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Original article

The interaction between resistance modifiers such as pyrido[3,2-g]quinoline, aza-oxafluorene and pregnane derivatives with DNA, plasmid DNA and tRNA

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Abstract

Various resistance mechanisms such as complex formation with DNA, tRNA and MDR1 *p*-glycoprotein were modified in bacteria and cancer cells in presence of pregnane, pyridoquinoline, and aza-oxafluorene derivatives. Interaction between the compounds, plasmid DNA and tRNA was shown and compared to the interaction with calf thymus DNA. Complex formation with MDR1 *p*-glycoprotein and drug accumulation increased in cancer cells. Both plasmid DNA and *p*-gp complex formation were related to the chemical structures of the resistance modifiers.

Keywords: Pregnanes; Pyridoquinolines; Aza-fluorenes; DNA interaction; Transfer RNA interaction; MDR1 *p*-glycoprotein

1. Introduction

It is well known that the denaturation of nucleic acids caused by elevated temperature will increase their UV absorbance due to the melting of double stranded regions (hyperchromic effect). By measuring the UV absorbance of nucleic acids at increasing temperature, the melting of nucleic acids can be monitored and the phase-transition temperature (thermal denaturation temperature, T_m) can be determined. Both positive and negative changes in T_m values can occur: an increase in T_m indicates stabilisation of the DNA helix and a decrease in T_m can mean the degradation of the nucleic acid by different chemical agents [1].

In order to confirm the evidence of the binding of antiplasmid compounds to nucleic acids, the UV spectrum of the ligands can be examined. When a ligand possessing a chromophore binds to nucleic acids, its UV spectra will be altered as a consequence of changing in its molecular environment. The wavelength of absorption undergoes a shift to longer wavelengths (bathochromic shift) with corresponding decrease in absorbance (hypochromic shift), which is a good indication of an interaction between the ligand and the nucleic acid [2].

2. Chemistry

2.1. General procedures for the preparation of the pregnane derivatives (Fig. 1)

2.1.1. Pregnane thioethers (P-S1 series) were prepared by a base catalysed Michael addition reaction from 3β-acetoxy-5,16-pregnadiene-20-one (PDA)

A solution of either H_2S or the appropriate mercaptan (H-SR; R = methyl, cyclohexyl, benzyl, phenyl) in dry ben-

Abbreviations: PTC, phase transfer catalysis; DMSO, dimethyl sulphoxide; T_m, thermal denaturation temperature; Tris, tris(hydroxymethyl)-aminomethane.

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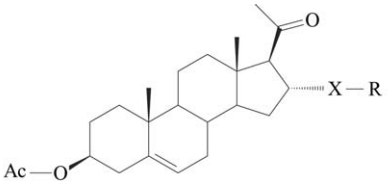


Compound	X	R	Clog P
P-SH	S	H	5.32
P-S1Me	S	CH_3	5.28
P-S1Ph	S	C_6H_5	6.35
P-S1Be	S	$CH_2C_6H_5$	7.13
P-S1cHe	S	C_6H_{11}	6.77
P-S2Me	S=O	CH_3	4.41
P-S2Ph	S=O	C_6H_5	5.63
P-S2Be	S=O	$CH_2C_6H_5$	6.16
P-S2cHe	S=O	C_6H_{11}	5.53
P-S3Me	O=S=O	CH_3	2.44
P-S3Ph	O=S=O	C_6H_5	3.28
P-S3Be	O=S=O	$CH_2C_6H_5$	3.72
P-S3cHe	O=S=O	C_6H_{11}	3.48
P-S3Am	O=S=O	$C_6H_5NH_2$	5.32
P-S3SuAm	O=S=O	NH_2	2.26
P-S3Na	O=S=O	OH	3.79

Fig. 1. Structures of pregnane derivatives investigated.

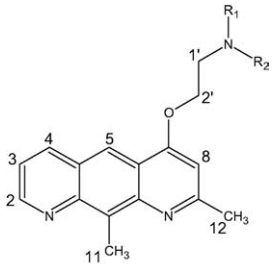
zene and piperidine was added to a solution of 3β-acetoxy-5,16-pregnadiene-20-one at 0 °C in dry benzene. The resulting solution was slowly warmed to room temperature and the mixture was stirred for 24 h. After the reaction had gone to completion, the solution was washed consecutively with dilute HCl, $NaHCO_3$ solution and water. The organic phase was dried and the solvent was removed in vacuo. The residue was purified by crystallisation to give pure products as white crystals.

2.1.2. Sulphoxides (P-S2 series) and sulphones (P-S3 series) were prepared by oxidation of the corresponding P-S1 product

The 16α-thioethers were dissolved in ethanol and oxidised by H_2O_2 (30% in water) at room temperature. After completion of the reaction (24–48 h) two products were detected by thin layer chromatography. The mixture was poured onto acidic water and the precipitate was filtered, washed, dried and separated by chromatography. The products of this reaction were the corresponding sulphoxide and sulphone derivatives.

*2.2. Monosubstituted pyrido[3,2-g]quinolines: **4a**, **4b**, **4c** and **4d** (Fig. 2)*

Monosubstituted pyrido[3,2-g]quinoline derivatives were obtained by alkylation of 2,10-dimethylpyridoquinoline-4-one under phase transfer catalysis (PTC) conditions. 2,10-Dimethylpyridoquinoline-4-one was obtained from commercial 2,6-diaminotoluene by a three-step synthesis. The first step, a Skraup reaction, gave 7-amino-8-methylquinoline [11,12].



4a R_1=R_2 = ethyl
4b R_1=R_2 = isopropyl
4c R_1=R_2 = pyrrolidinyl
4d R_1=R_2 = piperidinyl

Fig. 2. Structures of monosubstituted pyrido[3,2-g]quinolines (**4a–d**).

A Michael 1,4-addition of ethyl acetoacetate to the 7-amino-8-methylquinoline affords the intermediate imine (8-methyl-7-[(2′-carboethoxy-1′-methylvinyl)amino)]-quinoline).

The pyridoquinolinones were then obtained by a Conrad–Limpach thermocyclisation of this intermediate imine [13,14].

*2.3. Diazatetraasteranes (aza-oxofluorenes **Az1**, **Az2**, **Az3**) (Fig. 3)*

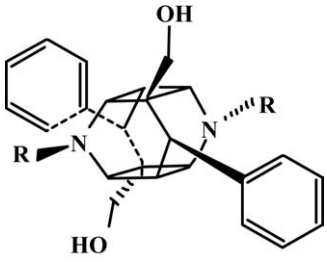
The synthesis of compounds **Az1**, **Az2**, and **Az3**; 3,9-dibenzyl-1,5,7,11-tetrahydroxymethyl-6,12-diphenyl-3,9-diazahexacyclo[6.4.0.$0^{2.7}$.$0^{4.11}$.$0^{5.10}$]dodecane (**Az1**) [15], 1,7-dihydroxymethyl-6,12-diphenyl-3,9-diphenoxycarbonyl 3,9-diazahexacyclo[6.4.0.$0^{2.7}$.$0^{4.11}$.$0^{5.10}$]dodecane (**Az2**) and 1,7-dihydroxymethyl-3,9-dimethoxycarbonyl-6,12-diphenyl-3,9-diazahexacyclo[6.4.0.$0^{2.7}$.$0^{4.11}$.$0^{5.10}$]dodecane (**Az3**) has been described [16].

3. Results and discussion

3.1. Thermal denaturation results

3.1.1. Pregnanes (Table 1)

If the temperature of a solution containing a helical nucleic acid is raised sufficiently, strand separation, or ‘melting’



R	
Az1	Benzyl
Az2	CO_2Phenyl
Az3	CO_2CH_3

Fig. 3. Structure of aza-fluorenes, **Az1–3**.

Table 1
Change in T_m of CT DNA, plasmid DNA and transfer RNA in the presence of pregnane derivatives

Pregnane	ΔT_m calf thymus DNA	ΔT_m plasmid DNA	ΔT_m tRNA
P-SH	-0.02		-5.45
P-S1Me	-1.07		-3.00
P-S1Ph	-1.23		-3.64
P-S1Be	-2.50	-4.10	-3.20
P-S1cHe	-0.58	-3.00	-3.40
P-S2Me	-0.60		-4.54
P-S2Ph	-2.80		-6.59
P-S2Be	-0.58	-0.83	-5.40
P-S2cHe	-2.66	-0.80	-5.20
P-S3Me	-2.50		-2.72
P-S3Na	-2.00		-11.82
P-S3Ph	1.95		-6.36
P-S3Be	1.34	-1.15	
P-S3cHe	-1.60	-3.00	
P-S3Am	-1.80		-10.68
P-S3SuAm	-2.00		-8.18

occurs [3]. The temperature that marks the midpoint of the melting process is called the melting temperature (or T_m). At the T_m, half the nucleic acid exists in the helical state and the other in the single-stranded state and the two species are in equilibrium.

Helical nucleic acid ↔ single-stranded nucleic acid

The difference between the T_m of the nucleic acid and the T_m observed in the presence of a ligand is termed ΔT_m. The ΔT_m value is related to the affinity of binding of the ligand to the nucleic acid. Ligands that bind to nucleic acids generally bind to the helical state with a greater affinity than to the single-stranded state. This stabilises the helical nucleic acid, the equilibrium shifts to the left and the T_m increases since more heat energy must be applied to the solution to dissociate the strands [4].

If the ligand binds to the single-stranded state more strongly than to the helical state, then the equilibrium shifts to the right and the T_m decreases and ΔT_m is negative. A cationic azoniacyclophane, for example, destabilises RNA duplexes because of the formation of an insertion complex with the bases of the single strand [5]. Cisplatin destabilises DNA resulting in negative ΔT_m values due to the formation of intrastrand covalent linkages between bases [6].

An analysis of the results in Table 1 on the effect of pregnane derivatives on the T_m of various nucleic acids shows some interesting variations. Largely negative ΔT_m values are observed, indicating a greater affinity for single-stranded forms of the nucleic acid. The variations in T_m produced both positive and negative values, which are relatively small (±2 °C) in the case of both calf thymus and plasmid DNA. However, with transfer RNA a large negative ΔT_m is observed (up to 11 °C). This indicates a much stronger affinity by the pregnane derivatives for transfer RNA as opposed to DNA and in particular for single-stranded regions of transfer RNA.

Chemically the pregnane derivatives investigated consist of three families with varying side chains within each family (Fig. 1). An analysis of the ΔT_m values shows that there is no obvious correlation, either between the three families or within the families, between the ΔT_m values obtained and the structural variations within the pregnanes investigated.

It must be assumed that the alteration of the oxidation state of the sulphur atom with the same side chains or the variations of the latter within the same thioether, sulphoxide or sulphone family produces both electronic and spatial differences. These competing variations would be expected to result in a complex structure activity pattern in these compounds. The results from log *P* calculations (Fig. 1) suggest that the polarity of the pregnanes increases in the order P-S1 < P-S2 < P-S3 and whereas this shows very little effect on the interaction with DNA which is anyway weak, it shows a marked effect on the interaction with tRNA (compare **P-S1Ph** ΔT_m = –3.64 °C with **P-S3Ph** ΔT_m = –6.36 °C). A further contributing effect is the size of the R group attached to the sulphur. Where R is small the effect on ΔT_m is minimised (compare **P-S1Me**, **P-S2Me** and **P-S3Me**). However, where R is large and/or electronegative in nature the P-S3 series shows a marked increase in interaction with tRNA (compare **P-S1Ph** ΔT_m = – 3.64 °C with **P-S3Ph** ΔT_m = –6.36 °C and **P-S3Am** ΔT_m = –10.68 °C). One possible explanation is that the interaction with the DNA/tRNA occurs through the 20-ketone and the 16-substituent at the same time. When it could form, the strength of the interaction perhaps depends on the distances between the interacting groups. Those distances are assumed essentially determined by the spatial arrangement of the 16-substituent and partly influenced by its hydrophobic/electronic effect.

3.1.2. Monosubstituted pyrido[3,2-g]quinolines

The interaction of the monosubstituted pyrido[3,2-g]quinolines with calf thymus DNA and plasmid DNA was studied by two different UV spectroscopic techniques. The results may be compared to the standard compounds, 9-aminoacridine, acridine orange, chlorpromazine and trifluperazine.

3.1.2.1. Thermal denaturation. All compounds raised the denaturation temperature of calf thymus DNA and plasmid DNA (Table 2). The increases in T_m, compared with the reference compounds, indicates that all four compounds interact strongly with calf thymus DNA and plasmid DNA. The interaction is probably by intercalation, given the planar structure and the presence of a protonatable nitrogen atom in the side chain of these compounds [2].

3.1.2.2. UV spectroscopic shift and binding affinity determination. All four compounds showed a bathochromic and hypochromic shift in the presence of calf thymus DNA indicating intercalation into DNA (Table 3). The four compounds were also titrated against calf thymus DNA and the data fitted to a one site ligand binding model using the Grafit version 5 curve fitting software (Erithacus Software).

Table 2
Change in T_m of CT DNA and plasmid DNA in the presence of pyrido[3,2-g]quinoline derivatives and aza-oxafluorenes derivatives

Compound	ΔT_m calf thymus DNA	ΔT_m plasmid DNA
Pyrido[3,2-g]quinoline derivatives		
4a	8.10	9.2
4b	10.90	11.5
4c	14.40	14.8
4d	14.40	15.5
Aza-oxafluorenes		
Az1	-1.50	2.90
Az2	0.00	1.50
Az3	1.00	1.50
9-Aminoacridine	10.70	12.00
Acridine orange	12.60	9.60
Phenothiazines		
Promethazine	1.00	5.10
Trifluoperazine	2.20	7.00

Table 3
UV spectral shift and binding affinity data for monosubstituted pyrido[3,2-g]quinolines binding to calf thymus DNA

Compound	Bathochromic shift (nm)	Isosbestic point	Binding capacity	Dissociation constant (M)
4a	1.0	404	0.25	0.22
4b	1.0	404	0.29	0.60
4c	1.0	405	0.56	1.70
4d	1.0	405	0.39	1.40
9-aminoacridine	4.0	442	0.14	0.09

All four compounds show strong binding to DNA as compared to 9-aminoacridine. On examining the structures of these compounds, all four compounds possess the same heterocyclic nucleus with variations in the side chain. The weaker binding of **4a** and **4b** may be rationalised by the bulky alkyl groups surrounding the side chain nitrogen that could cause some steric interference to binding. On the other hand **4c** and **4d** contain five and six membered pyrrolidine and piperidine ring structures in the side chain, which are less bulky and produce less interference. Alternatively the differences seen could be due to structural constraints of the side chain and access to the unshared electrons of the extracyclic nitrogen atom.

3.1.3. Aza-oxafluorenes

The three aza-oxafluorenes investigated showed only marginal interaction with calf thymus DNA but a slightly increased interaction with plasmid DNA (Table 2).

3.2. MDR reversal results (Tables 4 and 5)

Resistance modifiers are able to interact with the expression of particular genes and the protein responsible for drug resistance of bacteria and cancer cells. In our experiments several compounds were tested for interaction with both calf thymus and plasmid DNA and with transfer RNA as in vitro models. The monosubstituted pyridoquinoline compounds, phenothiazines and aza-fluorene, **Az3** were able to increase the T_m value of calf thymus DNA. Pregnane derivatives destabilised the nucleic acid structure by lowering the melting point (m.p.). The results were similar when the compounds were tested on pBR 322 and plasmid DNA but the interaction was more marked on tRNA.

Table 4
MDR reversal of some pregnane derivatives on mouse T-lymphoma cell lines

Compounds	Concentration (μg ml^{-1})	Fluorescence activity ratio
Verapamil	10	5.02
P-S1cHe	4	0.98
	8	1.03
	16	1.04
P-S2cHe	4	4.7
	8	18.61
	16	56.45
P-S3cHe	1	0.92
	2	2.75
	4	8.73
P-S1Be	4	1.1
	8	2.47
	16	4.04
P-S2Be	4	6.28
	8	10.66
	16	22.83
P-S3Be	1	3.89
	2	11.27
	4	45.37
DMSO control	20	0.7

Table 5
MDR reversal of some monosubstituted pyrido[3,2-g]quinolines (**4a–d**) and aza-oxafluorenes(**Az1–3**) on mouse T-lymphoma cell lines

Compounds	Concentration (μg ml^{-1})	Fluorescence activity ratio
Verapamil	10	6.06
4a	4	40.18
	40	43.55
4b	4	43.10
	40	49.83
4c	4	38.96
	40	47.55
4d	4	53.09
	40	43.12
Verapamil	10	7.27
Az1	4	30.19
	40	24.83
Az2	4	28.10
	40	30.87
Az3	4	1.11
	40	18.62
DMSO control	20	0.99

The biological activity, the efflux of *p*-gp 170 was studied in the presence of compounds mentioned above. Therefore, the efflux capacity of the human multidrug resistance gene encoded *p*-glycoprotein was measured by flow cytometry after 10 min treatment by measurement of the rhodamine accumulation. It was found that the majority of pyridoquinoline derivatives, aza-oxafluorenes, phenothiazines and the preg-

nane derivatives were able to reduce the activity of MDR efflux pump.

In the case of substituted pregnane derivatives, the following compounds were extremely effective inhibitors of the *p*-gp 170: **PS3-Be**, **PS2-cHe**, **PS2-Be**, **PS1-Be** and **PS1-cHe**, the last compounds were ineffective in low concentration from 1 to 16 μg ml^{-1}, however, at 40 μg ml^{-1} they greatly enhanced the drug accumulation. However two compounds, **PS2-cHe**, **PS3-cHe** were toxic at this highest concentration. It is difficult to see a direct relationship between the effects of these compounds on MDR and their interaction with nucleic acids. Probably the interaction with DNA is unrelated to the MDR reversal effect. However, the strong interaction with tRNA particularly with the pregnane derivatives may suggest either that the mechanism of MDR reversal is linked to disruption of the function of tRNA in resistant cells or that similar structural features are responsible for both the interaction with tRNA and the MDR reversal effect.

4. Conclusions

The MDR reversal activity of a series of novel pregnanes, pyridoquinolines and aza-fluorenes has been measured and related to the interaction of these compounds with nucleic acids. It was found that the majority of these compounds were able to reverse MDR in sensitive cells. However, significant differences were shown in the abilities of these types of compounds to interact with nucleic acids. The pyridoquinolines were able to intercalate strongly into DNA whereas the aza-fluorenes and the pregnanes showed little or no interaction. The pregnanes however showed significant ability to interact with single-stranded regions of transfer RNA, which may suggest that the mechanism of MDR reversal is linked to the disruption of tRNA function in resistant cells.

5. Experimental protocols

5.1. Chemistry

^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded with a Bruker Avance DRX 400 instrument at 25 °C in $CDCl_3$ solution and are reported as chemical shifts in ppm (δ) relative to TMS—m.p.s were determined with a Kofler instrument and are uncorrected—elemental analyses were performed with a Kovo (Czech Republic) CHN automatic analyser and found to be in good agreements with the calculated values.

5.1.1. Pregnanes (Fig. 1)

*5.1.1.1. 3-Acetoxy-16-benzylsulfanyl-pregn-5-ene-20-one [17] (**P-S1Be**).* Benzyl mercaptan (9.6 g, 77 mmol) and PDA (5.0 g, 14 mmol) were reacted as described by Romo et al. [17].

Yield: 5.2 g (78%), m.p.: 124–126 °C.

^{1}H NMR ($CDCl_3$) 0.60 (s, 3H, C-18), 0.99 (s, 3H, C-19), 2.02 (s, 3H, C-21), 2.05 (s, 3H, 3Ac), 2.52 (d, 1H, C-17), 3.67–3.73 (m, 1H 16, 2H, $BzCH_2$), 4.60 (m, 1H, C-3), 5.37 (m, 1H, C-6) 7.22–7.31 (m, 5H, aromatic).

^{13}C NMR ($CDCl_3$) 13.8, 19.2, 20.8, 21.3, 27.6, 31.3, 31.5, 31.6, 34.4, 36.5, 36.9, 37.1, 38.0, 38.6, 41.2, 45.2, 49.6, 54.8, 71.7, 73.7, 122.1, 126.9, 128.7, 138.2, 139.6, 170.4, 207.1.

Oxidation of 3-acetoxy-16-benzylsulfanyl-pregn-5-ene-20-one (***P-S1Be***): The reaction was carried out as described by Romo et al., but without the addition of Na_2CO_3.

Ten millilitre of H_2O_2 (30% in water) was added at room temperature to the **P-S1Be** (2.0 g, 4.16 mmol) dissolved in 150 ml of ethanol. The reaction was allowed to continue over 24 h and resulted in two products. The mixture was poured onto acidic water and the precipitate was filtered, washed and dried. The products were separated by column chromatography on silica and identified as:

a) 3-Acetoxy-16-benzylsulfinyl-pregn-5-ene-20-one (**P-S2Be**)

M.p.: 149–150 °C.

^{1}H NMR ($CDCl_3$) 0.64 (s, 3H, C-18), 1.02 (s, 3H, C-19), 2.03 (s, 3H, C-21), 2.14 (s, 3H, 3Ac), 3.00 (d, 1H, C-17), 3.79 (d, 1H, $BzCH_2$), 3.98 (d, 1H, $BzCH_2$), 4.60 (m, 1H, C-3), 5.38 (m, 1H, C-6), 7.27–7.38 (m, 5H, aromatic).

^{13}C NMR ($CDCl_3$) 14.0, 19.2, 20.8, 21.3, 22.3, 27.7, 31.3, 31.8, 36.5, 36.9, 38.0, 38.2, 45.2, 49.4, 55.3, 56.2, 57.6, 65.3, 73.7, 122.1, 128.3, 128.9, 129.8, 130.5, 130.7, 139.6, 170.4, 206.0.

b) 3-Acetoxy-16-benzylsulfonyl-pregn-5-ene-20-one (**P-S3Be**)

M.p.: 199–200 °C.

^{1}H NMR ($CDCl_3$) 0.61 (s, 3H, C-18), 1.00 (s, 3H, C-19), 2.03 (s, 3H, C-21), 2.18 (s, 3H, 3Ac), 3.17 (d, 1H, C-17), 4.12 (t 1H C-16), 4.16 (s, 2H, $BzCH_2$), 4.60 (m, 1H, C-3), 5.36 (m, 1H, C-6) 7.39–7.87 (m, 5H, aromatic).

^{13}C NMR ($CDCl_3$) 13.9, 19.2, 20.8, 21.3, 27.0, 31.2, 31.3, 31.4, 36.4, 38.2, 38.4, 44.6, 49.3, 55.3, 59.0, 57.6, 62.7, 63.9, 73.6, 121.8, 127.5, 128.5, 128.7, 129.1, 130.7, 139.6, 170.3, 205.1.

*5.1.1.2. 3-Acetoxy-16-phenylsulfanyl-pregn-5-ene-20-one (**P-S1Ph**).* This derivative was described by Sunthankar et al. [18]. However, here it was prepared by reacting thiophenol (7.5 g, 68 mmol) and PDA (5.0 g, 14 mmol) by the method used by Romo et al. [17].

Yield: 5.0 g (76%), m.p.: 151–154 °C.

^{1}H NMR ($CDCl_3$) 0.66 (s, 3H, C-18), 1.01 (s, 3H, C-19), 2.02 (s, 3H, C-21), 2.03 (s, 3H, 3Ac), 2.61 (d, 1H, C-17), 4.20 (t, 1H, C-16), 4.60 (m, 1H, C-3), 5.35 (m, 1H, C-6), 7.18–7.35 (m, 5H, aromatic).

^{13}C NMR ($CDCl_3$) 14.0, 19.2, 20.8, 21.3, 27.7, 31.2, 31.5, 31.7, 34.6, 36.5, 36.9, 38.0, 38.8, 43.6, 45.3, 49.7, 54.8, 70.9, 73.7, 122.0, 126.5, 128.7, 130.9, 136.1, 139.6, 170.4, 206.6.

Oxidation of 3-acetoxy-16-phenylsulfanyl-pregn-5-ene-20-one (***P-S1Ph***): Ten millilitre of H_2O_2 (30% in water) was

added at room temperature to the **P-S1Ph** (2.0 g, 4.29 mmol) dissolved in 150 ml of ethanol. The reaction was allowed to continue over 24 h and resulted in two products. The mixture was poured onto acidic water and the precipitate was filtered, washed and dried. The two products were separated by column chromatography on silica and identified as:

a) 3β-Acetoxy-16α-phenylsulfinyl-pregn-5-ene-20-one (**P-S2Ph**)

M.p.: 164–166 °C.

^{1}H NMR ($CDCl_3$) 0.66 (s, 3H, C-18), 1.00 (s, 3H, C-19), 2.02 (s, 3H, C-21), 2.03 (s, 3H, 3Ac), 2.62 (d, 1H, C-17), 4.22 (m, 1H, C-16), 4.63 (m, 1H, C-3), 5.38 (m, 1H, C-6), 7.19–7.50 (m, 5H, aromatic).

^{13}C NMR ($CDCl_3$) 14.0, 19.2, 20.8, 21.3, 27.5, 29.6, 31.3, 31.5, 31.6, 34.6, 36.5, 38.8, 43.6, 49.6, 54.8, 59.0, 60.8, 70.9, 73.7, 122.0, 124.4, 126.5, 128.7, 130.9, 136.1, 139.6, 170.4, 213.1.

b) 3-Acetoxy-16-phenylsulfonyl-pregn-5-ene-20-one (**P-S3Ph**)

M.p.: 272–274 °C.

^{1}H NMR ($CDCl_3$) 0.59 (s, 3H, C-18), 1.00 (s, 3H, C-19), 2.00 (s, 3H, C-21), 2.03 (s, 3H, 3Ac), 3.14 (d, 1H, C-17), 4.28 (t, 1H, C-16), 4.62 (m, 1H, C-3), 5.37 (m, 1H, C-6), 7.52-7.87 (m, 5H, aromatic).

^{13}C NMR ($CDCl_3$) 14.9, 19.9, 21.5, 22.0, 27.8, 28.3, 31.7, 32.0, 32.1, 37.2, 37.6, 38.6, 39.1, 45.9, 50.0, 56.0, 63.2, 64.6, 74.3, 122.5, 129.2, 129.9, 134.3, 139.2, 140.3, 171.1, 205.5.

*5.1.1.3. 3-Acetoxy-16-cyclohexylsulfanyl-pregn-5-ene-20-one (**P-S1cHe**).* Cyclohexyl mercaptan (8.6 g, 74 mmol) dissolved in dry benzene was added to a solution of PDA (5.0 g, 14 mmol) and piperidine (4.0 ml, 40 mmol) in dry benzene at 8 °C. The mixture was warmed to room temperature and stirred for 48 h. After completion of the reaction, the solution was washed with dilute HCl, and 5% $NaHCO_3$ solution, and finally with water. The organic phase was dried over anhydrous Na_2SO_4 and the solvent was removed in vacuo. The residue was purified by crystallisation from hexane to give pure product.

Yield: 5.4 g (82%), m.p.: 124–126 °C.

^{1}H NMR ($CDCl_3$) 0.64 (s, 3H, C-18), 1.01 (s, 3H, C-19), 2.03 (s, 3H, C-21), 2.15 (s, 3H, 3Ac), 2.53 (d, 1H, C-17), 2.59 (m, 1H, cyclohexyl-1), 3.76 (t, 1H, C-16), 4.61 (m, 1H, C-3), 5.37 (m, 1H, C-6).

^{13}C NMR ($CDCl_3$) 13.8, 19.3, 20.9, 21.4, 25.8, 26.1, 26.2, 27.7, 31.4, 31.6, 32.0, 33.7, 34.0, 36.1, 36.6, 36.9, 38.1, 38.7, 39.2, 44.3, 45.1, 49.7, 54.8, 72.1, 73.7, 122.1, 139.6, 170.5, 207.3.

*Oxidation of the 3-acetoxy-16-cyclohexylsulfanyl-pregn-5-ene-20-one (**P-S1cHe**)*: Ten millilitre of H_2O_2 (30% in water) was added at room temperature to the **P-S1cHe** (2.0 g, 4.23 mmol) dissolved in 150 ml of ethanol. The reaction was allowed to continue over 24 h and resulted in two products. The mixture was poured onto acidic water and the precipitate was filtered, washed, dried. The products were separated by column chromatography on silica and identified as:

a) 3-Acetoxy-16-cyclohexylsulfinyl-pregn-5-ene-20-one (**P-S2cHe**)

M.p.: 157–160 °C.

^{1}H NMR ($CDCl_3$) 0.7 (s, 3H, C-18), 1.02 (s, 3H, C-19), 2.03 (s, 3H, C-21), 2.22 (s, 3H, 3Ac), 3.26 (d, 1H, C-17), 3.92 (t, 1H, C-16), 4.60 (m, 1H, C-3), 5.36 (m, 1H, C-6).

^{13}C NMR ($CDCl_3$) 14.6, 19.3, 20.9, 21.3, 25.1, 25.3, 25.5, 26.4, 27.7, 29.9, 31.2, 31.5, 31.6, 36.6, 36.9, 38.0, 38.3, 44.8, 49.4, 53.2, 55.3, 58.3, 61.3, 73.7, 121.9, 139.7, 170.4, 206.9.

b) 3-Acetoxy-16-cyclohexylsulfonyl-pregn-5-ene-20-one (**P-S3cHe**)

M.p.: 170–172 °C.

^{1}H NMR ($CDCl_3$) 66 (s, 3H, C-18), 1.02 (s, 3H, C-19), 2.03 (s, 3H, C-21), 2.23 (s, 3H, 3Ac), 2.73 (t, 1H, cyclohexyl-1), 3.19 (d, 1H, C-17), 4.22 (t, 1H, C-16), 4.61 (m, 1H, C-3), 5.37 (m, 1H, C-6).

^{13}C NMR ($CDCl_3$) 14.1, 19.2, 20.8, 21.3, 24.9, 25.1, 25.3, 27.6, 27.8, 31.3, 31.4, 31.5, 36.5, 36.9, 37.9, 38.3, 44.5, 49.3, 55.6, 56.6, 60.6, 62.9, 73.7, 121.9, 139.6, 170.3, 205.6.

Log *P* values for the pregnane derivatives (Fig. 1) were calculated using interactive log *P* and log *W* predictor (ChemSilico http://www.logp.com).

*5.1.2. Monosubstituted pyrido[3,2-g]quinolines: **4a**, **4b**, **4c** and **4d** (Fig. 2)*

5.1.2.1. 8-Methyl-7-aminoquinoline. 2,6-Diaminotoluene (4.51 g, 36.9 mmol), glycerol (28.5 g, 0.41 mol) and a freshly prepared aqueous solution of arsenic (V) pentoxide (19.04 g in 15 ml of water) were heated at 100 °C with stirring. A solution of sulphuric acid (38 ml in 35 ml of water) was then added to the mixture. The temperature was raised to 150 °C for 4 h. After cooling, the mixture was poured into a large flask and ammonia was added until precipitation occured. The precipitate was filtered off, washed with water and dried. Yield: 3.9 g (67%), m.p. 34–136 °C. ^{1}H NMR ($CDCl_3$): δ = 8.9 (dd, J = 4.2 Hz, J = 1.0 Hz, 1H C-2), 8.0 (dd, J = 8.1 Hz, J = 1.0 Hz, 1H C-3), 7.6 (d, J = 8.7 Hz, 1H C-4), 7.2 (dd, J = 8.1 Hz, J = 4.3 Hz, 1H C-6), 7.0 (d, J=8.7 Hz, 1H C-5), 4.0 (m, 2H NH_2), 2.6 (s, 3H CH_3). ^{13}C NMR($CDCl_3$): 149.7 (d), δ = 144.9 (s), 135.2 (d), 122.6 (s), 126.3 (d), 119.9 (d), 118.5 (d), 117.4 (d), 115.1 (s), 10.4 (q).

5.1.2.2. 8-Methyl-7-[(2′-carboethoxy-1′-methylvinyl)amino)]-quinoline. 7-Amino-8-methylquinoline (4.87 g, 30.7 mmol), ethyl acetoacetate (4.0 g, 30.7 mmol), absolute ethanol (35 ml), anhydrous $CaSO_4$ (12 g) and few drops of acetic acid were heated at 80 °C with stirring for 5 days. After filtration, the solvent was removed in vacuo and the crude product was used without further purification for the next step.

^{1}H NMR ($CDCl_3$): 11.5 (s, 1H NH), 8.9 (d, J = 4.2 Hz, J = 1.6 Hz, 1H C-2), 8.1 (d, J = 8.2 Hz, J = 1.6 Hz, 1H C-6), 7.6 (d, J = 8.6 Hz, 1H C-5), 7.3 (dd, J = 8.2 Hz, J = 4.2 Hz, 1H C-3), 7.2 (d, J = 8.7 Hz, 1H C-4), 4.8 (s, 1H ethene H), 4.2 (q, J = 7.2 Hz, 2H ester CH_2), 2.8 (s, 3H ring CH_3), 1.9 (s, 3H ethene CH_3), 1.25 (t, J = 7.2 Hz, 3H ester CH_3).

5.1.2.3. 2,10-Dimethylpyrido-[3,2-g]quinoline-4-one. 8-Methyl-7-aminoquinoline (6 g, 22 mmol) and diphenylether (80 ml) were heated rapidly to 250 °C under nitrogen and held for 1 h at this temperature. After cooling to 60 °C, the mixture was poured onto petroleum ether (150 ml). The resulting red precipitate was filtered, washed thoroughly with ethylether and dried.

Yield: 1.95 g (56%), m.p.: 255 °C. ^{1}H NMR (dimethyl sulphoxide, DMSO-d_6): 10.5 (s, 1H OH), 9.0 (dd, J = 3.9 Hz, J = 1.8 Hz, 1H C-2), 8.6 (s, 1H C-8), 8.5 (dd, J = 8.3 Hz, J = 1.8 Hz, 1H C-3), 7.5 (dd, J = 8.3 Hz, J = 3.9 Hz, 1H C-4), 5.9 (s, 1H C-5), 3.0 (s, 3H C-12), 2.5 (s, 3H C-11). ^{13}C NMR($CDCl_3$): 149.7 (s), 144.9 (s), 135.2 (s), 122.6 (s), 126.3 (s), 119.9 (d), 118.5 (s), 99.9 (d), 67.4 (t), 51.04 (t), 48.1 (t), 27.0 (q), 12.4 (q), 12.1 (q).

*5.1.2.4. 2,10-Dimethylpyrido-[3,2-g]quinolines (**4a–d**).* 2,10-Dimethylpyrido-[3,2-g]quinoline-4-one (1 g, 4.46 mmol) was dissolved in toluene (70 ml) and either 1-chloro-2-diethylaminoethane hydrochloride (1.54 g, 8.92 mmol, for **4a**, 1-chloro-2-diisopropylaminoethane hydrochloride (1.1 g 8.92 mmol) for **4b**, 1-chloro-2-pyrrolidinoethane hydrochloride (1.2 g, 8.92 mmol) for **4c** or 1-chloro-2-piperidinoethane hydrochloride (1.3 g, 8.92 mmol) for **4d** was added. To this solution was added 50% aqueous KOH (35 ml) and tetrabutylammonium bromide (0.3 g, 0.9 mmol) and the solution was heated at 110 °C with stirring, for 24 h. The reaction mixture was then filtered whilst still warm. The aqueous phase was extracted three times with toluene. The organic layers were dried over anhydrous Na_2SO_4. After removal of the solvent, the oily product was refrigerated until solid and then washed with cold toluene.

For **4a** yield: 0.29 g (30%); m.p.: 142–144 °C. ^{1}H NMR ($CDCl_3$): 9.0 (dd, J = 3.9 Hz, J = 1.9 Hz, 1H C-2), 8.5 (s, 1H C-8), 8.3 (dd, J = 8.5 Hz, J = 1.8 Hz, 1H C-3), 7.3 (dd, J = 6.5 Hz, J = 3.9 Hz, 1H C-4), 6.5 (s, 1H C-5), 4.25 (t, J = 6.2 Hz, 2HC-2′), 3.3 (s, 3H C-12), 3.05 (t, 2H C-1′), 2.7 (s, 3H C-11), 2.7 (q, J = 6.3 Hz, 4H ethyl CH_2), 1.1 (t, J = 2.1 Hz, 6H ethyl CH_3). ^{13}C NMR ($CDCl_3$): 161.6 (s), 160.7 (s), 150.8 (d), 145.9 (s), 145.6 (s), 137.3 (d), 134.1 (s), 125.2 (s), 120.15 (s), 119.9 (d), 118.9 (d), 99.9 (d), 67.4 (t), 51.04 (t), 48.1 (t), 27.0 (q), 12.4 (q), 12.1 (q).

For **4b** yield: 0.19 g (20%); m.p.: 140 °C. ^{1}H NMR ($CDCl_3$): 9.0 (dd, 1H C-2), 8.6 (s, 1H C-8), 8.3 (dd, 1H C-3), 7.3 (dd, 1H C-4), 6.6 (s, 1H C-5), 4.2 (t, J = 7.1, 2H isopropyl CH), 3.4 (s, 3H C-12), 3.1 (m, 4H 1′,2′CH_2), 2.8 (s, 3H C-11), 1.1 (d, J = 6.5, 12H isopropyl CH_3). ^{13}C NMR($CDCl_3$): 161.6 (s), 160.7 (s), 150.6 (s), 145.9 (s), 145.5 (s), 137.2 (d), 133.9 (s), 125.1 (s), 120.0 (s), 119.9 (d), 118.9 (d), 99.8 (d), 69.7 (t), 49.6 (d), 44.1(t), 26.9 (q), 20.9 (q), 12.3 (q).

For **4c** yield: 0.29 g (30%); m.p.: 148–150 °C. ^{1}H NMR ($CDCl_3$): 9.0 (dd, J = 3.9 Hz, J = 1.9 Hz, 1H C-2), 8.5 (s, 1H C-8), 8.3 (dd, J = 8.5 Hz, J = 1.9 Hz, 1H C-3), 7.4 (dd, J = 8.5 Hz, J = 4.0 Hz, 1H C-4), 6.6 (s, 1H C-5), 4.4 (t, J = 6.2 Hz, 2H C-2′), 3.4 (s, 3H C-12), 3.0 (t, 2H C-1′), 2.8 (s, 3H C-11), 2.6 (m, 4H pyrrolidinyl CH_2), 1.6 (m, 4H pyrrolidinyl CH_2). ^{13}C NMR ($CDCl_3$): 161.4 (s), 160.5 (s), 150.6 (d), 145.8 (s), 145.5 (s), 137.2 (s), 134.0 (s), 125.1 (s), 120.0 (s), 119.8 (d), 118.8 (d), 99.7 (d), 66.8 (t), 57.5 (t), 55.1 (t), 26.0 (q), 24.1 (d), 12.3 (q).

For **4d** yield: 0.29 g (30%); m.p.: 135–1374 °C. ^{1}H NMR ($CDCl_3$): 9.0 (dd, J = 3.9 Hz, J = 1.9 Hz, 1H C-2), 8.5 (s,1H C-8), 8.3 (dd, J = 8.5 Hz, J = 1.9 Hz, 1H C-3), 7.4 (dd, J = 8.5 Hz, J = 4.0 Hz, 1H C-4), 6.6 (s, 1H C-5), 4.4 (t, J = 6.2 Hz, 2H C-2′), 3.4 (s, 3H C-12), 3.0 (t, 2H C-1′), 2.8 (s, 3H C-11), 2.6 (m, 4H piperidinyl CH_2), 1.6 (m, 6H piperidinyl CH_2). ^{13}C NMR ($CDCl_3$): 161.4 (s), 160.5 (s), 150.6 (d), 145.8 (s), 145.5 (s), 137.2 (s), 134.0 (s), 125.1 (s), 120.0 (s), 119.8 (d), 118.8 (d), 99.7 (d), 66.8 (t), 57.5 (t), 55.1 (t), 26.0 (q), 24.1 (d), 12.3 (q).

5.2. Biology

5.2.1. Materials

5.2.1.1. Nucleic acids. Highly polymerised type 1 calf thymus DNA sodium salt and type XXI transfer RNA were from the Sigma Chemical Corporation, pBR 322 plasmid DNA was prepared by Birnboim-Doly rapid alkaline extraction procedure [7,8].

5.2.1.2. Buffer solutions. For CT DNA and plasmid DNA: tris(hydroxymethyl)aminomethane (Tris, 0.003 M), NaCl (0.018 M) buffer adjusted to pH 7.0 was used.

For transfer RNA: Tris (0.1 M), NaCl (0.1 M), hydrated $MgCl_2$ (0.01 M) buffer adjusted to pH 7.5 was used.

5.2.1.3. Acridine-derivatives, phenothiazines. 9-Aminoacridine (Aldrich-Chemie, D-7924 Steinheim), Acridine-orange (Reanal Budapest, Hungary), Promethazine (EGIS Pharmaceutical Company, Budapest, Hungary), trifluoperazine (Sigma Chemical Co., St. Louis, USA) were used as internal standards.

Stock soutions of all compounds were prepared at a concentration of 1 mg ml^{-1}. 9-Aminoacridine, acridine-orange, promethazine and trifluoperazine were dissolved in deionised water, all other compounds were dissolved in DMSO.

5.2.2. Methods

5.2.2.1. Nucleic acid interactions: thermal denaturation studies. Calf thymus DNA (10 mg) was suspended in 0.03 M Tris hydrochloride buffer containing 0.018 M NaCl adjusted to pH 7.0 (10 ml) and the solution was kept at 4 °C for at least 3 days before use. This stock solution was then diluted to the working concentration immediately before use. Transfer RNA (10 mg) was suspended in 0.1 M Tris hydrochloride buffer containing 0.1 M NaCl and 0.01 M hydrated $MgCl_2$ adjusted to pH 7.5 (10 ml) and the solution was kept at 4 °C for at least 3 days before use. This stock solution was then diluted to the working concentration immediately before use. Plasmid DNA (1 mg) was suspended in 0.03 M Tris hydrochloride buffer containing 0.018 M NaCl adjusted to pH 7.0 (1 ml).

The concentrations of the polynucleotide solutions were determined spectrophotometrically in terms of nucleotide phosphate and calculated from an extinction coefficient at 260 nm of 6600 M^{-1} cm^{-1} for calf thymus DNA [9] and of 5.3×10^5 M^{-1} cm^{-1} for tRNA [10].

Measurement of the thermal denaturation profiles of ligand: DNA complexes were made for ligand: polynucleotide ratios of 1:10 and were recorded on a Cary Varian Model IE spectrophotometer using a Cary temperature controller connected to a Cary 1/3 multicell block. The solutions were allowed to equilibrate for 20 min before increasing the temperature and the temperature then increased at a rate of 0.5 °C min^{-1}. A cell containing polynucleotide solution alone was always measured along side cells containing ligand/polynucleotide mixtures to act as an internal standard. The blank cells in all measurements contained Tris buffer solution and all cells were stoppered with teflon caps, after debubbling and space was allowed for expansion of the solutions. The mid point of the thermal denaturation profile of the solutions (T_m) was determined by calculating the average absorbance using the instruments thermal application software [1].

5.2.2.2. Nucleic acid interactions: UV spectral shifts and binding affinity determination. In the case of the monosubstituted pyrido[3,2-g]quinolines: **4a**, **4b**, **4c** and **4d** which all exhibit a absorption maxima in the visible region well away from the absorption maxima of nucleic acids, it was possible to investigate the interaction of these compounds with nucleic acids by UV spectral shift analysis.

The spectra of a series of solutions of the monosubstituted pyrido[3,2-g]quinolines were measured in the presence of increasing amounts of either CT DNA or plasmid DNA. The spectra were recorded on a Cary Varian 1E spectrophotometer in 0.03 M Tris buffer containing 0.018 M NaCl at pH 7.00 for 2.50×10^{-5} M. The bathochromic shift (if any) was determined and a binding affinity constant (k) calculated from the hypochromic shift recorded at the λ_{max} of each compound using the Grafit version 5 curve fitting software (Erithacus Software).

5.2.2.3. Assay for reversal of multidrug resistance in tumour cells. The L2178 (parent) mouse T cell lymphoma cell line and its transformed subline with human MDR-1 gene were used. The cells were grown in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum, L-glutamine and antibiotics. The cells were adjusted to a concentration of 2×10^6 ml^{-1} and resuspended in serum-free McCoy's 5A medium and were distributed into 0.5 ml aliquots to Eppendorf centrifuge tubes. The tested compounds were added at various concentrations in different volume of the stock solutions and the samples were incubated for 10 min at room temperature. Then 10 μl (5.2 μM final concentration) of the indicator Rhodamine 123 was added to the samples and the cells were incubated for further 20 min at 37 °C, washed twice with phosphate buffered saline (PBS) and resuspended in 0.5 ml PBS for analysis. The fluorescence of the cell populations was measured by flow cytometry using a Beckton Dickinson FACScan fluorimeter. Verapamil was used as a positive control in the Rhodamine 123 exclusion experiments. The percentage of mean fluorescence intensity was calculated using the following equation on the basis of measured fluorescence values:

$$\text{R (fluorescence activity ratio)} = \frac{\text{MDR treated/MDR control}}{\text{Parental treated/Parental control}}$$

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