Synthesis and Biological Evaluation of Novel Chloroethylaminoanthraquinones with Potent Cytotoxic Activity against Cisplatin-Resistant Tumor Cells

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Novel 1- and 1,4-substituted chloroethylaminoanthraquinones with DNA binding and alkylating properties along with their respective hydroxyethylaminoanthraquinone intermediates were synthesized. Selected chloroethylaminoanthraquinones were shown to cross-link DNA and alkylate guanines (at low nM concentration) with a preference for reaction sites containing 5′-PyG. A compound (Alchemix) with the bis-chloroethyl functionality confined to one side chain alkylated but did not cross-link DNA. All the 1,4-disubstituted chloroethylaminoanthraquinones were potently cytotoxic (nM IC_{50}s) against cisplatin-resistant ovarian cancer cell lines.

Introduction

Detailed structure−activity studies have allowed a deep understanding of the mechanism of action of the anthracyclines and have rationalized the use of aminoanthraquinone congeners as synthetic cytotoxic agents. From this, mitoxantrone, a 1,4-disusbstituted hydroxyethylaminoanthraquinone, has emerged as a clinically effective anticancer drug. However, mitoxantrone does not have the broad spectrum of clinical activity associated with the anthracyclines, and in addition, both classes of agent are substrates for multidrug resistance mechanisms. The search for DNA intercalating anthraquinones with superior antitumor activity to mitoxantrone (and the anthracyclines) has led to a substantial library of compounds. In particular, previous work has established that within the 1,4-disubstituted series of anthraquinones a well-defined relationship exists between the basic alkylamino side chains and configuration of functional groups attached.[1−3] The anticancer activity of mitoxantrone is related to its ability to persistently trap the DNA−topoisomerase (topo) II ‘cleavable complex’ by continually reforming with its nuclear targets.[4] It is generally acknowledged that drugs that dissociate more slowly from DNA are likely to be more cytotoxic due to longer exposure time of the drug within the cell. Few attempts have been made to develop anthraquinones that covalently bind to DNA by irreversible inhibition of the topo II−DNA ‘cleavable complex’. Anthraquinones linked to melphalan[5] and mechlorethamine[6] have shown that the inclusion of an alkylating functionality into the side chain of the anthraquinone chromophore enhances its cytotoxicity compared to the respective noncovalent binding analogue. Given the substantial literature demonstrating the superiority of mitoxantrone over other substituted anthraquinones, it follows that covalently adducting compounds should be based on the 1,4-disubstituted aminoalkylamino-5,8-dihydroxyanthraquinone structure. In recognition of the therapeutic value of this configuration, this study primarily explores the effect of introducing alkylating functionalities to create a series of symmetrical (identical side chains) and nonsymmetrical (mixed side chains) 1,4-disubstituted chloroethylaminoanthraquinones (CAQs) as a novel class of hybrid compounds with DNA binding and DNA alkylating properties.

Results and Discussion

Chemistry. The synthesis of all the compounds used in this study (see Table 1) has not previously been reported. The 1-monosubstituted hydroxyethylaminoanthraquinones (HAQs) were prepared by the displacement of chloride from 1-chloroanthraquinone with an aminoalkylamino side chain in a polar solvent such as 2-methoxyethanol. The 1,4-disubstituted HAQs were synthesized using previously described methods[1,2] by the condensation of either leucoquinizarin or 5,8-dihydroxyleucoquinizarine (5,8,9,10-tetrahydroxyanthracene-1,4-dione) with an excess amount of *N*-alkyl-*N*-hydroxyalkylaminoalkylamine, which was synthesized according to literature methods.[7,8] The 1,4-disubstituted HAQs were normally isolated in a relatively low yield, between 20 and 50%, depending on the purity of the side chains used. Chlorination was subsequently achieved under mild conditions using triphenylphosphine−carbon tetrachloride complex (PPh_3−CCl_4). This method proved highly efficient for the conversion of HAQs to chloroethyl derivatives, with approximately 80% yields (synthetic scheme is included as Supporting Information).

DNA Binding and Alkylation Studies. The high DNA melting temperatures and close structural similarity of the HAQs (**2**−**8**) to mitoxantrone, a compound known to intercalate DNA,[4] suggests that these compounds also most likely intercalate with DNA. In support of this, **6**, the *N*-methyl derivative of mitoxan-

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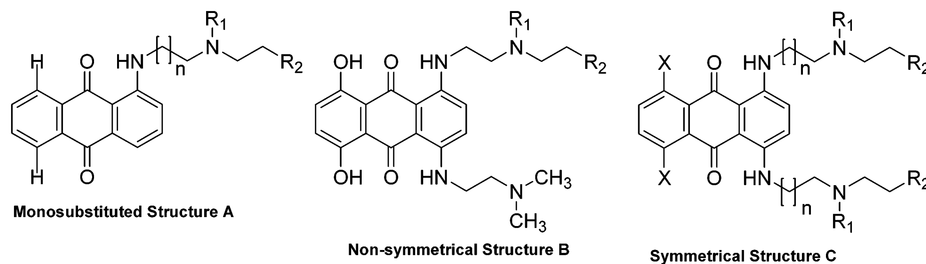
Table 1. Effect of Aminoanthraquinones on (i) Denaturation Temperature of Calf Thymus DNA and (ii) Growth Inhibition (IC_{50}, μM) against Human Ovarian Cancer Cell Lines



structure	R_1	R_2	*n*	X	ΔT_m[a]	A2780[b]	A2780cisR	RF[c]	CH1	CH1cisR	RF	SKOV3
					Hydroxyethylaminoanthraquinone							
1 C	CH_2CH_2OH	OH	1	H	–	3.3	3.5	1.1	3.9	3.3	0.8	18
2 C	CH_2CH_2OH	OH	2	H	4.0	3.5	3.4	1.0	2.2	2.4	1.1	6.7
3 C	CH_3	OH	1	H	11.3	0.72	0.58	0.8	0.68	0.8	1.2	3.3
4 C	CH_2CH_2OH	OH	2	OH	12.8	0.68	0.74	1.1	0.58	0.78	1.3	2.45
5 B	CH_3	OH	–	OH	10.5	0.005	0.005	1.0	0.024	0.045	1.9	0.3
6 C	CH_3	OH	1	OH	19.7	0.004	0.004	1.0	0.008	0.022	2.6	11
7 B	CH_2CH_2OH	OH	–	OH	13.5	0.013	0.013	1.0	0.030	0.046	1.6	0.26
8 C	CH_2CH_2OH	OH	1	OH	11.0	0.082	0.115	1.4	0.3	0.37	1.2	1.5
9 A	CH_3	OH	1	–	0.4	3.4	4.2	1.2	2.9	2.8	1.0	13
10 A	CH_3	OH	2	–	–	3.1	3.3	1.1	2.6	2.6	1.0	11.5
					Chloroethylaminoanthraquinone							
11 C	CH_2CH_2Cl	Cl	1	OH	–	0.12	0.17	1.4	0.054	0.11	2.0	1. 45
12 B	CH_2CH_2Cl	Cl	–	OH	10.1	0.016	0.019	1.2	0.018	0.019	1.0	0.155
13 C	CH_2CH_2Cl	Cl	2	OH	2.3	0.028	0.038	1.4	0.025	0.035	1.4	0.185
14 A	CH_2CH_2Cl	Cl	2	–	–	0.41	0.64	1.6	0.185	0.55	2.9	1.9
15 C	CH_3	Cl	1	OH	5.4	0.009	0.024	2.7	0.007	0.014	1.9	0.1
16 C	CH_3	Cl	1	H	7.2	0.135	0.355	2.6	0.11	0.145	1.3	0.77
17 A	CH_3	Cl	2	–	–	2.7	2.7	1.0	1.44	1.3	0.9	10.8
18 C	CH_2CH_2Cl	Cl	2	H	5.7	0.031	0.052	1.7	0.030	0.110	3.7	0.19
19 C	CH_2CH_2Cl	Cl	1	H	–	0.051	0.096	1.9	0.078	0.092	1.2	0.86
20 A	CH_3	Cl	1	–	1.7	4.6	8.0	1.7	2.5	3.1	1.2	13.5
chlorambucil	–	–	–	–	–	–	52.5	5.8	3.3	7.9	2.4	35
cisplatin	–	–	–	–	–	–	3.3	11	0.1	0.65	6.5	4.4

[a] T_m (°C) determined at the λ_{260} DNA and expressed as the mean (±SD) $n = 3$; T_m of calf thymus DNA = 70.5 ± 0.5 °C. [b] A2780, CH1 = wild-type ovarian cell lines and A2780cisR, CH1cisR = cisplatin-resistant variants; SKOV3 is an intrinsically resistant ovarian cell line. [c] RF = resistance factor (IC_{50} in the resistant cell line/IC_{50} in the parent cell line). See Methods for details.

trone, was the most effective agent (ΔT_m = 19.7 °C); see Table 1. With regard to the CAQs, their structural features are consistent with potential for intercalation as well as covalently binding to DNA. It was of interest therefore to determine their effect on DNA denaturation since, as potential cross-linking agents, they may have been expected to stabilize the DNA double helix. However, Table 1 shows that the DNA melting temperature in the presence of CAQs was generally less (ΔT_m = 2−10 °C) than the hydroxyethyl analogues (ΔT_m = 4−20 °C). It is possible that DNA cross-linking by the CAQs under the elevated temperature used in the thermal unwinding assay could result in depurination-mediated strand breakage.[9] To investigate this further, DNA interstrand cross-linking was examined (autoradiograph is included as Supporting Information) for the four most potent CAQs (**12**, **13**, **15**, and **18**) as identified from the cytotoxicity studies (see Table 1). Compounds with alkylating functionality on both sidearms (**13**, **15**, and **18**) were shown to inhibit DNA strand separation in the low nM range consistent with interstrand cross-linking. Previously, Hartley et al. demonstrated that mechlorethamine cross-linked DNA under the same conditions, but at a much higher concentration (10 μM).[21] This shows that the anthraquinone chromophore linked to chloroethyl functionality is significantly more potent at interstrand cross-linking consistent with a likely increase in DNA affinity of this class of agent. Compound **12**, which has the bischloroethyl functionality confined to one sidearm, showed no cross-linking of DNA even after prolonged (up to 24 h) incubation (results not shown). Interestingly, **12** exhibited the largest melting temperature increase (ΔT_m = 10.1 °C) of all the CAQs and comparable to that of the HAQs. This would suggest that under the conditions of the DNA melting assay **12** is not able to promote interstrand depurination. Instead stabilization of the DNA double helix to thermal denaturation is observed.

12, **13**, **15**, and **18** were investigated for their ability to covalently adduct double stranded DNA using the *Taq* polymerase assay[10] (Figure 1) and showed inhibition of DNA elongation consistent with DNA alkylation at a concentration as low as 5 nM. Comparison of the alkylation patterns with mechlorethamine, which has no intrinsic DNA affinity, revealed a difference in guanine selectivity. The CAQs demonstrated a preference for guanines adjacent to a 5′ pyrimidine (5′-PyG), particularly isolated 5′-PyGPy sites (e.g. base positions 530 and 566), which are only weak sites for alkylation by nitrogen mustards.[11] At the sequence 5′-CGGGGGA-3′ (bases 536−540) the CAQs preferentially alkylated at the 5′ guanine base while mechlorethamine alkylated this base the least. There was no apparent difference in selectivity between **12** and the three DNA cross-linking compounds **13**, **15**, and **18**. However, **13**, **15**, and **18** revealed more intense bands at certain isolated 5′-

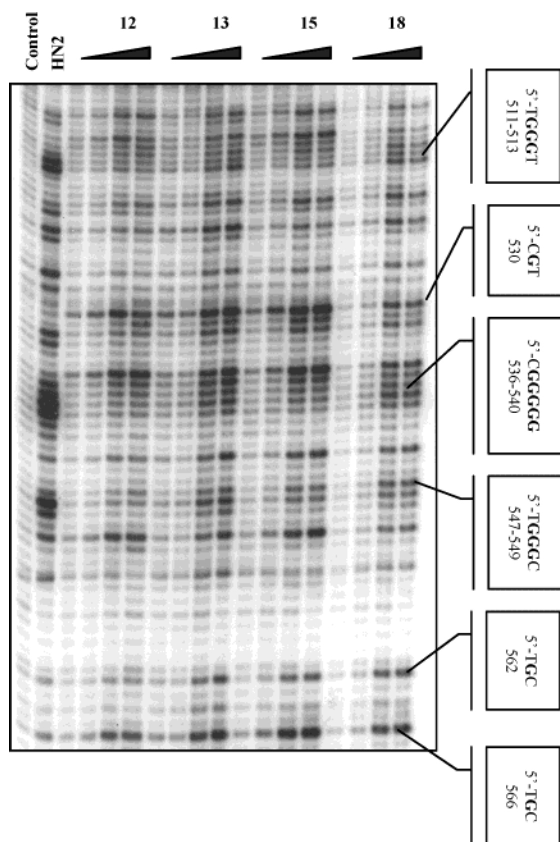


Figure 1. Effect of **12**, **13**, **15**, and **18** on *Taq* DNA polymerase elongation of plasmid DNA. Control: unmodified DNA; HN2 = mechlorethamine (2.5 μM); concentrations of chloroethylaminoanthraquinone, from left to right: 5, 10, 50, 100 nM. See methods for details.

TGC sites, e.g. at base positions 562 and 566, which probably shows sites where cross-linking had occurred.

Cytotoxicity Studies. The effect of the CAQs on cells with intrinsic or acquired resistance to conventional alkylating agents was an essential part of this study in order to justify the development of these new agents against established targets. For this purpose, all compounds were investigated in ovarian carcinoma cell lines A2780 and CH1 and compared with activity in the respective acquired cisplatin-resistant variants A2780cisR and CH1cisR, as well as SKOV3, an ovarian cell line that shows intrinsic resistance to cisplatin. Table 1 shows that for the noncovalent binding compounds **1**–**8**, the results are generally consistent with the known effects of 1-mono- versus 1,4-disubstituted aminoanthraquinones and the importance of the 5,8-dihydroxylated chromophore substitution.[1–3] Specifically, the cytotoxicity of the HAQs with a 5,8-dihydroxy-substituted chromophore was greater than that of the 5,8-dihydro analogues, which is consistent with previous studies on noncovalent binding aminoanthraquinones.[1–3] In contrast, the CAQ analogues based on 5,8-dihydro and 5,8-dihydroxy chromophores have similar cytotoxicity profiles. This is likely to be due to the alkylating ability of these compounds, which permits covalent linkage to DNA and hence eliminates the 5,8-substitution dependency of residence time as a limiting factor of cytotoxicity. This is consistent with DNA denaturation experiments which showed the CAQs with the 5,8-dihydro chromophore to be more effective in stabilizing double helical DNA than their 5,8-dihydroxy analogues (cf. **16** and **15**; **18** and **13**, Table 1). The CAQs were substantially more cytotoxic than their HAQ analogues. This is exemplified by comparing the 5,8-dihydro chromophore-based compound **19** with **1** and **18** with **2** (20–110 fold more potent), and the 5,8-dihydroxy chromophore-based compounds **13** with **4** and **11** with **8** (up to 15-fold more potent).

Importantly this study shows that following 24 h exposure, the novel CAQs retained most of their cytotoxicity in the cisplatin-resistant A2780cisR and CH1cisR cell lines despite their well-established resistance mechanism as an effect of exposure to cisplatin. A2780cisR has been characterized with elevated levels of glutathione, alterations in drug uptake/efflux and DNA repair due to acquired exposure of cisplatin.[12–14] CH1cisR has primarily been associated with enhanced removal of, or increased tolerance to, platinum–DNA adducts.[15] All compounds were shown to be less cytotoxic in the intrinsic resistant SKOV3 cells than in the acquired resistant cells, perhaps reflecting the ability of these cells to tolerate higher levels of DNA damage.[16] **12** exhibited no cross-resistance in the CH1cisR cells and was the least cross-resistant chloroethyl compound in the A2780cisR cells.

Conclusion

It is notable that of all the CAQs investigated, **12** is the only compound of the series with a bis-chloroethyl moiety confined to one side. Although **12** possesses a bis-alkylating functionality, uniquely it did not covalently cross-link DNA although it was a potent DNA alkylator. The alkylating and DNA binding properties of **12** may facilitate formation of a covalent linked drug/DNA/topoisomerase II ternary complex that persistently inhibits the enzyme and which is not subject to DNA mismatch repair. Alternatively, the formation of alkylated regions of DNA not recognized by repair mechanisms is also possible. In a recent study,[17] **12** (Alchemix, ZP281M) was shown to retain potent cytotoxicity in the doxorubicin resistant 2780AD cell line known to overexpress P-glycoprotein, indicating that this agent is not a substrate for MDR. In the same study, **12** was shown to possess potent antitumor activity against A2780 ovarian cancer xenografts completely resistant to either cisplatin or epirubicin. A more detailed investigation of the 1,4-disubstituted CAQs with alkylating functionality confined to one sidearm is therefore warranted.

Experimental Section

Chemistry. All aminoalkylamino side chains were prepared as previously described.[7,8] The synthesis of target compounds follow the procedure described for compound **7** and **12**.

1-[{2-[Dimethylamino]ethyl}amino]-4-[{2-[bis(2-hydroxyethyl)amino]ethyl}amino]-5,8-dihydroxyanthracene-9,10-dione (7). 5,8-Dihydroxyleucoquinizarin (0.2 g, 0.75 mmol) was added to a mixture of *N*,*N*-bis(2-hydroxyethyl)ethylenediamine (0.67 g, 3.6 mmol) and *N*,*N*-dimethylethylenediamine (0.194 g, 2.2 mmol) at 60 °C under N_2 and heated for 5 h. The reaction was cooled to room temperature, and aq NaOH (2 M, 0.2 cm^3) was added and stirred overnight exposed to air. The reaction was diluted with CH_2Cl_2 (80 cm^3), washed with water (3 × 80 cm^3), dried with $MgSO_4$, and concentrated. The crude solid was purified by chromatography (CH_3OH:CH_2Cl_2:NH_3, 4.5:94:0.5 increasing to 19.5:80:0.5). The resulting solid was further purified by redissolving in CH_3OH and precipitation with dry diethyl ether to give the product as dark blue solid (60.03 mg, 20% yield). mp 204.2–205 °C; ^{1}H NMR

($CDCl_3/CD_3OD$) δ: 2.4 (s, 6H, 2 × NC*H*$_3$), 2.7 (2 × t, 6H, 2 × NC*H*$_2$), 3.0 (t, 2H, C*H*$_2$N), 3.6 (q, 4H, 2 × C*H*$_2$NHAr), 3.7 (t, 4H, 2 × C*H*$_2$OH), 7.1(s, 2H, Ar*H*), 7.3 (s, 2H, Ar*H*), 10.6 (t, 2 × H, N*H*Ar); IR ν_{max} (KBr) cm^{-1} 3600−3300 (OH), 1580 (C=O), 1230 (N−H); E_λ = 7936 cm^{-1}; λ_{max} (608 nm); FAB-MS, *m*/*z* (M + H)$^+$ 473; Anal. ($C_{24}H_{32}N_4O_6$) C, H, N.

1-[{2-[Bis(2-chloroethyl)amino]ethyl}amino]-4-[{2-[dimethylamino]ethyl}amino]-5,8-dihydroxyanthracene-9,-10-dione dihydrochloride (12). Triphenylphosphine (0.11 g, 0.32 mmol) and then CCl_4 (0.19 g, 1.25 mmol) were added to a stirred solution of **7** (0.05 g, 0.10 mmol) in CH_2Cl_2 (5.0 cm^3) under N_2. The resulting suspension was allowed to stir at room temperature for 24 h. The residue was precipitated by the addition of dry ethereal HCl, isolated by filtration, and dried under vacuum. The crude product was dissolved in a minimum quantity of CH_2Cl_2/EtOH (1:1) at 60°C and isolated from the triphenylphosphine oxide byproduct and excess triphenylphosphine by precipitation with EtOH/EtOAc (1:1). The product was isolated as a dark blue solid (51.5 mg, 83%). mp 190.0−192.1°C; ^{1}H NMR ($CDCl_3/CD_3OD$) δ: 3.0 (s, 6H, 2 × NC*H*$_3$), 3.4 (t, 4H, 2 × C*H*$_2$N), 3.6 (t, 4H, 2 × C*H*$_2$N), 3.85 (t, 4H, 2 × C*H*$_2$NHAr), 3.9 (t, 4H, 4 × C*H*$_2$Cl), 7.1 (s, 2H, Ar*H*), 7.3 (s, 2H, Ar*H*), 10.6 (t, 2H, 2 × N*H*Ar); IR ν_{max} (KBr) cm^{-1}: 3600−3300 (OH), 1580 (C=O), 1230 (N−H); E_λ (CH_3OH/DMSO) = 14162 cm^{-1}; λ_{max} (620 nm); FAB-MS, *m*/*z* (M + H)$^+$ 509; Anal. ($C_{24}H_{30}N_4O_4Cl_2 \cdot 2HCl \cdot 2H_2O$) C, H, N.

Biology. Thermal Denaturation Studies. The temperature of a mixture of drug (8.5 × 10^{-8} M) in Tris buffer (0.05 M, pH 7.2,) and DNA (8.5 × 10^{-7} M) (DNA:drug ratio 10:1) was raised at a rate of 1 °C/min, and the absorbance of the DNA was monitored at 260 nm from 60 to 100 °C. This was compared to the absorbance change for DNA in the absence of drug.

Agarose Gel Cross-Link Assay. The experiments followed the method of Hartley et al.[18] Briefly, linearized and 5′-end ^{32}P-radiolabeled plasmid pB*R*322 DNA (10 μL, ~100 ng), drug (25 μL to give final concentrations of 5, 10, 50, and 100 nM), and TEOA buffer (15 μL) were incubated at 37 °C for 1 h. Samples were precipitated, dried, and resuspended in strand separation buffer and heated for 2 min at 95 °C before being chilled in ice. The samples were electrophoresed in 0.8% agarose gel at 40 V overnight, dried, and autoradiographed.

***Taq* Polymerase Stop Assay.** The experiments followed the method of Ponti et al.[10] Briefly, linearized plasmid DNA pB*R*322 (0.5 μg/10 μL per PCR reaction), TEOA buffer (5 μL, 1×), drug solution (1 μL, to give final concentrations of 5, 10, 50, and 100 nM), and dH_2O to make up a final volume of 50 μL were mixed and incubated for 1 h at 37 °C. The DNA was precipitated using sodium acetate (3 M, pH 3.3, 30 μL), dH_2O (40 μL), and EtOH (300 μL, 95%), cooled in a dry ice/ethanol bath, and lyophilized. The pellet was then washed twice with EtOH (100 μL, 75%) and dried by lyophilization. For the PCR, the following were mixed: ^{32}P-labeled primer (5 μL, 10 pM/μL), gelatin (5 μL, 0.2%), $MgCl_2$ (10 μL, 25 mM), buffer (10 μL, 10×), dNTP mix (10 μL, 2.5 nM), dH_2O (18 μL), drug-treated DNA (40 μL), and DNA *Taq* polymerase (2 μL, U/μL). The PCR was set to cycle 36 times at 94 °C for 1 min, 58 °C for 2 min, 72 °C for 1 min, and an additional 1 min per cycle, a total of 3 h. After precipitation and lyophilization, each dried sample was resuspended in formamide dye, heated to 95 °C for 2 min, and cooled in an ice-bath. The samples were electrophoresed 3 h at 3000 V and 55 °C, dried, and autoradiographed.

Cytotoxicity Studies. The effect of the aminoanthraquinones on viability of human ovarian carcinoma cell lines was used as a measure of cytotoxicity: Two wild-type cell lines A2780, CH1 and their cisplatin acquired resistant variants A2780cisR, CH1cisR. SKOV3, an intrinsically cisplatin-resistant cell, was also used. All cell lines were maintained as previously described.[15] The compounds were dissolved in DMSO immediately before adding aliquots to exponentially growing cell lines (5 × 10^4 cells/mL) and incubated at 37 °C for 24 h followed by replacement of the drug-containing medium with fresh medium and a 3 day growth period. Cell viability was assessed by the sulforhodamine B (SRB) assay, and the concentration that caused a 50% reduction in SRB color formation (IC_{50}) was determined.[19]

Supporting Information Available: Synthetic methods and a scheme covering the route to target compounds. Autoradiograph of DNA interstrand cross-linking by compound **13**, **15**, and **18**. This material is available free of charge via the Internet at http://pubs.acs.org.

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