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Diazoniapolycyclic Ions Inhibit the Activity of Topoisomerase I and the Growth of Certain Tumor Cell Lines

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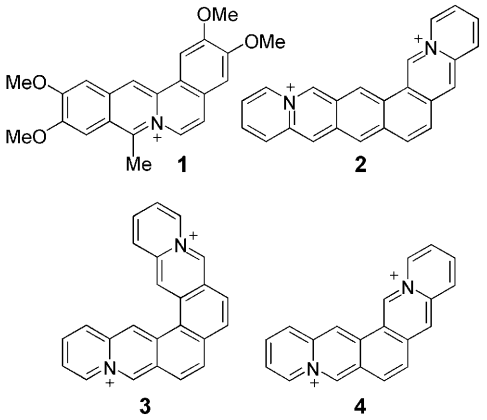
Topoisomerases are essential DNA-targeting enzymes that initially induce DNA strand cleavage; this process is followed by a reorganization and reconnection of the damaged DNA strand.[1] The result of these events is the relaxation of supercoiled DNA which is required during transcription or replication. It has been shown that DNA intercalators can interfere significantly with this physiological process.[1d,2] Ligands that occupy the topoisomerase binding site may suppress the association of topoisomerase with DNA, thus influencing topoisomerase activity. Moreover, a ternary complex between DNA, the intercalator, and topoisomerase can be formed, which is more stable than the DNA–topoisomerase assembly, leading to an extended lifetime of the initially cleaved DNA. As a consequence, re-ligation of the strands cannot take place and, because the strand breaks persist, topoisomerase acts as an endogenous poison under these circumstances. Therefore, DNA intercalators that inhibit topoisomerase activity or that form stabilized ternary complexes with DNA and topoisomerase have high potential as DNA-targeting anticancer drugs.[1d,2b,3]

Among the many organic compounds that have been tested for topoisomerase-inhibiting and topoisomerase-poisoning properties,[4] the protoberberine-type alkaloid coralyne (**1**) and derivatives thereof have been shown to inhibit topoisomerase I function.[5] These compounds have been postulated as a promising class of compounds for the design of efficient topoisomerase poisons with high potential for clinical application.[5b] Because coralyne represents an annelated quinolizinium ion, a class of nitrogen heterocycles with which we have gained much experience over the last decade, it seemed worthwhile to investigate other annelated quinolizinium derivates with respect to their interaction with DNA in the presence of topoisomerase. In particular, we focused our attention on a series of diazoniapolycyclic salts such as **2**, **3**, and **4**, which contain two quinolizinium fragments and thus an extended dicationic chromophore.[6] We have demonstrated that these compounds intercalate only partially into DNA, with a relatively large portion



of the π surface pointing out of the binding pocket. This non-intercalated portion of the ligand offers an additional anchoring point for the association of an appropriate receptor unit, so that a ternary complex may be formed. Indeed, the propensity of the diazoniapolycyclic intercalators **3** and **4** to stabilize ternary complexes with DNA and another host molecule has been demonstrated by the selective stabilization of triplex DNA by these ligands.[7] Herein we report that the diazoniapolycycles **2**–**4** are also able to affect the activity of topoisomerase I and to inhibit cell growth in selected tumor cell lines.

Supercoiled plasmid pBR322 (25 μM) was incubated with human topoisomerase I in the presence of compound **2**, **3**, **4**, or coralyne (**1**) at concentrations ranging from 0.5 to 200 μM. Coralyne was used as reference compound for better comparison. The unwinding of the plasmid was monitored by agarose gel electrophoresis and staining of the DNA bands with ethidium bromide after electrophoretic separation (Figure 1). The content of the supercoiled plasmid (form I) and the fully relaxed plasmid (form II) in the gel were quantified by densitometric analysis (Figure 1 G and H). Inhibition of plasmid relaxation by **1** (Figure 1 A) is similar to the results that were already reported for this compound,[5a] indicating comparable experimental conditions. At first sight, the diazoniapolycycles **2** and **3** exhibit a similar influence on topoisomerase activity; that is, at low concentrations of the ligand (**2**: <1 μM; **3**: <2.5 μM), the amount of relaxed plasmid increases as indicated by the increasing intensity of the DNA bands of decreased electrophoretic mobility (Figure 1 B and C). This observation usually indicates intercalation of the ligand into the DNA, resulting in its partial unwinding.[8] In the case of compound **3**, relaxation of the plasmid is almost fully suppressed at concentrations greater than 10 μM, which may be the result of topoisomerase inhibition by intercalation at the topoisomerase binding site. In

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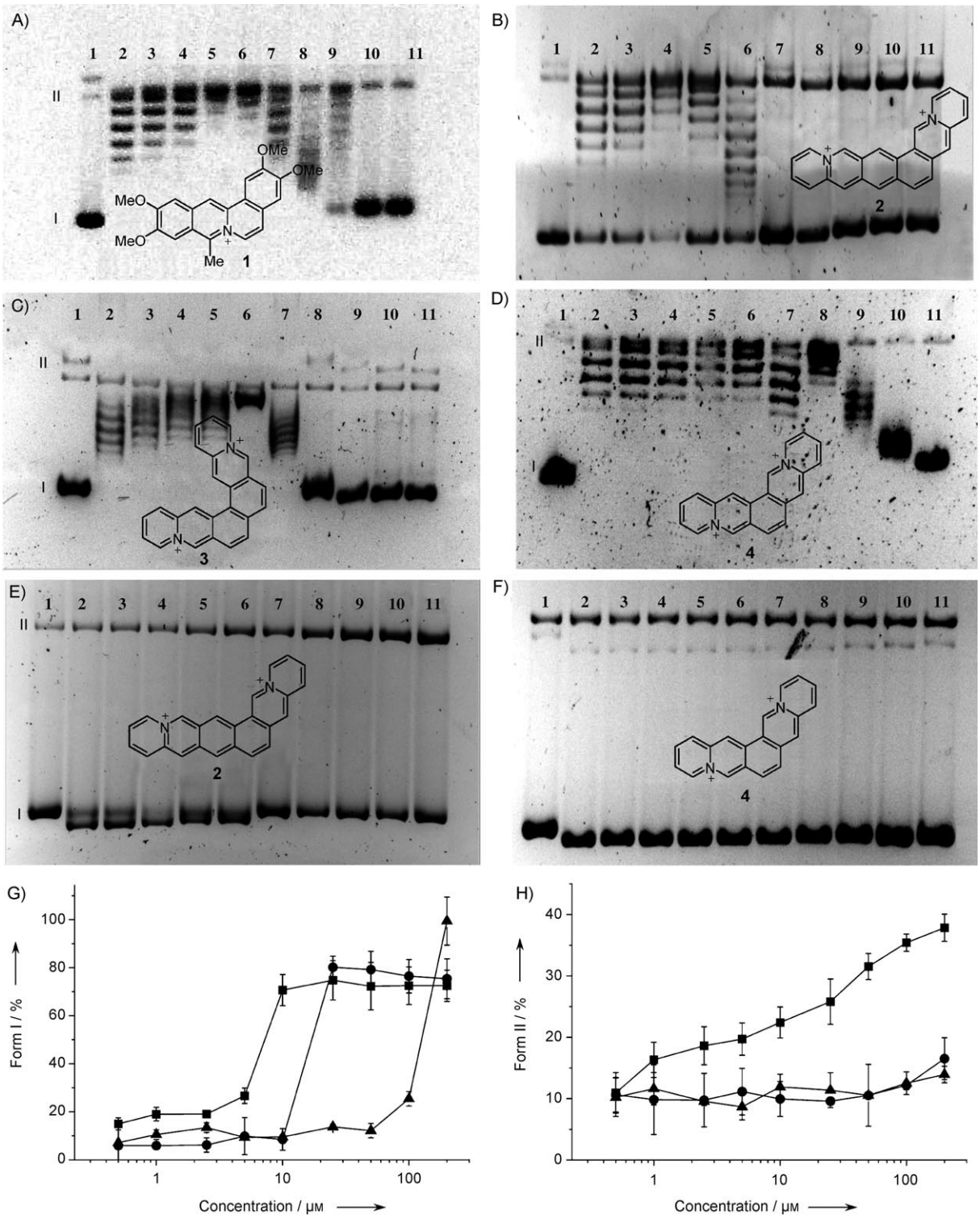

Figure 1. Effect of increasing concentrations of A) coralyne (**1**), B) and E) diazoniahexaphene **2**, C) diazoniaanthraanthracene **3**, and D) and F) diazoniapentaphene **4** on the relaxation of pBR322 plasmid DNA by human topoisomerase I; I = supercoiled plasmid; II = relaxed, open-circular plasmid. Supercoiled DNA (500 ng, $c = 25$ μM, form I) was incubated with 10 U topoisomerase I in the absence or presence of increasing concentrations of ligands **1**–**3**. The DNA samples were separated by electrophoresis on an agarose gel without (A–D) or with ethidium bromide (E and F). Lanes 1: plasmid, lanes 2: plasmid + topo I, lanes 3–11: plasmid + topo I + ligand (0.5, 1, 2.5, 5, 10, 25, 50, 100, and 200 μM, respectively). G) and H) Densitometric determination of the content of forms I and II of pBR322 (**2**: ■, **3**: ●, **4**: ▲); data are expressed as mean values ± SD of two independent experiments.

the case of ligand **2**, densitometric analysis indicates greater amounts of form I plasmid in the presence of compound **2** at concentrations between 5 and 10 μM. With increasing concentrations of **2**, however, the content of the open circular form of the plasmid also increased (Figure 1 B and H). It may be assumed that under these conditions, that is, at this particular ligand/DNA ratio, the ternary complex between the topoisomerase, DNA, and ligand **2** is significantly stabilized. The resulting extended lifetime of the topoisomerase cleavage complex leads to DNA single-strand cleavage and thus to an increased formation of the relaxed form of the plasmid. Notably, a significant retardation of some plasmid bands at higher drug loading takes place, presumably due to the high DNA binding affinity of the ligands, which cannot be removed even with thorough extraction with chloroform/isoamyl alcohol. This leads to a pronounced unwinding of the DNA at higher ligand concentrations.

Electrophoresis carried out in a gel already containing ethidium bromide before separation[9] offers a simple method to distinguish between the relaxed intact plasmid and the open circular DNA containing permanent single-strand breaks because the intercalation of ethidium bromide leads to unwinding of the relaxed topoisomers. At high ethidium concentrations this unwinding leads to formation of the positively supercoiled form and migration in the gel that is slightly faster than that of the initial negatively supercoiled form I.[9] In contrast, the nicked plasmid is essentially unaffected after association of ethidium and still gives the usual bands of form II in the gel. Accordingly, for compound **2**, the relaxation assay was repeated with an ethidium-containing gel (Figure 1 E). Under these conditions, the amount of nicked DNA (form II) increases significantly with increasing concentration of compound **2**. At the same time the amount of relaxed supercoiled plasmid decreases, as indicated by the disappearing DNA band in the gel. This experiment indicates DNA single-strand cleavage by the ternary complex of plasmid, topoisomerase, and ligand **2**.

Relative to compounds **2** and **3**, the diazoniapentaphene **4** exerts a less pronounced influence on topoisomerase activity (Figure 1 D). Topoisomerase-induced relaxation of the plasmid is assisted by **4** in a concentration range between 0.5 and 25 μM, whereas relaxation is inhibited only at relatively large concentrations (>100 μM). Moreover, analysis of the relaxation assay with an ethidium-containing gel (Figure 1 F) reveals that over the whole concentration range of compound **4** (0.5–200 μM), only small amounts of single-strand breaks are formed (note that the initial plasmid already contains form II). This observation indicates that compound **4** does not stabilize the topoisomerase cleavage complex.

To assess cell growth inhibition by the diazoniapolycyclic derivatives **2**, **3**, and **4**, a series of selected human tumor cell lines were tested, namely K562 (human erythroleukemia), Jurkat (human T-cell leukemia), HL-60 (human promyelocytic leukemia), A549 (small cell lung carcinoma), and MCF-7 (breast cancer carcinoma). Cells were incubated in the presence of various concentrations of compounds **2**, **3**, and **4** for 72 h, and cell viability was evaluated with the MTT test (Table 1). For comparison, coralyne (**1**) was also tested in this assay as reference compound. Indeed, the diazoniapolycycles **2**–**4** inhibit the growth of all five cancer cell lines assayed, and in most cases the GI_{50} value is similar or even slightly lower than that of coralyne. However, all three are significantly less efficient than the well-known topoisomerase poison camptothecin (GI_{50} = 0.004 μM against human lymphoblast RPMI 8402[5b]). Moreover, the viability of Jurkat cells in the presence of ligands **1**–**4** was monitored by the trypan blue exclusion assay (Figure 2). The results confirm that each ligand inhibits cell

Table 1. Cell growth inhibition of investigated compounds.

Compd	GI_{50} [μM][a] K562	Jurkat	HL-60	A549	MCF-7
1	9.5 ± 0.5	4.9 ± 0.1	3.9 ± 0.1	8.4 ± 0.5	21.1 ± 1.9
2	5.2 ± 0.3	3.1 ± 0.1	6.9 ± 1.1	4.1 ± 0.4	21.5 ± 1.5
3	7.5 ± 0.3	5.3 ± 0.2	6.7 ± 0.5	4.2 ± 0.3	9.7 ± 0.3
4	4.3 ± 0.2	3.2 ± 0.1	6.8 ± 0.2	2.9 ± 0.2	4.6 ± 0.1

[a] Data are mean values ± SE ($n \geq 3$).

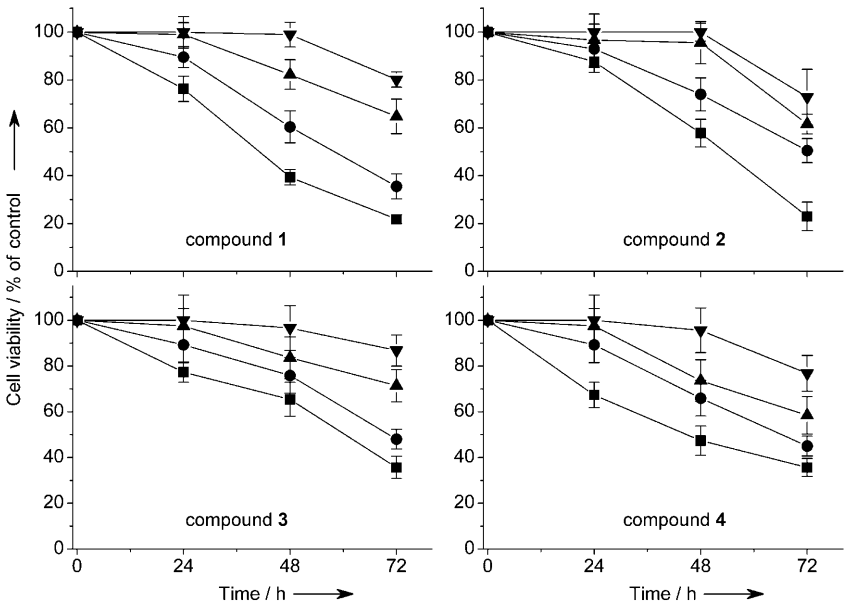

Figure 2. Time profiles of the inhibition of Jurkat cell growth with increasing concentrations (2.5 μM: ▼, 5.0 μM: ▲, 10 μM: ●, 20 μM: ■) of ligands **1**–**4** after 24, 48, and 72 h. Cell viability was determined by the trypan blue exclusion assay, and is given as percentage (mean ± SE, $n = 3$) of the control sample.

growth significantly and that cell viability decreases with increasing ligand concentration and incubation time.

From these data, detailed structure–activity relationships cannot be deduced; that is, there is no clear correlation between the topoisomerase-poisoning activity and cell toxicity of the ligands. Thus, the derivative **2** exhibits the greatest inhibition activity toward topoisomerase, but has essentially the same GI_{50} value as ligands **3** and **4**. On the other hand, the compound with the lowest activity toward topoisomerase, diazoniapentaphene **4**, has the greatest effect on the cell growth of lines K562, A549, and MCF-7. This lack of correlation between topoisomerase inhibition and cell growth inhibition has been observed for other compound classes, such as berberine derivatives,[5b,e] and is proposed to be due to the influence of other factors such as cell membrane permeability and availability of the ligand within the cell. Moreover, it must be considered that the cytotoxicity of intercalators **2**–**4** may originate from mechanisms other than topoisomerase inhibition or poisoning.

To gain more insight into the interactions of the diazoniapolycyclic ligands with topoisomerase, we performed a molecular modeling analysis of the structures of the potential ternary cleavage complexes of topoisomerase I, DNA, and ligands **1** and **2** by using molecular docking simulations. In general, ligands **1** and **2** form ternary complexes with topoisomerase and DNA with a negative free energy of binding ΔG_{bind}. Thus, local energy minima were observed for structures in which ligands **1** and **2** are accommodated in the DNA intercalation site by adopting a slightly different binding motif (Figure 3). Examination of the ternary cleavage complexes of ligand **2** reveals two docking structures with negative binding energy, that is, $\Delta G_{bind} < 0$ (represented as **2a** and **2b** in Figure 3). In each case, the large overlap of the aromatic ligands with the nucleotide bases indicates the significant influence of π–π stacking interactions on the orientation of the ligand within the intercalation site and on the overall binding energy. Most notably, the orientation of ligand **2a** in the ternary complex resembles that observed for topotecan (TPT) in the solid state

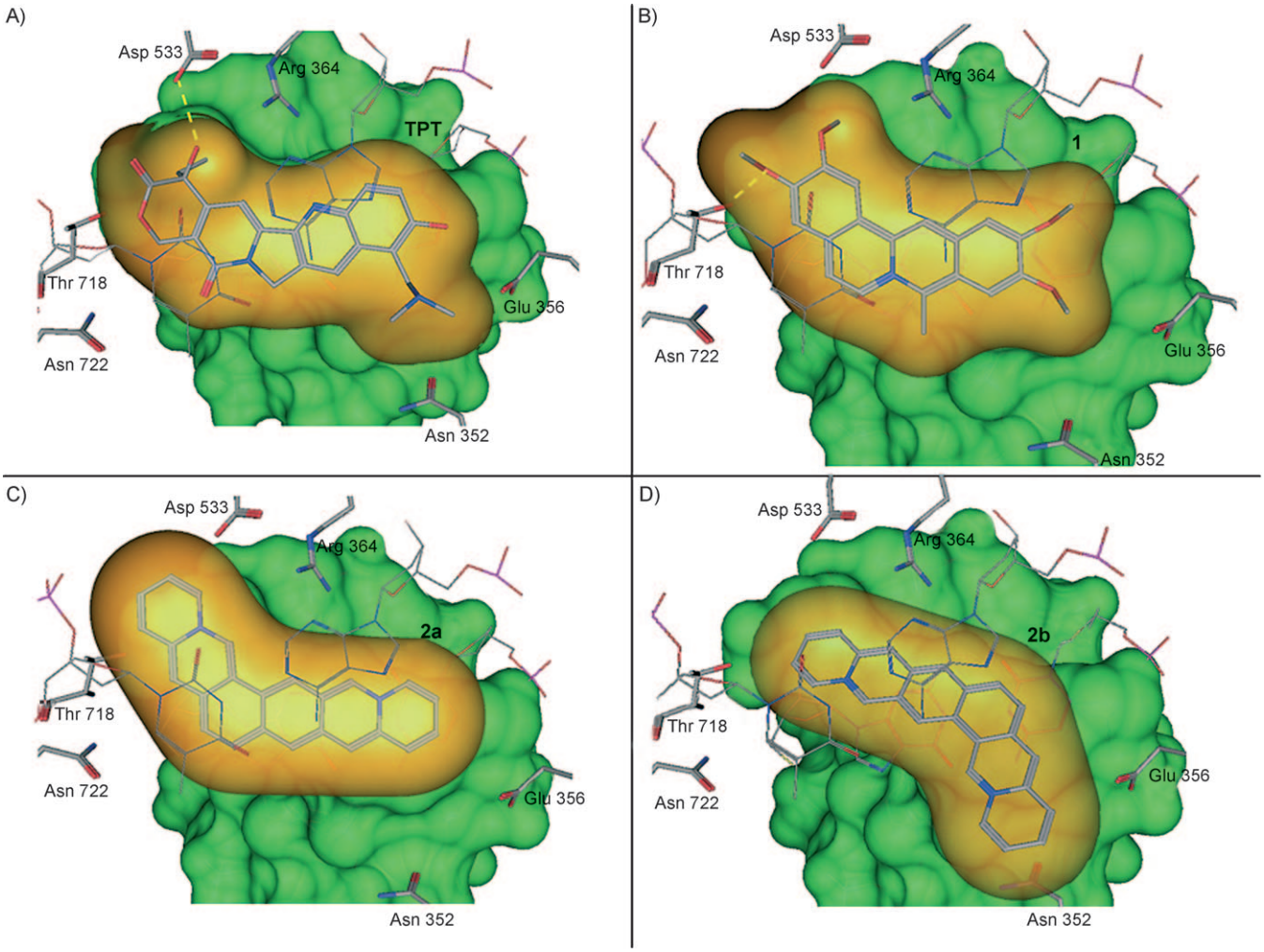

Figure 3. A) Structure of the ternary complex of topotecan (TPT), DNA, and topoisomerase (reference compound),[10] and docking positions of B) compound **1** and of the two lowest-energy docked orientations of **2** [C) **2a** and D) **2b**] in ternary complex with DNA and topoisomerase I. All complexes are displayed from the top of the DNA longitudinal axes. All DNA base pairs after the −1 position of the cleaved site are represented by the corresponding Connolly surface colored in green. Connolly surfaces of all intercalators are also highlighted in yellow. Hydrogen bonding interactions are indicated by dashed yellow lines.

(PDB code: 1K4T)[10] and the one calculated for coralyne (**1**). However, other than TPT and **1**, ligand **2** does not exhibit any additional attractive interactions, such as hydrogen bonding, with topoisomerase.

In summary, we have demonstrated that aromatic diazoniapolycyclic cations exert a significant influence on topoisomerase I activity along with pronounced inhibition of cell growth. Theoretical studies support the proposed stabilization of the topoisomerase I cleavage complex by the intercalated ligand. These results lead us to propose that structure **3** and particularly ligand **2** are promising platforms for the exploration of a new class of topoisomerase inhibitors and topoisomerase poisons. Notably, the parent compounds already show significant effects; considering the facile synthetic routes to these compounds, variations in the substitution pattern of these ligands should be readily realized. Therefore, extensive studies of a structure–activity relationship with this class of compounds may be performed.

Experimental Section

Materials. Coralyne chloride hydrate (**1**) was purchased from Acros Organics (Nidderau, Germany) and used without further purification. Compounds **2**–**4** were synthesized according to published protocols.[6] Human topoisomerase I was purchased from Calbiochem (Nottingham, UK). Plasmid pBR322 was obtained from Fermentas (Burlington, ON, Canada). All other materials were of the highest purity grade available.

Topoisomerase I relaxation assay.[9,11] At 0 °C, supercoiled plasmid pBR322 (0.25 μg) and human topoisomerase I (10 U) were added to an aqueous solution (20 μL, pH 7.5) of 50 mM Tris-HCl, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 0.1 mM EDTA, and 30 μg mL^{-1} bovine serum albumin (BSA). The mixture was incubated at 37 °C for 30 min, and pre-warmed SDS (final concentration 1%) was added, followed by proteinase K (final concentration 50 μg mL^{-1}). The mixture was incubated at 37 °C for 30 min. The digestion reaction was stopped by the addition of 2 μL aqueous loading buffer (0.25% bromophenol blue, 50% glycerol). The samples were successively extracted four times with a mixture of chloroform and isoamyl alcohol (24:1 *v/v*) prior to loading to the gel. The reaction mixtures were analyzed by electrophoresis on 1% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 20 V overnight and then stained with a solution containing 0.5 μg mL^{-1} ethidium bromide. DNA bands were visualized by UV light and photographed with a digital camera (Kodak DC256). Quantitation of the bands was carried out with the image analyzer software Quantity One (BioRad). Similar experiments were performed using ethidium-containing gels (0.5 μg mL^{-1}). Note: experiments with coralyne (**1**) must be performed in the absence of direct light irradiation (even sunlight) to avoid photoinduced damage of the plasmid.[12]

Cell culture. Human T-cell leukemia cells (Jurkat), human promyelocytic leukemia (HL-60) and human erythroblastic leukemia (K562) were grown in RPMI-1640 medium (Sigma, USA). Human non-small-cell lung carcinoma (A-549) and human breast carcinoma cells (MCF-7) were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma, USA). All media were supplemented with penicillin G (115 U mL^{-1}; Invitrogen, Milan, Italy), streptomycin (115 μg mL^{-1}; Invitrogen, Milan, Italy) and 10% fetal bovine serum (Invitrogen, Milan, Italy). Individual wells of a 96-well tissue culture microtiter plate (Falcon BD, Italy) were inoculated with 100 μL complete medium containing 5×10^3 cells. The plates were incubated at 37 °C in a humidified (5%) incubator for 18 h prior to the experiments. After removal of the medium, 100 μL drug solution, dissolved in DMSO and diluted with complete medium [final DMSO concentration: 0.5% (*v/v*)], were added to each well and incubated at 37 °C for 72 h. Cell viability was determined by the MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] test as described previously.[13]

Trypan blue exclusion assay.[14] Jurkat cells (10^5) were seeded into each well of a 24-well cell culture plate. After incubation overnight, various concentrations of the test compounds were added. After incubation for 24, 48, and 72 h, an aliquot of each cell suspension was taken, mixed with trypan blue, and counted with a hemocytometer under an optical microscope.

Molecular modeling. The structure of the ternary complex containing topoisomerase I, DNA, and topotecan was obtained from the Protein Data Bank (PDB code: 1K4T).[10] One molecule of polyethylene glycol (remaining in the crystal after vapor equilibration)[10] and topotecan were deleted. All atoms were then fixed according to the MOE atom types.[15] Hydrogen atoms were added and minimized using the AMBER 94s[16] force field and AMBER 94 charges[17] until the rms value of truncated Newton (TN) method reached <0.1 kcal mol^{-1} Å^{-1}. The structures of the coralyne **1** and annelated quinolizinium ions **2** were constructed in MOE and energy minimized with the MMFF 94 force field[16] and RHF/AM1-ESP charges.[18] Derivatives **1** and **2** were docked into the topotecan intercalation site by using the MOE-Dock tool, part of the MOE suite. Searching was conducted within a user-specified 3D docking box (the standard protocol selects all atoms inside a sphere of 12 Å radius from the center of mass of the binding cavity), using the Tabu Search[19] protocol (standard parameters: 1000 steps per run, 10 attempts per step, and 10 Tabu list length), and the MMFF 94 force field.[18] MOE-Dock performs a user-specified number of independent docking runs (50 in our case) and writes the resulting conformations and their energies in a molecular database file. The resulting docked complexes were subjected to MMFF 94 energy minimization until the rms of conjugate gradient was <0.1 kcal mol^{-1} Å^{-1}. Charges for the ligands were imported from the MOPAC output files. The binding free energies were calculated using the MM-GBSA free energy calculation method.[20] In this computational technique, the free energy of inhibitor binding, ΔG_{bind}, is obtained from the difference between the free energy of the receptor–ligand complex (G_{cpx}), and the unbound receptor (G_{rec}) and ligand (G_{lig}): $\Delta G_{bind} = G_{cpx} - (G_{rec} + G_{lig})$. The binding free energy (ΔG_{bind}) was evaluated in ternary complexes obtained as docking results. ΔG_{bind} was calculated as the sum of changes in the energy of three different contributions: 1) a force field term (E_{FF}) for bond, angle, torsional, van der Waals, and electrostatic potential energies; 2) a polar solvation free energy part (ΔG_{GB}), calculated according to the generalized Born approximation model;[21] and 3) a nonpolar contribution to the solvation free energy (ΔG_{NP}): a) $\Delta G_{bind} = E_{FF} + \Delta G_{GB} + \Delta G_{NP}$; b) $\Delta G_{NP} = \gamma$ SASA. SASA represents the solvent-accessible surface area of the solute, whereas γ is an empirical parameter of 0.005 kcal mol^{-1} Å^{-2}.

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