Biochemical Pharmacology, Vol. 38, No. 9, pp. 1395–1406, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

INTERRELATIONSHIP BETWEEN AFFINITY FOR DNA, CYTOTOXICITY AND INDUCTION OF DNA-BREAKS IN CULTURED L1210 CELLS FOR TWO SERIES OF TRICYCLIC INTERCALATORS

SIMPLIFIED ANALOGUES OF ELLIPTICINE DERIVATIVES

V. PIERSON,* A. PIERRE,† P. DE COINTET, C. H. NGUYEN‡, E. BISAGNI‡ and P. GROS§

Sanofi Recherche, 195 route d'Espagne, 31035 Toulouse Cedex; ‡ UA 533 CNRS, Laboratoire de Synthèse Organique, Institut Curie-Biologie, Bât. 110–112, 15 rue Georges Clémenceau, 91405 Orsay; and § Sanofi Recherche, rue du Professeur Blayac, 34082 Montpellier, France

(*Received* 17 *May* 1988; *accepted* 31 *August* 1988)

Abstract—The interrelationship between affinity for DNA, cytotoxicity and induction of single-strand DNA breaks in cultured L1210 cells was studied for 21 compounds belonging to two series of tricyclic intercalators: 1-amino-substituted 4-methyl-5*H*-pyrido[4,3-b]indoles (γCARB) and 1-amino-substituted 4-methyl-5*H*-pyrido[3′,4′:4,5]pyrrolo[2,3-c]pyridines (PPP), which are simplified analogues of Ellipticine derivatives obtained by deletion of one cycle. Adriamycin®, *m*-AMSA (4′-(9-acridinylamino) methanesulfon-*m*-anisidide), PZE (10-[diethylaminopropyl amino]-6-methyl-5*H*-pyrido[3′,4′:4,5]-pyrrolo[2,3-g] isoquinoline and RTE ([1-(3-diethylaminopropylamino)-9-methoxy ellipticine, bimaleate) are used as reference compounds. The intercalation of these compounds into DNA was strongly suggested by three experimental observations: (i) the competitive inhibition of ethidium bromide intercalation, (ii) bathochromic and hypochromic effects on absorption spectra induced by DNA, and (iii) drug-induced increase of the DNA length, measured by viscosimetry. PPP derivatives are generally less cytotoxic and induce DNA breaks less efficiently than the γCARB ones, both in terms of maximum breakage frequencies and required drug concentrations. The most active compounds induced SSB in the DNA of L1210 cells, in a bell-shaped manner: the SSB frequency increased, rose to a maximum and then decreased as the drug concentrations increased. The maximum SSB frequencies induced by the most active compounds are of the same order as those of reference compounds Adriamycin® and PZE. The structurally important requirements are essentially the same for both DNA breakage activity and cytotoxicity: (i) a N–CH_3 in the 5-position, (ii) a CH_3 in the 4-position, (iii) a hydroxy in the 8-position and (iv) the presence of an (aminoalkyl)amino side chain with preferentially a 3 carbon unit. There is no direct relationship between DNA affinity *in vitro* and induction of DNA breaks in cells, although a relatively high affinity seemed to be a necessary condition, since the most active compounds have the highest affinities and compounds having a very low affinity are totally inactive. The close correlation between cytotoxicity and extent of induction of DNA breaks suggests that these breaks may be in fact the lethal lesions responsible for cell death and thereby for the antitumor properties of these tricyclic intercalators.

Ellipticine and its analogs belong to the broad class of DNA-intercalating compounds which are important antitumor agents. The cytotoxic and antitumor activities of these compounds are probably closely related to their ability to induce DNA strand breaks in cells in culture [1–5]. These protein-associated breaks are due to the trapping of topoisomerase II–DNA complexes by the drug and can be demonstrated in isolated nuclei and in *in vitro* experiments using purified enzyme and DNA [6–8].

The existence of a relationship between this effect and the cytotoxic properties of antitumor intercalating drugs is suggested by three lines of evidence: (i) a majority of the most active drugs induce DNA-breaks; (ii) some cell lines, resistant to the topoisomerase II-interacting drugs exhibit lower frequencies of breaks when compared to the sensitive ones [9–11]; and (iii) some preliminary structure-relationships (SAR) have shown a direct correlation between these two parameters [12, 13]. However, among the SAR studies yet reported, only a few of them, involving a limited number of drugs, have led to conflicting conclusions.

A classical example of SAR result is the com-

* Supported by a grant from the Ministere de la Recherche et de l'Enseignement Supérieur. To whom requests for reprints should be addressed. Present address: Laboratoire de Pharmacologie et de Toxicologie Fondamentales du C.N.R.S., 205 route de Narbonne, 31078 Toulouse, France.

† Present address: Institut de Recherche Servier, 11 rue des Moulineaux, 92150 Suresnes, France.

‖ Abbreviations used: *m*-AMSA, 4′-(9-acridinylamino) methanesulfon-*m*-anisidide: NMHE, 2*N*-methyl 9-hydroxy ellipticinium acetate; DSB, double-strand breaks; SSB, single-strand breaks; BD40, PZE, 10-[diethylaminopropyl amino]-6-methyl-5*H*-pyrido[3′,4′:4,5]pyrrolo[2,3-g] isoquinoline; BD84, RTE, [1-(3-diethylamino-propylamino)-9-methoxy ellipticine, bimaleate]; PPP, 1-amino-substituted 4-methyl-5*H*-pyrido[3′,4′:4,5]-pyrrolo[2,3-c]pyridines; γCARB, 1-amino-substituted 4-methyl-5*H*-pyrido[4:3-b]indoles; EB, ethidium bromide.

parison between *m*-AMSA‖ and its ortho isomer: the former is more potent in causing both DNA cleavage and antitumor effects [13]. An earlier study performed with three structurally-different drugs (NMHE, *m*-AMSA and 5-iminodaunorubicin) suggested that there was little or no relationship between DNA breakage and cytotoxicity [6]. A more recent work, performed with four 9-aminoacridinecarboxamides, demonstrated a direct correlation between cytotoxicity and DNA breaks [12]. Likewise, Rowe *et al.* showed a strong correlation between the double strand breaks induced *in vitro* in pBR322 DNA and the cytotoxicity of *m*-AMSA and five congeners [13]. However, no direct relation was found with doxorubicin and 4 chromophore-modified analogues [14].

Thus, in the case of antitumor intercalating drugs, the availability of defined series of structurally-related analogues allowing quantitative structure–activity relationships remains an interesting way to study the correlation between DNA break induction and cytotoxicity.

We described, in recent papers [15–18], the synthesis and the preliminary *in vivo* evaluation of two structurally-related series of tricyclic analogues of ellipticine derivatives: 1-amino-substituted 4-methyl-5*H*-pyrido[3′,4′:4,5]pyrrolo[3,2-c]pyridines and 1-amino-substituted 4-methyl-5*H*-pyrido[4,3-b]indoles which were abbreviated PPP and γCARB respectively in this work. Among these compounds, two were highly cytotoxic for L1210 cells in culture, and exhibited potent antitumor activity in standard murine models (leukemia and solid tumors) when administered by the i.p. or the i.v. route [16]. Moreover, a good correlation between the cytotoxicity and the antitumor activity on the P388 leukemia was found [15, 16]. The cytotoxicity is thus a representative criterion of the major biological activity of these compounds.

The aim of the present work was first to demonstrate the intercalative binding of these compounds by viscosimetry and spectrophotometry and second to study the interrelationship between the affinity for DNA, the cytotoxicity for L1210 cells in culture and the ability to induce DNA breaks in these cells. For this purpose, we selected 21 compounds with a wide range of activities and studied them with comparison to two reference compounds, 10-[diethylaminopropylamino]-6-methyl-5*H* pyrido-[3′,4′:4,5]pyrrolo[2,3-g]isoquinoline (SR 95225, BD40, PZE in this paper) [19] and [1-(3-diethylaminopropylamino)9-methoxyellipticine] (SR 95325, BD84, RTE in this paper) [20]. These two drugs are two potent ellipticine derivatives, currently in clinical trials, and can be considered as the parent compounds of their simplified analogues PPP and γCARB, respectively. The two well known intercalators *m*-AMSA and Adriamycin® (doxorubicin hydrochloride)* were also used as reference compounds.

* Adriamycin is a registered trademark of Farmitalia Carlo Erba.

MATERIALS AND METHODS

Drugs. *m*-AMSA (4′-9-acridinylamino)methanesulfon-*m*-anisidide) was obtained from Laboratoires Substantia, Paris, France, and was dissolved at 10 mM in dimethylsulfoxide and stored at −20°. Adriamycin® was obtained from Laboratoires R. Bellon, Paris, France, and dissolved in distilled water just before use. PPP and γCARB derivatives were synthetized as described [15–18]. The hydrochlorides of 10[3-diethylamino-propylamino]-6-methyl-5*H*-pyrido[3′,4′:4,5]pyrrolo[2,3-g]isoquinoline (SR 95225, BD 40 or PZE in this paper) and of 1-(3-diethylaminopropylamino) 9-methoxy ellipticine (SR 95325, BD 84 or RTE in this paper) were synthetized by Sanofi Chimie, as already described [21, 22]. All these drugs were dissolved in distilled water at 1–2 mM just before use.

DNA binding. The apparent DNA binding constants (KAFF) were measured by competition with ethidium bromide as described [23], in 20 mM $Na_2HPO_4/NaHPO_4$, 0.1 M NaCl, 1 mM EDTA, pH 7.4 (PNE buffer), with calf thymus DNA (Sigma Chemical Co.) at 10 μg/ml. The KAFFs were calculated from Scatchard plots by regression analysis (correlation coefficient = 0.98–0.99) using three concentrations of the tested drug, in two separate experiments.

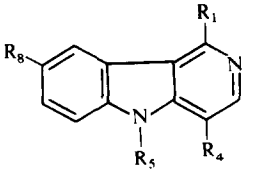
Absorption spectra. The spectra of the drug alone (10 μM) and with increasing concentrations of DNA (0–362 μM) were recorded. The extinction coefficient of the bound drug were measured in the presence of a saturating excess of DNA.

Viscosimetry. DNA was sonicated at 0° under nitrogen (15 cycles of one minute each) and was purified by gel filtration on a Sephacryl S 1000 column (Pharmacia). Fractions corresponding to the center of the peak were collected, precipitated with ethanol at 4°, dried, dissolved in PNE at 2 mg/ml and stored at −20°. The molecular weight, determined by a standard electrophoretic method, was found to be approximately 1×10^6. The thawed DNA was centrifuged before use to discard precipitates. Viscosimetric titrations were conducted in 3 ml of PNE in an Ubbelohde micro-viscosimeter (capillary no. 1, i.d. 0.32 mm) in a thermostatic bath at 25°. The flow times (three for each measurement) were automatically measured with the Schott Gerate AVS 300 unit. The value R corresponding to the average number of bound drug molecules per nucleotide was calculated from a quadratic equation, using the K_{aff} value measured in the same conditions, as described above. Data were then plotted according to Cohen and Einsenberg [24]. The slope of the straight line obtained when $(N/N_0)^{1/3}$ is expressed as a function of R was calculated by regression analysis. Given values of the slope were the means of values obtained in two separate experiments.

L1210 cells. Mouse leukemia L1210 cells were cloned and stored in liquid nitrogen; fresh aliquots were thawed every 2–3 months in order to rule out a possible shift in cell sensitivity to drugs. Cells were grown in suspension culture in RPMI 1640 medium supplemented with 10% foetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine. Cultures used to assess drug effects were in

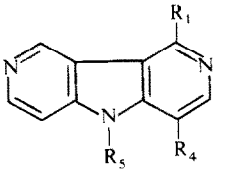
Table 1. Structures of the tricyclic analogues

(A) 1-amino-substituted 4-methyl-5*H*-pyrido[4:3-b]indoles (γCARB)



Compounds	R_1	R_4	R_5	R_8
1	$-NH-(CH_2)_3-N(Me)(Me)$	$-CH_3$	$-CH_3$	-OMe
2	$-NH-CH_2-CH(CH_3)-CH_2-N(Me)(Me)$	$-CH_3$	$-CH_3$	-OH
3	$-NH-(CH_2)_4-N(Me)(Me)$	$-CH_3$	$-CH_3$	-OMe
4	$-NH-(CH_2)_3-N$ (pyrrolidine)	$-CH_3$	$-CH_3$	-OH
5	$-NH-(CH_2)_3-N(Me)(Me)$	$-CH_3$	-H	-OH
6	$-NH-(CH_2)_3-N(Et)(Et)$	$-CH_3$	$-CH_3$	-OH
7	$-NH-(CH_2)_3-N(Me)(Me)$	$-CH_3$	$-CH_3$	-OH
8	$-NH-(CH_2)_3-N(Me)(Me)$	-H	$-CH_3$	-OH
9	$-NH-(CH_2)_3-N(Me)(Me)$	-H	-H	-OH
10*	H	$-CH_3$	$-CH_3$	-OH
11	$-NH-CH_2-CHOH-CH_2-N(Et)(Et)$	$-CH_3$	$-CH_3$	-OH

(B) 1-Amino-substituted 4-methyl-5*H*-pyrido[3′,4′:4,5]pyrrolo[2,3-c]pyridines (PPP)



Compounds	R_1	R_4	R_5
12	$-NH-(CH_2)_2-N(Et)(Et)$	$-CH_3$	-H
13	$-NH-(CH_2)_3-N$ (pyrrolidine)	$-CH_3$	$-CH_3$
14	$-NH-(CH_2)_4-N(Et)(Et)$	$-CH_3$	$-CH_3$
15	$-NH-(CH_2)_4-N(Et)(Et)$	$-CH_3$	-H
16	$-NH-(CH_2)_3-N(Me)(Me)$	$-CH_3$	$-CH_3$
17	$-NH-(CH_2)_3-N(Et)(Et)$	$-CH_3$	$-CH_3$
18	$-NH-(CH_2)_3-N(Et)(Et)$	$-CH_3$	-H
19	$-NH-(CH_2)_3-N(Me)(Me)$	$-CH_3$	-H
20	$-N(CH_3)-(CH_2)_3-N(Et)(Et)$	$-CH_3$	$-CH_3$
21	$-NH-(CH_2)_3-N(Et)(Et)$	-H	$-CH_3$

(A) Chemical structures of 1-amino-substituted 4-methyl-5*H*-pyrido[4:3-b]indoles, γCARB.

* This compound was not previously described. It has been obtained by dechlorination of 1-chloro-4,5-dimethyl-8-methoxy-5*H*-pyrido[4,3-b]indoles [16] (pd/c 10% in ethanol) which afford 71% of 4,5-dimethyl-8-methoxy-5*H*-pyrido[4,3-b]indole (m.p. 115–117°, cyclohexane) and subsequent demethylation by hydrobromic acid (47%, d. 1.47, 1.5 hr at reflux) followed by usual work up (yield = 83%, m.p. > 260°, acetonitrile).

(B) Chemical structures of 1-amino-substituted 4-methyl-5*H*-pyrido[3′,4′:4,5]-pyrrolo[2,3-c]pyridines, or PPP.

exponentional phase of growth (doubling time = 10–12 hr) and were routinely checked for the absence of mycoplasma contaminations.

Inhibition of L1210 cell proliferation was measured as described [15]. The drugs were added to cells in exponential phase of growth (0.8×10^5 cells/ml) for 48 hr. The cells were counted and results were expressed as the drug concentration which inhibited the growth by 50% (IC_{50}). The IC_{50} values were estimated by regression from the dose–response data and were the means of at least two separate experiments, differing by less than a factor 2.

The clonogenic assay was performed as described [25]. Cells (250 or 10^5, depending on the drug concentration) were treated for 1 hr, washed, plated in 35 mm culture dishes and incubated for 10 days at 37°. Colonies were stained with 3(4,5-dimethyl-thiazol-2-yl)-2,5 diphenyl-tetrazolium bromide or thiazolyl blue (Sigma Chemical Co.) and scored. Each point is the mean of triplicate dishes and was done at least three times in separate experiments. Plating efficiency was 40–80% in untreated cells.

Alkaline elution. Alkaline elution procedures were essentially as reported by Kohn *et al.* [26] and exactly as we reported in a recent paper [25]. Briefly, treated (or untreated control) [^{14}C]thymidine-labeled cells were mixed with [^{3}H]thymidine-labeled cells which had received 300 rad of X-ray irradiation to serve as internal standard cells, deposited onto polycarbonate membrane filters and lysed using a solution containing 0.1 M glycine, 0.025 M EDTA, 2% SDS and 0.5 mg/ml proteinase K. Elutions were performed with a solution of tetrapropylammonium hydroxide, 0.02 M EDTA, 0.1% SDS, pH 12.1. Single strand break (SSB) frequency was calculated at a retention of 60% of the tritiated DNA from irradiated internal standard cells. For SAR studies, at least four concentrations of each compound were used: 30, 10, 3 and 1 μM, in order to determine the maximum frequency of breaks. Experiments were done at least twice.

Double-strand breaks (DSB) were determined without internal standard. Two hundred and fifty thousand [^{14}C]thymidine labeled, treated cells or untreated control cells were deposited onto polycarbonate filters and elution was performed at pH 9.6. The retention of [^{14}C]DPM was calculated after 10 hr of elution. The DSB frequency was expressed as rad equivalents as determined from calibration curves that expressed retention of DNA vs radiation dose in rads [6].

RESULTS

Intercalative binding of the compounds

We first measured the apparent affinity constant for DNA of all the synthetized derivatives (structures shown in Table 1) by competition with ethidium bromide. The data were plotted according to Scatchard and a typical graph obtained with compound **6** is shown in Fig. 1. The curves are perfectly linear and intercept the abscissa axis at a R value close to 0.20, suggesting a competitive inhibition of EB binding. All the analogues gave similar curves (not shown). A large range of affinity constant values was obtained, from 0.003 to 0.46×10^6/M. The K_{AFF} values of the tricyclic analogues PPP were significantly lower (at least one order of magnitude) than that of the reference compound PZE (Table 2), although those of γCARB were close to RTE value.

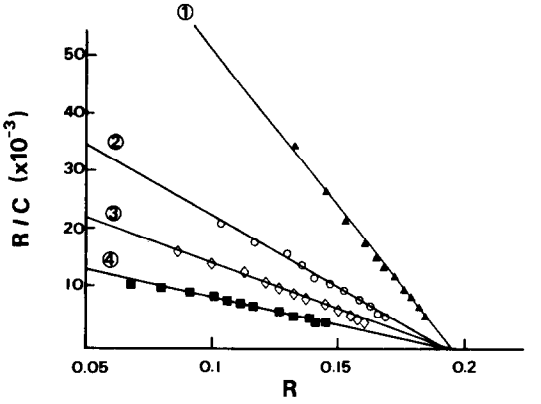


Fig. 1. Competitive inhibition of ethidium bromide intercalation by product **6** (Scatchard plot). 1: ethidium bromide alone (▲); 2: ethidium bromide + 5 μM drug (○); 3: ethidium bromide + 10 μM drug (◇); 4: ethidium bromide + 20 μM drug (■).

The interaction with DNA of some selected analogues having a relatively high K_{AFF} was studied by spectrophotometry. Addition of DNA induced three effects on the spectrum of the free drug, RTE: (i) a hypochromic effect; (ii) a bathochromic effect, and (iii) the presence of an isobestic point. These modifications had been already observed in the case of ellipticine [27] and some derivatives [28] and are characteristic of an intercalative binding. The modifications induced by the tricyclic analogues were significantly different: the bathochromic effect was very low, and there was no peak of absorption corresponding to the bound drug at a wavelength superior to that of the isobestic point (Table 3). The same kind of modifications in the absorption spectra, induced by intercalators have also been previously described [29].

In order to confirm unambiguously the intercalative binding of these compounds, we also measured the increase of sonicated DNA length by viscosimetry. This effect is generally considered as the strongest indication of an intercalative binding. All the tested drugs increased the length of sonicated DNA as measured by viscosimetry. When the data are plotted according to Cohen and Eisenberg a value of 2 is expected for the slope of the straight line obtained, but experimental values are generally below this value [30]. For example, Adriamycin® included as a reference compound gave a slope of 1.3. All these results are summarized in Table 3 and strongly suggest the intercalative binding of these compounds (PPP and γCARB), as is the case for PZE and RTE.

Cytotoxicity

The IC_{50} values are reported in Table 2 and ranged from 0.01 μM to 52 μM. The compounds belonging to the PPP series were generally less cytotoxic than the γCARBs. PZE was 10 times more cytotoxic than its best tricyclic analogue (compound **17**), although

Table 2. Biochemical data of the tricyclic intercalators and reference compounds

Compounds	K_{AFF}* (10^6/M)	IC_{50}† (μm)	F_{max}‡ (SSB rad eq.)
γCARB			
1	0.360	0.13	195
2	0.136	0.01	1499
3	0.460	1.3	84
4	0.370	0.62	167
5	0.210	0.09	480
6	0.240	0.03	1274
7	0.270	0.01	1194
8	0.120	0.19	371
9	0.070	11.2	145
10	0.029	9.2	0
11	0.230	0.06	1007
PPP			
12	0.012	19.5	24
13	0.060	1.6	154
14	0.040	2	84
15	0.030	12	91
16	0.040	0.3	629
17	0.040	0.13	523
18	0.030	0.34	224
19	0.020	0.16	567
20	0.003	52	0
21	0.017	1.6	90
Reference compounds			
PZE	0.460	0.018	1784 at 16 μM
RTE	0.700	0.09	823 at 50 μM
Adriamycin®	3.600	0.02	1607 at 12.5 μM
m-AMSA	0.040	0.015	3068 at 0.25 μM

Biochemical data of the tricyclic intercalators in comparison with two internal reference compounds (PZE, RTE) and two external reference compounds (Adriamycin®, *m*-AMSA).

* Values of the apparent DNA affinity constant in 10^6/M.

† The micromolar concentration of drug that reduced the proliferation of treated L1210 cells in culture to 50% of the control (mean of values obtained in two independent experiments differing by less than a factor 2).

‡ Maximum SSB frequency induced in the L1210 DNA at 10 μM for γCARB and at 30 μM for PPP, determined by alkaline elution using proteinase K.

in the γCARB series five compounds (**2**, **5**, **6**, **7**, **11**) were as active as (or more active than) RTE. Thus, as is the case for DNA affinity, the deletion of one cycle does not have the same consequences if a nitrogen atom is present in the 9 position in the ellipticine nucleus. The cytotoxicity of the more active compounds and the reference molecules was also measured by the clonogenic assay (not shown). The IC_{90} values increased in the following order: RTE < PZE < compound **6** < compound **17** (2.7, 3.1, 5.1, 49 μM, respectively). Thus, and in terms of relative drug potency, the results are essentially the same in the clonogenic and in the standard proliferation assay, except for RTE.

As shown in Fig. 2, there is no direct relationship between DNA affinity and cytotoxicity.

Alkaline elution

The production of DNA breaks (SSB) due to the action of the well-known intercalators Adriamycin® and *m*-AMSA used here as external references and of some studied compounds is shown in Fig. 3A. As previously described [31], *m*-AMSA induced a large amount of SSB that increased with increasing drug concentrations. A concentration of 0.25 μM *m*-AMSA gave a frequency of 3068 ± 325 rad eq. On the contrary, the number of SSB produced by Adriamycin® rose to a maximum (1607 ± 242 rad eq. at 12.5 μM) and then decreased as the concentration increased (Fig. 3A). This effect has been previously described by Potmesil *et al.* [32]. Likewise, PZE and RTE were self-inhibitory at high concentrations (Fig. 3A). PZE was as active as Adriamycin®, although RTE was considerably less efficient, both in the number of breaks and in the concentration required for maximum effect. The rate of SSB was maximum at 16 μM (1784 ± 370 rad eq.) for PZE and at 50 μM (823 ± 217 rad eq.), for RTE.

The bell-shaped curves obtained with compounds **6** and **17** are shown in Fig. 3B. From this figure, the rate of SSB was maximum at 12.5 μM for compound **6** and at 40 μM for compound **17**. Compound 20 was totally inactive in inducing DNA breaks as it was devoid of cytotoxic efficacy.

Table 3. Viscosimetric and spectrophotometric results

Compound	Slope	λ_{free} (nm)	ξ_{free} (/M)	λ_{bound} (nm)	ξ_{bound} (/M)	IP (nm)
RTE	1.3	299	48000	317	20000	313
5	1.4	332	7400	340	4800	343
6	1.2	335	8400	338	4700	355
PZE	1.6	293	28000	315	16000	298
17	1.7	322	5900	326	4400	373
18	1.3	315	5300	317	3900	369

Slope: slope of the straight line obtained when $(N/N_0)^{1/3}$ is plotted as a function of real R.

λ_{free}, λ_{bound}: wavelengths of the maximum absorbance for the compounds, free in solution or bound to an excess of DNA, respectively.

ξ_{free}, ξ_{bound}: extinction coefficients of the compounds free or bound to DNA, respectively, determined at λ_{free}.

IP: wavelength of the isobestic points.

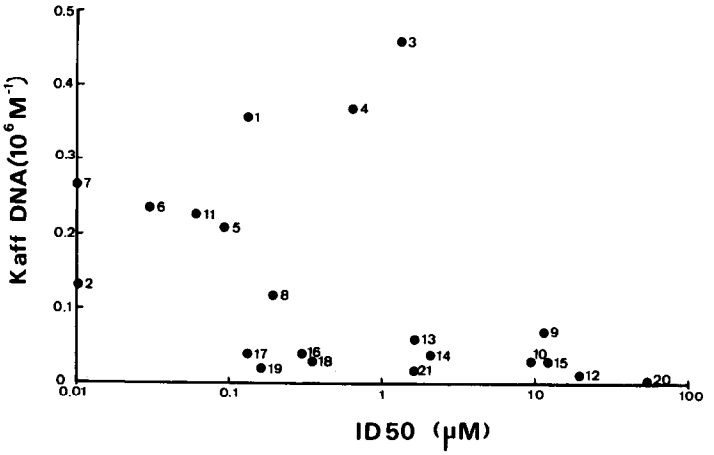


Fig. 2. Lack of correlation between the apparent affinity for DNA and the cytotoxicity for L1210 cells in the series of PPP and γCARB.

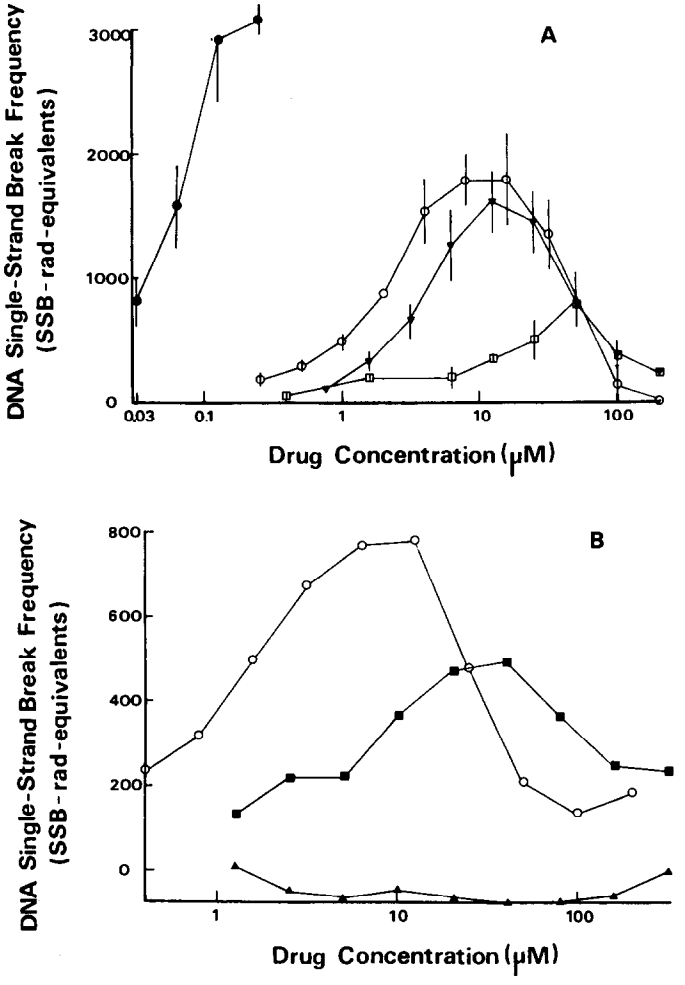


Fig. 3. Dependence of SSB frequency on drug concentration in L1210 cells. After a 1 hr drug exposure, cells were washed by centrifugation at 4° and then assayed by alkaline elution under deproteinizing conditions. (A) PZE (○), Adriamycin® (▼), *m*-AMSA (●) or RTE (□). Error bars represent ±SEM for at least three independent experiments, shown when larger than symbol size. (B) Compound **6** (○), compound **17** (■) or compound **20** (▲). Means of values obtained in two separate experiments, differing by less than a factor 2.

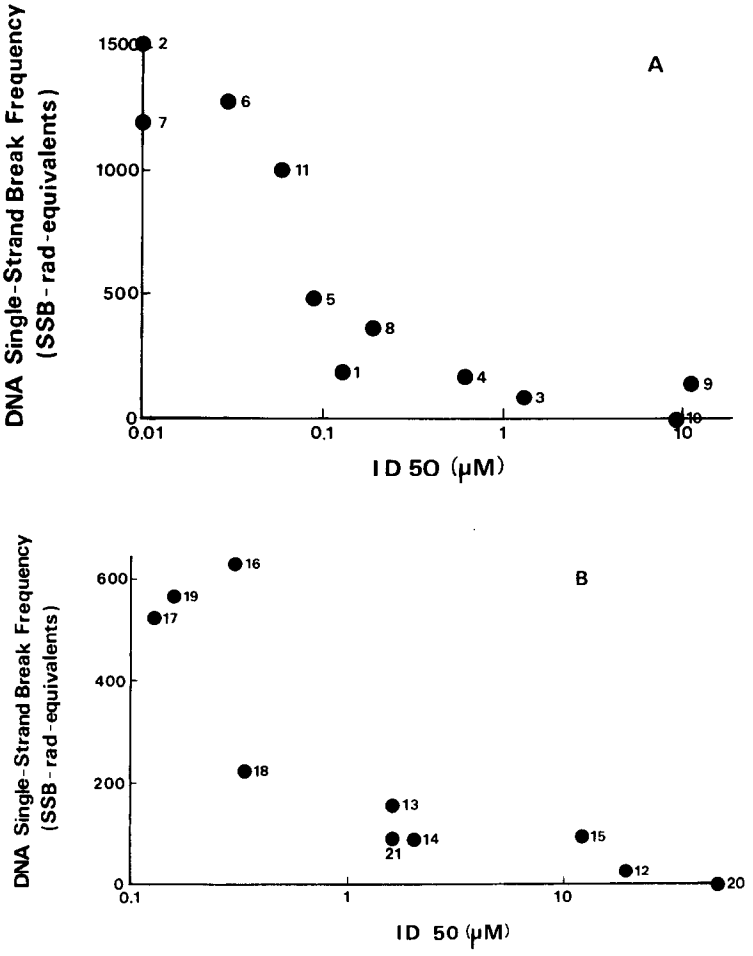


Fig. 4. Correlation between cytotoxicity for L1210 cells and DNA single-strand break frequency in the tricyclic simplified analogues of ellipticine derivatives: (A) γCARB; (B) PPP.

The inhibition of SSB formation at high drug concentrations by all the active compounds (not shown) and the fact that the concentration giving the maximum of breaks can be different for each drug led us to perform the SAR study with at least four concentrations. Two parameters could be determined: the concentration giving the maximum of breaks (C_{max}) and the maximum frequency, in rad eq. obtained at this concentration (F_{max}). It appeared that, at least with the 21 tested tricyclic intercalators, the concentrations giving the maximum of breaks (i.e. the top of the bell-shaped curve) depended on the presence or not of a nitrogen atom in the 8 position: F_{max} was obtained at 6–12 μM for γCARB and at 20–50 μM for PPP. In order to facilitate the interpretation of the results for the structure–activity relationship studies, we choose to quantify the DNA breaking efficiency by the frequency (F_{max}) obtained at 10 μM in the series of γCARB and at 30 μM in the series of PPP.

A wide range of SSB frequencies were obtained, from 0 to 1600 rad eq. (Table 2). Compounds in the PPP series induce DNA breaks less efficiently than those in the γCARB, both in terms of maximum frequencies and of concentration required.

PPP derivatives were also less active than the internal reference molecule PZE. In contrast, the most active γCARB analogues (**2**, **6**, **7**, **11**) were more efficient than RTE. Thus, in this latter case, tricyclic intercalators can be at least as active (*in vitro*) as the corresponding tetracyclic ones.

As we discussed previously, there is no direct relationship between DNA affinity and cytotoxicity, but we found a good correlation between cytotoxicity and *in vivo* antitumor activity, in the two series [15, 16]. The results presented in Table 2 show that the cytotoxicity is closely related to the capacity to induce DNA breaks. The most cytotoxic compounds induced the highest SSB frequencies (compounds **2**, **6**, **7**, **11** and **16**, **17**, **19**) and compounds devoid of cytotoxicity were unable to induce significant SSB frequencies (compounds **10**, **12**, **20**). This relationship is illustrated in Fig. 4A and B, where the SSB frequencies were plotted as a function of IC_{50} values. The two series (γCARB and PPP) were plotted separately, since the concentrations giving the maximum frequencies were different. The induction of DNA breaks, measured by the alkaline elution technique, is thus fairly predictive for cytotoxicity and for the antitumor activity, in these two series.

The structural requirements important for the DNA breakage activity were essentially the same as those for cytotoxicity [15, 16]:

(i) Compounds having a N–CH_3 at their 5-position were generally more cytotoxic and induced more breaks in the CARB series (compare compounds **7** vs **5** or **8** vs **9**).

(ii) A CH_3 at the 4 position increased both cytotoxicity and DNA breaks (compare compounds **7** versus **8** or **17** vs **21**).

(iii) The replacement of a methoxy group in the 8 position by a hydroxy group (compare compounds **1** vs **7**) led to more cytotoxic and more efficient compounds in the series of γCARB.

(iv) The existence of an (aminoalkyl)amino side chain plays a crucial role. Compounds lacking this chain were considerably less active (data not shown), or totally inactive, in these series (compound **10**). The replacement of the dimethylamino or diethylamino groups by a more bulky cyclic amino group (compare compounds **6** and **7** vs **4** or **16** and **17** vs **13**) strongly decreased cytotoxicity and SSB frequencies. There are no important differences when the terminal diethylamino substituent is replaced by a dimethylamino group (compare compounds **6** and **7**, **16** and **17**).

The length of the side chain is also an important parameter: a 3 carbon unit seemed to be optimal, since the C-2 compound **12** and the C-4 compound **3** were less cytotoxic and broke DNA to a lesser extent than the corresponding C-3 compounds (**18** and **1** respectively).

As we mentioned above, there is no direct relationship between DNA affinity and induction of DNA breaks. However, a relatively high affinity for DNA seemed to be a necessary condition for inducing DNA breaks and cytotoxicity, since the more active γCARB derivatives have also the highest DNA affinity and we did not find compounds with a low affinity and inducing a high SSB frequency.

The presence of a side chain is important for DNA affinity too: the introduction of a side chain in the 1-position of compound **10** led to compounds **6** and **7** having a 10-fold higher affinity, an IC_{50} lower by two orders of magnitude and a good capacity to induce DNA breaks. This side chain thus seems to stabilize the intercalation of the planar nucleus into DNA, this stable interaction being probably a necessary condition for induction of DNA breaks and cytotoxicity. However, compound **4**, which bears a side chain with a bulky substituent, has a high affinity but is much less active on L1210 cells and is a weak inducer of DNA breaks. Thus, one can postulate that another kind of interaction than intercalation is required to induce high DNA breaks frequencies.

The two reference compounds (PZE and RTE) and the more active product of each series (compounds **6** and **17**) were tested for their ability to induce double-strand breaks (DSB) in cultured L1210 cells. For these four drugs, the true single strand breaks over the double strand breaks ratios (s/d ratio) were calculated as described in Materials and Methods. The results are reported in Table 4. Like PZE and in contrast to Ellipticine and NMHE [2] which induced exclusively DSB, the two tested drugs (compounds **6** and **17**) were able to induce both DSB and true SSB. RTE, at low concentration induced almost as many DSB as SSB, but at higher

Table 4. Estimates of true single-strand to double-strand break ratios in drug treated L1210 cells.

Compound	[SSB]* (rad eq.)	[DSB] (rad. eq.)	[SSB]/[DSB]	$\frac{s}{d}$
PZE				
16 μM	1784 ± 370†	9325 ± 1254	0.191	2.40
4 μM	1528 ± 240	6214 ± 1127	0.246	3.65
1 μM	473 ± 84	2491 ± 440	0.190	2.37
17				
40 μM	397 ± 48	2325 ± 385	0.170	1.91
20 μM	401 ± 35	1991 ± 333	0.201	2.62
10 μM	356 ± 10	1991 ± 666	0.178	2.09
RTE				
50 μM	823 ± 217	769 ± 111	1.070	22.61
25 μM	491 ± 145	436 ± 111	1.126	23.90
12.5 μM	344 ± 43	2325 ± 385	0.148	1.40
6				
12.5 μM	1150 ± 371	3325 ± 769	0.346	5.95
6.25 μM	1045 ± 258	4213 ± 444	0.248	3.70
3.12 μM	846 ± 98	4214 ± 618	0.200	2.62

* [SSB], SSB frequency in SSB rad equivalents; [DSB], DSB frequency in DSB rad equivalents; s, frequency of true SSB (not including those arising from DSB); d, true DSB frequency; s/d calculated according to the equation:

$$\frac{s}{d} = \frac{\text{Krs}}{\text{Krd}} \frac{[\text{SSB}]}{[\text{DSB}]} - 2, \text{ assuming } \frac{\text{Krs}}{\text{Krd}} = 23 \text{ [6]}.$$

† Mean ± SEM for at least three independent experiments.

concentration (25 μM), this latter drug produced much less DSB per SSB.

DISCUSSION

The aim of the present study was to confirm the intercalation into DNA and to study the mechanisms of cytotoxicity of two series of tricyclic antitumor intercalators: 1-amino-substituted 4-methyl-5*H*-pyrido[3′,4′:4,5]pyrrolo[2,3-c]pyridines (PPP) and 1-amino-substituted 4-methyl-5*H*-pyrido[4:3-b]-indoles (γCARB). The intercalation of these compounds into DNA was strongly suggested by three pieces of experimental evidence: (i) competitive inhibition of ethidium bromide intercalation, (ii) bathochromic and hypochromic effects induced by DNA on absorption spectra, and (iii) drug-induced increase of the DNA length, measured by viscosimetry. This latter effect is generally considered as the strongest indication of an intercalative binding. However, some differences were found when results were compared with those of the parent compounds, the tetracyclic intercalators PZE and RTE: the modifications of the absorption spectra were not identical and the slopes of the straight line obtained in the Cohen and Eisenberg representation were slightly lower. The mode of interaction of tricyclic intercalators could thus be slightly different from that of tetracyclic ones.

Many different studies have shown that DNA breakage is a common effect of antitumor intercalating drugs [2, 4, 33]. These breaks are due to the trapping by the drug of topoisomerase II–DNA complexes, in which the enzyme is covalently bound to the 5′ end of the broken DNA [34, 35]. Although this interaction is now well documented and can be studied *in vitro* with purified topoisomerase II, the sequence of events leading to cell death remains largely unknown, addressing the actual role of the DNA breaks in the cytotoxic properties of these drugs.

Some SAR studies, involving only few drugs, concluded to a lack of correlation between cytotoxicity and DNA breakage potency [6] while others found some correlation between these two parameters [12]. Many important factors (repair of lesions, DSB/SSB ratio and sequence specificity) that could modulate the consequence of the interaction with topoisomerase II are probably different in the various series of drugs, thus complicating the interpretation of the results. Hence such a study should be performed with analogues belonging to the same series.

We thus took advantage of the availability of more than 50 tricyclic intercalators belonging to Sanofi Recherche in order to study the most active PPP and γCARB derivatives; we investigated their efficiency as DNA-break inducers and the interrelationship between affinity for DNA, cytotoxicity and induction of breaks.

As expected, the most active compounds (γCARB derivatives) were able to induce breaks in the DNA of L1210 cells in culture, with a maximum frequency and at concentrations of about the same order than those of reference compounds (Adriamycin®, RTE and PZE). In addition, the bell-shaped aspects of the dose–response curve (in which SSB frequencies are plotted as a function of drug concentration) were similar to those of Adriamycin®, PZE and RTE.

This inhibition of induction of DNA breaks at high drug concentrations is consistent with results of Potmesil *et al.* with Adriamycin® [32], Pierson *et al.* with PZE [25] and Pommier *et al.* with NMHE, a quaternized ellipticine derivative, on isolated nuclei [36]. This effect is also observed *in vitro*, in experiments using purified topoisomerase II and DNA [37]. The precise mechanism of this effect is unknown. High concentrations of intercalators may stabilize an intermediate in which the topoisomerase II is dissociated from DNA or bound to DNA without inducing cleavage, or a high degree of drug intercalation may induce DNA-structure alterations which inhibit the binding of topoisomerase II to DNA [8].

It must be pointed out that, although the concentrations giving the maximum frequencies of breaks might be different for not structurally related compounds, the range was the same for all the compounds inside each series (PPP and γCARB).

In each of these two series, a good correlation was found between cytotoxicity and maximum of break frequencies. We did not find compounds having a very low cytotoxicity and inducing high SSB frequencies or highly cytotoxic compounds which lacked DNA breaking activity. This correlation suggests that the DNA breaks are closely related to the lethal lesions induced by these drugs. The most probable mechanism for the formation of these breaks is the interaction of PPP and γCARB with topoisomerase II–DNA complexes, which has already been demonstrated for different ellipticine derivatives [25, 37].

In terms of structure–activity relationships, the most important parameter is probably the presence of a dimethylaminopropylamino or diethylaminopropylamino side chain in position 1. This kind of side chain appears to be an essential determinant for cytotoxicity in the ellipticine derivatives series [38] as well as in other series of antitumor intercalating drugs [39, 40]. This side chain can exert its effect through at least three different types of interaction: (i) by stabilizing the intercalation of the planar nucleus through an outside binding, (ii) by increasing the lipophilicity, facilitating the membrane diffusion of the drug, (iii) by a direct interaction with the enzyme topoisomerase II, thus stabilizing the complex topo II–DNA and increasing the number of breaks. As we discussed above in the results section, the first effect is obvious, but probably not sufficient by itself, since drugs having slightly modified chains, are less cytotoxic and less efficient in inducing breaks. The latter assumption is attractive and was recently proposed to explain the effects of a bulky side chain in the 9-aminoacridine series [41].

In conclusion, we found, for two series of closely structurally-related tricyclic intercalators, a good correlation between cytotoxicity and induction of DNA breaks. This correlation suggests that these breaks, probably induced by the interaction of the drugs with the topoisomerase II–DNA complex, are actually the lethal lesions responsible for the cell death and, consequently, for the antitumor properties of these drugs.

Acknowledgements—V.P. was supported by a grant from the Ministere de la Recherche et de l'Enseignement Supérieur. We thank Claudine Marcy, Nicole Baudry, Arlette Spinazze for technical assistance and Daniel Boutin for computer analysis. We wish to thank also Dr K. W. Kohn and Dr Y. Pommier for introducing us to the alkaline elution technique.

REFERENCES

1. Paoletti C, Lesca C, Cros S, Malvy C and Auclair C, Ellipticines and derivatives induce breakage of L1210 cells DNA *in vitro*. *Biochem Pharmacol* **28**: 345–350, 1979.
2. Zwelling LA, Michaels S, Kerrigan D, Pommier Y and Kohn KW, Protein-associated deoxyribonucleic acid strand breaks produced in mouse leukemia L1210 cells by Ellipticine and 2-methyl-9-hydroxy ellipticinium. *Biochem Pharmacol* **31**: 3261–3267, 1982.
3. Zwelling LA, Michaels S, Erickson LC, Ungerleider RS, Nichols M and Kohn KW, Protein-associated deoxyribonucleic acid strand breaks in L1210 cells treated with the deoxyribonucleic acid intercalating agents 4′-(9-acridinylamino) methanesulfon-*m*-anisidide and Adriamycin. *Biochemistry* **20**: 6553–6563, 1981.
4. Zwelling LA, Kerrigan D and Michaels S, Cytotoxicity and DNA strand breaks by 5-iminodaunorubicin in mouse leukemia L1210 cells: comparison with Adriamycin and 4′-(9-Acridinylamino) methanesulfon-*m*-anisidide. *Cancer Res* **42**: 2687–2691, 1982.
5. Pommier Y, Zwelling LA, Kao-Shan CS, Whang-Peng J and Bradley MO, Correlation between intercalators-induced DNA strand breaks and sister chromatid exchangers, mutations and cytotoxicity in Chinese hamster cells. *Cancer Res* **45**: 3143–3149, 1985.
6. Pommier Y, Schwartz RE, Kohn KW and Zwelling LA, Formation and rejoining of deoxyribonucleic acid double-strand breaks induced in isolated nuclei by antineoplastic intercalating agents. *Biochemistry* **23**: 3194–3201, 1984.
7. Tewey KM, Rowe TC, Yang L, Halligan BD and Liu LF, Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. *Science* **226**: 466–468, 1984.
8. Tewey KM, Chen GL, Nelson EM and Liu LF, Intercalative antitumor drugs interfere with the breakage-reunion reaction of mammalian DNA topoisomerase II. *J Biol Chem* **259**: 9182–9187, 1984.
9. Pommier Y, Schwartz RE, Zwelling LA, Kerrigan D, Mattern M, Charcosset R, Jacquemin-Sablon A and Kohn KW, Reduced formation of protein-associated DNA strand breaks in Chinese hamster cells resistant to topoisomerase II inhibitors. *Cancer Res* **46**: 611–616, 1986.
10. Goldenberg GJ, Wang H and Blair GW, Resistance to Adriamycin: relationship of cytotoxicity to drug uptake and DNA single- and double-strand breakage in cloned cell lines of Adriamycin-sensitive and resistant P388 leukemia. *Cancer Res* **46**: 2978–2983, 1986.
11. Zijlstra JG, de Vries EGE and Mulder NH, Multifactorial drug resistance in an Adriamycin-resistant Human small cell lung carcinoma cell line. *Cancer Res* **47**: 1780–1784, 1987.
12. Denny WA, Roos IAG and Wakelin LPG, Interrelations between anti-tumour activity, DNA breakage and DNA binding kinetics for 9-aminoacridine-carboxamide anti-tumour agents. *Anti-cancer Drug Design* **1**: 141–147, 1986.
13. Rowe TC, Chen GL, Hsiang YH and Liu LF, DNA damage by antitumor acridines mediated by mammalian DNA topoisomerase II. *Cancer Res* **46**: 2021–2026, 1986.
14. Capranico G, Soranzo C and Zunino F, Single-strand DNA breaks induced by chromophore modified anthracyclines in P388 leukemia cells. *Cancer Res* **46**: 5499–5503, 1986.
15. Nguyen CH, Bisagni E, Pépin O, Pierré A and de Cointet P, 1-amino-substituted 4-methyl-5*H*-pyrido[3′,4′:4,5]pyrrolo[3,2-c]pyridines: a new class of antineoplastic agents. *J Med Chem* **30**: 1642–1647, 1987.
16. Bisagni E, Nguyen CH, Pierré A, Pépin O, de Cointet P and Gros P, 1-amino-substituted 4-methyl-5*H*-pyrido[4,3-b]indoles (γ carbolines) as tricyclic analogues of ellipticines: a new class of antineoplastic agents. *J Med Chem* **31**: 398–405, 1988.
17. Bisagni E and Nguyen CH, Première synthèse de méthyl-4-5*H*-pyrido[3′,4′:4,5]pyrrolo[3,2-c]-pyridines, analogues tricycliques des aza-9-ellipticines. *Tetrahedron* **42**: 2311–2318, 1986.
Nguyen CH and Bisagni E, Autre voie d'accès aux 5*H*-pyrido[3′,4′:4,5]pyrrolo[3,2-c]pyridines et leur transformation en dérivés N-5 et N-8 substitués. *Tetrahedron* **42**: 2303–2309, 1986.
18. Nguyen CH and Bisagni E, Synthèse d'analogues tricycliques des ellipticines: les méthyl-4,5*H*-pyrido[4,3-b]indoles (γ-carbolines) diversement substitués sur leur sommet 1, 5 et 8. *Tetrahedron* **43**: 527–535, 1987.
19. Lidereau R, Chermann JC, Gruest J, Montagnier L, Ducrocq C, Rivalle C and Bisagni E, Antitumoral activity of dipyrido(4,3-b)(3,4-f)indoles on L1210 leukemia. *Bull Cancer (Paris)* **67**: 1–8, 1980.
20. Ducrocq C, Wendling F, Tourbez-Perrin M, Rivalle C, Tambourin P, Pochon F and Bisagni E, Structure-activity relationship in series of newly synthetised 1-amino-substituted Ellipticine derivatives. *J Med Chem* **23**: 1212–1216, 1980.
21. Ducrocq C, Bisagni E, Rivalle C and Lhoste JM, Synthesis of 10-substituted 5*H*-pyrido[3′,4′:4,5]-pyrrolo[2,3-g]isoquinolines. *J Chem Soc Perkin* **I**, 142–145, 1979.
22. Bisagni E, Ducrocq C, Lhoste JM, Rivalle C and Civier A, Synthesis od 1-substituted ellipticines by a new route to pyrido[4,3-b]carbazoles. *J Chem Soc Perkin* **I**: 1706–1711, 1979.
23. Le Pecq JB and Paoletti C, A fluorescent complex between ethidium bromide and nucleic acids. Physical-chemical characterisation. *J Mol Biol* **27**: 87–106, 1967.
24. Cohen G and Einsenberg H, Viscosity and sedimentation study of sonicated DNA-proflavine complexes. *Biopolymers* **8**: 45–55, 1969.
25. Pierson V, Pierré A, Pommier Y and Gros P, Production of protein-associated DNA breaks by 10-diethylaminopropylamino-6-methyl-5*H*-pyrido[3′,4′:4,5]-pyrrolo[2,3-g]isoquinoline in cultured L1210 cells and in isolated nuclei. Comparison with other topoisomerase II inhibitors. *Cancer Res* **48**: 1404–1409, 1988.
26. Kohn KW, Ewig RAG, Erickson LC and Zwelling LA, Measurement of strand breaks and crosslinks by alkaline elution. In: *DNA Repair, a Laboratory Manual of Research techniques* (Eds. Friedberg EC and Hanawalt PC), pp. 379–401. Marcel Dekker, New York, 1981.
27. Kohn KW, Waring MJ, Glaubiger D and Friedman CA, Intercalative binding of Ellipticine to DNA. *Cancer Res* **35**: 71–76, 1975.
28. Tourbez-Perrin M, Pochon F, Ducrocq C, Rivalle C and Bisagni E, Intercalative binding to DNA of new antitumoral agents: dipyrido[4,3-b][3,4-f]indoles. *Bull Cancer (Paris)* **67**: 9–13, 1980.
29. Shan-Fong Yen, Gabbay J and Wilson WD, Interaction of aromatic imides with deoxyribonucleicacid. Spectrophotometric and viscosimetric studies. *Biochemistry* **21**: 2070–2076, 1982.

30. Wilson WD, Keel RA, Jones RL and Mosher CW, Viscosimetric analysis of the interaction of bisphenanthridinium compounds with closed circular supercoiled and linear DNA. *Nucleic Acid Res* **10**: 4093–4105, 1982.
31. Pommier Y, Kerrigan D, Schwartz RE and Zwelling LA, The formation and rescaling of intercalator-induced DNA strand breaks in isolated L1210 cell nuclei. *Biochem Biophys Res Comm* **107**: 576–583, 1982.
32. Potmesil M, Kirschenbaum S, Israel M, Levin M, Khetarpal VK and Silber R, Relationship of Adriamycin concentrations to the DNA lesions induced in hypoxic and euoxic L1210 cells. *Cancer Res* **43**: 3528–3533, 1983.
33. Ross WE, Glaubiger DL and Kohn KW, Protein-associated DNA breaks in cells treated with Adriamycin or ellipticine. *Biochim Biophys Acta* **519**: 23–30, 1978.
34. Nelson EM, Tewey KM and Liu LF, Mechanism of antitumor drug action: poisoning of mammalian DNA topoisomerase II on DNA by 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide. *Proc Natl Acad Sci USA*, **81**: 1361–1365, 1984.
35. Pommier Y, Mattern MR, Schwartz RE and Zwelling LA, Changes in deoxyribonucleic acid linking number due to treatment of mammalian cell nuclei with the intercalating agent 4′-(9-acridinylamino)methanesulfon-*m*-anisidide. *Biochemistry* **23**: 2927–2932, 1984.
36. Pommier Y, Schwartz RE, Zwelling LA and Kohn KW, Effects of DNA intercalating agents on topoisomerase II induced DNA strand cleavage in isolated mammalian cell nuclei. *Biochemistry* **24**: 6406–6410, 1985.
37. Pommier Y, Minford JK, Schwartz RE, Zwelling LA and Kohn KW, Effects of the DNA intercalators 4′(9-acridinylamino)methanesulfon-*m*-anisidide and 2-methyl-9-OH ellipticinium on topoisomerase II mediated DNA strand cleavage and strand passage. *Biochemistry* **24**: 6410–6416, 1985.
38. Auclair C, Pierré A, Voisin E, Pépin O, Cros S, Colas C, Saucier MP, Verschuere B, Gros P and Paoletti C, Physicochemical and pharmacological properties of the antitumor ellipticine derivative 2-(diethylamino-2-ethyl)9-hydroxy ellipticinium-chloride, HCl. *Cancer Res* **47**: 6254–6260, 1987.
39. Atwell G J, Rewcastle GW, Baguley B C and Denny WA, Potential antitumor agents: 48. 3′-dimethylamino derivatives of Ansacrine: redox chemistry and *in vivo* solid tumor activity. *J Med Chem* **30**: 652–658, 1987.
40. Atwell G J, Rewcastle GW, Baguley BC and Denny WA, Potential antitumor agents: 50. *In vivo* solid tumor activity of derivative of N-2-(diethylamino)ethyl acridine-4-carboxamide. *J Med Chem* **30**: 664–669, 1987.
41. Pommier Y, Covey J, Kerrigan D, Mattes W, Markovits J and Kohn KW, Role of DNA intercolation in the inhibition of purified mouse leukemia (L1210) DNA topoisomerase II by 9-aminoacridines. *Biochem Pharmacol* **36**: 3477–3486, 1987.