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Molecular Modelling and Cytotoxicity of Substituted Anthraquinones as Inhibitors of Human Telomerase

Donald Cairns,[a,*] Evangelia Michalitsi,[a] Terence C. Jenkins[b] and Simon P. Mackay[c]

[a]*Institute of Pharmacy and Chemistry, University of Sunderland, Sunderland SR1 3SD, UK*
[b]*YCR Laboratory of Drug Design, University of Bradford, Bradford, West Yorkshire BD7 1DP, UK*
[c]*Strathclyde Institute of Biomedical Sciences, University of Strathclyde, Glasgow G4 0NR, UK*

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Abstract—Molecular modelling has been carried out for a number of amine-functionalised anthraquinone derivatives to determine their extent of binding to G-tetraplex DNA and their ability to inhibit the enzymes telomerase and *Taq* polymerase. The results are compared to data obtained from a modified TRAP assay and show good correlation between the two methods. The findings suggest that anthraquinone derivatives of this type inhibit telomerase by stabilisation of four-stranded tetraplex structures associated with guanine-rich telomeric DNA regions.

The 3′-ends of eukaryotic chromosomes consist of polynucleotides comprising several kilobases with tandem repeats of the same base sequence (e.g., 5′-TTAGGG in humans).[1,2] This region, termed the telomere, is ribonucleoprotein in nature and serves to protect the chromosomal termini from end-to-end recombination and attack by exonuclease enzymes.[3,4] The observation that the telomere region of DNA is not conserved during cell division led to the hypothesis that the length of the telomere correlates with the capacity of cells to replicate.[5,6] Most human cells can only divide 50–60 times before senescence occurs and the cells enter a cycle of programmed cell death (apoptosis), leading to the conclusion that telomere length is a 'biological clock' capable of determining the proliferative capacity of most human somatic cells.[7,8] Certain cell types (e.g., germ cells, stem cells and essentially immortal cells such as those found in neoplasms) have been shown to regulate their telomeres to nearly constant length by expressing a specialised RNA-dependent DNA polymerase called telomerase. Interest in this enzyme arose from work carried out by Kim and colleagues[9] who showed that telomerase was expressed in >85% of cancers, including breast and gastric cancer while the enzyme was not detectable in healthy somatic cells.[10,11] Consequently, telomerase became an obligate focus for anti-cancer research as it promised a unique target for chemotherapy without the associated side effects of conventional chemotherapeutics (for recent reviews, see refs 12 and 13).

DNA sequences that are rich in guanines can adopt unusual secondary DNA structural forms. In particular, four guanine bases can associate in a planar, hydrogen-bonded assembly called a G-tetrad (or quartet) where each guanine simultaneously accepts and donates two hydrogen bonds in a reverse-Hoogsteen arrangement. Successive layers of G-tetrads allow such single-stranded DNA to adopt a high-order foldback structure in solution termed a G-tetraplex (or 'quadruplex').[14,15] Work pioneered by Zahler et al.[16] and later by Hurley's group[17] showed that telomerase requires access to a single-stranded region of telomeric DNA in order to bring about the required elongation. These findings suggested that telomere extension by telomerase could be inhibited if agents could be found that acted to stabilise the folded G-tetraplex structure.

As part of work in our laboratories on the use of anthraquinones as anti-cancer agents, a number of mono- and bis-(amino)-substituted derivatives have been synthesised and fully characterised as potential telomerase inhibitors.[18,19] In this paper, we report findings from a molecular modelling study where the ability of these compounds to stabilise G-tetraplex DNA structures was examined. Computed results are compared with experimental data for telomerase inhibition and cytotoxicity with these agents.

*Corresponding author. Tel.: +44-191-515-2544; fax: +44-191-515-2502; e-mail: donald.cairns@sunderland.ac.uk

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1-(5-Hydroxypentylamino)-9,10-anthraquinone (**1A**) was prepared in good yield by refluxing 1-chloroanthraquinone with an excess of 5-aminopentanol. The symmetrically disubstituted compounds **1,5AA** and **1,8AA** were prepared by analogous reaction with 1,5- and 1,8-dichloroanthraquinone, respectively. The asymmetric bis-substituted derivatives, **1,5AC, 1,4AC,** and **1,8AC** were synthesised from the appropriate dichloroanthraquinone in two steps by (i) reflux with 5-aminopentanol to yield the mono-substituted derivative, followed by isolation and purification, before (ii) subsequent attachment of the second side-chain with 1-amino-2-diethylaminoethane. 1,4-Bis(5-hydroxypentylamino)-9,10-anthraquinone (**1,4AA**) was prepared from quinizarin by reduction to the leuco-form and attachment of 5-aminopentanol (Fig. 1). All compounds were purified by flash column chromatography and fully characterised using infra-red, NMR spectroscopy and mass spectrometry methods.[18]

Modified Telomeric Repeat Amplification Protocol (TRAP) Assay

This procedure was carried out by the method of Perry et al.[20] using extracts from exponentially growing A2780 human ovarian carcinoma cells. Briefly, telomerase-mediated extension of a primer was initiated followed by addition of protein and incubation of the mixture with the anthraquinone compound. Controls containing no protein, heat-inactivated protein or protein at a concentration of 50% were included in each assay. *Taq* DNA polymerase activity was determined by heating the reaction mixture to 80 °C (to inactivate telomerase) followed by addition of a reverse CX primer and *Taq* polymerase. A three-step PCR was performed, and the presence or absence of telomerase-extended PCR products was determined using acrylamide gel electrophoresis.

Growth Inhibition Assay

Growth inhibition was determined in three human ovarian carcinoma cell lines (A2780, CH1 and SKOV-3) using a sulforhodamine B assay by the method of Kelland et al.[21] The anthraquinone derivatives were tested as water-soluble mono- or di-hydrochloride acid addition salts at concentrations of 4 to >100 μM. Following incubation, cells were stained with 1% SRB in acetic acid and the optical absorbance at 540 nm was measured. This value was expressed as a percentage compared to the value for the untreated control cells, and IC_{50} values were determined for each compound.

Results from the modified TRAP assay are summarised in Table 1. These data show that all compounds tested inhibited telomerase at concentrations of 10 μM, with **1,8AC** effecting a strong 43.3% inhibition. Importantly, no compound inhibited the related *Taq* polymerase enzyme used in this assay at <50 μM concentrations, indicating a selective inhibition of telomerase. The telomerase inhibitory data for two compounds (**1,8AC** and **1,5AA**) at 50 μM are unusual in that inhibition does not increase at the higher 50 μM dose; this behaviour is attributed to poor aqueous solubility for these derivatives.

Table 1. Inhibition of *Taq* polymerase and telomerase by the mono-/di-(amino)-anthraquinones

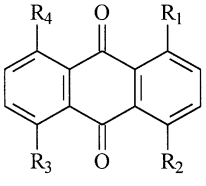
Compd	*Taq* inhibition[a]		% Telomerase inhibition	
	10 μM	50 μM	10 μM	50 μM
1A	NEG	NEG	6.4	36.1
1,4AA	NEG	NEG	22.0	—[c]
1,5AA	NEG	NEG	31.5	29.9
1,8AA	NEG	NEG	—[b]	24.5
1,4AC	NEG	NEG	18.1	93.5
1,5AC	NEG	NEG	27.8	57.3
1,8AC	NEG	NEG	43.3	52.8

[a]NEG, negligible detection of *Taq* polymerase inhibition.
[b]Insufficient sample for the modified TRAP assay.
[c]Compound not sufficiently soluble.

Table 2. In vitro cytotoxicity of the agents in human carcinoma cell lines

Compd	IC_{50} (μM)[a]				
	A2780	A2780*cis*^{R}	CH1	CH1*cis*^{R}	SKOV-3
1A	>100	>100	>100	>100	>100
1,4AA	>100	>100	>100	>100	>100
1,5AA	>100	>100	>100	>100	>100
1,8AA	>100	>100	>100	>100	>100
1,4AC	4.3	10.5	6.8	2.7	10.5
1,5AC	12.5	13.0	17.0	8.0	23.5
1,8AC	>100	>100	>100	11.5	>100

[a]A2780/A2780*cis*^{R} and CH1/CH1*cis*^{R} refer to the wild-type cells and their derived cisplatin-resistant counterparts, respectively, for each cell line.



1A $R_1 = NH(CH_2)_5OH$ $R_2 = R_3 = R_4 = H$

1,4AA $R_1 = R_2 = NH(CH_2)_5OH$ $R_3 = R_4 = H$

1,5AA $R_1 = R_3 = NH(CH_2)_5OH$ $R_2 = R_4 = H$

1,8AA $R_1 = R_4 = NH(CH_2)_5OH$ $R_2 = R_3 = H$

1,4AC $R_1 = NH(CH_2)_5OH$ $R_2 = NH(CH_2)_2NEt_2$ $R_3 = R_4 = H$

1,5AC $R_1 = NH(CH_2)_5OH$ $R_3 = NH(CH_2)_2NEt_2$ $R_2 = R_4 = H$

1,8AC $R_1 = NH(CH_2)_5OH$ $R_4 = NH(CH_2)_2NEt_2$ $R_2 = R_3 = H$

Figure 1. Structures of the amino-functionalised anthraquinones.

Table 3. Effect of compounds on growth of human carcinoma cells

Compd	% Cell growth at 100 μM dose[a]				
	A2780	A2780*cis*^{R}	CH1	CH1*cis*^{R}	SKOV-3
1A	84.6	86.3	94.0	71.5	89.1
1,4AA	92.8	98.4	104.1	102.0	98.8
1,5AA	98.6	100.1	99.6	101.1	102.1
1,8AA	97.0	98.8	104.2	116.0	99.2
1,4AC	0.5	6.4	20.3	2.4	6.7
1,5AC	10.9	12.5	36.9	1.2	48.8
1,8AC	94.5	96.9	89.4	12.9	107.4

[a]Relative growth at a fixed 100 μM drug dose compared to untreated control cells.

In vitro cytotoxicity and cell growth inhibitory data for the functionalised anthraquinones are collected in Tables 2 and 3. All compounds with the exception of compounds **1,4AC** and **1,5AC** are remarkably non-toxic in the ovarian carcinoma cell lines used. It appears that compounds with the 2-(diethylamino)ethylamino side-chain (i.e., **1,4AC** and **1,5AC**) are more cytotoxic than the corresponding (5-hydroxypentyl)amino-substituted compounds. This may in part be due to the presence of a protonatable nitrogen in the side-chains of **1,4AC** and **1,5AC**, which would stabilise interaction with the polyanionic backbone of DNA to promote binding to double-stranded DNA and thereby induce a greater toxicity response.

The 1,8-difunctionalised compound **1,8AC** appears anomalous in that it shows significant inhibition of human telomerase at 10 μM with no associated cytotoxicity, despite the presence of a protonatable 2-(diethylamino)ethylamino side chain. This is attributed to the fact that 1,8-disubstituted anthraquinones can only intercalate DNA with the long axis of the planar chromophore aligned parallel to the base-pair axis. Other disubstituted compounds are unable to intercalate in this way.[22] Alternatively, the telomerase inhibition of these compounds may be mediated through a non-specific inhibition of the enzyme that is unrelated to intercalation.

Molecular Modelling Methods

Molecular modelling was carried out on a Silicon Graphics Octane R12000 workstation using the Insight-II 2000 graphics interface and Discover 98.0 simulation software (Molecular Simulations Inc., Cambridge, UK). The cvff all-atom force-field was used for all energy calculations, minimisations and dynamics simulations. Solvent effects were represented by using the explicit water TIP3P model in all calculations. Atomic coordinates for the human telomeric G-tetraplex DNA structure[23] were obtained from the Brookhaven Protein Database (PDB ref. 143D), and solvated with two 10-Å layers of water to prevent evaporation during simulations. Explicit Na^+ counterions were included, placed at a 6 Å distance from each phosphate–oxygen bisector prior to solvation. The solvated complex was minimised using the conjugate gradients algorithm until an energy convergence criterion of 0.1 kcal mol^{-1} A^{-1} was reached. Molecular dynamics (MD) at 300 K were then performed on the solvated system for a 10 ps equilibration and 100 ps of production employing a 1-fs time step, from which 100 structures were sampled at 1-ps intervals and averaged. The final averaged structure was then minimised as described.

A model intercalation site was generated between the diagonal T_2A loop and the first G-tetrad of the tetraplex structure (i.e., the 5′-ApG step) by breaking the local phosphodiester backbones and separation of the bases to 6.8 Å.[24] Counterion positions were modified accordingly. The backbones were then reconnected and molecular mechanics (MM) energy minimisation was then used to relieve any steric distortion in the chains due to the separation, unwinding and connection. Positional restraints were applied to each G-tetrad during this process, and convergence was achieved at 1 kcal mol^{-1} A^{-1}. Models for each anthraquinone molecule were constructed (Insight-II) and partial atomic charges were approximated from a single-point PM3 calculation using the MOPAC program a semi-empirical method which calculates charge sets comparable to high-level calculations, but is less computationally expensive.[25] Atom potentials were assigned according to parameters defined within the cvff force-field. Each drug structure was then individually docked into the tetraplex intercalation site, with optimal orientations obtained by computing non-bonded interactions between the reactants. Alterations to ligand side-chain conformations were performed in order to minimise close intermolecular contacts with the host DNA tetraplex.

Each DNA–ligand complex was then resolvated and minimised as before. The optimised models were subjected to MD simulation (10 ps equilibration and 100 ps production with 1-fs timesteps) with initial atomic velocities taken from a Maxwell–Boltzmann distribution at 300 K. The time-averaged structures from 100 samples taken at 1-ps intervals were minimised.

Ligand structures were sampled for conformational averaging in water by immersion in a solvent box of 25 $Å^3$ and MD subject to periodic boundary conditions. Non-bonded interactions for van der Waals were accounted for by the Ewald-summation method, with electrostatic contributions treated separately using an atom-based cut-off distance of 12 Å. An MD equilibration period of 5 ps at 300 K was used followed by a 50-

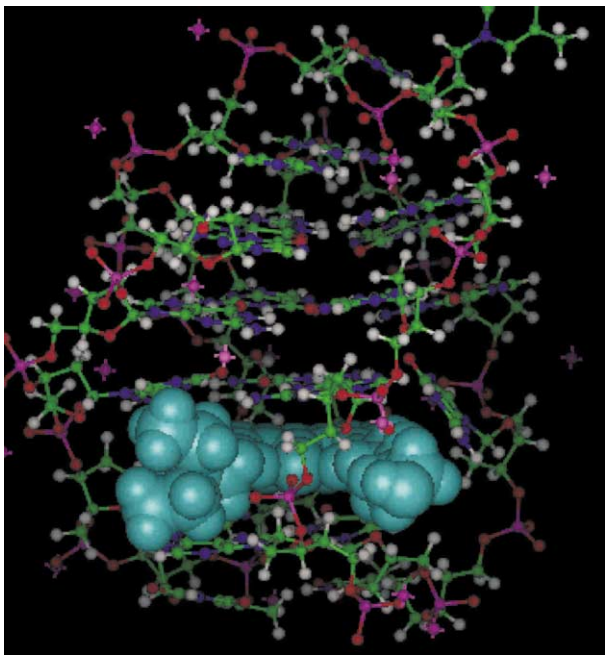


Figure 2. Image of minimised average structure of **1,8AC** intercalated into G-tetraplex. Carbon atoms are coloured in green, nitrogens in blue, oxygens in red and hydrogens in white. Phosphorus and sodium counterions are shaded in purple. The drug is drawn as a CPK space-filled model and is shaded light blue.

Table 4. Computed enthalpy terms (kcal mol^{-1}) for the 1:1 G-tetraplex–anthraquinone complexes[a]

Ligand	E_{bind}	$E_{complex}$	E_{AQ}
1A	−30.05	775.44	112.82
1,4AA	−39.58	788.38	135.29
1,5AA	−47.68	771.35	126.36
1,8AA	−34.41	791.98	133.72
1,4AC	−31.06	805.61	144.00
1,5AC	−40.29	795.76	143.38
1,8AC	−41.06	793.20	141.59

[a]Where $E_{DNA} = 692.67$ kcal mol^{-1} for the host telomeric DNA tetraplex.

ps production phase with a 1-fs time step. Atomic trajectories saved every 1 ps were used for conformational averaging. The latter structures were finally submitted for minimisation to an energy convergence criterion of 0.1 kcal mol^{-1} Å^{-1}.

Binding enthalpies were determined by calculation of the potential energy of the DNA tetraplex–ligand complex that included contributions from bonded and non-bonded van der Waals and electrostatic energies and subtracting from this value the sum of the energies of the unbound DNA and anthraquinone. The latter values were obtained from the minimised conformational average of the structures simulated free in solution. While solvation energy contributions were not included in binding enthalpy calculations for the complexes, the use of explicit water in production of the structures is influential in determining the final conformations adopted by all reactant molecules. Binding enthalpies (E_{bind}) were calculated using $E_{bind} = E_{complex} - [E_{AQ} + E_{DNA}]$, where $E_{complex}$, E_{AQ} and E_{DNA} are the computed potential energies for the minimised average AQ-complex, free AQ and free tetraplex, respectively. This modelling strategy has been used to successfully predict DNA-binding sites for berenil and bis(amidine) ligands with lengthy *tyrT* fragments[26] or defined duplex sequences on the basis of enthalpic interaction terms.[27] It should be stressed that this approximation neglects any entropic contributions to the net free energy of binding. Nevertheless, good correlations between experimental and computed data have been obtained for classes of DNA groove-binding or intercalating ligands, particularly for variants within a series of structurally-related molecules.[23,26,28]

The results in Tables 1 and 4 show that, with the exception of **1,5AA** and **1,8AC** (where the order is transposed), our modelling calculations are in excellent agreement with experimental data obtained from the TRAP assay. This is reassuring from the viewpoint of drug design as the results validate our modelling protocol. More importantly, this correlation suggests that anthraquinone derivatives of this type achieve their inhibition of telomerase by TpG intercalation and stabilisation of the G-tetraplex structures associated with telomeric DNA. In doing so, they stabilise the host tetraplex and prevent access of the unfolded DNA primer to the RNA template region of telomerase. Compound **1,8AC**, which shows the greatest inhibition of telomerase at 10 μM, appears to have the optimal substitution pattern for intercalation into G-tetraplex structures (Fig. 2). It is our experience that non-specific cytotoxicity associated with agents of this type is caused by promiscuous binding of these agents to duplex DNA.[12,19] The way forward for telomerase inhibitors of this class lies in maximising the tetraplex-binding affinity while minimising their capacity for duplex binding. Such design exercises are currently underway in our laboratories.

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