Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry 11 (2003) 399–405

BIOORGANIC & MEDICINAL CHEMISTRY

Rational Design, Synthesis and Biological Evaluation of Thiadiazinoacridines: A New Class of Antitumor Agents

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Received 14 June 2002; accepted 6 September 2002

Abstract—A series of potential DNA-binding antitumor agents, 3-[ω-(alkylamino)alkyl]-6-nitro-[1,2,6]thiadiazino[3,4,5-*kl*]acridines **12** and 1,3-di[ω-(alkylamino)alkyl]-6-nitro-[1,2,6]thiadiazino[3,4,5-*kl*]acridines **13**, has been prepared by cyclization with $SOCl_2$ of 1-{[ω-(alkylamino)alkyl]amino}-9-imino-4-nitro-9,10-dihydroacridines **16** or 1-{[ω-(alkylamino)alkyl]amino}-9-[ω-(alkylamino)alkyl]imino-4-nitro-9,10-dihydroacridines **17**, respectively. The non-covalent DNA-binding properties of **12, 13** have been examined using a fluorometric technique. In vitro cytotoxic potencies of these derivatives toward six tumor cell lines, including human colon adenocarcinoma (HT29) and human ovarian carcinoma (A2780 sensitive, A2780cisR cisplatin-resistant, CH1, CH1cisR cisplatin-resistant, and SKOV-3) cells, are described and compared to that of reference drugs. In vivo antitumor activity of some selected derivatives, endowed with relevant cytotoxic activity against murine leukemia P388 are reported. The 3-[2-(dimethylamino)ethyl]-6-nitro-2,7-dihydro-3*H*-2λ^4-[1,2,6]thiadiazino[3,4,5-*kl*]acridin-2-one (**12d**) has been identified as a new lead in the development of anticancer tetracyclic acridine derivatives.

Introduction

The acridine skeleton fused with a five or six-membered heterocyclic ring yields polycyclic derivatives which play an important role in the class of DNA-intercalating anticancer drugs. They are structurally characterized by the presence of a planar or semi-planar chromophore portion, possibly capable of intercalation into DNA. A noticeable example, in phase II clinical trial, is constituted by the 5-nitropyrazolo[3,4,5-*kl*]acridines (**1**, Fig. 1).[1,2]

We have previously studied a number of polycyclic acridine derivatives with interesting antitumor properties, including the pyrimido[5,6,1-*de*]acridines,[3,4] the pyrazolo[3,4,5-*kl*]acridine-5-carboxamides,[5] the pyrazolo[3,4,5-*mn*]pyrimido[5,6,1-*de*]acridines,[6] and, in particular, the pyrimido[4,5,6-*kl*]acridines (**2**–**11**, Fig. 1).[7,8]

As shown in Figure 1, the pyrimido[4,5,6-*kl*]acridines (**2**–**11**, X = H, OMe, OH; R = alkylaminoalkyl) were designed and synthesized as analogues related to the pyrazoloacridines **1**. They are formally derived by substitution of the pyrazole ring with a pyrimidine ring in the chromophore moiety. This substitution leads to derivatives that retain a noticeable in vitro cytotoxic activity.

Thus, we decided to further investigate a novel chromophore constituted by an acridine skeleton fused with a six-membered heterocyclic ring to form the new [1,2,6]thiadiazino[3,4,5-*kl*]acridines **12** and **13** depicted in Figure 2. Compounds **12** and **13** are structurally related to the 5-nitropyrazolo[3,4,5-*kl*]acridines **1** and, especially, to the pyrimido[4,5,6-*kl*]acridines **2** and **10**. Prompted by the above rationale, we synthesized compounds **12a**–**k** and **13a,b** investigating some biologically relevant properties such as (i) mode and affinity of DNA-binding, (ii) in vitro cytotoxicity, and (iii) in vivo antitumor activity.

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PII: S0968-0896(02)00442-X

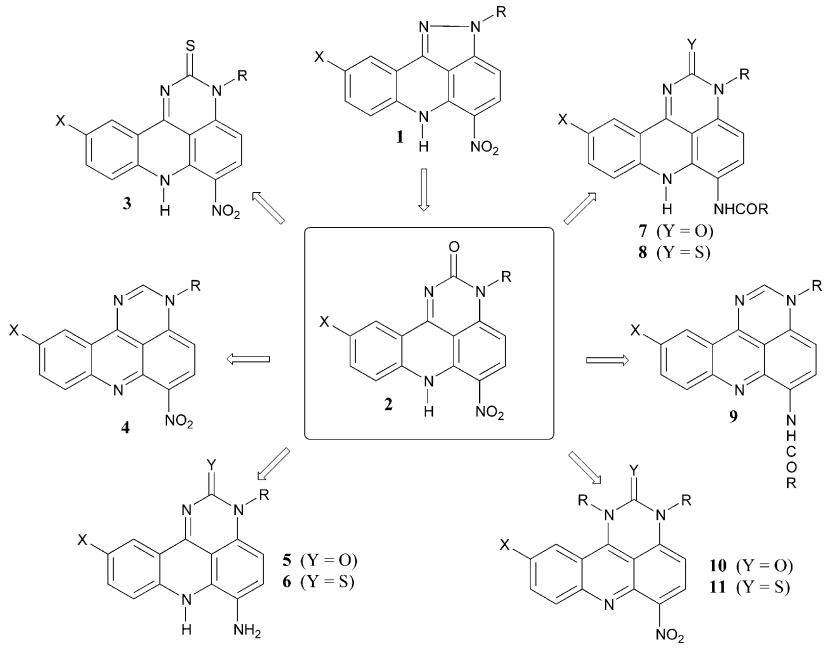


Figure 1. Rational design of pyrimido[4,5,6-*kl*]acridines from 5-nitropyrazolo[3,4,5-*kl*]acridines.

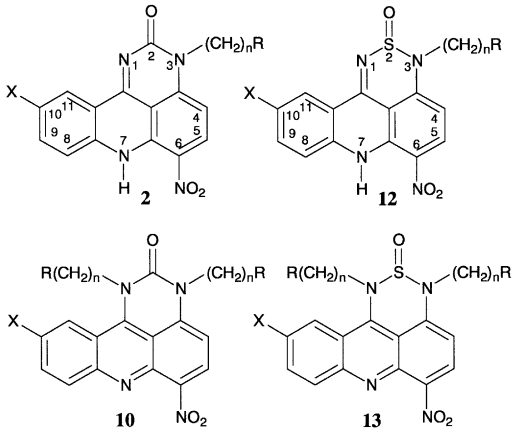


Figure 2. Rational design of [1,2,6]thiadiazino[3,4,5-*kl*]acridines **12,13** from pyrimido[4,5,6-*kl*]acridines **2** and **10**. For *X*, *n*, and *R* see Scheme 1.

Results and Discussion

Chemistry

Scheme 1 shows two synthetic pathways leading to derivatives **12** and **13**. The new 1-{[ω-(alkylamino)alkyl]-amino}-9-imino-4-nitro-9,10-dihydroacridines (**16g–k**) were obtained from the 1-chloro-4-nitro-9,10-dihydro-9-acridinone (**14**)[9] or from the 1-chloro-7-methyl-4-nitro-9,10-dihydro-9-acridinone (**15**)[10] in a manner similar to that reported previously for the already described[9] derivatives **16a–f**. Thus, **14** or, alternatively, **15** were refluxed in an excess of $POCl_3$ with a catalytic amount of DMF (*N,N*-dimethylformamide). Evaporation of the volatiles gave a solid, which was purified and then heated at 80 °C in phenol with ammonium carbonate. After cooling, a solution of NaOH was added to yield a precipitate, which was purified and treated with the appropriate amine in DMF at 100 °C to afford **16g–k**.

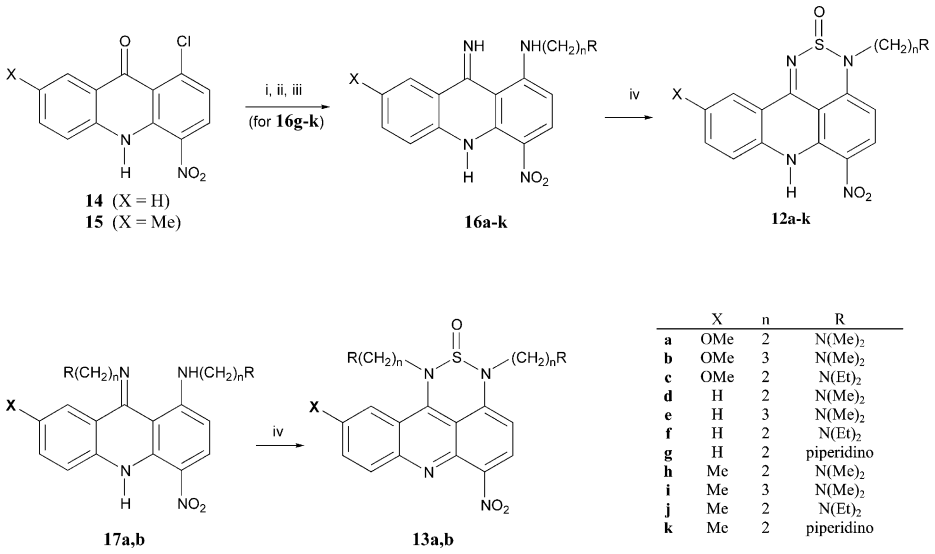
The cyclization of **16a–k** or **17a,b**[9] with $SOCl_2$ in chloroform at room temperature yielded the target [1,2,6]thiadiazino[3,4,5-*kl*]acridines **12a–k** and **13a,b**, respectively.

To examine the DNA-binding properties and the antineoplastic activity of these agents, the free base forms of the target derivatives were converted into their water-soluble maleates by the usual methods.

DNA-binding properties

'Apparent' binding constant (K_{app}) values were determined using a competitive fluorometric ethidium displacement method that has been used extensively for other DNA-binding ligands, particularly intercalants.[11–13] In this assay, the relative K_{app} affinity for calf thymus DNA (CTDNA) is defined by K_{app} (drug) = K_{app} (ethidium)/C_{50}, were C_{50} represents the concentration (μmol) of added compound required to reduce the fluorescence of a ethidium–DNA complex (containing 1.35 μmol or CT–DNA or A–T or G–C and 1 μmol ethidium bromide) by 50%, and the K_{app} (ethidium) binding constant is taken as $10^7\,M^{-1}$.[11,13] In the present study, fluorescence displacement assays were performed at pH 7 to enable comparison in biological conditions.

On these bases, the K_{app} values can be regarded as indicative of the strength and extent of binding to this 'pseudo-random' DNA sequence, but not of the mode of interaction (e.g., intercalation and/or groove binding mechanism). However, Table 1 shows that all compounds **12** have a significant binding with DNA, very similar to that of parent compound **2** with the same *X*, *n*, and *R* (Fig. 2). The K_{app} values are growing in the series: 10-unsubstituted ≅ 10-methyl < 10-methoxy derivatives. Potent binding is shown by the bis(amine-



	X	n	R
a	OMe	2	$N(Me)_2$
b	OMe	3	$N(Me)_2$
c	OMe	2	$N(Et)_2$
d	H	2	$N(Me)_2$
e	H	3	$N(Me)_2$
f	H	2	$N(Et)_2$
g	H	2	piperidino
h	Me	2	$N(Me)_2$
i	Me	3	$N(Me)_2$
j	Me	2	$N(Et)_2$
k	Me	2	piperidino

Scheme 1. Reagents: (i) $POCl_3$; (ii) PhOH, $(NH_4)_2CO_3$, 80 °C; (iii) $H_2N(CH_2)_nR$; (iv) $SOCl_2$.

Table 1. DNA binding and cytotoxicity (HT29) of target compounds **12a–k** and **13a,b** in comparison with corresponding parent compounds **2** and **10**[a]

Compd	$K_{app} \times 10^{-7}\,M^{-1}$			Binding site preference	IC_{50} (μM)
	CT–DNA	A–T	G–C		HT29
12a	0.27	0.19	0.13	A–T (1.5)	0.087
2a	0.24	0.22	0.29	None (0.76)	0.62
12b	0.10	0.24	0.11	A–T (2.2)	0.60
2b	0.19	0.27	0.32	None (0.84)	0.26
12c	0.10	0.075	0.086	None (0.87)	0.13
2c	0.14	0.29	0.23	None (1.26)	0.29
12d	0.038	0.19	0.031	A–T (61)	0.027
2d	0.056	0.053	0.11	G–C (0.45)	0.76
12e	0.088	0.12	0.046	A–T (26)	0.56
2e	0.081	0.084	0.088	None (0.95)	0.77
12f	0.12	0.12	0.070	A–T (17)	0.25
2f	0.064	0.069	0.073	None (0.95)	0.86
12g	0.019	0.14	0.024	A–T (58)	0.28
12h	0.095	0.17	0.054	A–T (31)	0.24
12i	0.14	0.16	0.063	A–T (25)	0.50
12j	0.059	0.18	0.017	A–T (110)	0.64
12k	0.051	0.060	0.023	A–T (2.6)	0.28
13a	6.9	7.7	8.5	None (0.91)	0.033
10a					5.6
13b	11.2	26.5	22	None (1.20)	2.1
10b					4.8

[a]For the meaning of K_{app}, binding site preference, and IC_{50} see text. Data for compounds **2** and **10** are from refs 7 and 8.

functionalized) derivatives **13**, which are 7–10 times more DNA-affinic than ethidium itself, suggesting that a second basic side chain is important for binding as we found for other acridine derivatives.[4,14,15]

An equivalent competitive fluorometric assay method was similarly used to assess the binding of derivatives **12** and **13** to the synthetic polynucleotides $[poly(dAdT)]_2$ (AT) and $[poly(dGdC)]_2$ (G-C) in order to examine the possible binding site preferences and to compare **12** to the parent compound **2** with the same *X*, *n*, and *R* (Fig. 2). The K_{app} values determined for the compounds with each duplex are collected in Table 1, together with the binding side preferences and, in parentheses, the A–T/G–C affinity ratio. The possible binding site selectivity is considered to be significant only for an A–T/G–C affinity ratio differing by more than 30% from the sequence-neutral unity value (i.e., <0.7 or >1.3). It can be observed that, also in these cases, the K_{app} values of compounds **12**, even inferior to that of ethidium, are indicative of a binding with these synthetic DNA. The site-dependent behavior is significantly (10-methoxy derivatives) or very markedly (10-unsubstituted or 10-methyl derivatives) A–T preferential, differently from parent compounds **2**, which show various and, in many cases, not significative binding site preference. Again, bis(amine-functionalized) derivatives **13** show excellent affinity, much higher than that of

ethidium, but the second basic side chain in position 1 makes the compounds neutral regarding the binding site preference.

In vitro cytotoxic activity

HT29 human colon adenocarcinoma cell line. In vitro cytotoxic potencies of target [1,2,6]thiadiazino[3,4,5-*kl*]acridines **12a–k** and **13a,b** in comparison with parent compound **2** and **10** with the same *X*, *n*, and *R* (Fig. 2) against human colon adenocarcinoma cell line (HT29) are reported in Table 1. The IC_{50} represents the drug concentration (μM) required to inhibit cell growth by 50%.

The results indicate that: (a) **12a,d** and **13a** emerge as the most potent among the new derivatives with IC_{50} values of 87, 27, and 33 nM, respectively; (b) all compounds **12** possess a good antiproliferative activity in the submicromolar range; (c) **12d** and **13a** posses potency similar to that of doxorubicin (Dx, $IC_{50} = 26$ nM) in similar assays.[5,6] The data obtained allow us to formulate some structure–activity relationships for both side chains and substituents in position 10 of **12** and some considerations in the comparison with parent derivatives: (i) the optimal distance between the two nitrogen atoms is of two methylene units, as indicated by the difference in potency especially between the pairs **12a**,**b**, **12d**,**e**, and **13a**,**b**, but also between the pair **12h**,**i**; (ii) bulky substituents at the terminal nitrogen atoms decrease the cytotoxicity, as can be seen from the IC_{50} values of the pairs **12a**,**c**, **12d**,**f**, **12d**,**g**, and **12h**,**j**, but less than what observed at point (i); (iii) both these behaviors are in contrast with what reported for parent compounds **2** and **10**; (iv) generally, the target compounds **12** and **13** are more active than corresponding **2** and **10**, as is particularly evident in the pairs **12a**–**2a**, **12d**–**2d**, and **13a**–**10a**; (v) substitution pattern in position 10 of compounds **12** leads to different results; the most interesting behavior is for the compounds in which $n = 2$ and $R =$ Me were the IC_{50} goes from 27 nM (**12d**, 10-non substituted), to 87 nM (**12a**, 10-methoxy), to 240 nM (**12h**, 10-methyl); (vi) the second (aminoalkyl)amino side chain in position 1 of derivatives **13** yield contrasting results; if we compare **13a** with **12a**, confer a superior antiproliferative action, but the contrary happens if we compare **13b** with **12b**; (vii) this behavior was not observed with related derivatives **2** and **10**, where the second side chain in position 1 leads always to a diminished cytotoxic activity.[8]

There is no correlation between DNA-binding and antiproliferative activity against HT29 cell line. The most cytotoxic agent **12d** in the series, is one of the weakest CT–DNA ligand; **13a**, which also shows marked cytotoxicity, is very affinic to CT–DNA, while **13b**, the most efficient CT–DNA ligand, is the least cytotoxic. As we previously noted,[5,6] these facts clearly indicate that to bind DNA is not the only determinant for antiproliferative action, but other factors, such as cellular uptake or enzyme inhibition, may also contribute.

Human ovarian carcinoma cell line panel. In vitro cytotoxic potencies of new acridine derivatives **12, 13** and of reference drug Cisplatin (Cs) against five human ovarian carcinoma cell lines, A2780 (sensitive), A2780/Cs (cisplatin resistant), CH1 (sensitive), CH1/Cs (cisplatin resistant), and SKOV-3, are shown in Table 2. In the resistant cell line columns, besides the IC_{50} values, are reported, in parentheses, the resistance index ($RI = IC_{50}$ ratio of resistant line on sensitive one) values.

Also on these cell line panels the best compounds seem to be **12d** and **13a**, which possess activity superior to that of reference drug Cs; in addition **12d** and, especially, **13a** are not cross resistant with Cs on A2780/Cs cell line. On the contrary, **12a** does not seem to be very cytotoxic against this cell line panel, but has IC_{50} values in the μM range, similarly to the other target derivatives. Compounds **12** and **13** are not or scarcely cross resistant with Cs on A2780/Cs cell line, especially the bis(alkylamino)alkyl functionalized **13**. Instead, **12** and **13** show some grade of cross resistance on CH1/Cs cell line.

In vivo antitumor activity

Some of the target derivatives, endowed of relevant cytotoxic activity, were selected for in vivo studies. In Table 3, antitumor activity of **12a**,**d**,**e** and **13a** against murine leukemia P388 are reported. The mean survival time (MST) for each treatment group (eight mice/group) and for each single dose was calculated and the percent T/C determined by using the following formula:% T/C MST treated/MST control × 100 (% T/C < 86 indicates toxicity,% T/C > 120% demonstrate moderate activity,% T/C > 175% demonstrate significant activity). The drug toxicity was measured as number of toxic death/total number of animals in group.

Compounds **12a** and **12e** show border line activity at optimal dose of 100 mg/kg, **13a** shows no activity, and only **12d** possesses significant antitumor action at 25 mg/kg dose.

Table 2. In vitro cytotoxic activity of target compounds **12** and **13** against a human ovarian carcinoma cell line panel in comparison with reference drug Cs[a]

Compd	A2780	A2780/Cs	CH1	CH1/Cs	SKOV-3
12a	2.6	4.0 (1.5)	2.0	13 (6.5)	7.7
12b	2.3	2.1 (0.9)	2.0	6.6 (3.3)	3.9
12c	1.6	1.9 (1.2)	1.9	12 (6.3)	2.0
12d	0.31	0.54 (1.7)	0.22	0.89 (4.0)	0.69
12e	0.62	0.76 (1.2)	0.68	3.9 (5.7)	1.3
12f	0.63	1.7 (2.7)	0.66	3.2 (4.8)	2.2
12g	2.4	2.2 (0.9)	2.9	>25 (>8)	5.4
12h	2.0	2.2 (1.1)	1.4	11 (7.9)	3.2
12i	2.8	3.2 (1.1)	2.3	10 (4.3)	4.2
12j	2.6	2.9 (1.1)	2.4	11 (4.6)	4.0
12k	2.8	2.3 (0.8)	3.5	>25 (>7)	>25
13a	0.68	0.49 (0.7)	0.63	7.7 (12)	0.96
13b	1.8	0.66 (0.37)	1.4	10 (7.1)	1.9
Cs	0.89	3.4 (3.8)	0.15	2.4 (16)	3.4

[a]See text.

Table 3. Antitumor activity of selected compounds against P388 murine leukemia[a]

Compd	Dose (mg/kg)	%T/C	Tox
12a	12.5	120	0/8
	25	120	0/8
	50	120	0/8
	100	130	1/8
12d	6.24	120	0/8
	12.5	140	0/8
	25	170	0/8
	50	80	2/8
12e	12.5	100	0/8
	25	100	0/8
	50	109	0/8
	100	127	0/8
	200	64	8/8
13a	12.5	100	0/8
	25	100	0/8
	50	118	0/8
	100	118	0/8
	200	64	8/8

[a]See text.

Conclusions

It can be concluded that the target 6-nitro-2,7-dihydro-3*H*-2λ^4-[1,2,6]thiadiazino[3,4,5-*kl*]acridin-2-ones constitute a new class of acridine derivatives endowed with noticeable cytotoxic activity. The substitution of a pyrimidinic ring of compounds **2** and **10** with a [1,2,6]thiadiazinic one of derivatives **12** and **13** yielded an increase in antiproliferative activity and in DNA-binding. In particular the 3-[2-(dimethylamino)ethyl]-6-nitro-2,7-dihydro-3*H*-2λ^4-[1,2,6]thiadiazino[3,4,5-*kl*]acridin-2-one (**12d**), which possesses the most relevant cytotoxicity in the series and a significant in vivo antitumor activity, can be regarded as a lead in the development of anticancer tetracyclic acridine derivatives.

Experimental

Chemistry

Melting points were determined on a Büchi 510 apparatus and are uncorrected. Thin-layer chromatography (TLC) was accomplished using plates precoated with silica gel 60 F-254 (Merck). All ^{1}H NMR spectra were recorded on a Varian VXR 300 instrument. Chemical shifts are reported as δ values (ppm) downfield from internal Me_4Si in the solvent shown. The following NMR abbreviations are used: br (broad), s (singlet), d (doublet), t (triplet), m (multiplet), ar (aromatic proton), ex (exchangeable with D_2O). Elemental analyses were performed on a Model 1106 elemental analyzer (Carlo Erba Strumentazione).

1-[2-(Piperidinoethyl)amino]-9-imino-4-nitro-9,10-dihydroacridine (16g). Example of general procedure for the preparation of 16g–k. The 1-chloro-4-nitro-9,10-dihydro-9-acridinone (**14**)[9] (1 g, 3.6 mmol) with a catalytic amount DMF (0.1 mL) was refluxed in $POCl_3$ (10 mL) for 4 h, then the solvent was evaporated. The obtained solid was stirred in dioxane (10 mL) for 10 min, put into diluted $(NH_4)OH$ and crushed ice and vigorously stirred for 10 min to yield a precipitate, which was filtered, washed successively with water and acetone, and dried at room temperature. This solid was poured in freshly distilled phenol (15 g, 0.16 mol) and ammonium carbonate (0.4 g, 4 mmol) and the resulting mixture was gradually heated at 80 °C, under stirring, for 2 h. After cooling, NaOH 3 N (80 mL) was added and the mixture stirred for 10 min to afford a precipitate which was washed with water until neutral. The dried crude solid obtained was put in DMF (10 mL) with 2-piperidinoethylamine (1.5 g, 11.7 mmol) and the resulting mixture was stirred at 100 °C for 2 h. To the hot reaction mixture, NaOH 2 N (35 mL) was added and stirring was continued for 10 min. The precipitated solid was collected by filtration, washed with water and crystallized from acetone–water to yield pure **16g** (62%): mp 175–177 °C; ^{1}H NMR ($CDCl_3$) δ 1.40–1.70 (m, 6H, 3 × CH_2), 2.50 (t, 4H, 2 × CH_2), 2.70 (t, 2H, CH_2), 3.45–3.60 (m, 2H, CH_2), 6.30 (d, 1H, ar), 7.20–7.30 (m, 2H, ar), 7.55 (t, 1H, ar), 7.80 (d, 1H, ar), 8.25 (d, 1H, ar), 9.18 (s, 1H, NH ex), 12.21 (s, 1H, NH, ex), 13.60 (br s, 1H, NH, ex).

Derivatives **16h–k** were prepared in a similar manner.

1-[2-(Dimethylamino)ethyl]amino-9-imino-7-methyl-4-nitro-9,10-dihydroacridine (16h). (60%); mp 218–220 °C; ^{1}H NMR (DMSO-d_6) δ 2.28 (s, 6H, 2 × CH_3), 2.45 (s, 3H, CH_3), 2.65 (t, 2H, CH_2), 3.55 (t, 2H, CH_2), 6.65 (d, 1H, ar), 7.45 (d, 1H, ar),7.60 (d, 1H, ar), 8.10–8.20 (m, 2H, ar), 9.95 (s, 1H, NH ex), 12.25 (s, 1H, NH ex), 14.0 (br s, 1H, NH ex).

1-[3-(Dimethylamino)propyl]amino-9-imino-7-methyl-4-nitro-9,10-dihydroacridine (16i). (62%); mp 152–154 °C; ^{1}H NMR ($CDCl_3$) δ 1.80–2.02 (m, 2H, CH_2), 2.30 (s, 6H, 2 × CH_3), 2.40–2.55 (m, 5H, $CH_3 + CH_2$), 3.40 (t, 2H, CH_2), 6.30 (d, 1H, ar), 7.10 (d, 1H, ar), 7.30 (d, 1H, ar), 7.50 (s, 1H, ar), 8.18 (d, 1H, ar), 8.90 (s, 1H, NH ex), 12.10 (s, 1H, NH ex), 13.60 (br s, 1H, NH ex).

1-[2-(Diethylamino)ethyl]amino-9-imino-7-methyl-4-nitro-9,10-dihydroacridine (16j). (54%); mp 138–139 °C; ^{1}H NMR ($CDCl_3$) δ 1.10 (t, 6H, 2 × CH_3), 2.47 (s, 3H, CH_3), 2.60–2.75 (m, 4H, 2 × CH_2), 2.90 (t, 2H, CH_2), 3.50 (t, 2H, CH_2), 6.30 (d, 1H, ar), 7.15 (d, 1H, ar), 7.33 (d, 1H, ar), 7.60 (s, 1H, ar), 8.22 (d, 1H, ar), 9.00 (s, 1H, NH ex), 12.20 (s, 1H, NH ex), 13.60 (br s, 1H, NH ex).

1-[2-(Piperidinoethyl)amino]-9-imino-7-methyl-4-nitro-9,10-dihydroacridine (16k). (45%); mp 166–168 °C; ^{1}H NMR ($CDCl_3$) δ 1.50–1.75 (m, 6H, 3 × CH_2), 2.45 (s, 3H, CH_3), 2.50 (t, 4H, 2 × CH_2), 2.58 (t, 2H, CH_2), 3.57 (t, 2H, CH_2), 6.35 (d, 1H, ar), 7.20 (d, 1H, ar), 7.38 (d, 1H, ar), 7.60 (s, 1H, ar), 8.25 (d, 1H, ar), 9.05 (s, 1H, NH ex), 12.20 (s, 1H, NH, ex), 13.60 (br s, 1H, NH, ex).

3-[2-(Dimethylamino)ethyl]-10-methoxy-6-nitro-2,7-dihydro-3*H*-2λ^4-[1,2,6]thiadiazino[3,4,5-*kl*]acridin-2-one (12a). Example of general procedure for the preparation of 12

and 13. To a solution of **16a**[9] (0.3 g, 0.84 mmol) and anhydrous triethylamine (0.29 mL, 2.1 mmol) in $CHCl_3$ (10 mL) stirred at 0 °C, a solution of $SOCl_2$ (0.08 mL, 1 mmol) in $CHCl_3$ (10 mL) was added dropwise. The stirring was protracted for 3 h at room temperature, after which the reaction mixture was partitioned between $CHCl_3$ (50 mL) and an excess of 1 M aqueous Na_2CO_3 (2 × 50 mL). The organic layer was worked up to give a residue which was eluted with $CHCl_3$/MeOH (19:1) in a silica gel column to afford pure **12a** (0.2 g, 59%): mp 232–234 °C, maleate 190–192 °C dec; ^{1}H NMR ($CDCl_3$) δ 2.40 (s, 6H, 2 × CH_3), 2.83 (t, 2H, CH_2), 3.93 (s, 3H, CH_3), 4.04–4.30 (m, 2H, CH_2), 6.70 (d, 1H, ar), 7.30 (d, 1H, ar), 7.40 (d, 1H, ar), 7.88 (s, 1H, ar), 8.60 (d, 1H, ar), 12.20 (s, 1H, NH ex). Anal. calcd for $C_{18}H_{19}N_5O_4S$: C, 53.86; H, 4.77; N, 17.45; S, 7.99. Found: C, 54.00; H, 5.09; N, 17.30; S, 7.54.

Derivative **12b–k** and **13a**,**b** were prepared in a similar manner.

3-[3-(Dimethylamino)propyl]-10-methoxy-6-nitro-2,7-dihydro-3*H*-2λ^4-[1,2,6]thiadiazino[3,4,5-*kl*]acridin-2-one (12b). (77%); mp 238–240 °C, maleate 223–224 °C dec; ^{1}H NMR ($CDCl_3$) δ 1.90–2.10 (m, 2H, CH_2), 2.28 (s, 6H, 2 × CH_3), 2.40 (t, 2H, CH_2), 3.90 (s, 3H, CH_3), 4.12 (t, 2H, CH_2), 6.77 (d, 1H, ar), 7.28–7.48 (m, 2H, ar), 7.94 (s, 1H, ar), 8.60 (d, 1H, ar), 12.30 (s, 1H, NH ex). Anal. calcd for $C_{19}H_{21}N_5O_4S$: C, 54.93; H, 5.09; N, 16.86; S, 7.72. Found: C, 54.88; H, 5.21; N, 17.03; S, 7.84.

3-[2-(Diethylamino)ethyl]-10-methoxy-6-nitro-2,7-dihydro-3*H*-2λ^4-[1,2,6]thiadiazino[3,4,5-*kl*]acridin-2-one (12c). (69%); mp 193–194 °C, maleate 151–152 °C dec; ^{1}H NMR ($CDCl_3$) δ 1.08 (t, 6H, 2 × CH_3), 2.50–2.75 (m, 4H, 2 × CH_2), 2.90 (t, 2H, CH_2), 3.93 (s, 3H, CH_3), 4.13 (t, 2H, CH_2), 6.75 (d, 1H, ar), 7.32 (d, 1H, ar),7.44 (d, 1H, ar), 7.90 (s, 1H, ar), 8.60 (d, 1H, ar), 12.28 (s, 1H, NH ex). Anal. calcd for $C_{20}H_{23}N_5O_4S$: C, 55.93; H, 5.40; N, 16.31; S, 7.46. Found: C, 56.09; H, 5.29; N, 16.38; S, 7.24.

3-[2-(Dimethylamino)ethyl]-6-nitro-2,7-dihydro-3*H*-2λ^4-[1,2,6]thiadiazino[3,4,5-*kl*]acridin-2-one (12d). (50%); mp 208–210 °C, maleate 154–156 °C dec; ^{1}H NMR ($CDCl_3$) δ 2.43 (s, 6H, 2 × CH_3), 2.90 (t, 2H, CH_2), 4.04–4.40 (m, 2H, CH_2), 6.82 (d, 1H, ar), 7.39 (d, 1H, ar),7.43 (d, 1H, ar), 7.78 (t, 1H, ar), 8.52–8.66 (m, 2H, ar), 12.20 (s, 1H, NH ex). Anal. calcd for $C_{17}H_{17}N_5O_3S$: C, 54.98; H, 4.61; N, 18.86; S, 8.63. Found: C, 54.87; H, 4.89; N, 18.91; S, 8.44.

3-[3-(Dimethylamino)propyl]-6-nitro-2,7-dihydro-3*H*-2λ^4-[1,2,6]thiadiazino[3,4,5-*kl*]acridin-2-one (12e). (49%); mp 182–184 °C, maleate 183–185 °C dec; ^{1}H NMR ($CDCl_3$) δ 1.90–2.12 (m, 2H, CH_2), 2.28 (s, 6H, 2 × CH_3), 2.40 (t, 2H, CH_2), 4.10 (t, 2H, CH_2), 6.80 (d, 1H, ar), 7.40–7.52 (m, 2H, ar),7.73 (t, 1H, ar), 8.55–8.70 (m, 2H, ar), 12.25 (s, 1H, NH ex). Anal. calcd for $C_{18}H_{19}N_5O_3S$: C, 56.09; H, 4.97; N, 18.17; S, 8.32. Found: C, 55.89; H, 5.09; N, 18.30; S, 8.17.

3-[2-(Diethylamino)ethyl]-6-nitro-2,7-dihydro-3*H*-2λ^4-[1,2,6]thiadiazino[3,4,5-*kl*]acridin-2-one (12f). (29%); mp 165–166 °C, maleate 132–134 °C dec; ^{1}H NMR ($CDCl_3$) δ 1.02 (t, 6H, 2 × CH_3), 2.50–2.70 (m, 4H, 2 × CH_2), 2.90 (t, 2H, CH_2), 4.10 (t, 2H, CH_2), 6.80 (d, 1H, ar), 7.40–7.50 (m, 2H, ar),7.78 (t, 1H, ar), 8.62–8.64 (m, 2H, ar), 12.22 (s, 1H, NH ex). Anal. calcd for $C_{19}H_{21}N_5O_3S$: C, 57.13; H, 5.30; N, 17.53; S, 8.03. Found: C, 56.94; H, 5.11; N, 17.32; S, 7.94.

6-Nitro-3-(2-piperidinoethyl)-2,7-dihydro-3*H*-2λ^4-[1,2,6]thiadiazino[3,4,5-*kl*]acridin-2-one (12g). (34%); mp 188–189 °C, maleate 209–210 °C dec; ^{1}H NMR ($CDCl_3$) δ 1.40–1.80 (m, 6H, 3 × CH_2), 2.40–2.60 (m, 4H, 2 × CH_2), 2.75 (t, 2H, CH_2), 4.00–4.25 (m, 2H, CH_2), 6.75 (d, 1H, ar), 7.38–7.55 (m, 2H, ar), 7.77 (t, 1H, ar), 8.55–8.70 (m, 2H, ar), 12.20 (s, 1H, NH, ex). Anal. calcd for $C_{20}H_{21}N_5O_3S$: C, 58.38; H, 5.14; N, 17.02; S, 7.79. Found: C, 58.22; H, 5.09; N, 17.30; S, 7.64.

3-[2-(Dimethylamino)ethyl]-10-methyl-6-nitro-2,7-dihydro-3*H*-2λ^4-[1,2,6]thiadiazino[3,4,5-*kl*]acridin-2-one (12h). (73%); mp 238–240 °C, maleate 220–222 °C dec; ^{1}H NMR ($CDCl_3$) δ 2.40 (s, 6H, 2 × CH_3), 2.50 (s, 3H, CH_3), 2.78–2.90 (m, 2H, CH_2), 4.00–4.25 (m, 2H, CH_2), 6.78 (d, 1H, ar), 7.38 (d, 1H, ar), 7.58 (d, 1H, ar), 8.40 (s, 1H, ar), 8.63 (d, 1H, ar), 12.22 (s, 1H, NH ex). Anal. calcd for $C_{18}H_{19}N_5O_3S$: C, 56.09; H, 4.97; N, 18.17; S, 8.32. Found: C, 56.31; H, 5.19; N, 17.98; S, 8.04.

3-[3-(Dimethylamino)propyl]-10-methyl-6-nitro-2,7-dihydro-3*H*-2λ^4-[1,2,6]thiadiazino[3,4,5-*kl*]acridin-2-one (12i). (54%); mp 215–216 °C, maleate 178–180 °C dec; ^{1}H NMR ($CDCl_3$) δ 1.88–2.13 (m, 2H, CH_2), 2.27 (s, 6H, 2 × CH_3), 2.33 (t, 2H, CH_2), 2.48 (s, 3H, CH_3), 4.12 (t, 2H CH_2), 6.80 (d, 1H, ar), 7.35 (d, 1H, ar), 7.55 (d, 1H, ar), 8.40 (s, 1H, ar), 8.60 (d, 1H, ar), 12.22 (s, 1H, NH ex). Anal. calcd for $C_{19}H_{21}N_5O_3S$: C, 57.13; H, 5.30; N, 17.53; S, 8.03. Found: C, 57.00; H, 5.19; N, 17.31; S, 7.84.

3-[2-(Diethylamino)ethyl]-10-methyl-6-nitro-2,7-dihydro-3*H*-2λ.^{4}-[1,2,6]thiadiazino[3,4,5-*kl*]acridin-2-one (12j) (59%); mp 191–194 °C, maleate 143–145 °C dec; ^{1}H NMR ($CDCl_3$) δ 1.10 (t, 6H, 2 × CH_3), 2.50 (s, 3H, CH_3), 2.6–2.7 (m, 4H, 2 × CH_2), 2.90 (t, 2H, CH_2), 4.00–4.20 (m, 2H, CH_2), 6.78 (d, 1H, ar), 7.39 (d, 1H, ar), 7.50 (d, 1H, ar), 8.40 (s, 1H, ar), 8.60 (d, 1H, ar), 12.22 (s, 1H, NH, ex). Anal. calcd for $C_{20}H_{23}N_5O_3S$: C, 58.10; H, 5.61; N, 16.94; S, 7.75. Found: C, 58.24; H, 5.49; N, 17.10; S, 7.54.

10-Methyl-6-nitro-3-(2-piperidinoethyl)-2,7-dihydro-3*H*-2λ^4-[1,2,6]thiadiazino[3,4,5-*kl*]acridin-2-one (12k). (40%); mp 248–249 °C, maleate 211–213 °C dec; ^{1}H NMR ($CDCl_3$) δ 1.40–1.70 (m, 6H, 3 × CH_2), 2.40–2.63 (m, 7H, 2 × CH_2 + CH_3), 2.80 (t, 2H, CH_2), 4.05–4.30 (m, 2H, CH_2), 6.80 (d, 1H, ar), 7.39 (d, 1H, ar), 7.55 (d, 1H, ar), 8.45 (s, 1H, ar), 8.61 (d, 1H, ar), 12.28 (s, 1H, NH, ex). Anal. calcd for $C_{21}H_{23}N_5O_3S$: C, 59.28; H, 5.45; N, 16.46; S, 7.53. Found: C, 59.09; H, 5.39; N, 16.30; S, 7.54.

1,3-Di[2-(dimethylamino)ethyl]-10-methoxy-6-nitro-2,3-dihydro-1*H*-2λ^4-[1,2,6]thiadiazino[3,4,5-*kl*]acridin-2-one

(13a). (65%); mp 173–174 °C, maleate 115–117 °C dec; ^{1}H NMR ($CDCl_3$) δ 2.07 (s, 6H, 2 × CH_3),.2.23 (s, 6H, 2 × CH_3), 2.40–2.77 (m, 4H, 2 × CH_2), 3.52–3.62 (m, 2H, CH_2)3.65–3.95 (m, 5H, $CH_3 + CH_2$), 6.65 (d, 1H, ar), 7.33–7.42 (m, 2H, ar), 7.95 (d, 1H, ar), 8.08 (d, 1H, ar). Anal. calcd for $C_{22}H_{28}N_6O_4S$: C, 55.92; H, 5.97; N, 17.78; S, 6.78. Found: C, 55.89; H, 6.28; N, 17.77; S, 6.34.

1,3-Di[3-(dimethylamino)propyl]-10-methoxy-6-nitro-2,3-dihydro-1*H*-2λ^4-[1,2,6]thiadiazino[3,4,5-*kl*]acridin-2-one (13b). (47%); mp 138–139 °C, maleate 92–94 °C dec; ^{1}H NMR ($CDCl_3$) δ 1.70–2.16 (m, 10H, 2 × CH_2 + 2 × CH_3), 2.23–2.37 (m, 8H, CH_2 + 2 × CH_3), 2.49 (t, 2H, CH_2), 3.58–3.70 (m, 2H, CH_2), 3.95–4.07 (m, 5H, $CH_3 + CH_2$), 6.85 (d, 1H, ar), 7.37 (s, 1H, ar), 7.54 (d, 1H, ar), 8.12–8.24 (m, 2H, ar). Anal. calcd for $C_{24}H_{32}N_6O_4S$: C, 57.58; H, 6.44; N, 16.79; S, 6.40. Found: C, 57.69; H, 6.29; N, 16.68; S, 6.25.

Fluorescence binding studies. The fluorometric assays have been described previously.[16] The C_{50} values for ethidium displacement from CT–DNA, A–T, and G–C were determined using aqueous buffer (10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.0) containing 1.26 μM ethidium bromide and 1 μM CT–DNA, A–T, and G–C, respectively.[16–18]

All measurements were made in 10-mm quartz cuvettes at 20 °C using a Perkin-Elmer LS5 instrument (excitation at 546 nm; emission at 595 nm) following serial addition of aliquots of a stock drug solution (~5 mM in DMSO). The C_{50} values are defined as the drug concentrations which reduce the fluorescence of the DNA-bound ethidium by 50%, and are calculated as the mean from three determinations.

In vitro cytotoxicity. HT29 human colon adenocarcinoma. Details of HT29 human colon adenocarcinoma carcinoma cell line assay have been previously described.[7] Drug solutions of appropriate concentration were added to a culture containing HT29 cells at 2.5 × 10^4 cells/mL of medium and the drug exposure was protracted for 144 h. All assays were performed in triplicate, as previously described.[7]

Human ovarian carcinoma experimental protocol

Established details and biological properties of human ovarian carcinoma cell lines (A2780, A2780cisR, CH1, CH1cisR, and SKOV-3) have been described previously.[19] The sulforhodamine B (SRB) experimental protocol used has been described previously.[16,19] Cells were plated (100–5000 cells) in 96-well microtiter plates and left overnight to adhere prior to drug treatment. Aqueous drug solutions at pH 7.0 were then added to the cells at various concentrations following dilution of a stock DMSO solution. After 96 h continuous drug exposure at 37 °C, growth inhibition was assessed using SRB protein staining. IC_{50} values, as mean of two independent assays, (drug dose required for 50% growth inhibition compared to drug-free controls) were determined by comparing treated and untreated cells.

In vivo antitumor activity. P388 Murine leukemia

Murine P388 leukemia was obtained from the Institute of Immunology and Experimental Therapy of Polish Academy of Sciences. For test purpose, CDF_1 mice were injected ip with 10^6 P388 lymphatic leukemia cells on day 0 and treated ip on days 1–5 in accordance with the protocols described by the National Cancer Institute.[20] The treated and the control group consisted of 8 animals.

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