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Activity of the pyrazoloacridines against multidrug-resistant tumor cells

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Summary. A series of 2-aminoalkyl-5-nitropyrazolo [3,4,5-kl]acridines (pyrazoloacridines) were tested in vitro against a panel of multidrug-resistant cell lines comprising Adriamycin-resistant P388 leukemia, B16 melanoma, and mammary adenocarcinoma 16c. This new class of anticancer agents, particularly the 9-substituted methoxy derivatives, exhibited significant activity against all of the lines tested. The degree of cross-resistance to these compounds ranged from zero to 8-fold in the 138-fold Adriamycin-resistant P388/ADR line and was greatly diminished in the B16/ADR and 16c/ADR lines. Selected pyrazoloacridines were subsequently tested in vivo against B16 and B16/ADR cells established as solid tumors from the tissue culture line and shown to retain a significant degree of Adriamycin resistance. Whereas the B16/ADR line exhibited 2 logs less net tumor-cell kill than the B16 parent in response to Adriamycin treatment, the resistant tumor was completely sensitive to the pyrazoloacridines tested and proved in some experiments to be collaterally sensitive. The favorable activity of the pyrazoloacridines against these Adriamycin-resistant tumor lines points to the potential efficacy of these compounds against multidrug-resistant tumors encountered clinically.

Introduction

The occurrence of drug resistance is frequently a major obstacle to successful tumor treatment. Tumors are often either inherently resistant or develop acquired resistance in response to treatment. The multidrug-resistant (MDR) phenotype ensues when tumor cells treated with a DNA-binding drug, for instance, eventually acquire resistance to the primary agent and also become resistant to other DNA binders [6] as well as structurally dissimilar compounds such as the natural product vincristine [20].

One approach to the problem of multidrug resistance lies in the development of active antitumor agents equally effective against MDR tumor cells. New intercalating agents such as amsacrine and mitoxantrone have shown disappointing activity against MDR tumors [8, 9].

The present study was undertaken to evaluate the activity of another new class of DNA binders, the pyrazoloacridines, against MDR tumors. The pyrazoloacridines, which are a new class of acridine antitumor agents, have recently been reported [18] to show selectivity against solid tumors relative to leukemias in vitro and to be selectively cytotoxic to hypoxic cells. An additional property is their broad-spectrum in vivo activity [17]. This report describes both in vitro and in vivo testing of the pyrazoloacridines against Adriamycin-resistant tumor lines exhibiting the MDR phenotype. The data reported indicate that this new class of DNA intercalators exhibits significant activity against MDR tumors. Preliminary reports of this work have previously been presented [13].

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Materials and methods

The pyrazoloacridines studied were synthesized as previously reported [1]. Adriamycin and vincristine were purchased from Sigma Chemical Co. (St. Louis, Mo).

Cell lines. P388 murine leukemia cells and an Adriamycin-resistant subline (P388/ADR) originated from the Southern Research Institute (Birmingham, Ala) and were cultured from the ascites fluid of tumor-bearing DBA-2 mice. The growth medium used for the P388 lines was Fischer's medium containing 10% horse serum, 10 μ*M* 2-mercaptoethanol, and gentamycin (50 μg/ml). The B16/BL6 murine melanoma cell line was obtained from Dr. Ralph Bernacki, Roswell Park Memorial Institute (Buffalo, NY) and is a variant of the parental B16/F10 line selected for increased invasive capacity and a greater number of pulmonary metastases [12]. The B16/ADR subline was developed in vitro as previously described [5]. Both of these melanoma lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and gentamycin (50 μg/ml). The mammary adenocarcinoma 16c and Adriamycin-resistant 16c lines were obtained from the Southern Research Institute and were cultured from tumor breis as previously reported [16] using RPMI 1640 medium supplemented as noted above. The overgrowth of Adriamycin-resistant cells during treatment of 16c lines with Adriamycin, cyclophosphamide, and 5-fluorouracil (5-FU) led to the establishment of the 16c/ADR tumor line, which also proved to be markedly resistant to vincristine [3, 15].

Growth inhibition assays were carried out in suspension cultures by the continuous exposure of cells for 72 h using 24-well plates. The number of cells was assessed based on Coulter counts at the end of the treatment period.

Growth inhibition assays were carried out in monolayer cultures using 24-well plates (for the B16 lines) or 96-well microtiter plates (for the 16c lines). Drug exposure was continuous for either 72 or 96 h to normalize for growth rate differences. The number of cells was then quantitated by trypsinizing and carrying out Coulter counts for B16 or by using the dye-release method previously described by Finlay et al. [4] for 16c. In all assays, activity was expressed as the amount of drug required to decrease final cell counts to 50% of the untreated control values.

Tumor passage. Tumor passage of the B16 lines was carried out in C57BL/6 mice. Tumors were routinely passaged at 2-week intervals and inoculated i.p. with 0.5 cc brei (0.2% and 10% for B16 and B16/ADR lines, respectively). Passage mice bearing the Adriamycin-resistant tumor were treated on day 1 with Adriamycin at 7 mg/kg i.p. to provide selective pressure.

Experimental chemotherapy. Life-span assays to assess anticancer activity were evaluated in $B_6C_3F_1$ mice weighing 18–22 g. Mice were randomized and implanted i.p. with 0.5 cc 10% brei containing either B16 or B16/ADR melanoma cells on day 0. Drug treatments were given on days 1, 5, and 9, and animals were held for 60 days. Anticancer activity was assessed on the basis of life-span extension (% T/C) and net logs of tumor-cell kill. The % T/C for the life-span assay was calculated as the ratio (multiplied by 100) of the median life span of treated mice vs the median life span of control mice. Net log tumor-cell kill is the estimated reduction in tumor burden over the course of treatment and accounts for tumor regrowth between injections. Net logs of tumor-cell kill were calculated as previously described by Schabel et al. [14] from the tumor doubling time, which was determined from a log-linear least-squares fit of the tumor titration for each experiment. Doubling times were calculated to be 1.6 days for the parent B16 line and 2.1 days for the Adriamycin-resistant B16 subline. A positive net log cell kill indicates that the tumor burden at the end of treatment was lower than at the beginning of therapy. Negative values for net cell kill indicated that the tumor grew during the treatment regimen, albeit possibly more slowly than the untreated control tumors.

Results

The compounds chosen for this study (Table 1, Fig. 1) were selected from a group of more than 40 compounds with significant in vivo activity against P388 leukemia. They were chosen on the basis of unique structural features and notable differences in their responses in an array of in vitro assays [18].

Table 1. Structural features of the pyrazoloacridines studied

PD number	Z	R
110,334	–H	$-(CH_2)_2N(CH_2CH_3)_2$
113,377	9–OCH_3	$-(CH_2)_2N(CH_2CH_3)_2$
114,245	9–OCH_3	$-(CH_2)_2N(CH_3)_2$
114,541	9–OH	$-(CH_2)_2N(CH_2CH_3)_2$
115,934	9–OCH_3	$-(CH_2)_3N(CH_3)_2$
117,802	9–$O_2CC(CH_3)_3$	$-(CH_2)_3N(CH_3)_2$

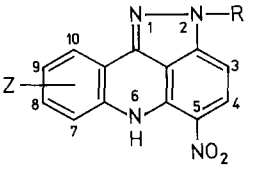

Fig. 1. Structure of the pyrazoloacridine nucleus (see Table 1 for R and Z)

Activity against an in vitro panel of MDR cell lines

Initial in vitro screening of the pyrazoloacridines against the P388 and P388/ADR lines in tissue-culture growth inhibition assays suggested that these compounds possessed favorable activity against MDR cells. Each pyrazoloacridine was assayed two or three times, and mean IC_{50} values are summarized in Table 2, which illustrates that the degree to which P388/ADR cells responded to the pyrazoloacridines varied with the structural substitutions characterizing these compounds. The P388/ADR line, which was 138-fold resistant to Adriamycin, exhibited no cross-resistance to any of the 9-methoxy-substituted compounds (PD 113,377, PD 114,245, and PD 115,934). The unsubstituted derivative (PD 110,334) was slightly less effective (1.6-fold cross-resistance), followed by the 9-hydroxy-substituted PD 114,541 and PD 117,802, containing a pivaloyl ester at the 9-position and resulting in the highest degree of cross-resistance (10-fold) of all of the pyrazoloacridines tested.

The finding of favorable activity for this series against the P388/ADR cell line led us to examine the effectiveness in vitro of the same compounds against other MDR tumor lines developed for use as potential solid tumor models for Adriamycin resistance. The lines chosen for study were Adriamycin-resistant lines of B16 melanoma and 16c adenocarcinoma. The B16/ADR line, which is 20-fold resistant to Adriamycin, proved to be completely sensitive to the 9-methoxy pyrazoloacridines and slightly less responsive (2-fold cross-resistance) to all other compounds tested in this series. The same response patterns were observed for the 16c/ADR-1 line, which was 7-fold resistant to Adriamycin but retained complete and perhaps collateral sensitivity to the 9-methoxy-substituted PD 113,377 and PD 115,934. As indicated in Table 2, both assays of PD 113,377 against 16c/ADR-1 cells showed collateral sensitivity; the same cell line was collaterally sensitive to PD 115,934 in one of two assays.

The clonogenic survival of the B16 and B16/ADR lines was also assessed after continuous exposure to either Adriamycin or PD 115,934. As shown in Fig. 2, the Adriamycin-resistant line was significantly insensitive to Adriamycin. The IC_{50} values for the parent and resistant lines were 0.011 and 0.25 μM, respectively, amounting to 23-fold resistance. In contrast, the two lines were equally sensitive to PD 115,934, with IC_{50} values of 0.060 and 0.053 μM for the sensitive and resistant lines, respectively.

In vitro assays with excised tumors

To address the potential of the sensitive and resistant B16 lines to serve as a useful in vitro/in vivo MDR solid tumor model, cells from both sublines were propagated in tissue culture and implanted into mice as described in *Materials and methods.* Resulting tumors were either passaged and

Table 2. In vitro activity of the pyrazoloacridines against multidrug-resistant cell lines

Compound	IC_{50} in μM[a]:								
	P388	P388/ADR	R/S[b]	B16	B16/ADR	R/S[b]	16c	16c/ADR	R/S[b]
PD 110,334	0.44	0.70	1.6 (1.3–1.9)	0.051	0.096	1.9 (1.4–2.4)	0.047	0.15	3.2 (2.4–3.7)
PD 113,377	1.0	0.99	1.0 (0.91–1.0)	0.11	0.16	1.5 (1.1–2.0)	0.025	0.018	0.7 (0.59–0.85)
PD 114,245	0.42	0.46	1.1 (0.94–1.2)	0.17	0.21	1.2 (1.0–1.4)	0.019	0.042	2.2 (2.1–2.4)
PD 114,541	0.0035	0.0093	2.7 (2.1–3.6)	0.0014	0.0030	2.1 (1.8–2.5)	0.0016	0.0041	2.6 (2.4–2.9)
PD 115,934	1.1	1.2	1.1 (1.0–1.2)	0.22	0.31	1.4 (1.0–2.0)	0.045	0.039	0.9 (0.75–1.0)
PD 117,802	0.0055	0.053	9.6 (8.8–11)	0.0037	0.0067	1.8 (1.1–1.9)	0.0018	0.0064	3.6 (3.3–3.9)
Adriamycin	0.064	8.8	138 (118–165)	0.023	0.39	17 (15–21)	0.017	0.12	7.1 (5.0–8.8)

[a] Mean of 2–3 determinations with the exception of Adriamycin, which was tested a minimum of 8 times; IC_{50} values for a given compound agreed within 2-fold of the mean

[b] R/S = IC_{50} (resistant)/IC_{50} (sensitive); the range of resistance indices observed from multiple determinations is indicated in parentheses

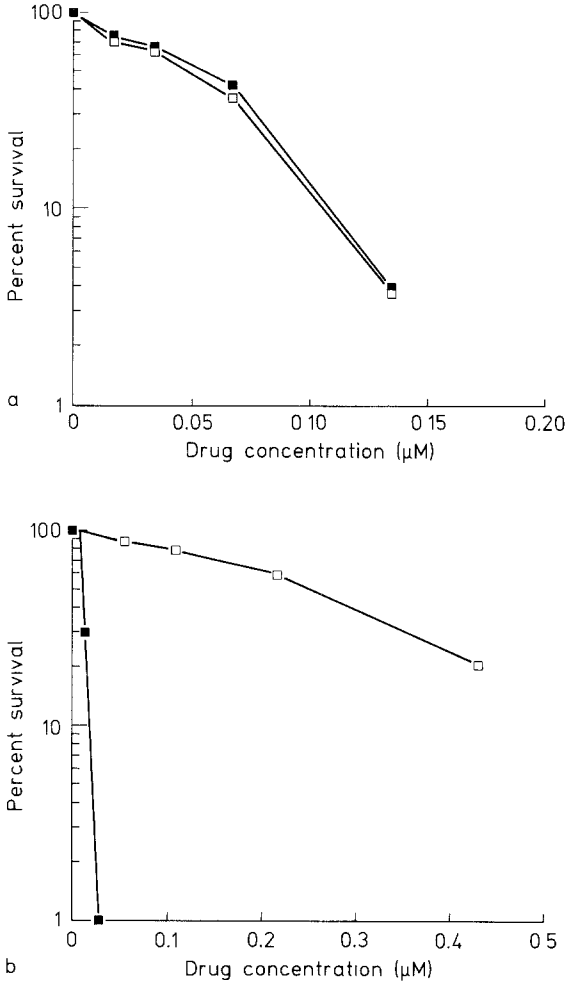

Fig. 2. Activity of PD 115,934 **(a)** and Adriamycin **(b)** against B16 (■) and B16/ADR (□) tumors. Exponentially growing cells were treated by continuous exposure in a clonogenic survival assay. Plotted values represent the mean of two determinations

subsequently treated to assess in vivo activity, which will be described in a later section of this report, or tested in the following in vitro assays.

Freshly excised tumor from B16- and B16/ADR-bearing mice were trypsinized and plated in continuous-exposure clonogenic assays with the test agents Adriamycin, vincristine, or PD 115,934. As summarized in Table 3, when tested against the B16 tumor in this assay system, B16/ADR proved to be 9-fold resistant to Adriamycin, cross-resistant (12-fold) to vincristine, and completely sensitive to the pyrazoloacridine as indicated by a resistance index of 1.2.

In vitro clonogenic assays were also carried out in solid tumor material excised 24 h after in vivo treatment with the above three compounds. Mice bearing equal tumor burdens of B16 and B16/ADR were treated with a single i.p. injection of drug. Each drug was tested at three doses (three mice/group), starting at the maximum tolerated dose and decreasing in 2-fold increments. Individual tu-

Table 3. Clonogenic survival of B16 and B16/ADR tumors treated in vitro[a]

Compound	IC_{50} (nM)[b]:		
	B16	B16/ADR	R/S[c]
Adriamycin	7.6 (5.2–9.8)	66 (48–83)	8.7
Vincristine	5.5 (4.8–6.5)	65 (49–76)	12
PD 115,934	93 (86–97)	110 (67–142)	1.2

[a] Plating efficiencies were 38% ± 2.2% and 11% ± 1.5% for B16 and B16/ADR tumors, respectively. Comparatively, in vitro cell lines of B16 and B16/ADR gave plating efficiencies of 59% ± 5.6% and 50% ± 6.0% (based on 4 determinations)

[b] Values represent the mean of 4 four tumors per group (2 tumors/assay × 2 tests). The range of values is indicated in parentheses

[c] R/S = IC_{50} (resistant)/IC_{50} (sensitive)

Table 4. Clonogenic survival of B16 and B16/ADR tumors treated in vivo

Compound	Dose (mg/kg)	Plating efficiency (% untreated control)[a]: B16	B16/ADR	R/S[b]
Adriamycin	20	1.4 (1.2–1.6)	16 (14–18)	11
	10	2.8 (0.3–5.3)	18 (10–27)	6.4
	5	1.5 (0.8–2.2)	35 (31–38)	23
Vincristine	4	10 (5.6–13)	30 (25–35)	3.1
	2	27 (25–29)	45 (44–47)	1.7
	1	63 (60–66)	77 (61–93)	1.2
PD 115,934	400	31 (28–34)	27 (10–43)	0.9
	200	46 (38–54)	34 (13–54)	0.7
	100	59 (53–65)	44 (42–46)	0.7

[a] Data are expressed as the mean of 2 determinations (3 tumors each), with the range of values indicated in parentheses. The plating efficiency of untreated controls was 44%±8.1% for B16 and 14%±3.7% for B16/ADR

[b] Plating efficiency (B16/ADR)/plating efficiency (B16)

mors were plated for clonogenic survival. The results are tabulated in Table 4 and confirm those of previous studies in which resistance of the B16/ADR line to both Adriamycin and, to a lesser degree, vincristine was observed, as was complete sensitivity to PD 115,934. The data in Table 4 clearly suggest that PD 115,934 is less potent than Adriamycin against the sensitive B16 line; however, these data also indicate that the activity of PD 115,934 is not diminished in the resistant line. Uncertainty regarding the predictive value of these studies led us to conduct the in vivo life-span assays described below.

In vivo activity assessed in life-span assays

Mice bearing i.p. implanted B16 and B16/ADR tumors were treated daily on days 1, 5, and 9 after tumor implantation with an i.p. injection of either Adriamycin, vincristine, or the pyrazoloacridines PD 114,541 or PD 115,934. Full drug dose-response studies were carried out, with the doses ranging from frankly toxic to ineffective levels. The optimal dose was defined as that which produced the highest T/C value. In these life-span assays activity was calculated on the basis of % T/C and net logs of tumor-cell kill, where the difference in tumor burden was determined immediately following the last treatment and immediately prior to the first treatment. The results obtained at the optimal doses are summarized in Table 5.

On the basis of net log kill, the B16/ADR line was 2 logs resistant to Adriamycin and 2 logs cross-resistant to vincristine. With i.p. administration of either pyrazoloacridine, 2 logs of enhanced cell kill resulted in the resistant tumor relative to the sensitive line. The increase in life span seen with PD 115,934 against the Adriamycin-resistant tumor was comparable to that observed when the B16 parent line was treated with Adriamycin. This result surpasses the effect seen when the B16 tumor was treated with PD 115,934, suggesting collateral sensitivity of the B16/ADR line to this pyrazoloacridine. Both pyrazoloacridines also retained full activity against s.c. B16/ADR implants, indicating their ability to cross physiological barriers; however, collateral sensitivity was no longer observed.

Limited in vivo testing was also carried out in the P388/ADR line. However, as exemplified by PD 115,934, these agents are not as active against this ADR-resistant ascites leukemia: at the optimal dose of 35 mg/kg on a day 1–5 schedule (i.p./i.p), T/C values of 290 vs 147 were observed for the sensitive and resistant lines, respectively. Diminished activity against P388/ADR cells relative to the parent P388 line was also seen with other 9-methoxy derivatives (PD 113,377 and PD 114,245) as well as the 9-hydroxy-substituted PD 114,541.

Discussion

The present study was undertaken to evaluate the activity of a new series of acridine derivatives, the pyrazoloacridines, against MDR tumor cells. Like Adriamycin, the pyrazoloacridines exhibit favorable activity against a broad spectrum of tumors [13]. A major limitation to suc-

Table 5. In vivo activity of selected pyrazoloacridines against B16 melanoma and B16/ADR tumors

Compound	Assay number[a]	Dose (mg/kg per injection)	Drug route	B16 MLS[a]	B16 % T/C	B16 Net log kill	B16/ADR MLS[a]	B16/ADR % T/C	B16/ADR Net log kill	S-R[b]
None (controls)	1	–	–	14.3	–	–	16.0	–	–	–
	2	–	–	13.8	–	–	18.3	–	–	–
	3	–	–	13.4	–	–	18.3	–	–	–
Adriamycin	1	5	i.p.	33.0	231	2.1	25.0	156	0.2	1.9
	2	5	i.p.	26.5	192	1.0	23.1	126	−0.5	2.0
	3	5	s.c.	30.0	223	1.3	24.3	132	−0.3	1.9
Vincristine	1	1.7	i.p.	24.8	173	0.5	16.0	100	−1.8	2.3
PD 115,934	1	120	i.p.	25.0	175	0.5	34.8	218	2.5	−2.0
	2	75	i.p.	16.9	122	−1.0	34.0	185	1.2	−2.2
	3	75	s.c.	24.0	179	0.4	28.0	153	0.3	0.1
PD 114,541	2	15	i.p.	20.8	150	−0.2	36.5	199	1.6	−1.8
	3	15	s.c.	26.3	196	0.7	30.0	163	0.6	0.1

[a] Median life span in days; 10 mice/group

[b] S-R = degree of resistance defined as net log kill (B16) − net log kill (B16/ADR)

cessful tumor management with Adriamycin is the frequent emergence of resistant cell populations that concurrently become refractory to other DNA-binding agents and structurally unrelated compounds such as vincristine.

This report presents data indicating that Adriamycin-resistant tumors may not be subject to cross-resistance to the pyrazoloacridines. In vitro evidence for this contention was derived from a panel of MDR lines developed as Adriamycin-resistant sublines of P388 leukemia, B16 melanoma, and 16c mammary adenocarcinoma. Total lack of cross-resistance to the 9-methoxy-substituted pyrazoloacridines was observed in all three cell lines. This finding was quite striking for the P388/ADR line, in which the degree of Adriamycin resistance amounted to 138-fold.

However, in vivo testing of selected pyrazoloacridines against the P388/ADR line did not agree with the in vitro results; although PD 115,934 was more active than Adriamycin in the resistant line, significant cross-resistance was noted when it was tested against P388/ADR cells. The reason for the failure of the in vitro P388 lines to predict for the in vivo response is unclear at present. Reestablishment of ascites cells in culture and subsequent testing indicated that the in vitro and in vivo lines had not diverged, i.e., the newly established in vitro P388/ADR line was again completely sensitive to the pyrazoloacridines (M. J. Havlick, unpublished data). The importance of pharmacologic factors for the observed discrepancy was pursued by evaluating the activity of PD 115,934 againt P388 and P388/ADR cells in culture with 1-h treatment. However, collateral sensitivity to this compound was still apparent in the resistant line relative to the sensitive line after the shorter treatment period, i.e., R/S as defined in Table 2 was <1.0 (M. J. Havlick, unpublished data). Thus, it is unlikely that rapid clearance of this compound in vivo explains its lack of activity against the P388/ADR line.

Our data would suggest that the P388/ADR model in vitro is a poor prognostic indicator of the in vivo actvity of DNA binders against the same tumor, which has also been observed for the anthrapyrazoles [11]. The diminished activity of the pyrazoloacridines against the P388/ADR line may reflect a multifactorial basis for multidrug resistance. That is, in addition to membrane alterations affecting drug transport, a nuclear defect may characterize P388/ADR cells, rendering them less susceptible to DNA-damaging agents [2, 11]. Unexplained at present is the reason why the in vitro and in vivo P388/ADR lines exhibit such different responses to DNA binders despite, presumably, the same nuclear defect.

In contrast, significant activity of the pyrazoloacridines was observed both in vitro and in vivo against the B16/ADR line, which exhibits membrane alterations in drug accumulation but is not known to possess a nuclear, i.e., topoisomerase II, defect (J. S. Sebolt and M. J. Havlick, unpublished data). Adriamycin resistance as well as complete sensitivity to the 9-methoxy pyrazoloacridines was observed for B16/ADR cells in every in vitro assay reported, whether the treated samples were derived from tissue culture lines or excised solid tumors. In addition to confirming the excellent activity of the pyrazoloacridines against MDR tumors, these data support the use of the B16/ADR line as a useful in vitro/in vivo model system for screening other DNA binders against the transport component of multidrug resistance.

We are continuing to explore the basis for the favorable activity of the pyrazoloacridines seen in the present study. Klohs et al. [10] have reported that the activity of another class of DNA intercalators, the anthrapyrazoles, against P388/ADR cells correlates with the degree of lipophilicity of the compound, i.e., increased lipophilicity results in improved activity against the resistant line. As a series, the pyrazoloacridines might be expected to be more lipophilic than the anthrapyrazoles, based on their 5-NO_2 substituent vs the basic side chain at C-5 in the anthrapyrazole series. Preliminary data (Sebolt et al., unpublished data) lend support to this contention.

In general, the 9-methoxy-substituted pyrazoloacridines are superior to other members in this series in their effect against multidrug resistance. It is interesting that the 9-methoxy compounds also show preferential activity against solid tumor lines vs leukemias in vitro as well as hypoxic and quiescent cells; furthermore, they preferentially inhibit RNA synthesis [18]. In this respect these compounds resemble aclacinomycin, which has also been reported to show preferential inhibition of RNA synthesis [21] while exhibiting favorable activity against MDR cells [19]. However, aclacinomycin primarily possesses antileukemic activity [22].

Taken together, the data point to two subclasses of pyrazoloacridines with very different profiles: the 9-OH-substituted derivatives have increased potency, conferring antileukemic activity as revealed by testing of three leukemia lines [18], whereas the 9-methoxy compounds have lowered potency and an RNA-directed effect, resulting in solid tumor selectivity and excellent activity against quiescent cells [18]. These data suggest the existence of more than one mechanism of cytotoxicity, which may indicate different effects of compounds within this series against topoisomerase II [7] and perhaps topoisomerase I. Furthermore, these different mechanisms of cytotoxicity may play a key role in our understanding of the basis for the response of MDR tumors to the pyrazoloacridines, since the degree of response of pleiotropically resistant cells to these agents also falls into two structural classes.

Based on the results of the present study, the pyrazoloacridines represent a new class of anticancer compounds that, in addition to their known favorable activity against solid tumors and the refractory cell populations therein, also show promising activity against MDR tumors. Therefore, these compounds emerge as interesting candidates for clinical evaluation.

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