 (1.6)	2.0	2.9 (1.5)
12	5.3	3.0	10.6 (3.5)	9.0	9.2 (1.0)
13	2.9	4.0	11.3 (2.8)	3.1	10.7 (3.4)
14	ND	8.2	45 (5.5)	ND	ND
15	ND	1.1	1.6 (1.5)	0.91	1.2 (1.3)
19b	ND	1700	4600 (2.7)	ND	6500

Continuous cytotoxicity (5-day; IC_{50} nM) with MTT assay in different cancer cell lines. The values are an average of at least three different experiments. Errors between 5 and 10%. The differential toxicity values are given in brackets. ND, not determined.

[a]The numbers in brackets are ratios of the IC_{50} values for H596:H460.

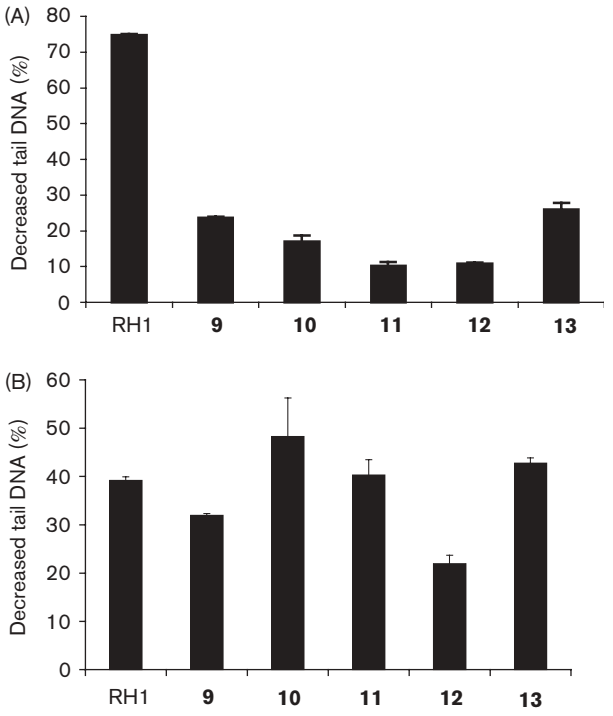
[b]Cytotoxicity ratios for BE:HT29.

Comet studies and DNA interstrand cross-link formation

Studies were carried out to measure the relative cross-linking ability of **9–13** on the high-DTD H460 cell line after 24 h treatment at concentrations of $50 \times IC_{50}$ and at 20 nM. RH1, a strong DNA cross-linker, was also used for comparison. In this manner a comparison can be made of the cross-linking efficiency at both equitoxic and equidose amounts of the drugs. The 20 nM concentration was chosen as it has been previously shown in our laboratories that RH1 appears to produce its maximum cross-linking efficiency in H460 cells at this concentration (data not shown). The results are summarized in Figure 3(A). At 20 nM concentrations, the agents **9–13** were between 3 and 4 times less efficient in cross-linking DNA than RH1 (which had 75% of decreased tail DNA compared to untreated irradiated control). It could be argued that the instability in cell culture medium observed with **10** (Fig. 2A) would lower the amount of agent actually available for cross-linking and therefore this could contribute to the lower cross-linking efficiency observed with this type of agents. However, it should be also noted that RH1 is about 24 times more toxic in the H460 cell line than either **9** or **10**, which are the most toxic DZQ–acridine compounds (Table 2). These results suggest that the DNA cross-linking efficiency may not play a main role in determining the differences in toxicity between RH1 and the acridine compounds. Indeed, when equitoxic concentrations were used ($50 \times IC_{50}$), most of the DZQ–acridine compounds tested gave the same, if not slightly higher, levels of cross-links than RH1 (Fig. 3B). It should also be noted that at this equitoxic dose, the levels of DNA cross-linking differed by at the most a factor of 2. It could be argued, however, that if these compounds had some other underlying toxicity mechanisms, the levels of DNA cross-linking at equitoxic doses would be quite different.

The cross-linking efficiency of **9–13** was also investigated in H596 cells which have a point mutation resulting in a lack of DTD activity [42]. In a similar manner as above, H596 were treated for 24 h with equidose (50 nM) concentrations of the drugs (data not shown). RH1, which is selectively toxic for high-DTD cell lines used as

Fig. 3



(A) Cross-links in the H460 cell line at 20 nM drug concentrations after 24 h. Cells were irradiated at 30 Gy. (B) Cross-links in the H460 cell line at $50 \times IC_{50}$ drug concentrations after 24 h. Cells were irradiated at 30 Gy. The data are representative of at least two different experiments.

reference compound [43]. As expected, on comparing the DNA cross-linking efficiencies from the H460 (high-DTD) cell line with those from H596 (low-DTD), RH1 showed a significant difference in the two cell lines with a dramatic decrease in cross-link observed in H596 cells. This is despite the fact that a higher concentration of drug had to be used for the H596 cells (50nM compared to 20 nM used in the H460 cells). The higher dose used for the H596 cells was due to the difficulty encountered in measuring the DNA cross-linking with some of **9–13** at 20 nM. Consistent with the low cytotoxicity differentials for **9–13** (Table 2), the amounts of DNA cross-links in the H460 cell lines were not significantly higher than those in the H596 cells. Indeed, in some cases, the amounts appeared to be lower.

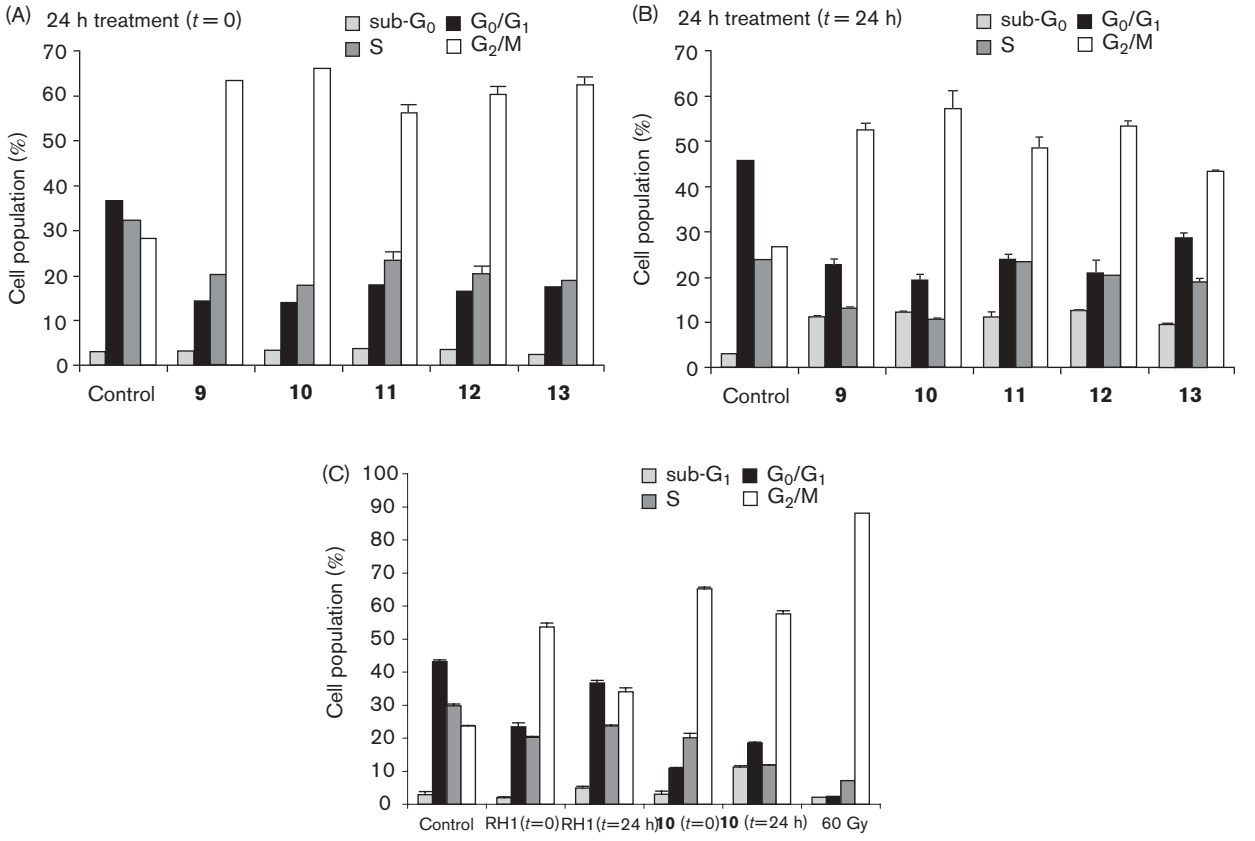
Compounds **9–13** gave a dose-dependent cross-linking efficiency of up to 60–75% in the K562 cells (data not shown). Interestingly, RH1 only gave an efficiency of 40% under the same conditions. K562 cells have low levels of DTD, but they also have active one-electron reducing enzymes [44]. It could therefore be argued that as the activation of RH1 is strongly dependent on the levels of DTD, whereas the DZQ–acridine compounds are not as dependent (see above), the relatively lower levels of cross-linking observed with RH1 are predictable. However, this argument does not fully explain the fact that RH1 is much more cytotoxic than all of **9–13** in the K562 cells (Table 2).

Cell cycle studies

The cell cycle studies were carried out with propidium iodide (PI) using the general method described in the experimental section. The object of the study was to evaluate whether agents **9–13** were able to affect the cell cycle in a permanent way causing a cytotoxic action rather than producing a temporary block in cell growth. K562 cells were treated with **9–13** at concentrations of 10 times the IC_{50} values for 5-day continuous exposure for 24 h. One set of samples were processed through the FACS immediately while a second set of samples were left at 37°C for a further 24 h in drug-free medium to allow re-cycling. The results are shown in Fig. 4. A comparative study with agent **10** and RH1 was also carried out under the same conditions (Fig. 4C).

Results from the cell cycle studies confirm that **9–13** damage DNA through formation of cross-links. The K562 cells treated for 24 h at concentrations of $10 \times IC_{50}$, showed a block in the G_2/M phase of the cell cycle

Fig. 4



(A and B) Cell cycle studies on K562 treated with compounds **9**–**13**. Cells were incubated with the compounds at $10 \times IC_{50}$ for 24 h. A set of cells was analyzed straight after drug treatment (top chart), whereas the second set of samples was left to recover for 24 h at 37°C in drug-free medium after incubation with the drugs (bottom chart). The data are representative of two experiments. (C) Cell cycle studies on K562 treated with **10** and RH1. Cells were incubated with the compounds at $10 \times IC_{50}$ for 24 h. A set of cells was analyzed immediately after drug treatment ($t = 0$), whereas the second set of samples was left to recover at 37°C for 24 h in drug-free medium after incubation with the drugs ($t = 24$ h). Irradiated cells (60 Gy) were left re-cycling in the incubator for a total of 48 h and analyzed with the other samples. The data are representative of two experiments.

compared to untreated control (Fig. 4A and B). This accumulation of cells in G_2/M appeared to last up to a further 24 h after the drug was removed from the medium (Fig. 4B). Interestingly, RH1 gave a lower and more temporary accumulation of cells in G_2/M compared to **10** (Fig. 4C). These results are consistent with the Comet data wherein it was shown that, under the same experimental conditions, RH1 produced less cross-links than **10** (data not shown).

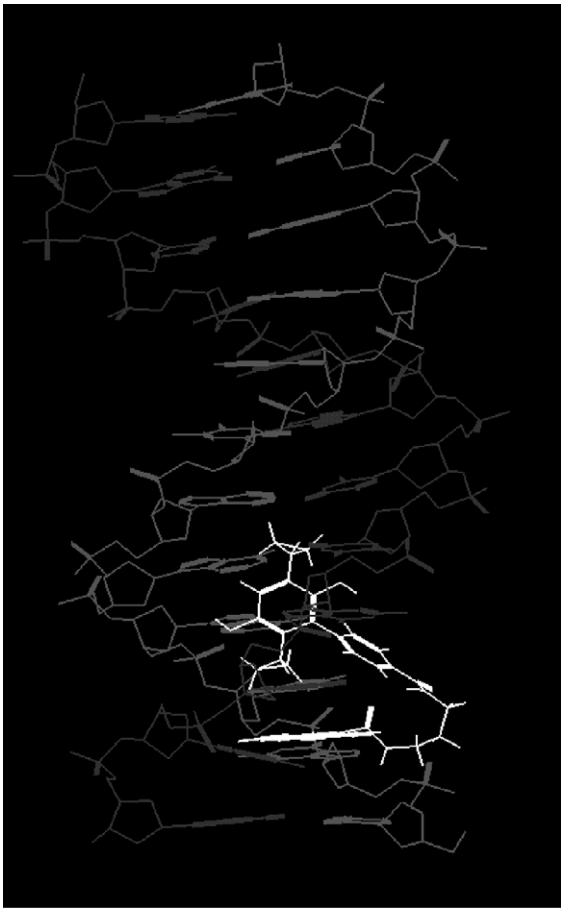
Molecular modeling studies

A molecular modeling experiment was conducted to determine whether the acridine–DZQ conjugates could bind to DNA in such a way that the acridine moiety would intercalate between DNA base pairs and the DZQ moiety be positioned to covalently react with guanine-N^7 groups in the major groove. The results from a study performed using QUANTA 4.0 and CHARMm *22.2* on the binding of **10** to a small DNA fragment in the B-conformation is depicted in Figure 5. The energy-minimized computer model suggests that the acridine can intercalate into DNA while the flexible methylene linker can allow the aziridines to come close to the N^7 positions of guanine, the main site of alkylation for the aziridinylquinones. However, in some respects this model actually suggests that the DZQ–acridine compounds probably do not adopt this conformation. This is because the model shows that the length of the methylene spacers should strongly influence the location of the aziridines and hence, presumably, the extent of DNA cross-link. However, it can be seen from the Comet data (Fig. 3A and B) that the extent of DNA cross-link produced by the DZQ–acridine compounds do not vary dramatically between these analogs.

In vivo studies

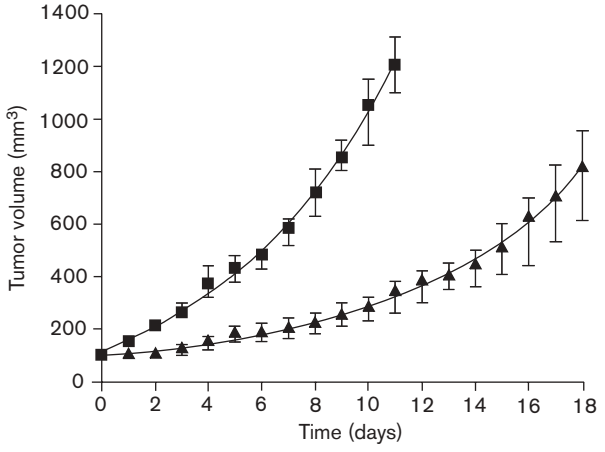
One of the most exciting aspects of this work comes from the *in vivo* studies, which demonstrates the potential

Fig. 5



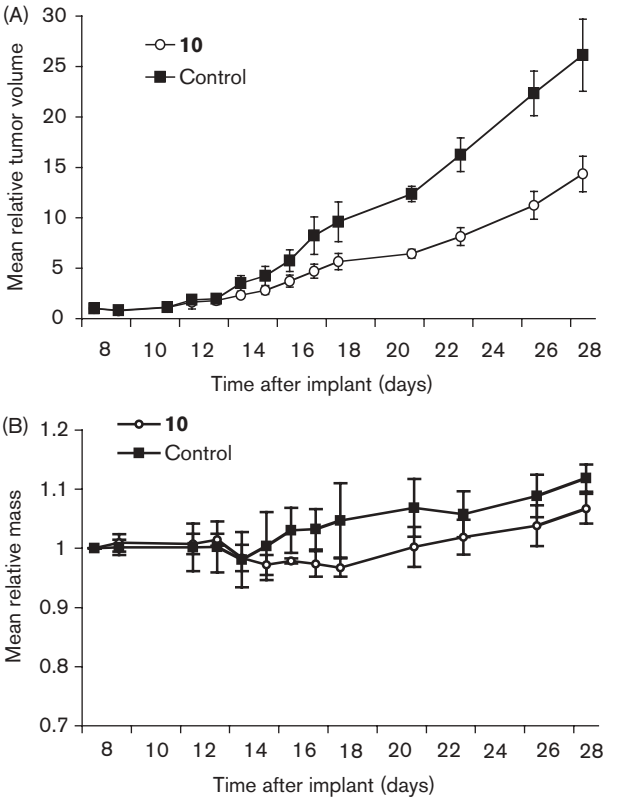
Energy-minimized computer model of the hydroquinone of **10** (in white) and duplex DNA.

Fig. 6



Tumor growth inhibition study on mouse mammary carcinoma in BDF1 mice. After tumors were established, mice were treated with **10**, 2 and 5 mg/kg at day 1 and 7, respectively (triangles). Untreated mice were used as control (squares).

Fig. 7



(A) Tumor xenografts (H460) treated with **10** (1.5 mg/kg/day for 5 consecutive days, from day 13 to 17). (B) Mean relative weight of mice with tumor xenografts (H460) treated with compound **10** at 1.5 mg/kg/day for 5 consecutive days, from day 13 to 17. The control mice for both experiments are indicated.

application of acridine–DZQ conjugates as potential anticancer drugs. In the first *in vivo* study conducted using a mouse mammary carcinoma, 30 BDF1 (all male) animals were employed and **10** was selected as a representative. The compound was injected via an i.p. route using arachis oil as carrier and three doses (5, 10 and 15 mg/kg) were used. The drugs were administered at the beginning of the experiment and at day 8. During the experiment, the body weight of each animal was measured and no detectable weight loss was observed, indicating that unlike RH1 and other DZQ compounds the conjugates described in this paper are less toxic to the animals. At the end of the experiment after 18 days, the animals were sacrificed and the liver weight was measured, and no significant difference in the liver weight were seen for the treated and control animals. The results given in Figure 6 demonstrate an ability of **10** to inhibit the tumor growth. Specifically, a 9–10 day tumor growth delay was seen at the end of the experiment. For comparison, cyclophosphamide, at a dosage equivalent to 250 mg/kg of body weight, produced a 12-day growth

delay of the same mouse mammary carcinoma in mice (unpublished results). Based on these positive results, further *in vivo* anticancer activity studies were conducted. The results for the H460 tumor xenografts with **10** (1.5 mg/kg/day given for 5 consecutive days) are shown in Figure 7(A). These animals were inspected and weighed daily. There was no loss of weight or signs of stress (Fig. 7B). Compound **10** showed a growth delay of 7 days (based on the EORTC guidelines) after the tumor had reached a mean relative volume of 10 mm^3. With **9**, the estimated growth delay was slightly less than that for **10** (data not shown).

The *in vivo* studies on **9** and **10** showed that both the drugs, and in particular, **10**, are able to delay tumor growth in H460 xenografts at doses at which no apparent toxicity was detected. Moreover, in a different long-term toxicity study, Compound **10** did not show any toxicity even at a dose of 10 mg/kg (data not shown). Some recent, preliminary studies suggest that this compound may be tolerated at yet higher (15 mg/kg) doses. It is worthy to note that the tumor growth inhibitions for **9** and **10** were obtained at similar concentrations to that used for RH1. However, RH1 is very toxic at doses of around 2–3 mg/kg. Hence, **9** and **10** are potentially better anticancer drugs.

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