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Structure–Activity Relationships and Optimisation of the Selective MDR Modulator 2-(3,4-Dimethoxyphenyl)-5-(9-fluorenylamino)-2-(methylethyl) Pentanenitrile and Its *N*-Methyl Derivative

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Abstract—Several ring-substituted derivatives of previously studied MDR inhibitors 2-(3,4-dimethoxyphenyl)-5-(9-fluorenylamino)-2-(methylethyl)pentanenitrile and 2-(3,4-dimethoxyphenyl)-5-[(9-fluorenyl)-*N*-methylamino]-2-(methylethyl)pentanenitrile have been synthesised and studied with the aim of optimising activity and selectivity. The results show that MDR inhibition is scarcely sensitive to modulation of the electronic properties of the fluorene ring. Even if dramatic improvement was not obtained, one of the compounds (**2**) showed improved potency and selectivity with respect to the leads and appears to be a better candidate for drug development.

Introduction

The resistance of human malignancy to chemotherapeutic agents remains a major obstacle to efficient cancer therapy. Multidrug resistance (MDR) is a kind of resistance of cancer cells to multiple classes of chemotherapic drugs that can be structurally and mechanistically unrelated.[1] Classic MDR[2] is concerned with altered membrane transport, which results in lower cell concentrations of cytotoxic drug and is related to the over expression of a variety of proteins that act as ATP-dependent extrusion pumps. P-glycoprotein (P-gp)[3,4] and MRP1[5] are the most important and widely studied members of the family that belong to the ABC superfamily of transporters. It is important to notice that, besides their role in cancer cell resistance, these proteins seem to have multiple physiological functions as well,[6,7] since they are expressed also in many important non tumoral tissues and similar transporting proteins of the ABC superfamily are largely present in procariotic organisms.[8] Due to the unprecedented variety of substrates extruded, the mechanism of action of these transporters is still controversial. As a matter of fact, the dominant 'drug pump' model has been questioned and, at the moment, the exact molecular mechanism for P-gp's function is far from being clearly understood.[9,10]

Even if the mechanism of action of transporter proteins such as P-gp is still obscure, it was found that some drugs (first generation chemosensitizers) are able to block their functions.[11] This finding was soon considered a promising approach to overcome MDR in cancer therapy and has paved the way to the design and synthesis of a variety of new molecules (second generation of chemosensitizers).[12,13] Hopefully, these compounds can maintain the MDR-reversal activity while lacking the side effects associated with the first generation, which are often known drugs and used for other purposes.

Recently, we reported the MDR-modulating activity of compounds 2-(3,4-dimethoxyphenyl)-5-(9-fluorenylamino)-2-(methylethyl)pentanenitrile (SC11, labelled as **5** in the reference) and 2-(3,4-dimethoxyphenyl)-5-[(9-fluorenyl) - *N* - methylamino] - 2 - (methylethyl)pentane - nitrile (SC17, labelled as **12** in the reference)[14] (Chart 1)

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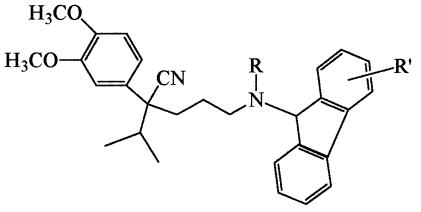
that showed interesting potency and selectivity. Compound SC11 is a potent reverter of MDR ($[I]_{0.5} = 0.25\,\mu M$) but maintains some negative chronotropic activity ($EC_{30} = 0.65\,\mu M$), compound SC17 is less potent ($[I]_{0.5} = 1.0\,\mu M$) but does not show any residual cardiovascular action. $[I]_{0.5}$ and EC_{30} are defined in the pharmacological part. Since potency and side effects are two major issues in MDR-inhibiting activity, we have begun a study aimed at optimising the pharmacological properties of our leads through modulation of the electronic properties of the fluorene nucleus. Therefore, we have synthesised the series of ring substituted analogues shown in Chart 1 and tested them as MDR modulators. The most potent compounds were then tested for cardiovascular action to check their selectivity.

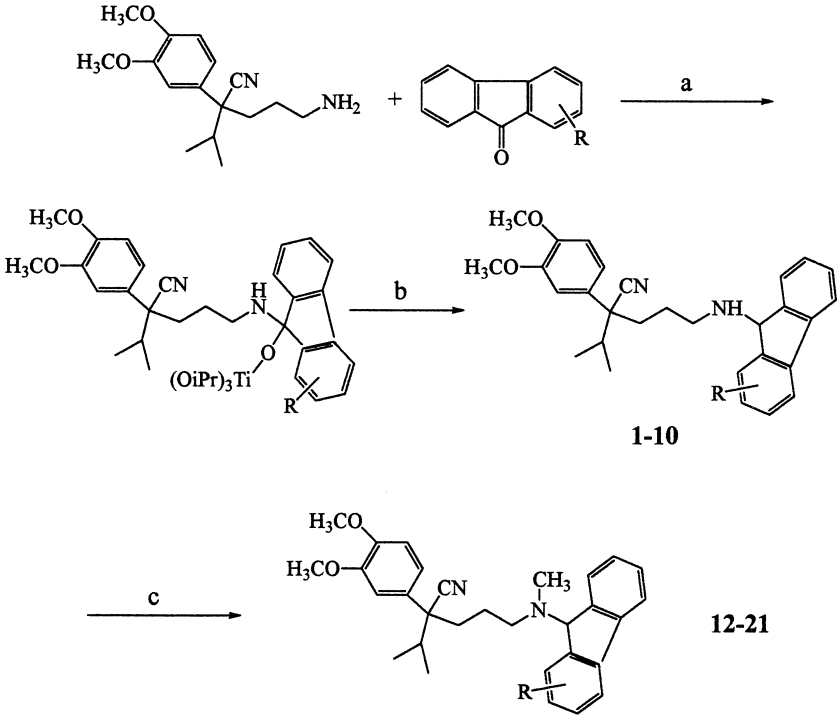
Chemistry

Secondary amines were obtained by reaction of 5-amino-2-(3,4-dimethoxyphenyl)-2-(methylethyl)pentanenitrile[14] with the suitable fluorenone (Scheme 1). In fact, in previous studies, we observed that functionalization of fluorene in position 9 could give instable derivatives that turn rapidly into fluorenone. So the high stability of 9-fluorenone and the commercial availability of many substituted fluorenones prompted us to look for synthetic pathways that involve these intermediates. 2-F-, 2-Br-, 2,7-dibromo-, 2-NO_2–, 2-OH– and 2-$N(CH_3)_2$–9-fluorenones are commercially available; 3,4-dimethoxy-9-fluorenone was synthesised according to Ladd.[15] 2-Methoxy-9-fluorenone **23** was obtained, as previously described by Kruber,[16] from the commercially available hydroxy derivative, but *O*-alkylation was not performed with dimethylsulfate, as described in the reference, but rather with CH_3I in KOH, according to a general procedure described by Johnstone.[17] Since this reaction is very simple and has an almost quantitative yield, we used the same pathways to also obtain the 3- and 4-methoxy-9-fluorenones **24** and **25**, that had previously been synthesized by more complicated routes.[18,19] Thus, starting from the commercially available 4-amino-9-



R = H; R' = H **SC11**
R = CH_3; R' = H **SC17**
R = H, CH_3 R' = Br, F, OH, OCH_3, NO_2, NH_2, $N(CH_3)_2$ **1-22**

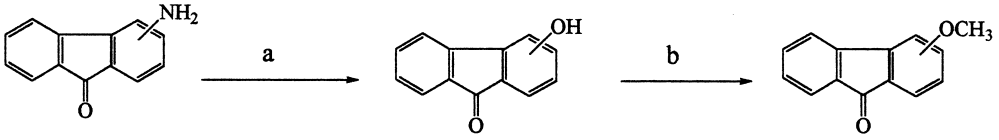
Chart 1.



1-10

12-21

Scheme 1. (a) Titanium (IV) isopropoxide; (b) $NaBH_3CN$; (c) HCHO/HCOOH.



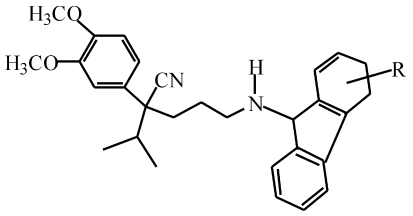
3-NH_2
4-NH_2

2-OH
3-OH
4-OH

2-OCH_3 **23**
3-OCH_3 **24**
4-OCH_3 **25**

Scheme 2. HCl 12 N/H_2O/$NaNO_2$ at 100 °C; (b) CH_3I/DMSO/KOH.

Table 1. Chemical and physical characteristics and MDR modulating activity of compounds **1**–**11**



N	R	Yield (%)[a]	Eluent[b]	Mp (°C)[c]	MDR activity			Analysis[d]
					$[I]_{0.5}$ (μM) (SEM)	α_{max}	K_I (μM) (SEM)	
1	2-F	48.2	A	203–207	0.30 (0.06)	0.75	0.1 (0.02)	$C_{29}H_{32}ClFN_2O_2$
2	2-Br	63.8	B	179–181	0.25 (0.05)	0.65	0.08 (0.01)	$C_{29}H_{32}BrClN_2O_2$
3	2,7-Br	36.9	A	51–53[a]	0.50 (0.1)	0.53	0.17 (0.4)	$C_{29}H_{30}Br_2N_2O_2$
4	2-NO_2	37.0	B	208–211	0.30 (0.06)	0.80	0.10 (0.01)	$C_{29}H_{32}ClN_3O_4$
5	2-OH	60.5	C	95–100	0.40 (0.08)	0.80	0.1 (0.02)	$C_{29}H_{33}ClN_3O_4$
6	2-$N(CH_3)_2$	43.8	A	90–93[e]	0.40 (0.08)	0.66	0.15 (0.02)	$C_{33}H_{39}N_3O_6$
7	2-OCH_3	54.8	A	78–80[e]	0.44 (0.09)	0.75	0.26 (0.05)	$C_{32}H_{36}N_2O_7$
8	4-OCH_3	50.9	C	110–112[e]	0.20 (0.04)	0.87	0.12 (0.03)	$C_{32}H_{36}N_2O_7$
9	3-OCH_3	51.1	C	121–123	1.10 (0.2)	0.90	0.36 (0.05)	$C_{30}H_{35}ClN_2O_3$
10	3,4-OCH_3	66.2	C	86–90[e]	0.38 (0.07)	1	0.15 (0.04)	$C_{33}H_{35}N_2O_8$
11	2-NH_2	54.8	—	229–230	0.50 (0.1)	0.90	0.15 (0.02)	$C_{29}H_{34}ClN_3O_2$
SC11	H		—	—	0.25 (0.05)	0.80	0.15 (0.04)	—
VRP	—		—	—	1.6 (0.3)	0.80	1.0 (0.1)	—

[a]As free base.
[b]A = $CHCl_3$ 99/MeOH 01; B = CH_2Cl_2 99/MeOH 01; C = CH_2Cl_2 98/MeOH 02.
[c]As hydrochloride, unless otherwise stated. Recrystallization solvent: absolute ethanol + petroleum ether.
[d]All compounds have been analyzed for C, H, N after vacuum drying at a temperature below the melting point; the results obtained range within ±0.4% of the theoretical values. IR and ^1H NMR spectra are consistent with the proposed structures.
[e]As oxalate.

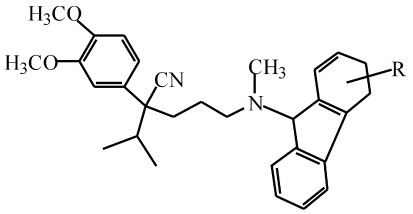
fluorenone, the corresponding 4-hydroxy derivative was obtained[20] that was then methylated with CH_3I. In the same way the 3-methoxy intermediate was also synthesised (Scheme 2).

The secondary amines **1**–**10** were obtained, according to the Mattson procedure,[21] by reductive alkylation of the 5-amino-2-(3,4-dimethoxyphenyl)-2-(methylethyl)pentanenitrile,[14] with the substituted 9-fluorenone, using titanium (IV) isopropoxide as Lewis acid catalist and $NaBH_3CN$ as reducing agent (Scheme 1; yields are reported in Table 1). This reaction always gave acceptable to good yields. Other procedures such as the one-pot formation and reduction with $NaBH_3CN$ of the Schiff bases in presence of molecular sieves, or the traditional synthesis of the Schiff bases with a Dean–Stark trap in presence of *p*-toluenesulfonic acid and subsequent reduction with $NaBH_4$, gave the worst results.

N-Methyl derivatives **12**–**21** were obtained from the corresponding secondary amines **1**–**10** by Eschweiler–Clarke methylation (Scheme 1; yields are reported in Table 2).

In the case of 2-NH_2 fluorenyl derivatives **11** and **22**, it was necessary to protect the 2-NH_2 function of the commercially available fluorenone in advance. We performed the protection both with di-*t*-butyldicarbonate (BOC) and with dibenzyldicarbonate (CBZ) (Scheme 3). The protected derivatives **26** and **27** were then reacted with 5-amino-2-(3,4-dimethoxyphenyl)-2-(methylethyl)pentanenitrile[14] as previously described; from the resulting BOC-protected amine **28** the secondary amine **11** was easily obtained. Methylation of **28**, on the contrary, gave poor results: in this case reductive methylation by HCOOH/HCHO could not be used because of the acidic conditions that deprotect and methylate the 2-NH_2 fluorenyl function. On the other hand, use of HCHO and sodium cyanoborohydride gave **30**, that after deprotection by acidic hydrolysis gave **22**, only in a very small yield (19%). Then for the synthesis of the *N*-methyl derivative **22** we performed the same reaction pathway on the CBZ-protected derivatives. Compound **29** was easily methylated with HCOOH/HCHO, and the resulting product was deprotected by acidic hydrolysis to give **22** in good yields.

Table 2. Chemical and physical characteristics and MDR activity of compounds **12–22**



N	R	Yield (%)[a]	Eluent[b]	Mp (°C)[c]	MDR activity			Analysis[d]
					$[I]_{0.5}$ (μM) (SEM)	α_{max}	K_I (μM) (SEM)	
12	2-F	67.3	A	243–246	1.2 (0.2)	0.80	0.20 (0.03)	$C_{30}H_{34}ClFN_2O_2$
13	2-Br	82.4	B	199–200	2.0 (0.4)	0.70	0.60 (0.1)	$C_{30}H_{34}BrClN_2O_2$
14	2,7-Br	65.2	C	212–215	0.5 (0.1)	0.57	0.46 (0.11)	$C_{30}H_{33}Br_2ClN_2O_2$
15	2-NO_2	79.0	A	149–153	—	0.40	0.20 (0.05)	$C_{30}H_{34}ClN_3O_4$
16	2-OH	75.5	D	104–106	3.8 (0.8)	0.75	1.6 (0.2)	$C_{30}H_{35}ClN_2O_3$
17	2-$N(CH_3)_2$	61.4	E	118–120	0.60 (0.12)	0.81	0.50 (0.07)	$C_{32}H_{40}ClN_3O_2$
18	2-OCH_3	64.6	F	58–60[e]	—	0.41	0.10 (0.03)	$C_{33}H_{38}N_2O_7$
19	4-OCH_3	70.1	E	62–64	1.2 (0.3)	0.83	0.80 (0.07)	$C_{31}H_{37}ClN_2O_3$
20	3-OCH_3	86.3	G	57–60	0.7 (0.14)	0.97	0.28 (0.04)	$C_{31}H_{37}ClN_2O_3$
21	3,4-OCH_3	78.7	E	75–78	0.84 (0.17)	0.86	0.33 (0.07)	$C_{32}H_{39}ClN_2O_4$
22	2-NH_2	80.7	—	100–103	1.0 (0.2)	0.70	0.40 (0.05)	$C_{30}H_{36}ClN_3O_2$
SC17	—		—	—	1.0 (0.2)	0.80	0.60 (0.2)	—
VRP	—		—	—	1.6 (0.3)	0.80	1.0 (0.1)	—

[a]As free base.
[b]A = $CHCl_3$ 99/MeOH 01, B = CH_2Cl_2 100, C = hexane 50/Et_2O 50, D = CH_2Cl_2 97/MeOH 03, E = CH_2Cl_2 98/MeOH 02, F = CH_2Cl_2 99/MeOH 01, G = CH_2Cl_2 95/ethyl acetate 05.
[c]As hydrochloride, unless otherwise stated. Recrystallization solvent: absolute ethanol + petroleum ether.
[d]All compounds have been analyzed for C, H, N after vacuum drying at a temperature below the melting point; the results obtained range within ±0.4% of the theoretical values. IR and ^1H NMR spectra are consistent with the proposed structures.
[e]As oxalate.

The compounds of this series possess two chiral centers and therefore are mixtures of racemic diastereoisomers. However, at this stage of the research, considering also the modest impact of stereochemistry in the MDR activity of verapamil derivatives,[22–24] we decided to neglect this problem, postponing any decision to the biological evaluation of the compounds.

Pharmacology

MDR-reverting activity

The ability of the examined compounds to revert MDR was evaluated on anthracycline-resistant erythroleukemia K562 cells measuring the uptake of THP-adriamycin (pirarubicin) by continuous spectrofluorometric monitoring of the decrease of the anthracycline fluorescence at 590 nm (λ_{ex} = 480 nm) after incubation with cells, following the protocols reported in previous papers.[14,25] More details are reported in the Experimental.

MDR-modulatory activity is described by (i) α, which represents the ratio of intracellular concentrations in MDR cells versus drug-sensitive cells of pirarubicin in the presence of the MDR-reverting agent and varies between 0 (in the absence of the inhibitor) and 1 (when the amount of pirarubicin in resistant cells is the same as in sensitive cells); (ii) α_{max}, which expresses the efficacy of MDR-modulator and is the maximum increase that can be obtained in the nuclear concentration of pirarubicin in resistant cells with a given inhibitor; (iii) $[I]_{0.5}$ which measures the potency of MDR-reverting agent and represents the concentration of the inhibitor that causes a half-maximal increase in nuclear concentration of pirarubicin at $\alpha = 0.5$; (iv) K_I which is the dissociation constant for the complex formed between P-gp and the inhibitor.

In more detail, the inhibition produced may be expressed by calculating the fractional activity:

$$r = V_a^i / V_a$$

where V_a and V_a^i stand for the rate of active efflux of the chemotherapic drug in the absence and presence of inhibitor, respectively. At low substrate concentrations, V_a can be written as $k_a.C_i$ and V_a^i as $k_a^i\ C_i$, therefore fractional activity as

$$r = k_a^i / k_a$$

C_i is the intracellular free drug concentration and k_a and k_a^i the active efflux coefficient in the absence and presence of inhibitor, respectively.[26]The P-gp-mediated efflux of anthracyclines by verapamil or by its derivatives is non-competitive.[25,27] For non-competitive inhibition r can be simply written as a function of the inhibitory constant K_I and the inhibitor concentration [I].[28]

$$r = K_I / ([I] + K_I)$$

Cardiovascular activity

Inotropic and chronotropic activities were tested on guinea pig isolated atria preparations, and vasodilator activity was tested on guinea pig aortic strip preparations following standard procedures, details of which have previously been reported.[29]

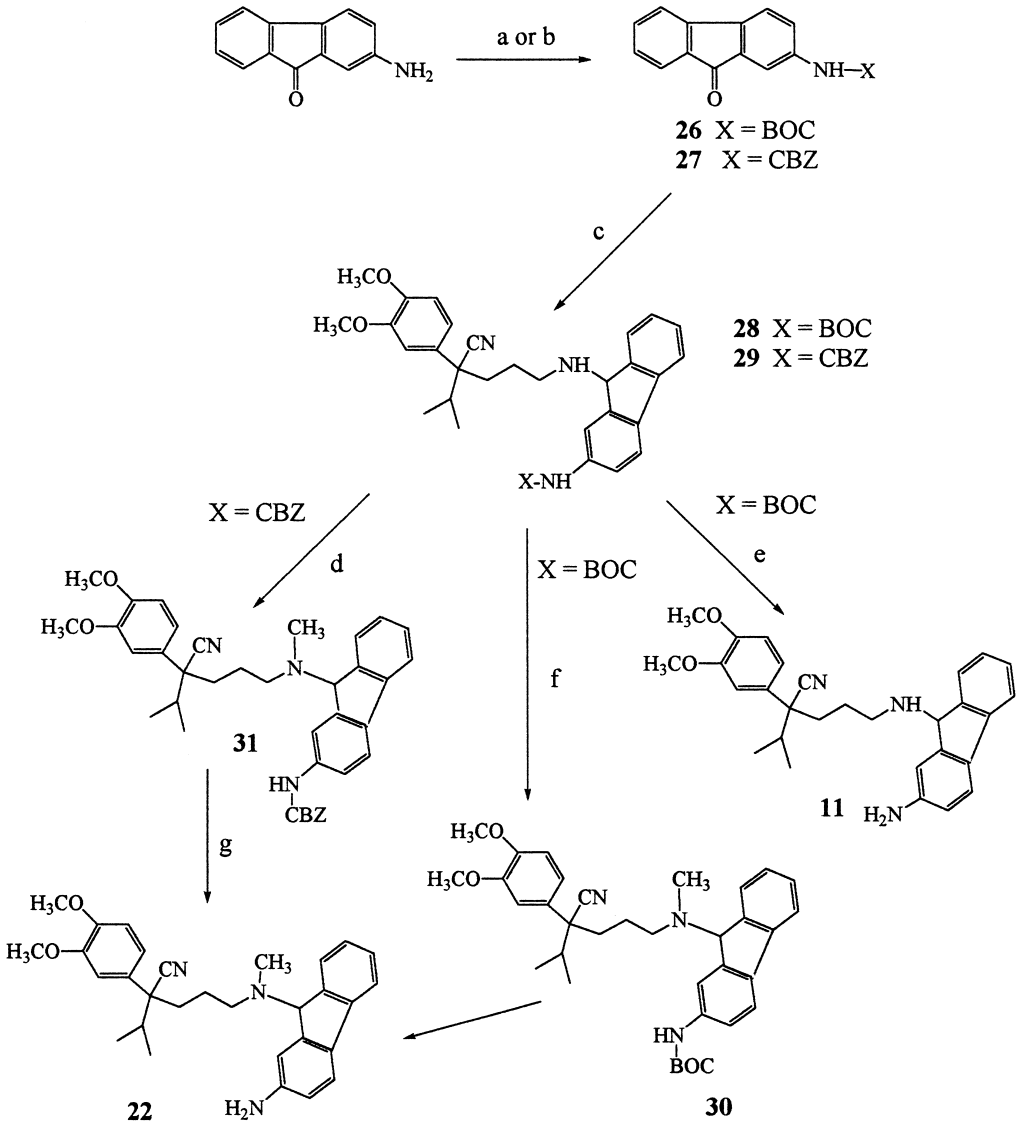
Potency of the drugs is defined as EC_{50} (concentration that gives 50% of the maximum negative inotropic effect), IC_{50} (concentration that gives 50% of maximum vasodilator effect), and EC_{30} (concentration that gives 30% of the maximum negative chronotropic effect). Activity is defined as the percent decrease in developed tension on isolated left atrium (negative inotropic activity), percent decrease in atrial rate of spontaneously beating isolated right atrium (negative chronotropic activity), and percent inhibition of calcium-induced contraction on K^+-depolarized aortic strips (vasodilator activity) at the concentrations indicated in the footnotes of Table 3.

Results and Discussion

The MDR-reverting activity of the compounds synthesised is reported in Tables 1 and 2. In Table 3 is reported the cardiovascular action of the compounds that have shown the most promising MDR-reverting activity. In all cases the corresponding values for verapamil (VRP), SC11 and SC17 were reported as reference.

As discussed in the Introduction, the preceding research evidenced two leads for further development.

The desmethyl derivative SC11 had a good potency but also possessed some negative chronotropic activity that was only 10 times lower than that of verapamil.



Scheme 3. (a) Di-*tert*-butyl dicarbonate, Et_3N, dioxane, 100 °C; (b) dibenzyl dicarbonate, NaOH 1 N, dioxane, 65 °C; (c) titanium isopropoxide, $NaBH_3CN$, 5-amino-2-(3,4-dimethoxyphenyl)-2-(methylethyl)pentanenitrile; (d) CH_2O, HCOOH; (e) ethyl acetate, HCl 3 N; (f) CH_2O, $NaBH_3CN$; (g) HBr/AcOH 33%.

Table 3. Cardiovascular activity of selected compounds

N	Cardiovascular activity					
	Negative inotropy		Negative chronotropy		Vasorelaxant activity	
	Ia%[a] (±SEM)	EC_{50} (μM)[b] (95% cl)	Ia%[c] (±SEM)	EC_{30} (μM)[b] (95% cl)	Ia%[d] (±SEM)	IC_{50} (μM)[b] (95% cl)
2	41 (2.3)	—	43 (1.8)	—	18 (0.7)	—
6	68 (0.6)	4.48 (3.65–5.38)	85 (2.5)	0.97 (0.75–1.26)	30 (1.5)	—
8	38 (2.2)	—	61 (4.1)	1.56 (1.20–2.03)	15 (0.4)	—
10	80 (3.5)	1.75 (1.35–2.28)	78[e] (3.2)	0.63 (0.48–0.82)	43 (3.8)	—
17	55 (2.1)	2.40 (1.95–2.90)	38 (2.1)	0.65	5 (0.2)	—
SC11	33 (1.2)	—	74 (4.5)	0.65 (0.57–0.72)	46 (2.1)	—
SC17	36 (2.6)	—	26 (1.4)	—	15 (0.9)	—
VRP	84[f] (2.1)	0.61 (0.40–0.80)	94[g] (3.4)	0.07 (0.05–0.10)	95[f] (1.7)	0.38 (0.20–0.70)

[a]Decrease in developed tension in isolated guinea pig left atrium at 5×10^{-5} M, expressed as percent changes from the control ($n=5$–6). The left atria were driven at 1 Hz. 5×10^{-5} M gave the maximum effect for most compounds.
[b]Calculated from log concentration–response curves (Probit analysis by Litchfield and Wilcoxon with $n=5$–7). When the maximum effect was $<50\%$, the EC_{50} ino., EC_{30} chrono., IC_{50} values were not calculated.
[c]Decrease in atrial rate on guinea pig spontaneously beating isolated right atrium at 5×10^{-5} M, expressed as percent changes from the control ($n=7$–8). Pretreatement heart rate ranged from 165 to 190 beats/min. 5×10^{-5} M gave the maximum effect for most compounds.
[d]Percent inhibition of calcium-induced contraction on K^+-depolarized guinea pig aortic strip at 5×10^{-5} M ($n=5$–6). 5×10^{-5} M gave the maximum effect for most compounds.
[e]At 5×10^{-6} M.
[f]At 10^{-5} M.
[g]At 10^{-6} M.

Modulation of the electronic properties of the fluorene moiety through ring substitution afforded compounds that, in general, show a potency in the same range of that of the lead without impressive improvement (Table 1). The most interesting among them are **2**, **6** and **8**, which are almost equivalent to the lead in terms of potency, efficacy and affinity. On the other hand, as shown in Table 3, while **6** and **8** maintain the undesired cardiovascular action of SC11, compound **2** is apparently devoid of negative chronotropic and inotropic action and of vasorelaxant activity as well. Therefore, **2** may represent a new lead toward the discovery of potent and selective MDR inhibitors. Finally, compound **10**, which is slightly less potent but is able to completely restore the original sensitivity of the cell line to the chemotherapic ($\alpha_{max}=1$) is still endowed with definite cardiovascular action.

Compound SC17 was less potent than SC11, but presented a fairly good selectivity as it was devoid of cardiovascular activity. Therefore, in this case the issue was that of improving potency while maintaining selectivity. Unfortunately, this goal was not reached, as ring substitution produced only a slight improvement in potency in the series (Table 2). This confirms our previous finding that *N*-methyl derivatives have reduced MDR-inhibitory activity.[14,30] Moreover, the results concerning cardiovascular activity were also disappointing in this series, as one of the most potent compounds, **17**, was endowed with a slight negative inotropic activity (Table 3) thus less selective than the lead.

In general, it is apparent that modulation of the electronic properties of the aromatic moiety through ring substitution does not produce any substantial variation in both MDR and cardiovascular activities. Accordingly, we could not evidence any significant correlation between the nature of the substituent and MDR inhibition. Nevertheless, the discovery of compound **2** is a partial success of this research. In fact, even if this compound is less potent than the most interesting product (MM36) described by us previously,[14] it shows good potency ($[I]_{0.5}=0.25$ μM) and affinity ($K_I=0.08$ μM) for the Pgp and is completely devoid of cardiovascular action, whereas MM36 maintains some cardiovascular activity.

Experimental

Chemistry

All melting points were taken on a Büchi apparatus and are uncorrected. Infrared spectra were recorded with a Perkin–Elmer 681 spectrophotometer in Nujol mull for solids and neat for liquids. NMR spectra were recorded on a Gemini 200 spectrometer. Chromatographic separations were performed on a silica gel column by gravity chromatography (Kieselgel 40, 0.063–0.200 mm; Merck) or flash chromatography (Kieselgel 40, 0.040–0.063 mm; Merck). Yields are given after purification, unless otherwise stated. Where analyses are indicated by symbols, the analytical results are within ±0.4% of the theoretical values.

General procedure for the synthesis of fluorene derivatives (1–10)

A mixture of 5-amino-2-(3,4-dimethoxyphenyl)-2-(methylethyl)pentanenitrile[14] (300 mg, 1.09 mmol), the suitable fluorenone (1.09 mmol) and titanium (IV) isopropoxide (0.45 mL, 1.36 mmol) was stirred with a drying tube at room temperature for 1–4 h, depending on the starting compound. After the suitable time, the IR spectrum of the mixture showed no ketone band, and the viscous solution was diluted with abs ethanol

(2 mL). Sodium cyanoborohydride (60 mg, 0.74 mmol) was added, and the solution was stirred for 20 h. Water (0.5 mL) was then added, the resulting inorganic precipitate was filtered and washed with ethanol, and the filtrate was concentrated in vacuo. The crude product was dissolved in chloroform, filtered to remove the solids, washed with a solution of $NaHCO_3$ and water, dried over Na_2SO_4 and concentrated in vacuo. The crude substance was then purified by column chromatography and transformed into the corresponding hydrochloride or oxalate. The chemical and physical characteristics of the compounds **1**–**10** are reported in Table 1. Their IR and ^1H NMR spectra are consistent with the proposed structures.

The spectra of 2-(3,4-dimethoxyphenyl)-5-[9-(2-fluoro)-fluorenylamino]-2-(methylethyl)pentanenitrile (**1**) are reported as an example. IR (neat) ν 3370 cm^{-1} (NH), 2240 cm^{-1} (CN). ^1H NMR ($CDCl_3$) δ 7.64–7.50 (m, 3H, fluorene); 7.40–7.22 (m, 3H, fluorene); 7.10–7.06 (m, 1H, fluorene); 6.87–6.81 (m, 3H, phenyl); 4.83 (s, 1H, 9-H fluorene); 3.89 (s) and 3.88 (s) (3H, OCH_3); 3.87 (s) and 3.86 (s) (3H, OCH_3); 2.34–2.26 (m, 2H, CH_2–N); 2.18–2.01 (m, 2H, CH_2–C–Ph); 1.79 (bs, 1H, NH); 1.82–1.72 (m, 1H, C–CH*H*–C); 1.58–1.38 (m, 1H, C–C*H*H–C); 1.18–1.01 (m, 1H, CH_3–*CH*); 1.17 (d) and 1.16 (d) ($J = 6.60$ Hz, 3H, *CH_3*–CH); 0.78 (d, $J = 6.60$ Hz, 3H, *CH_3*–CH).

2-(3,4-Dimethoxyphenyl)-5-[[9-(2,7-dibromo)fluorenyl]-*N*-methylamino]-2-(methylethyl)pentanenitrile (14). 90 mg (0.15 mmol) of **3** were dissolved in 3 mL of abs ethanol, and to this solution 3 mL of HCOOH and 3 mL of HCHO were added. The mixture was refluxed at 80 °C for 2 h and concentrated in vacuo. The residue was then dissolved in CH_2Cl_2 and the organic layer was washed with a saturated solution of Na_2CO_3 and with water, dried over Na_2SO_4 and concentrated in vacuo. The crude product was then purified by column chromatography using hexane/ether (50:50) as eluting system. Compound **14** was obtained as an oil (60 mg, 65.2% yield). IR (neat) ν 2240 cm^{-1} (CN). ^1H NMR ($CDCl_3$) δ 7.68–7.66 (m, 2H, fluorene); 7.50–7.47 (m, 4H, fluorene); 6.96–6.82 (m, 3H, phenyl); 4.74 (s, 1H, 9-H fluorene); 3.88 (s, 3H, OCH_3); 3.87 (s, 3H, OCH_3); 2.46–2.40 (m, 2H, CH_2–N); 2.16 (s, 3H, CH_3–N); 2.15–2.02 (m, 2H, CH_2–C–Ph); 1.94–1.82 (m, 1H, C—CH*H*–C); 1.61–1.42 (m, 1H, C–C*H*H–C); 1.23 (d, $J = 6.60$ Hz, 3H, *CH_3*–CH); 1.21–1.11 (m, 1H, CH_3–*CH*); 0.81 (d, $J = 6.60$ Hz, 3H, *CH_3*–CH).

The oily product was transformed into the hydrochloride that recrystallized from abs ethanol/anhydrous ether. Mp 212–215 °C. Anal. ($C_{30}H_{33}Br_2ClN_2O_2$) C, H, N.

Compounds **12**, **13** and **15**–**21** were obtained in the same way; their chemical and physical characteristics are reported in Table 2 and their IR and ^1H NMR spectra are consistent with the proposed structures.

2-Methoxy-9-fluorenone (23). 230 mg (4.08 mmol) of grounded KOH were added to 2 mL of DMSO. The resulting suspension was stirred for 5 min, then 200 mg (1.02 mmol) of 2-hydroxy-9-fluorenone and 0.13 mL (2.04 mmol) of iodomethane were added. The mixture was stirred at rt for 15 min, then was diluted with 20 mL of water and treated with CH_2Cl_2 in order to extract the title compound. The organic layer was washed with water, dried over Na_2SO_4 and concentrated in vacuo. Title compound was obtained in an almost quantitative yield (230 mg). Mp 77–78 °C (ethanol). IR (neat) ν 1720 cm^{-1} (C=O). ^1H NMR ($CDCl_3$) δ 7.55 (d, $J = 7.33$ Hz, 1H, aromatics); 7.39–7.30 (m, 3H, aromatics); 7.19–7.12 (m, 2H, aromatics); 6.94–6.87 (dd, $J = 8.25$ Hz, $J = 2.38$ Hz, 1H, aromatics); 3.81 (s, 3H, OCH_3).

Following the same procedure, 3-methoxy-9-fluorenone (**24**) and 4-methoxy-9-fluorenone (**25**) were obtained, starting from the corresponding hydroxy derivatives.[20] Their IR and ^1H NMR spectra are consistent with the proposed structure.

2-(*N*-*ter*-Butoxycarbonyl)-amino-9-fluorenone (26). 0.54 mL (3.84 mmol) of triethylamine and 570 mg (2.61 mmol) of di-*ter*-butyldicarbonate (BOC) were added to a solution of 500 mg (2.56 mmol) of 2-amino-9-fluorenone in 8 mL of 1,4-dioxane. Reaction was maintained for 48 h at 100 °C. In any case (TLC) the starting fluorenone did not disappear. The solvent was then removed under reduced pressure and the residue dissolved in ethyl acetate, and washed with water. After drying with Na_2SO_4 the organic layer was concentrated in vacuo and the residue purified by column chromatography using $CHCl_3$ 100 as the eluent. Title compound was obtained with 41% yield (310 mg). IR (nujol) ν 3300 cm^{-1} (NH); 1735 cm^{-1} (C=O); 1700 cm^{-1} (C=O). ^1H NMR ($CDCl_3$) δ 7.64–7.57 (m, 3H, aromatics); 7.47–7.41 (m, 3H, aromatics); 7.26–7.21 (m, 1H, aromatics); 1.54 (s, 9H, CH_3). Anal. ($C_{18}H_{17}NO_3$) C, H, N.

2-(*N*-Benzyloxycarbonyl)-amino-9-fluorenone (27). A solution of 740 mg (2.58 mmol) of dibenzyldicarbonate (CBZ) in 2.6 mL of 1,4-dioxane was dropwise added to a solution of 500 mg (2.56 mmol) of 2-amino-9-fluorenone in 5.2 mL of 1,4-dioxane/NaOH 1 N (1:1). The mixture was kept 36 h at 65 °C. Check of the reaction (TLC) showed that the starting fluorenone did not disappear. The solvent was then removed by reduced pressure, and the resultant residue was dissolved in H_2SO_4 1 N at pH 2 and extracted with ethyl acetate. The organic layer, after washing with water and drying with Na_2SO_4, was concentrated in vacuo giving a residue that was purified by flash chromatography (cyclohexane/ethyl acetate 9:1). 510 mg of the title compound were obtained (62.8% yield). IR (nujol) ν 3360 cm^{-1} (NH); 1710 cm^{-1} (C=O); 1610 cm^{-1} (C=O). ^1H NMR ($CDCl_3$) δ 7.69–7.27 (m, 11H, aromatics); 7.81–7.66 (m, 1H, aromatic); 2.19 (s, 2H, CH_2); 1.58 (bs, 1H, NH). Anal. ($C_{21}H_{15}NO_3$) C, H, N.

2-(3,4-Dimethoxyphenyl)-5-[[9-[2-(*N*-*ter*-butoxycarbonyl)-amino]-fluorenyl]-amino]-2-(methylethyl)pentanenitrile (28). Following the general procedure reported for

compounds **1**–**10**, the title compound was synthesized, using 270 mg (0.98 mmol) of 5-amino-2-(3,4-dimethoxyphenyl)-2-(methylethyl)pentanenitrile,[14] 290 mg (0.98 mmol) of **26**, 0.405 mL (1.23 mmol) of titanium (IV) isopropoxide and 50 mg (0.79 mmol) of sodium cyanoborohydride. The crude product was purified by column chromatography using CH_2Cl_2/MeOH 98:2 as the eluent. Title compound was obtained as an oil (360 mg, 66.4% yield). ^{1}H NMR ($CDCl_3$) δ 7.67–7.21 (m, 7H, aromatics, fluorene); 6.90–6.72 (m, 3H, aromatics, phenyl); 4.82 (s, 1H, 9-H fluorene); 3.87 (s, 3H, OCH_3); 3.86 (s, 3H, OCH_3); 2.31–1.67 (m, 6H, Ph–C–CH_2–CH_2–CH_2–N); 1.58 (bs, 1H, NH); 1.54 (s, 9H, CH_3); 1.15 (d, J = 6.60 Hz, 3H, CH–CH_3); 1.16–1.03 (m, 1H, CH–CH_3); 0.77 (d, J = 6.60 Hz, 3H, CH–CH_3). Anal. ($C_{34}H_{41}N_3O_4$) C, H, N.

2-(3,4-Dimethoxyphenyl)-5-[[9-(2-amino)-fluorenyl]-amino]-2-(methylethyl)pentanenitrile (11). To a solution of 200 mg (0.361 mmol) of **28** in 2 mL of ethyl acetate, 1.55 mL of HCl 3N were added under vigorous stirring. After 4 days at rt the solution was basified with NaOH 33% and extracted with $CHCl_3$. The organic layer was dried over Na_2SO_4 and concentrated in vacuo. The resulting crude product was purified by column chromatography using CH_2Cl_2/MeOH 98:2 as eluting system. 90 mg of the title compound were obtained (oil, 54.8% yield). IR (neat) ν 3470 cm^{-1}, 3395 cm^{-1}, 3230 cm^{-1} (NH); 2260 cm^{-1} (CN). ^{1}H NMR ($CDCl_3$) δ 7.55–7.14 (m, 7H, aromatics, fluorene); 6.90–6.65 (m, 3H, aromatics, phenyl); 4.77 (s, 1H, 9-H fluorene); 3.88 (s) and 3.87 (s) (3H, OCH_3); 3.86 (s), 3.85 (s) (3H, OCH_3); 2.38–1.86 (m, 6H, C–CH_2–CH_2–CH_2–N and NH_2); 1.81–1.66 (m, 1H, C–C*H*H–C), 1.58–1.40 (m, 1H, C–CH*H*–C); 1.15 (d, J = 6.60 Hz, 3H, CH–CH_3); 1.16–1.01 (m, 2H, CH–CH_3 and NH); 0.78 (d, J = 6.60 Hz, 3H, CH–CH_3).

The oily compound was transformed into the corresponding hydrochloride. Mp 229–230 °C. Anal. ($C_{29}H_{34}ClN_3O_2$) C, H, N.

2-(3,4-Dimethoxyphenyl)-5-[[9-[2-(*N*-benzyloxycarbonyl)-amino]-fluorenyl]-amino]-2-(methylethyl)pentanenitrile (29). Following the general procedure reported for compounds **1**–**10**, and using 300 mg (1.09 mmol) of 5-amino-2-(3,4-dimethoxyphenyl)-2-(methylethyl)pentanenitrile,[14] 350 mg (1.10 mmol) of **27**, 0.45 mL (1.36 mmol) of titanium (IV) isopropoxide, and 50 mg (0.79 mmol) of sodium cyanoborohydride, 450 mg of title compound were obtained, without further purification. ^{1}H NMR ($CDCl_3$) δ 7.62–7.18 (m, 12H, aromatics, fluorene and CBZ); 6.94–6.78 (m, 3H, aromatics, phenyl); 5.22 (s, 2H, CH_2 CBZ); 4.80 (s, 1H, 9-H fluorene); 3.87 (s, 3H, OCH_3); 3.86 (s, 3H, OCH_3); 2.40–1.98 (m, 6H, Ph–C–CH_2–CH_2–CH_2–*NH* and NH); 1.83–1.68 (m, 1H, C–C*H*H–C), 1.56–1.32 (m, 1H, C–CH*H*–C); 1.18 (d, J = 6.60 Hz, 3H, CH–CH_3); 1.16–1.01 (m, 1H, CH–CH_3); 0.78 (d, J = 6.60 Hz, 3H, CH–CH_3). Anal. ($C_{37}H_{39}N_3O_4$) C, H, N.

2-(3,4-Dimethoxyphenyl)-5-[[9-[2-(*N*-*ter*-butoxycarbonyl)-amino]-fluorenyl]-*N*-methylamino]-2-(methylethyl)pentanenitrile (30). 150 mg of **28** (0.27 mmol) in a mixture of H_2O (1 mL) and AcOH (0.1 mL) were treated with 37% HCHO (0.2 mL) and $NaBH_3CN$ (60 mg, 0.74 mmol). The stirred solution was treated with additional AcOH as necessary to maintain the acidity at approximately pH 5. The mixture was kept at 80 °C for 2 h, then HCl 2N (0.13 mL) was added, and the mixture was extracted with EtOAc. The aqueous phase was made basic with K_2CO_3 and extracted with chloroform. The organic layer was dried over Na_2SO_4 and concentrated in vacuo, and the residue was chromatographed ($CHCl_3$/hexane/MeOH 59:40:1) to afford 30 mg of an oil (yield 19.5%). ^{1}H NMR ($CDCl_3$) δ 7.63–7.21 (m, 7H, aromatics, fluorene); 6.97–6.80 (m, 3H, aromatics, phenyl); 4.74 (s, 1H, 9-H fluorene); 3.87 (s, 6H, OCH_3); 2.46–1.61 (m, 9H, Ph–C–CH_2–CH_2–CH_2–N and N–CH_3); 1.54 (s, 9H, CH_3); 1.27–1.16 (m, 1H, CH–CH_3); 1.23 (d) and 1.20 (d) (3H, J = 6.60 Hz, CH–CH_3); 0.80 (d, J = 6.60 Hz, 3H, CH–CH_3). Anal. ($C_{35}H_{43}N_3O_4$) C, H, N.

2-(3,4-Dimethoxyphenyl)-5-[[9-[2-(*N*-benzyloxycarbonyl)-*N*-methylamino]-fluorenyl]-amino]-2-(methylethyl)pentanenitrile (31). Following the same procedure as described for **14**, and using 450 mg (0.78 mmol) of **29**, 12 mL of abs EtOH, 12 mL of HCOOH and 12 mL of HCHO, 680 mg of crude product were obtained, and purified by column chromatography using CH_2Cl_2/MeOH 99:1 as the eluent. Title compound was obtained as a yellow oil (270 mg). ^{1}H NMR ($CDCl_3$) δ 7.77–7.20 (m, 12H, aromatics, fluorene and CBZ); 7.00–6.80 (m, 3H, aromatics, phenyl); 5.22 (s, 2H, CH_2 CBZ); 4.80 (s, 1H, 9-H fluorene); 3.86 (s, 3H, OCH_3); 3.84 (s, 3H, OCH_3); 2.49–2.40 (m, 2H, CH_2–N); 2.19 (s) and 2.15 (s) (3H, N–CH_3); 2.13–1.88 (m, 4H), 1.63–1.42 (m, 1H) (Ph–C–CH_2–CH_2 and NH); 1.24–1.12 (m, 1H, CH–CH_3); 1.22 (d) and 1.19 (d) (3H, J = 6.60 Hz, CH–CH_3); 0.82 (d, J = 6.60 Hz, 3H, CH-CH_3). Anal. ($C_{38}H_{41}N_3O_4$) C, H, N.

2-(3,4-Dimethoxyphenyl)-5-[[9-(2-amino)-fluorenyl]-*N*-methylamino]-2-(methylethyl) pentanenitrile (22).

Procedure A. Following the same procedure as described for **11** and using 30 mg (0.053 mmol) of **30** dissolved in 1 mL of ethyl acetate and 0.46 mL of HCl 3 N, 20 mg of a pure product were obtained (oil, 80.7% yield).

Procedure B. To 250 mg (0.085 mmol) of **31** dissolved in 0.5 mL of anhyd CH_2Cl_2, 0.24 mL of HBr in 33% acetic acid were added, maintaining the mixture in ice-bath and with a drying tube. After 3 h, the solvent was removed by reduced pressure and the residue dissolved in CH_2Cl_2. The organic layer was washed with 10% NaOH and with water, dried over Na_2SO_4 and concentrated in vacuo. 150 mg of a pure product were obtained (oil, yield 68%). ^{1}H NMR ($CDCl_3$) δ 7.55–7.16 (m, 7H, aromatics, fluorene); 6.95–6.67 (m, 3H, aromatics, phenyl); 4.79 (s) and 4.71 (s) (1H, 9-H fluorene); 3.88 (s, 3H, OCH_3); 3.87 (s, 3H, OCH_3); 2.51–2.45 (m, 2H, CH_2–N); 2.18 (s), 2.17 (s) (3H, N–CH_3); 2.18–

1.82 (m, 3H), 1.61–1.48 (m, 1H) (Ph–C–CH_2–CH_2); 1.30–1.19 (m, 1H, *CH*–CH_3); 1.27 (bs, 2H, NH_2); 1.22 (d, $J = 6.60$ Hz, 3H, CH–*CH_3*); 0.81 (d, $J = 6.60$ Hz, 3H, CH–*CH_3*).

The oily product was transformed into the corresponding hydrochloride (abs EtOH/anhyd ether) mp 100–103 °C. Anal. ($C_{30}H_{36}ClN_3O_2$) C, H, N.

Pharmacology

Drugs and chemicals

Purified pirarubicin was provided by Laboratoire Roger Bellon (France). Concentrations were determined by diluting the stock solutions to approximately 10^{-5} M and using $\varepsilon_{480} = 11\,500\ M^{-1}\,cm^{-1}$. Stock solutions were prepared just before use. Buffer solutions were HEPES buffer containing 5 mM HEPES, 132 mM NaCl, 3.5 mM $CaCl_2$, 5 mM glucose, at pH 7.25.

Cell lines and cultures

K 652 is a human leukemia cell line.[31] Cells resistant to doxorubicin were obtained by continuous exposure to increasing doxorubicin concentrations and were maintained in medium containing doxorubicin (400 nM) until 1–4 weeks before experiments. This subline expresses a unique membrane glycoprotein with a molecular weight of 180 000 Da.[32] Doxorubicin-sensitive and-resistant erythroleukemia K 562 cells were grown in suspension in RPMI 1640 (Sigma) medium supplemented with L-glutamine and 10% FCS at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Cultures, initiated at a density of 10^5 cells/mL, grew exponentially to $8–10\times10^5$ cells/mL in 3 days. For the spectrofluorometric assays, in order to have cells in the exponential growth phase, culture was initiated at 5×10^5 cells/mL, and cells were used 24 h later, when the culture had grown to about $8–10\times10^5$ cells/mL. Cell viability was assessed by trypan blue exclusion. The cell number was determined by Coulter counter analysis.

Cellular drug accumulation

The uptake of pirarubicin in cells was followed by monitoring the decrease in the fluorescence signal at 590 nm ($\lambda_{ex} = 480$ nm) following the method previously described.[33] Using this method it is possible to accurately quantify the kinetics of the drug uptake by the cells and the amount of anthracycline intercalated between the base pairs in the nucleus at the steady state, as incubation of the cells with the drug proceeds without compromising cell viability. All experiments were conducted in 1-cm quartz cuvettes containing 2 mL of buffer at 37 °C using a circulating thermostated water bath. Cells, 2×10^6, were suspended in 2 mL of glucose containing HEPES buffer at pH 7.3, under vigorous stirring; 20 μL of the stock anthracycline solution was quickly added to this suspension yielding an anthracycline concentration C_T equal to 1 μM. The decrease of the fluorescence intensity F at 590 nm was followed as a function of time. After about 20 min, the curve $F = f(t)$ reached a plateau and the fluorescence intensity was equal to F_n. The drug-cells system was thus in a steady state and the overall concentration C_n of drug intercalated between the base pairs in the nucleus was $C_n = C_T.\ (F_0 - F_n)/F_0$. Once the steady state was reached, the inhibitor at concentration [i] ([i] was varied from 0.05 to 10 μM), was added and a new steady state was reached, the fluorescence intensity being F^i_n. The overall concentration C^i_n of drug intercalated between the base pairs in the nucleus was then $C^i_n = C_T.\ (F_0 - F^i_n)/F_0$. An aliquot of the solution was then taken away and cell viability was assessed by trypan blue exclusion. Cell membranes were then permeabilized by the addition of 0.05% Triton X-100 yielding the equilibrium state which was characterised by a new value F_N of the fluorescence intensity. The overall concentration C_N of drug intercalated between the base pairs in the nucleus was then $C_N = C_T.\ (F_0 - F_N)/F_0$.We checked that tested compounds did not affect the fluorescence of THP-adriamycin. α was measured with the following expression: $\alpha = (C^i_n - C_n)/(C_N - C_n)$.

References and Notes

1. Kane, S. E. In *Advances in Drug Research*; Academic: 1996, Vol. 24, pp 181–252.
2. Volm, M. *Anticancer Res.* **1998**, *18*, 2905.
3. Bosch, I.; Croop, J. *Biochim. Biophys. Acta* **1996**, *1288*, F37.
4. Ueda, K.; Yoshida, A.; Amachi, T. *Anti-Cancer Drug Des.* **1999**, *14*, 115.
5. Borst, P.; Evers, R.; Kool, M.; Wijnholds, J. *Biochim. Biophys. Acta* **1999**, *1461*, 347.
6. Johnstone, R. W.; Ruefli, A. A.; Tainton, K. M.; Smyth, M. J. *Leuk. Lymphoma* **2000**, *38*, 1.
7. Johnstone, R. W.; Ruefli, A. A.; Smyth, M. J. *TIBS* **2000**, *25*, 1.
8. Hrycyna, C. A.; Gottesman, M. M. *Drug Resist. Update* **1998**, *1*, 81.
9. Zhu, B. T. *Mol. Carcinogenesis* **1999**, *25*, 1.
10. Roepe, P. D. *Curr. Pharmmaceut. Des.* **2000**, *6*, 241.
11. Tsuruo, T.; Iida, H.; Tsukagoshi, S.; Sakurai, Y. *Cancer Res.* **1981**, *41*, 1967.
12. Ecker, G.; Chiba, P. *Exp. Opin. Ther. Patents* **1997**, *7*, 589.
13. Norman, B. H. *Drug of the Future* **1998**, *23*, 1001.
14. Teodori, E.; Dei, S.; Quidu, P.; Budriesi, R.; Chiarini, A.; Garnier-Suillerot, A.; Gualtieri, F.; Manetti, D.; Romanelli, M. N.; Scapecchi, S. *J. Med. Chem.* **1999**, *42*, 1687.
15. Ladd, D. L.; Weinstock, J.; Wise, M.; Gessner, G. W.; Sawyer, J. L.; Flaim, K. E. *J. Med. Chem.* **1986**, *29*, 1904.
16. Kruber, O. *Chem. Ber.* **1936**, *69*, 107.
17. Johnstone, R. A. W.; Rose, M. E. *Tetrahedron* **1979**, *35*, 2169.
18. Horner, L.; Baston, D. W. *Liebigs Ann. Chem.* **1973**, 910.
19. House, H. O.; Gannon, W. F.; Ro, R. S.; Wluka, D. J. *J. Am. Chem. Soc.* **1960**, *82*, 1463.
20. Graebe, C.; Schestakow, P. *Liebigs Ann. Chem.* **1895**, *284*, 306.
21. Mattson, J.; Pham, K. M.; Leuck, D. J.; Cowen, K. A. *J. Org. Chem.* **1990**, *55*, 2552.
22. Haussermann, K.; Benz, B.; Gekeler, V.; Schumacher, K.; Eichelbaum, M. *Eur. J. Clin. Pharmacol.* **1991**, *40*, 53.

23. Raderer, M.; Scheithauer, W. *Cancer* **1993**, *72*, 3553.
24. Hollt, V.; Kouba, M.; Dietel, M.; Vogt, G. *Biochem. Pharmacol.* **1992**, *43*, 2601.
25. Pereira, E.; Teodori, E.; Dei, S.; Gualtieri, F.; Garnier-Suillerot, A. *Biochemical Pharmacology* **1995**, *50*, 451.
26. Marbeuf-Gueye, C.; Salerno, M.; Quidu, P.; Garnier-Suillerot, A. *Eur. J. Pharmacol.* **2000**, *391*, 207.
27. Pereira, E.; Borrel, M.-N.; Fiallo, M.; Garnier-Suillerot, A. *Biochem. Biophys. Acta* **1994**, *1225*, 209.
28. Wingler, P. W. *Biochem. Biophys. Res. Commun.* **1999**, *257*, 410.
29. Bellucci, C.; Gualtieri, F.; Scapecchi, S.; Teodori, E.; Budriesi, R.; Chiarini, A. *Il Farmaco* **1989**, *44*, 1167.
30. Teodori, E.; Dei, S.; Romanelli, M. N.; Scapecchi, S.; Gualtieri, F.; Budriesi, R.; Chiarini, A.; Lemoine, H.; Mannhold, R. *Eur. J. Med. Chem.* **1994**, *29*, 139.
31. Lozio, C. B.; Lozzio, B. B. *Blood* **1975**, *45*, 321.
32. Tsuruo, T.; Iida, H.; Kawataba, H.; Oh-hara, T.; Hamada, H.; Utakoji, T. *Jpn. J. Cancer Res.* **1986**, *77*, 682.
33. Mankhetkorn, S.; Garnier-Suillerot, A. *Eur. J. Pharmacol.* **1998**, *343*, 313.