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Design, synthesis, and antiproliferative activity of some novel aminosubstituted xanthenones, able to overcome multidrug resistance toward MES-SA/Dx5 cells

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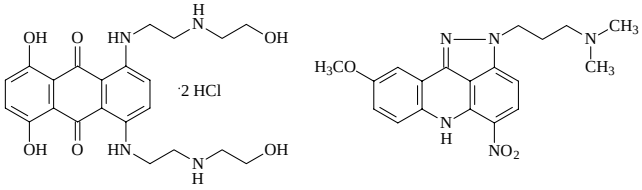
Abstract—A series of novel xanthenone aminoderivatives and their pyrazole-fused counterparts possessing structural analogy to the potent anticancer agent 9-methoxypyrazoloacridine (PZA) reported. These compounds exhibited an interesting cytotoxic activity against a panel of cell lines. Most noticeably, they retain activity against the multidrug resistant MES-SA/Dx5 subline, showing resistant factors close to 1.

DNA-interacting agents are widely used against a number of malignant diseases. Among them, the anthracycline glycosides doxorubicin and daunomycin are extensively used in clinical practice.[1]

Structure–activity studies of the anthracyclines rationalized the use of aminoanthraquinone congeners as antitumor agents and resulted in the development of the clinically useful anthracenedione mitoxantrone (**1**, Fig. 1).[2,3] Numerous analogues of this agent are studied to eliminate its side effects, mainly cardiotoxicity[4] and the development of multidrug resistance (MDR).[5] Cumulative cardiotoxicity has limited the prolonged use of quinone derivatives and led to the development of anthrapyrazoles,[6] which due to the presence of a quasi-iminoquinone group, possess reduced cardiotoxicity.[7] Many compounds belonging to this class are currently under preclinical and clinical evaluation,[8] and a number of structurally related acridine derivatives have also been developed.[9]



1 Mitoxantrone dihydrochloride **2** PZA

Figure 1. Structures of mitoxantrone and PZA.

Unfortunately, as most anticancer drugs behave, anthrapyrazoles are subjected to the development of acquired or intrinsic resistance (MDR) of certain tumor cell populations, which results in a limited potential for cancer cure.[10] One of the most investigated strategies to reverse MDR is the development of pharmacological agents that are able to modulate the function of P-glycoprotein (Pgp), an ATP-binding membrane protein, responsible for the active efflux of cytotoxic drugs out of resistant cells.[11] For anthracenediones and the related acridine cytostatics, it has been postulated that the incorporation into the chromophore of a fused five- or

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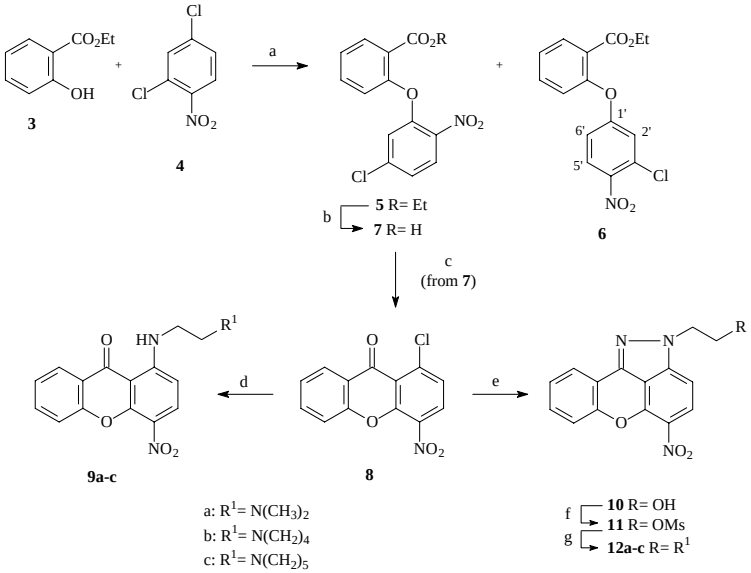
six-membered heterocyclic ring has led to compounds that are able to overcome MDR.[12] 9-Methoxypyrazoloacridine (PZA, **2**, Fig. 1) was selected for clinical evaluation from a series of compounds that combine the DNA-complexing activity of the acridine chromophore with the potential hypoxic cell-selectivity derived from a reducible nitro group substitution on the ring.[13,14] PZA retains full activity against resistant cell lines that exhibit the MDR phenotype.[15] Recent findings suggest that this compound targets both topoisomerases simultaneously, but without stabilization of the topoisomerase-DNA cleavable complexes.[16] The results obtained from different studies suggest a novel mechanism of action, which needs further exploitation and maybe additional clinical combination studies of PZA with other chemotherapeutic agents, with the aim of delaying or circumventing drug resistance.[8,13,17]

We have previously studied the synthesis and antiproliferative activity study of a number of cytotoxic xanthenone aminoderivatives.[18–20] On the other hand, some pyrazole-fused pyranoxanthenones, bearing a dialkylaminoethyl substitution, showed strong cytotoxic activity against the murine leukemia L1210 cell line, as well as against some human solid tumor cell lines.[21] As a continuation of this study, we present here the synthesis and biological evaluation of some novel xanthenone aminoderivatives and their pyrazole-fused counterparts, with direct structural similarity to PZA.

For the synthesis of the target compounds, we have used ethyl salicylate (**3**) as starting material (Scheme 1), which was treated with 2,4-dichloronitrobenzene (**4**) to result in a mixture of the isomeric esters **5** and **6**. These esters were separated by column chromatography and their structure was unambiguously established by ^{1}H and ^{13}C NMR spectroscopy, using both direct and long-range heteronuclear correlation experiments (HMBC and HMQC sequences). Structural discrimination resulted from the observation that C-1′ of compound **6** exhibits ^{2}J coupling with two aromatic protons, namely H-2′ and H-6′, and ^{3}J coupling with H-5′, while in the case of compound **5** the corresponding C-1′ possesses ^{2}J coupling only with H-6′ and ^{3}J coupling with H-3′. Compound **5** was then saponified under mild conditions since the presence of a nitro group facilitates the easy cleavage of the ether linkage. The resulting carboxylic acid **7** was isolated in good purity and was ring-closed upon treatment with PPA, to provide 1-chloro-4-nitro-9*H*-xanthen-9-one (**8**) in good yield. The target compounds **9a–c** were prepared by the nucleophilic substitution of the chloro group of **8** by appropriately substituted 2-(dialkylamino)ethylamines.

The next step concerns the preparation of the corresponding pyrazole-fuzed aminoderivatives. For this purpose, **8** was reacted with commercially available 2-hydroxyethylhydrazine to provide the carbinol **10** (Scheme 1). The structural assignment for the carbinol was confirmed using NOESY experiments. The side-chain methylene, which is adjacent to the pyrazole ring, exhibited NOEs only with the 3-aromatic proton. Additional evidence for the structure of compound **10** was obtained by a gradient inverse-detected long-range $^{1}H–^{15}N$ correlation experiment at natural abundance, where clear cross-peaks were observed, correlating N-2 with the adjacent side-chain methylene and the 3-aromatic proton, confirming the N-2 NMR signal assignment (N-2, H-3 cross-peak) and the position of the side-chain. Conversion of the carbinol **10** to the mesylate **11**, followed by nucleophilic substitution of the readily displaced mesyloxy group by appropriately substituted secondary amines, resulted in the benzopyrano[4,3,2-*cd*]indazoles **12a–c**.



Scheme 1. Synthesis of compounds **9a–c** and **12a–c**. Reagents and conditions: (a) K_2CO_3, Cu_2O, DMF, 110 °C, 8 h; (b) EtOH, NaOH 40%, rt, 30 min; (c) PPA 110 °C, 1 h; (d) $H_2NCH_2CH_2R^1$, pyridine, reflux, 1–3 h; (e) $H_2NNHCH_2CH_2OH$, pyridine, rt 12h; (f) CH_3SO_2Cl, CH_2Cl_2, Et_3N, rt, 4 h; (g) secondary amine, EtOH abs., reflux, 10–12 h.

For comparative reasons, as regards the structure–activity relationship studies, we have also prepared the corresponding aminoderivatives that lack the nitro group. We have thus used salicylic acid (**13**, Scheme 2), which was treated with 1,3-cyclohexanedione to result in the diketone **14**. The preparation of **14** has already been reported and was effective through the ring closure of 2-fluorobenzoic acid 3-oxo-cyclohex-1-enyl ester.[22]

However, the procedure presented herein is very simple and high-yielding. The diketone **14** was subsequently oxidized by the use of DDQ in boiling toluene to provide the phenol **15**, which was then converted to the corresponding tosylate **16**. Compound **16** was used for the synthesis of the amines **17a–c** upon reaction with the suitably substituted diamines. On the other hand **16** was treated with 2-hydroxyethylhydrazine, the resulting carbinol **18** was converted to the mesylate **19**, which reacted with the suitable secondary amines to provide the pyrazole-fused derivatives **20a–c**.

For biological evaluation purposes, the free base forms of the target amines were converted into their water-soluble hydrochloride addition salts by treatment with hydrochloric acid in methanol.

The in vitro cytotoxic activity of the new compounds was evaluated in the established model of the murine leukemia cell line L1210, and in three human solid tumor cell lines: colorectal adenocarcinoma HT-29, uterine sarcoma MES-SA, as well as its variant MES-SA/Dx5, reported to be 100-fold resistant to doxorubicin.[23] The results, including reference compounds mitoxantrone and doxorubicin, are presented in Tables 1 and 2.

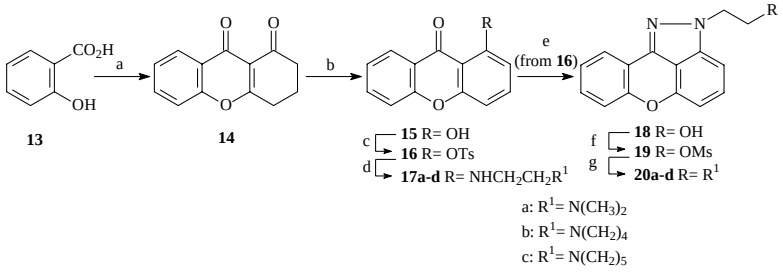
The derivatives possess strong cytotoxic activity against all the tested cell lines, and their IC_{50} values vary typically within the range of 0.8–20 μM.

The aminoderivatives bearing the nitro group (**9a–c**) appear to be considerably more active when compared with the corresponding non-substituted analogues (**17a–c**). On the other hand, the difference in activity between the substituted and non-substituted derivatives in the pyrazole-fused analogues is less pronounced.

With regard to the activity against the L1210 cell line, compounds **9b**, **12a**, **20a**, and **20b** possess interesting cytotoxicity, with IC_{50} values within the range of 1.17–1.81 μM, while the corresponding value for PZA has been reported to be 0.424 μM.[26]

The new derivatives, with the exception of compound **9b**, possess a rather diminished antiproliferative activity against the colorectal adenocarcinoma HT-29 cell line.

On the contrary, the nitro substituted derivatives exhibit cytotoxicity against the uterine sarcoma MES-SA cell line. The majority of them possess an interesting profile of IC_{50} values, below 2 μM, the most cytotoxic compound being **9b**, which shows an antiproliferative activity in the submicromolar range (IC_{50} value: 0.78 μM). Furthermore, from a direct comparison of the activity toward the sensitive and resistant cell lines, it is evident that the compounds appear to be active against MES-SA,



Scheme 2. Synthesis of compounds **17a–c** and **20a–c**. Reagents and conditions: (a) PPA, 1,3-cyclohexanedione, 150 °C, 2h; (b) DDQ, toluene, 90 °C, 30 min; (c) 4-toluenesulfonylchloride, acetone, Na_2CO_3, reflux, 6 h; (d) $H_2NCH_2CH_2R^1$, pyridine, reflux, 16 h; (e) $H_2NNHCH_2CH_2OH$, pyridine, reflux 10 h; (f) CH_3SO_2Cl, CH_2Cl_2, Et_3N, rt, 4 h; (g) secondary amine, EtOH abs., reflux, 10 h.

Table 1. Inhibition of proliferation of the aminosubstituted xanthenone derivatives (IC_{50} values in μM[a])

Compound	R^1	L1210	HT-29	MES-SA	MES-SA/Dx5	RF[b]
9a	$N(CH_3)_2$	3.13 (±0.31)	4.66 (±0.84)	1.56 (±0.62)	0.95 (±0.20)	0.61
9b	$N(CH_2)_4$	1.48 (±0.67)	2.53 (±0.39)	0.78 (±0.11)	0.55 (±0.07)	0.70
9c	$N(CH_2)_5$	5.88 (±1.47)	6.49 (±2.29)	1.59 (±0.18)	1.56 (±0.12)	0.98
17a	$N(CH_3)_2$	6.58 (±1.65)	21.6 (±7.51)	9.03 (±2.61)	6.62 (±1.96)	0.73
17b	$N(CH_2)_4$	9.00 (±1.88)	26.6 (±2.55)	14.61 (±4.16)	7.76 (±1.80)	0.53
17c	$N(CH_2)_5$	4.59 (±0.97)	26.5 (±5.11)	8.31 (±2.19)	6.04 (±1.75)	0.73
Mx		0.077 (±0.010)	0.020 (±0.004)	0.003 (±0.000)	0.030 (±0.020)	11.55
Dx		0.080 (±0.005)	0.320 (±0.180)	0.016 (±0.008)	1.56 (±0.10)	98.25

[a] The results represent means (± standard deviation) of three independent experiments and are expressed as IC_{50}, the concentration that reduced by 50% the optical density of treated cells with respect to untreated controls.

[b] IC_{50} resistant cells/IC_{50} sensitive cells.

Table 2. Inhibition of proliferation of the pyrazole-fuzed xanthenone derivatives (IC_{50} values in μM[a])

Compound	R[1]	L1210	HT-29	MES-SA	MES-SA/Dx5	RF[b]
12a	$N(CH_3)_2$	1.81 (±0.41)	7.49 (±0.31)	1.03 (±0.58)	0.63 (±0.25)	0.61
12b	$N(CH_2)_4$	3.52 (±0.35)	15.8 (±2.38)	1.13 (±0.07)	1.47 (±0.21)	1.29
12c	$N(CH_2)_5$	10.7 (±1.85)	21.6 (±7.99)	4.05 (±0.75)	5.38 (±1.26)	1.33
20a	$N(CH_3)_2$	1.17 (±0.20)	20.9 (±9.45)	2.79 (±0.69)	2.76 (±1.08)	0.99
20b	$N(CH_2)_4$	1.79 (±0.21)	11.5 (±1.89)	1.75 (±0.16)	1.58 (±0.12)	0.90
20c	$N(CH_2)_5$	2.74 (±0.70)	9.92 (±3.41)	4.40 (±0.90)	3.88 (±0.35)	0.88
Mx		0.077 (±0.010)	0.020 (±0.004)	0.003 (±0.000)	0.030 (±0.020)	11.55
Dx		0.080 (±0.005)	0.320 (±0.180)	0.016 (±0.008)	1.56 (±0.10)	98.25

[a] The results represent means (± standard deviation) of three independent experiments and are expressed as IC_{50}, the concentration that reduced by 50% the optical density of treated cells with respect to untreated controls.
[b] IC_{50} resistant cells/IC_{50} sensitive cells.

Table 3. Cell cycle-phase distribution (%)[a]

Compound	G0/G1	S	G2/M
9b	41.70 (±2.12)	20.79 (±3.67)	37.41 (±5.51)
17b	59.41 (±3.71)	32.66 (±4.12)	7.93 (±1.49)
12a	30.00 (±4.90)	43.40 (±8.12)	26.60 (±5.75)
20a	33.06 (±3.08)	20.87 (±4.21)	46.08 (±3.60)
20b	22.63 (±1.19)	14.81 (±0.91)	62.57 (±3.88)
Control	54.70 (±4.80)	35.57 (±2.84)	9.74 (±1.96)

[a] Mean (± standard deviation) of three independent experiments.

simultaneously possess generally comparable cytotoxicity against the doxorubicin resistant MES-SA/Dx5 cell line. The ability of all the tested compounds to overcome multidrug resistance of the MES-SA/Dx5 cell line is clearly indicated by the resistant factor (RF) values, which are all practically equal to 1. These results suggest that the novel compounds seem to be hardly recognized by the protein mechanisms controlling multidrug resistance. Worth mentioning, the RF to doxorubicin was found to be 98.25, as expected,[23] while the RF to mitoxantrone was 11.55.

Cell-cycle perturbations induced after incubation of exponentially growing MES-SA uterine sarcoma cells with a number of new compounds for 24 h are given in Table 3. The studied compounds provoke, in general, a G2/M arrest, expected on account of their structural similarity to pyrazoloacridines and mitoxantrone, which have also been reported to block the cell cycle in the G2 phase.[24,25] Compound **17b**, with an IC_{50} higher than the concentration used for FACS analysis, had, indeed, no significant effect on the cell cycle-phase distribution.

In conclusion, we have prepared a novel series of xanthenone aminoderivatives, bearing structural analogy to PZA, which inhibit the proliferation of several cancer cell lines. Most noticeably, this class of compounds exhibits a very promising ability to overcome the multidrug resistance of the MES-SA/Dx5 cell line.

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