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# Synthesis, DNA binding and in vitro antiproliferative activity of purinoquinazoline, pyridopyrimidopurine and pyridopyrimidobenzimidazole derivatives as potential antitumor agents

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**Abstract** – In the search for new antitumor agents, 8,10-dimethylpurino[7,8-a]quinazoline-5,9,11(6H,8H,10H)-triones **1**, 8,10-dimethylpyrido[2′,3′:4,5]pyrimido[1,2-f]purine-5,9,11(6H,8H,10H)-triones **2**, and 5,7-dihydro-5-oxopyrido[3′,2′:5,6]pyrimido[1,2-a]benzimidazoles **3**, a series of new planar heteropolycyclic compounds, were synthesized. The approach to understanding their structure–activity relationship involved a physico-chemical investigation of the binding process of these molecules to DNA, considered to be an important target for drug action, and an examination of their biological activity. Thermodynamic parameters of the DNA binding process, intrinsic binding constant and exclusion parameter were determined. The mode of interaction was additionally investigated by means of linear flow dichroism studies. Evaluation of the biological activity included cell growth inhibition in human tumoral cell lines and the ability to induce DNA cleavage in the presence of eukaryotic topoisomerase II. Only compounds of the purinoquinazoline series **1**, which are able to form a complex with DNA and to inhibit the topoisomerase II, show antiproliferative activity. © Elsevier, Paris

**purinoquinazolines / pyridopyrimidopurines / pyridopyrimidobenzimidazoles / antiproliferative properties / DNA binding agents**

## 1. Introduction

Most of planar polycyclic derivatives show an antiproliferative activity in vitro and some of them are important anticancer drugs [1, 2]. The biological activity of these compounds is related to their ability to form a complex with DNA, leading to cell death by inhibition of replicative enzymes and DNA repair systems or interfering with topoisomerases [3]. In the search for new compounds with an antiproliferative activity, in this paper we report the synthesis of angular derivatives containing the purine or benzimidazole nuclei as a part of their tetracyclic system. Three classes of derivatives were obtained and are represented in *figure 1*: 8,10-dimethylpurino[7,8-a]quinazoline-5,9,11(6H,8H,10H)-triones **1a–d**, 8,10-dimethylpyrido[2′,3′:4,5]pyrimido[1,2-f]purine-5,9,11(6H,8H,10H)-triones **2a–d** and 5,7-dihydro-5-oxopyrido[3′,2′:5,6]pyrimido[1,2-a]benzimidazoles **3b–d**. All compounds are characterized by having an alkylamino substituted side chain, since this has frequently been associated with improved DNA binding properties. An evaluation of their antiproliferative activity was carried out using two human tumor cell lines. Furthermore, to better understand the mode of action of these molecules, a physical–chemical characterization of the DNA binding process along with their ability to stimulate topoisomerase II-mediated DNA cleavage, was undertaken. This approach appeared to represent a reasonable way to gain insight into the structure–activity relationships for these classes of molecules.

## 2. Chemistry

The intermediates for the synthesis of alkylated compounds **1a–d** and **2a–d** were represented by the hetero-

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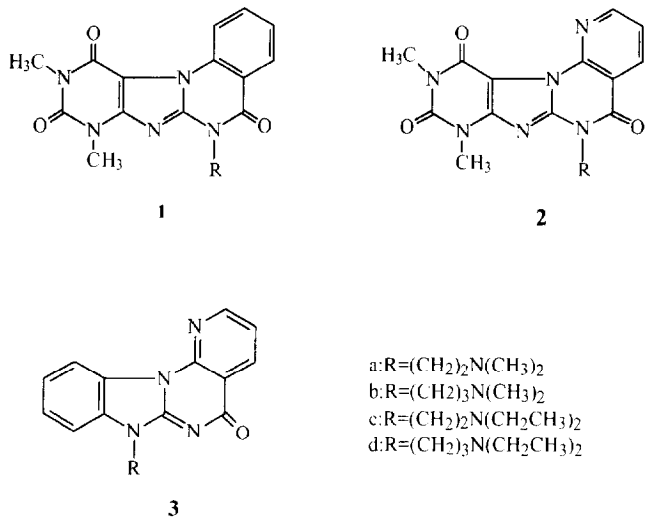



**Figure 1**. Chemical structure of the compounds examined.

cycles 8,10-dimethylpurino[7,8-a]quinazoline-5,9,11 (6H,8H,10H)-trione **4** (*figure 2*), previously described by us [4], and by 8,10-dimethylpyrido[2′,3′:4,5]pyrimido [1,2-f]purine-5,9,11(6H,8H,10H)-trione **5**, which represents a new heterocyclic ring system never described before.

Compound **5** was prepared by condensation between 8-aminotheophylline [5, 6] with 2-chloronicotinic acid, performed in Ullmann conditions, in the presence of anhydrous potassium carbonate and a catalytic amount of cuprous bromide (*figure 2*). Compound **5** was also obtained, even if with a low yield, heating the same reagents with PPA at 200 °C for 6 h. Analytical IR, $^1$H-NMR and mass ($M^+$, *m/z* 298) spectral data were consistent with the proposed structure. The assignment of an angular structure to compound **5** was supported principally by considerations emerging from an examination of the UV spectra. In fact, the UV spectra of **5**, which showed an absorption maximum at $\lambda$ = 220 nm, $\varepsilon$ = 16267, appeared to be closely related to that of the angular 8,10-dimethylpurino[7,8-a]quinazoline-5,9,11 (6H,8H,10H)-trione **4** [4] ($\lambda_{max}$ = 222 nm, $\varepsilon$ = 29088), and different from that of the linear tetracyclic 1,3-dimethylpurino[8,7-b]quinazoline-2,4,6-(1H,3H,11H)-trione [4] ($\lambda_{max}$ = 245 nm, $\varepsilon$ = 21805) (unpublished results). Although the cromophores of compounds **4** and **5** were different, these data supported the angular structure proposed for **5**. Moreover, cyclization on the N(9) of theophylline is not likely, due to the steric hindrance of the 3-methyl group [7].

By reaction with dimethyl sulfate in acetonitrile solution, in the presence of potassium carbonate [8], compound **5** afforded **6** in a good yield. Further evidence for the structure of **5** derived from an examination of the $^1$H-NMR spectra of compound **6**. In fact, in the previous paper [4] it was observed that the methyl group in the 11 position of the linear 1,3,11-trimethylpurino[8,7-b]

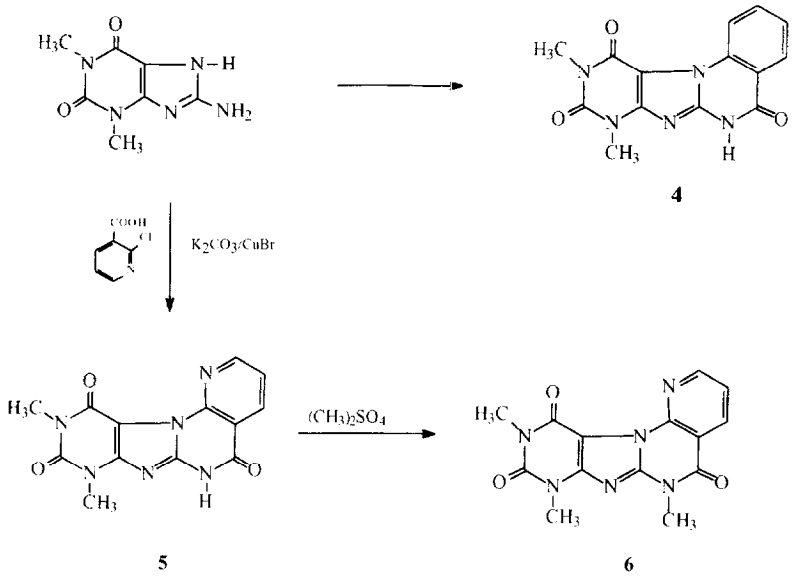



**Figure 2.**

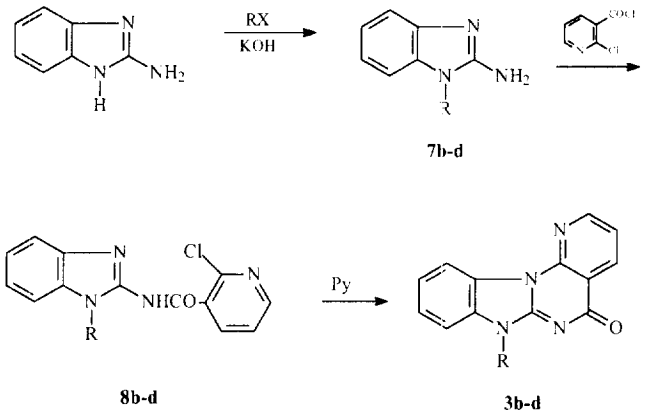



**Figure 3.**

quinazoline-2,4,6-(1H,3H,11H)-trione resonates at δ 3.96, while the corresponding methyl in the 6 position of the angular heterocyclic 6,8,10-trimethylpurino[7,8-a]quinazoline-5,9,11(6H,8H,10H)-trione resonates at δ 3.67. The 6-methyl of product **6** resonating at δ 3.67 confirmed the proposed angular structure for **5**.

The heterocyclic compounds **4** and **5** were then converted to the desired aminoalkyl derivatives **1a–d** and **2a–d**, respectively, by reaction with the appropriate dialkylaminoalkyl chloride hydrochlorides in DMF solution, in the presence of sodium hydride. All products **1a–d** and **2a–d** were purified by recrystallization and their structures were confirmed by IR, $^1$H-NMR and elemental analyses (*tables I* and *II*).

The 1-aminoalkyl-2-aminobenzimidazoles **7b–d**, which were conveniently obtained from 2-aminobenzimidazole using experimental conditions similar to those described in literature [12] for 1-alkyl compounds, represented the starting products employed for the preparation of the 7-substituted-5,7-dihydro-5-oxopyrido[3′,2′:5,6]pyrimido[1,2-a]benzimidazoles **3b–d.** The appropriate 1-alkyl-2-aminobenzimidazoles **7b–d** were allowed to react with 2-chloropyridine-3-carbonyl chloride in anhydrous THF, in the presence of triethylamine, to obtain the amides **8b–d** in good yields, which were then cyclized to the desired heterocyclic derivatives **3b–d** by reflux in pyridine for 48 h (*figure 3*). Physical and spectral data are reported in *table III*.

## 3. Results and discussion

### *3.1. Biological evaluation*

All substances were evaluated for cytotoxic potency in vitro using the human promyelocytic leukemia (HL-60) and cervix adenocarcinoma (HeLa) cell lines. Doxorubicin and ellipticine were used as reference compounds. The data are shown in *table IV* as $IC_{50}$ values. The purinoquinazoline derivatives **1** showed a significant potency: in particular, compounds **1a** and **1c** exhibited $IC_{50}$ values lower to that of ellipticine in HL-60 cells, and comparable in HeLa cells. The other two series of synthesized compounds **2** and **3** proved to be practically devoid of biological activity.

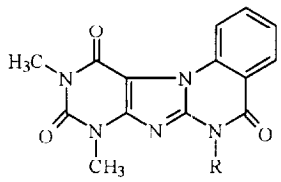
### *3.2. DNA binding studies*

A first indication of the ability of the compounds tested to interact with DNA was obtained by means of denaturation studies. Relative binding behavior was determined on the basis of the thermal stabilization of double-stranded DNA induced by each ligand compound. The extent to which ligands stabilize the helix coil transition can be taken as an overall indication of the strength of binding [13]. However, it should be stressed that $\Delta T_m$ values can provide only a qualitative ranking order for DNA interaction. The data shown in *table V* show that the purinoquinazolines **1a–d** and pyridopyrimidobenzimidazoles **3b–d** produced fairly high $\Delta T_m$ values, indicating a significant stabilization of the double helix. On the contrary, very low $\Delta T_m$ values were observed for pyridopyrimidopurine derivatives **2a–d**. The nature of the chromophore, the position and the characteristics of substituents modulated binding ability for each class of compounds.

A second indication about the complex formed by the macromolecule and the compounds tested was obtained by performing circular dichroism experiments.

*Figure 4* shows a representative CD spectrum of solutions of compound **1b** at different [DNA]/[Drug]

**Table I.** Physical and spectral data of compounds **1a–d**.



| No. | R | Yield (%) | M.p., °C (recryst. solv.) | $^1$H-NMR, DMSO-$d_6$ (δ ppm) | MS, *m/z* ($M^+$) | Formula |
|---|---|---|---|---|---|---|
| **1a** | $(CH_2)_2N(CH_3)_2$ | 85 | 209–211 (DMF) | 2.25 (s, 6H, $N(CH_3)_2$), 2.69 (t, 2H, $CH_2CH_2$ $N(CH_3)_2$), 3.35 (s, 3H, 10-$CH_3$), 3.53 (s, 3H, 8-$CH_3$), 4.39 (t, 2H, $CH_2CH_2N(CH_3)_2$), 7.58 (ddd, 1H, H-2), 7.94 (ddd, 1H, $J_{2,3}$ = 7.17 Hz, H-3), 8.26 (dd, 1H, $J_{1,2}$ = 7.96 Hz, $J_{1,3}$ = 1.55 Hz, H-1), 9.66 (dd, 1H, $J_{3,4}$ = 8.62 Hz, $J_{2,4}$ = 0.91 Hz, H-4). | 368 | $C_{18}H_{20}N_6O_3$ |
| **1b** | $(CH_2)_3N(CH_3)_2$ | 33 | 178–180 (DMF) | 1.92 (m, 2H, $CH_2CH_2CH_2N(CH_3)_2$), 2.16 (s, 6H, $N(CH_3)_2$), 2.37 (t, 2H, $CH_2CH_2CH_2N(CH_3)_2$), 3.33 (s, 3H, 10-$CH_3$), 3.51 (s, 3H, 8-$CH_3$), 4.31 (t, 2H, $CH_2CH_2CH_2N(CH_3)_2$), 7.55 (ddd, 1H, H-2), 7.89 (ddd, 1H, $J_{2,3}$ = 7.17 Hz, H-3), 8.23 (dd, 1H, $J_{1,2}$ = 7.91 Hz, $J_{1,3}$ = 1.57 Hz, H-1), 9.60 (dd, 1H, $J_{3,4}$ = 8.62 Hz, $J_{2,4}$ = 0.91 Hz, H-4). | 382 | $C_{19}H_{22}N_6O_3$ |
| **1c** | $(CH_2)_2N(CH_2CH_3)_2$ | 52 | 198–200 (DMF) | 0.94 (t, 6H, $N(CH_2CH_3)_2$), 2.55 (q, 4H, $N(CH_2CH_3)_2$), 2.80 (t, 2H, $CH_2CH_2N(C_2H_5)_2$), 3.32 (s, 3H, 10-$CH_3$); 3.50 (s, 3H, 8-$CH_3$), 4.31 (t, 2H, $CH_2CH_2N(C_2H_5)_2$), 7.56 (ddd, 1H, H-2), 7.90 (ddd, 1H, $J_{2,3}$ = 7.19 Hz, H-3), 8.23 (dd, 1H, $J_{1,2}$ = 7.92 Hz, $J_{1,3}$ = 1.57 Hz, H-1), 9.60 (dd, 1H, $J_{3,4}$ = 8.62 Hz, $J_{2,4}$ = 0.89 Hz, H-4). | 410 | $C_{20}H_{24}N_6O_3$ |
| **1d** | $(CH_2)_3N(CH_2CH_3)_2$ | 40 | 162–166 (DMF) | 0.94 (t, 6H, $N(CH_2CH_3)_2$), 1.92 (m, 2H, $CH_2CH_2CH_2N(C_2H_5)_2$), 2.42–2.57 (m, 6H, $CH_2CH_2CH_2N(CH_2CH_3)_2$), 3.31 (s, 3H, 10-$CH_3$), 3.49 (s, 3H, 8-$CH_3$), 4.29 (t, 2H, $CH_2CH_2CH_2N(C_2H_5)_2$), 7.54 (ddd, 1H, H-2), 7.88 (ddd, 1H, $J_{2,3}$ = 7.18 Hz, H-3), 8.22 (dd, 1H, $J_{1,2}$ = 7.91 Hz, $J_{1,3}$ = 1.60 Hz, H-1), 9.57 (dd, 1H, $J_{3,4}$ = 8.62 Hz, $J_{2,4}$ = 0.84 Hz, H-4). | 409 | $C_{21}H_{26}N_6O_3$ |

ratios. Measurements in the DNA absorption region, for compounds **1a–d** and **3b–d**, showed that the positive double helix band increased rapidly as the DNA-drug ratio decreased, indicating a significant interaction with the macromolecule. In addition, a weak induced circular dichroism was observed in the chromophore absorption region; this dichroic band, negative for all the compounds examined, suggests a common geometry of interaction. No appreciable shifts were observed in the negative or positive band induced by the drug.

Compounds **2a–d** had no effect on the DNA CD spectrum either in the DNA or in the chromophore absorption regions.

To understand better the geometry of the DNA-drug complex, linear flow dichroism measurements were performed. *Figure 5* shows representative absorption (A), linear dichroism (LD) and reduced linear dichroism ($LD_r$) spectra of a solution of compound **1d,** in the presence of DNA. Linear flow dichroism spectra showed a negative band in the chromophore absorption region, indicating an intercalative mode of binding for compounds **1a–d** and **3b–d**. Moreover, the $LD_r$ values determined at the wavelength region where the added drug absorbs, were close to that observed for the DNA absorption band (260 nm). From these results and using equation (2) (see experimental protocols), it was possible to estimate the average

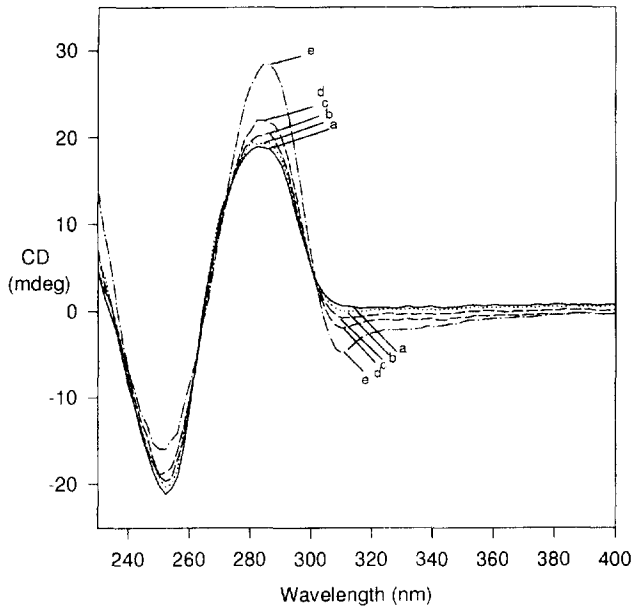



**Figure 4**. Circular dichroism spectra for compound **1b** at various [DNA]/[Drug] ratios: a = 0; b = 50; c = 25; d = 12.5; e = 5; [DNA] = $2.3 \times 10^{-4}$ M.

angle α, between the drug transition dipole moment and the orientation direction, represented by the helix axis [14, 15]. The value calculated is equal to 90° for all the compounds examined (*table V*). No reliable data were obtained for compounds **2a–d,** due to their low affinity for the macromolecule.

Thermodynamic parameters of binding with the nucleic acid were obtained for compounds **1a–d** and **3b–d** by fluorimetric titration (*table V*). Formation of the DNA-drug complex causes almost total quenching of the fluorescence signal. The binding isotherm obtained, represented as Scatchard plots [16], allowed us to determine, by non-linear fitting, using the McGhee–vonHippel equation, the intrinsic binding constant $K_i$, and the exclusion parameter $n$ [17]. *Figure 6* shows representative binding curves for compounds **1c** and **3b**.

As shown in *table V*, in conditions of low ionic strength, the compound in the purinoquinazoline series **1** bearing a dimethylaminoethyl side chain (compound **1a**) exhibited the highest affinity toward DNA. The affinity decreased slightly for the analogous compounds having a diethylaminoethyl, dimethylaminopropyl or diethylaminopropyl side chains (compounds **1c**, **1b** and **1d**, respectively). For compounds **3b–d** analogous values of $K_i$ were observed. Replacement of the angular benzene ring with a pyridine nucleus abolishes fluorescence properties, hence it was not possible to evaluate the binding con-

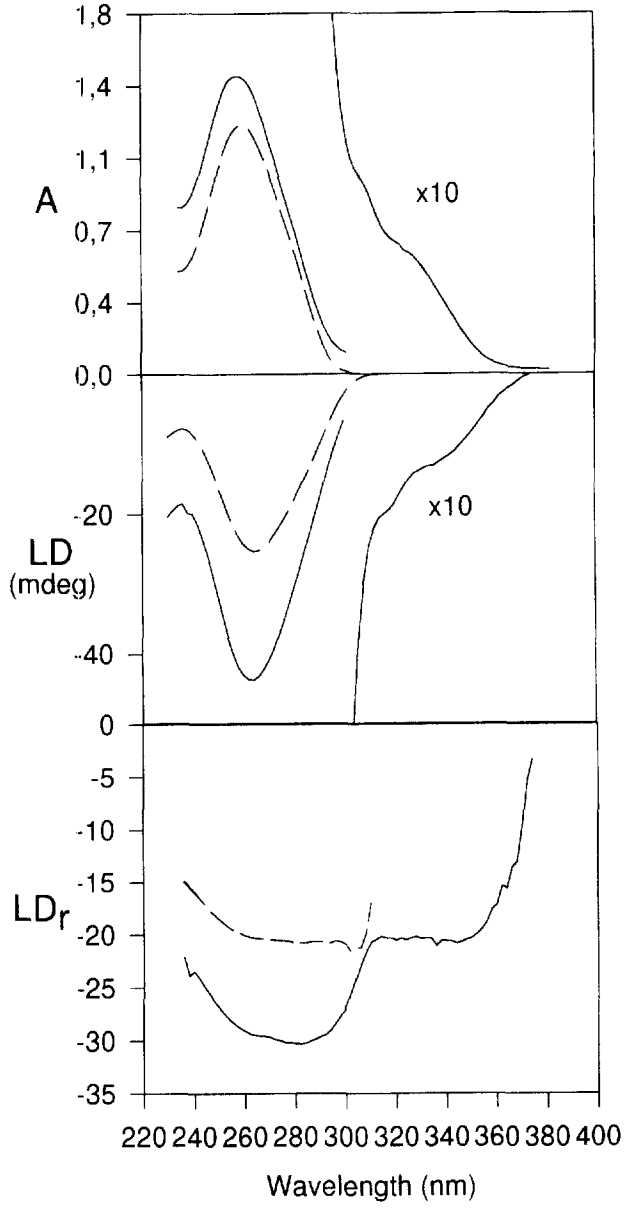



**Figure 5**. Absorbance (A), linear dichroism (LD) and reduced linear dichroism ($LD_r$) spectra of compound **1d** in the presence of DNA at a [DNA]/[Drug] ratio = 12.5 (solid curves). Absorbance, linear dichroism and reduced linear dichroism spectra of free DNA (broken curves).

stants for compounds **2a–d**. The $K_i$ calculated are in the range of $1.0–1.6 \times 10^{-5}$ molar base pairs and the exclusion parameters $n$ are in the range of 4.5–7.4 base pairs. These values indicate an interaction with DNA less effective than that of true intercalators such as doxorubicin or ellipticine [18, 19].

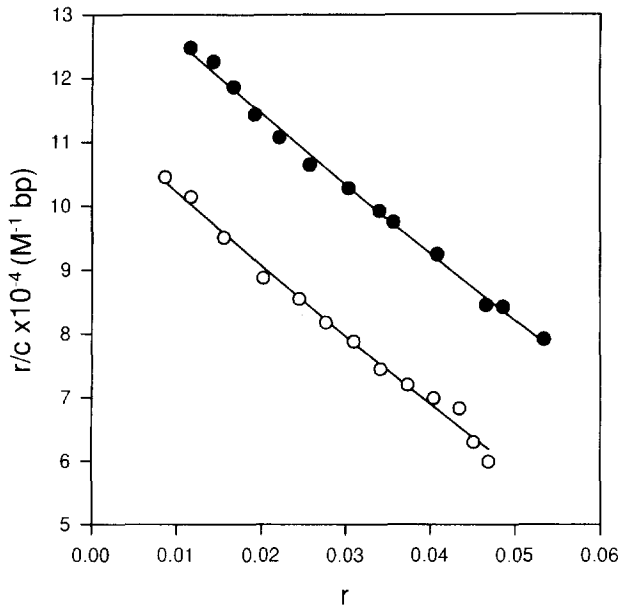



**Figure 6.** Scatchard plots for the binding of compounds **1c** (filled circles) and **3b** (open circles) to DNA at 25 °C, obtained by fluorescence titrations. Smooth curves are the least-squares fit to the equations of McGhee and von Hippel [17].

Additional experiments were performed to investigate a possible selectivity of the purinoquinazoline derivatives **1** toward DNA sequences. Fluorimetric titrations were performed using DNA from various sources as well as synthetic polynucleotides. The results are summarized in *table VI*, which clearly shows the preference of these series of compounds for GC-rich sequences, typical of many intercalating agents [20–22]. It may be observed that the affinity towards the GC pairs decreases with the lengthening and the dimension of the basic nitrogen substituents of the side chain, as shown by the ratio $K_{AT}/K_{GC}$. It is known that the presence of a cationic charge, either on the chromophore itself or on the side chain, makes an important contribution to the stabilization of the drug-DNA complex. In this case, moving the positive charge away from the chromophore, the distribution occurs more evenly along the DNA chain. Further studies (e.g. footprinting) are needed to examine the sequence specificity in the binding of these derivatives to DNA.

### *3.3. Topoisomerase II-mediated cleavable complex*

DNA topoisomerases are a unique class of enzymes that change the topological state of DNA by breaking and re-uniting the phosphodiester backbone of the nucleic acid. Some antitumor drugs interfere with the activity of mammalian DNA topoisomerase II by trapping a key covalent reaction intermediate, called the cleavable complex. The treatment of this complex with a strong protein denaturant such as SDS results in DNA breakage. It is generally recognized that the effects of drug-stabilized topoisomerase cleavable complex are most probably responsible for the cytotoxicity and antitumor activity of these compounds [23, 24].

*Figure 7*, lanes C–K, illustrates the effect on human topoisomerase II activity of compounds **1a**, **1c** and **3b** respectively, at three different concentrations, as indicated. The new purinoquinazolines, **1a** and **1c**, clearly promote DNA cleavage to the same extent at the higher concentration taken into consideration (50 μM). Conversely, the pyridopyrimidobenzimidazole **3b**, despite a behaviour similar to that of the purinoquinazolines with respect to affinity toward DNA, does not interfere with topoisomerase II activity. This fact could explain its lower biological activity on cell lines.

The other two compounds **1b** and **1d**, belonging to the purinoquinazolines series, are also able to inhibit topoisomerase II activity at the same concentration as the analogous **1a** and **1c** (lanes M and N, respectively). None of the pyridopyrimidobenzimidazoles **3b–d** produced any cleavage site, even at the highest concentration (100 μM), (lanes O–Q). Lane L shows the effect of doxorubicin, taken as the reference drug.

## 4. Conclusions

On the basis of the above results, we concluded that only compounds of the purinoquinazoline series **1** retained a significant cytotoxic activity in vitro against the solid tumor (HeLa) and promyelocytic human leukemia (HL-60) cell lines. The optimal length of the dialkylamino alkyl side chain in position 6 was two methylene units for both tumoral cell lines tested. Also the presence of two methyl or ethyl substituents in the basic nitrogen of the side chain influences cytotoxicity: the compound with two methyl substituents, **1a**, exerts a cytotoxic activity three times higher than the analogous one with two ethyl substituents, **1c**. The presence of three methylene units in the dialkylamino alkyl side chain in position 6 (**1b**, **1d**) diminishes the biological activity, and in this case the substituents in the basic nitrogen of the side chain did not influence the cytotoxicity appreciably. Replacement of the angular benzene ring with a pyridine nucleus (series **2**) abolished cytotoxic properties. This substitution also produced a dramatic decrease in the DNA binding ability, as shown by $\Delta T_m$ values and circular and linear dichroism data. For these compounds

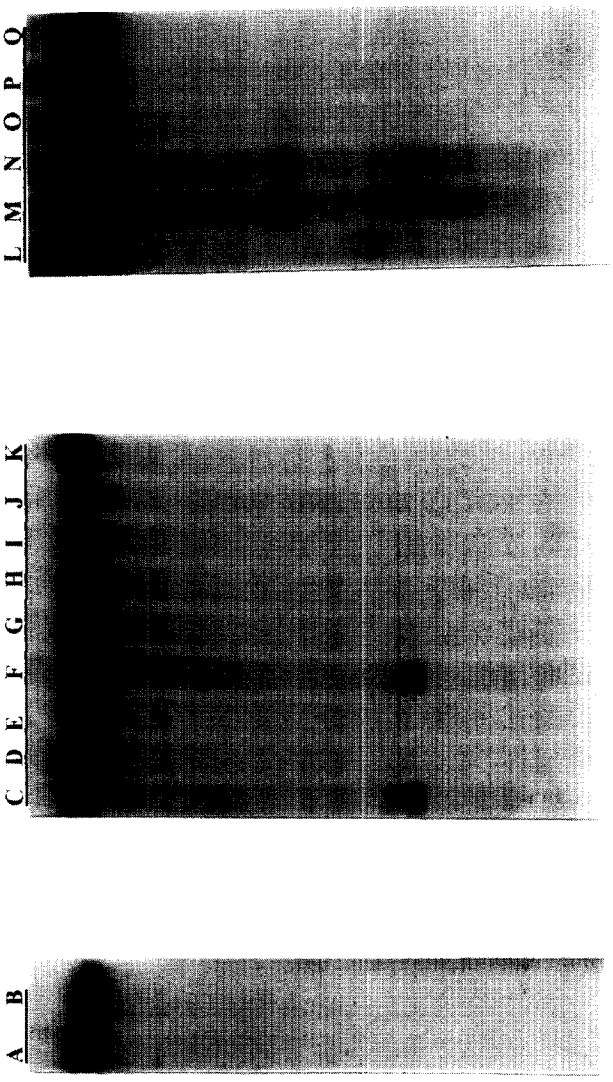



**Figure 7.** Topoisomerase II-mediated cleavage of pBR322 DNA. Cleavage reactions were carried out as described in the experimental protocols. Lane A: pBR322 DNA control (no enzyme); lane B: pBR322 DNA and topoisomerase II (no drug); lanes C–E: same as lane B, with 50, 5 and 0.5 μM **1a**, respectively; lanes F–H: same as lane B, with 50, 5 and 0.5 μM **1c**, respectively; lanes I–K: same as lane B, with 50, 5 and 0.5 μM **3b**, respectively; lane L: same as lane B with 0.2 μM doxorubicin; lanes M and N: same as lane B, with 50 μM **1b** and **1d**, respectively; lanes O–Q: same as lane B, with 100 μM **3b**, **3c** and **3d**, respectively.

it appeared that the lack of biological activity was correlated with a low affinity for DNA. The last series of derivatives considered, **3b–d,** was also practically devoid of any biological activity, although the values of the physico-chemical parameters were comparable to those of the active compounds.

The ability to induce topoisomerase II-associated DNA breaks was evaluated for the two series of compounds (**1** and **3**) that were able to form a molecular complex with DNA. Only the compounds belonging to series **1** promote DNA cleavage. The pyridopyrimidobenzimidazoles **3b–d** do not exert any effect and this could justify their biological inactivity.

These results confirm that the ability to inhibit topoisomerase II requires specific structural conditions. This fact underlines the complexity of the interactions which occur in the formation of the ternary complex and the key biological role of this nuclear enzyme.

## 5. Experimental protocols

### *5.1. Chemistry*

Melting points were determined using a Reichert Köfler hot-stage apparatus and are uncorrected. Infrared spectra were obtained on a PYE/UNICAM mod. PU 9561 spectrophotometer in Nujol mulls. Nuclear magnetic resonance spectra were recorded on a Bruker AC 200 spectrometer, unless otherwise reported, using tetramethylsilane (TMS) as an internal standard. Mass spectra were obtained on a Hewlett-Packard 5988 A spectrometer using a direct injection probe and an electron beam energy of 70 eV. Magnesium sulfate was always used as the drying agent. Evaporation were made in vacuo (rotating evaporator). Analytical TLC was carried out on Merck 0.2 mm precoated silica gel aluminium sheets (60 F-254). Elemental analyses were performed by our Analytical Laboratory and agreed with theoretical values to within ±0.4%.

#### *5.1.1. 8,10-Dimethylpyrido[2',3':4,5]pyrimido[1,2-f]purine-5,9,11(6H,8H,10H)-trione* **5**

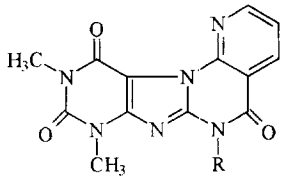
*Procedure a*: A suspension of 8-aminotheophylline (1.95 g, 10 mmol), 2-chloronicotinic acid (1.60 g, 10 mmol), anhydrous potassium carbonate (1.66 g, 12 mmol) and 0.08 g of cuprous bromide in 5 mL of nitrobenzene was heated at 220 °C for 16 h. After cooling, the reaction mixture was diluted with water and acidified with concentrated hydrochloric acid. The crude product was collected and purified by sublimation (270–300 °C, 0.4 mm Hg) and successive recrystallization from DMF, to give 1.10 g (37% yield) of pure **5**, m.p. > 300 °C. $^1$H-NMR (DMSO-$d_6$) δ: 3.30 (s, 3H, 10-$CH_3$), 3.49 (s, 3H, 8-$CH_3$), 7.60 (dd, 1H, H-3), 8.55 (dd, 1H, $J_{3,4}$ = 7.82 Hz, H-4), 8.86 (dd, 1H, $J_{2,3}$ = 4.70 Hz, $J_{2,4}$ = 1.89 Hz, H-2); MS: *m/z* 298 ($M^+$); Anal. $C_{13}H_{10}N_6O_3$ (C,H,N).

*Procedure b*: A mixture of 8-aminotheophylline (1.0 g, 5 mmol), 2-chloronicotinic acid (0.80 g, 5 mmol) and 12 g of polyphosphoric acid was heated at 200 °C for 6 h. After cooling, the reaction mixture was diluted with ice-cold water and supplemented with $NH_4OH$ aqueous solution until the pH was 1. The solid was collected, washed with water and purified by recrystallization from DMF to give 0.086 g (5.8% yield) of cyclized compound **5**.

#### *5.1.2. 6,8,10-Trimethylpyrido[2',3':4,5]pyrimido[1,2-f]purine-5,9,11(6H,8H,10H)-trione* **6**

A solution of dimethylsulfate (0.38 mL, 4 mmol) in acetone (2 mL) was added dropwise, at 0 °C, to a suspension of **5** (0.300 g,

**Table II**. Physical and spectral data of compounds **2a–d**.



| No. | R | Yield (%) | M.p., °C (recryst. solv.) | $^1$H-NMR, DMSO-$d_6$ (δ ppm) | MS, *m/z* ($M^+$) | Formula |
|---|---|---|---|---|---|---|
| **2a** | $(CH_2)_2N(CH_3)_2$ | 26 | 230–232 (DMF) | 2.29 (s, 6H, $N(CH_3)_2$), 3.21 (t, 2H, $CH_2CH_2$ $N(CH_3)_2$), 3.31 (s, 3H, 10-$CH_3$), 3.52 (s, 3H, 8-$CH_3$), 4.38 (t, 2H, $CH_2CH_2N(CH_3)_2$), 7.63 (dd, 1H, H-3), 8.60 (dd, 1H, $J_{3,4}$ = 7.86 Hz, H-4), 8.89 (dd, 1H, $J_{2,3}$ = 4.69 Hz, $J_{2,4}$ = 1.88 Hz, H-2). | 369 | $C_{17}H_{19}N_7O_3$ |
| **2b** | $(CH_2)_3N(CH_3)_2$ | 27 | 221–224 (DMF) | 1.93 (m, 2H, $CH_2CH_2CH_2N(CH_3)_2$), 2.16 (s, 6H, $N(CH_3)_2$), 2.39 (t, 2H, $CH_2CH_2CH_2N(CH_3)_2$), 3.31 (s, 3H, 10-$CH_3$), 3.53 (s, 3H, 8-$CH_3$), 4.31 (t, 2H, $CH_2CH_2CH_2N(CH_3)_2$), 7.63 (dd, 1H, H-3), 8.61 (dd, $J_{3,4}$ = 7.84 Hz, H-4), 8.89 (dd, 1H, $J_{2,3}$ = 4.71 Hz, $J_{2,4}$ = 1.89 Hz, H-2). | 383 | $C_{18}H_{21}N_7O_3$ |
| **2c** | $(CH_2)_2N(CH_2CH_3)_2$ | 34 | 235–237 (DMF) | 0.96 (t, 6H, $N(CH_2CH_3)_2$), 2.56 (q, 4H, $N(CH_2CH_3)_2$), 2.80 (t, 2H, $CH_2CH_2N(C_2H_5)_2$), 3.30 (s, 3H, 10-$CH_3$), 3.51 (s, 3H, 8-$CH_3$), 4.31 (t, 2H, $CH_2CH_2N(C_2H_5)_2$); 7.63 (dd, 1H, H-3), 8.60 (dd, $J_{3,4}$ = 7.86 Hz, H-4), 8.88 (dd, 1H, $J_{2,3}$ = 4.69 Hz, $J_{2,4}$ = 1.88 Hz, H-2). | 396 | $C_{19}H_{23}N_7O_3$ |
| **2d** | $(CH_2)_3N(CH_2CH_3)_2$ | 42 | 223–225 (DMF) | 0.93 (t, 6H, $N(CH_2CH_3)_2$), 1.92 (m, 2H, $CH_2CH_2CH_2N(C_2H_5)_2$), 2.42–2.58 (m, 6H, $CH_2CH_2CH_2N(CH_2CH_3)_2$), 3.32 (s, 3H, 10-$CH_3$), 3.52 (s, 3H, 8-$CH_3$), 4.31 (t, 2H, $CH_2CH_2CH_2N(C_2H_5)_2$), 7.64 (dd, 1H, H-3), 8.61 (dd, $J_{3,4}$ = 7.86 Hz, H-4), 8.90 (dd, 1H, $J_{2,3}$ = 4.69 Hz, $J_{2,4}$ = 1.89 Hz, H-2). | 411 | $C_{20}H_{25}N_7O_3$ |

1 mmol) and anhydrous potassium carbonate (0.138 g, 1 mmol) in acetonitrile (5 mL). The reaction mixture was stirred at room temperature for 8 h. The solid was then collected, washed with water and purified by recrystallization from DMF to give 0.198 g (64% yield) of pure **6**, m.p. 289–290 °C. $^1$H-NMR (DMSO-$d_6$) δ: 3.32 (s, 3H, 10-$CH_3$), 3.54 (s, 3H, 8-$CH_3$), 3.67 (s, 3H, 6-$CH_3$), 7.64 (dd, 1H, H-3), 8.61 (dd, 1H, $J_{3,4}$ = 7.86 Hz, H-4), 8.90 (dd, 1H, $J_{2,3}$ = 4.66 Hz, $J_{2,4}$ = 1.88 Hz, H-2); MS: *m/z* 312 ($M^+$); Anal. $C_{14}H_{12}N_6O_3$ (C,H,N).

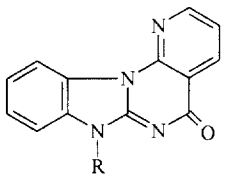
*5.1.3. 6-Dialkylaminoalkyl-8,10-dimethylpurino[7,8-a]quinazoline-5,9,11-(6H,8H,10H)-triones* ***1a–d*** *and 6-dialkylaminoalkyl-8,10-dimethylpyrido[2′,3′:4,5]pyrimido[1,2-f]purine-5,9,11-(6H,8H,10H)-triones* ***2a–d****: general procedure*

Sodium hydride in 50% dispersion in mineral oil (0.045 g, 1.875 mmol) was added in small portions to a stirred suspension of 0.673 mmol of compound **4** or **5** in 8 mL of anhydrous DMF. The reaction mixture was stirred at room temperature for 1 h, then supplemented with 0.997 mmol of the appropriate dialkylaminoalkyl chloride hydrochloride and left at room temperature with stirring for 72–96 h (with the exception of compounds **2a,b** for which the reaction mixture was heated at reflux for 24 h), until disappearance of the starting reagents (TLC analysis). Compounds **1a,c** and **2a–d** were recovered from the reaction mixture as insoluble material, washed with water and purified by recrystallization. Compounds **1b,d** were obtained by evaporation to dryness of the DMF solution and worked up as above (*table I* and *II*).

*5.1.4. 1-(Dialkylaminoalkyl)-2-aminobenzimidazoles* ***7b–d****: general procedure*

The appropriate ω-chloroalkyldialkylamine hydrochloride (7.5 mmol) was added to a stirred solution of 2-aminobenzimidazole (1.0 g, 7.5 mmol) and potassium hydroxide (1.00 g, 18 mmol) in ethanol (50 mL). The mixture was stirred at room temperature for 5 h and then the inorganic material (KCl) was

**Table III.** Physical and spectral data of compounds **3b–d**.



| No. | R | Yield (%) | M.p., °C (recryst. solv.) | $^1$H-NMR DMSO-$d_6$ (δ ppm) [a] | MS, *m/z* ($M^+$) | Formula |
|---|---|---|---|---|---|---|
| **3b** | $(CH_2)_3N(CH_3)_2$ | 65 | 150–152 (Benzene-Pet.ether 60–80°) | 2.5–2.9 (m, 2H, $CH_2CH_2CH_2N(CH_3)_2$), 3.2 (s, 6H, $N(CH_3)_2$), 3.6 (t, 2H, $CH_2CH_2CH_2N(CH_3)_2$), 4.9 (t, 2H, $CH_2CH_2CH_2N(CH_3)_2$), 7.7–8.3 (m, 4H, ArH), 8.9–9.5 (m, 3H, ArH). | 321 | $C_{18}H_{19}N_5O$ |
| **3c** | $(CH_2)_2N(CH_2CH_3)_2$ | 60 | 174–177 (Benzene-Pet.ether 60–80°) | 1.6 (t, 6H, $N(CH_2CH_3)_2$), 3.4–4.3 (m, 6H, $CH_2CH_2N(CH_2CH_3)_2$), 5.2 (t, 2H, $CH_2CH_2N(C_2H_5)_2$), 7.7–8.3 (m, 4H, ArH), 8.9–9.5 (m, 3H, ArH). | 335 | $C_{19}H_{21}N_5O$ |
| **3d** | $(CH_2)_3N(CH_2CH_3)_2$ | 66 | 158–159 (Benzene-Pet.ether 60–80°) | 1.5 (t, 6H, $N(CH_2CH_3)_2$), 2.3–2.9 (m, 2H, $CH_2CH_2CH_2N(C_2H_5)_2$), 3.2–3.9 (m, 6H, $CH_2CH_2CH_2N(CH_2CH_3)_2$), 4.8 (t, 2H, $CH_2CH_2CH_2N(C_2H_5)_2$), 7.7–8.2 (m, 4H, ArH), 8.9–9.5 (m, 3H, ArH). | 349 | $C_{20}H_{23}N_5O$ |

[a] Recorded on a Varian EM 360 A.

**Table IV.** Cytotoxic activity of examined compounds against HL60 and HeLa cells.

| Compounds | $IC_{50}$ (μM) [a] | |
|---|---|---|
| | HL60 | HeLa |
| **1a** | 0.072 ± 0.002 | 0.15 ± 0.016 |
| **1b** | 0.47 ± 0.04 | 0.91 ± 0.07 |
| **1c** | 0.19 ± 0.02 | 0.53 ± 0.05 |
| **1d** | 0.46 ± 0.04 | 1.5 ± 0.05 |
| **2a** | > 10 | > 10 |
| **2b** | > 10 | > 10 |
| **2c** | > 10 | > 10 |
| **2d** | > 10 | > 10 |
| **3b** | 4.8 ± 0.5 | > 10 |
| **3c** | 3.2 ± 0.2 | > 10 |
| **3d** | 4.6 ± 0.4 | > 10 |
| doxorubicin | 0.0036 ± 0.0001 | 0.035 ± 0.004 |
| ellipticine | 0.64 ± 0.02 | 0.31 ± 0.01 |

[a] Drug concentration required to inhibit the cell growth by 50% after 72 h of incubation. Data represent mean values ± SE for three separate experiments.

filtered off. The solution was evaporated to dryness and the residue obtained was purified by recrystallization.

*1-(Dimethylaminopropyl)-2-aminobenzimidazole* ***7b***: m.p. 145–148 °C (acetone); 30% yield; $^1$H-NMR (Varian EM 360 A, $CDCl_3$) δ: 1.7–2.4 (m, 4H, $CH_2CH_2CH_2N(CH_3)_2$), 2.3 (s, 6H, $N(CH_3)_2$), 4.1 (t, 2H, $CH_2CH_2CH_2N(CH_3)_2$), 6.4 (br s, 2H, $NH_2$ exch. $D_2O$), 7.0–7.6 (m, 4H, ArH); MS: *m/z* 204 ($M^+$); Anal. $C_{12}H_{18}N_4$(C,H,N).

*1-(Diethylaminoethyl)-2-aminobenzimidazole* ***7c***: m.p. 132–133 °C (acetone); 36% yield; $^1$H-NMR (Varian EM 360 A,

**Table V.** Drug-DNA binding parameters in ETN buffer (IS 0.01 M, pH = 7, 25 °C) and orientation angle α of the examined compounds bound to DNA.

| Compounds | $K_i \times 10^{-5}$ [a] | $n$ [b] | $\Delta T_m$ (°C) [c] | $\alpha_L$ ($\lambda_{nm}$) [d] |
|---|---|---|---|---|
| **1a** | 1.58 | 4.5 | 6.8 ± 0.3 | 90° (308) |
| **1b** | 1.28 | 4.9 | 5.8 ± 0.7 | 90° (308) |
| **1c** | 1.32 | 4.5 | 6.5 ± 0.2 | 90° (308) |
| **1d** | 1.2 | 7.4 | 6.5 ± 0.5 | 90° (308) |
| **2a** | nd | nd | 0.5 ± 0.1 | nd |
| **2b** | nd | nd | 1.2 ± 0.1 | nd |
| **2c** | nd | nd | 1.1 ± 0.1 | nd |
| **2d** | nd | nd | 1.0 ± 0.1 | nd |
| **3b** | 1.08 | 6.1 | 6.0 ± 0.1 | 90° (310) |
| **3c** | 1.26 | 6.6 | 6.3 ± 0.2 | 90° (310) |
| **3d** | 1.02 | 6.8 | 6.2 ± 0.2 | 90° (310) |

[a] Intrinsic binding constant expressed as molar base pairs, mean ± 5% from at least four determinations; [b] exclusion parameter (base pairs); [c] determined at the ratio [DNA]/[drug] = 5 (see text); [d] determined from $LD_r$ at the given wavelength, at the ratio [DNA]/[drug] = 12.5.

**Table VI.** Drug-DNA binding with DNAs from different sources and synthetic polynucleotides: affinity and selectivity for purinoquinazoline derivatives.

| Compounds | M. Lysodeictikus | | C. Perfrigens | | Poly[dA–dT]$_2$ | | Poly[dG–dC]$_2$ | | $K_{AT}/K_{GC}$ [c] |
|---|---|---|---|---|---|---|---|---|---|
| | $K \times 10^{-5}$ [a] | $n$ [b] | $K \times 10^{-5}$ | $n$ | $K \times 10^{-5}$ | $n$ | $K \times 10^{-5}$ | $n$ | |
| **1a** | 1.70 | 4.6 | 0.92 | 8.6 | 1.06 | 4.2 | 4.70 | 2.0 | 0.22 |
| **1b** | 1.18 | 4.9 | 0.86 | 15.1 | 1.54 | 2.9 | 2.52 | 3.6 | 0.61 |
| **1c** | 1.28 | 7.6 | 0.91 | 11.2 | 1.18 | 5.9 | 3.22 | 5.7 | 0.36 |
| **1d** | 1.54 | 5.0 | 0.92 | 7.7 | 1.26 | 4.1 | 2.14 | 3.5 | 0.58 |

[a] Binding affinity constants expressed as molar base pairs, mean value ± 5% from three determinations; [b] exclusion parameter (base pairs); [c] $K_{AT}/K_{GC}$ is the ratio between binding constants for Poly[dA–dT]$_2$ and Poly[dG–dC]$_2$.

$CDCl_3$) δ: 0.9 (t, 6H, $N(CH_2CH_3)_2$), 2.7 (m, 6H, $CH_2CH_2N(CH_2CH_3)_2$), 4.1 (t, 2H, $CH_2CH_2N(C_2H_5)_2$), 6.2 (br s, 2H, $NH_2$ exch. $D_2O$), 7.0–7.7 (m, 4H, ArH); MS: *m/z* 218 ($M^+$); Anal. $C_{13}H_{20}N_4$ (C,H,N).

*1-(Diethylaminopropyl)-2-aminobenzimidazole* ***7d***: m.p. 144–146 °C (acetone); 43% yield; $^1$H-NMR (Varian EM 360 A, $CDCl_3$) δ: 1.0 (t, 6H, $N(CH_2CH_3)_2$), 1.8–2.2 (m, 2H, $CH_2CH_2CH_2N(C_2H_5)_2$), 2.4 (t, 2H, $CH_2CH_2CH_2N(C_2H_5)_2$), 2.7 (q, 4H, $N(CH_2CH_3)_2$), 4.1 (t, 2H, $CH_2CH_2CH_2N(C_2H_5)_2$), 6.3 (br s, 2H, $NH_2$ exch. $D_2O$), 7.1–7.7 (m, 4H, ArH); MS: *m/z* 232 ($M^+$); Anal. $C_{14}H_{22}N_4$ (C,H,N).

*5.1.5. N-(1-Dialkylaminoalkylbenzimidazol-2-yl)-2-chloropyridine-3-carboxamides* ***8b–d****: general procedure*

A solution of 2-chloropyridine-3-carboxylic acid (0.63 g, 4.0 mmol) and thionyl chloride (5 mL, 40 mmol) in anhydrous benzene was refluxed for 12 h. The solution was evaporated to dryness and the oily residue obtained was dissolved in anhydrous THF (10 mL), and added to a suspension of the appropriate 1-(dialkylaminoalkyl)-2-aminobenzimidazole **7b–d** (4.0 mmol) and triethylamine (0.6 mL, 4.4 mmol) in 40 mL of anhydrous THF. The reaction mixture was left under stirring at room temperature for 12 h. After filtering off the insoluble triethylamine hydrochloride, the solution was concentrated to dryness. The oily residue was dissolved in $CHCl_3$ and the solution was washed with $NaHCO_3$ 10% aqueous solution and then with water. The organic layer was evaporated to dryness to give the crude products **8b–d**, which were purified by recrystallization.

*N-(1-Dimethylaminopropylbenzimidazol-2-yl)-2-chloropyridine-3-carboxamide* ***8b***: m.p. 107–109 °C (Benzene-Pet.ether 60–80°); 41% yield; $^1$H-NMR (Varian EM 360 A, DMSO-$d_6$) δ: 1.7–2.6 (m, 4H, $CH_2CH_2CH_2N(CH_3)_2$), 2.2 (s, 6H, $N(CH_3)_2$), 4.2 (t, 2H, $CH_2CH_2CH_2N(CH_3)_2$), 7.2–7.8 (m, 5H, ArH), 8.3–8.7 (m, 2H, ArH); MS: *m/z* 357 ($M^+$); Anal. $C_{18}H_{20}ClN_5O$ (C,H,N).

*N-(1-Diethylaminoethylbenzimidazol-2-yl)-2-chloropyridine-3-carboxamide* ***8c***: m.p. 119–120 °C (Ethanol–water); 47% yield; $^1$H-NMR (Varian EM 360 A, DMSO-$d_6$) δ: 0.8 (t, 6H, $N(CH_2-CH_3)_2$), 2.5 (q, 4H, $N(CH_2CH_3)_2$), 2.8 (t, 2H, $CH_2CH_2N(C_2H_5)_2$), 4.2 (t, 2H, $CH_2CH_2N(C_2H_5)_2$), 7.1–7.8 (m, 5H, ArH), 8.2–8.6 (m, 2H, ArH); MS: *m/z* 371 ($M^+$); Anal. $C_{19}H_{22}ClN_5O$ (C,H,N).

*N-(1-Diethylaminopropylbenzimidazol-2-yl)-2-chloropyridine-3-carboxamide* ***8d***: m.p. 103–104 °C (Benzene-Pet.ether 60–80°); 57% yield; $^1$H-NMR (Varian EM 360 A, DMSO-$d_6$) δ: 1.1 (t, 6H,

$N(CH_2-CH_3)_2$), 1.7–2.7 (m, 8H, $CH_2CH_2CH_2N(CH_2CH_3)_2$), 4.2 (t, 2H, $CH_2CH_2CH_2N(C_2H_5)_2$), 7.2–7.8 (m, 5H, ArH), 8.2–8.7 (m, 2H, ArH); MS: *m/z* 385 ($M^+$); Anal. $C_{20}H_{24}ClN_5O$ (C,H,N).

*5.1.6. 5,7-Dihydro-7-dialkylaminoalkyl-5-oxopyrido[3',2':5,6] pyrimido[1,2-a]benzimidazoles* **3b–d**: *general procedure*

A suspension of the appropriate carboxamide **8b–d** (2.5 mmol) in 10 mL of pyridine was refluxed for 48 h. After cooling, the solution was evaporated in vacuo and the residue obtained was washed with $NaHCO_3$ 10% aqueous solution and purified by recrystallization to give compounds **3b–d** (*table III*).

*5.2. Biochemicals*

Salmon testes DNA (46% GC, sodium salt $\varepsilon_{260} = 6600\ M^{-1}\ cm^{-1}$), Micrococcus lisodeikticus DNA (72% GC $\varepsilon_{260} = 6900\ M^{-1}\ cm^{-1}$), Clostridium perfrigens DNA (31% GC $\varepsilon_{260} = 6700\ M^{-1}\ cm^{-1}$), Poly $[dA–dT]_2$, (poly deoxyadenylic-thymidilic acid, sodium salt $\varepsilon_{260} = 6300\ M^{-1}\ cm^{-1}$), Poly-$[dG–dC]_2$, (poly deoxyguanylic-cytidylic acid sodium salt $\varepsilon_{260} = 7100\ M^{-1}\ cm^{-1}$) were purchased from SIGMA Co. Nucleic acid concentrations, expressed with respect to mononucleotides, were determined spectrophotometrically using the above-reported values for the molar absorption coefficients at 260 nm. All experiments, unless otherwise indicated, were performed using Salmon testes DNA. Doxorubicin hydrochloride and ellipticine were purchased from Aldrich Chem. Co.

*5.3. DNA binding studies*

Light absorption spectra were recorded on a Perkin-Elmer Lambda15 spectrophotometer. Fluorescence spectra were recorded on a Perkin-Elmer LS50B luminescence spectrophotometer. Experiments were performed by addition of small amounts of the free ligand to a solution containing DNA-ligand complex, ([DNA]/[drug] ratio = 150). The concentration of the free ligand was the same as that of the complex; in this way different [DNA]/[drug] ratios were obtained at a constant ligand concentration. Generally, all measurements were carried out in ETN buffer (TRIS 10 mM, EDTA 2 mM, and NaCl to adjust the ionic strength to 0.01 M) at pH 7 and 25 °C. The amounts of bound and free ligand ($C_b$ and $m$) were determined from emission readings at a fixed wavelength, corresponding to the maximum of emission, in accordance with the equations:

$$C_b = (F - F_f / F_b - F_f)C_0$$
$$m = C_0 - C_b$$

where $F_f$ is the emission fluorescence of the free compound, $F_b$ is the emission fluorescence of the bound compound, $F$ is the emission fluorescence of the free and bound compound and $C_0$ is the total concentration of the compound. The binding data thus obtained were evaluated in accordance with the method of McGhee and von Hippel [17] to obtain the intrinsic binding constant and the exclusion parameter.

*5.4. Circular dichroism*

Circular dichroism (CD), is the differential absorption of circularly polarized light:

$$CD(\lambda) = A_L(\lambda) - A_R(\lambda)$$

where $A_L(\lambda)$ and $A_R(\lambda)$ are the absorption spectra measured with left and right circularly polarized light. CD measurements were performed on a Jasco J500A spectropolarimeter equipped with an IBM PC and a Jasco J interface. Spectra were recorded in ETN (0.01 M), pH = 7 at 25 °C at different [DNA]/[drug] ratios.

*5.5. Linear flow dichroism*

Linear dichroism (LD) measurements were performed on a Jasco J500A spectropolarimeter equipped with an IBM PC and a Jasco J interface. Linear dichroism is defined as the differential absorption of the orthogonal form of linearly polarized light:

$$LD = A_{//} - A_{\perp}$$

where $A_{//}$ corresponds to the absorbance of the sample when the light is polarized parallel to the orientation of flow, and $A_{\perp}$ is the perpendicular absorbance. By dividing by the absorbance another unoriented sample at rest, $A_{iso}$, the 'reduced' linear dichroism:

$$LD_r = LD / A_{iso}$$

is defined, which quantity may be related to the orientation of DNA (S) and the angle between the respective light-absorbing transition moment and DNA helix axis according to Norden et al. [14, 15]:

$$LD_r = 3/2 \times S(3\cos^2\alpha - 1) \qquad (1)$$

The orientation is produced by a device designed by Wada and Kozawa [25] at a shear gradient of 500–700 rpm. Assuming a value of $\alpha = 90°$ for the DNA bases for a ligand bound to it, it follows that:

$$\alpha_L = \arccos[1/3 - (LD_r)_L/3(LD_r)_{DNA}]^{1/2} \qquad (2)$$

where $(LD_r)_L$ is the reduced linear dichroism for the ligand, $(LD_r)_{DNA}$ is the reduced LD for DNA and $\alpha_L$ defines the ligand–DNA relative orientation. For intercalated system, $\alpha_L \approx 90°$ and $(LD_r)_L \approx (LD_r)_{DNA}$.

*5.6. Thermal transition studies*

Absorbance–temperature profiles were determined on a Perkin-Elmer 554 spectrophotometer with a temperature programmer. Samples were heated at a rate of 0.5 °C $min^{-1}$ in a temperature range 25–100 °C. Absorbance was monitored constantly at 260 nm. The melting profiles of DNA alone or drug-DNA complex, at a ratio [DNA]/[drug] = 5 in ETN (0.01 M), were analyzed and the transition melting temperature $T_m$ was determined at the midpoint of the curves. The $\Delta T_m$ values can be defined as the difference between $T_m$ for DNA alone and DNA-drug complex.

*5.7. Biological activity*

HL-60 and HeLa cell lines were grown respectively in RPMI 1640 (Sigma Co.) supplemented with 15% foetal calf serum (Mascia Brunelli–Italy) and Nutrient Mixture F-12 (HAM, Sigma) supplemented with 10% foetal calf serum (Mascia Brunelli–Italy). 100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL

amphotericin B (Sigma) were added in both media. The cells were cultured in a moist atmosphere of 5% carbon dioxide in air. Cells ($3 \times 10^4$) were seeded into each well of a 24-well microtiter plate. After incubation for 24 h, various concentrations of the test agents were added in complete medium and incubated for a further 72 h. The trypan blue assay was performed to determine cell viability. Cytotoxicity data were expressed as $IC_{50}$ values, i.e. the concentration of the agent (μM) inducing 50% reduction in cell numbers compared with control cultures.

### *5.8. Topoisomerase II-mediated DNA cleavage*

The Topoisomerase II-mediated DNA cleavage assay was performed as reported by Tewey et al. [26] with some modifications. Briefly, pBR322 plasmid DNA (Sigma) was linearized with Eco RI (Sigma) and then end-labelled at the 3′ termini with the Klenow fragment of DNA polymerase I (Sigma) in the presence of [α-$^{32}$P]dATP (Amersham). Unincorporated triphosphates were removed by ethanol precipitation in the presence of 2M ammonium acetate. The linear plasmid was further digested with Hind III restriction endonuclease (Sigma) to remove a 31-base pair small fragment.

The cleavage reaction was performed in a reaction mixture containing 40 mM Tris-HCl (pH = 7.6), 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA, 30 μg/mL bovine serum albumin, 1.6 mM ATP, 50 ng of the uniquely end-labelled pBR322 DNA, 20 ng of human type II topoisomerase (TopoGEN, Inc.) in the absence or in the presence of the drug tested. Reactions were incubated at 37 °C for 30 min and then terminated by the addition of 0.5% SDS and 75 μg/mL proteinase K (Sigma). DNA samples were analyzed on a 1% agarose gel in TBE buffer (89 mM Tris-borate (pH = 8.3) and 2 mM EDTA). Gels were dried and autoradiographed for 1 or 2 days.

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