Antitumor Polycyclic Acridines. 8.[1] Synthesis and Telomerase-Inhibitory Activity of Methylated Pentacyclic Acridinium Salts

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Two short routes to novel methylated pentacyclic quinoacridinium salts have been devised. New compounds display telomerase-inhibitory potency (<1 μM) in the TRAP assay. 3,11-Difluoro-6,8,13-trimethyl-8*H*-quino[4,3,2-*kl*]acridinium methosulfate (**12d**, RHPS4, NSC 714187) has a higher selectivity for triplex and quadruplex DNA structures than the 3,6,8,11,13-pentamethyl analogue (**12c**, RHPS3, NSC 714186) and a low overall growth-inhibitory activity in the NCI 60 cell panel (mean GI_{50} 13.18 μM); in addition, the activity profile of **12d** does not COMPARE with agents of the topoisomerase II class. Compound **12d** is soluble in water, stable in the pH range of 5−9, efficiently transported into tumor cells, and is currently the lead structure for further elaboration in this new class of telomerase inhibitor.

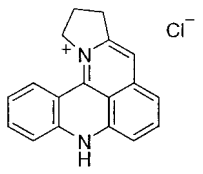
Introduction

Activation of the enzyme telomerase (hTERT), a reverse transcriptase that is responsible for annealing hexanucleotide telomeric repeats (TTAGGG) to the ends (telomeres) of chromosomes, has been shown to represent a vital step in human epithelial tumor evolution.[2,3] Normal cells, with the exception of hematopoetic T and B cells,[4] do not express telomerase, whereas in ∼85% of human cancers telomerase is activated and functions to maintain telomere integrity, usually at relatively short lengths in the region of 3−8 kB.[5] Target validation experiments employing dominant negative constructs of hTERT transfected into cells have shown that telomerase inhibition is paralleled by inhibition of cellular growth and abrogation of tumorigenicity in nude mice; significantly, the timing of these effects is related to initial cellular telomere length.[6,7] Thus, telomerase has emerged as a promising molecular target in the search for more selective antitumor agents than hithertofore available.[8,9]

Telomerase has a multicompartmental structure comprising an RNA template, a catalytic protein domain (hTERT), and various chaperoning and regulating proteins.[10] In addition to its enzymatic activity, occupation (capping) of telomeres by telomerase and other proteins may protect eroding chromosome ends from activating DNA damage response mechanisms.[11] Chemotherapeutic strategies employing antisense molecules (oligonucleotides, ribozymes, PNAs) targeted to the RNA template and reverse transcriptase inhibitors of hTERT (e.g., AZT) have not led to major breakthroughs. There is a burgeoning interest in small molecules that stabilize the G-rich single-stranded telomeric overhang in a G-quadruplex DNA polymorphic form; these agents have been shown also to inhibit telomerase, generally at the micromolar level. Drugs eliciting this effect include inter alia: disubstituted anthraquinones,[12] fluorenones,[13] and acridines[14] and heteroaromatic tetracyclic[15] and polycyclic systems,[16] including porphyrin derivatives.[17] More potent G-quadruplex stabilizing telomerase inhibitors built on ethidium derivatives,[18] quinolines,[19] and trisubstituted acridine frameworks,[20] with nanomolar IC_{50} values in the TRAP assay, have been described recently.

In the preceding part in this series,[1] we described the synthesis and topoisomerase II-inhibitory properties of tetra- and pentacyclic compounds analogous in structure to some marine natural products. The most potent agent, the indolizino[7,6,5-*kl*]acridinium salt (**1**) forms a transitory intercalated "hot spot" within 5′-CpG sequences in duplex DNA as adduced by ^{1}H NMR studies.[21] This close molecular encounter is sufficiently tangible to be sensed by the DNA damage recognition machinery of the cell and to provoke apoptotic cell death.[22]

We now report the synthesis of new compounds of related structure with potent telomerase-inhibitory properties. Our priorities have been to develop novel agents with three qualities: accessibility by simple synthetic pathways; potent telomerase-inhibitory activity through selective binding to higher ordered DNA structures (e.g., quadruplex); and possession of robust pharmaceutical properties including chemical and metabolic stability.



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Chemistry

The starting point for the preparation of the quinoacridinium salt (**6**) without peripheral substituents was the

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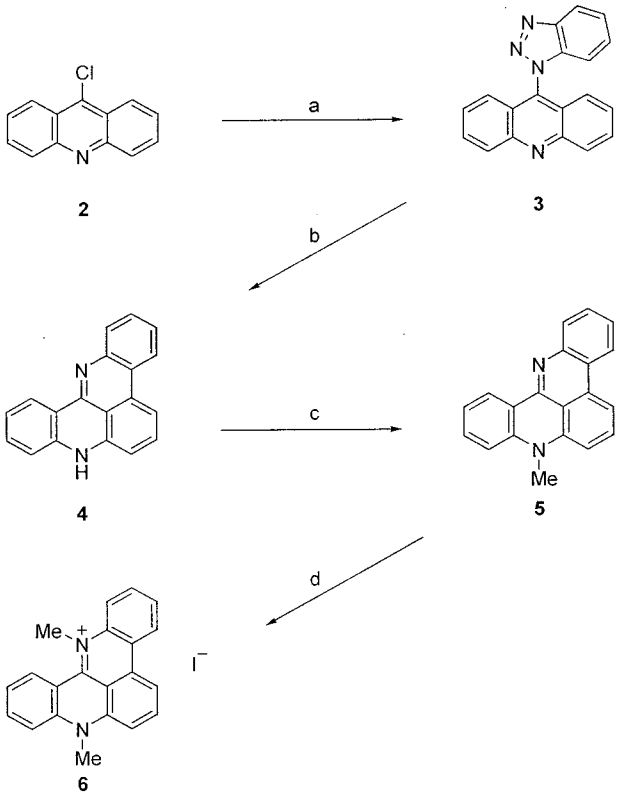
Scheme 1[a]



[a] Reagents: (a) NaH, 1*H*-1,2,3-benzotriazole, DMF, 140 °C; (b) 259 °C in diphenyl ether; (c) NaH, dimethyl sulfate, DMF, 25 °C; (d) MeI.

interaction of 9-chloroacridine (**2**) and the anion of 1,2,3-benzotriazole, which is a more efficient route (92%) to the 9-(1,2,3-benzotriazol-1-yl)acridine (**3**) than previously devised methods.[23,24] The benzotriazole was then thermolyzed at 269 °C (Graebe−Ullmann reaction) to the pentacycle (**4**).[24] Methylation of this base with NaH/dimethyl sulfate afforded the 8-methyl-quinoacridine (**5**), which, on further treatment with methyl iodide, yielded the required 8,13-dimethyl-quinoacridinium iodide (**6**) (Scheme 1).

Two further examples of this novel type of quinoacridinium salt (**12a**,**b**) have been described by Polish chemists from a "one-pot" reaction of *N*-ethyl-2-methylquinolinium iodides (**7a**,**b**) in ethanolic piperidine.[25,26] We have exploited this extraordinary reaction to prepare the salts (**12c**−**f**) from the quinolinium methosulfates (**7c**−**f**). A proposed mechanism (Scheme 2) requires the conversion of the 2-methyl group of the substrate (**7**) to the carbanion (**8**) by the base piperidine; the carbanion could then attack the electron deficient C-2 of another molecule of quinolinium salt (**7**). Opening of the dihydroquinoline ring of the adduct (**9**) would generate an unsaturated system (**10**) templated for an electrocyclization to the tetrahydro-quinoacridine (**11**). Oxidation, presumably under the reaction conditions, would generate the observed salts (**12**).

We have not been able to reproduce the high yields claimed for the iodides (**12a**, 63%[25] and **12b**, 78.5%)[26] in our syntheses of the analogues. Choice of the quinolinium methosulfate salts, rather than iodides, as starting materials was dictated by the efficiency of synthetic routes to the precursors (**7c**−**f**); yields of pentacyclic acridinium salts (**12c**−**f**) were generally <25% (unoptimized). The mechanism proposed (Scheme 2) requires oxidation of a tetrahydro-acridinium intermediate (**11**). As no improvement in yield was obtained by bubbling air through the reaction mixture, it is unlikely that molecular oxygen provides the oxidizing equivalents; similarly, yields in our hands were not enhanced by incorporating various oxidants (I_2, chloranil, $FeCl_3$, MnO_2, S or Se, and nitrobenzene) in the reaction mixture. Therefore, it seems likely that intermediates in the reaction pathway must act as oxidants and this would significantly reduce the experimental yield of products (**12**). Use of tertiary bases (triethylamine, Hunig's base, 4-methylmorpholine) or basic alumina as a replacement for piperidine gave evidence of some pentacycle formation (thin-layer chromatography) but did not lead to improved yields. We concur with the views of the discoverers of this intriguing reaction[25] who indicated that the mechanism "needs clarification".

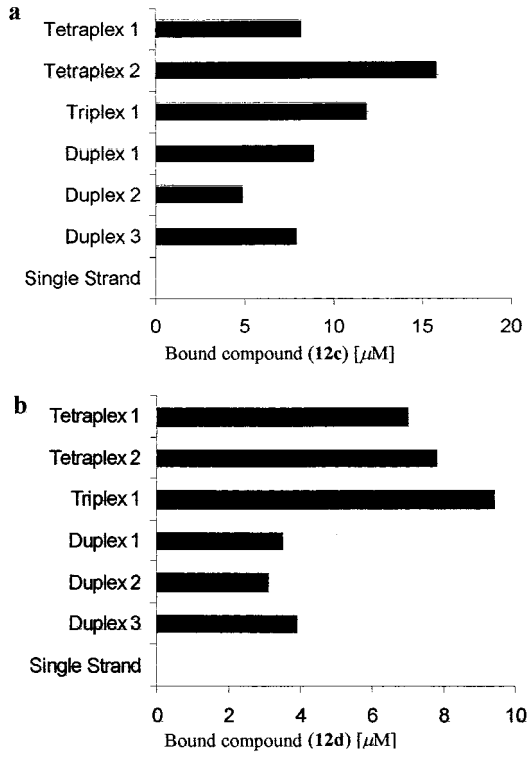
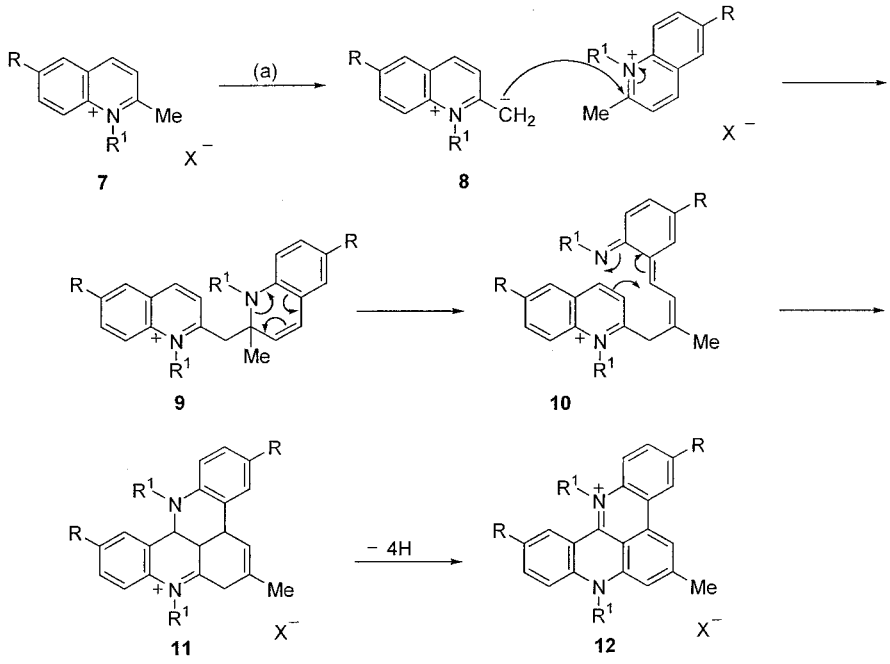


Figure 1. Relative affinities of polycyclic acridinium salts for different DNA structures measured by differential dialysis:[27] a, compound **12c**; b, compound **12d**. Tetraplex 1, $[T_2G_{20}T_2]_4$; tetraplex 2, $[AG_3(T_2AG_3)_3]$; triplex 1, poly[dT]*poly[dA].poly-[dT]; duplex 1, poly[dA-dT]$_2$; duplex 2, poly[dG].poly[dC]; duplex 3, salmon testis DNA; single strand, poly[dT]. Note the different scales for drug concentration.

Biophysical Studies

The structure of the original Polish salt (**12a**) has been confirmed by an X-ray determination,[25] and the ^1H, ^{13}C, and ^{15}N NMR spectra of **12a**,**b** have been analyzed.[26] We have used the differential dialysis technique developed by Ren and Chaires[27] to probe the relative affinities of polycyclic acridinium ligands (**12c**,**d**) for different DNA structures (Figure 1). These compounds were selected because they are, respectively, the most and least potent at inhibiting cell growth in a 60 cell panel of human tumor cells as measured by mean GI_{50} values (see later). The results show that both

Scheme 2[a]



	R	R^1	X
a:	H	Et	I
b:	Me	Et	I
c:	Me	Me	$MeOSO_3$ (RHPS3)
d:	F	Me	$MeOSO_3$ (RHPS4)
e:	H	Me	$MeOSO_3$
f:	Cl	Me	$MeOSO_3$

[a] Reagents: (a) Piperidine in EtOH, reflux.

compounds bind to a range of quadruplex, triplex, and duplex structures but point to subtle differences in selectivity. Compound **12c** is a better ligand overall for all of the DNA structures but binds approximately equieffectively to duplex, triplex, and quadruplex DNA. The difluoro compound (**12d**) binds more selectively to higher ordered structures, i.e., triplex and quadruplex DNA structures. These patterns have been confirmed by DNA melting studies; **12c** increased the T_m of salmon testis DNA by 16.6 °C (only 10.8 °C for **12d**) confirming that **12c** is the better duplex ligand (data not shown). Overall, these results imply that the two compounds, **12c**,**d**, although closely related structurally, might have different biological fingerprints.

Biological Results and Discussion

In Vitro Activities of Pentacyclic Acridinium Salts Against Human Cell Lines. Although the polycyclic acridinium salt (**12a**) has been evaluated in the NCI 60 cancer cell screen in vitro (NSC 629622),[28] there have been no further biological studies reported, apparently. As the NCI screen involves only a 2 day exposure to drugs,[29] it is unlikely that activity fingerprints reflect telomerase inhibition within the cells. The mean GI_{50} values of examples of pentacyclic acridinium salts in the cell panel are shown in Table 1. We have reported previously on the activity of the indolizino-[7,6,5-*kl*]acridinium salt (**1**);[1] this compound was the most growth-inhibitory overall of the series (GI_{50} 0.09 μM) and ~10-fold more potent than compound (**12a**). Also noted in our work on **1** was its selectivity toward cells of melanoma origin at the LC_{50} level,[1] a feature also shown by **12a** (data not shown). Compounds **12c** and **12d** differ only in the electronic nature of substituents in the 3 and 11 positions: pentacycle (**12c**), with two electron-donating methyl groups, was much more growth-inhibitory overall (GI_{50} 0.40 μM) than **12d**, with electron-withdrawing fluoro groups (GI_{50} 13.18 μM). The enhanced activity of **12c** may be attributed to its promiscuous interactions with all DNA polymorphic structures as reported in the biophysical studies (above).

COMPARE Analysis. Discrete differences in mechanisms of action within this series of compounds are revealed by a COMPARE analysis[30] of cell line activities of four of the pentacycles with GI_{50} profiles of clinical agents of "known" mechanistic class; in this analysis, a Pearson correlation coefficient (PCC) > 0.60 is considered significant. The two most growth-inhibitory agents (**1** and **12c**) share high PCC correlations with DNA interactive agents, particularly topoisomerase II inhibitors; contrastingly, pentacyclic salts (**12a**,**d**) show no PCCs > 0.60 to topoisomerase II inhibitors. The presence of phyllanthoside with a PCC > 0.60 in the COMPARE analyses of the salts (**12a**,**c**,**d**) implies that these agents may be related biomechanistically. Also, molecular targets COMPARE analysis[30] indicates that **12c**, but not **12d**, is a substrate for P-glycoprotein-mediated drug efflux.

Telomerase-Inhibitory Activities of Polycyclic Acridinium Salts in the TRAP Assay. As the poly-

Table 1. Biological Properties of Pentacyclic Acridinium Salts

cmpd	mean GI_{50} (μM)[a]	telomerase IC_{50} (μM)[b]	SI[c]	COMPARE[d] chemical name	COMPARE[d] PCC[e]
1	0.09	NT[f]		*N*,*N*-dibenzyldaunomycin	0.70
				amonafide	0.645
				3-deazauridine	0.63
				MX2·HCl	0.625
				daunomycin	0.62
6	NT	0.38			
12a	1.1	2.0	0.55	phyllanthoside	0.61
				paclitaxel	0.61
12c (RHPS3)	0.41	0.25	1.8	MDR RHOD30	0.81
				phyllanthoside	0.78
				actinomycin D	0.70
				bactobolin	0.68
				bisantrene	0.68
				chromomycin	0.65
12d (RHPS4)	13.18	0.33	40	phyllanthoside	0.63
12e	1.29	0.76	1.7		
12f	4.17	0.25	17		

[a] In the NCI 60 cell panel (ref 28). [b] TRAP assay (ref 13). [c] Selectivity index: mean GI_{50}/telomerase IC_{50}. [d] Refs 27−29. [e] All PCC values >0.6 included. [f] Not tested.

merase chain reaction (PCR)-based TRAP assay relies on the use of Taq polymerase,[13] compounds were originally assayed as inhibitors of the polymerase. Of the compounds evaluated, only the indolizino[7,6,5-*kl*]acridinium salt (**1**) inhibited Taq polymerase (at 10 μM) and so was excluded from evaluation in the TRAP assay. The activities of polycyclic acridinium salts as telomerase inhibitors are shown in Table 1.

The salt (**12a**) is the least potent (IC_{50} 2 μM) possibly because of the greater deviations from coplanarity elicited by the bulky *N*-ethyl residues.[25] The five analogues (**6** and **12c**−**f**) are approximately equiactive as telomerase inhibitors (IC_{50} < 1 μM). We have shown recently that compound **12d** elicits a marked reduction in cell growth after 15 days in two cell lines (breast 21NT and vulval A431) possessing relatively short telomeres. Furthermore, the agent induced a reduction in cellular telomerase activity and a lower expression of the hTERT gene.[31]

The selectivity index (SI: mean $GI_{50}/IC_{50telomerase}$) of the acridinium salt (**12a**) is 0.55, whereas the 3,11-dimethyl analogue (**12c**) has an SI of 1.8. Significantly, the 3,11-difluoro-quinoacridinium salt (**12d**) has the most favorable selectivity profile (SI 40) of all polycyclic acridinium salts reported here. Accordingly, **12d** was selected for preliminary pharmaceutical evaluation.

Pharmaceutical Properties of 3,11-Difluoro-6,8,-13-trimethyl-8*H*-quino-[4,3,2-*kl*]acridinium Methosulfate (12d). Compound (**12d**) is a quaternary salt with no possibilities for protonation or deprotonation within a physiologically relevant pH range. The compound is soluble in water at >5 mg/mL, and when solutions of 0.025 mg/mL were incubated at 37 °C in buffers ranging from pH 5.0 to pH 9.0, there were no changes in the UV/visible spectrum of the compound or absorbance changes at the long wavelength band (484 nm) over 21 days (data not shown).

Compound **12d** is polar with a log P (octanol/water) of −0.80. Contrary to expectation, the salt readily traffics into human mammary MCF-7 and nonsmall cell lung A549 cell lines in vitro. Presumably, the hydrophobic periphery of the molecule "blankets" the cationic charge, which is delocalized within the core of the molecule. Uptake of the amphiphilic and fluorescent

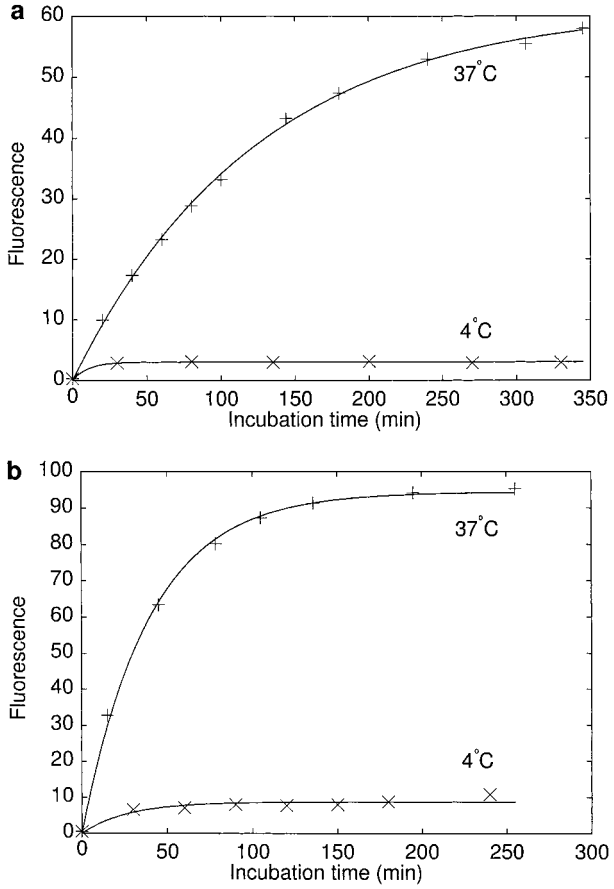


Figure 2. Uptake of **12d** (1 μM) at 4 and 37 °C measured by flow cytometry (see Experimental Section): a, MCF-7 cells; b, A549 cells.

drug (excitation wavelength 488 nm; emission wavelength 575 nm) was monitored by flow cytometry (Figure 2); at 37 °C, internalization of drug in both cell lines was saturated at ∼6 h, and at 4 °C, uptake was very slow. Drug accumulation in cells fits a simple passive transport model. Localization of **12d** (30 μM) within the cytoplasm, nuclear membrane, and nucleoli of MCF-7 (Figure 3a) was evident at 45 min as determined by confocal microscopy. Uptake into A549 cells was faster (Figure 2a,b); after 15 min, a pattern of

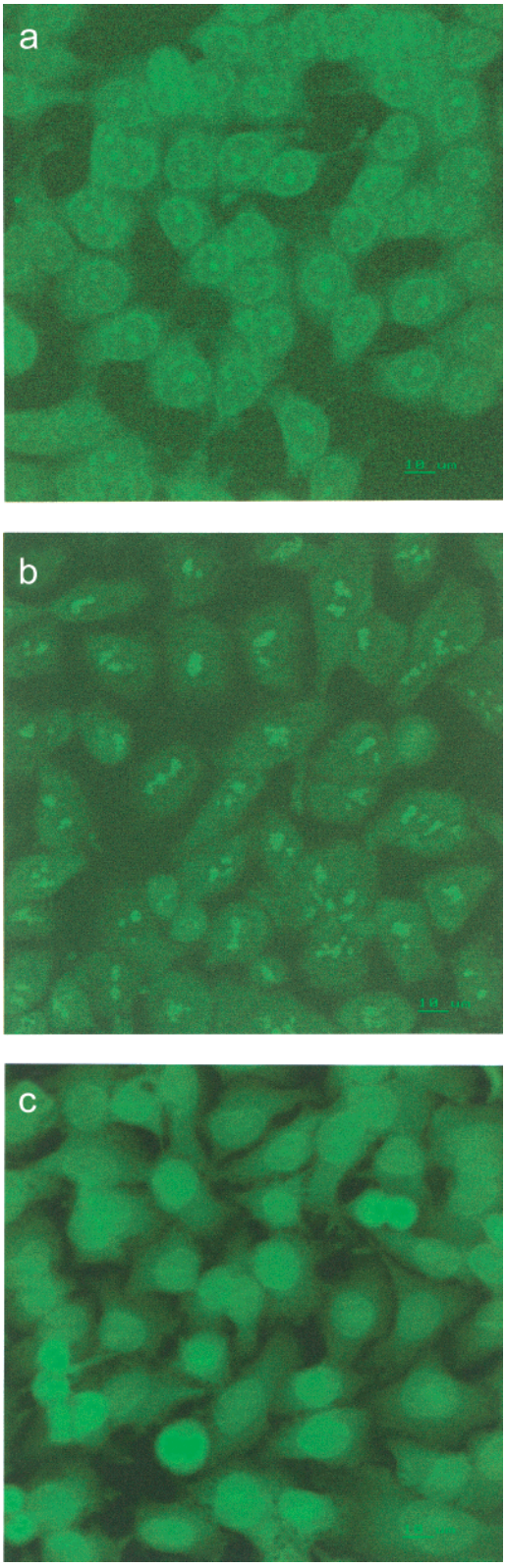


Figure 3. Distribution of **12d** (30 μM) into cell nuclei: a, MCF-7 cells after 45 min; b, A549 cells after 15 min; c, A549 cells after 45 min.

distribution in the cytoplasm, nucleus, and nucleoli (Figure 3b) was evident, and after 45 min (Figure 3c), a uniform distribution in the nucleus was observed. Compound **12a** is similarly transported rapidly into MCF-7 cells and distributes within the cytoplasm and nucleus (data not shown).

Stability studies (by high-performance liquid chromatography, HPLC) on **12d** in culture media harboring MCF-7, A549, and colon HT-29 cells over 7 days show no evidence of degradation or metabolism, another potentially favorable pharmaceutical quality of the drug.

Conclusion

In this paper, we have shown that novel pentacyclic quinoacridinium salts can be synthesized by two short synthetic routes. Compounds **12c** (RHPS3, NSC 714186) and **12d** (RHPS4, NSC 714187) are among the most potent telomerase inhibitors yet disclosed, with IC_{50} values < 0.5 μM. Although of comparable potency as telomerase inhibitors, **12c** is 30-fold more growth-inhibitory than **12d** in the NCI 60 cell panel. This may reflect the fact that **12c**, with electron-donating methyl groups in the 3 and 11 positions, is a promiscuous binder for all DNA structures (duplex, triplex, and quadruplex), whereas **12d**, with electron-withdrawing fluoro groups, binds more selectively to higher ordered triplex and quadruplex architectures.

Pentacycle **12d** is a stable water-soluble compound, which is readily taken up into cells where it locates within the nucleus; also, it is metabolically robust in the presence of tumor cells in vitro. The lead structure has that desirable conjunction of properties that we sought at the outset: synthetic accessibility, potent and selective telomerase-inhibitory activity, and an exploitable array of pharmaceutical properties.

We have constructed a molecular model for the stabilization of G-quadruplex DNA structures by quinoacridinium salts.[32] Information from this model, which has been corroborated by ^{1}H NMR studies,[33] is now guiding rational lead optimization work in this new series of compounds. In particular, we anticipate that selection of appropriate substituents attached around the aromatic periphery of the pentacyclic framework will reveal compounds with enhanced selectivity for G-quadruplex binding and hence potency as telomerase inhibitors but without compromising pharmaceutical priorities.

Experimental Section

Synthetic Chemistry. 9-(1*H*-1,2,3-Benzotriazol-1-yl)-acridine (3). 1*H*-1,2,3-Benzotriazole (1.90 g, 16 mmol) was added to a mixture of sodium hydride (60% dispersion in mineral oil, 18 mmol) and dry dimethylformamide (DMF, 60 mL). 9-Chloroacridine (**2**, 3.43 g, 16 mmol) was added, and the mixture was heated to 100 °C for 2 h. Addition of excess water gave a precipitate of the benzotriazolylacridine (92%), identical (mp, IR, ^{1}H NMR, and ^{13}C NMR) to an authentic sample.[24]

8-Methyl-8*H*-quino[4,3,2-*kl*]acridine (5). Sodium hydride (1.34 g, 0.056 mol) was suspended in DMF (30 mL) under nitrogen. A sample of 8*H*-quino[4,3,2-*kl*]acridine (**4**, 5.0 g, 0.019 mol) (obtained by thermolysis of **3** at 269 °C)[24] in DMF (50 mL) was added, and the reaction mixture was stirred at 25 °C for 60 min. Dimethyl sulfate (3.53 g, 0.028 mol) was added, and the reaction mixture was stirred for a further 60 min and then quenched with water (100 mL). The product was collected, dried, and crystallized from ethanol to give a mustard colored solid (4.9 g, 93%); mp 208−210 °C (from ethyl acetate). UV (EtOH) λ_{max}, nm: 205, 237, 287, 421, 436. IR (cm^{-1}): 1580, 1557, 1491, 1458, 1359, 745. ^{1}H NMR (DMSO-d_6): δ 8.78 (1H, dd, J = 1.5, 7 Hz, H-12), 8.54 (1H, dd, J = 1.5, 8 Hz, H-4), 8.13 (1H, d, J = 8 Hz, H-5), 7.91 (1H, dd, J = 1, 8 Hz, H-1), 7.86 (1H, m, H-6), 7.63 (3H, m, H-2,9,10), 7.50 (1H, m, H-3), 7.38 (1H, d, J = 8 Hz, H-7), 7.28 (1H, dt, J = 1.5, 7 Hz, H-11), 3.69 (3H, s, CH_3). ^{13}C NMR (DMSO-d_6): δ 149.3 (C), 145.2 (C),

141.5 (C), 141.4 (C), 134.1 (C), 132.5 (CH), 132.3 (CH), 129.45 (CH), 128.8 (CH), 125.3 (CH), 125.2 (CH), 123.3 (CH), 122.9 (C), 121.5 (CH), 121.4 (C), 115.9 (C), 115.15 (CH), 111.6 (CH), 33.6 (CH_3). HRMS−FAB (*m*/*z*): calcd for $C_{20}H_{14}N_2$, 283.1235; found, 283.1212. Anal. Calcd for $C_{20}H_{14}N_2$: C, H, N.

8,13-Dimethyl-8*H*-quino[4,3,2-*kl*]acridinium Iodide (6). 8-Methyl-8*H*-quino[4,3,2-*kl*]acridine (1 g, 3.5 mmol) was dissolved in methyl iodide (20 mL) and heated in a sealed tube for 72 h. The precipitate was collected, washed with ether (20 mL), and crystallized from methanol to give red crystals (1.1 g, 73%); mp 213-214 °C. IR (cm^{-1}): 1613, 1578, 1530, 1248, 748. ^{1}H NMR (DMSO-d_6): δ 8.75 (1H, d, J = 7.5 Hz), 8.51 (2H, m), 8.35 (1H, t, J = 8.5 Hz), 8.13 (4H, m), 7.94 (1H, t, J = 7 Hz), 7.77 (1H, t, J = 7.5 Hz), 7.63 (1H, t, J = 8 Hz), 4.40 (3H, s, CH_3), 4.18 (3H, s, CH_3). EIMS (*m*/*z*): M^+ 297.4. Anal. Calcd for $C_{21}H_{17}N_2I$: C, H, N.

3,6,8,11,13-Pentamethyl-8*H*-quino[4,3,2-*kl*]acridinium Methosulfate (12c, RHPS3). 1,2,6-Trimethylquinolinium methosulfate (**7c**, 2.0 g) and piperidine (1 mol equiv) were heated under reflux in ethanol (20 mL) for 5 days. Solvent was removed by vacuum evaporation, and the residue was crystallized from acetone to yield the methosulfate (14%); mp 240−242 °C. UV (EtOH) λ_{max}, nm: 257, 294, 325, 485, 514. IR (cm^{-1}): 1616, 1576, 1534, 1250, 1227, 1009, 729. ^{1}H NMR (DMSO-d_6): 8.53 (1H, s), 8.34 (1H, s), 8.23 (1H, s), 8.03 (1H, d, J = 9 Hz), 8.0 (1H, d, J = 9 Hz), 7.9 (2H, m), 7.73 (1H, dd, J = 2, 8 Hz), 4.35 (3H, s, CH_3), 4.11 (3H, s, CH_3), 3.39 (3H, s, CH_3), 2.76 (3H, s, CH_3), 2.58 (3H, s, CH_3), 2.56 (3H, s, CH_3). ESMS (*m*/*z*): M^+ 339.3. Anal. Calcd for $C_{25}H_{26}N_2O_4S$: C, H, N.

3,11-Difluoro-6,8,13-trimethyl-8*H*-quino[4,3,2-*kl*]acridinium Methosulfate (12d, RHPS4). 1,2-Dimethyl-6-fluoroquinolinium methosulfate (**7d**, 26.4 g) and piperidine (9.1 g) were heated under reflux in ethanol (1.2 L) for 5 days. Solvent was removed by vacuum evaporation, and the residue was crystallized from acetone to yield the methosulfate (11.5%); mp 256−258 °C. UV (EtOH) λ_{max}, nm: 258, 294, 325, 485, 514. IR (cm^{-1}): 1620, 1584, 1532, 1252, 1225, 1013, 721. ^{1}H NMR (DMSO-d_6): 8.60 (1H, dd, J = 3, 9 Hz), 8.42 (1H, s), 8.29 (1H, dd, J = 3, 8.5 Hz), 8.23 (1H, t, J = 5 Hz), 8.12 (1H, q, J = 5 Hz), 8.0 (1H, dt, J = 9 Hz), 7.95 (1H, s), 7.85 (1H, dt, J = 9 Hz), 4.37 (3H, s, CH_3), 4.17 (3H, s, CH_3), 3.77 (3H, s, CH_3), 3.38 (3H, s, CH_3). HRMS−FAB (*m*/*z*): calcd for $C_{23}H_{20}F_2N_2O_4S$, 347.1360; found, 347.1388.

6,8,13-Trimethyl-8*H*-quino[4,3,2-*kl*]acridinium Methosulfate (12e). Prepared similarly from 1,2-dimethylquinolinium methosulfate (**7e**) and piperidine in ethanol, the acridinium methosulfate (20%) had mp 190−192 °C. UV (EtOH) λ_{max}, nm: 257, 294, 325, 485, 514. IR (cm^{-1}): 1611, 1530, 1466, 1250, 1245, 1009, 760. ^{1}H NMR (DMSO-d_6): 8.60 (1H, d, J = 8 Hz), 8.39 (1H, d, J = 9 Hz), 8.24 (1H, s), 8.08−7.98 (3H, m), 7.87 (1H, t, J = 8 Hz), 7.80 (1H, s), 7.70 (1H, t, J = 7 Hz), 7.60 (1H, dt, J = 1, 8), 4.36 (3H, s, CH_3), 4.15 (3H, s, CH_3), 3.31 (3H, s, CH_3), 2.83 (3H, s, CH_3). EIMS (*m*/*z*): calcd for $C_{23}H_{22}N_2O_4S$, 311.1; found, 311.1.

3,11-Dichloro-6,8,13-trimethyl-8*H*-quino[4,3,2-*kl*]acridinium Methosulfate (12f). Prepared from **7f** and piperidine in ethanol (as above), the dichloro-acridinium methosulfate (36%) had mp 240−242 °C. UV (EtOH) λ_{max}, nm: 257, 294, 325, 485, 514. IR (cm^{-1}): 1610, 1595, 1520, 1220, 1210, 1000, 750. ^{1}H NMR (DMSO-d_6): 8.87 (1H, d, J = 2 Hz), 8.55−8.52 (2H, m), 8.24−8.09 (3H, m), 8.00−7.96 (2H, m), 4.37 (3H, s, CH_3), 4.18 (3H, s, CH_3), 3.38 (3H, s, CH_3), 2.78 (3H, s, CH_3). EIMS (*m*/*z*): 379.5, 381.5, 383.6. Anal. Calcd for $C_{23}H_{20}Cl_2N_2O_4S$: C, H, N.

Biophysical Studies. The DNA from salmon testes (ST-DNA: highly polymerized sodium salt; 58.8% A−T and 41.2% G−C), single-stranded poly[dT], double-stranded alternating purine−pyrimidine poly[dA−dT]·poly[dA−dT] and poly[dG−dC]·poly[dG−dC] and nonalternating poly[dA]·poly[dT] and poly[dG]·poly[dC] were purchased from Sigma Chemical Co., Dorset, England and used without further purification. Tetraplex DNA 5′-$T_2G_{20}T_2$ and 5′-$AG_3(T_2AG_3)_3$ were purchased HPLC-purified from Sigma-Genosys Ltd., Cambridge, England. A nondenaturing polyacrylamide gel electrophoresis gel confirmed the existence of single DNA species.

Differential dialysis was conducted according to the procedure of Ren and Chaires[27] with the following adaptations. A concentration of 2 μM of compounds RHPS3 and RHPS4 was used. Dialysis was carried out for 48 h at 4 °C. The extinction coefficients for RHPS3 were ϵ_{512} = 11 500 M^{-1} cm^{-1} and for RHPS4 ϵ_{512} = 10 300 M^{-1} cm^{-1}, respectively, determined in 6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM EDTA, 185 mM NaCl, and 1% sodium dodecyl sulfate.

Biological Studies. In Vitro Screening. In vitro screening of quinoacridinium salts against the NCI 60 human tumor cell panel[29] and COMPARE analyses[30] were conducted by published methods.

***Taq* Polymerase Assay.** The method for the evaluation of compounds in the *Taq* polymerase and TRAP telomerase assays followed the literature procedure of Perry et al.[13,31] Briefly, for assessment of nonspecific inhibition of *Taq* polymerase, concentrations of drugs at 1, 5, 10, and 20 μM were added to a PCR 50 μL master mix containing 10 ng of pCl-neo mammalian expression vector and forward (GGAGTTCCGCGTTACATAAC) and reverse (GTCTGCTCGAAGCATTAACC) primers (200 nM). The product of approximately 1 kb was visualized using ethidium bromide after separation by electrophoresis on a 2% w/v agarose gel following amplification (30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2.5 min) using a MBS thermal cycler (Hybaid, U.K.).

TRAP Telomerase Assay. For the TRAP assay, telomerase was prepared from extracts of exponentially growing A2780 cells by lysing for 30 min on ice in a CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate)-based buffer [0.5% w/w CHAPS, 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 5 mM 2-mercaptoethanol, 10% v/v glycerol]with 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride freshly added. The lysate was then centrifuged at 12 000 rpm for 30 min at 4 °C, and the supernatant was collected and stored frozen in aliquots at −80 °C for up to 3 months. Total cellular protein was then determined, and the TRAP assay was performed in two steps using 40 ng protein.

Step 1: Telomerase-mediated extension of a nontelomeric oligonucleotide forward TS primer (5′-AATCCGTCGAGCAGAGTT) (0.1 μg) contained in a 40 μL reaction mix comprising TRAP buffer (20 mM Tris-HCl, pH 8.3, 68 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 0.05% Tween 20), 0.05 μg of bovine serum albumin, 50 μM of each deoxynucleotide triphosphate, and 3 μCi of [α-^{32}P]dCTP (Amersham, U.K.). Protein was then incubated with the reaction mix, with or without test compounds at various final concentrations, for 20 min at 25 °C. A lysis buffer (no protein) control, heat-inactivated (85 °C for 10 min) protein control, and 25% protein control (10 ng) were included in each experiment.

Step 2: Samples were heated at 85 °C for 5 min to inactivate telomerase, and during this time, 0.1 μg of reverse CX primer (3′-AATCCCATTCCCATTCCC-ATTCCC) and 2 units of *Taq* polymerase ("red hot", Advanced Biotechnologies) were added. A 3 step PCR was then performed: 31 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min. Telomerase-extended PCR products, with or without test compounds, were then measured either by electrophoretic separation (8% w/w acrylamide denaturing gels, Sequagel, National Diagnostics) and visualization by phosphoimaging or by harvesting on Whatman filters (25 mm) by TCA precipitation and then analysis by liquid scintillation counting.

Pharmaceutical Studies. Stability Studies. Buffers used to assess the stability of **12d** were the following: pH 4.97, 0.2 M NaOAc (49 mL) and 0.2 M AcOH (21 mL); pH 6.01, 0.2 M KH_2PO_4 (50 mL) and 0.2 M NaOH (5.6 mL) to 100 mL with distilled water; pH 7.06, 0.2 M KH_2PO_4 (50 mL) and 0.2 M NaOH (29.1 mL) to 100 mL with distilled water; pH 7.96, 0.2 M KH_2PO_4 (50 mL) and 0.2 M NaOH (46.1 mL) to 100 mL with distilled water; pH 9.00, 0.2 M glycine (25 mL) and 0.2 M NaOH (4.4 mL) to 100 mL with distilled water.

Solutions of **12d** (0.025 mg/mL) were incubated at 37 °C in buffers for up to 21 days. The full UV/visible spectrum of the

compound and absorbance changes at the long wavelength band (484 nm) were monitored.

log P Determination. The log P (octanol/water) of **12d** was determined according to the Shake Flask Method.[34]

Drug Uptake Studies. Cells (MCF-7 or A549) were grown to approximately 80% confluence, harvested with trypsin, collected in tissue culture medium (RPMI 1640 supplemented with 10% fetal bovine serum), centrifuged at 1100 rpm for 5 min, and resuspended in serum free medium (RPMI 1640). Aliquots of 1 mL in 12 mm × 77 mm tubes were equilibrated on ice (4 °C) or at 37 °C for 20 min. Compound **12d** was added to a final concentration of 1 μM and incubated at 4 or 37 °C for periods ranging from 30 min to 6 h. Drug uptake was analyzed by flow cytometry (Coulter Epics XL MCL flow cytometer) for fluorescence emission at 575 nm (excitation wavelength, 488 nm). An average value for 5000 cells was recorded, and fluorescence increase was interpreted as drug uptake. Cells incubated at the same temperatures and for the same times in the absence of **12d** showed no increase in fluorescence emission.

Confocal Microscopy. Cells (MCF-7 and A549) were grown on cover slips overnight. **12d** was added to a final concentration of 30 μM, and treated cells were incubated at 37 °C for 30 min (5% CO_2 incubator). The coverslips were rinsed in PBS, and cells were fixed in ice-cold MeOH for 5 min. Cells were again rinsed with PBS and mounted onto microscope slides using glycerol−PBS and stored in the dark before being viewed on a Leica TDS 4D confocal microscope. Under blue light, **12d** emitted a green fluorescence.

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