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2,7-Dihydro-3*H*-pyridazino[5,4,3-*kl*]acridin-3-one derivatives, novel type of cytotoxic agents active on multidrug-resistant cell lines. Synthesis and biological evaluation

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Abstract—We have earlier postulated that the presence of a pyridazone ring fused with an anthracenedione moiety resulted in the analog's ability to overcome multidrug resistance of tumor cells [*J. Med. Chem.* **1999**, *42*, 3494]. High cytotoxic activity of obtained anthrapyridazones [*Bioorg. Med. Chem.* **2003**, *11*, 561] toward the resistant cell lines, prompted us to synthesize the similarly modified acridine compounds. A series of pyridazinoacridin-3-one derivatives (**2b–h**) were prepared from the reaction of 9-oxo-9,10-dihydroacridine-1-carboxylate with $POCl_3$, followed by addition of the appropriate (alkylamino)alkylhydrazines. In vitro cytotoxic activity toward sensitive and resistant leukemia cell lines: L1210, K562, K562/DX, HL-60, HL-60/VINC, and HL-60/DX, with various type of multidrug resistance (MDR and MRP) was determined. The compounds studied exhibited in comparison to the reference cytostatics (DX, MIT) desirable very low resistance indexes (RI). Variations have been observed depending upon the substituent and the type of drug exporting pump. The cytotoxic activities of examined compounds, as well as of model anthrapyridazone derivative PDZ, were lower than those of reference drugs (DX, MIT) due to their diminished affinity to DNA.

1. Introduction

The prolonged use of antitumor agents often leads to the appearance of cell populations, which are crossresistant to many cytostatic agents.[1–3] This effect known as multidrug resistance has recently become a major cause of failures in antitumor chemotherapy. This phenomenon depends on the overexpression of plasma membrane drug efflux pumps. Many efforts have been directed toward the design of new cytotoxic agents with the ability to overcome multidrug resistance, including rather popular antitumor agents of anthraquinone and acridine groups. We have earlier postulated that the essential structural factor in anthracenedione and acridine cytostatics, enabling the compounds to overcome multidrug resistance of tumor cells, is the presence of a fused five or six-membered heterocyclic ring(s) as part of the molecule.[4–8] We have evidenced that the mechanism of this effect is based on the increased rate of diffusive drug influx surpassing its export mediated by efflux pumps in the multidrug-resistant tumor cells.[9–11] Some of these analogs of the anthraquinone family are anthrapyridazones (**1**), characterized by the presence of an additional pyridazone ring fused to the anthracenedione moiety (Fig. 1).[5] These compounds exhibit good activity on multidrug-resistance tumor cell lines as of yet, the analogous tetracyclic acridine compounds have not been prepared. Known acridine polycyclic analogs active on multidrug-resistant tumor cell lines comprise compounds with fused pyrazole and dihydropyrimidine rings in tetracyclic and pentacyclic systems.[7,8] Now, we report the synthesis and the biological evaluation of

Abbreviations: MIT, Mitoxantrone; DX, Doxorubicin; VINC, Vincristine; PDZ, 6-chloro-2-[2-(dimethylamino)ethyl]-2,7-dihydro-3*H*-dibenzo[*de*,*h*]-cinnoline-3,7-dione; MDR, P-gp dependent multidrug resistance; MRP, multidrug resistance associated protein dependent resistance.

Keywords: Antitumor compounds; Acridine analogs; Cytotoxic activity; Multidrug resistance.

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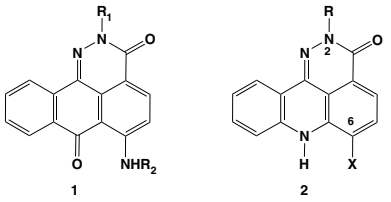


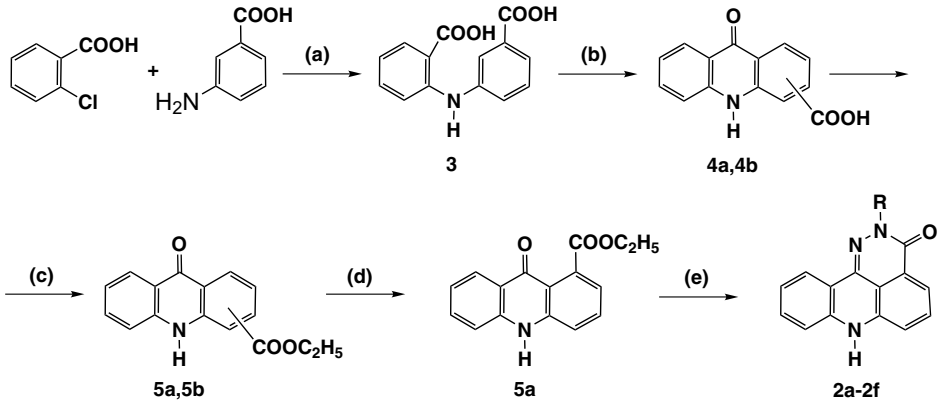
Figure 1. The structures of anthrapyridazones (**1**) and pyridazoacridines (**2**).

pyridazinoacridines (**2**), in which an acridine moiety is fused with the pyridazone ring to form a tetracyclic system (Fig. 1). The pyridazone ring is substituted, as in active anthrapyridazones, at position 2 with different (alkylamino)alkyl side chains. Also an analog with a chlorine atom at the C_6 has been synthesized. The model, analogous anthrapyridazone compound (PDZ), was also used in these studies for comparative purposes.

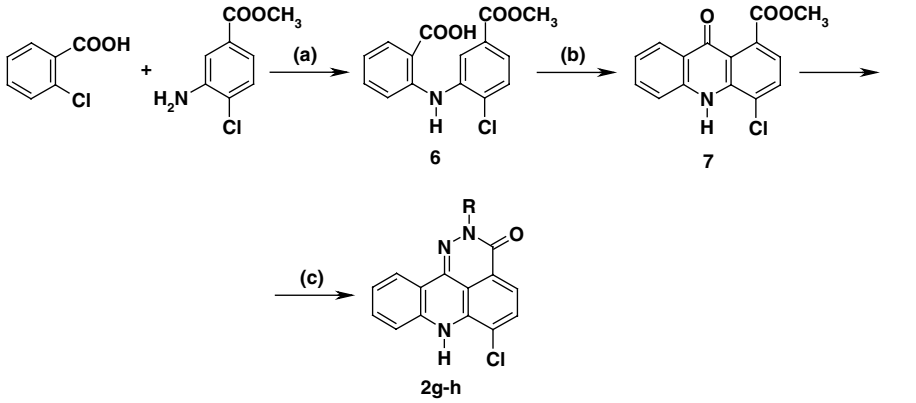
Pyridazinoacridin-3-one derivatives (**2b**–**g**) were evaluated for their in vitro cytotoxic potency toward sensitive and multidrug resistant tumor cell lines representative for two major types of drug exporting pumps, MDR (P-gp dependent multidrug resistance) and MRP (multidrug resistance associated protein dependent resistance). The applied panel was comprised of leukemia cell lines: sensitive murine L1210 and human K562 and HL-60, as well as resistant sublines K562/DX (MDR-type resistance), HL-60/VINC (MDR-type resistance), and HL-60/DX (MRP-type resistance). For the examined compounds the DNA binding test was performed.

2. Chemistry

Schemes 1 and 2 show the applied synthetic pathways. The 2-[3-(carboxy)anilino]benzoic acid **3** is most conveniently prepared by the Jourdan–Ullmann condensation of 2-chlorobenzoic acid and 3-aminobenzoic acid.[12] 9-Oxo-9,10-dihydro-1-acridinecarboxylic **4a** and 9-oxo-9,10-dihydro-3-acridinecarboxylic acids **4b** were prepared together as an isomeric mixture from **3** by acid catalyzed cyclization with concentrated H_2SO_4. An initial attempt to separate the two isomers by recrystallization was rather tedious because of their insolubility properties. Therefore, to facilitate the separation of iso-

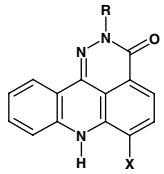


Scheme 1. Synthetic route for compounds **2a**–**f**. Reagents and conditions: (a) Cu powder, K_2CO_3, *i*-$C_5H_{11}OH$, reflux/3 h; (b) concd H_2SO_4, 100 °C/1 h; (c) EtOH/dry HCl, reflux/4 h; (d) chromatography on silica gel; (e) for **2a**: $NH_2NH_2xH_2O$, 125 °C/6 h; for **2b**–**f**: 1. $POCl_3$, 125 °C/1 h; 2. NH_2NHR, 125 °C/2.5–3 h.



Scheme 2. Synthetic route for compounds **2g**–**h**. Reagents and conditions: (a) *N*-methylpyrrolidinone and *N*,*N*-diisopropylethylamine, $Cu(OAc)_2xH_2O$, 130 °C/3 h; (b) PPE, 100 °C/1 h; (c) for **2g**: 1. $POCl_3$, reflux/0.5 h, 2. $NH_2NH(CH_2)_3N(CH_3)_2$, DMA, 130 °C/3 h; for **2h**: $NH_2NH_2xH_2O$, DMA, 125 °C/2 h.

Table 1. Structure and characteristics of compounds **2b–g**



Compound	R	X	Yield (%)	Mp (°C)	Molecular formula
2b	$CH_2CH_2N(CH_3)_2$	H	45	258–259	$C_{18}H_{18}N_4O$
2c	$CH_2CH_2CH_2N(CH_3)_2$	H	40	251–253	$C_{19}H_{20}N_4O$
2d	$CH_2CH_2N(CH_2CH_3)_2$	H	51	248–250	$C_{20}H_{22}N_4O$
2e	CH_2CH_2-*c*-$N(CH_2)_4O$	H	43	236–238	$C_{20}H_{20}N_4O_2$
2f	CH_2CH_2-*c*-$N(CH_2)_5$	H	35	231–233	$C_{21}H_{22}N_4O$
2g	$CH_2CH_2CH_2N(CH_3)_2$	Cl	25	185–186	$C_{19}H_{19}N_4OCl$

mers, isomeric mixture of **4a** and **4b** was esterified with acidic ethanol under anhydrous conditions. The isomer esters were separated by chromatography with silica gel as adsorbent using $CHCl_3$/MeOH as eluent to give **5a** and **5b**.

Compound **2a** (R = H) was obtained by prolonged heating of **5a** with hydrazine by a procedure similar to that described for the synthesis of anthrapyridazones.[5] However, this procedure failed to give the 2-*N*-alkyl derivatives of the pyridazinoacridin-3-one chromophore. Therefore, a different procedure was required for producing compounds **2b–f** from **5a**. Compound **5a** was dissolved in $POCl_3$ and heated to reflux to afford the 9-chloroderivative, which was immediately treated with the respective (alkylamino)alkylhydrazine.

Compound **2g**, the 6-chloro analog of **2c**, was prepared from **7** under similar reaction conditions described for the preparation of **2b–f**. The last one was prepared in the following way: 2-chlorobenzoic acid and methyl-3-amino-4-chlorocarboxylate were condensed in a modified Jourdan–Ullmann reaction[13] to give the half ester **6**, followed by ring closure with polyphosphate ester to afford compound **7**. Finally, cyclization was achieved with hydrazine to give **2h**.

Attempts to substitute the chlorine atom at position 6 with an (aminoalkyl)amino nucleophile in compound **2g** or **2h** under various conditions were unsuccessful.

The structure, molecular formulas, and melting points of compounds **2b–g** are presented in Table 1. For biological evaluations, the free bases were converted into the hydrochloride salts.

3. Results and discussion

Tetracyclic anthraquinone analogs with fused pyridazone ring (anthrapyridazones) are able to overcome multidrug resistance of tumor cells.[5] Presently we have synthesized and examined a group of analogous tetracyclic acridine analogs (pyridazinoacridines). The results of cytotoxic activities and DNA binding data for compounds **2b–g** are presented in Table 2. Doxorubicin (DX), Mitoxantrone (MIT), and representative anthrapyridazone closely related to **2g**, the 6-chloro-2-[2-(dimethylamino)ethyl]-2,7-dihydro-3*H*-dibenzo[*de*,*h*]-cinnoline-3,7-dione (PDZ),[5] were used as reference compounds.

All compounds tested, depending on their structure, exhibited varied cytotoxic activities on three sensitive cell lines (L1210, K562, HL-60). These activities, including those of PDZ, is generally lower than those of reference compounds DX and MIT. Cytotoxic activity of compounds are influenced by the type of side chain. The highest activity was recorded for compounds with terminal dimethylamino moiety (**2c**, **2b**). The length of alkyl moiety (two or three methylene groups; compounds **2b** and **2c**, respectively) has no significant influence. The lowest activity is exhibited by compounds **2d** and **2f** with terminal *N*-diethyl and piperydyl moieties, respectively. Somewhat higher activity is exhibited by **2e**, bearing terminal morpholine moiety in the side chain. The replacement of hydrogen atom by chlorine (**2g** vs **2e**) does not seem to have an effect on cytotoxicity. We did not find any essential correlation between the cytotoxic activity of examined tetracyclic compounds and DNA binding measured by ethidium bromide displacement method. The essential difference was observed only between these compounds and highly active reference DX and MIT.

The novel group of pyridazinoacridines, however of rather low cytotoxic activity against sensitive cell lines, was designed to investigate the effect of structural factors of tetracyclic acridine analogs on their ability to overcome multidrug resistance of tumor cells. In this respect the dramatic improvement of cytotoxicity against resistant K562/DX, HL-60/VINC, and HL-60/DX cell lines was noticed in comparison with the activity of reference DX and MIT. It is of importance that these novel compounds are able to overcome MDR, as well as, MRP types of resistance. However, the activity against the MRP cell line is somewhat lower. The cytotoxic activity of tested pyridazinoacridines toward cells with MDR-type resistance is not significantly influenced by modification of substituents. The resistance indexes are within range ca. 1–4. Much more interesting is the activity of these compounds toward tumor cells with

Table 2. Cytotoxic activity in vitro in a panel of sensitive and resistant leukemia cell lines and DNA binding of compounds **2b–g**, in comparison with reference compounds: PDZ, Doxorubicin (DX), and Mitoxantrone (MIT)

	Compound								
	Tested						Reference		
	2b	**2c**	**2d**	**2e**	**2f**	**2g**	PDZ[a]	DX	MIT
Cytotoxic activity									
Cell line[b]	IC_{50}[c] (nM) ± SEM[d]/RI[e]								
L1210	48 ± 4	39 ± 5	398 ± 54	93 ± 13	300 ± 23	15 ± 1	839 ± 40	19 ± 1	1.4 ± 0.1
K562	308 ± 68	234 ± 20	1189 ± 69	380 ± 64	960 ± 44	320 ± 36	2018 ± 373	42 ± 3	21 ± 4
K562/DX	256 ± 16	178 ± 24	2188 ± 313	574 ± 79	2294 ± 211	451 ± 62	4140 ± 388	7031 ± 542	554 ± 50
RI	*0.83*	*0.76*	*1.84*	*1.51*	*2.39*	*1.41*	*2.05*	*167*	*26.4*
HL-60	79 ± 2	73 ± 4	1484 ± 36	91 ± 4	755 ± 59	49 ± 3	666 ± 69	18 ± 1	2.2 ± 0.3
HL-60/VINC	245 ± 7	255 ± 15	3097 ± 185	407 ± 20	1419 ± 89	110 ± 9	472 ± 39	523 ± 33	56.5 ± 4.2
RI	*3.10*	*3.49*	*2.09*	*4.47*	*1.88*	*2.24*	*0.71*	*29.0*	*25.7*
HL-60/DX	931 ± 54	1035 ± 53	3801 ± 364	1061 ± 85	2159 ± 129	744 ± 76	710 ± 98	3869 ± 59	1179 ± 203
RI	*11.78*	*14.2*	*2.56*	*11.7*	*2.86*	*15.2*	*1.07*	*215*	*536*
DNA binding									
C_{50}[f] (μM)	2.95	3.75	6.24	3.12	2.92	2.83	1.76	0.08	0.05
K_{app}[g]	0.43	0.34	0.20	0.40	0.43	0.44	0.72	15.00	25.20
R^2[h]	0.9939	0.9935	0.9936	0.9895	0.9812	0.9797	0.9850	0.9933	0.9677

[a] 6-Chloro-2-[2-(dimethylamino)ethyl]-2,7-dihydro-3*H*-dibenzo[*de*,*h*]-cinnoline-3,7-dione.[5]
[b] L1210—murine lymphocytic leukemia; K562—human myelogenous leukemia and Doxorubicin resistant (MDR type) subline K562/DX; HL60—human promyelocytic leukemia, and Vincristine resistant (MDR type) subline HL-60/VINC, and Doxorubicin resistant (MRP type) subline HL-60/DX.
[c] IC_{50}—the concentration of compound causing 50% inhibition of cell growth after 48 h (L1210) or 72 h (other lines) continuous exposure.
[d] SEM—standard error of the mean.
[e] RI—resistance index; the ratio of IC_{50} value for resistant cell line to IC_{50} value for sensitive cell line.
[f] C_{50}—the micromolar drug concentration of compound to give a 50% drop in fluorescence of ethidium bound to DNA.
[g] K_{app} ($\times 10^{-7}$ M^{-1})—apparent equilibrium binding constant.
[h] R^2—curvefit coefficient.

MRP-type resistance. In this case the effect of structure on RI value is much more expressed. The compounds **2d** and **2f**, as more lypophilic, exhibited the highest activity (lowest RI). These compounds exhibit most dramatic differences in the ability to overcome MRP-type resistance in regard to reference DX and MIT.

Comparison of pyridazinoacridines with analogous anthrapyridazones concerning their ability to overcome multidrug resistance of tumor cells (PDZ vs **2g**) point to the latter group as more effective in this respect.

The results obtained broaden our knowledge on cytotoxic activity of acridine compounds with fused heterocyclic rings and bring new data supporting our earlier hypothesis[4] that anthraquinone and acridine analogs with fused heterocyclic rings, when properly substituted, are able to overcome multidrug resistance of tumor cells.

4. Experimental

4.1. Chemistry

Melting points were determined with a Boeticus PHMK05 apparatus and are uncorrected. Combustion analyses are within ±0.4% of the theoretical values and were carried out on a Carlo Erba CHNS-O-EA1108 instrument for C, H, N. A Beckman 3600 spectrophotometer was used for UV spectral determination. IR spectra were recorded on a UR 10 Zeiss spectrometer from KBr pellets; ^{1}H NMR spectra were taken on a Varian 300-MHz or 500-MHz spectrometer using tetramethylsilane as an internal standard. The following NMR abbreviations are used: ex (exchangeable with D_2O), d ex (exchangeable with D_2O, but with difficulty). MS spectra were recorded on a Quadrupolic Mass Spectrophotometer Trio-3 (FAB technique). Thin layer chromatography (TLC) was carried out on silica gel (Kieselgel 60 plates, Merck), column chromatography on silica gel (Kieselgel 70–230 mesh Merck, and Sephadex LH-20, Pharmacia).

4.2. Typical procedure for the preparation of hydrochloride salt of 2b–g

To a stirred solution of the compound (as free base) in $CHCl_3$ or $CHCl_3$/MeOH, a slightly molar excess of hydrogen chloride in absolute ethanol was added dropwise at 5 °C. Anhydrous diethyl ether was added and a precipitate formed, which was collected by filtration. The solid was purified by column chromatography (Sephadex LH-20) eluting with MeOH and crystallized from MeOH/Et_2O.

4.3. Ethyl 9-oxo-9,10-dihydro-acridine-1-carboxylate (5a)

A suspension of 2-chlorobenzoic acid (7.8 g, 50 mmol), 3-aminobenzoic acid (6.8 g, 50 mmol), and K_2CO_3 (7 g, 60.6 mmol) in isopentyl alcohol (150 mL) was stirred at reflux point for 0.5 h during which time one-tenth

of the solvent was allowed to boil off. Copper powder (0.25 g, 3.9 mmol) was added and then the reaction mixture was heated at reflux for 3 h. After that time 1 M aqueous K_2CO_3 (100 mL) was added and then the reaction mixture was filtered to remove insoluble material. The aqueous layer was separated and acidified to pH 5 with 2 N HCl to form a precipitate, which was filtered, suspended in boiling water (200 mL), filtered again, and washed with EtOH to give crude product **3** (60% yield), which was used for the next step.

A sample of **3** (2.6 g, 10 mmol) was heated with concentrated H_2SO_4 (10 mL) for 1 h at 90 °C. The mixture was poured on ice. A precipitate formed, which was filtered and washed with hot water, followed with hot MeOH to give the crude isomeric mixture of **4a** and **4b** (yield 85%). The isomeric mixture was suspended in EtOH saturated with dry HCl (150 mL) and refluxed for 4 h. The reaction mixture was concentrated in vacuo and then the residue was poured into a cold excess aqueous ammonia solution to provide a precipitate, which was filtered, washed with water, and dried (yield 95%). The isomeric mixture (ca. 1.8:1 ratio of **5a**:**5b**) was chromatographed three times on silica gel using $CHCl_3$/MeOH (10:1, v/v; 10:1, v/v; 5:1,v/v) as eluent.

Compound **5a** was isolated as a pale brown solid with strong fluorescence at 366 nm. Mp 218–219 °C. ^{1}H NMR (DMSO-d_6): δ 1.25 (t, 3H, J = 3.5 Hz); 4.28 (q, 2H, J = 3.5 Hz); 7.15 (dd, 1H, J = 6.8 Hz, J = 1.1 Hz); 7.28 (t, 1H, J = 7.0 Hz); 7.56 (d, 1H, J = 8.0 Hz); 7.68 (dd, 1H, J = 9.0 Hz, J = 1.1 Hz); 7.68–7.8 (m, 2H); 8.18 (dd, 1H, J = 8.0 Hz, J = 1.1 Hz); 11.95 (s, 1H, ex).

The faster eluting compound **5b** is a yellow solid. Mp 301–302 °C. ^{1}H NMR (DMSO-d_6): δ 1.65 (t, 3H, J = 3.5 Hz); 4.39 (q, 2H, J = 3.5 Hz); 7.31 (t, 1H, J = 3.6 Hz); 7.55 (d, 1H, J = 4.0 Hz); 7.71–7.86 (m, 2H); 8.21 (d, 1H, J = 2.5 Hz); 8.26 (s, 1H); 8.33 (1H, d, J = 4 Hz); 12.0 (s, 1H, ex).

4.4. 3-(2-Carboxy-phenylamino)-4-chlorobenzoic acid methyl ester (6)

A suspension of methyl 3-amino-4-chlorobenzoate (1.6 g, 8.6 mmol), 2-chlorobenzoic acid (1.3 g, 8.6 mmol) and Cu(OAc)xH$_2$O (1.7 g, 8.6 mmol) in a mixture of methyl-2-pyrrolidinone (10 mL) and *N*,*N*-diisopropylethylamine (20 mL) was stirred for 3 h at 130 °C. After cooling, dilution with water and acidification to pH 2, the precipitate was washed with hot water and chromatographed (eluent $CHCl_3$/MeOH 15:1) to give **6** (yield 52%). Mp 216 °C.

^{1}H NMR (DMSO-d_6): δ 3.82 (s, 3H); 6.95 (t, 1H, J = 7.0 Hz); 7.28 (d, 1H, J = 8.0 Hz); 7.48 (d, 1H, J = 8.5 Hz); 7.56 (dd, 1H, J = 8.3 Hz, J = 1.8 Hz); 7.66 (d, 1H, J = 6.5 Hz); 7.97 (dd, 1H, J = 8.0 Hz, J = 1.5 Hz); 8.02 (d, 1H, J = 8.0 Hz); 9.95 (s, 1H, ex); 13.2 (s, 1H, ex).

4.5. Methyl 4-chloro-9-oxo-9,10-dihydro-acridine-1-carboxylate (7)

A sample of **6** (1 g, 3.3 mmol) and PPE (polyphosphate ester) (40 g) in $CHCl_3$ (50 mL) were stirred at reflux until all solid was dissolved. The $CHCl_3$ was allowed to evaporate to give oil, which was heated for 1 h at 100 °C. The cooled mixture was diluted with water and basified to pH 9. The insoluble product was collected, washed with water, dried and crystallized from EtOH/ether. Yield 55%. Mp 228–229 °C.

^{1}H NMR (DMSO-d_6): δ 3.84 (s, 3H); 7.19 (d, 1H, J = 7.3 Hz); 7.35 (t, 1H, J = 7.5 Hz); 7.8 (t, 1H, J = 7.2 Hz); 7.97 (d, 1H, J = 7.3 Hz); 8.06 (d, 1H, J = 8.4 Hz); 8.16 (dd, 1H, J = 8.1 Hz, J = 1.1 Hz); 11.2 (s, 1H, d ex).

4.6. 2,7-Dihydro-3*H*-pyridazino[5,4,3-*kl*]acridin-3-one (2a)

To a solution of **5a** (0.26 g, 1 mmol) in DMA (3 mL) hydrazine hydrate ($NH_2NH_2xH_2O$) (3 mL) was added and the reaction mixture was heated for 6 h at 125 °C. The mixture was neutralized with dilute HCl to form a precipitate, which was filtered, washed with acetone, and crystallized from DMF/acetone to give **2a** as a light yellow powder (yield 30%). Mp >300 °C.

^{1}H NMR (DMSO-d_6): δ 7.08 (t, 1H, J = 7.3 Hz); 7.16 (d, 1H, J = 7.3 Hz); 7.3 (d, 1H, J = 7.8 Hz); 7.42 (t, 1H, J = 7.5 Hz); 7.50 (d, 1H, J = 7.3 Hz); 7.70 (t, 1H, J = 8.2 Hz); 8.04 (d, 1H, J = 7.8 Hz); 10.75 (s, 1H, d ex); 12.23 (s, 1H, d ex). MS *m*/*z* (relative intensity, %): 235 [(M)$^+$, 100].

4.7. 2-[3-(Dimethylamino)ethyl]-2,7-dihydro-3*H*-pyridazino[5,4,3-*kl*]acridin-3-one (2b)

A sample of **5a** (0.26 g, 1 mmol) was refluxed in $POCl_3$ (2 mL) for 0.5 h. The reaction mixture was evaporated in vacuo to give an oily residue, which was dissolved in DMA (2 mL). An excess of 2-(dimethylamino)ethylhydrazine (0.5 mL) was added dropwise and the solution of reagents were heated for 3 h at 130 °C. The reaction mixture was diluted with $CHCl_3$ and washed carefully with water. The organic layer was dried over Na_2SO_4, evaporated in vacuo and the crude product purified by column chromatography (eluent $CHCl_3$/MeOH 5:1) to afford **2b** as pale yellow powder (yield 45%). Mp 258–259 °C (xHCl 293–295 °C).

^{1}H NMR (DMSO-d_6): δ 2.20 (s, 6H, 2 × CH_3); 2.69 (t, 2H, J = 6.5 Hz); 4.29 (t, 2H, J = 6.5 Hz); 7.09 (t, 1H, J = 7.3 Hz); 7.17 (d, 1H, J = 8.3 Hz); 7.29 (d, 1H, J = 8.3 Hz); 7.43 (t, 1H, J = 7.5 Hz); 7.51 (d, 1H, J = 7.3 Hz); 7.70 (t, 1H, J = 8.0 Hz); 8.07 (d, 1H, J = 7.8); 10.83 (s, 1H, d ex). MS *m*/*z* (relative intensity, %): 306 [(M)$^+$, 100]. Found: C, 70.26; H, 5.83; N, 18.01. Calcd for $C_{18}H_{18}N_4O$: C, 70.57; H, 5.92; N, 18.29.

Compounds **2c**–**g** were prepared by the procedure described for the preparation of **2b** using the appropriate

(alkylamino)alkylhydrazines. Compounds **2c–f** and **2g** were prepared from **5a** and **7**, respectively.

4.8. 2-[3-(Dimethylamino)propyl]-2,7-dihydro-3*H*-pyridazino[5,4,3-*kl*]acridin-3-one (2c)

Yield 40%. Mp 251–253 °C (*x*HCl 271–272 °C dec). ^{1}H NMR (DMSO-d_6): δ 1.89 (m, 2H); 2.15 (s, 6H, $2\times CH_3$); 2.28 (t, 2H, J = 7.0 Hz); 4.12 (t, 2H, J = 7.0 Hz); 7.07 (t, 1H, J = 7.3 Hz); 7.15 (d, 1H, J = 8.3 Hz); 7.27 (d, 1H, J = 8.3 Hz); 7.40 (t, 1H, J = 7.5 Hz); 7.49 (d, 1H, J = 7.3 Hz); 7.68 (t, 1H, J = 8.1 Hz); 8.05 (d, 1H, J = 7.7); 10.80 (s, 1H, ex). Found: C, 70.92; H, 6.14; N, 17.26. Calcd for $C_{19}H_{20}N_4O$: C, 71.23; H, 6.29; N, 17.49.

4.9. 2-[2-(Diethylamino)ethyl]-2,7-dihydro-3*H*-pyridazino[5,4,3-*kl*]acridin-3-one (2d)

Yield 51%. Mp 248–250 °C (*x*HCl 273–275 °C dec). ^{1}H NMR (DMSO-d_6): δ 1.12 (t, 6H, J = 3.5 Hz); 2.60–2.75 (m, 6H); 4.25 (t, 2H, J = 6.5 Hz); 7.09 (t, 1H, J = 7.3 Hz); 7.15 (d, 1H, J = 8.3 Hz); 7.28 (d, 1H, J = 8.3 Hz); 7.39 (d, 1H, J = 7.5 Hz); 7.50 (t, 1H, J = 7.3 Hz); 7.70 (t, 1H, J = 8.0 Hz); 8.06 (d, 1H, J = 7.9 Hz); 10.81 (s, 1H, ex). Found: C, 71.47; H, 6.53; N, 16.48. Calcd for $C_{20}H_{22}N_4O$: C, 71.83; H, 6.63; N, 16.75.

4.10. 2-(2-Morpholinoethyl)-2,7-dihydro-3*H*-pyridazino[5,4,3-*kl*]acridin-3-one (2e)

Yield 43%. Mp 236–238 °C (*x*HCl 260–262 °C dec). ^{1}H NMR (DMSO-d_6): δ 2.6 (4H, t, J = 4.6 Hz); 2.75 (4H, t, J = 6.3 Hz); 3.24 (2H, t, J = 5.8 Hz); 4.5 (2H, t, J = 5.8 Hz); 7.10 (t, 1H, J = 7.2 Hz); 7.15 (d, 1H, J = 8.2 Hz); 7.27 (d, 1H, J = 8.3 Hz); 7.40 (t, 1H, J = 7.5 Hz); 7.50 (d, 1H, J = 7.2 Hz); 7.68 (t, 1H, J = 8.0 Hz); 8.08 (d, 1H, J = 7.9 Hz); 10.86 (s, 1H, ex). Found: C, 68.75; H, 5.81; N, 16.07. Calcd for $C_{20}H_{20}N_4O_2$: C, 68.95; H, 5.79; N 16.08.

4.11. 2-(2-Piperidinoethyl)-2,7-dihydro-3*H*-pyridazino[5,4,3-*kl*]acridin-3-one (2f)

Yield 35%. Mp 231–233 °C (*x*HCl 225–227 °C dec). ^{1}H NMR (DMSO-d_6): δ 1.38–1.62 (m, 6H); 2.47 (m, 4H); 2.70 (t, 2H, J = 6.5 Hz); 4.30 (t, 2H, J = 6.5 Hz); 7.12 (t, 1H, J = 7.3 Hz); 7.17 (d, 1H, J = 8.3 Hz); 7.27 (d, 1H, J = 8.5 Hz); 7.45 (t, 1H, J = 7.5 Hz); 7.55 (d, 1H, J = 7.4 Hz); 7.72 (t, 1H, J = 8.0 Hz); 8.10 (d, 1H, J = 7.9 Hz); 10.78 (s, 1H, ex). Found: C, 72.50; H, 6.45; N, 16.01. Calcd for $C_{21}H_{22}N_4O$: C, 72.81; H, 6.40; N, 16.17.

4.12. 6-Chloro-2-[2-(dimethylamino)propyl]-2,7-dihydro-3*H*-pyridazino[5,4,3-*kl*]acridin-3-one (2g)

Yield 25%. Mp 185–186 °C (*x*HCl 257–258 °C dec). ^{1}H NMR (DMSO-d_6): δ 1.92 (m, 2H); 2.18 (s, 6H, $2\times CH_3$); 2.68 (t, 2H, J = 6.5 Hz); 4.27 (t, 2H, J = 6.5 Hz); 7.16 (t, 1H, J = 7.5 Hz); 7.45 (t, 1H, J = 7.5 Hz); 7.54 (d, 1H, J = 8.3 Hz); 7.68 (d, 1H, J = 8.2 Hz); 7.85 (dd, 1H, J = 8.2 Hz, J = 1.1 Hz); 8.09 (d, 1H, J = 8.2 Hz); 10.17 (s, 1H, ex). Found: C, 64.25; H, 5.42; N, 15.71. Calcd $C_{19}H_{19}N_4OCl$ for: C, 64.31; H, 5.40; N, 15.79.

4.13. 6-Chloro-2,7-dihydro-3*H*-pyridazino[5,4,3-*kl*]acridin-3-one (2h)

Compound **2h** was prepared from **7** and $NH_2NH_2xH_2O$ (98%) by the procedure described for the preparation of **2b** and was worked up by the procedure described for the preparation of **2a**. Yield 55%. Mp >300 °C. ^{1}H NMR (DMSO-d_6): δ 7.15 (t, 1H, J = 7.5 Hz); 7.45 (t, 1H, J = 7.5 Hz); 7.53 (d, 1H, J = 8.0 Hz); 7.66 (d, 1H, J = 8.0 Hz); 7.84 (d, 1H, J = 8.0 Hz); 8.06 (d, 1H, J = 8.0 Hz); 10.0 (s, 1H, ex); 12.45 (s, 1H, d ex).

5. Biophysical and biological evaluation

5.1. Fluorescence binding studies

The fluorometric assays were performed as described previously.[14] The C_{50} values for ethidium displacement from CT-DNA were determined using aqueous buffer (10 mM Na_2HPO_4, 10 mM Na_2HPO_4, 1 mM EDTA, pH 7.0, room temperature) containing 1.26 μM ethidium bromide and 1 μM CT-DNA (type II, Sigma).[14–16] Examined compounds were added to this solution to yield several concentrations. Measurements were made using a PerkinElmer LS55 instrument (excitation at 546 nm; emission at 595 nm). The C_{50} values are defined as the drug concentrations, which reduce the fluorescence of the DNA-bound ethidium by 50%. Data from three independent determinations were fitted to second polynomial order curves or linear curve in the case of MIT by Microsoft Excel Program, and C_{50} values were calculated. Apparent equilibrium binding constants were calculated from the C_{50} (in μM) values using the following equation: $K_{app} = (1.26/C_{50}) \times K_{ethidium}$ where $K_{ethidium} = 10^7\ M^{-1}$ for ethidium bromide[15] and 1.26 is concentration (in μM) of ethidium in ethidium–DNA complex.

5.2. Biological evaluation

5.2.1. Cell lines. Murine L1210 lymphocytic leukemia cells were grown in RPMI 1640 medium supplemented with 5% FBS (foetal bovine serum), penicillin G (100,000 units/L), and streptomycin (100 mg/L). Human myelogenous leukemia sensitive cell line K562 and Doxorubicin resistant subline K562/DX (ICIG, Villejuif, France) were grown in RPMI 1640 medium supplemented with 10% FBS, penicillin G (100,000 units/L), streptomycin (100 mg/L), and 2 mM L-glutamine. Reselection of the resistant cell lines was performed once a month by exposure to 500 nM Doxorubicin. Human promyelocytic leukemia sensitive cell line HL-60 and resistant sublines: Vincristine resistant HL-60/VINC and Doxorubicin resistant HL-60/DX (Kansas State University, Manhattan, KS, USA), were grown in RPMI 1640 medium supplemented with 10% FBS peni-

cillin G (100,000 units/L), streptomycin (100 mg/L). Reselection of the resistant cell lines was performed once a month by exposure to 200 nM Doxorubicin and 1 μM vincristine for HL-60/DX and HL-6-/VINC, respectively. Cell lines were grown in a controlled (air–5% CO_2) humidified atmosphere at 37 °C and were transplanted three times a week. For the experiments the cells in logarithmic growth were suspended in the growth medium to give a final required density. The resistant cell lines were maintained without the reselection drugs at least one week before the experiments.

5.2.2. In vitro cytotoxic evaluation. Cells of required density were seeded and different concentrations of the drugs were added. The experiments were carried out in a controlled (air–5% CO_2) humidified atmosphere at 37 °C. The exposure times were: 48 h for L1210 cells and 72 h for other cell lines. The cytotoxic activity (IC_{50} values) of the compounds was defined as their in vitro concentrations causing 50% inhibition of cell growth after continuous exposure to the drug, as measured by cell counting with Z2 Cell Analyzer (Beckman Coulter) or by the protein content of the cells as described previously.[17] Results are given as the mean of at least three independent experiments ± standard error of the mean (SEM). The resistance index was defined as the ratio of IC_{50} value for resistant cell line to IC_{50} value for sensitive cell line.

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