Antitumor Polycyclic Acridines. 7.[1] Synthesis and Biological Properties of DNA Affinic Tetra- and Pentacyclic Acridines

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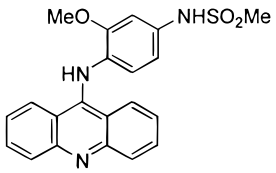
New synthetic routes to a series of tetra- and pentacyclic acridines related in structure to marine natural products are reported. The novel water-soluble agent dihydroindolizino[7,6,5-*kl*]-acridinium chloride **14** has inhibitory activity in a panel of non-small-cell lung and breast tumor cell lines exceeding that of *m*-AMSA. The salt inhibited the release of minicircle products of kDNA confirming that disorganization of topoisomerase II partly underlies the activity of the compound. COMPARE analysis of the NCI mean graph profile of compound **14** at the GI_{50} level corroborates this conclusion with Pearson correlation coefficients (>0.6) to clinical agents of the topoisomerase II class: however, this correlation was not seen at the LC_{50} level. The inhibitory action of **14** on *Saccharomyces cerevisiae* transfected with human topoisomerase II isoforms showed a 3-fold selectivity against the IIα isoform over the IIβ isoform. Unlike *m*-AMSA, **14** is not susceptible to P-glycoprotein-mediated drug efflux and retains activity in lung cells with derived resistance to the topoisomerase II inhibitor etoposide.

Introduction

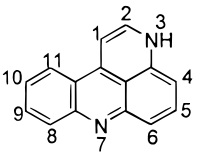
The ease of synthesis, attractive coloration, and crystallinity of acridine derivatives has long attracted the attention of medicinal chemists.[2] The basic tricyclic framework can be decorated with appropriate substituents to confer specificity against both prokaryotic and eukaryotic targets which have given acridines a respectable reputation in the history of chemotherapy in the 20th century.[3] In recent decades the focus of research into the role of acridines in cancer treatment has centered in Oceania where New Zealand scientists have developed a range of derivatives, such as the prototypic *m*-AMSA (**1**) (Chart 1), and variants, which are now known to target the enzyme topoisomerase II (topo II).[4−6] Some of the most familiar drugs in the cancer chemotherapeutic armamentarium are thought to act, in part, by disorganizing the functions of this protein:[7] however, common physical properties of basicity and partial planarity give these agents avid DNA-binding properties which may interplay at the single-strand, duplex, or triplex levels. Thus, their true mode of action as antitumor agents is difficult to define unequivocally.

To be considered for clinical evaluation, newly designed agents of this class would need to demonstrate unique biological properties, possibly by modulating specific protein−DNA recognition, stabilizing DNA triplexes in a sequence-directed sense, or selectively engaging apoptotic pathways without eliciting overall cytotoxicity. This is a tall order. In following our interest in polycyclic acridines, we have taken some inspiration from the natural world, particularly marine organisms from two phyla, the tunicates and sponges, which elaborate highly colored alkaloids based on the pyrido-[2,3,4-*kl*]acridine nucleus **2**. The rich diversity of structures[8,9] presumably evolved as chemical warfare agents to deter marine predators, and these agents exhibit a

Chart 1

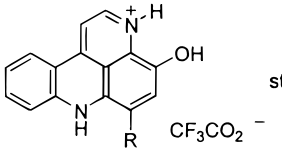


1 *m*-AMSA



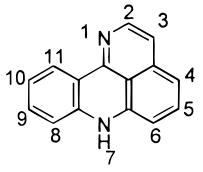
3*H*-pyrido[2,3,4-*kl*]acridine

2



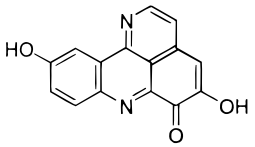
styelsamines A-D

3a R = $CH(OH)CH_2NH_3^+$ $CF_3CO_2^-$
3b R = $(CH_2)_2NHAc$
3c R = CHO
3d R = $(CH_2)_2\overset{+}{N}H_3$ $CF_3CO_2^-$



7*H*-pyrido[4,3,2-*kl*]acridine

4



necatorone

5

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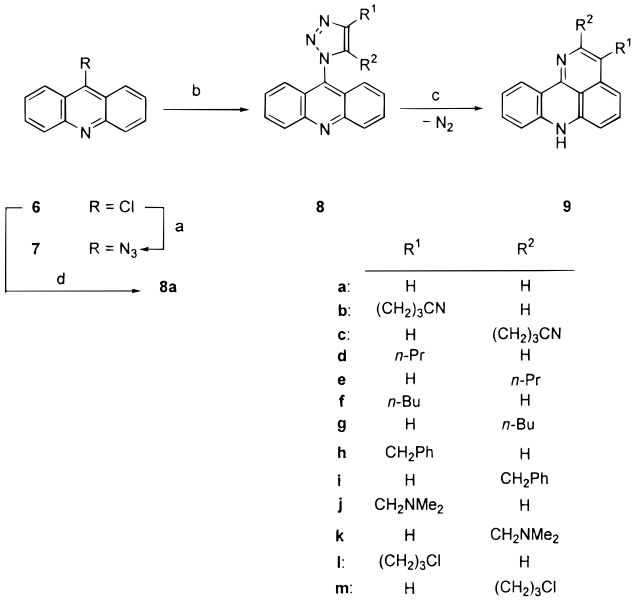
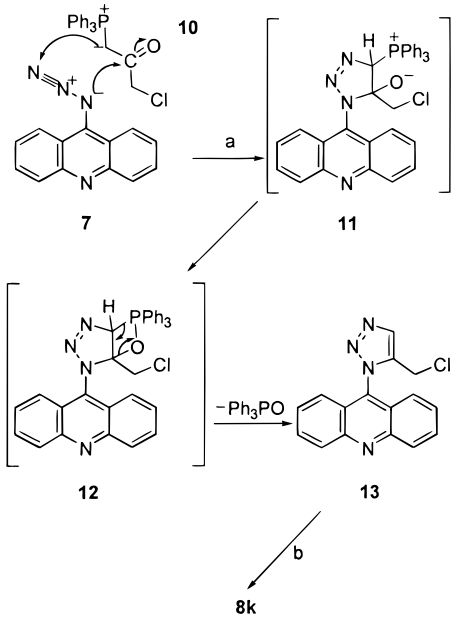
Scheme 1[a]



[a] Reagents and conditions: (a) NaN_3, aq acetone, reflux; (b) for **7** only, $R^1C{\equiv}CR^2$, toluene, 60 °C, 24 h; (c) diphenyl ether, 210−230 °C; (d) NaH in DMF, 1,2,3-triazole and **6**, 25 °C.

Scheme 2[a]



[a] Reagents and conditions: (a) reflux in benzene, 2 h; (b) Me_2NH in THF, 30−40 °C, 5 days.

range of intriguing biological properties, including topo II inhibition. Most recently, a series of new alkaloids, styelsamines A−D **(3a−d),** have been isolated from the Indonesian Ascidian, *Eusynstyela latericius*, which inhibit human colon HCT-116 cells in vitro in the 1−100 μM range.[10]

We have concentrated on developing new synthetic routes to derivatives of the isomeric pyrido[4,3,2-*kl*]-acridine ring system **4**. This tetracycle is found in nature only as the alkaloid necatorone **(5)** and some analogues, including dimers, elaborated by the toadstool *Lactarius* necator,[11] and apart from our own synthetic work (and references therein)[12−14] there is only one further reported example of this ring system in the literature.[15] As compounds of this type undoubtedly bind to DNA,[16,17] we have set ourselves the challenging task of tracking the sequence of events following the initial DNA encounter[1] which eventually leads to the death of cells.

In this paper we survey routes developed to synthesize pyrido[4,3,2-*kl*]acridines and benzo-annealed modifications, together with improvements over published methods, and give the first account of some of their biological properties.

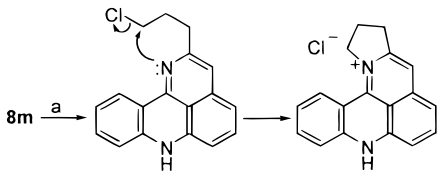
Chemistry

9-Chloroacridine **(6)**, a convenient starting point for the synthesis of the target polycyclic acridines, was converted efficiently to the azidoacridine **7** with sodium azide in aqueous acetone.[18] The azide participated in cycloaddition reactions with 1-alkylalkynes to yield regioisomeric 9-(triazol-1-yl)acridines **8a−i** (Scheme 1).[13] Alternatively, the unsubstituted 9-(1,2,3-triazol-1-yl)acridine **(8a)** can be prepared by reacting 9-chloroacridine with the anion of 1,2,3-triazole[12] or employing trimethylsilylacetylene in the cycloaddition reaction followed by removal of the TMS protecting group.[13] The former route gave the highest yield despite the fact that the required triazole was accompanied by the unwanted 9-(1,2,3-triazol-2-yl)acridine isomer. Generally, in the cycloaddition reaction with simple alkylalkynes, the least sterically hindered triazole was the major product as exemplified in the reaction of 5-cyanopent-1-yne and **7** which gave 9-[4-(3-cyanopropyl)-1,2,3-triazol-1-yl]acridine **(8b)** (51%) and 9-[5-(3-cyanopropyl)-1,2,3-triazol-1-yl]acridine **(8c)** (27%). However, the disadvantage of the cycloaddition method is that chromatographic separation of the mixture of isomeric triazoles is always required.

In the present work, two new triazoles were prepared from **7** and 1-dimethylaminoprop-2-yne: the products were separated laboriously into 9-[4-(dimethylaminomethyl)-1,2,3-triazol-1-yl]acridine **(8j)** (58%) and the isomer **8k** (22%). In our previous work we have shown that 9-(1,2,3-triazol-1-yl)acridines can be prepared in a regiospecific manner from 9-azidoacridine and reactive methylenic compounds.[14] In a variation of this process, reaction of **7** and the chloroacetonyltriphenylphosphorane ylide **10** gave exclusively 9-[5-(chloromethyl)-1,2,3-triazol-1-yl]acridine **(13)** in 67% yield. The mechanism of this cycloaddition reaction involves initial formation of the tetrahedral adduct **11** and cyclization to the oxaphosphetane intermediate **12**, followed by elimination of triphenylphosphine oxide (Scheme 2) in the manner of a Wittig olefination reaction. Incubation of **13** with dimethylamine at 30−40 °C in THF gave access to pure **8k** (72%) which was only the minor isomer from the azide−alkyne cycloaddition route (see above).

The triazoles **8a−i** have been thermolyzed in hot diphenyl ether to generate reactive diradical or carbene species which cyclized generally in good yields to 7*H*-pyrido[4,3,2-*kl*]acridines **9a−i** (Scheme 1).[13] Exceptions were the thermolyses of **8j** and **8k** which gave the new tetracycles **9j** and **9k** in 60% and 58% yields, respectively. An interesting variant of this synthesis is seen in the different behavior of the regioisomeric chloropropyltriazolylacridines **8l** and **8m**: predictably, isomer **8l** cyclized to 3-(3-chloropropyl)pyridoacridine **9l**, whereas

Scheme 3[a]



[a] Reagents and conditions: (a) diphenyl ether, 210−230 °C.

isomer **8m** gives the deep maroon pentacyclic indolizino-[7,6,5-*kl*]acridinium salt **14** (77%), presumably via intramolecular alkylation by the unstable chloropropyl intermediate **9m** (Scheme 3).[13] We have improved this reaction to furnish **14** in 86% yield. Because the salt **14** was one of the most biologically interesting of the polycyclic acridines examined (see later), a more convenient "one-pot" procedure starting from azidoacridine **7** and 5-chloropent-1-yne was sought which would take advantage of the polar nature of **14** to facilitate a simple separation from nonpolar contaminants by solvent partitioning. After many attempts, a procedure involving initial incubation of **7** and 5-chloropent-1-yne in diphenyl ether at 60 °C for 24 h (to effect synthesis of a mixture of triazoles **8l** and **8m**) and brief heating to 210−230 °C to complete cyclization to polycyclic acridines followed by water extraction of polar materials gave only a 6% yield of **14**, together with much insoluble black material. The latter was probably 1,2-bis(acridin-9-yl)-diazene,[18] the product of thermal degradation of the 9-azidoacridine unconsumed in the initial cycloaddition step. Clearly, the "one pot" process is much less efficient than the two-step procedure (above).

9-Anilinoacridines **15a**−**d**, prepared from 9-chloroacridine **(6)** and *o*-phenylenediamines, underwent nitrosative cyclization to 9-(benzotriazolyl)acridines **16a**−**d** required as precursors to the pentacyclic acridines **17a**−**d**.[12] An alternative synthesis of **16a** has been reported from 9-azidoacridine **(7)** and benzyne (47%).[19] However, we now report the most efficient route to **16a** (69%), by reacting **6** directly with the sodio derivative of benzotriazole in boiling DMF. Because of the ambident nature of a substituted benzotriazolyl anion, this approach is not appropriate for the synthesis of the substituted benzotriazoles **16b**−**d**. Thermolysis of the benzotriazoles **16a**−**d** in boiling diphenyl ether (259 °C) afforded 8*H*-quino[4,3,2-*kl*]acridines **17a**−**d** in reasonable yields[12] (Scheme 4), but entrainment with solvent necessitated purification of products by vacuum sublimation. The most convenient process for the preparative-scale decomposition of **16a** utilized boiling triethylene glycol dimethyl ether (triglyme, bp 216 °C) or diethylene glycol (bp 245 °C) as the thermolytic medium, despite the fact that these temperatures are below those at which decomposition is observed by differential scanning calorimetry:[12] the pentacyclic product **17a** was collected in 87% and 93% yields, respectively, after diluting the reaction medium with water. Formation of **17a** from **16a** by the thermal nitrogen-extrusion route (above) was more efficient than the corresponding photochemical cyclization[20] or radical cyclization of 9-(2-iodoanilino)-acridine.[12] Although flash vacuum pyrolysis of **16a** at 590 °C gave a 83% yield of **17a** on a 100-mg scale, the process was not amenable to efficient scale-up. Thermolysis of the nitrobenzotriazolylacridine **16d** to the 2-nitroquinoacridine **17d** was also conveniently effected (74%) in refluxing triglyme, but because of the insolubility of the pentacyclic acridine in diphenyl ether, it could be recovered efficiently from the latter thermolysis medium simply by dilution and washing with hexane.

Catalytic hydrogenation of the nitroquinoacridine **17d** over a palladium−charcoal catalyst has given the corresponding amine **17e** in only moderate yield (<60%).[12] In the present work reduction of **17d** with Raney nickel−hydrazine hydrate in ethanol or tin(II) chloride dihydrate in refluxing ethanol was no more efficient: the optimum conditions (95% yield) were tin(II) chloride in 12 M HCl. A more elegant approach to the amine **17e** was considered feasible if a nitrenium ion (**19**) ↔ π-carbocation (**19′**) reactive species could be generated from a suitable precursor such as the azidoaniline **18d**: a sequence nitroanilinoacridine **18a** → hydroxylamine **18b** → reactive species **19** → intermediate **20** → **17e** (Scheme 4) is typical of intramolecular cyclizations developed especially by Abramovitch.[21] Encouragingly, reduction of nitroarenes with zinc dust in a mixture of TFSA and TFA at 0 °C has been utilized by Ohta[22] as a method of generating such reactive intermediates. The *m*-nitroanilinoacridine **18a** was prepared from **6** and *m*-nitroaniline and subjected to zinc dust/TFSA/TFA degradation. None of the required amine **17e** was detected (TLC), and the only product isolated was the *m*-aminoanilinoacridine **18c** (70%). In an alternative approach the azidoanilinoacridine **18d** was decomposed at 0 °C in a mixture of TFSA, TFAA, and TFA. A vigorous evolution of nitrogen was observed, but only minor amounts of the pentacyclic amine **17e**, together with the *m*-aminoanilinoacridine **18c** (combined yield < 30%), were formed. Although this type of reaction often affords high yields of products,[21] the poor outcome in the present example can be explained by protonation of the acridine in the highly acidic conditions which would deactivate the acridinium system for nucleophilic addition to the π-carbocation reactive intermediate.

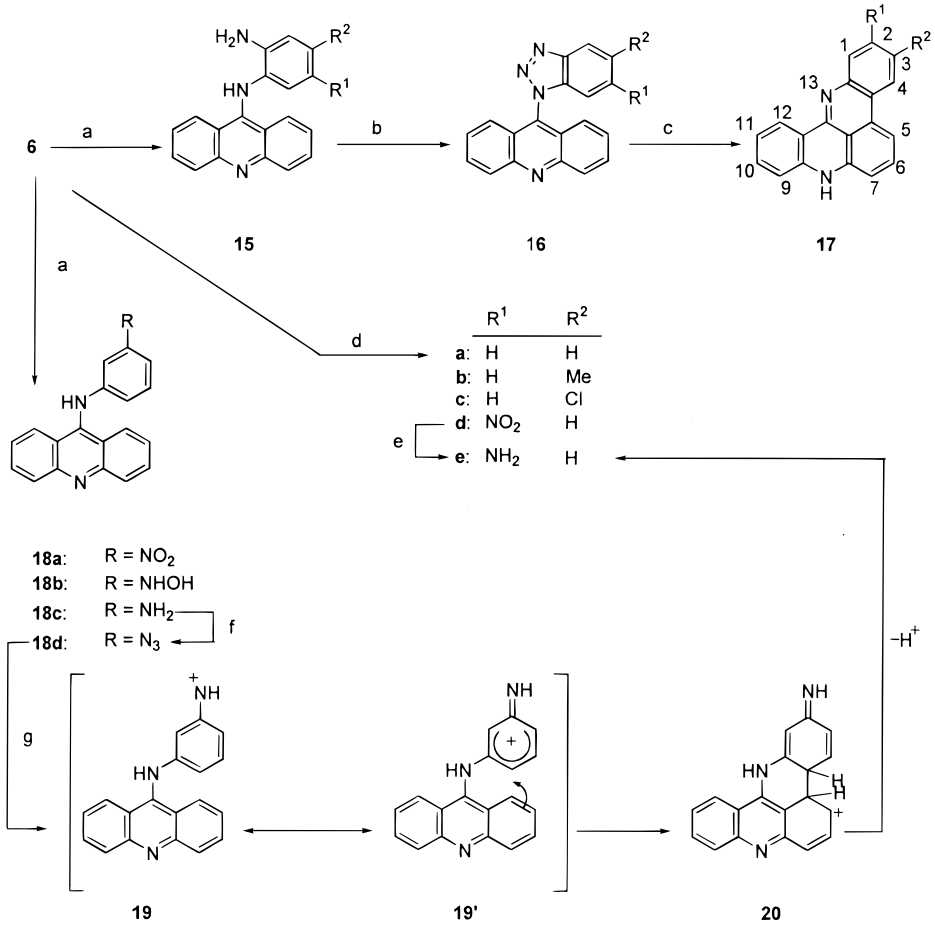
The weakly basic aminoquinoacridine **17e** (pK_a = 6.18)[16] formed water-soluble methanesulfonic, ethanesulfonic, and tetrafluoroborate acid salts which were more suitable for biological studies: protonation was accompanied by a bathochromic shift of the long wavelength absorption in the visible spectrum from 490 nm in the base to 518−520 nm, confirming that (mono)-protonation takes place on the quinoacridine ring system, presumably at N-13.

Biological Results and Discussion

In Vitro Cytotoxicities of Polycyclic Acridines in Panels of Human Non-Small-Cell Lung and Breast Cell Lines. One of the ways of determining the possible molecular target of novel compounds with structural resemblances to agents with a known, or assumed, mode of action is by testing the activity of new agents in a panel of cancer cell lines expressing different levels of the molecular target. Previous studies have shown that the potency of topo II inhibitors, whether topo II poisons or topo II catalytic inhibitors,[23] is dependent on the intracellular protein levels.

As a prelude to detailed investigation of the biological properties of polycyclic acridines, their cytotoxicities

Scheme 4[a]



[a] Reagents and conditions: (a) substituted aniline, MeOH, reflux; (b) $NaNO_2$, 2 M HCl, 0 °C; (c) diphenyl ether, reflux, 2 h; (d) benzotriazole, NaH, DMF, reflux; (e) for **17d** only, $SnCl_2 \cdot 2H_2O$, 10 M HCl, 25 °C; (f) $NaNO_2$, 2 M HCl, then NaN_3; (g) TFSA/TFAA/TFA, 0 °C.

Table 1. Inhibitory Activity of Polycyclic Acridines and Standard Agents in a Panel of Human NSCL Cancer Cell Lines[a]

	IC_{50} values (μM)[b] of cell lines[c]						
compd	NCI-H647[d]	A549[e]	NCI-H226[d]	NCI-H358[d]	NCI-H460[e]	NCI-H322[d]	mean IC_{50}
m-AMSA, **1**	0.07	0.03	1.01	0.15	0.06	0.21	0.26
9a	2.20	2.14	2.34	1.42	2.06	1.86	2.02
9b	2.92	2.57	2.72	2.38	2.56	2.27	2.57
9c	1.68	1.33	1.21	2.01	1.17	1.53	1.49
14	0.12	0.16	0.22	0.11	0.18	0.17	0.16
17a	0.54	0.49	0.75	0.46	0.52	0.49	0.54
17b	0.80	0.81	1.22	0.60	0.71	0.84	0.83
17c	5.30	7.10	>10.0	6.03	3.90	>10.0	>7.0
17d	0.95	>10.0	>10.0	9.50	>10.0	>10.0	>8.4
17e	0.08	0.08	0.17	0.40	0.07	0.14	0.16
etoposide	0.08	0.16	0.24	1.50	0.10	1.59	0.61
doxorubicin	<0.001	0.004	0.008	0.13	0.01	0.03	<0.03

[a] 7-day MTT assay. [b] IC_{50} values are the mean of at least 3 separate determinations. [c] Cell lines are presented in order of decreasing topo IIα content from left to right. The ranking of topo IIβ is: NCI-H226 > A549 > NCI-H358 > NCI-H322 > NCI-H460 > NCI-H647 (data from ref 24). Other molecular characteristics: [d] mutant p53; [e] wild-type p53.

against the adenosquamous NCI-H647, a non-small-cell lung (NSCL) line which is known to exhibit elevated topo IIα levels,[24] were assessed in 4-day MTT assays. Potency rankings followed the general order: indolizino-[7,6,5-*kl*]acridinium salt **14** > *m*-AMSA **(1)** > pentacyclic acridines **17** > tetracyclic acridines **9** > triazoles **8** (data not shown).

Some of the most active agents, together with reference compounds, were then evaluated in a larger panel of NSCL (Table 1) and breast (Table 2) cancer cell lines with varying levels of topo IIα and β isoforms. The cells had been characterized for certain other molecular targets (see footnotes to the tables).[24,25] The results showed that the NSCL cell lines were more resistant than the breast cell lines, and there were no striking differences in activity between representative tetracyclic acridines **9a**−**c** and the pentacyclic series **17a**−**e**. Results corroborate the rankings in the prescreen, confirming that the indolizino[7,6,5-*kl*]acridinium salt **14** and 2-aminoquinoacridine **17e** were the most potent

Table 2. Inhibitory Activity of Polycyclic Acridines and Standard Agents in a Panel of Human Breast Carcinoma Cell Lines[a]

	IC_{50} values (μM)[b] of cell lines[c]						
compd	SKBr-3[d]	MCF-7wt[e]	MDA-231[f]	T47D[g]	ZR-75-1[h]	MDA-468[i]	mean IC_{50}
m-AMSA, **1**	0.06	0.075	0.14	0.22	0.18	0.49	0.19
9a	0.58	0.83	1.73	2.10	1.55	1.37	1.36
9b	0.42	0.27	0.96	1.31	0.72	0.32	0.66
9c	3.45	3.79	2.05	3.59	2.70	1.87	2.90
14	0.12	0.15	0.19	0.16	0.17	0.18	0.16
17a	0.15	0.27	0.54	0.69	0.85	0.09	0.43
17b	0.45	0.18	0.55	2.13	0.39	0.17	0.65
17c	1.03	1.67	5.45	5.55	5.59	0.31	3.27
17d	0.12	0.55	>10	>10	0.03	0.04	>3.5
17e	0.02	0.05	0.23	0.27	0.03	0.04	0.11
etoposide	0.30	0.085	0.15	0.16	0.24	0.60	0.26
doxorubicin	0.02	0.001	0.006	0.007	0.009	0.005	0.008

[a] 7-day MTT assay. [b] IC_{50} values are the mean of at least 3 separate determinations. [c] Cell lines are presented in order of decreasing topo IIα content from left to right. The ranking of topo IIβ content is: MCF-7wt > SKBr-3 > T47D > MDA468 > ZR-75-1 >MDA-231 (data from ref 25). Other molecular characteristics: [d] ER^-, amplified erbB2, mutant p53; [e] ER^+, PgR^+, wild-type p53; [f] ER^-, $EGFR^+$, mutant p53; [g] ER^+, mutant p53; [h] ER^+, PgR^+, mutant p53; [i] ER^-, amplified EGFR, mutant p53.

of the polycyclic acridines across the two panels. These agents were slightly more active than *m*-AMSA and etoposide but considerably less cytotoxic than doxorubicin. There were strong correlations between the antitumor activity of doxorubicin and topo IIα isoform expression in the NSCL lines (Spearman rank correlation coefficient: $r = 0.829$, $p = 0.042$) and of *m*-AMSA to topo IIα in the breast cell lines ($r = 0.943$, $p = 0.005$), but there was no apparent correlation between activity and topo IIα or β isoform expression for any of the new polycyclic acridines in the in vitro assays.

The polycyclic acridines were also evaluated in the NCI in vitro 60-cell line panel.[26] An unusual feature of the dose−response relationships of the most potent agent **14** against all cell lines (mean GI_{50} of 0.09 μM) was the extended concentration range (0.01−10 μM) at which the compound elicited first growth inhibitory (GI) and then cytocidal (LC) effects. This was especially noticeable in the melanoma subpanel where cell lines LOX IMVI, MALME-3M, SK-MEL-5, and UACC-62 gave LC_{50} values of 0.7−3 μM against a mean LC_{50} in the full 60-cell panel of 25 μM.

In Vitro Cytotoxicities of Polycyclic Acridines in Resistant NSCL and Breast Cancer Cell Lines. The antitumor activities of polycyclic acridines were evaluated in cell lines with acquired resistance to known topo II inhibitors. Results for new agents and reference compounds against the NCI-H460 lung line, and the etoposide-resistant counterpart H460pv8[24] in which topo IIα is down-regulated, are shown in Table 3. H460pv8 cells not maintained in etoposide showed a resistance factor (RF) to etoposide of 4, but when these cells were maintained in 1 μM etoposide (H460pv8/eto) there was a 2-fold increase in RF, and this variant line showed increased resistance to the pentacyclic acridine **17e** and doxorubicin, but not to the indolizino[7,6,5-*kl*]acridinium salt **14**. These results suggest that topo IIα may well be the intracellular target for the standard agent doxorubicin, in accordance with published work,[27] but that the lead compound **14** is not operating as a topo II inhibitor in these cell lines. *m*-AMSA was approximately equiactive against both resistant cell lines, possibly because of its ability to target the isoform topo IIβ which is not down-regulated in these lines.[24]

Ideally, activity of a new agent considered for clinical trial should not be subverted by classic P-glycoprotein-modulated resistance which confers the clinically significant multidrug resistance (MDR) phenotype to tumors. The antitumor activity of polycyclic acridines against parental MCF-7wt cells and their MDR counterparts MCF-7/adr, which were derived following prolonged exposure of MCF-7wt to adriamycin (doxorubicin),[28] is shown in Table 4. Significantly, the MCF-7/adr cell line was cross-resistant to *m*-AMSA with a RF of 15.5 and etoposide (RF = 74) but not the acridinium salt **14** (RF = 1.1), confirming that the polar acridinium salt is not a substrate for P-glycoprotein drug efflux. Modest levels of cross-resistance (RF = 2.2−6.8) were observed with other neutral polycyclic acridines (Table 4).

Table 3. Inhibitory Activity of Polycyclic Acridines and Standard Agents against Etoposide-Resistant NCI-H460 Cells[a]

	IC_{50} values (μM)[b]			resistance factors	
compd	NCI-H460	H460pv8	H460pv8/eto	*c*	*d*
m-AMSA, **1**	0.06	0.20	0.04	3.3	0.6
9a	2.06	1.10	1.07	0.5	0.5
9b	2.56	2.06	1.09	0.8	0.4
9c	1.17	1.12	1.02	0.8	0.8
14	0.18	0.13	0.10	0.7	0.5
17a	0.52	0.32	0.34	0.7	0.7
17b	0.71	0.49	0.49	0.7	0.7
17c	3.90	3.77	6.47	0.7	1.3
17e	0.07	0.05	0.12	0.8	1.7
etoposide	0.10	0.39	0.74	4.0	7.6
doxorubicin	0.01	0.004	0.02	0.4	2.0

[a] 7-day MTT assay. [b] IC_{50} values are the mean of 3 separate determinations. [c] Resistance factor is defined as: (IC_{50} H460pv8)/(IC_{50} NCI-H460). [d] Resistance factor is defined as: (IC_{50} H460pv8/eto)/(IC_{50} NCI-H460).

Table 4. Activity of Polycyclic Acridines against MCF-7wt and MCF-7/adr Cell Lines[a]

	IC_{50} values (μM)[b]		
compd	MCF-7wt	MCF-7/adr	resistance factor[c]
m-AMSA, **1**	0.075	1.165	15.5
9b	0.27	0.61	2.2
14	0.15	0.165	1.1
17a	0.27	0.63	2.4
17b	0.18	1.21	6.8
17c	1.67	6.31	3.8
17e	0.05	0.355	6.6

[a] 7-day MTT assay. [b] IC_{50} values are the mean of 3 separate determinations. [c] Resistance factor is defined as: (IC_{50} MCF-7/adr)/(IC_{50} MCF-7wt).

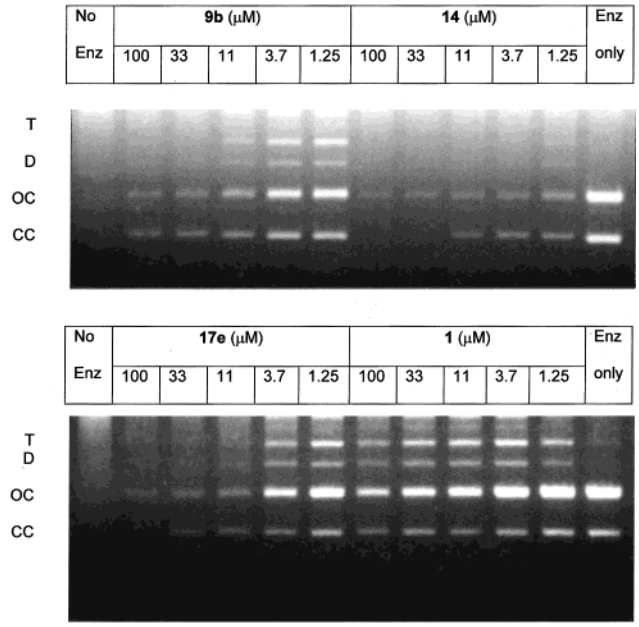


Figure 1. Inhibition of the DNA decatenation activity of human topo IIα by compounds **1**, **9b**, **14**, and **17e**. Assays were performed as described in the Experimental Section. The release of minicircle products of kDNA networks is decreased in the presence of each drug. Product identities: T = trimer; D = dimer; OC = open circular DNA; CC = closed circular DNA.

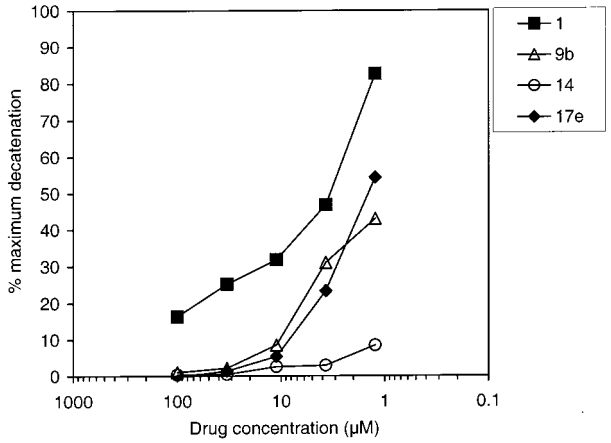


Figure 2. Inhibition of the DNA decatenation activity of human topo IIα by compounds **1**, **9b**, **14**, and **17e**. Assays were performed as described in the Experimental Section, and results for each drug are plotted as the percentage of decatenated products detected when compared with drug-free controls. The release of minicircle products of kDNA networks is decreased in the presence of each drug.

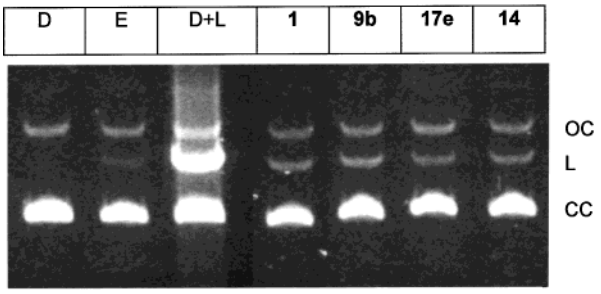


Figure 3. Induction of double-strand DNA cleavage by compounds **1**, **9b**, **14**, and **17e** in the presence of human topo IIα. Assays were performed as described in the Experimental Section; each drug was present at 1 μM. DNA cleavage is identified as an increase in the amount of linear DNA band in a reaction. Quantitation of these results reveals 2−4 times more DNA cleavage in drug-treated samples. D = DNA only; E = enzyme only; D + L = DNA plus a marker of linear pBR322; OC = open circular DNA; CC = closed circular DNA; L = linear product.

Inhibition of Topo II. In a preliminary topo II assay, compounds were evaluated as inhibitors of the enzyme from HeLa cells which can relax supercoiled pBR322. New agents were assayed in the concentration range 0.3−100 μM, and compound activity was compared to that of *m*-AMSA by determining the concentrations at which relaxation of pBR322 was inhibited by 100%. The most potent compounds were the indolizino-[7,6,5-*kl*]acridinium salt **14** and the methanesulfonic acid salt of 2-aminoquinoacridine **17e** which were equiactive (IC_{100} 3 μM). The 3-(3-cyanopropyl)pyridoacridine **9b**, the most cytotoxic of the tetracyclic compounds, gave an IC_{100} of 30 μM (cf. *m*-AMSA IC_{100} 30 μM).

A detailed investigation of the ability of the polycyclic acridines **9b**, **14**, and **17e** to inhibit decatenation of kDNA by human topo IIα was conducted using the enzyme overexpressed in *S. cerevisiae* strain JEL-1 from the plasmid pYEpWob6.[29] All three compounds inhibited the release of minicircle products of kDNA (Figure 1). Quantification of the results (Figure 2) showed that all are more active than *m*-AMSA and that the indolizinoacridinium salt **14** was the most potent with IC_{50} < 1 μM. Each compound was also capable of inducing DNA double-strand cleavage in the presence of topo IIα. When tested at 1 μM, each compound revealed 2−4 times more DNA cleavage than with enzyme alone (Figure 3). In this experiment the most potent compound was the 3-(3-cyanopropyl)pyridoacridine **9b** which was approximately equiactive with *m*-AMSA.

Compound **14** was also tested for its ability to inhibit the growth of *S. cerevisiae* transfected with recombinant human topo IIα and β isoforms.[30] The IC_{50} value for killing the topo IIα-expressing yeast (22 μM) was 3-fold lower than the comparable value (66 μM) of the IIβ-expressing yeast suggesting a slight selectivity for the topo IIα isoform. Comparable published values for *m*-AMSA, doxorubicin, and etoposide are 20/26, 3/15, and 39/131 μM.[30]

NCI COMPARE Analysis. COMPARE is the COMputerized PAttern REcognition algorithm used in evaluation of data generated in the NCI in vitro cell panel. This analysis can determine the degree of similarity, or lack thereof, of mean graph fingerprints of new compounds with patterns of activity of standard agents. Compounds matched with Pearson correlation coefficients (PCCs) > 0.6 frequently share related biochemical mechanisms of action.[31] Three polycyclic acridines were subjected to COMPARE analysis, and results are shown in Table 5. At the GI_{50} level, with the salt **14** as seed compound, five of the top six standard agents were in the mechanistic category of topo II inhibitors, the exception being 3-deazauridine (PCC = 0.633), an inhibitor of cytosine triphosphate synthase. A similar pattern was shown for the pentacyclic acridine free base **17a**. However, at the LC_{50} level (equating to cytocidal activity), the top six COMPAREs for compound **14** were headed by homoharringtonine, mitozolomide, and mi-

Table 5. NCI COMPARE Analysis with Polycyclic Acridines **14**, **17a**, and **17e** as Seed Compounds

	top six standard agents[a] (PCC)[b]	
seed compd	GI_{50}	LC_{50}
14	*N,N*-dibenzyldaunomycin (0.701)	homoharringtonine (0.694)
	amonafide (0.645)	mitozolomide (0.675)
	3-deazauridine (0.633)	mitomycin C (0.675)
	mitoxantrone (0.625)	doxorubicin (0.656)
	daunomycin (0.616)	bruceantin (0.643)
	pyrazoloacridine (0.614)	amonafide (0.634)
17a	pyrazoloacridine (0.755)	trimetrexate (0.642)
	N,N-dibenzyldaunomycin (0.698)	phosphotrienin (0.624)
	3-deazauridine (0.691)	emofolin sodium (0.608)
	pyrazofurin (0.662)	D-tetrandrine (0.600)
	dichloroallyllawsone (0.639)	*c*
	brequinar (0.633)	
17e	*d*	tamoxifen (0.810)
		o,p'-DDD (0.744)
		hycanthone (0.737)
		teroxirone (0.718)
		penclomedine (0.717)
		m-AMSA (0.670)

[a] For structures, see the NCI DTP web pages at: http://epnws1.ncifcrf.gov:2345/dis3d/dtp.html. [b] For definitions, see ref 31. [c] Only four compounds with PCC values > 0.6 at LC_{50} level. [d] No compounds with PCC > 0.6 at GI_{50} level.

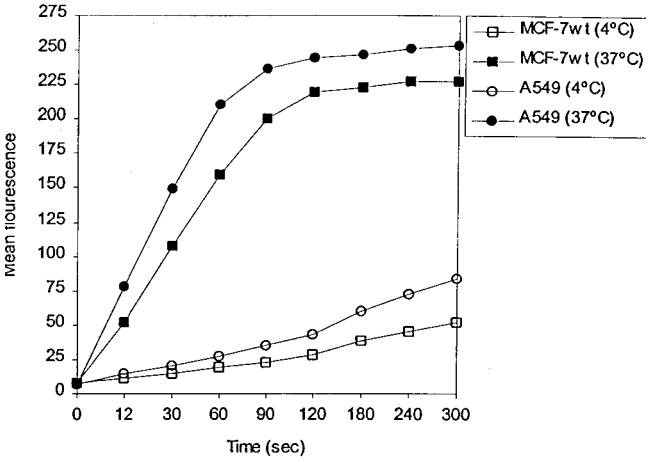


Figure 4. Uptake of indolizino[7,6,5-*kl*]acridine **14** (0.05 μM) in MCF-7wt and A549 cells at 4 and 37 °C.

tomycin C, agents not classified as topo II inhibitors. The amino-substituted pentacyclic acridine **17e** did not reveal any PCC values above 0.6 at the GI_{50} level but high PCC values (>0.7) at the LC_{50} level to a group of agents of disparate mechanisms.

Drug Uptake and Intracellular Localization. The high fluorescence of compound **14** when excited at 488 nm allowed for the use of fluorescence-activated cell sorting (FACS) to study the kinetics of drug uptake. At 37 °C compound **14** achieved plateau levels in 2 min within breast MCF-7 and NSCLC A549 cells (Figure 4); at 4 °C entry was much slower. The subcellular distribution of the drug is shown in fluorescence images (Figure 5). After 30 min of incubation of drug (10 μM) with cells, compound **14** localized predominantly in the nuclei of both cell types, although in MCF-7 cells there appeared to be some heterogeneity of fluorescence within the nuclei. The tetracyclic acridine **9b** and pentacyclic derivative **17e** showed similar rapid nuclear localization (data not shown).

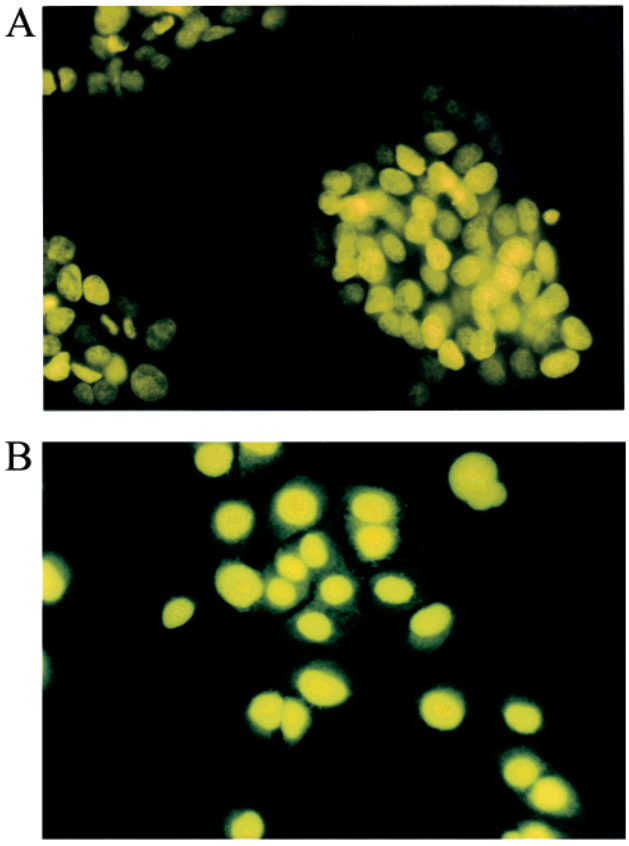


Figure 5. Fluorescence micrographs (magnification ×400; reproduced at 60% of original for publication) of (A) MCF-7 wt and (B) A549 cells incubated for 30 min in 10 μM indolizino-[7,6,5-*kl*]acridine **14** at 37 °C. Yellow fluorescence indicates localization of drug predominantly in the nuclei of cells.

Conclusions

In this paper we have disclosed the first biological results on a readily accessible series of novel polycyclic acridines, where the core heterocyclic framework is structurally related to bioactive marine natural products. Potentially the most interesting compound is the indolizino[7,6,5-*kl*]acridinium salt **14** which forms a binding 'hot spot' in DNA with the planar pyridoacridine moiety intercalating at G-C sequences and the pyrrolidinium fragment occupying minor or major grooves.[1] Clearly, inhibition of topo II is a component of the overall activity of this compound, although there is a distinctive fingerprint distinguishing the compound from other agents of this type. Thus the sensitivities of a panel of NSCL (Table 1) and breast (Table 2) cancer cell lines are not related to their topo IIα isoform expression; lung cells NCI-H460pv8 and H460pv8/eto which are resistant to etoposide are not cross-resistant to **14** (Table 3). Unlike *m*-AMSA, **14** is not a substrate for P-glycoprotein-mediated cellular efflux (Table 4), and its water solubility could be pharmaceutically advantageous.

The NCI COMPARE analysis raises the intriguing prospect that **14** might trigger different inhibitory molecular mechanisms at the growth inhibition (GI_{50}) and cytocidal (LC_{50}) levels. Growth inhibition induced by **14** involves inhibition of topo II, and the localization of the drug in the nuclei of sensitive cells supports the disorganization of a nuclear target by this agent. In contrast, cytocidal activity appears to be unrelated to

topo II inhibition. We have shown that compound **14** is a potent inducer of apoptosis in lung and breast cell lines irrespective of their p53 status (wild type or mutant).[32] The influence of this compound on cell cycle events, and regulation of genes involved in the apoptotic cascade, will be the subject of the next paper in this series.

Experimental Section

Synthetic Chemistry. All melting points were recorded on a Gallenkamp melting point apparatus and are uncorrected. UV spectra were measured in 95% EtOH on a Cecil 1020S spectrometer. IR spectra were recorded on a Mattson 2020 GALAXY series FT-IR spectrometer as KBr disks. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker ARX250 spectrometer at 250.1 and 62.9 MHz, respectively, in solvents as specified, with tetramethylsilane or residual protic solvents as internal standard, *J* values being in Hz. Low-resolution mass spectra were recorded on an AEI MS-902 or VG Micromass 7070E. High-resolution mass spectra (HRMS) were performed by the EPSRC Mass Spectrometry Service Centre, Swansea. Silica gel TLC was performed on 60F-254 precoated sheets (E. Merck) and column chromatography on silica gel C60 (60−120 mesh).

Synthesis of 9-(1,2,3-Triazol-1-yl)- and 9-(1,2,3-Benzotriazol-1-yl)acridines. Compounds **8a**−**i**,[13] **9a**−**i**,[13] and **16a**−**d**[12] were prepared by published methods.

9-(4-Dimethylaminomethyl-1,2,3-triazol-1-yl)acridine, 8j. 9-Azidoacridine **(7)** (1.10 g) and 1-dimethylamino-2-propyne (1.24 g) were dissolved in dry toluene (10 mL) and heated to 60 °C under nitrogen for 24 h. Removal of solvent (vacuum evaporation) gave a residue which was chromatographically fractionated on silica gel using ethyl acetate−ethanol (1:1) as eluent. Evaporation of the orange band gave the dimethylaminotriazolylacridine **8j** (0.88 g, 58%), as orange crystals (from ethanol): mp 121−123 °C (dec at 218 °C); UV 211.6, 249.4, 359 nm; IR 3442, 2773, 1552, 1450, 1384, 1227, 1041, 807 cm^{-1}; δ_H (DMSO-d_6) 8.80 (s, 1H, H-5′), 8.35 (d, 2H, J = 8.8, H-4,5), 7.99 (m, 2H, J = 1.4, H-3,6), 7.74 (m, 2H, J = 1.1, 8.8, H-2,7),), 7.38 (d, 2H, J = 8.4, H-1,8), 3.77 (s, 2H, CH_2), 2.29 (s, 6H, 2 × CH_3); δ_C (DMSO-d_6) 149.5 (C), 145.3 (C), 137.8 (C), 132.1 (CH), 130 3 (CH), 128.4 (CH), 128.2 (CH), 123.1 (CH), 122.2 (C), 52.8 (CH_2), 45.6 (CH_3); *m*/*z* (CI) 304 (MH^+). Anal. ($C_{18}H_{17}N_5$) C, H, N.

9-(5-Dimethylaminomethyl-1,2,3-triazol-1-yl)acridine, 8k. The pale yellow band (from ethyl acetate) from the previous experiment afforded triazolylacridine **8k**: mp 192−193 °C (0.33 g, 22%); UV 211.3, 250.1, 359 nm; IR 3442, 2789, 1514, 1451, 1384, 1237, 1027, 754 cm^{-1}; δ_H (DMSO-d_6) 8.35 (d, 2H, J = 8.9, H-4.5), 8.18 (s, 1H, H-4′), 7.98 (td, 2H,J = 1.0, 8.8, H-3,6), 7.71 (t, 2H, J = 8.6, H-2,7), 7.28 (d, 2H, J = 8.9, H-1,8), 3.27 (s, 2H, CH_2), 1.83 (s, 6H, 2 × CH_3); δ_C (DMSO-d_6) 149.7 (C), 134.8 (C), 132.1 (CH), 130.3 (CH), 129.4 (CH), 128.2 (CH), 123.1 (CH), 122.8 (C), 52.8 (CH_2), 45.1 (CH_3); *m*/*z* (CI) 304 (MH^+). Anal. ($C_{18}H_{17}N_5$) C, H, N.

9-(5-Chloromethyl-1,2,3-triazol-1-yl)acridine, 13. 3-Chloro-1-triphenylphosphoranylidene-2-propanone **(10)**[33] (5.7 g) was added to a stirred solution of 9-azidoacridine **(7)** (3.56 g) in dry benzene (40 mL) and the mixture was refluxed under N_2 for 2 h. The evaporated mixture was purified by chromatographic fractionation on a silica gel column with ethyl acetate: hexane (1:1) as eluting solvent. The yellow band gave the triazolylacridine **13** (3.28 g, 67%): mp 181−182 °C (yellow crystals, from ethyl acetate); δ_H (DMSO-d_6) 8.39 (s, 1H, H-4′), 8.37 (d, 2H, J = 8.7, H-4,5), 8.00 (m, 2H, H-3,6), 7.73 (m, 2H, H-2,7), 7.26 (d, 2H, J = 8.7, H-1,8), 4.70 (s, 2H, CH_2Cl); δ_C ($CDCl_3$) 149.2 (C), 137.1 (C), 135.4 (C), 134.0 (CH), 130.9 (CH), 129.9 (CH), 128.5 (CH), 122.7 (C), 122.0 (CH), 31.8 (CH_2). Anal. ($C_{16}H_{11}ClN_4$) C, H, N.

A solution of **13** (0.83 g) and dimethylamine (2.25 g) in THF (25 mL) was stirred at 30−40 °C for 5 days. Solvent was evaporated and the residue crystallized from ethyl acetate to give **8k** as yellow crystals (0.61 g, 72%): mp 192−193 °C; identical (IR, ^{1}H and ^{13}C NMR) to the sample (above).

9-(1,2,3-Benzotriazol-1-yl)acridine, 16a. 1,2,3-Benzotriazole (1.90 g) was added to a mixture of sodium hydride (1.1 mol equiv as a 60% dispersion in mineral oil) in dry DMF (60 mL). 9-Chloroacridine **(6)** (3.43 g) was added and the mixture was refluxed (3.5 h). Addition of excess water precipitated the benzotriazolylacridine (3.40 g, 71%), identical (UV, IR, ^{1}H and ^{13}C NMR) to an authentic sample.[12]

Synthesis of Tetracyclic Acridines. 3-Dimethylaminomethyl-7*H*-pyrido[4,3,2-*kl*]acridine, 9j. A suspension of 9-(4-dimethylaminomethyl-1,2,3-triazol-1-yl)acridine **(8j)** (0.15 g) in diphenyl ether (5 mL) was maintained at 225 °C for 0.5 h during which effervescence of nitrogen was observed. The mixture was fractionated on a short silica gel column to remove diphenyl ether (eluted with hexane) followed by elution of the product with ethyl acetate−ethanol. The pyridoacridine (0.077 g, 60%) had: mp 200 °C dec; UV 227.5, 262, 318.5, 423 nm; IR 3389, 2944, 1638, 1555, 1466, 1339, 1154, 766 cm^{-1}; δ_H ($CDCl_3$) 8.50 (dd, 1H, J = 1.5, 8.0, H-11), 8.17 (s, 1H, H-2), 7.52 (t, 1H, J = 8.2, H-5), 7.35 (m, 1H, NH), 7.20−7.40 (m, 2H, H-4, H-5), 7.11(m, 1H, J = 1.0, 6.8, H-10), 6.93 (d, 1H, J = 8.0, H-8), 6.71 (dd, 1H, J = 1.0, 7.8, H-6), 3.57 (s, 2H, CH_2), 2.30 (s, 6H, 2 × CH_3); δ_C ($CDCl_3$) 153.4 (C), 151.6 (C), 140.0 (C), 139.9 (C), 131.8 (CH), 131.5 (CH), 131.5 (CH), 125.8 (CH), 121.8 (CH), 118.9 (C), 115.7 (CH), 115.3 (CH), 114.1 (CH), 105.0 (CH), 66.3 (CH_2), 46.0 (CH_3); *m*/*z* (CI) 276 (MH^+). Anal. ($C_{18}H_{17}N_3$) C, H, N.

2-Dimethylaminomethyl-7*H*-pyrido[4,3,2-*kl*]acridine, 9k. Prepared (58%) from 9-(5-dimethylaminomethyl-1,2,3-triazol-1-yl)acridine **(8k)** in boiling triglyme (1.5 h), the pyridoacridine had: mp 200 °C dec; UV 228, 267.5, 318.5, 429 nm; IR 3424, 1638, 1561, 1476, 1449, 1333, 1096, 802 cm^{-1}; δ_H ($CDCl_3$) 8.54 (dd, 1H, J = 1.5, 8.0, H-11), 7.38 (t, 1H, J = 7.8, H-5), 7.31 (m, 1H, H-9), 7.22 (s, 1H, H-3), 7.04 (td, 1H, J = 0.8, 8.2, H-10), 7.00 (d, 1H, J = 8.0, H-4), 6.90 (dd, 1H, J = 1.0, 8.0, H-8), 6.62 (dd, 1H, J = 0.8, 7.5, H-6), 3.71 (s, 2H, CH_2), 2.45 (s, 6H, 2 × CH_3); δ_C ($CDCl_3$) 146 (CH), 139.9 (C), 139.7 (C), 137.0 (C), 132.0 (CH), 131.5 (CH), 125.4 (CH), 124.3 (C), 122.1 (CH), 115.2 (CH), 112.0 (CH), 106.1 (CH), 61.8 (CH_2), 46.0 (CH_3); *m*/*z* (CI) 276 (MH^+). Anal. ($C_{18}H_{17}N_3$) C, H, N.

Synthesis of Pentacyclic Acridines. 1*H*-2,3-Dihydroindolizino[7,6,5-*kl*]acridinium Chloride, 14. A suspension of 9-[5-(3-chloropropyl)-1,2,3-triazol-1-yl]acridine **(8m)** (1.80 g) in diphenyl ether (35 mL) was maintained at reflux temperature for 0.5 h under nitrogen. The melt was triturated with ethyl acetate and the red salt **14** (1.42 g, 86%) was collected and washed free of diphenyl ether with more ethyl acetate. The product was identical (UV, IR, ^{1}H and ^{13}C NMR) with an authentic sample.[13]

8*H*-Quino[4,3,2-*kl*]acridine, 17a. A suspension of 9-(1,2,3-benzotriazol-1-yl)acridine **(16a)** (1.0 g) in triglyme (5 mL) was boiled (4 h) and the cooled solution was diluted with excess water. The product was collected and washed with water to yield the quinoacridine (87%) which was identical (mp, UV, IR, ^{1}H and ^{13}C NMR) with an authentic sample.[12] Using diethylene glycol as thermolysis medium afforded the same quinoacridine (93%).

2-Nitro-8*H*-quino[4,3,2-*kl*]acridine, 17d. 9-(6-Nitro-1,2,3-benzotriazolyl)acridine **(16d)** (3.9 g) was stirred with diphenyl ether (20 mL) under nitrogen to form a mustard-colored suspension which was refluxed for 6 h. To the cooled melt was added hexane (100 mL), with stirring, and the quinoacridine **17d** (2.96 g, 82%) was collected. The product was identical (UV, IR, ^{1}H and ^{13}C NMR) with an authentic sample.[12]

The same quinoacridine (74%) was formed when 9-(6-nitro-1,2,3-benzotriazolyl)acridine **(16d)** was thermolyzed in refluxing triglyme (3.5 h) and the solution diluted with water to precipitate the product.

2-Amino-8*H*-quino[4,3,2-*kl*]acridine, 17e. (i) 2-Nitro-8*H*-quino[4,3,2-*kl*]acridine **(17d)** (1.0 g) was added portionwise to a solution of tin(II) chloride dihydrate (2.88 g, 4 mol equiv) in 10 M hydrochloric acid (15 mL) at 25 °C to give a dark brown solution which was stirred at 25 °C for 60 h. Basification of

the mixture to pH >12 with excess ice-cold 10 M sodium hydroxide solution liberated the aminoquinoacridine base (0.85 g, 95%) as a mustard-colored solid: mp 285−295 °C dec (lit.[12] mp > 300 °C dec). The product was identical (UV, IR, ^{1}H and ^{13}C NMR) to an authentic sample.[12]

(ii) 9-(3-Aminoanilino)acridine **(18c)**[34] was converted to 9-(3-azidoanilino)acridine **(18d)** by sequential diazotization/azidation. The azide free base (82%) was crystallized from ethyl acetate−MeOH: mp 196−200 °C dec; UV 203.5, 225.9, 246.5, 410 nm; IR 3023 (NH), 2116 (N_3), 1584, 1485 cm^{-1}; *m*/*z* (EI) 311 (M^+).

The azide (0.15 g) was added in small portions to a stirred mixture of TFSA (0.5 mL), TFA (0.6 mL) and TFAA (0.15 mL) at 0 °C. The mixture was maintained at 0 °C for 0.5 h, then allowed to warm to 25 °C and stirred for a further 3 h. The reaction mixture was basified with concentrated aqueous ammonia−ice and products were extracted in ethyl acetate (3 × 150 mL). The washed, dried ($MgSO_4$) organic fraction was evaporated to give a brown oil which was chromatographically fractionated on silica gel (ethyl acetate−hexane−MeOH) to give 2-amino-8*H*-quino[4,3,2-*kl*]acridine **(17d)** (0.02 g) and 9-(3-aminoanilino)acridine **(18c)** (0.022 g), identical to authentic samples.

Synthesis of Salts of 2-Amino-8*H*-quino[4,3,2-*kl*]acridine, 17e. The **methanesulfonic acid salt**, prepared (75%) from **17e** free base and methanesulfonic acid (1.1 mol equiv) in THF, had: mp > 300 °C dec (from aqueous 2-propanol); UV 207.4, 237.5, 276.3, 324.3, 461.9, 518.3 nm; δ_H (DMSO-d_6) 12.94 (s, 1H, NH), 12.80 s, 1H, NH), 8.82 (d, 1H, J = 8.5, H-12), 8.22 (d, 1H, J = 9.0, H-4), 7.96 (m, 3H, H-5,6,10), 7.68 (d, 1H, J = 8.5, H-9), 7.54 (t, 1H, J = H-11), 7.39 (d, 1H, J = 7.6, H-7), 7.14 (d, 1H, J = 1.7, H-1), 6.91 (dd, 1H, J = 8.8, H-3), 6.31 (brs, 2H, NH_2), 2.35 (s, 3H, CH_3); δ_C (DMSO-d_6) 151.2 (C), 147.25 (C), 140.3 (C), 140.1 (C), 136.6 (CH), 136.4 (C), 135.4 (CH), 134.1 (C), 125.1 (CH), 124.7 (CH), 123.4 (CH), 118.3 (CH), 116.2 (CH), 112.0 (C), 116.7 (C), 111.5 (C), 111.1 (CH), 110.9 (CH), 40.2 (CH_3). Anal. ($C_{20}H_{17}N_3O_3S$) C, H, N.

The **ethanesulfonic acid salt**, similarly prepared from **17e** and ethanesulfonic acid, had: mp > 300 °C dec (from aqueous ethanol); UV 206.6, 236.7, 275.9, 325.5, 457.1, 519.0 nm. Anal. ($C_{21}H_{19}N_3O_3S$) C, H, N.

The **tetrafluoroboric acid salt**, from **17e** and 48% fluoroboric acid, had: mp 245 °C dec (from aqueous 2-propanol); UV 207.5, 238.4, 276.6, 326.1, 520.0 nm.

In Vitro Cytotoxicities of Polycyclic Acridines. All the tested polycyclic acridines and *m*-AMSA were dissolved in DMSO to make stock solutions of 10 mM. Stock solutions of etoposide (10 mM) and doxorubicin (1 mM) were prepared in saline (0.9%). All drug solutions were stored at −20 °C.

NSCLC and breast cancer cells were grown in RPMI 1640 culture medium supplemented with 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin and 10% heat-inactivated FCS. Initial seeding densities per well for 7-day cytotoxicity assays were: A549 (250), NCI-H460 (250), NCI-H647 (300), NCI-H226 (1000), NCI-H322 (1000), NCI-H358 (1000), NCI-H460pv8 (500), MCF-7 (300), SKBr-3 (1000), T47D (1000), MDA-231 (800), ZR-75-1 (1000), MDA-468 (800), MCF-7/adr (800).

MTT Colorimetric Assays. The fraction of viable cells after drug treatment was determined by the ability of cells to metabolize MTT. To each well was added 50 μL of MTT solution (2 mg/mL) to yield a final concentration of 0.4 mg/mL and the plates were further incubated at 37 °C (95% air, 5% CO_2) for 4 h to allow viable cells to convert soluble MTT to insoluble purple formazan. The medium containing MTT was aspirated and the formazan was dissolved by adding DMSO (100 μL) and glycine buffer (25 μL). The absorbance of the formazan solutions was determined at 550 nm using an Anthos Labtec System plate reader. The IC_{50} values (concentration of drug to produce a 50% reduction in the absorbance of the drug untreated controls) were determined from the dose−response curves.

NCI in Vitro Cytotoxicity Assays. Sulforhodamine B assays were used for assessing the cytotoxicity of test agents in a panel of 60 cell lines.[35] Briefly, cell lines were inoculated into a series of 96-well microtiter plates, with varied seeding densities depending on the growth characteristics of specific cell lines. Following a 24-h drug-free incubation, test agents were added routinely at five 10-fold dilutions with a maximum concentration of 10^{-4} M. After 2 days of drug exposure, the change in protein stain optical density allowed the inhibition of cell growth to be analyzed.

Inhibition of Topo II. Topo IIα was overexpressed in *S. cerevisiae* strain JEL-1 from the plasmid pYEpWob6.[29] Enzyme was isolated to >95% purity from yeast nuclear extracts by a four-stage chromatographic process.[36] Compounds were assayed for inhibition of topo IIα decatenation using kDNA (Topogen) as a substrate and for DNA cleavage using pBR322 (gift from A. Howells, University of Leicester, U.K.).

Decatenation mixtures (20 μL) were prepared using topo assay buffer (50 mM Tris-HCl, pH 7.5; 130 mM NaCl; 10 mM $MgCl_2$; 2 mM DTT; 0.5 mM EDTA; 0.25 mg kDNA; 1.2 nM topo IIα) and contained drug at the indicated concentrations. Reactions were started by addition of ATP to 1.25 mM, incubated for 15 min at 37 °C and stopped by addition of 10 μL of STEB (100 mM Tris-HCl, pH 8.0; 40% w/v sucrose; 0.1 M EDTA; 0.5 mg/mL bromophenol blue).

Cleavage assay mixtures (10 μL) contained drug at the indicated concentrations in cleavage buffer (50 mM Tris-HCl, pH 7.5; 50 mM NaCl; 10 mM $MgCl_2$; 2 mM DTT, 0.5 mM EDTA, 2.5 mM ATP; 0.125 mg pBR322; 30 nM topo IIα). Reactions were incubated for 10 min at 25 °C and stopped by addition of 1 μL of 10% SDS. Mixtures were then incubated with proteinase K (0.1 mg/mL) for 30 min at 37 °C, 10 μL of STEB was added and samples were finally extracted with 50 μL of phenol−$CHCl_3$−isoamyl alcohol (25:24:1).

Completed reactions were analyzed by 0.8% agarose gel electrophoresis with gels containg 40 μg/mL ethidium bromide. Gels were scanned and DNA was quantitated using a video camera linked to a Syngene gel analysis program.

Activity of Compounds against *S. cerevisiae* Expressing Recombinant Human Topo II. This experiment used logarithmically growing yeast strain JN394 *top*2-4 transformed with a human TOP2α, human TOP2β or yeast TOP2 plasmid, and activity of compound **14** was assayed according to the method of Meczes et al.[30]

Drug Uptake and Intracellular Localization. Cells were trypsinized and collected in fresh RPMI 1640 medium and the cell density was adjusted to 5 × 10^5−10^6 cells/mL. The cells were kept on ice or 37 °C prior to introduction of drugs. To 1 mL of cells in 12 × 75-mm tubes, compound **14** was added to a final concentration of 50 nM. The average fluorescence (Fl1) of 2000 cells was recorded using a Becton-Dickinson FACScan flow cytometer at diffrent time periods. The kinetic uptake of drugs was expressed as the change of mean cellular fluorescence with time.

For determination of drug intracellular distribution in adherent cells A549 and MCF-7 cells were grown on cover slips in 60 × 10-mm Petri dishes containing 10 mL of RPMI medium at 37 °C in a 5% CO_2 and 95% air atmosphere. Cells were incubated with compound **14** (10 μM) for 30 min. Cells were washed free of drugs with cold PBS and the coverslips were mounted onto microscope slides using Fluoromount mountant (BDH Chemicals). Slides were visualized under blue light (503−530 nm) using a Leitz Dialux 20 fluorescence microscope. Fluorescence micrographs were captured using 400 or 800 ASA film.

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