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Evaluation of by disubstituted acridone derivatives as telomerase inhibitors: the importance of G-quadruplex binding

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Abstract—The synthesis and evaluation of a group of 2,6-, 2,7- and 3,6-bis-aminoalkylamido acridones are reported, which show a similar level of activity against telomerase in vitro compared to their acridine counterparts. Computer modelling and calculations of relative binding energies suggest an equivalent binding mode to human intramolecular G-quadruplex DNA, but with significantly reduced affinity, as a result of the limited delocalisation of the acridone chromophore compared to the acridine system. Thermal melting studies on acridone and acridine quadruplex complexes using a FRET approach support these predictions. Long-term cell proliferation studies at sub-cytotoxic doses with two representative acridones using the SKOV3 cell line, show that neither compound produces growth arrest, in contrast with the effects produced by the tri-substituted acridine compound BRACO-19. It is concluded that telomerase inhibitory activity is a necessary though by itself insufficient property in order for cellular growth arrest to occur at sub-toxic concentrations, and that tight quadruplex binding is also required.

The reverse transcriptase enzyme telomerase is responsible for the maintenance of telomere length in over 80% of all tumours, and is not expressed in normal somatic cells.[1] Its potential as an anti-cancer target[2] has been demonstrated in a number of studies, using dominant negative mutants,[3] antisense to the template region of telomerase RNA,[4] and with active site inhibitors.[5] A more indirect approach[6] exploits the requirement of the single-stranded 3′ overhang of the primer strand of telomeric DNA to maintain its single-stranded conformation[7] in order to effectively hybridise with the RNA template of the RNA (hTR) domain of telomerase. Folding the single-stranded DNA overhang primer into a four-stranded quadruplex using small molecules to bind and stabilise quadruplex DNA inhibits telomerase in vitro.[8] Many such compounds have now been described,[9] and the more potent produce replicative senescence and telomere shortening in cell culture.[10] We have previously reported inter alia the synthesis and biological evaluation of G-quadruplex-binding bis-aminoalkylamido molecules, based on the acridine scaffold.[11] Structure–activity relationships in these series show that the amidoalkylamino side chains $NHCOCH_2CH_2N^+R_2$, are important for G-quadruplex stabilisation and telomerase inhibition. A trisubstituted series of acridine derivatives have more recently been designed using computer modelling,[12] which shows high potency against telomerase. The lead compound BRACO-19, in combination with taxol, has in vivo antitumour activity against the A431 ovarian tumour xenografts.[13]

We report here on three regioisomeric series of structurally simple acridone derivatives (Fig. 1). The route to their synthesis has been established previously for the synthesis of 3,6-disubstituted acridine derivatives.[11] The syntheses of key intermediates for these and the 2,6- and 2,7-series follows in part earlier studies[14,15] on acridine chemistry. Diphenylmethane was converted to the 3,6-diaminoacridone by nitration, oxidation and subsequent reductive cyclisation. Acylation with 3-chloropropionylchloride (CPC) afforded the intermediate

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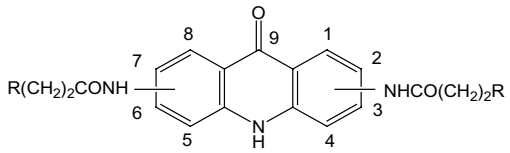

Figure 1. Chemical formula and numbering scheme of the disubstituted acridones.

chloropropionamide in good yield. Subsequent aminolysis by reflux treatment with the appropriate secondary amine gave the product acridones. 2,7- and 2,6-acridone derivatives were obtained via a cyclisation method, by which the appropriate diamino aniline was coupled under Ullmann conditions with an aryl chloride. Subsequent cyclisation under acid conditions provided a nitro-amino acridone, which by reduction of the nitro group provided 2,6- or 2,7-diaminacridone intermediates. Acylation with 3-CPC and amination furnished the 2,6- and 2,7-acridone derivatives. In all cases the acid addition salts were prepared to improve water solubility for biological and biophysical analysis.

Molecular orbital considerations suggest that the acridone chromophore, by contrast with acridine or anthraquinone, is not a fully delocalised system. The involvement of the charged central ring acridine nitrogen atom in delocalisation is a major contributor to effective π–π stacking interactions with adjacent base pairs, and so it was anticipated that quadruplex affinity of acridones would be substantially reduced compared to their disubstituted acridine counterparts. Molecular modelling studies, using as a starting point the crystal structure[16] of the 22-mer intramolecular G-quadruplex formed from the human telomeric DNA sequence, have been undertaken to examine plausible interactions with several acridone derivatives. Relative binding energies have been calculated for solvated species, and compared with that for the pyrrolidine di-substituted acridine BSU6039 (Table 1). The simulations predict that the acridones will stack onto and between G-quartet bases, with the side chains residing in opposite quadruplex grooves (Fig. 2). The morpholino compound **5** has a significantly reduced binding energy, due to the smaller electrostatic contribution that arises from the weakly basic morpholino group. Computed binding energies to the quadruplex structure are significantly greater for the disubstituted acridine ligand compared to all three other acridones, largely due to the superior electrostatic energy contribution of the acridine moiety to the overall total energy (Table 1).

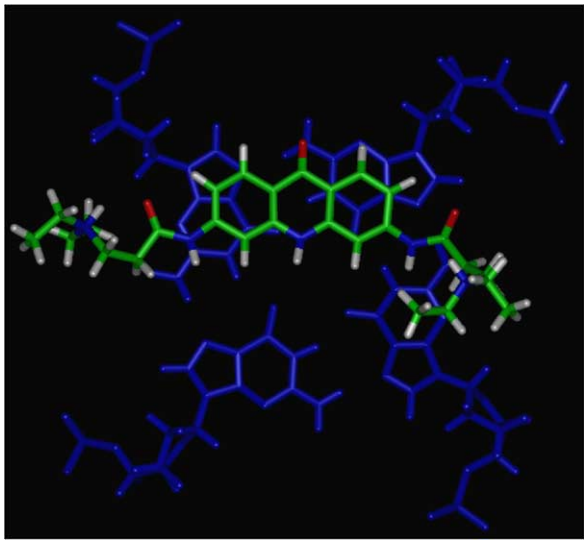

Figure 2. A view from the molecular modelling study, of the minimum-energy structure of the complex of compound **4** bound to the human quadruplex, viewed onto the G-quartet plane.

All compounds were evaluated for in vitro short-term (96 h) acute cytotoxicity against a panel of three human carcinoma cell lines (A2780, CH1 and SKOV-3) using the sulforhodamine B (SRB) assay (Table 2). Prior to their evaluation in a PCR-based modified TRAP telomerase assay, the compounds were tested for their ability to inhibit *Taq* polymerase, in part in order to ensure that no false positives were obtained in the subsequent TRAP assay. TRAP assays were then performed at concentrations lower than which *Taq* polymerase inhibition was observed, using extracts from the A2780 cell line as a source of telomerase. None of the acridone derivatives showed *Taq* polymerase inhibition at levels <50 μM. The concentrations required to inhibit telomerase activity by 50% ($^{tel}EC_{50}$ values) are reported in Table 2. We have also examined the binding of compound **1** to the intramolecular quadruplex formed from four repeats of the human telomeric DNA sequence d(TTAGGG), using fluorescent resonance energy transfer (FRET) methods to examine the melting behaviour of the complex. Under the conditions used, compound **1** at a 1 μM concentration produces an increase in melting temperature ΔT_m, of 11.0 °C relative to the control. Other substituted acridones from Table 2 gave similar values (data not shown). This ΔT_m value may be compared with that for the analogous 3,6-bis-pyrrolidino acridine derivative (BSU3069), which gave a ΔT_m of 22.7 °C under the same conditions and concentration, and the potent

Table 1. Calculated relative binding energies (ΔE, in kcal mol^{-1}) for interactions of representative disubstituted acridones and the disubstituted acridine 3,6-bis-(pyrrolinido)propionamido-acridine, (BSU6039) with the human G-quadruplex structure

Ligand	G4 DNA + ligand in water box			Ligand in waterbox			ΔE
	vdW	Electrostatic	Total	vdW	Electrostatic	Total	
2	−87.20	−123.19	−203.13	−14.71	−21.17	−34.95	−168.18
3	−81.80	−132.13	−213.94	−11.38	−21.09	−32.49	−181.45
4	−91.02	−114.88	−205.91	−4.46	−8.91	−13.10	−191.81
5	−83.62	−12.63	−94.43	−3.44	−2.19	−5.65	−90.78
BSU6039	−85.06	−195.42	−271.40	−1.42	−4.22	−5.62	−265.78

Table 2. Telomerase inhibition ($^{tel}EC_{50}$) and acute cytotoxicity (IC_{50}) values (both in μM) for regioisomeric acridones

Compound	Isomer	$^{tel}EC_{50}$	A2780	CH1	SKOV3	Substituent
1	3,6	8.1	>25	>25	>25	Pyrrolidine
2	3,6	5.9	19	13	21	Piperidine
3	3,6	4.3	>25	>25	>25	Dimethylamine
4	3,6	5.7	>25	>25	>25	Diethylamine
5	3,6	49	>25	>25	>25	Morpholine
6	3,6	1.7	3	3	5.6	4-Me-piperidine
7	3,6	1.7	>25	>25	>25	4-OH-piperidine
8	3,6	2.3	13	11.8	12	2-Eth-piperidine
9	3,6	2.3	13	11	10.5	2-Me-piperidine
10	3,6	1.9	11.8	11	10.5	N-Eth-piperazine
11	2,7	5.8	>25	>25	>25	Pyrrolidine
12	2,7	1.9	>25	>25	>25	Piperidine
13	2,7	0.6	>25	>25	>25	Dimethylamine
14	2,7	1.9	>25	>25	>25	N-Me-piperazine
15	2,7	1.5	>25	>25	>25	2-Me-piperidine
16	2,7	2.3	>25	>25	>25	4-OH-piperidine
17	2,6	0.2	11.2	25	25	2-CH_2OH-piperidine
18	2,6	0.7	16	25	25	Diethylamine
19	2,6	0.4	5.6	25	25	Piperidine
20	2,6	0.2	>25	25	25	4-Me-piperidine
21	2,6	0.2	3.5	25	25	4-OH-piperidine
Ethidium	—		0.1	0.4	4.5	—
Acridine	3,6	5.8	0.6	2.5	2.3	Diethylamine
Acridine	3,6	5.2	2.7	8.2	2.6	Pyrrolidine
Acridine	3,6	2.8	1.7	2.3	0.5	Piperidine
BRACO-19	3,6,9	0.095	10.0	10.1	13.0	Pyrrolidine

All experiments were performed in triplicate. The disubstituted acridines are described in Ref. 11 and the trisubstituted acridine BRACO-19 in Ref. 12.

3,6,9-trisubstituted acridine compound[12] BRACO-19, which produces a ΔT_m of 29.1 °C at 1 μM.

Almost all the acridone isomers examined show significant in vitro telomerase inhibition. The 2,7- and 3,6-acridone derivatives have $^{tel}EC_{50}$ values between 0.6 and 8.1 μM, with the exception of compound **5**. This contains a morpholino group at the terminus of each substituent side chain, in accord with previous observations,[11] reinforcing the conclusion that strongly cationic terminal substituents on the side chains are required for telomerase potency. The $^{tel}EC_{50}$ values are all comparable to those for the equivalent acridine[11] derivatives. The 2,6 derivatives are overall the most potent acridones, with $^{tel}EC_{50}$ values ranging between 0.2 and 0.7 μM. A general trend of low to moderate levels of acute cytotoxicity (10 to >25 μM) is apparent for the three acridone series, in all three carcinoma cell lines tested. No compounds reported here in the 2,6- or 2,7-series have IC_{50} values less than 25 μM in the cell lines examined. The effects of acridones **1** and **3** on the growth of SKOV-3 cells over a 28-day period are shown in Figure 3a and b. Neither compound has a significant effect on cell growth relative to the controls at either concentration used, contrasting with the responses to BRACO-19 (Fig. 3c). In this instance, cell growth starts to diminish within a few days of administration; after day 14 cells progressively die, and the whole population of cells disappears by day 28 due to apoptosis (data not shown here).

The telomerase activity of these acridones, by itself is not exceptional since a large number of ligands have been reported with comparable levels of potency.[9,10] Table 2 shows there are no significant differences in $^{tel}EC_{50}$ values between the acridones and equivalently

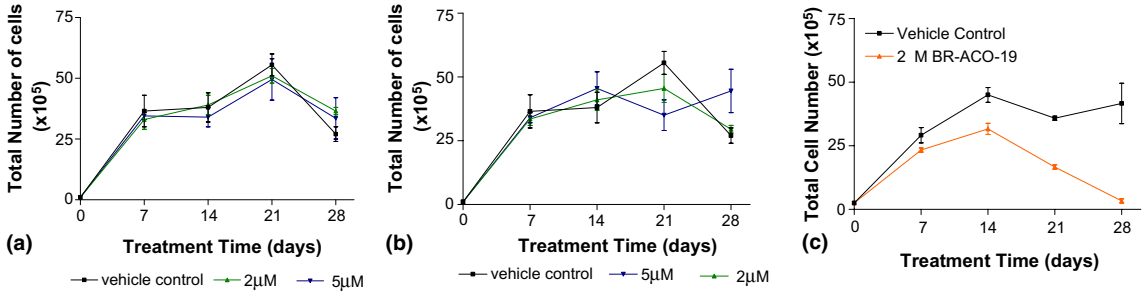

Figure 3. Plots showing the response of growing SKOV-3 cells over a 28-day period to treatment with twice-weekly dosages of (a) acridone compound **1**, (b) acridone compound **3** and (c) acridine BRACO-19. In each case, the effects are shown compared to treatment with a vehicle control (water).

substituted acridines. This is unexpected since (i) the qualitative modelling and the calculated relative binding energies for the acridone–quadruplex complexes predict less affinity than the analogous acridine compounds, and (ii) in accord with this, the limited FRET study reported here suggests that acridones have lower affinity for quadruplexes than acridines. The long-term cell proliferation experiments with the two acridones **1** and **3** show that they do not produce any significant cell growth arrest at concentrations of up to 5 μM after 28 days exposure, in contrast with the potency of the 3,6,9-trisubstituted acridine derivative BRACO-19, which has enhanced telomerase potency and quadruplex affinity[12] compared to the disubstituted acridines. This work, taken with studies in several other laboratories[9,10] have all supported the hypothesis that quadruplex affinity correlates with telomerase inhibitory activity. The anomalous results reported here, suggest that the in vitro telomerase inhibition of the acridones is insufficient by itself to affect cell growth, at least at the concentrations used here, and is by implication, insufficient to affect telomere maintenance in this cell line. We suggest that this latter feature requires a compound to both have telomerase inhibitory potency *and* a level of quadruplex affinity beyond that shown by the acridones. Dissociation (i.e., uncapping) of telomerase from the telomere ends, which is an initial signal of DNA damage to the cell, may thus require a more stable quadruplex complex. We cannot at this stage exclude other mechanisms by which the acridones inhibit telomerase (e.g., interacting with the telomerase RNA domain hTR[17]), or possibly, that the experimental models used here for the intramolecular human quadruplex structure, do not adequately represent the actual folded state of telomeric DNA in this instance. The cellular stability of the acridones is comparable to that of the acridines,[18] indicating that the differences in biological response are not due to any differences in metabolism.

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18. 21NT cells were grown in culture medium with 10 μM of an acridone present. Aliquots of the growth medium were withdrawn at 0, 16, and 64 h, filtered to remove cells (cells were not lysed), and analysed by hplc. After 16 h 92% of the compound remained intact, and after 64 h 64%.