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REVIEW

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Biological activities of pyridoacridines

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Covering: 1983–2003

This review consolidates biological activity data reported for pyridoacridine **1** molecules in the literature from 1983–2003 into several tables with brief discussions of assays used and results obtained. This review summarizes recent progress in structure activity relationships for analogues of amphimedine **2** and ascididemin **3** classes of pyridoacridines and correlates reported molecular mechanisms of action with biological activities.

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1 Introduction

The purpose of this review is to provide a convenient summary of the biological activities of the pyridoacridine **1** class of natural products and some synthetic derivatives. This work follows three excellent reviews by Molinski[1], Ding *et al.*[2], and Delfourne and Bastide[3], which while summarizing biological activities, have focused mainly on the structural relatedness of members of the class. As a class, the pyridoacridines are considered DNA binding molecules and have been characterized largely on the basis of their cytotoxicity. Our review is warranted because recent work has demonstrated that the individual pyridoacridines can vary dramatically in their molecular mechanism of cell killing, suggesting a diversity of possible applications not previously appreciated. In addition, we will discuss in depth the implications of recent work accomplished with amphimedine **2**, ascididemin **3** and some of their analogues.

Marine natural products are molecules rich in diverse biological activities. Assessment of the chemical diversity contained in the oceans is an established field with a great deal of research focused on extraction of chemicals from sessile invertebrates. These organisms, including ascidians, sponges and corals, live in a highly competitive environment. They produce a wide variety of toxic chemicals in order to mediate spatial competition as well as to prevent parasitism and predation.[2,4] The toxic chemicals include pyridoacridines **1a** and pyridoacridones **1b**, referred to here simply as pyridoacridines. Pyridoacridines are a group of nitrogen containing aromatic alkaloids that are often cytotoxic. Early work by Burres and colleagues attributed their cytotoxicity primarily to their DNA binding activity.[5] This concept has found support in many subsequent studies, but does not apply

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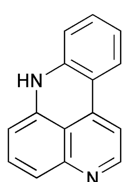
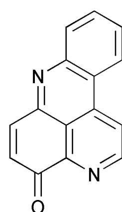
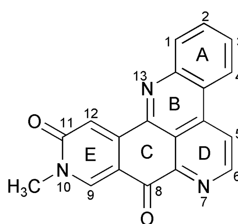


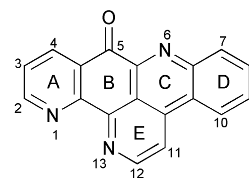
1a 11*H*-pyrido[4,3,2-*mn*]-acridine skeleton



1b 4*H*-pyrido[2,3,4-*kl*]-acridone skeleton



2 Amphimedine



3 Ascididemin

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universally. While pyridoacridines can often intercalate DNA, a generally non-selective mechanism of cytotoxicity, they can also have surprisingly selective effects in living systems. As detailed below, they have anti-bacterial, anti-fungal, anti-viral, anti-parasitic and insecticidal activities.[6–18] They inhibit topoisomerases, produce reactive oxygen species, cause the release of calcium from the sarcoplasmic reticulum, induce neuronal differentiation and bind nucleotide receptors.[5,10,12,16, 18–31] Pyridoacridines may also be immunosuppressant, and are known to alter cell surface differentiation markers associated with leukemias.[17,32–35] Furthermore, recently there has been significant progress in strategies for the synthesis of pyridoacridines. This progress in combination with the myriad of biological activities being identified provides the medicinal chemist with a unique opportunity to establish meaningful structure activity relationships.

The first pyridoacridine identified was amphimedine **2** in 1983.[36] Since then well over a hundred have been identified or synthesized. The biological activity most often reported for new pyridoacridine structures is mammalian cell cytotoxicity. While cytotoxicity remains the most universally reported biological measure of the pyridoacridines, other biological activities have been documented. An inclusive summary of these activities is presented in Tables 1–21, accompanied by brief explanatory paragraphs.

Table 1 lists marine derived pyridoacridines, their source, if a synthetic scheme has been published for them and if they have been reported as cytotoxic in mammalian cells. Chemical structures provided in this review are indicated by their number in bold.

2 Review of pyridoacridine biological activity

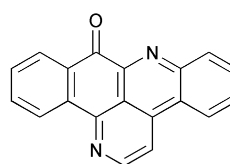
Almost all of the pyridoacridines tested have strong cytotoxic activity *in vitro*. As mentioned above, this activity is often attributed to their ability to intercalate DNA and thereby interact with, or inhibit, DNA metabolizing enzymes. Pyridoacridines have also proved effective in several additional assays, demonstrating anti-microbial, anti-viral, fungicidal, and other activities. Some pyridoacridines which are cytotoxic to cultured mammalian tumor cell lines have demonstrated excellent anti-tumor activity in various models, while others have proven too toxic to be useful.

2.1 Anti-bacterial and anti-fungal activity

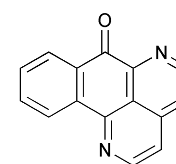
The appearance of multi-drug resistant bacteria has highlighted the need for antibiotics of new and unusual activity. While many of the compounds reviewed here have anti-microbial activity, in many cases their potential anti-microbial use would be limited by their toxicity to mammalian cells.

Anti-microbial activity is determined using several different assays. These assays generally measure activity against microbial cells grown in culture. Disk diffusion-zone of inhibition studies were performed for many of the molecules discussed here. This is a common screening technique and is assumed to be the method of analysis in manuscripts which did not explicitly state their methods. In disk diffusion-zone of inhibition studies, a filter paper or disk is permeated with the drug and placed upon a lawn of microbial cells. If the drug is active, a zone of inhibition is apparent by a clear area around the perimeter of the disk. The radius of the zone of inhibition provides a useful measure of the anti-microbial activity and can readily be compared to control compound disks on the same culture plate. Several different disks may be evaluated on the same culture plate which increases the through-put of this assay and makes it suitable for industrial screening.

Using this approach, anti-microbial activity was reported for **3**, benzo[4,5]sampangine **4** (deazaascididemin) and two synthetic precursors against *Escherichia coli*, *Bacillus subtilis*, *Candida albicans*, and *Cladisporium resinae*, with an MIC (see below) of 0.39 μg **4** mL^{-1} against *C. albicans*.[6] Sampangine **5** and several analogues possessed potent anti-microbial activity against the opportunistic infection organisms *C. albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, and *Mycobacterium intracellulare*.[7] Anti-microbial activity was reported for **27** against *E. coli* and *Staphylococcus aureus*.[8]



4 Benzo[4,5]sampangine



5 Sampangine

A more quantitative measure of anti-microbial activity (Table 2) is called the minimal inhibitory concentration (MIC). To determine MIC, serial dilutions of drug are added to the culture media of various test microbes. The culture tubes are then inoculated with a given titer of microbial cells and incubated for 24 to 48 hours. The MIC is the concentration of compound that inhibits microbial growth (usually determined by optical absorption). Similarly, the minimal fungicidal concentration (MFC) can be determined if no viable fungi are detected in treated cultures. In general it is more informative to compare MIC values obtained from various groups, rather than zones of inhibition, because of the uniformity of the protocols.

An extensive study of the anti-microbial activities of meridine **6**[10], assessing activity *via* determination of MIC and MFC was conducted and results are shown in Tables 2 and 3. **6** appeared to exert its anti-fungal activity through a fungicidal mechanism since its MICs and MFCs were so similar. A pH study showed **6** to be about twice as effective above pH 5.0, but this difference was not considered significant.[10] The pK_a of **6** was not provided. A study of *C. albicans* macromolecular synthesis inhibition showed the inhibitory effect of **6** on DNA synthesis was achieved at much lower concentrations than was needed to inhibit other synthetic pathways.

The biochemical prophage induction assay (BIA) relies on the induction of the SOS response in *E. coli* as a surrogate indicator of DNA damage (Table 4). The SOS response in *E. coli* is characterized by the induction of a diverse set of genes in response to DNA damage or interference with DNA replication. These genes are normally suppressed by LexA protein. Activation of RecA in response to the above signals stimulates the degradation of LexA and the induction of the SOS response. The response includes phage and bacterium

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Table 1 Sources of marine derived pyridoacridines

Pyridoacridine	Source	Published synthesis	Cytotoxic	Ref.
Amphimedine **2**	*Amphimedon* sp. sponge (Guam)	Yes	Yes	20,36,75
	Xestospongia cf. *carbonaria*, *X*. cf. *exigua* (Indopacific)			27,66
9-Aminobenzo[*b*]pyrido[4,3,2-*de*]-[1,10]-phenanthrolin-8(8*H*)-one **31**	*Biemna fortis* sponge (Indonesia)		Yes	30
Arnoamine A	*Cystodytes* sp. ascidian (Arno Atoll, Rep. Marshall Is.)	Yes	Yes	26,76
Arnoamine B	*Cystodytes* sp. ascidian (Arno Atoll, Rep. Marshall Is.)	Yes	Yes	36,76
Ascididemin **3**	*Didemnum rubeum* ascidian (Indonesia)	Yes	Yes	6
	Didemnum sp. (Okinawa)			28
	Cystodytes dellechiajei ascidian (Mediterranean, Spain)			25
	Unidentified ascidian (Singapore)			77
Biemnadin **37**	*Biemna fortis* sponge (Indonesia)			30
2-Bromoleptoclinidinone **10**	*Leptoclinides* sp. ascidian (Truk Lagoon)	Yes	Yes	24,65
Calliactine	*Calliactis parasitica* sea anemone			78,79
Cycloshermilamine	*Cystodytes violatinctus* ascidian (Comoros Islands)			80
Cyclodercitin	*Dercitus* sp. sponge (Bahamas)		Yes	32
Cystodytin A **32**	*Cystodytes* sp. ascidian (Fiji)	Yes	Yes	19,29
	Cystodytes dellechiajei ascidian (Okinawa)			81–83
Cystodytin B **33**	*Cystodytes dellechiajei* ascidian (Okinawa)	Yes	Yes	29,81–83
Cystodytin C **34**	*Cystodytes dellechiajei* ascidian (Okinawa)	Yes	Yes	29,81–83
Cystodytin D	*Cystodytes dellechiajei* ascidian (Okinawa)		Yes	82,83
Cystodytin E	*Cystodytes dellechiajei* ascidian (Okinawa)		Yes	82,83
Cystodytin F	*Cystodytes dellechiajei* ascidian (Okinawa)		Yes	82,83
Cystodytin G	*Cystodytes dellechiajei* ascidian (Okinawa)		Yes	82,83
Cystodytin H	*Cystodytes dellechiajei* ascidian (Okinawa)		Yes	82,83
Cystodytin I	*Cystodytes dellechiajei* ascidian (Okinawa)		Yes	82,83
Cystodytin J **27**	*Cystodytes* sp. ascidian (Fiji)	Yes	Yes	19,82
	Lissoclinum notti ascidian (New Zealand)		Yes	13,84
Cystodytin K	*Lissoclinum notti* ascidian (New Zealand)		Yes	13
Dehydrokuanoniamine **23**	*Cystodytes* sp. ascidian (Fijian)		Yes	19
Debromopetrosamine	*Xestospongia* cf. *carbonaria*			1
Debromoshermilamine A **24** = Shermilamine B	*Eudistoma* sp. ascidian (Red Sea)			85,86
Deoxyamphimedine **20**	*Xestospongia* sp. sponge (Philippines)		Yes	27
Dercitin **8**	*Dercitus* sp. sponge (Bahamas)	Yes	Yes	5,12,17,32,40,86
Dercitamide = Kuanoniamine C **17**	*Stelletta* sp. sponge (Bahamas)	Yes	Yes	12,17,40
	Oceanapia sagittaria sponge (Micronesia, Palau)		Yes	11,87
	Dercitus sp. sponge (Bahamas)		Yes	32
	Chelynotus semperi mollusc and unidentified ascidian (Pohnpei)			88
	Ascidian (Singapore)			77
	Cystodytes sp. ascidian (Pohnpei)			12
Dercitamine **39**	*Stelleta* sp. sponge			12
	Dercitus sp. sponge (Bahamas)		Yes	32
Diplamine **28**	*Diplosoma* sp. ascidian (Fiji)	Yes	Yes	8
	Cystodytes sp. ascidian (Fiji)	Yes	Yes	19,84,86
	Lissoclinum notti ascidian (New Zealand)		Yes	13
Eilatin **7**	*Cystodytes* sp. ascidian (Fijian)	Yes	Yes	19
	Eudistoma sp. ascidian (Eilat)			81,85,86
Eudistone A	*Eudistoma* sp. (Seychelles)		Yes	89
Eudistone B	*Eudistoma* sp. (Seychelles)			89
11-Hydroxyascididemin = Cystodamine **74**	*Leptoclinides* sp. ascidian (Truk Lagoon)	Yes	Yes	90
	Cystodytes dellechiajei ascidian (Mediterranean, Spain)			
Isodiplamine	*Lissoclinum notti* ascidian (New Zealand)		Yes	13
Isosegoline A	*Eudistoma* sp. ascidian (Red Sea)			81,85,86
Kuanoniamine A **9**	Pacific ascidian (unidentified)	Yes	Yes	40,91
	Chelynotus semperi mollusc and unidentified ascidian (Pohnpei)			88
	Unidentified ascidian (Singapore)			70
Kuanoniamine B	*Chelynotus semperi* mollusc			88
	Unidentified ascidian (Pohnpei)	Yes		17
Kuanoniamine C **17** = see dercitamide				
Kuanoniamine D **18**	*Oceanapia* sp. (Micronesia)	Yes	Yes	11,17
	Chelynotus semperi mollusc and unidentified ascidian (Pohnpei)			88
	Cystodytes sp. ascidian (Fiji, Pohnpei)	Yes		12,19,86
	unidentified ascidian (Singapore)			77
Kuanoniamine E	unidentified ascidian (Singapore)			77
Kuanoniamine F	unidentified ascidian (Singapore)			77
N-Deacyl kuanoniamine derivative **19**	*Oceanapia* sp. (Micronesia)		Yes	11

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Table 1 (*Contd.*)

Pyridoacridine	Source	Published synthesis	Cytotoxic	Ref.
Labuanine A **35**	*Biemna fortis* sponge (Indonesia)			30
Lissoclin A	*Lissoclinum* sp. ascidian (Australia)			9
Lissoclin B	*Lissoclinum* sp. ascidian (Australia)			9
Lissoclin C	*Lissoclinum* sp. ascidian (Australia)			9
Lissoclinidine	*Lissoclinum notti* ascidian (New Zealand)		Yes	13
Meridine **6**	*Amphicarpa meridiana* ascidian (S. Australia)	Yes	Yes	90
	Leptoclinides sp. sponge (Truk Lagoon)			90
	Corticium sp. sponge (Bahamas)			10
Meridine regioisomer **36**	Biemna fortis sponge (Indonesia)			30
5-Methoxyneoamphimedine **40**	*Xestospongia* cf. *carbonaria*, *X.* cf. *exigua* (Indopacific)		Yes	66
Neoamphimedine **16**	*Xestospongia* sp. sponge (Philippines)		Yes	20,48
	Xestospongia cf. *carbonaria* (Micronesia)			27
	Xestospongia cf. *carbonaria*, *X.* cf. *exigua* (Indopacific)			66
Neoamphimedine Y **41**	*Xestospongia* cf. *carbonaria*, *X.* cf. *exigua* (Indopacific)		Yes	66
Neoamphimedine Z **42**	*Xestospongia* cf. *carbonaria*, *X.* cf. *exigua* (Indopacific)		Yes	66
Nordercitin **38**	*Stelletta* sp. sponge	Yes	Yes	12,40
	Dercitus sp. sponge (Bahamas)			67
Norsegoline **21**	*Eudistoma* sp. ascidian (Red Sea)		Yes	85,86
Pantherinine	*Aplidium pantherinum* ascidian (S. Australia)		Yes	92
Petrosamine	*Petrosia* sp. sponge (Caribbean)			93
Sagitol	*Oceanapia sagittaria* sponge (Palau)		Yes	87
Sebastianines A	*Cystodytes dellechiajei* ascidian (Brazil)		Yes	94
Sebastianines B	*Cystodytes dellechiajei* ascidian (Brazil)	Yes	Yes	29,94
Segoline A	*Eudistoma* sp. ascidian (Red Sea)			80,85,86
Segoline B	*Eudistoma* sp. ascidian (Red Sea)			80,85,86
Segoline C	*Eudistoma bituminis* ascidian (Indian Ocean)			80,95
Shermilamine A **29**	*Trididemnum* sp. ascidian (Guam)			82,96
Shermilamine B **24** = Debromoshermilamine A	*Trididemnum* sp. ascidian (Guam)	Yes		82,84
	Chelynotus semperi mollusc and unidentified ascidian (Pohnpei)			97
	Cystodytes sp. ascidian (Fiji)		Yes	2,19
Shermilamine C **25**	*Cystodytes* sp. ascidian (Fiji)		Yes	2,19
Shermilamine D	*Cystodytes violatinctus* ascidian (Comoros Islands)		Yes	3,80
Shermilamine E	*Cystodytes violatinctus* ascidian (Comoros Islands)			3,80
Stellettamine	*Stelletta* sp. sponge		No	12
Styelsamine A	*Eusynstyela latericius* ascidian (Indonesia)		Yes	98
Styelsamine B	*Eusynstyela latericius* ascidian (Indonesia)	Yes	Yes	86,95,98
Styelsamine C	*Eusynstyela latericius* ascidian (Indonesia)		Yes	98
Styelsamine D	*Eusynstyela latericius* ascidian (Indonesia)	Yes	Yes	98
Varamine A	*Lissoclinum vareau* ascidian (Australia)		Yes	9,14
Varamine B	*Lissoclinum vareau* ascidian (Australia)		Yes	9,14

hyper-mutability, phage induction and increased DNA repair.[37] In the BIA assay λ-lacZ-dependent induction of β-galactosidase is a measure of DNA damage. Several agents that cause or interfere with the repair of DNA damage can induce the SOS response. However, not all DNA damaging agents induce this response.[37] McDonald *et al.* tested a series of eight pyridoacridines and determined that intercalation alone was not sufficient for a positive BIA response. Only eilatin **7** gave a positive response.[19]

2.2 Anti-viral activity

With the emergence of AIDS and the renewed recognition of the morbidity associated with many viral infections, a new emphasis has been placed on the discovery of anti-viral compounds. Furthermore, the recent successes of anti-human immunodeficiency virus (HIV) and anti-herpes simplex virus (HSV) drugs have given new hope to the field. Ideal anti-viral agents should inhibit viral reproduction at concentrations that are non-toxic to the host cells or organisms. Therefore, drugs are frequently tested for the ability to protect cells from viral-mediated killing at minimally- or non-toxic concentrations. Cell survival at the end of the incubation period is determined by a plaque forming assay[16] or by trypan blue exclusion (viable cells with intact membranes exclude indicator dyes and can be counted) or a colorimetric assay based on cellular metabolism (*e.g.*, MTT assay).[20,38,39] The binding of virus to the cell surface can be measured using fluorescent antibodies to viral proteins using fluorescence activated cell sorting or other fluorescence detection methodology.

Dercitin **8** exhibited strong inhibition of HSV-1 at 5 μg mL^{-1} with moderate cytotoxicity. It also completely inhibited murine A59 coronavirus at 1 μg mL^{-1} with no cytotoxicity.[17] **4** possessed anti-viral activity while **3** and kuanoniamine A **9** appeared inactive. **4** showed complete inhibition of HSV-1 at 80 μg mL^{-1} with no host cell toxicity (BSC-1 green monkey cells). It also displayed activity against polio virus type 1 (partial inhibition at 80 μg mL^{-1} with no detectable cytotoxicity to the Pfizer vaccine strain) and HIV-1 (46% protection at 0.7 μM with host cell toxicity at 10 μM).[15] Considerable anti-HIV activity was described for regioisomers of **8** and **9**, though none of the activities were found to be superior to azidothymidine.[40] Luedtke *et al.* have reported strong anti-HIV activity for **7**–Ru(II) complexes in CD4+ HeLa cells and human peripheral blood monocytes. They demonstrated the importance of planarity for this activity and provided evidence that the **7** complex is

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Table 2 Anti-microbial activity

	Cc[a]	Ec	Bs	Pa	Ca	Tm	Cr	Sa	Cn	Af	Mi	Ref.
MIC[b]												
Benzo[4,5]sampangine **4**					0.39				1.56	0.39	0.39	7
Sampangine **5**					1.56				0.78	1.56	0.78	7
Meridine **6**		–[c]	3.1	–		6.2			0.8			10
Amphotericin B					0.78				0.39	0.39	NT[d]	7
Rifampin					NT				NT	NT	0.78	7
Zone of inhibition[e]												
Ascididemin **3**		10	14	–	11	–	10					6
Benzo-1,3oxathiazoline						–						9
Benzo[4,5]sampangine **4**		8	–	–	12	10	–					6,7
Cystodytin J **27**		4	6		10	4						13
Cystodytin K		NT	5		NT	3						13
Diplamine **28**		+[f]						+				8
Diplamine **28**		3	9		–	12						13
Isodiplamine		–	4		4	1						13
Kuanoniamine C **17**	–	–	–					–				11
Kuanoniamine D **18**	–	–	–					–				11
Kuanoniamine *N*-deacyl derivative **19**	–	–	–					–				11
Lissoclin A						–						9
Lissoclin B						–						9
Lissoclin C						–						9
Lissoclinidine		6	8		9	6						13
Stellettamine				–								12
Varamine A					–							14
Varamine B					–							14

[a] *Cladosperium cucumenerinum* (Cc), *Escherichia coli* (Ec), *Bacillus subtilis* (Bs), *Pseudomonas aeruginosa* (Pa), *Candida albicans* (Ca), *Trichophyton mentagrophytes* (Tm), *Cladisporium resinae* (Cr), *Staphylococcus aureus* (Sa), *Cryptococcus neoformans* (Cn) *Aspergillus fumigatus* (Af), *Mycobacterium intracellulare* (Mi). [b] Minimum inhibitory concentration (μg mL^{-1}). [c] Drug was inactive (–). [d] Not tested (NT). [e] Zone of inhibition (mm) of anti-microbial and anti-fungal activity. [f] Activity was reported (+), but no concentrations were given.

interfering with Rev-RRE activity, resulting in the anti-HIV activity.[16] See Table 5.

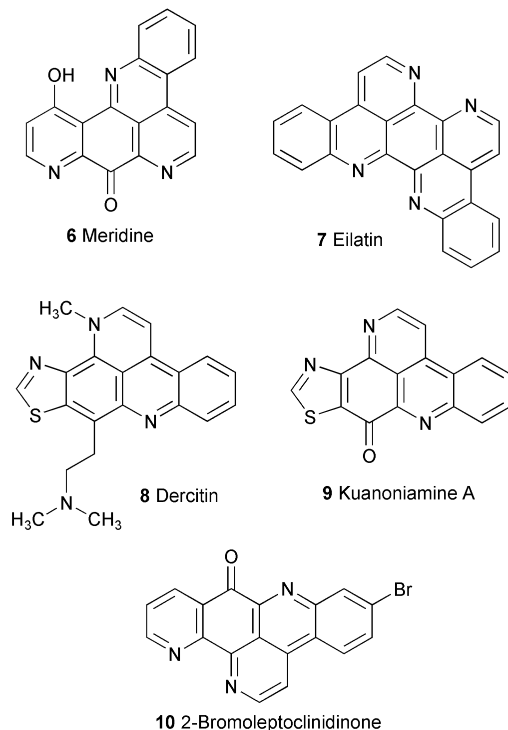


Table 3 Anti-fungal activity of meridine **6**

	Growth medium	MIC[a]	MFC	Ref.
Ca[b]	Sabouraud dextrose broth	3.1	3.1	10
Ca	YNBG	2.5	NT[c]	10
Ca	RPMI-1640	0.2	0.2	10
Cn	Emmon's SDB	0.8	6.2	10
Tm	Sabouraud dextrose broth	6.2	NT	10
Ef	Sabouraud dextrose broth	1.6	NT	10
Mg	Sabouraud dextrose broth	25	NT	10
Tb	Sabouraud dextrose broth	>25	NT	10
Ss	Sabouraud dextrose broth	>25	NT	10
Sb	Sabouraud dextrose broth	>25	NT	10

[a] MIC: minimal inhibitory concentration and MFC: minimum fungicidal concentration (μg mL^{-1}). [b] *Candida albicans* (Ca), *Cryptococcus neoformans* (Cn), *Trichophyton mentagrophytes* (Tm), *Epidermophyton floccosum* (Ef), *Microsporum gypseum* (Mg), *Trichosporon beigellii* (Tb), *Sporothrix schenkii* (Ss), *Scopulariopsis brevicaulis* (Sb). [c] NT, not tested.

Table 4 BIA[a] activity

	SOS induction	Ref.
Dehydrokuanoniamine **23**	Inactive	19
Shermilamine C **25**	Inactive	19
Cystodytin J **27**	Inactive	19
Kuanoniamine D **18**	Inactive	19
Shermilamine B **24**	Inactive	19
Eilatin **7**	+ > 2 μg/disk	19
Diplamine **28**	Inactive	19

[a] Biochemical prophage induction assay.

2.3 Anti-parasitic activity

Parasitic infections are responsible for millions of deaths each year in developing countries and for untold morbidity. Malaria alone claims over 2.7 million lives a year, one death every 13 seconds.[41,42] The search for new anti-parasitic drugs is therefore ongoing, although with less emphasis than oncological drug discovery. Recent work, discussed later, has demonstrated that many of the pyridoacridine alkaloids generate potentially cytotoxic reactive oxygen species (ROS) within cells. These are of particular interest as potential anti-parasitics, since ROS generation appears to be an effective mechanism of inhibition.

Table 5 Anti-viral activity

	HSV-1[a]	PV	A59	HIV	Ref.
Amphimedine **2**				–[b]	[c]
Ascididemin **3**	–	–		–	15
Benzo[4,5]-sampangine **4**[d]	+++	+		+, T	[c], 15
Dercitin **8**	++		+++		17
Deoxyamphimedine **20**				T	[c]
Eilatin **7**				+++	16
Kuanoniamine A **9**	–	–		–	15
Neoamphimedine **16**				T	[c]

[a] HSV-1, herpes simplex virus-1; PV, polio virus; A59, murine coronavirus; HIV, human immunodeficiency virus. [b] Relative activities are reported as active (+) most active being (+++), inactive (–) or too toxic to cells before effect was observed (T). [c] Unpublished data – Dr L. R. Barrows' group. [d] Benzo[4,5]sampangine **4** = deazaascididemin.

Table 6 Anti-parasitic activity

	P. falciparum strains		*L. donovani*		*T. cruzi*	*T. b. rhodes*	Mammalian cell lines		
	K1	NF54	Extracellular	Intracellular	Intracellular	Intracellular	L6[c]	RAW	Ref.
Ascididemin **3**	0.022[a]	0.020	8.70	ne[b]	19.400	4.000	1.70	>100	18
2-Bromolepto-clinidone **14**	4.420	0.020	>100	nd	0.220	1.760	0.25	>100	18
Benzo[4,5]-sampangine **4**	0.016	0.029	6.80	ne	0.680	0.002	1.90	>100	18
Sampangine **5**	0.160	0.180	48.00	35.00	0.230	2.000	0.63	3.14	18
11	0.450	0.330	>100	nd	0.056	0.015	1.10	>100	18
BC109 **12**	0.210	0.130	>100	nd	0.024	0.015	0.95	>100	18
13	0.024	0.034	3.40	ne	1.9000	6.610	5.10	>100	18
14	4.410	7.160	5.40	10.00	15.600	0.550	5.4	>100	18
15	0.480	0.970	5.70	ne	0.640	0.070	7.30	>100	18

[a] Values are 50% inhibitory concentration in μg mL^{-1}. [b] ne = no evaluation possible, nd = not determined. [c] L6, myoblast cell line and RAW, macrophage-like cell line.

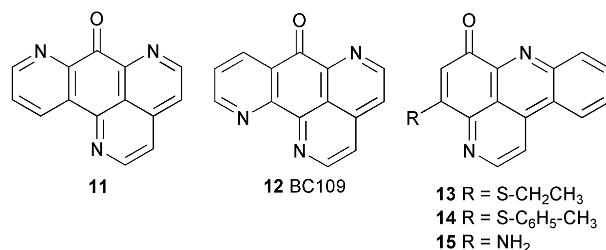
The data suggest, however, that other molecular mechanisms also contribute to pyridoacridine anti-parasitic activity.

Plasmodium falciparum anti-plasmodial activity is often compared amongst strains of normal and resistant drug sensitivity. The work of Copp and colleagues employed the *P. falciparum* NF54 (normal) and K1 (chloroquine and pyrimethamine resistant) strains.[18] Intra-erythrocytic inhibition of parasite growth is commonly determined by measuring the incorporation of radioactive (or fluorescent alternative) hypoxanthine. For this assay, human red blood cells were infected with *P. falciparum* in hypoxanthine-deficient culture medium and exposed to replicate drug dilutions in microtiter plates for a period of two days. Labelled hypoxanthine was then added for another 24 hours. The drug concentration required to achieve 50% inhibition of maximal hypoxanthine incorporation was then calculated.

Trypanosoma brucei inhibition can also be quantified in microtiter culture. Replicate drug dilutions were added to small volumes of the bloodstream forms of *T. brucei*. Cultures were then grown in cell culture medium for three days, after which time, surviving parasites were quantified using a metabolic indicator, such as Alamar blue.

Rodent skeletal myoblasts can form "feeder layers" for *Trypanosoma cruzi* in culture assays that can be performed in microtiter plates. Copp and colleagues utilized a *T. cruzi* strain transfected with a β-galactosidase gene reporter construct. After infection of the myoblasts, the culture wells were supplied with replicate drug dilutions in culture medium and incubated for approximately 4 days. At that time, a chromophore substrate was added and the inhibition of trypomastigote growth calculated.

Pyridoacridine activity against *Leishmania* sp. parasites has been reported using a metabolic assay. *Leishmania* promastigotes were grown and treated in culture and pelleted by centrifugation. Promastigotes were lysed by osmotic shock, homogenized and centrifuged to yield a crude mitochondrial pellet. Mitochondrial NADH-fumarate reductase activity is a measure of the fumarate-dependent increase in the rate of NADH-oxidation, which was measured spectrophotometrically in 1 mL cuvettes. Data provided by Copp and co-workers demonstrated the potency of several analogues of **3** against cultured parasites (Table 6). **3** was one of the most potent of 18 analogues tested against malaria and leishmania, while its synthetic deazaanalogue, **4**, had comparable activity. Analogues lacking the D ring also showed good activity, though slightly less. These same analogues were among the most active against the trypanosomal lines as well; however the rank order of their potency was different. The authors concluded that the therapeutic window for these drugs is probably too small. However, the parasitic selectivity was approximately 50 fold for several of the most active anti-malarial compounds, as compared to the most sensitive mammalian cell line. We believe that this may afford enough selectivity against blood-borne parasites, especially for the treatment of blood reserves extra-corporally. Further inspection suggests that the molecular mechanism of action that appears to correlate best with anti-parasitic activity in this system is simple DNA binding, because the most potent DNA binders in the series (reviewed below) have the best anti-malarial and anti-leishmanial activity.



In collaboration with Dr Cyrus Bacchi, Pace University, we have tested **2** and neoamphimedine **16** against *T. brucei brucei* (unpublished data, L. R. Barrows' group). This was performed because **16** has a unique mechanism of top2 inhibition (reviewed

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Table 7 Insecticidal activity of the kuanoniamines

	Kuanoniamine C **17**	Kuanoniamine D **18**	*N*-Deacyl derivative **19**	Ref.
Sl[a]	156 ppm	59 ppm	>934 ppm	11
As	37 μg mL^{-1}	19 μg mL^{-1}	>100 μg mL^{-1}	11

[a] *Spodoptera littoralis* (Sl)-neonatal larvae, *Artemia salina* (As)-brine shrimp.

below), that might selectively impact parasites of the order Kinetoplastida. In this study, **2** had no activity against *T. b. brucei* while **16** was quite active with an IC_{50} of 0.065 μg mL^{-1} (~0.21 μM).

2.4 Insecticidal activity

There is limited information in the literature concerning pyridoacridine insecticidal activity.[11] Kuanoniamines were studied using neonatal *Spodoptera littoralis* larvae. Three different kuanoniamines were fed to the larvae for 6 days and the 50% lethal concentration (LC_{50}) was determined. Brine shrimp 24 h lethality data from the same reference are provided for comparison (Table 7).

2.5 *In vivo* anti-tumor activity

Much of the impetus for natural products research has derived from the search for new anti-cancer agents. Primary screens have tended to test compounds for cytotoxicity against cultured tumor cell lines. Some molecules that have shown unusual activity in cultured mammalian or human cell lines have been tested further in animal models. The two *in vivo* assays used to assess pyridoacridines have been the hollow fiber assay developed by Plowman and colleagues[43,44] and the tumor implant assays developed over the years by Skipper and coworkers.[45,46] Hollow fibers are tubes produced from permeable membranes that are capable of supporting the growth of cultured tumor cells installed into the lumen. These cell-filled fibers may be implanted into the peritoneal cavity, or subcutaneously into the flanks or backs of immunosuppressed mice. Following implantation and treatment of the animals, the fibers are removed, the tumor cells retrieved and their viability determined. The data are reported as %T/C, representing the % viability of the tumor cells retrieved from the fibers of treated animals over the viability of tumor cells retrieved from control animals. Thus, injected drugs may be tested for activity against several tumor lines in one animal, at sites distal to injection.

Allograft or xenograft tumor implant models allow testing of drugs for activity against actual rodent or human leukemias and tumors. Typically, immunosuppressed mice are injected with one to several million cultured cancer cells that are capable of growing in the rodent host. The life span of the animal or the tumor volume is monitored. Two of the principal types of these tumor models have been used to assess pyridoacridines. In the first, mouse leukemia cells (*e.g.*, 10^6 P338 cells) are injected into the peritoneum of DBA/2 mice. These mice live for approximately 10 days. The tumor bearing mice are then treated with the test drug, which is administered i.p. in the data reported here. Response is measured as increased life span (%ILS). Different groups accept different ILS values as significant; typically from 130% to 170%. Cultured human tumor cells (10^6 to 10^7) are injected s.c. on the sides or backs of immunosuppressed mice for the xenograft implant model. Tumor growth is monitored by orthogonal external measurement with calipers, and tumor volume is calculated. Treatment, usually multiple drug administrations, begins after the tumors reach a detectable volume of approximately 50 mm^3. The mice are treated (usually i.p., i.v., p.o. or s.c.) with the drug and their body weight and tumor size are measured periodically.[20–22,44,47] %T/C is defined as percent of treated tumor volume over control tumor volume. The i.p./i.p. mouse leukemia system is considered less rigorous than the flank tumor model for two reasons. First, leukemia cells are generally more responsive to drugs than solid tumors, and second, delivering the drug into the same compartment that houses the tumor cells does not require drug absorption and distribution to distal sites of action.

2 was the first pyridoacridine isolated and it was reported to be cytotoxic[36], but no *in vivo* data were reported until recently.[20,48] **8**, however, yielded early positive *in vivo* data, providing a precedent of activity for the class.

2-Bromoleptoclinidinone **10** had excellent *in vitro* cytotoxicity, but when tested in xenograft models, proved too toxic to yield significant anti-tumor responses (Table 8).[49] **3** was also tested *in vivo* as a consequence of its observed *in vitro* potency and subpanel selectivity.[49,50] It was tested at the USA National Cancer Institute against twelve human tumor cell lines in a preliminary *in vivo* hollow fiber assay.

As shown in Table 8, **3** exhibited significant activity (%T/C < 50) against six of the cell lines. Notably, it demonstrated activity at a site remote (s.c.) from the drug delivery point (i.p.) in the LOX IMVI (melanoma), and NCI-H23 (non-small cell lung cancer) tumors and also resulted in net cell kill. These results satisfied the criteria for **3**'s testing in subcutaneous xenograft assays. It was tested against HCT 116 (colon), U251 (CNS), OVCAR-3 (ovarian), LOX IMVI (melanoma), and two breast cell lines MDA-MB-435 and MDA-MB-231. Three doses were used with each tumor system.

Unfortunately, **3** showed only weak activity in the xenograft models with the best response, 58% T/C, being achieved in the HCT 116 human colon tumor xenograft at a dose of 8 mg kg^{-1} i.p. on days five and nine.[21] Although this level of response was not considered significant, HCT 116 is a relatively refractory tumor and new understanding of structure–cytotoxicity relationships of **3** may improve the spectrum of *in vivo* anti-tumor activity observed. Although not significantly active in the other tumor lines, maximally tolerated doses were obtained only in the HCT 116 bearing animals. It is conceivable that at higher doses, significant anti-cancer activity could have been achieved in the other systems (personal communication, B. R. Copp).

Recent work by our group with **2** confirmed the earlier reported lack of activity *in vivo*. An analogue of **2**, deoxyamphimedine **20**, is a potent ROS generator and DNA binder (see below). **20** also failed to show anti-cancer activity, toxicity limiting the preliminary dose escalation study at 25 mg kg^{-1} i.p. in nude mice (unpublished data, L. R. Barrows' group). **16**, on the other hand, demonstrated potent anti-tumor activity in two different xenograft models, providing an interesting contrast in activity to **20** and **2** that could be correlated with its contrasting *in vitro* activities (Table 8). No toxicity was observed in nude mice treated with **16** up to the highest dose tested, 50 mg kg^{-1}.[20]

2.6 *In vitro* anti-tumor activity

The effects of several pyridoacridines on markers of leukemia and tumor growth have been assessed in *in vitro* or explant systems (Table 9). Longley and colleagues evaluated **8** and **6** cytotoxicity and found they are approximately equally potent against a solid human lung cancer line and a mouse leukemia line.[33] Also, a mouse lymphoblast line was used as a model of cell adhesion. In this model, the presence of phorbol-12-myristae-

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Table 8 *In vivo* anti-tumor activity

Ascididemin **3**	Dose/mg kg^{-1}	Hollow fiber (% T/C[a])		Xenograft	Ref.
Cell line[b]	12	i.p.[c] fiber	s.c. fiber		
OVCAR-3		10*[d]	76	NS[e]	21
SF-295		22*	81	NS	21
MDA-MB-435		30*	80	NS	21
MDA-MB-231		40*	100	NS	21
NCI0H23		77	44*	NS	21
LOX IMVI		97	29*	NS	21
Tumor xenografts					
2-Bromoleptoclinidinone **10**					
Cell line	Dose/mg kg^{-1}	% T/C		Drug deaths	
SW-620	10	Toxic		5	49
	6.7	Toxic		4	49
	4.5	90		1	49
HCT 116	10	Toxic		6	49
	6.7	Toxic		5	49
	4.5	97		1	49
A498	16.7	83		1	49
	11.2	85		0	49
	7.5	93		0	49
LOX IMVI	10	Toxic		6	49
	6.7	50		2	49
	4.5	82		0	49
Dercitin **8**					
Cell line	Dose/mg kg^{-1}	% T/C		%ILS[f]	
P388	5			170	5
	2.5			160	5
	1.25			145	5
	0.625			140	5
B16	5			109	5
	2.5			116	5
	1.25			125	5
	0.625			120	5
Lewis lung	5	0.59			5
	2.5	0.58			5
	1.25	0.49			5
	0.625	0.82			5
Amphimedine **2**	Dose (mg kg^{-1})	%T/C			
KB	50	NE[g]			20
Deoxyamphimedine **20**					
KB	25	NE			[h]
	12.5	NE			[h]
Neoamphimedine **16**					
KB	50	35*			20
HCT 116	50	36*			20
	25	30*			20
	12.5	36*			20
Etoposide					
KB	50	31*			20
9-Aminocamptothecin					
HCT 116	1	26*			20

[a] % T/C, percent of treated tumor volume divided by control tumor volume. [b] Cell lines are as follows: OVCAR-3 (ovarian tumor), SF-295 (CNS tumor), MDA-MB-435 and MDA-MB-231 (breast tumors), NCI0H23 (non-small cell lung tumor), LOX IMVI (melanoma), SW-620 and HCT 116 (colon tumors), A498 (renal tumor), P388 (leukaemia), B16 (melanoma), Lewis lung carcinoma and KB (nasopharyngeal carcinoma). [c] i.p., intra-peritoneal; s.c., subcutaneous. [d] * = Significant difference observed ($p < 0.05$). [e] NS, not significant in xenograft models. [f] %ILS, percent increased lifespan. [g] NE, no anti-tumor effect. [h] Unpublished data – L. R. Barrows' group.

13-acetate (PMA) causes the cells to adhere to culture substrate in a protein kinase C dependent manner. **8** inhibited the PMA stimulated activity, while **6** stimulated adhesion.

7 and norsegoline **21** were tested against acute myeloid leukemia (AML) cell lines and fresh isolates for the ability to differentially affect cells proliferating in suspension *versus* those grown in agar.[34] This assay was developed with cytosine arabinoside (Ara-C), the most active drug against AML, and Ara-C was preferentially toxic to cells grown in suspension. Cell growth in agar is purported to mimic the proliferatively inert terminal divisions of blast cells, while growth in suspension reflects "self renewal". Both **7** and **21** were more toxic to cells in suspension, **21** having effects equivalent or superior to Ara-C. **7** and **21** were then compared to Ara-C for the ability to regulate cell surface markers of differentiation. All three molecules reduced cell surface expression of CD34 (a stem cell marker) and increased the expression of CD11 (an integrin family molecule) and CD14 (a lipopoly-saccharide receptor).[34]

7 was studied further for the ability to target myeloid progenitor cells from chronic myeloid leukemia (CML) patients in blast crisis.[35] **7** was found to be equally effective with interferon-α and Ara-C in inhibiting colony formation in samples from CML patients in blast crisis. Furthermore, all of these agents demonstrated selectivity toward blast crisis samples compared to samples from patients in chronic phase leukemia. **7** was found to be approximately twice as effective at eliminating cells containing the characteristic Philadelphia chromosome *Bcr/Abl* fusion gene (by FISH analysis) from blast crisis or

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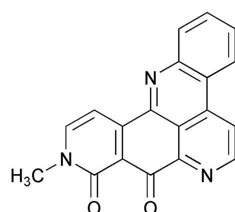
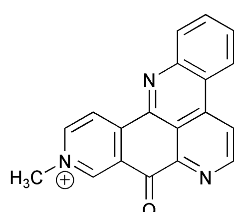
Table 9 *In vitro* anti-tumor activity

Cell adherence				
	EL-4.IL-2 (IC_{50})			Ref.
Dercitin **8**	Inhibitor <6.0 µg mL^{-1}			33
Meridine **6**	Inducer 8.8 µg mL^{-1}			33
Cell surface expression[a]				
	CD11	CD14	CD34	
Eilatin **7**				
HL-60	↑	↑	↓	34
AML-3	↑	↑	↓	34
AML-4	↑	↑	↓	34
Blast[b]	↑	↑	ND[c]	34
Norsegoline **21**				
HL-60	↑	↑	↓	34
AML-3	↑	↑	↓	34
AML-4	↑	↑	↓	34
Blast	↑	↑	ND	34
Ara-C				
HL-60	↑	↑	↓	34
AML-3	↑	↑	↓	34
AML-4	↑	↑	ND	34
Blast	↑	↑	ND	34
Inhibition of primary colony formation by CD34+ cells				
	% Inhibition			
	Agar	Suspension		
Blastic crisis				
Eilatin **7** 10^{-7} M	50	44		35
IFN-α 500 U/mL	23	24		35
Ara-C 10-9 M	31	16		35
Chronic phase				
Eilatin **7** 10-7 M	38	40		35
IFN-α 500 U/mL	17	20		35
Ara-C 10-9 M	31	27.5		35
Percent of BCR/ABL fusion product[d]				
	Blastic	Chronic		
Control	94.6	94.6		35
Eilatin **7**	48.6	61.0		35
IFN-α	60.6	66.0		35
Ara-C	76	64.6		35

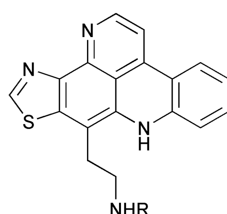
[a] Cell surface expression compared to control cells. [b] Blast, primary AML blasts. [c] ND, not enough cells to make a meaningful interpretation. [d] Observed in cells expressing 100% Ph+ on day one, treated for 7 days.



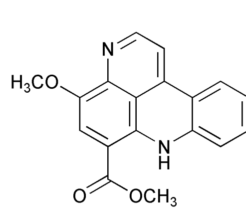
16 Neoamphimedine



20 Deoxyamphimedine



17 R = COC_2H_6 (Kuanoniamine C)
18 R = $COCH_2$ (Kuanoniamine D)
19 R = H (*N*-Deacyl derivative)
22 R = COCH=CMe_2 (Kuanoniamine B)



21 Norsegoline

chronic phase leukemic cells as either interferon-α or Ara-C.[35] Further work by Einat and coworkers showed that hematopoetic progenitors, following exposure to **7** or Ara-C, were readily stimulated to recovery expansion by IL-11 or stem cell factor in conjunction with IL-3 and granulocyte macrophage colony stimulating factor.[51]

3 Molecular mechanisms of activity

Discussions to this point have focused on pyridoacridine cytotoxicity in various systems. Recent work has demonstrated that some pyridoacridines possess unique and selective molecular mechanisms of toxicity. Section 3 will focus on activities reported for the pyridoacridines at the cellular or molecular level.

3.1 DNA-directed activity

As mentioned earlier, pyridoacridine toxicity has often been attributed to the ability to intercalate into DNA. Pyridoacridines are generally planar compounds that are able to interpose between stacked base pairs. This intercalation may alter DNA topology and change the way DNA metabolizing enzymes interact with their substrate. This may result in inhibition of many DNA metabolizing processes, including synthesis or topoisomerization. DNA intercalation may also allow pyridoacridines that generate ROS to release their radicals in close proximity to DNA resulting in greater oxidative damage.

3.1.1 DNA binding. The ability to bind or intercalate DNA often becomes apparent when the presence of a drug alters the mobility of DNA subjected to electrophoresis. Intercalation into closed circular DNA will alter the apparent linking number (supercoiling) and increase or decrease the movement of DNA through the gel upon electrophoresis. Charged molecules that bind DNA in other ways can render similar effects. Sophisticated "footprint" type analyses also can be applied to relatively small

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DNA molecules, and provide much information about their DNA binding preferences. In these assays, DNA radiolabeled at one end is subjected to a treatment that will uniformly degrade the polymer and generate an even distribution of fragments over a given stretch of DNA. This yields an even pattern of bands when analyzed by polyacrylamide gel electrophoresis and autoradiography. Specific information about a drug's DNA binding can be inferred if the presence of drug protects a particular region of DNA from fragmentation or increases fragmentation in another.

The ability to displace ethidium bromide (EtBr) from DNA is another frequently used measure of intercalation. Although strictly speaking it is a measure of DNA binding and additional evidence is usually required to prove intercalation (*e.g.*, shift in lambda max, DNA unwinding, absorbance quench, *etc.*). The K_{disp} is most conveniently defined as the concentration of compound needed to decrease DNA-bound EtBr fluorescence by 50%. Common conditions begin with approximately 0.5 µM EtBr and 0.5 µM DNA. The displacement of EtBr from DNA is easily quantified by measuring the decrease in fluorescence around 600 nm (excitation around 530). Furthermore, through the use of defined DNA substrates, an appreciation of the DNA sequence requirements for drug binding can be obtained.

Thus, DNA intercalation is considered prototypic of the pyridoacridines and many pyridoacridine bioactivities correlate with their DNA binding. Signature bioactivities include the ability to unwind supercoiled DNA, inhibit DNA polymerase I and decrease DNA mobility in a gel electrophoresis assay. Early on, **8** was found to exert these activities. It was found that DNA in solution quenched **8** absorbance (514 nm) while an equimolar concentration of unpolymerized dNTPs had no effect. In fact, the presence of extracellular DNA in culture medium protected P388 cells from **8** cytotoxicity.[5] Luedtke and coworkers demonstrated that **7**–Ru(II) complexes had affinity for nucleic acids when using a very small percentage of EtBr intercalation to start with.[16] Kuanoniamine B **22** also appears to be a DNA intercalator as its fluorescence is quenched in the presence of DNA (Table 10).[12]

Pyridoacridines vary in their affinity for DNA, as determined by an EtBr displacement assay. **7**, dehydrokuanoniamine B **23**, shermilamine B **24** and shermilamine C **25** all required more than 100 µM concentrations to displace 2.5 µM EtBr from 50 µg mL^{-1} bovine DNA (although **7** was shown to be a DNA intercalator by fluorescence quenching).[19] AK36 **26** is a synthetic analogue synthesized by Dr Kashman, Tel Aviv University, Israel, that did not displace EtBr from DNA.[23] Thus, not all pyridoacridines are good intercalators. Furthermore, the ability to displace EtBr does not always correlate with an increase in cytotoxic potency. In some cases intercalation strength appears to correlate with cytotoxic potency[18,20], in others it appears that intercalation is necessary but not sufficient for cytotoxicity[19], and in other cases, it appears that cytotoxicity is unrelated to intercalation.[23,31]

In a sophisticated study of **3**–DNA interactions, **3** UV absorbance and λ maxima were quenched and shifted, respectively, as would be expected of a DNA intercalator.[25] Electric linear dichroism suggested a parallel insertion of the heterocycle into the base pairing of the DNA helix in both poly AT and poly GC templates. However, DNase I protection studies suggested a greater affinity for GC base pairs. The disturbance of the DNA helix could be detected over several neighboring base pairs extending from the site of intercalation, a result seen with other DNA intercalating molecules.[25] Using EtBr displacement to rank the intercalation of **3** and several analogues, we found those of **3** and **5**, with K_{disp}s around 1 µM, were significantly stronger DNA binders than **3**'s azaanalogue (**4**), with K_{disp} around 10 µM, which, in turn, was a significantly stronger binder than **5**'s deazaanalogue (**12**), with K_{disp} of approximately 20 µM (unpublished data, L. R. Barrows' group). In a separate study using different concentrations of EtBr (approximately 8 times the concentration of EtBr), a K_{disp} of 70 µM was determined for **4**.[23] **2** is a poor intercalator compared to **16**. On the other hand, **20** is a much more potent DNA binder, possibly due to its positive charge.

Table 10 DNA binding activity

	DNA affinity (K_{disp})[a]	Ref.
	calf thymus DNA	
Cystodytin J **27**	54	19
Dehydrokuanoniamine B **23**	>100	19
Dercitin **8**	3.1	5
Diplamine **28**	21	19
Eilatin **7**	>100	19
Eilatin–Ru(II)[b]	0.1, 0.4	16
Kuanoniamine D **18**	62	19
Shermilamine B **24**	>100	19
Shermilamine C **25**	>100	19
Adriamycin	16.5 (bovine DNA)	5
Ascididemin **3**	+[d]	25
Dercitin **8**	9.4 (*C. perfringes*)	5
Kuanoniamine B	+	12
	salmon testes sperm DNA	
AK36 **26**	>100	23
AK144 **43**	1.12	[c]
AK165 **35**	1.59	[c]
AK199 **45**	1.26	[c]
Amphimedine **2**	ND[e]	[c]
Benzo[4,5]sampangine **4**	20, 70.8	23,[c]
Deoxyamphimedine **20**	1.5	[c]
Neoamphimedine **16**	100	[c]
	% inhibition	
Ascididemin **3**	100% at 10 µM	21
Benzo[4,5]sampangine **4**	not complete at 20 µM	21
BC109 **12**	not complete at 20 µM	21
Sampangine **5**	100% at 20 µM	21

[a] DNA affinity K_{disp}/µM (50% EtBr displacement). [b] Eilatin–bipyridine–ruthenium complexes (Λ and Δ conformations, respectively). [c] Unpublished data – L. R. Barrows's group. [d] K_{disp} not reported (+). [e] Not determined (ND).

3.1.2 Inhibition of macromolecule synthesis. The inhibition of cellular processes, such as the synthesis of critical macromolecules, can provide insight into the cellular targets of drug action. One simple procedure to assess this is to seed mammalian cells into culture dishes at a constant number and treat them with the toxin at a given concentration (*i.e.*, LC_{50} or LC_{90}). The cell cultures are then exposed to radioactive precursors of DNA, RNA or protein (*e.g.*, thymidine, uracil or leucine) for short periods of time. The radioactive precursor is then removed from the medium and the radioactivity is chased into full length macromolecules during a period of further cell growth. The cells are usually lysed in SDS and the macromolecules trapped on glass filters while the small molecular weight precursors are rinsed away. Liquid scintillation counting quantifies the amount of radioactive precursor incorporated into cellular macromolecules during the pulse period. Early decreases of precursor incorporation into DNA or RNA are consistent with the DNA-directed effects characteristic of the marine metabolites discussed here.

The ability of pyridoacridines to inhibit macromolecular synthesis has only been examined in this way (Table 11). **8** at a high concentration (1.5 µM, compared to an IC_{50} for the same cells of 81 nM) inhibited DNA, RNA and protein synthesis by more than 90% over the first 30 minutes of exposure. A lower concentration partially inhibited DNA, RNA and protein synthesis, with the latter being least affected.[5] As mentioned above, the inhibitory effect of **6** on DNA synthesis in *C. albicans* was achieved at much lower concentrations than was needed to inhibit the other synthetic pathways.[10] Cystodytin J **27** and diplamine **28** were also examined for their ability to inhibit macromolecular synthesis. This protocol used equitoxic

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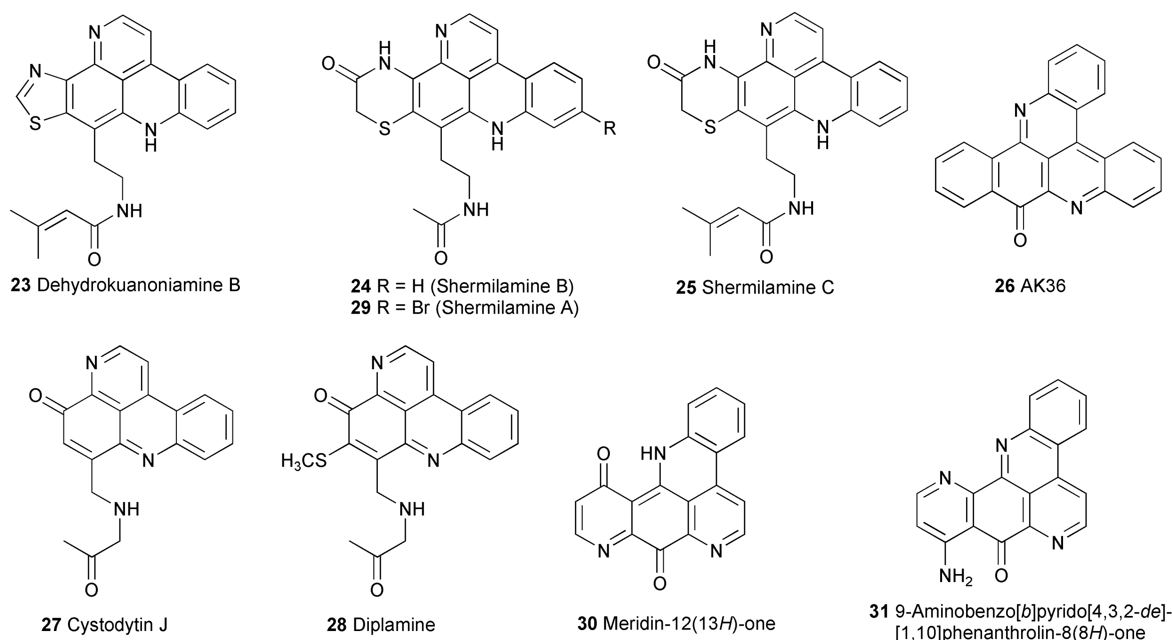

Table 11 Inhibition of DNA, RNA and protein synthesis

Compound	Concentration	Synthesis inhibition (%)[a] DNA		RNA		Protein	Ref.
Dercitin **8**	0.4 μM	61		83		23	5
	1.5 μM	99		95		92	5
Actinomycin	10 μM	94		95		42	5
1-*β*-D-Arabino-furanosylcytosine	10 μM	98		0		0	5
Daunomycin	0.5 μM	49		49		0	5
Emetine	1 μM	72		49		100	5
		DNA		RNA		Protein	
		1–3 h	6–9 h	1 h	9 h	9 h	
Cystodytin J **27**	10 μg mL^{-1}	50%	90%	50%	90%	No effect	19
Diplamine **28**	10 μg mL^{-1}	50%	90%	50%	90%	No effect	19
Concentration (μg mL^{-1}) to decrease incorporation of radiolabeled substrate by 50% compared to control[b]							
	Adenine	Leucine	Glucose	Acetate	Ref.		
Meridine **6**	1	50	>100	>100	10		
5-Fluorouracil[c]	6	>100	>100	>100	10		
Blasticidin	>100	6	>100	>100	10		

[a] Determined using (^{3}H) radiolabeled precursors: thymidine (DNA), uridine (RNA) and leucine (protein). [b] Determined using (^{14}C) radiolabeled precursors: adenine (DNA and RNA synthesis), leucine (protein), glucose (general metabolism), and acetate (membrane transport). [c] 5-Fluorouracil, inhibitor of nucleic acid synthesis and blasticidin, inhibitor of protein synthesis.

concentrations (approximately IC_{90}) of the drugs and assessed DNA, RNA and protein synthesis at 1, 3, 6 and 9 hours. The earliest observed effects were on DNA and RNA synthesis with protein synthesis being unaffected in this protocol at the time points tested.[19]

3.1.3 Inhibition of the topoisomerase enzymes. Type 1 topoisomerases (top1 and top3) produce ATP-independent single strand DNA breaks that allow the rotation of the scissile strand of the DNA helix around the uncut strand of DNA. Type 2 topoisomerases (top2 α and β and top4) are ATP-dependent enzymes that catalyze transient double strand DNA cleavage and the passage of one DNA helix through another. Currently top1 and top2 are the only clinically relevant topoisomerase targets used in cancer chemotherapy. The short lived breaks created by top1 or top2 can be stabilized by drugs resulting in DNA breakage and eventually cell death. Most of the top2 poisons in the clinic are DNA-binding planar quinone molecules. Several pyridoacridines inhibit top2 (Table 12).

Several of the different assays have been employed to screen drugs for topoisomerase-directed drug activity. These include the use of purified enzyme systems or modified mammalian- or yeast-cell lines.[20–22,52–54]

Many DNA binding or intercalative compounds can inhibit the catalytic function of topoisomerases. Fortunately, assays for topoisomerase inhibition are relatively simple and specific. Topoisomerases are the only enzymes known which relieve topological strain (supercoiling) in DNA. Their activity is readily visualized as the production of topoisomers, or totally relaxed circular DNA, from supercoiled plasmid DNA that has been exposed to enzyme. These DNA isomers are easily resolved by agarose gel electrophoresis and visualized. The inhibition of enzyme is obvious by the absence of relaxed DNA topoisomers and provides a qualitative/semi-quantitative measure of a compound's inhibitory potency.

A specific measure of top2 activity can be obtained by assessment of DNA decatenation. Top2 induces a DNA double strand break as a normal intermediate step in its catalytic cycle. The enzyme cuts one helix of DNA, passes another helix through the cut and then ligates the ends back together. Kinetoplast DNA from the insect parasite trypanosome *Crithidia fasciculata* is composed of a "chain mail" of interlinked DNA circles. Type

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Table 12 Topoisomerase inhibition activity

	Top2	Top1	Ref.
Amphimedine **2**	–[a]	–	20,24
Arnoamine A	+ > 90 μM		26
Arnoamine B	+ > 90 μM		26
Ascididemin **3**	140	140	25
Ascididemin **3**	75		24
Benzo[4,5]sampangine **4**	+	+	23
Meridin-12(13*H*)-one **30**	–		24
Neoamphimedine **16**	–	–	20
Sampangine **5**	+	+	23
Shermilamine A **29**	–		24
Shermilamine B **24**	30		24
	Top2 (IC_{90})[b]		
Cystodytin J **27**	8.4		19
Dehydrokuanoniamine **23**	115		19
Diplamine **28**	9.2		19
Eilatin **7**	ND		19
Etoposide	68		19
Kuanoniamine D **18**	127		19
m-AMSA	33		19
Mitoxantrone	1.1		19
Shermilamine B **24**	118		19
Shermilamine C **25**	138		19

[a] Inhibition was observed (+) or not seen (–). [b] Concentration required to inhibit 90% of the topoisomerase activity.

II topoisomerases are the only known enzymes that can release intact closed circular monomers from this kinetoplast substrate.

Inhibition of topoisomerase catalysis is not the ultimate indicator of a molecule's anti-cancer potential. It was recognized about 20 years ago that the most clinically useful topoisomerase inhibitors were able to stabilize the covalent enzyme–DNA reaction intermediate.[55] This is commonly referred to as the cleavable complex, or the cleavage complex, and is the step of catalysis in which topoisomerase has cut and is covalently linked to the DNA. These stabilized cleavage complexes result in DNA breakage in the cell and their production can be detected in cell lines deficient in DNA repair. Chinese hamster ovary lines such as the xrs-6, which is ku80 deficient, and thus recombination-repair deficient, is supersensitive to killing by top2 poisons.[56] The CHO line EM9 is DNA ligase III deficient and thus deficient in single strand break repair and supersensitive to killing by top1 poisons.[57]

Inhibition of topoisomerase catalysis in the absence of cleavage complex stabilization is likely a measure of non-specific DNA-directed toxicity and suggests that the chemical's interactions with DNA might inhibit many DNA metabolizing enzymes. Unfortunately, nonspecific inhibitors of topoisomerases have not yet proved to be clinically useful anti-cancer agents.

Engineered yeast strains are a powerful tool to study topoisomerase drugs. Yeast possess DNA repair pathways (complementation groups) and type I and II topoisomerases somewhat similar to those of higher eukaryotes. The advantage of yeast systems over mammalian ones is that culture procedures are simpler and mutants are much easier to isolate. These advantages have been exploited in a way to provide a specific screen for topoisomerase active molecules.[20,52,53] *Sacharomyces cerevisiae Rad* 52 mutants are deficient in the repair of DNA strand breaks (such as those caused by cleavage complex stabilizing topoisomerase poisons) and meiotic recombination. A *Rad* 52 strain that over expresses yeast top2 has been isolated that exhibits supersensitivity to top2 cleavage complex stabilizers. Additionally, a *Rad* 52 top1 deleted strain has been developed. Compounds that are supertoxic to the parental *Rad* 52 (topoisomerase normal) strain, but much less so to the *Rad* 52 top1 deleted strain, are likely top1 poisons. Topoisomerase directed activity can be quantified in these yeast strains by measuring zones of inhibition around chemical-treated disks, by determination of minimal inhibitory concentrations or by titering.

A more quantitative measure of DNA cleavage can be obtained by liquid scintillation counting of the cleavage products. In contrast to analysis by DNA sequencing technology, the cleavage of plasmid DNA is quantified following agarose gel electrophoretic fractionation of the reaction products. When plasmid DNA is subjected to gel electrophoresis in the presence of EtBr, four distinct bands can be resolved. These bands contain nicked circular DNA, linearized DNA, negatively supercoiled DNA, or fully relaxed but intact circular DNA which has become positively supercoiled by the intercalation of EtBr in the running buffer. If the plasmid DNA is radiolabeled, the individual DNA bands can be cut from the DNA gel and quantified by liquid scintillation counting. This technique is superior to DNA sequencing analysis for quantification of drug-DNA cleavage, either direct or topoisomerase induced, because cleavage over a much longer stretch of DNA is measured (plasmid length *versus* the 200 or 300 base pairs visualized on a sequencing gel). It also requires fewer manipulations of the fragmented DNA reaction products than are needed for polyacrylamide gel electrophoresis.

The ability to inhibit top2 was reported for **24** and **3** in a study including **2**, shermilamine A **29**, meridian-12(13*H*)-one **30** and **10**. These assays were performed at SmithKline French laboratories and the precise protocol was not reported.[24] In a study of pyridoacridines from a *Cystodytes* sp. ascidian, all six of the molecules tested inhibited top2 catalysis.[19] However, only **27** and **28** inhibited the activity at concentrations comparable to the positive control drugs mitoxantrone and *m*-AMSA. **5**, **4** and **24** have been reported to inhibit top2 at relatively low concentrations in plasmid relaxation assays.[12,21,23] **27** and **28** inhibited top2 decatenation at low μM concentrations as well.[19] Clearly some pyridoacridines have the capacity to inhibit topoisomerases.

3 has been the subject of the closest scrutiny when it comes to topoisomerase interaction with four different groups reporting on it. **3** can inhibit both top1 and top2 catalysis at low μM concentrations and it probably has the ability to produce cleavage complexes, albeit to an extent much less than prototypic top2 poisons, etoposide or doxorubicin (Table 13). Bonnard and colleagues reported partial cleavage of DNA substrates by **3** in the presence of top1 or top2 at concentrations of 140 μM. They concluded that topoisomerases were not likely to be the primary cellular target of **3**.[25]

Two synthetic deazaanalogues of **3** (**5** and **4**) have the unusual ability to stabilize top1 cleavage complexes at low μM concentrations and produce single strand breakage in cells, consistent with top1 cleavage complex stabilization. These are the only pyridoacridines discovered to have this capacity.[23] The pyridoacridine, **4**, found to stabilize a DNA top1 cleavable complex was identified by its activity in a cell based screen (EM9). The unusual cytotoxicity profile of **4** (greatly enhanced activity towards the EM9 mutant CHO cell line) suggested top1 as the possible mediator of DNA damage (Table 14). **4** was found to inhibit top1 catalysis between 2.5 μM and 5 μM, in comparison to an inhibitory concentration of approximately 1 μM for camptothecin.[58] Analysis in a top2 catalysis inhibition assay revealed the ability of **4** to inhibit top2 catalysis at a concentration of 10 μM. Therefore, the ability of **4** to produce top1-mediated DNA cleavage was pursued further.

4 was found to stabilize DNA-top1 cleavage complexes in a time and concentration dependent manner similar to 9-aminocamptothecin. It produced topoisomerase I-DNA cleavage comparable to 9-aminocamptothecin, but required about 100 times the concentration. It is characteristic of top1 cleavage complexes to be reversible by high salt concentration or temperature. This was true for both 9-aminocamptothecin and **4**, however, different relaxation states of DNA were observed after reversal. Following reversal of 9-aminocamptothecin, stabilized

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Table 13 DNA cleavage and catenation

	DTT[a]	Top1	Top2	Top2 catenation	Ref.
Amphimedine **2**	−[b]	−	−/+	−	20,48
Arnoamine A			−		26
Arnoamine B			−		26
Ascididemin **3**	+	−	+	−	21,22
BC109 **12**					
Benzo[4,5]-sampangine **4**	−	+	−	−	21–23
Deoxyamphimedine **20**	+	+	+		27
Neoamphimedine **16**			−/+	+	20,48
Sampangine **5**	−	+	−	−	21–23
Doxorubicin		−	+	−	[c]
Etoposide		−	+	−	20

[a] Dithiothreitol, DTT. [b] No cleavage or catenation was observed (−), minimal or no cleavage was observed (−/+), cleavage or catenation was observed (+). [c] Unpublished data – L. R. Barrows' group.

Table 14 CHO cell differential representative of DNA damage[a]

	EM9[b]	xrs-6	Ref.
Amphimedine **2**		100 μM (1)	20
Ascididemin **3**	0.4 μM (7.7)	0.7 μM (4.4)	21
BC109 **12**	0.3 μM (27)	1.4 μM (6)	21
Benzo[4,5]-sampangine **4**	4 μM (4.5)		23
Cystodytin J **27**		135.6 μM (1)	19
Dehydro-kuanoniamine B **23**		80 μM (1)	19
Deoxyamphimedine **20**	6 μM (4)		27
Diplamine **28**		71.2 μM (1)	19
Kuanoniamine D **18**		88.9 μM (2)	19
Neoamphimedine **16**		1.6 μM (1.5)	20
Shermilamine C **25**		8.1 μM (1)	19
Shermilamine D		14.9 μM (1)	19
Top1 drug			
Camptothecin	0.3 μM (5)		[c]
Top2 drugs			
Etoposide		0.14 μM (7)	19
m-AMSA		0.24 μM (4)	19
Mitoxantrone		0.001 μM (9)	19

[a] CHO, Chinese hamster ovary cells mutated to detect single strand (EM9) and double strand (xrs-6) DNA breaks. [b] Values are given in micromolar with the differential toxicity to the parental cell line in parentheses. [c] Unpublished data – L. R. Barrows' group.

complexes of the released plasmid DNA relaxed fully. In contrast, reversal of **4** cleavage complexes released supercoiled plasmid DNA. These data suggest that **4** may inhibit the ability of top1 to relax DNA following DNA scission, implying that top1–DNA relaxation may be more regulated than the proposed simple swivel model.[23] The fact that **4** is a relatively potent inhibitor of top2 catalysis as well as top1 may possibly contribute to its toxicity in some cellular systems.

Recent work with **16** shows that it possesses a top2 dependent mechanism of toxicity but does not stabilize cleavage complexes. Both **16** and etoposide are supertoxic to yeast that over express top2. Unlike etoposide, however, there is no evidence for the production of top2 complexes by **16** in mammalian cells. **16** does not show enhanced cytotoxicity in the xrs-6 CHO cell line (Table 14).[20] **16** also contrasts with etoposide in that it inhibits the cell cycle in S rather than G_2 phase (unpublished data, L. R. Barrows' group). See Table 15. G_2 inhibition is indicative of DNA damaging agents. S phase arrest may be indicative of a distinct effect of **16** in mammalian cells. *In vitro* purified enzyme systems show that etoposide produces significant top2 dependent DNA cleavage while **16** does not. **16** in the presence of human top2 however, produces a covalently linked high molecular weight complex of plasmid DNA *in vitro*. This is most likely due to its ability to cause DNA

Table 15 Cell cycle arrest

	Phase[a]	Ref.
Ascididemin **3**	S	[b]
BC109 **12**	S	[b]
Benzo[4,5]sampangine **4**	S	[b]
Sampangine **5**	S	[b]
Amphimedine **2**	NE[c]	[b]
Etoposide	G2/M	[b]
Neoamphimedine **16**	S	[b]
9-Aminobenzo[*b*]pyrido[4,3,2-*de*]-[1,10]phenanthrolin-8(8*H*)one **31**	G2/M	30
Etoposide	G2/M	30

[a] S,S-phase (DNA synthesis); G_2/M, G_2/M-phase (period between DNA synthesis and mitosis). [b] Unpublished data – L. R. Barrows' group. [c] No effect up to 100 μM.

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aggregation *in vitro*. **2** was inactive in all these systems at tested concentrations.[20]

Cell cycle arrest is characteristic of some DNA interacting molecules. Etoposide, a prototype top2 cleavage complex stabilizer, arrests treated cells in G_2. Aoki *et al.* recently reported cell cycle data for a new pyridoacridine, 9-aminobenzo[*b*]pyrido[4,3,2-*de*][1,10]-phenanthrolin-8(8*H*)one **31**, comparing it to etoposide. Both were able to arrest the cell cycle in the G_2 phase (see Table 15).[30] Matsumoto and colleagues analyzed cell cycle arrest data for **3** and analogues **12**, **4** and **5**, finding that all of these compounds partially arrested cells in the S-phase (unpublished data, L. R. Barrows' group).

3.1.4 Sequence specific cleavage. Sequence specific cleavage analysis is frequently performed on compounds with the potential to cleave DNA directly or those that induce topoisomerase to do the same (*i.e.*, stabilize the cleavage complex). Radioactive DNA, typically end labelled by any of a number of means, is dissolved in topoisomerase or other reaction buffer. Individual cleavage reactions are conducted in the absence or presence of a drug. If topoisomerase is the actual DNA cleaver, the reactions are stopped with the addition of detergent and proteinase in order to trap any topoisomerase–DNA cleavage complexes present and transform them into cleaved DNA fragments. The DNA fragments generated by such reactions are then subjected to polyacrylamide gel electrophoresis along side DNA sequencing reactions of the same substrate DNA. DNA fragments are apparent as dark bands following autoradiography. Sequence specificity of the drug–DNA or topoisomerase–drug–DNA interaction can be determined from the DNA sequence lanes. The relative intensity of the bands visualized by this procedure provides a semi-quantitative measure of drug-induced DNA cleavage. Efforts in our laboratory to determine nucleotide specific cleavage of DNA substrates by **3**, in the presence or absence of topoisomerases, have only revealed random cleavage, such as that produced by ROS (unpublished data, L. R. Barrows' group).

3.1.5 Reactive oxygen species. Reactive oxygen species (ROS) occur naturally within a cell during aerobic respiration and are often associated with their negative effects (*e.g.*, DNA and protein oxidation).[59] When molecular oxygen is reduced by one electron, a superoxide ($O_2^{-\bullet}$) or perhydroxyl ($HOO^{-\bullet}$) is produced in a pH dependent manner. The superoxide itself may then undergo further reduction by an additional electron to form H_2O_2. H_2O_2, in turn, can spontaneously break down into the hydroxyl radical ($HO^{-\bullet}$), which may be reduced further to form water (H_2O).[60,61] Superoxide, hydrogen peroxide and the hydroxyl radical can directly damage DNA by producing modified nucleotides that result in single strand breaks and abasic sites.[59,60] Locally multiple damaged sites in DNA, areas of DNA with multiple ROS-induced lesions, frequently resolve into frank DNA double strand breaks.[21,22] Cellular defences against ROS damage involve anti-oxidant molecules and enzymes. Anti-oxidant enzymes include superoxide dismutase, catalase and glutathione peroxidase. Superoxide dismutase catalyzes the conversion of two equivalents of superoxide into molecular oxygen and hydrogen peroxide. Catalase and glutathione peroxidase catalyze the direct two electron reduction of hydrogen peroxide into water, bypassing the reactive hydroxyl intermediate.[47,59–62]

ROS also result when quinones and semiquinones undergo redox reactions or when metals are oxidized and promote electron transfer to other molecules in the cell. Metals like iron can undergo reduction-oxidation cycling in the presence of a reducing agent under aerobic conditions (*e.g.*, bound to a drug like bleomycin). This redox cycling can continue indefinitely. Redox cycling and the damage resulting from ROS production can be attenuated using iron chelators and/or anti-oxidants.[59–61,63] DNA intercalating quinones may be reduced directly to produce semiquinone radicals that ultimately generate ROS. In addition, hydroquinones are well described iron chelating structures and can also complex iron and produce ROS.[60,64] ROS production often correlates positively with oxygen levels.

3, **12**, and **20** have been reported to damage DNA *via* production of ROS (Table 16). These compounds, in aerobic conditions and in the presence of the reducing agent DTT, were capable of cleaving DNA *in vitro*[21,22,27], although the analogues **5**, **4**, **2** and **16** could not. Redox cycling requires a source of oxygen and a reducing agent. Theoretically, DNA cleavage would be prevented under anoxic conditions or attenuated under hypoxic conditions. It would also be prevented in the absence of a reducing agent. **3** and **12** failed to stimulate DNA cleavage in the absence of oxygen, indicating the necessity of oxygen for **3** and **12** induced DNA cleavage. In addition, **3** and **12** required a reducing agent (DTT) to generate DNA cleavage *in vitro*.[21,22] Likewise the DNA cleavage achieved by **20** was greatly increased in the presence of DTT in a concentration dependent manner. However, when hypoxic conditions were employed for **20**, the percentage of induced DNA cleavage was attenuated

Table 16 Drug induced DNA cleavage and protection

DNA cleavage in the presence or absence of a reducing agent			
	Ascididemin [a]	BC109	Deoxyamphimedine
No DTT	– [b]	–	–
DTT	+++	+++	+++
Anoxic/hypoxic	–	–	–/+
Effect of added metal salts or metal chelators on DNA cleavage			
$FeSO_4$ [c]	NC [d]	NC	ND
NiCl	Slight ↓	NC	NT
EDTA	NC	NC	NC
Desferoxamine	NT	NT	ND
Ferrozine	NT	NT	ND
DNA cleavage protection from anti-oxidant enzymes and anti-oxidants			
Superoxide dismutase	None	None	None
Catalase	Strong	Strong	Strong
Benzoic acid	Moderate	Moderate	Strong
Glutathione	Strong	Strong	Moderate
N-Acetylcysteine	Slight	Slight	Moderate
Ethanol	Slight	Slight	NT

[a] Ascididemin **3** and BC109 **12** data (refs 21 and 22), deoxyamphimedine **20** data is from ref. 48 and unpublished data-L. R. Barrows' group. [b] Relative cleavage activity is reported as cleaving DNA (+), most active being (+++), minimal to no activity (±) or inactive (–). [c] Other metal salts tested ($CuSO_4$, $MgCl_2$ and $MnCl_2$) did not further stimulate DNA cleavage induced by **3** or **12**. [d] NC: no change, ND: not determined because cleavage was too high, NT: not tested.

Table 17 Induction of HO-1[a]

		HO-1 induction		
AA8[b]	EM9[c]	Ref.		
Ascididemin **3**	IC_{50}	Strong	Strong	21
	IC_{80}	Strong	Weak	21
Benzo[4,5]sampangine **4**	IC_{50}	ND[d]	Weak	21
	IC_{80}	ND	ND	21
BC109 **12**	IC_{50}	Strong	Strong	21
	IC_{80}	Strong	Weak	21
Sampangine **5**	IC_{50}	Weak	Weak	21
	IC_{80}	Weak	Weak	21
Sodium arsenite	(μM)			
	6.25	Strong	Weak	21
	25	Strong	Strong	21

[a] HO-1: heme oxygenase-1. [b] CHO cell lines, wild type (AA8) and mutated (EM9) Chinese hamster ovary cells. [c] EM9 data is unpublished from L. R. Barrows' group. [d] ND = not detected.

(unpublished data, L. R. Barrows' group). Further, it was shown that the peroxide destroying enzyme catalase was able to protect DNA from cleavage in this *in vitro* system. Additionally, various radical scavengers could protect DNA from **3**, **12**[21,22] or **20** cleavage *in vitro* (unpublished data, L. R. Barrows' group).

Three separate lines of evidence showed that ROS production probably contributes to the cytotoxicity of **3** and **12** in mammalian cells. First, it was shown that **3** and **12** both showed enhanced toxicity towards the EM9 CHO cell line, a cell line that is sensitive to killing by DNA single strand breaks generated by ROS. Second, it was shown that elevation of intracellular levels of reduced glutathione, an intracellular anti-oxidant, protected CHO cells from **3** and **12** toxicity. Third, cells treated with **3** and **12** induced the expression of the oxygen stress protein, heme oxygenase-1 (HO-1) (Table 17).[21] These data provide compelling evidence that the production of ROS contributes significantly to the cytotoxicity of **3** and **12** in mammalian cells.

Many of the pyridoacridines exhibit structural similarity to 1,10-phenanthroline and thus might be expected to chelate metals in the same way. There are data in the literature showing that some pyridoacridines bind metal. NMR signals from H-2, H-3 and H-11 of kuanoniamine D **18** were broadened at low concentrations of Zn^{++} and then sharpened at a 2 : 1 alkaloid to metal ratio. The quenching of **18** fluorescence intensity and shifting of emission maxima as the drug was titered with increasing amounts of metal ion (Co^{2+}, Cu^{2+}, Fe^{2+} and Zn^{2+}) yielded binding ratios and stability constants for the drug–metal coordination complexes.[12] A previous report found no evidence for Fe^{2+} binding to **10**.[65] Luedtke *et al.* reported a **7**–Ru(II) complex that was necessary for its anti-HIV activity discussed earlier in this review.[16]

One hypothesis tested in the literature was that metals binding to pyridoacridines were responsible for ROS generation. Therefore, different metal chelators were tested for the ability to protect DNA from pyridoacridine generated ROS. The overall result of this work was that the chelators tested (desferoxamine, EDTA, ferrozine) did not significantly protect from **20**, **3** or **12** induced DNA cleavage *in vitro*. Furthermore, the addition of metal salts ($FeSO_4$, $NiCl_2$, $CuSO_4$, $MgCl_2$ and $MnCl_2$) at concentrations that did not induce DNA damage by metal alone, did not synergize with the pyridoacridines to produce more DNA cleavage than pyridoacridine alone.[21,22,27]

Inductively-coupled plasma-mass spectrometry (ICP-MS) was performed with **20** in DMSO to determine if stock solutions contained appreciable metal ion concentrations. The results suggest evidence for some metal binding in the parts per million range, but large amounts of contaminating metals were not detected (unpublished data, L. R. Barrows' group).

Metal catalyzed ROS production did not seem to be primarily responsible for DNA cleavage by **3**. The alternative hypothesis that direct reduction of the iminoquinone was responsible for the ROS generation was tested using cyclic voltammetry and electron paramagnetic resonance spectroscopy (EPR). Cyclic voltammetry was used to measure the reduction potential of **3**, **12**, **5** and **4** (Table 18). Matsumoto *et al.* found that **3** and **12** were reducible at potentials consistent with bioreduction within a cell (∼−200mV). The reduction-oxidation potentials of **5** and **4** were from 10 to 40% higher than those of **3** and **12**.[21] Whether this quantitative difference is significant enough to explain the qualitative difference in ROS production amongst these molecules remains to be determined.

Table 18 Cyclic voltammetry

	Ag/ACl[a]	Ref.
Ascididemin **3**	−0.226	21
Benzo[4,5]sampangine **4**	−0.284	21
BC109 **12**	−0.251	21
Sampangine **5**	−0.318	21

[a] Measured in volts (V).

EPR uses electromagnetic radiation to measure production of radical species in solutions and was employed to elucidate differences between the ROS generating **3** and **12** and the non-ROS generating molecules **4** and **5**. This technique can confirm the presence of discrete radical states based on their corresponding energies. The integrated intensity signal is proportional to the sum of the active species within the sample. EPR spectra reported by Matsumoto *et al.* supported the concept that **3** and **12** could produce a variety of radical states, while **4** and **5** could not.[21]

3.2 Other activities

There are several miscellaneous activities reported for pyridoacridines in the literature. This section presents those disparate data.

A few pyridoacridines can facilitate calcium release from the sarcoplasmic reticulum.[28,29] Calcium serves as an important intracellular regulator, controlling activation of various cellular pathways *via* calmodulin and other calcium-sensitive proteins. Calcium release from the sarcoplasmic reticulum also plays an important role in apoptosis. Sometimes calcium is released as a general effect of cell death. Caffeine was used as the positive control in these experiments. Cystodytin A **32** and cystodytin B **33** showed exceptional activity in this assay, while cystodytin C **34** and **3** were not as effective (Table 19).

Eder and colleagues reported the affinity of three kuanoniamines for adenosine and GABA receptors compared to theophylline and caffeine (Table 20).[11] Kuanoniamine C **17**, **18**, and *N*-deacyl kuanoniamine **19** were active in competitive binding experiments studying the ability to inhibit the binding of radiolabeled adenosine ligands to the A_1 and A_{2A} receptors in rat brain cortical and striatal membranes, respectively. They also showed inhibition of radiolabeled diazepam to the $GABA_A$ receptor in rat brain cortical membranes. Only partial inhibition was determined in these binding experiments, with **18** being the most effective yielding 68% inhibition of N^6-cyclohexyladenosine (CHA) to the A_1 receptor.

The ability of pyridoacridines to induce neuronal differentiation was demonstrated by Aoki and coworkers.[30] They

Table 19 Ca^{++} release from the sarcoplasmic reticulum

Compound	Ca^{++} release	Ref.
Ascididemin **3**	7 X caffeine	29
Cystodytin A **32**	36 X caffeine	28
Cystodytin B **33**	13 X caffeine	28
Cystodytin C **34**	No effect	28

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Table 20 Affinity[a] of kuanoniamines for adenosine and GABA receptors

	Kuanoniamine C **17**	Kuanoniamine D **18**	*N*-Deacyl derivative **19**		Theophylline	Caffeine	Ref.
	25 µM	25µM	25 µM	100 µM			
% inhibition of A_1 receptor ligand binding in rat brain cortical membranes	34 ± 4% (nd)[b]	68 ± 10% (2.94 ± 0.02)		36 ± 1% (nd)	(26)	(17)	11
% inhibition of A_{2A} receptor ligand binding in rat brain striatal membranes	43% (nd)	56 ± 1% (13 ± 0.6)		46 ± 5% (nd)	(22)	(9.4)	11
% inhibition of $GABA_A$ receptor ligand binding at the rat brain cortical membrane	39%	32%	30%		(565)	(376)	11

[a] Affinity determined by measuring inhibition of radiolabeled ligand (^{3}H-CHA for A_1 receptor, ^{3}H CGS21680 for the A_{2A} receptor or ^{3}H-diazepam for the $GABA_A$ receptor). [b] K_I in µM (expressed in parentheses) ± SEM (standard error of the mean); nd, not determined.

Table 21 Neuronal differentiation

Compound	Concentration	% neurite bearing cells appearing	Ref.
9-Aminobenzo[*b*]-pyrido[4,3,2-*de*]-[1,10]phenan-throlin-8(8*H*)-one **31**[a]	0.03 µM	85	30
Biemnadin **37**	3 µM	90	30
Labuanine **35**	1 µM	80	30
Merdine regioisomer **36**	3 µM	75	30

[a] **30** was cytotoxic at concentrations greater than 0.3 µM.

used **31**, labuanine A **35**, 9-hydroxybenzo[*b*]-pyrido[4,3,2-*de*][1,10]phenanthrolin-8(8*H*)-one (meridine regioisomer) **36**, and biemnadin **37**. While none of these compounds induced neurite outgrowth in rat pheochromocytoma PC12 cells, they were all able to induce neuronal differentiation in the murine neuroblastoma cell line Neuro-2A (Table 21). This differentiation was determined by measuring the neuritogenic activity, seen as cell processes growing larger than the diameter of the cell body. Cells were treated for 72 hours and neurotogenic activity was determined. **31** was the most potent and also increased the neuronal marker, acetylcholine esterase activity, 4 fold when neurons were exposed to 0.03 µM for 48 hours. The authors concluded that the neuronal differentiation induced by these pyridoacridines likely acted by pathways different than neuronal growth factor (NGF), an endogenous differentiation inducer. NGF causes neuronal cells to arrest in G_1. As mentioned earlier, **31** arrested cells in G_2. Etoposide, which induces multipolar neuritogenesis in the Neuro-2A cells, also arrests cells in G_2. The authors suggested that the neuronal differentiation observed for these G_2 arresting compounds may be related to top2 interactions.[30]

Immunosuppressant activity has also been reported for pyridoacridine molecules. **18**, at 10 ng mL^{-1}, completely prevented the murine mixed lymphocyte response (MLR).[17] The MLR is an *in vitro* model of the antigen-specific initiation phase of the allotransplantation reaction. Mouse spleen cell suspensions exposed to antigenic stimuli will respond depending on the type of stimulus. The strongest proliferative stimulus is generated by class II incompatibility. CD4 T lymphocytes are the principal responding cells in the MLR, but CD8 cytotoxic T cells are stimulated as are natural killer cells. Nordercitin **38**, **17**, and dercitamine **39** have also been reported to have significant immunosuppressant activity but no data were reported.[32]

4 SAR studies of amphimedine and ascididemin

The amphimedine and ascididemin classes of compounds have promising anti-tumor activity. Recently there have been large synthetic efforts based on these prototype skeletons. The analogues of **2** first reported from marine sources include **16** and **20** from *Amphimedon* and *Xestospongia* spp. sponges.[20,27] Thale and coworkers have also identified **2**, **16**, **20** from a *Xestospongia* sp. sponge, and isolated the new analogues 5-methoxy-neoamphimedine **40**, neoamphimedine Y **41**, and neoamphimedine Z **42**.[66] Brahic and colleagues have synthesized several toxic analogues of **2**, **43–48**.[68] While **2** itself has relatively weak cytotoxicity *in vitro*, **16** and **20** have relatively potent cytotoxicity.

16 is the regioisomer of **2** where the carbonyl at the 11 carbon position has been changed to the C-9 position. As mentioned earlier, **16** facilitates a top2-dependent death in cells and induces top2-dependent catenation of plasmid DNA *in vitro*. It intercalates DNA and has significant anti-cancer activity in xenograft models. In the case of **20**, the carbonyl at the C-9 position of **2** was removed and the nitrogen at the 10 position became charged. These changes increased the cytotoxic potency and DNA affinity of **20** compared to **16** and also resulted in **20** being a powerful generator of ROS when in the presence of a reducing agent. Due to the inherent DNA cleavage activity of **20**, it has not yet been possible to determine if it induces top2 DNA cleavage as well (unpublished data, L.R. Barrows' group).

Comparative toxicity studies by Thale and colleagues of **40**, **41** (with hydroxy groups attached to carbons 8, 11 and 12) and **42** (with methoxy groups attached to carbons 8, 11 and 12) were performed using a disk diffusion assay that compared the differences in "kill zones" detected with cells established from solid tumors *versus* leukemic cell lines.[66,67] Larger kill zones (>250 units) are taken as indicative of solid tumor selectivity. While **40** had dramatic activity in the murine solid *versus* leukemic comparison panel (>700 units), it showed the reverse trend in the human solid *versus* leukemic panel.[66]

Of the analogues of **2** synthesized by Brahic and colleagues, none had the E ring carboxyl of **2** or **16**, nor the E ring *N*-methyl of **20**.[68] The first set consisted of tetracyclic compounds that had the original A ring removed from the parent compound. The nitrogen at the 8 position was switched with the carbon at the 9 position for compounds **43a–c**. Compounds that did not have this switch were **44a–c**. **43b** and **44b** had a methoxy group attached to the 4 carbon, while **43c** and **44c** had fluorine attached to the 4 carbon. Of the second set of pentacyclic amphimedine

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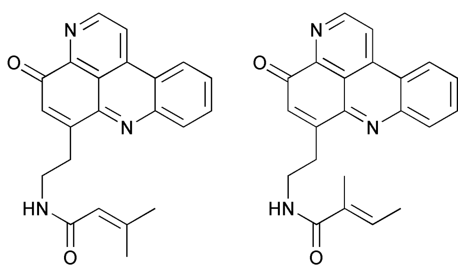
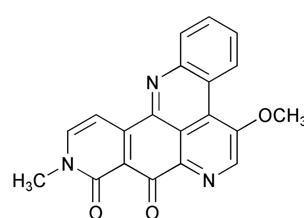
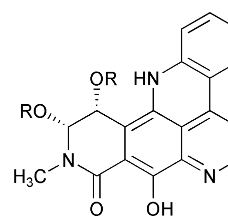


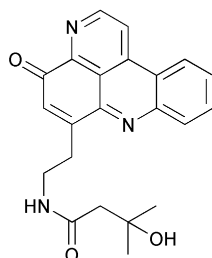
32 Cystodytin A **33** Cystodytin B



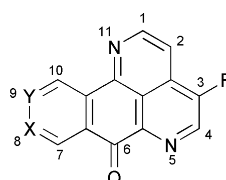
40 5-Methoxyneoamphimedine



41 R = H (Neoamphimedine Y)
42 R = CH_3 (Neoamphimedine Z)

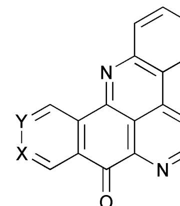


34 Cystodytin C

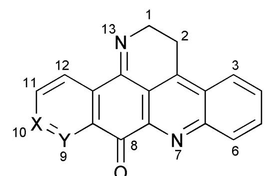


43 Y = N, X = CH
a R = H
b R = OMe
c R = F

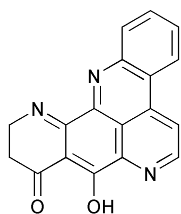
44 X = N, Y = CH
a R = H
b R = OMe
c R = F



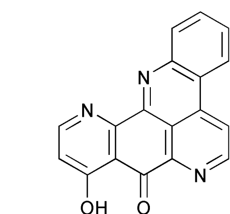
45 Y = N, X = CH
46 X = N, Y = CH



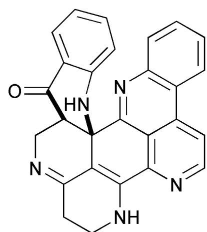
47 X = N, Y = CH
48 Y = N, X = CH (Ascididemin regioisomer)



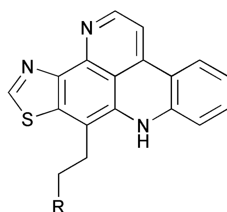
35 Labuanine A



36 9-Hydroxybenzo[*b*]pyrido[4,3,2-*de*][1,10]phenanthrolin-8(8*H*)-one



37 Biemnadin



38 R = $N(CH_3)_2$ (Nordercitin)
39 R = $NHCH_3$ (Dercitamine)

analogues, **45** had its E ring nitrogen shifted from the 10 position to the 11 position, while **46** did not. The A ring was removed and an aromatic ring was placed next to the D ring for compounds **47** and **48**. However, the nitrogen at the 10 position was changed to the 9 position on **48**. Results indicated that the nitrogen in the 9 or 10 position (**48** or **47**, respectively) was important for toxicity. The fluorine additions to C-4 did not dramatically affect the toxicity of **43c** or **44c**. Moving the aromatic ring in the pentacyclic compounds decreased cytotoxicity. All of these analogues were cytotoxic to some degree in the cell lines tested.

The laboratory of Dr Copp synthesized **3** and **10**, and several analogues of **4**, **5**, **11–15**.[18,22] Koller *et al.* synthesized **4**, and the related molecules **26**, **49–73**.[31] The naturally occurring **3**, **10**, **5**, 11-hydroxy-ascididemin **74** and neocalliactine **75** have also been synthesized by Delfourne *et al.*, along with many analogues **11**, **12**, **76–112**.[69–71] The regioisomer of **3** (**48**) was very similar to **6** and Delfourne and colleagues also created a series of analogues of **6**, **113–121**. As detailed above, these molecules display an amazing spectrum of biological activities.

Copp's group and ours, in collaboration, have done extensive work with his series of analogues.[6,15,18,21,22] **3** was initially tested in the standard *in vitro* assay at the NCI using 60 human tumor cell lines, which are derived from nine cancer types (leukemia, lung, colon, CNS, melanoma, ovarian, renal, prostate and breast) (personal communication B. R. Copp). The compound was tested over the concentration range of 10^{-4} to 10^{-8} M against all of the cell lines in the panel, and the three response parameters GI_{50} (50% growth inhibition), TGI (total growth inhibition) and LC_{50} (50% lethal concentration) calculated. **3** was moderately active, with sub-μM GI_{50} and TGI values being observed, with sub-panel sensitivity observed against melanoma, colon and non-small cell lung cancer cells.

In some of Copp's analogues the nitrogen in the 1 position of **3** was replaced by CH to generate **4**. When the D ring on **4** or **3** was removed, **5** and **12** were created, the former also lacking the 1 position nitrogen. **11** similarly lacked the D ring, but had the nitrogen at the 1 position moved to the 4 position. Another set of tetracyclic analogues were created when the A ring was deleted and the 1 position was replaced with a sulfur ethyl **13**, a thiol alkyl **14**, or an amine **15**. **10** was also included in these structure–activity relationship studies. The minor changes described here had profound effects on the pharmacodynamics of these molecules. The cytotoxicity of **3** and **12** was linked to their ability to generate ROS, while **4** and **5** were found to inhibit top1 and top2 catalysis and stabilize top1 cleavable complexes.[21–23]

Koller *et al.* also synthesized a series of **3** related molecules and found that a sixth ring, attached to the E ring of **3** (**26**), significantly increased cytotoxicity against P388 mouse leukemia cells while the addition of methoxy groups (**49**), to increase the electron density of the ring systems, significantly decreased potency.[31] Our group did not find **26** to be appreciably cytotoxic to human colon tumor cells, nor did we find it to displace EtBr from DNA at 100 μM.[23] Table 22 combines data generated on this series of compounds.

Delfourne and colleagues have produced several series of tetracyclic, pentacyclic and hexacyclic analogues of **3** and **6**.[69–72] We have averaged the cytotoxicities reported from the tumor

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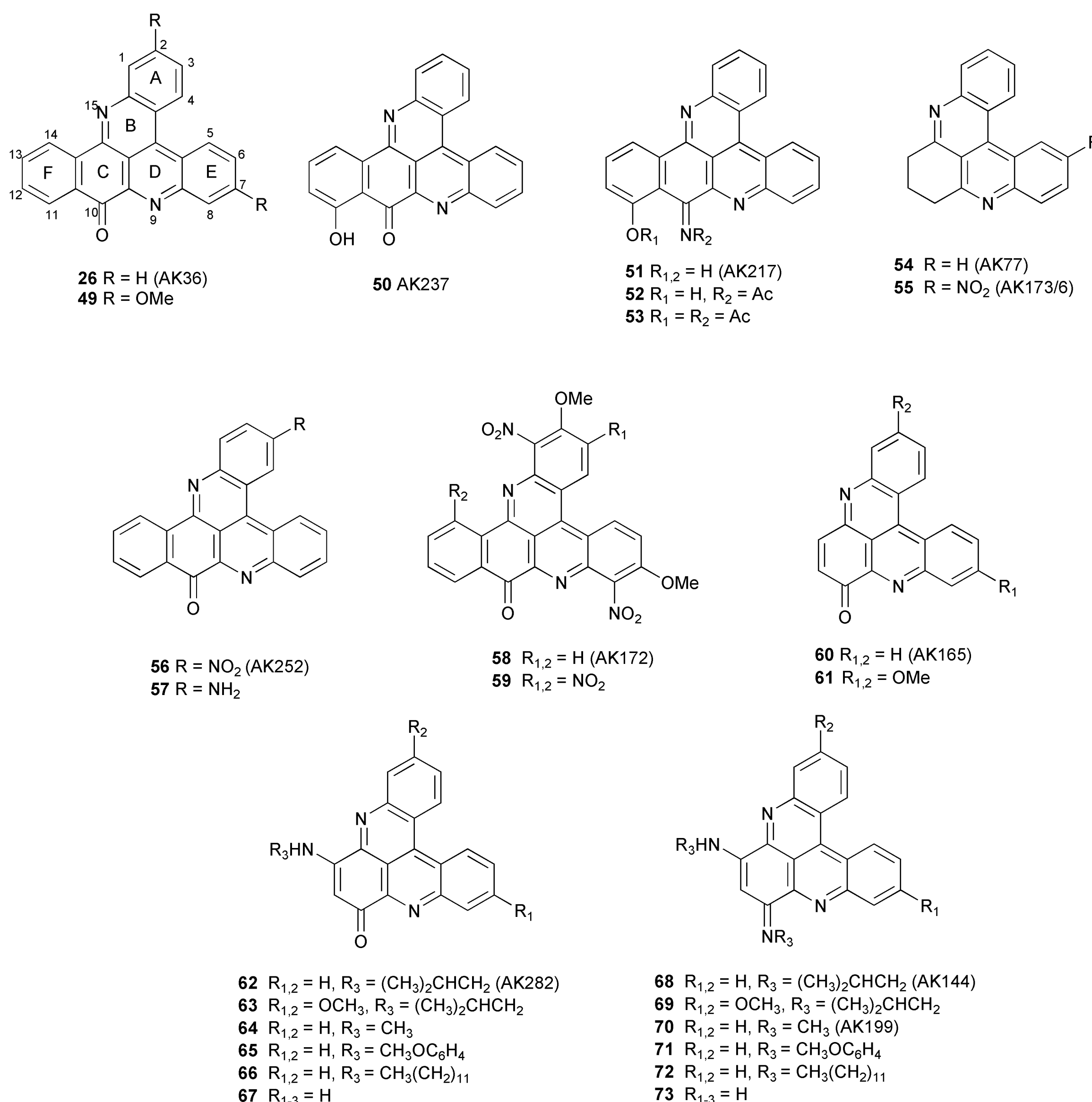

cell line panel they used. This panel consisted of 12 human tumor cell lines including 3 glioblastoma, 2 colon, 2 non-small cell lung, 2 bladder, 1 prostate, and 2 breast. Many of the pyridoacridine analogues possessed more than 3 orders of magnitude differential in cytotoxicity depending on the cell line. In addition, the average cytotoxic potency amongst the various analogues varied by several logs. The authors hypothesized that the cytotoxicities may relate to ascididemin-line iminoquinone cycling and the generation of ROS, top2 inhibition, or DNA intercalation.

The most cytotoxic analogues of **6** (**114** and **117**) had minimal A ring substitutions (Table 23). The most active molecules were unsubstituted or substituted with a methoxy group at the R_1.[72] These analogues had approximately 10 000 times greater potency *in vitro* than **6**, approximately 10 times that of **3**.[72]

In the second report of this series of studies, Delfourne and colleagues showed several D ring substitutions that could be made to **3** (**10**, **74**–**90**) without losing cytotoxicity (Table 24).[69] The authors cited a study[73] that hypothesized a quantitative linear relationship between spectronigrin reduction potentials and the rate at which the analogues degrade DNA *in vitro*. Eleven of Delfourne's D ring modified analogues exhibited more potent cytotoxicity and improved MTD when compared to the parent compounds **3** and **10**.

Two additional studies of modified **3** molecules (**11**, **12**, **49**, **91**–**112**) were published by Delfourne and colleagues in 2003 (Tables 25 and 26).[70,71] **3**'s A ring nitrogen regioisomer (**48**) retained cytotoxic potency close to **3** (within about 1 log), other analogues were slightly less potent. Placing a nitro group on the D ring greatly decreased the cytotoxicity of **3**'s A ring nitrogen regioisomer.[71] The other study focused on D ring deleted analogues and a number of quinone and tricyclic analogues.[70] A ring modification consisting of a methoxy to the *para* position of **3**'s A ring nitrogen regioisomer yielded the most potent product **110**, which was 3–10 times more cytotoxic than **3** itself. The authors tested the analogues for maximally tolerated doses in mice, but found "no clear cut relationship between cytotoxicity against the different cell lines and toxicity".[70]

Debnath and colleagues recently analyzed a series of analogues of **3** related to those created by Delfourne *et al.*, although there is some confusion about the presence of the iminoquinone carbonyl which was present in the analogues of Delforne *et al.*[69], but was missing in the structure presented by Debnath *et al.*[74] These were analyzed using a mathematical approach that evaluated the physicochemical parameters of the molecules, including the electrotopological state of component atoms as a means to quantify the structure–cytotoxicity potency relationships. The importance of R_1 and R_3 was demonstrated by showing cytotoxicity could be improved when an electron withdrawing group (NO_2 or NH_2) was placed at the R_1 position or when a hydrogen or alkyl group was positioned at R_3. The work also demonstrated the importance of having a hydrogen at R_5. When other moieties were substituted at R_5 (OH, OCH_3), the cytotoxicity was decreased dramatically.

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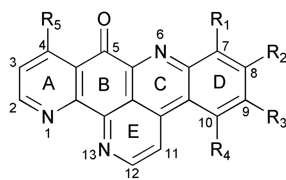
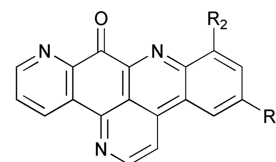
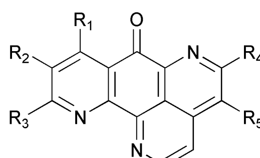


3 R_{1-5} = H (Ascididemin)
10 R_2 = Br, $R_{1,3-5}$ = H (2-Bromoleptoclinidinone)
74 R_5 = OH, R_{1-4} = H (11-Hydroxyascididemin)
75 R_3 = OH, $R_{1,2,4,5}$ = H (Neocalliactine)
76 R_5 = OMe, R_{1-4} = H (11-Methoxyascididemin)
77 R_1 = NO_2, R_{2-4} = H (7-Nitroascididemin)
78 R_3 = NO_2, $R_{1,2,4,5}$ = H (5-Nitroascididemin)

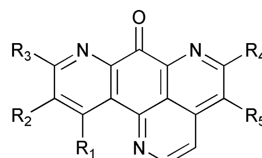
79 R_1 = NH_2, R_{2-5} = H
80 R_3 = Br, $R_{1,2,4,5}$ = H
81 R_3 = NH_2, $R_{1,2,4,5}$ = H
82 R_3 = $NHCH_2CH_2Cl$, $R_{1,2,4,5}$ = H
83 R_3 = $N(CH_2CH_2Cl)_2$, $R_{1,2,4,5}$ = H
84 R_3 = $NHCH_2CH_2NMe_2$, $R_{1,2,4,5}$ = H
85 R_3 = Cl, $R_{1,2,4,5}$ = H
86 R_3 = Me, $R_{1,2,4,5}$ = H
87 R_3 = OMe, $R_{1,2,4,5}$ = H
88 R_3 = NMe_2, $R_{1,2,4,5}$ = H
89 R_3 = NHBn, $R_{1,2,4,5}$ = H
90 R_3 = NH_2, R_4 = Br, $R_{1,2,5}$ = H



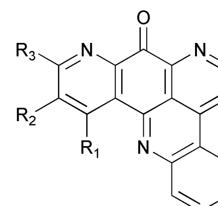
48 $R_{1,2}$ = H (Ascididemin regioisomer)
91 R_1 = H, R_2 = NO_2
92 R_1 = H, R_2 = NH_2
93 R_1 = Br, R_2 = H
94 R_1 = NH_2, R_2 = H
95 R_1 = NMe_2, R_2 = H



12 R_{1-5} = H
96 R_1 = Cl, R_{2-5} = H
97 R_1 = OMe, R_{2-5} = H
98 R_5 = OMe, R_{1-4} = H
99 R_3 = OMe, $R_{1,2,4,5}$ = H
100 $R_{1,5}$ = OMe, R_{2-4} = H



11 R_{1-5} = H
101 R_1 = OMe, R_{2-5} = H
102 R_1 = OH, R_{2-5} = H
103 R_1 = NMe_2, R_{2-5} = H
104 R_5 = OMe, R_{1-4} = H
105 R_2 = CO_2Et, $R_{1,3-5}$ = H
106 R_3 = OH, $R_{1,2,4,5}$ = H
107 R_3 = OMe, $R_{1,2,4,5}$ = H
108 $R_{1,3}$ = OMe, $R_{2,4,5}$ = H
109 R_1 = NMe_2, R_3 = Cl, $R_{2,4,5}$ = H
110 $R_{1,5}$ = OMe, R_{2-4} = H
111 R_1 = NMe_2, R_5 = OMe, R_{2-4} = H
112 $R_{3,5}$ = OMe, $R_{1,2,4}$ = H



6 R_1 = OH, $R_{2,3}$ = H (Meridine)
113 R_1 = Cl, $R_{2,3}$ = H
114 R_1 = OMe, $R_{2,3}$ = H
115 R_1 = NH_2, $R_{2,3}$ = H
116 R_1 = Br, $R_{2,3}$ = H
117 R_{1-3} = H
118 R_1 = NO_2, $R_{2,3}$ = H
119 R_1 = NMe_2, $R_{2,3}$ = H
120 R_2 = CO_2Et, $R_{1,3}$ = H
121 R_3 = OH, $R_{1,2}$ = H

Table 22 Cytotoxic activity for analogues of ascididemin **3**

	HCT 116[a]	K_{disp}	P388[b]	Ref.
4	30	70.8	0.1	23,31
26	>100	>100	0.05	31,35
49			10	31
50	>100			[c]
51	>100		>10	[c],31
52			5	31
53			10	31
54	>100			[c]
55	>100			[c]
56	>100		0.5	[c],31
57			10	31
58	>100			[c]
59			2.5	31
60	4	1.59	1	[c],31
61			2.5	31
62	>100		0.25	[c],31
63			1	31
64			0.25	31
65			1	31
66			2.5	31
67			1	31
68	4.2	1.12	0.1	[c],31
69			0.1	31
70	4	1.26	0.1	[c],31
71			0.5	31
72			0.5	31
73			>10	31

[a] IC_{50} values for HCT 116 and K_{disp} values for EtBr displacement are in μM. [b] IC_{50} values for P388 are in μg mL^{-1}. [c] Unpublished data, L. R. Barrows' group.

5 Conclusion

Pyridoacridines are a promising class of biologically active molecules. As can be seen from this summary, a wide variety of analyses have yielded an uneven and incomplete body of biological data on them. The inconsistency of the data often places the

Table 23 Cytotoxic activity for A ring substituted meridine **6** analogues

	IC_{50}[a]		IC_{50}		IC_{50}	Ref.
6	0.97	**115**	0.23	**118**	1.3	72
113	0.74	**116**	2.8	**119**	0.27	72
114	0.014	**117**	0.014	**120**	1.7	72

[a] Average of the IC_{50} values obtained from a 12 line cell panel, values are expressed in μM; IC_{50} of **121** was not determined.

Table 24 Cytotoxic activity for D ring substituted ascididemin analogues

	IC_{50}[a]	MTD[b]		IC_{50}	MTD	Ref.
3	343	20	**82**	185	>160	69
10	288	40	**83**	494	>160	69
74	4250	>160	**84**	442	40	69
75	NT[c]		**85**	473	>160	69
76	883	>160	**86**	155	20	69
77	141	20	**87**	208	>160	69
78	NT		**88**	92.1	>160	69
79	373	>160	**89**	363	>160	69
80	251	>160	**90**	545	>160	69
81	219	>160				69

[a] Average of the IC_{50} values obtained from a 12 line cell panel, values are expressed in nM (these averages differ from the averages reported in reference 60). [b] MTD, maximum tolerated dose (mg kg^{-1}). [c] NT, not tested.

Table 25 Cytotoxic activity for D ring substituted ascididemin analogues

	IC_{50}[a]		IC_{50}		IC_{50}	Ref.
48	1.30	**91**	>6.08	**93**	>3.62	71
3	0.347	**92**	2.86	**94**	>4.36	71

[a] Average of the IC_{50} values obtained from a 12 line cell panel, values are expressed in μM; **95**, not tested.

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Table 26 Cytotoxic activity for A and D ring substituted ascididemin and meridine **6** analogues

	IC_{50}[a]	MTD[b]		IC_{50}	MTD	Ref.
11	2.5	10	**104**	0.68	>160	70
48	2.3	10	**105**	NT	NT	70
96	2.2	20	**106**	NT	NT	70
97	1.2	10	**107**	5.9	40	70
98	1.7	>160	**108**	5.3	40	70
99	>6.2	>160	**109**	>6.2	40	70
100	2.5	80	**110**	0.035	20	70
101	0.78	5	**111**	4.3	>160	70
102	>6.5	10	**112**	1.3	>160	70
103	2.3	10				70

[a] Average of the IC_{50} values obtained from a 12 line cell panel, values are expressed in μM. [b] MTD, maximum tolerated dose (mg kg^{-1}).

researcher in the position of 'comparing apples and oranges', and forces them to rely on experience or intuition to predict or interpret results. Further studies remain to be performed to better understand these molecules in terms of structure activity relationships, especially for cytotoxicity against mammalian or microbial cells. Clearly some mechanisms of cell killing will have more potential for use against human cancer, while others may have more potential for combating microbial infections or other diseases. Research indicates that DNA binding, the inhibition of DNA metabolizing enzymes, and the production of ROS may all play a role in the cytotoxicity of the pyridoacridines. This complicates matters because the simple end point of cell death does not reveal the principle underlying mechanism. For this reason the authors urge continued collaboration amongst chemists and pharmacologists so the molecular mechanisms that underlie particularly useful prototypes of this class can be better understood.

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