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Synthesis and biological properties of a new series of *N*-pyrido substituted tetrahydrocarbazoles

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Abstract

A series of methyl and ethyl quaternary pyridiniumtetrahydrocarbazoles was synthesized and studied in comparison with ellipticine, chosen as a reference. In general, their antiproliferative activity, tested in different biological substrates, appeared to be higher than that of the corresponding non-quaternarized compounds. This fact could be attributed to the introduction of a positive charge in the molecule, which can stabilize the molecular complex they form with DNA. In a prokaryotic system, the T2 bacteriophage, both quaternarized and non-quaternarized compounds inhibited its infectivity moderately, in a similar way to ellipticine. This effect seemed to be connected to a direct activity on the virions rather than on the indicator bacteria. In mammalian cells, the pyridiniumtetrahydrocarbazoles were more effective. In particular, they appeared to be very active in inhibiting DNA synthesis in Ehrlich ascites cells; some of them were as effective as ellipticine. However, pyridiniumtetrahydrocarbazoles were less active in comparison with ellipticine when their capacity for inhibiting the clonal growth in Chinese hamster ovary (CHO) cells was tested. A similar picture was obtained studying the formation of chromosome aberrations and of sister chromatid exchanges in the same cells. These different responses can be explained considering that the data on DNA synthesis reflect effects only on DNA replication within a short time, without considering any later consequences; on the contrary, in the long-term tests, other events, which lead to cell killing or genotoxicity, can take place. Pyridiniumtetrahydrocarbazoles damage DNA, inducing double-strand breaks efficiently. These observations, together with the data already obtained on unsubstituted derivatives, suggest the pyridiniumtetrahydrocarbazoles induce antiproliferative and genotoxic effects, very probably by inhibiting topoisomerase II.

Keywords: Pyridotetrahydrocarbazoles; T2 bacteriophage; DNA synthesis; Chromosome aberrations; Sister chromatid exchanges; Double-strand breaks

1. Introduction

We have already described the antiproliferative activity of some unsubstituted angular pyridotetrahydrocarbazoles (PyCs) and some aspects of their ability to interact with DNA [1]. These compounds are analogues of ellipticine, a well-known alkaloid with antitumor activity, which has been extensively modified to obtain efficient therapeutic agents [2–4]. These tetracyclic derivatives form molecular complexes with DNA in vitro and are able to damage DNA of mammalian cells by inducing double-strand breaks and DNA–protein cross-links [1]. The data obtained suggest that these ellipticine derivatives can interfere with the activity of topoisomerase II.

The presence of a positive charge on the nitrogen atom in the ellipticine derivatives increases the stability of their complex with DNA and, as a consequence, their biological activity [4,5]. In addition, the positive charge increases the water solubility of such compounds, which in general is very poor. In fact, the low solubility of ellipticine limits its clinical use [6]. Thus, several N_2-quaternarized derivatives have been designed. On the basis of these observations, we have prepared and studied the corresponding quaternary compounds of the PyCs as ethyl and methyl iodides (QPyCs; see Fig. 1). In this paper we describe their synthesis and some aspects of their antiproliferative and genotoxic activity.

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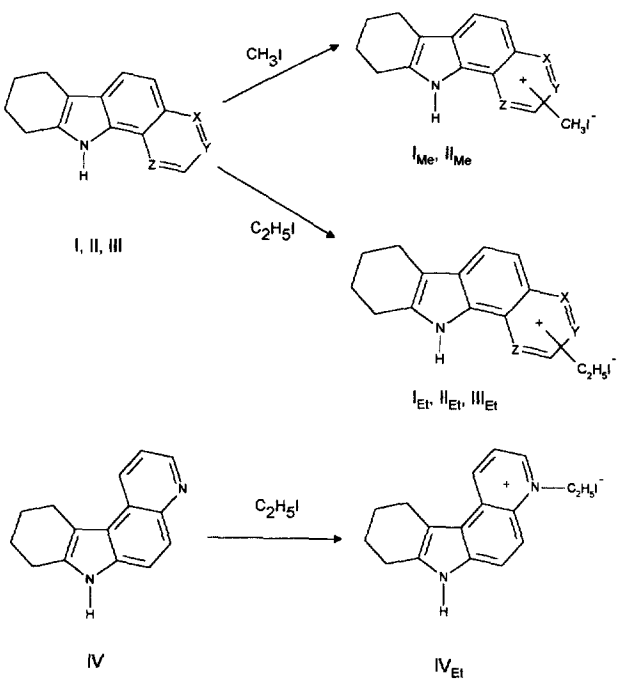

Fig. 1. Molecular structure of the compounds studied: **I**: X=N; Y=Z=C; 1*N*,5*H*-pyrido[2,3-a]-6,7,8,9-tetrahydrocarbazole; $\mathbf{I}_{Me}$ and $\mathbf{I}_{Et}$ are the methyl and ethyl iodide derivatives, respectively. **II**: Y=N; X=Z=C; 2*N*,5*H*-pyrido[2,3-a] 6,7,8,9-tetrahydrocarbazole; $\mathbf{II}_{Me}$ and $\mathbf{II}_{Et}$ are the methyl and ethyl iodide derivatives, respectively. **III**: Z=N; X=Y=C; 4*N*,5*H*-pyrido[2,3-a]-6,7,8,9-tetrahydrocarbazole; $\mathbf{III}_{Et}$ is the ethyl iodide derivative. **IV**: 4*N*,7*H*-pyrido[2,3-c]-8,9,10,11-tetrahydrocarbazole; $\mathbf{IV}_{Et}$ is the ethyl iodide derivative.

2. Materials and methods

2.1. Chemistry

Melting points were determined on a Gallenkamp MFB 595-010M/B capillary melting point apparatus, and are not corrected. Infrared spectra were recorded on a Perkin-Elmer 1760 IFTR spectrometer as potassium bromide pressed disks; values are expressed in cm^{-1}. ^1H NMR spectra were recorded on a Varian Gemini apparatus (200 MHz) using the indicated solvents: chemical shifts are reported (in ppm) downfield from tetramethylsilane as internal reference. *J* values are given in Hz. In the case of multiplets, the chemical shift quoted was measured from the approximate centre. Integrals corresponded satisfactorily to those expected on the basis of compound structure. Elemental analyses were performed in the Microanalytical Laboratory of the Department of Pharmaceutical Sciences of the University of Padua, using a Perkin-Elmer elemental analyser model 240B; results fell in the range $\pm 0.3\%$ with respect to calculated values. Column flash chromatography was carried out on Merck silica gel (250–400 mesh ASTM), and thin-layer chromatography (TLC) was performed on Merck silica gel F254 plates. Solutions were concentrated in a rotary evaporator under reduced pressure.

PyCs were synthesized as described in Ref. [1]. Ellipticine, used as a reference, was obtained from Sigma, St. Louis, MO, USA. Compounds were dissolved in dimethyl sulfoxide (4.5 mM) and the solutions were stored frozen in plastic tubes. Just before the experiments, a calculated amount of the drug solution was added to phosphate buffer saline (PBS) or to the growth medium containing cells to a final solvent concentration of 0.5%.

^3H-thymidine (4.77 TBq mM^{-1}) was obtained from Amersham International (UK). Proteinase K was obtained from Boehringer Mannheim (Germany). Tetrapropylammonium hydroxide, 1 M aqueous solution, was purchased from Sigma, St Louis, MO (USA).

2.2. T2 bacteriophage and Escherichia coli B48 inactivation

T2 bacteriophage was grown in *Escherichia coli* B48 cultures using nutrient broth. Virus suspensions (10^8–10^9 particles per ml) in 1 mM $MgSO_4$ containing the compound to be tested were incubated at 37°C for 60 min. After appropriate dilutions, virus titres were obtained according to Adams [7], using *E. coli* B48 as indicator bacteria.

The effect of PyCs and QPyCs on bacteria growth was determined by incubating aliquots of 10^4 *E. coli* B48 cells in their presence for two hours and then checking cell density in comparison with the untreated control and a positive one treated with chloramphenicol (2.5 μg ml^{-1}). Bacterial cell density was determined by light scattering at 440 nm.

2.3. DNA synthesis in Ehrlich cells

Ehrlich ascites tumor cells (Lettrè strain, from Heidelberg) were routinely transferred by injecting 2×10^6 cells per animal intraperitoneally into NCL mice. The tumor cells, collected on the 6th–7th days after transplant, were processed as already described [1,8].

2×10^7 cells per ml in Hank's solution containing the compound to be studied were incubated at 37°C for 60 min; then, ^3H-thymidine (40 kBq ml^{-1}), in a small volume of the same medium, was added and the cells were further incubated at 37°C for 30 min. The acid-insoluble fraction was precipitated by adding 5% ice-cold trichloroacetic acid and then filtered through Whatman GF/C filters. After several washings with cold 1% trichloroacetic acid, the filters were dried and counted. The results were calculated as the percentage of radioactivity incorporated into the DNA of untreated control cells (about 3-6 MBq); filtration were carried out by a Sample Manifold apparatus (Millipore, Bedford, MA, USA).

2.4. Chinese hamster ovary (CHO) cell culture

Cells were grown as monolayers in nutrient mixture F12 Ham medium (Sigma) supplemented with 10% foetal calf serum (Biological Industries, Kibbutz Beth Haemek, Israel) and the antibiotics penicillin (50 units ml^{-1}) and streptomycin (50 μg ml^{-1}). Trypsin (0.25%, Boehringer Mannheim) was routinely used for subcultures.

2.5. Chromosome preparations

The method used to assay chromosomal aberrations and sister chromatid exchanges (SCEs) has been previously described [9,10]. Briefly, suspensions of CHO cells (2×10^6 per ml) from log-phase cultures were diluted with growth medium in Petri dishes (100 mm in diameter). About 8×10^5 cells in 10 ml of medium were incubated for 24 h; then, the medium was replaced by a pre-warmed complete growth medium containing the compounds to be studied, and the cells were incubated further for 24 h. The drugs were removed by washing the cells twice with 10 ml of PBS. Colchicine (0.4 μg ml^{-1}, Merck, Darmstadt, Germany) was added before fixing the preparations, during the last 2 h.

All the cultures were treated with bromodeoxyuridine (BrdUrd) so that sister chromatid exchanges and chromosomal aberrations were scored in the same cell preparations: first- and second-division metaphases could be distinguished because double-stranded BrdUrd-substituted chromatids give a paler stain with Giemsa [11]. BrdUrd was added (9 mg l^{-1}) 24 h after cell seeding and the cells allowed to incorporate the analogue for 30 h. Metaphase cells were dislodged by gently pipetting the overlaying medium and pelleting the suspensions at 1000 rpm for 8 min. The cell pellet was suspended in 5 ml hypotonic buffer (0.075 M KCl) at 37°C for 10 min; then cells were fixed in cold methanol/acetic acid (3:1). Metaphase spreads were stained in 5% Giemsa.

2.6. Clonogenic survival

200 CHO cells were seeded in 60-mm dishes in growth medium (4 ml). Triplicate cultures were established for each treatment. After 24 h, the dishes were washed with PBS and the medium was replaced with fresh medium containing the compound to be studied and then incubated for 7 days at 37°C in the dark in a 5% carbon dioxide atmosphere. At this time the colonies were stained and counted, discounting colonies with less than 50 cells. The efficiency of the clonal growth, i.e. the ratio between the number of colonies formed and the number of cells seeded, was then calculated.

2.7. Detection of DNA damage

DNA damage was detected by neutral elution, carried out according to Kohn [12]. CHO cells in exponential growth were labelled by overnight incubation in the presence of ^{3}H-thymidine (7.4 KBq ml^{-1}). The radioactive medium was removed and replaced by a freshly grown one containing the compound to be studied or containing 0.5% dimethyl sulfoxide for the controls; in both cases, the cells were incubated for 3 h in the dark. The cells were then washed with PBS.

About $0.5–1.0 \times 10^6$ treated ^{3}H-cells were deposited on a polycarbonate filter (pores 2 μm in diameter, Nucleopore, Pleasanton, CA, USA) placed in a Swinnex-25 filter holder (Millipore, Bedford, MA, USA) and immediately lysed with 2% sodium dodecylsulfate, 0.1 M glycine, 0.025 M Na_2EDTA, pH 9.6, (5 ml); then, the solution was allowed to flow out by gravity. A 2 ml aliquot of the same solution containing 0.5 mg ml^{-1} of proteinase K was gently poured on the filter, followed by 40 ml of the eluting solution (tetrapropylammonium hydroxide, EDTA, 0.1% sodium dodecyl sulphate, pH 9.6). Elutions were carried out with a Gilson Minipuls peristaltic pump, at a flow of 0.03–0.04 ml min^{-1}. In the fractions, collected with a Gilson fraction collector (approximately 3.5 ml per fraction), the radioactivity was then determined.

The results obtained with the neutral elution assay were expressed as K values according to the formula [13]

$$K = \frac{V}{-\ln(r)}$$

where K is the average elution rate constant of DNA, r is the fraction of DNA retained on the filter and V is the eluted volume. The formula reflects the assumption of a first-order kinetics for DNA elution, as a first approximation [12].

2.8. Radiochemical determinations

The radioactivity measurements were performed using Instagel (Packard Instruments, Meriden, CT, USA) as a scintillation fluid. All determinations were carried out with a Packard A 300 CD spectrometer.

2.9. Calculations

The data relating to DNA synthesis were elaborated using probit analysis, thus obtaining the ID_{50}, i.e. the drug concentration which induces a 50% inhibition of DNA synthesis. As is known, this method calculates a regression using the probability units (probits), ascribing the highest statistical weight to the data approaching 50%.

3. Results

3.1. Chemical

3.1.1. Synthesis of methyl- and ethyl-pyridiniumtetrahydrocarbazole iodides

3.1.1.1. General procedure

300 mg (1.35 mm) of starting tetracyclic unsubstituted compound, dissolved in 4 ml of dimethylformamide (DMF), were treated with 13.7 mm of methyl or ethyl iodide and the mixture heated for 6–12 h at 60°–70°C. The resulting red solutions were evaporated to dryness, and the raw materials crystallized from methanol. The yields were in the range of 60–80%.

3.1.1.2. Features of the new quaternary compounds

1*N*-1-ethyl-5*H*-pyridinium[2,3-a](6,7,8,9)-tetrahydrocarbazole iodide ($\mathbf{I}_{Et}$): m.p. 239°C dec. (CH_3OH); ^{1}H NMR

(DMSO-d_6): δ 11.58 (1H, s, NH), 9.41 (1H, d, $J_{2,3}=8.4$ Hz, HC-2), 9 (1H, d, $J_{4,3}=5.9$ Hz, HC-4), 8.3 (1H, d, $J_{11,10}=9.1$ Hz, HC-10), 8.11 (1H, dd, $J_{3,4}=5.8$ and $J_{3,2}=8.4$ Hz, HC-3), 8.00 (1H, d, $J_{10,11}=9.1$ Hz, HC-10), 5.07 (2H, q, $J=7.2$ Hz, CH-2), 2.9 (2H, m, H_2C-6), 2.78 (2H, m, H_2C-9), 1.88 (4H, m, H_2C-7 and H_2C-8), 1.61 (3H, t, $J=7.1$ Hz, CH_3).

1*N*-1-methyl-5*H*-pyridinium[2,3-a](6,7,8,9)-tetrahydrocarbazole iodide ($\mathbf{I}_{Me}$): m.p. 250°C (CH_3OH); ^1H NMR (DMSO-d_6): δ as before, 4.53 (3H, s, CH_3).

2*N*-2-ethyl-5*H*-pyridinium[2,3-a](6,7,8,9)-tetrahydrocarbazole iodide ($\mathbf{II}_{Et}$): m.p. 259–261°C dec. (CH_3OH); ^1H NMR (methanol-d_3): δ 12.68 (1H, s, NH), 9.55 (1H, s, HC-1), 8.48 (1H, dd, $J_{3,2}=1.5$ and $J_{3,4}=7.0$ Hz, HC-3), 8.35 (1H, d, $J_{4,3}=7.0$ Hz, HC-4), 7.86 (1H, d, $J_{11,10}=8.7$ Hz, HC-11), 7.71 (1H, d, $J_{10,11}=8.6$ Hz, HC-10), 4.68 (2H, q, $J=8.3$ Hz, HC2), 2.89 (2H, t, H_2C-6), 2.73 (2H, m, H_2C-9), 1.94 (4H, m, H2C-7 and H_2C-8), 1.69 (3H, t, $J=7.2$ Hz, CH_3).

2*N*-2-methyl-5*H*-pyridinium[2,3-a](6,7,8,9)-tetrahydrocarbazole iodide ($\mathbf{II}_{Me}$): m.p. 260°C dec. (CH_3OH); ^1H NMR (DMSO-d_6): δ as before, 4.34 (3H, s, CH_3).

4*N*-4-ethyl-5*H*-pyridinium[2,3-a](6,7,8,9)-tetrahydrocarbazole iodide ($\mathbf{III}_{Et}$): m.p. 275–277°C dec. (CH_3OH); ^1H NMR (DMSO-d_6): δ 9.1 (2H, m, HC-1 and HC-3), 8.08 (1H, d, $J_{11,10}=8.6$ Hz, HC-11), 7.87 (1H, d, $J_{10,11}=8.6$ Hz, HC-10), 7.82 (1H, m, HC-2), 5.22 (2H, q, $J=7.4$ Hz, CH2), 3.04 (2H, m, H_2C-6), 2.89 (2H, m, H_2C-9), 2.0 (4H, m, H2C-7 and H_2C-8), 1.77 (3H, t, $J=7.4$ Hz, CH3).

4*N*-4-ethyl-7*H*-pyridinium[2,3-c](8,9,10,11)-tetrahydrocarbazole iodide ($\mathbf{IV}_{Et}$): m.p. 246–248°C dec. (CH_3OH); ^1H NMR (methanol-d_3): δ 12.19 (1H, s, NH), 9.31 (2H, m, HC-1 and HC-3), 8.28 (1H, m, HC-2), 8.24 (1H, d, $J_{5,6}=9.1$ Hz, HC-5), 8.06 (1H, d, $J_{6,5}=9.1$ Hz, HC-6), 5.09 (2H, q, $J=7.0$ Hz, CH_2), 3.10 (2H, m, H_2C-8), 2.89 (2H, m, H_2C-11), 1.92 (4H, m, H_2C-9 and H_2C-10), 1.62 (3H, t, $J=7.0$ Hz, CH_3).

3.2. Biological

3.2.1. T2 bacteriophage inactivation

Fig. 2 shows the results obtained studying T2 bacteriophage inactivation. PyCs exhibited a moderate activity. Quaternary derivatives appeared to be less active than the parent compounds. This behaviour is more evident with **I** and **III** compounds, which, contrary to $\mathbf{I}_{Me}$, $\mathbf{I}_{Et}$ and $\mathbf{III}_{Et}$, induced a marked reduction of the surviving fraction. Compounds **II**, **IV** and their derivatives were all weakly active. As already observed, ellipticine, used as a reference, showed a little activity on T2 bacteriophage [1]. We also performed some experiments to check the effect of both PyCs and QPyCs on *E. coli* B48, following the bacterial growth after adding the drugs to the cultures in the same range of concentrations used for T2 bacteriophage. All compounds, used at the same concentrations employed with T2 bacteriophage, failed to induce any significant decrease in bacterial growth.

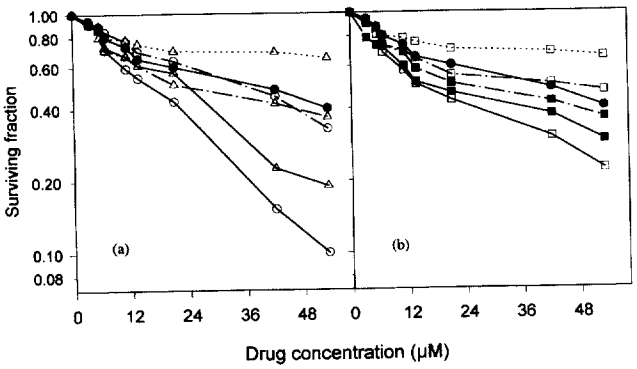

Fig. 2. T2 bacteriophage inactivation by pyridocarbazoles and their quaternary derivatives; virus particles were incubated at 37°C in the presence of increasing drug concentrations and then the number of plaque-forming units was determined. Unsubstituted pyridotetrahydrocarbazoles, ———; methyl iodide derivatives: · · ·; ethyl iodide derivatives: – · –. The symbols are: (a) △, **I**; ○, **III**; (b) □, **II**; ■, **IV**. Ellipticine: – ● –.

3.2.2. Inhibition of DNA synthesis in Ehrlich cells

The data relating to DNA synthesis in Ehrlich ascites tumor cells are reported in Fig. 3. In general, all quaternary derivatives are more effective than their parent compounds. The increase of the capacity of inhibiting DNA synthesis seems to be higher for derivatives arising from the less effective parent ones. The quaternarization of **I**, a compound which induced a moderate inhibition of DNA synthesis, yields very active derivatives; in particular, compound $\mathbf{I}_{Et}$ appeared to be as effective as ellipticine, used as a reference. Compounds $\mathbf{III}_{Et}$ and $\mathbf{IV}_{Et}$ are also much more active than their parent compounds. On the contrary, for **II** the differences with its quaternary derivatives are small. We elaborated these data by probit analysis, thus calculating the ID_{50}, i.e. the drug concentration capable of inducing a 50% inhibition of DNA synthesis. The results are reported in Table 1. Based on this approach, $\mathbf{I}_{Me}$ and $\mathbf{I}_{Et}$ are, respectively, three and nine times more active than **I**. An increase in activity was also obtained with $\mathbf{III}_{Et}$ and $\mathbf{IV}_{Et}$, which are, respectively, three and four times more effective than the parent compounds.

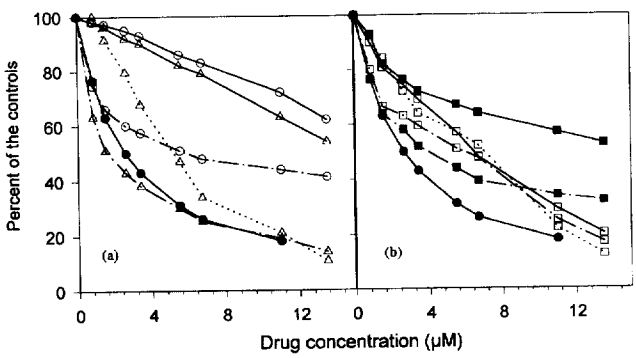

Fig. 3. Inhibition of DNA synthesis in Ehrlich cells by pyridotetrahydrocarbazoles and their quaternary derivatives; the tumor cells were incubated in the presence of increasing drug concentrations and of ^3H-thymidine and then the acid-insoluble radioactivity was determined. Unsubstituted pyridotetrahydrocarbazoles: ———; methyl iodide derivatives: · · ·; ethyl iodide derivatives: – · –. The symbols are: (a) △, **I**; ○, **III**; (b) □, **II**; ■, **IV**. Ellipticine: – ● –.

Table 1
Inhibition of DNA synthesis in Ehrlich cells expressed as ID_{50}

Comp.	$ID_{50} \pm$ S.E. (μM)	Relative activity	Comp.	$ID_{50} \pm$ S.E. (μM)	Relative activity
I	15.84 ± 3.0	1	**III**	19.95 ± 5.4	1
$\mathbf{I}_{Me}$	5.07 ± 1.7	3.12			
$\mathbf{I}_{Et}$	1.74 ± 2.31	9.10	$\mathbf{III}_{Et}$	6.19 ± 1.6	3.22
II	5.74 ± 1.6	1	**IV**	16.94 ± 2.5	1
$\mathbf{II}_{Me}$	5.29 ± 1.6	1.08			
$\mathbf{II}_{Et}$	4.32 ± 1.6	1.32	$\mathbf{IV}_{Et}$	3.83 ± 1.6	4.42
Ellipticine	2.52 ± 1.9	–			

ID_{50} (i.e. the drug concentration which induces a 50% inhibition of DNA synthesis) was determined by probit analysis; S.E. = standard deviation. The relative activities were calculated assuming as the unit the activity of the parent compound.

3.3. Clonogenic survival

The clonogenic assay was used to determine the cytotoxicity of PyCs and QPyCs in CHO cells. Fig. 4 shows the results using a 5 μM concentration. As observed in Ehrlich cells, the introduction of an alkyl chain at the nitrogen increases the activity of the drugs. In fact, derivatives of compounds **I**, **II** and **III** appeared to be much more active than the parent compounds; with $\mathbf{IV}_{Et}$ the increase is very much higher, about 14 times. Methyl derivatives showed a similar activity to the ethyl ones. In these experiments also, ellipticine was used as a reference; it appeared to be about two orders of magnitude more effective than all compounds.

3.4. Chromosome damage

Table 2 shows the types and frequencies of chromosomal aberrations observed in CHO cells incubated for 24 h in the presence of the drug, studied at 5 μM concentration. The number of chromosome aberrations is significantly increased by treatment with all tested compounds; however, the increase is much lower than that induced by ellipticine, used as a positive control for the response of our cell system. A detailed analysis of chromosome aberrations shows that single chromatid gaps, breaks and interchanges prevail; however, dicentric chromosomes, isochromatid breaks, chromosome and chromatid rings are also induced.

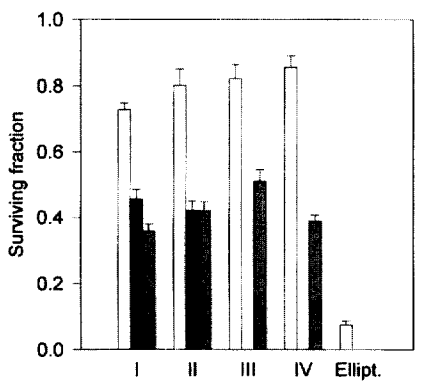

Fig. 4. Inhibition of clonal growth in CHO cells by pyridotetrahydrocarbazoles and their quaternary derivatives; the cells were incubated for 7 days in the presence of the drugs (5 μM) and their clonal growth ability was then determined. Unsubstituted pyridotetrahydrocarbazoles and ellipticine: white bars; methyl iodide derivatives: dark grey bars; ethyl iodide derivatives: light grey bars.

The response of CHO cells in the induction of SCEs is shown in Table 3. The frequencies of SCEs in second-division metaphases are significantly increased after treatment with all derivatives. In this case, also, QPyCs were more effective than their parent compounds. Treatment with ellipticine under the same experimental conditions (5 μM) did not allow the frequency of the SCEs to be analysed.

3.5. DNA damage

The induction of double-strand breaks (DSBs) in the DNA of CHO cells was studied by neutral elution after incubation (3 hours) in growth medium in the presence of the drug. The results are shown in Table 4. In every case, DNA from treated cells eluted faster than that from untreated controls; this result suggests that all derivatives are capable of inducing DSBs into DNA. In these experiments also, we have the same picture observed in the other tests. Quaternarized derivatives are more effective than the parent compounds; except for compound $\mathbf{I}_{Me}$, all other derivatives yielded faster elution rates. As expected, DNA from cells treated with ellipticine under the same experimental conditions, used as a reference also in these experiments, eluted very fast.

4. Discussion

We studied some aspects of the antiproliferative and genotoxic activity of a new group of QPyCs, which were prepared starting from the corresponding compounds already studied [1]. The quaternarization, accomplished by preparing the methyl and ethyl quaternary derivatives, was carried out with the aim of improving their antiproliferative activity. Actually, it is well known that the quaternarization of the N_2 of ellipticine improves the antiproliferative activity [5]. We studied the features of such compounds using various biological substrates and in comparison with ellipticine, chosen as a reference compound.

The most simple system used was T2 bacteriophage. The parent compounds appeared to be more effective in killing this virus than the quaternary ones, which were only moderately active. We also assayed the activity of such drugs on *E. coli* B48 (the indicator bacteria) using the same drug concentrations employed in the T2 bacteriophage test, but we found them entirely insensitive. Therefore, we can conclude that neither PyCs nor QPyCs act on the *E. coli* machinery. Scoring the number of infecting virions after the drug treatment according to the protocol used, the virus suspensions are extensively diluted before plating on indicator bacteria; this makes the drug concentration negligible. Therefore, the

Table 2
Chromosomal aberrations induced in CHO cells exposed to the tested compounds (5 μM)

	1st-division metaphases (%)	Percent of total aberrations	Chromosome aberrations		Chromatid aberrations				
			Dicentric	Ring	Gap	Break	Ring	Interchange	Isochromatid break
I	41	17	2	–	1	4	1	8	1
$\mathbf{I}_{Me}$	25	16	4	5	–	1	–	5	1
$\mathbf{I}_{Et}$	24	5	–	–	2	–	–	3	–
II	32	10	3	2	1	1	–	3	–
$\mathbf{II}_{Me}$	16	4	1	–	3	–	–	–	–
$\mathbf{II}_{Et}$	24	5	2	–	3	–	–	–	–
Ellipticine	96	93	12	1	11	23	3	36	7

For analysis 100 metaphases (1st- and 2nd-division metaphases) were counted. The total aberration frequency observed in untreated control cells was 17 (1st-division metaphases: 14 chromosome aberrations: 1 ring; chromatid aberrations: 1 gap, 5 breaks, 3 interchanges and 1 isochromatid break). The data reported in the table are the values observed in the treatments minus the corresponding value obtained in control cells. All compounds were tested at 5 μM.

Table 3
Sister chromatid exchanges (SCEs) induced in CHO cells exposed to the tested compounds (5 μM)

Treatment	Metaphases scored [a]	SCEs per metaphase (±S.E.)
I	59	1.38±0.2
$\mathbf{I}_{Me}$	75	2.29±0.3
$\mathbf{I}_{Et}$	76	1.86±0.4
II	68	1.44±0.2
$\mathbf{II}_{Me}$	73	1.42±0.2
$\mathbf{II}_{Et}$	73	1.96±0.25

[a] 2nd-division metaphases.
The SCEs observed in untreated control cells were 5.97±0.23. The data reported in the table are the values observed in the treatments minus the corresponding value obtained in control cells carried out in parallel. All compounds were tested at 5 μM. The treatment with ellipticine did not yield scorable second-generation metaphase cells.

Table 4
Detection of double-strand breaks in CHO by neutral elution exposed to the tested compounds (5 μM)

Comp.	Elution rate ($\times 10^{-2}$) (±S.E.)	Comp.	Elution rate ($\times 10^{-2}$) (±S.E.)
I	0.556±0.07	**III**	0.006±0.002
$\mathbf{I}_{Me}$	0.028±0.004		
$\mathbf{I}_{Et}$	0.577±0.05	$\mathbf{III}_{Et}$	0.019±0.002
II	0.272±0.094	**IV**	0.10±0.02
$\mathbf{II}_{Me}$	0.009±0.001		
$\mathbf{II}_{Et}$	0.51±0.11	$\mathbf{IV}_{Et}$	0.375±0.027
Ellipticine	2.75±0.26		

The elution rate observed in untreated control cells was $0.92\times10^{-2}\pm0.11$. The data reported in the table are the values observed in the treatments minus the corresponding value obtained in control cells carried out in parallel. All compounds were tested at 5 μM.

observed inhibition of T2 infectivity is certainly due to a direct action on the virions. Considering the properties of the non-quaternarized compounds [1], we can suppose the toxic effect is related to the capacity of these drugs to interact with DNA, but at present we have no evidence to support this hypothesis. On the other hand, as has been reported, ellipticine is also scarcely capable of inhibiting T2 growth [1].

Studying QPyCs in mammalian cells, we obtained a different picture: all derivatives exhibited strong antiproliferative and genotoxic effects. In each case, nitrogen quaternarization increased the activity. This effect is general, even if some differences, from a quantitative point of view, exist according to the compounds and to the tests considered. We did not observe significant differences between the methyl and ethyl derivatives. It seems simply that the quaternarization improves the activity, independently of the length of the chain introduced.

We tested the antiproliferative activity in mammalian cells using two different tests: the inhibition of DNA synthesis in Ehrlich cells and clonogenic survival of CHO cells cultivated in vitro. The first part is a short-term test (cells are kept in contact with the drug for only 1 hour), while in the clonogenic test, survival was determined after 7 days of incubation in the presence of the drug. Thus, the results obtained on DNA synthesis tell us about the effect of the drugs on DNA replication, without considering any later consequences, i.e. cell killing or genotoxic events. This explains some data obtained in Ehrlich cells, e.g. the observation of very high efficiencies of DNA synthesis inhibition, comparable with that obtained with ellipticine. The inhibition of incorporation of labelled thymidine is probably a consequence of the drug interaction with DNA. In fact, we have already observed that unsubstituted PyCs are capable of intercalating into DNA, forming a molecular complex [1]. Thus, it is reasonable to think that the quaternarized derivatives are also able to form a molecular complex, which can act on DNA synthesis in the same way. Thus, very probably, these data reflect the ability of such complexes to block the replication fork.

However, the block to DNA synthesis may be bypassed and DNA elongation may be restored. But, this may result in the formation of chromosome aberrations and SCEs; alternatively, it can yield cell killing. All of these consequences require a certain time to be induced, much longer than that employed in the test on DNA synthesis. In fact, in the long-

term tests, PyCs and their quaternarized derivatives appeared to be much less active than ellipticine. For example, in the clonogenic test, ellipticine is at least one order of magnitude more active than all these drugs. A similar conclusion can be drawn considering the data on chromosome aberrations and SCEs. In this connection the picture arising from the types of chromosome aberrations induced by QPyCs is comparable to that generated by ellipticine. A certain parallelism was also observed when studying the damage induced in DNA (double-strand breaks).

These observations, together with the data already obtained in the study on the unsubstituted PyCs [1], let us to suppose that, similarly to ellipticine, their quaternarized derivatives inhibit the activity of topoisomerase II, even if with a lower efficiency than the reference. This is in agreement with the observation that some N_2-quaternarized derivatives, such as N_2-methyl ellipticine, are characterized by low antitumor activities compared with the parent alkaloid. In contrast, the 9-hydroxylated drugs from the N_2-quaternarized series show significant antitumor activity which could be related to their ability to stabilize the cleavable complexes in the presence of topoisomerase II [14].

5. Conclusions

The introduction of a positive charge in the molecule by quaternarization of the pyridine nitrogen improves both the antiproliferative and genotoxic activity of PyCs, as already observed for ellipticine [4,5]. The different angular structure of these compounds certainly influences their activity, but the presence in the molecule of a positive nitrogen deeply changes its effect. At the same time the dimension of the alkyl chain linked to the nitrogen atom seems not to be determinant. However, this molecular structure, in spite of the presence of a non-aromatic ring, yields compounds capable of interfering with the activity of topoisomerase II, an important target of antitumor drugs. This leads us to investigate further the activity of other derivatives belonging to this class of compounds with the aim of obtaining new effective antiproliferative drugs.

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