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Synthesis and biological evaluation of new cyclic amidine analogs of chlorambucil

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Abstract

A number of novel cyclic amidine analogs of chlorambucil were synthesized and examined for cytotoxicity in breast cancer cell cultures and for inhibition of topoisomerases I and II. Evaluation of the cytotoxicity of these compounds employing a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and inhibition of [^{3}H]-thymidine incorporation into DNA in both MDA-MB-231 and MCF-7 breast cancer cells demonstrated that these compounds were more active than chlorambucil. The degree to which these compounds inhibited cell growth breast cancer cells was directly correlated to DNA-binding affinity. These studies indicate that cyclic amidine analogs of chlorambucil are a potent catalytic inhibitor of topoisomerase II but not topoisomerase I. The highest degree of DNA binding and cytotoxicity in both MDA-MB-231 and MCF-7 breast cancer cells was observed for the compound, which possess a 4,5-dihydro-1*H*-imidazol moiety.

Keywords: Chlorambucil; MCF-7; MDA-MB-231; DNA topoisomerase II; DNA binding; Cytotoxicity

1. Introduction

Alkylating agents are a major class of anticancer drugs for the treatment of various cancers including hematological malignancies [1,2]. Similar to other alkylating agents, the dose-limiting factor of chlorambucil is hematologic suppression [2]. A series of minor groove-binding bifunctional nitrogen mustards and their corresponding mono-analogs using this carrier have been reported [3–8]. Targeting reactive moieties to DNA by attachment of a DNA minor groove-binding carrier such as distamycin or netropsin reduces the loss of active drug due to reaction with other cell components and makes it possible to direct the alkylation both sequence specifically and regiospecifically [3–8].

In our previous papers we reported the synthesis and structure–activity studies of amidine analogs of chlorambucil derivatives, which appeared to be a new class of cytotoxic minor groove binders and topoisomerase II inhibitors [9]. The promising biological data obtained with these compounds have encouraged the synthesis of a novel series of chlorambucil derivatives bearing a cyclic amidine moiety (Fig. 1). We synthesized compounds that have two moieties in the structure. One is a cyclic amidine moiety to acquire DNA minor groove-binding activity, and another is an *N,N-bis(*2-chloroethyl)amino residue for DNA alkylation. The DNA-binding ability of these compounds were studied employing the topoisomerase I/II inhibition assay and ethidium displacement assay using calf thymus DNA, T4 coliphage DNA, poly(dA–dT)$_2$ and poly(dG–dC)$_2$. The biological activities of the new compounds were compared to those of chlorambucil and its amidine analog **7** (Fig. 1) which was previously found to be the most active against breast cancer MCF-7 cells [9].

2. Experimental

2.1. Chemistry

The structures of all the compounds were confirmed by ^{1}H and ^{13}C NMR spectra recorded on Brucker AC 200F apparatus (^{1}H: 200 MHz and ^{13}C: 50 MHz) in DMSO-d$_6$. Melting points were determined on Büchi 535 melting-point apparatus and were uncorrected. Elemental analysis of C, H and N was performed on a Perkin-Elmer 240 analyzer and satisfactory results within ±0.4% of calculated values were obtained. Chemical shifts are expressed in δ value (ppm). Multiplicity

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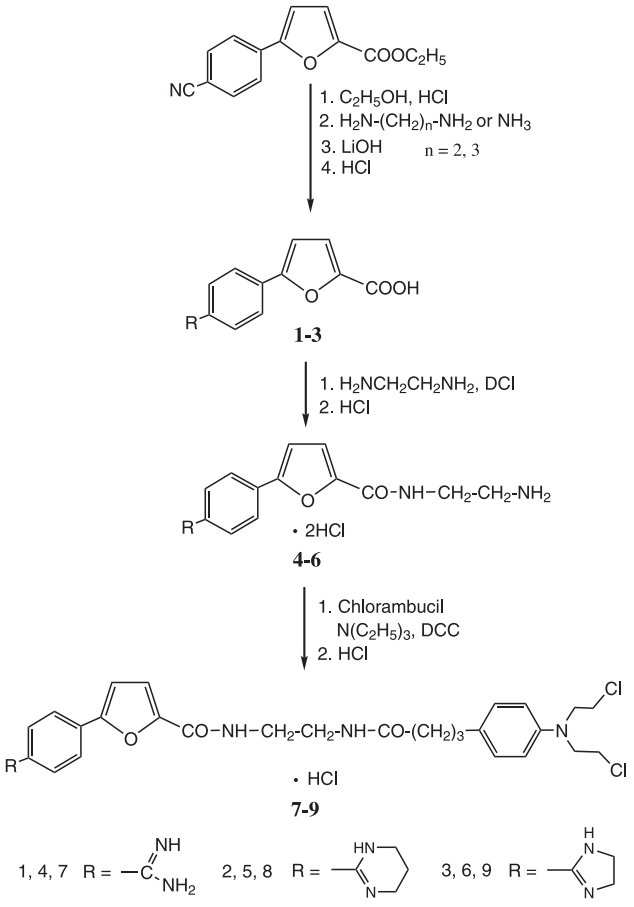

Fig. 1. Synthesis of compounds **1–9.**

of resonance peaks are indicated as singlet (s), doublet (d), triplet (t), quarter (q) and multiplet (m). Chlorambucil, tetrahydrofuran (THF), dimethylformamide (DMF), acetone, triethylamine, *N,N′*-dicyclohexylcarbodiimide (DCC), *N,N′*-carbonyldiimidazole (DCI), LiOH, 1,3-propanediamine and ethylenediamine were purchased from Sigma Chemical Co (St. Louis, MO, USA).

The compounds **1, 4, 5** and **7** have been prepared and described previously [9,10], and synthesis of all new compounds are given below.

*2.1.1. Ethyl 5-[4-(3,4,5,6-tetrahydro-*1H*-pyrimidine-2-yl)-phenyl]-2-furancarboxylate hydrochloride (**2**)*

Ethyl 5-(4-cyanophenyl)-2-furancarboxylate **2** (1.81 g, 7.5 mmol) [10] was suspended in 22 ml of an absolute ethanol, cooled in an ice-salt bath and dry HCl was passed for 20 min. The stoppered flask was stirred at room temperature for 36 h. The solution was diluted with dry ether. The imidate ester hydrochloride, which precipitated as a white solid was filtred, washed with ether and dried under vacuum at room temperature overnight. The imidate ester was used directly without further characterization. Freshly distilled 1,3-propanodiamine (0.5 ml, 6 mmol) was added to suspension of the imidate ester hydrochloride **3** (2 g, 6 mmol) in 15 ml of absolute ethanol. The reaction mixture was stirred at room temperature for 12 h and refluxed for next 12 h. The reaction mixture was diluted with ether. A white solid was filtered, and dried under vacuum to yield 1.4 g of **6.**

Yield 71%; m.p. 238–240 °C; ^{1}H NMR (DMSO-d_6) δ 9.52 (br, 1H), 8.05 (d, 2H), 7.87 (d, 2H), 7.47 (d, 1H), 7.42 (d, 1H), 4.34 (q, 2H), 3.50 (t, 4H), 3.29 (t, 4H), 2.00 (m, 2H), 1.82 (m, 2H); ^{13}C NMR (DMSO-d_6) δ 158.4, 157.6, 154.8, 144.1, 132.8, 128.5, 128.2, 124.5, 120.2, 110.3, 60.7, 38.7, 17.6, 14.1. Anal. (C, H, N): $C_{17}H_{18}N_2O_3$•HCl•$2H_2O$ (370.5).

*2.1.2. 5-[4-(3,4,5,6-tetrahydro-*1H*-pyrimidine-2-yl)phenyl]-2-furancarboxylic acid hydrochloride (**3**)*

Ethyl 5-[4-(3,4,5,6-tetrahydro-1*H*-pyrimidine-2-yl)phenyl]-2-furancarboxylate hydrochloride **2** (1.4 g, 4 mmol) was suspended in a mixture of 10 ml of THF and 10 ml of 1 M LiOH. The reaction was allowed to stir at room temperature until no starting ester could be detected by TLC (ligroine/chloroform/methanol, 5:3:3). The basic mixture was acidified to pH 3 with 2 M hydrochloric acid (HCl) and diluted with acetone. The precipitated acid was filtered and dried in vacuo at room temperature to afford 1.1 g of **3.**

Yield 85%; m.p. 249–250 °C; ^{1}H NMR (DMSO-d_6) δ 10.13 (br, 1H), 8.03 (d, 2H), 7.90 (d, 2H), 7.37 (d, 1H), 7.35 (d, 1H), 3.49 (t, 4H), 2.01 (m, 2H); ^{13}C NMR (DMSO-d_6) δ 158.9, 158.3, 154.4, 144.1, 132.0, 128.4, 128.2, 124.3, 119.6, 110.2, 38.6, 17.6. Anal. (C, H, N): $C_{15}H_{14}N_2O_3$•HCl•$2H_2O$ (342.5).

2.1.3. N*-(2-aminoethyl)-5-[4-(3,4,5,6-tetrahydro-*1H*-pyrimidine-2-yl)phenyl]-2-furancarboxamide dihydrochloride (**5**)*

To a solution of 5-[4-(3,4,5,6-tetrahydro-1*H*-pyrimidine-2-yl)phenyl]-2-furancarboxylic acid hydrochloride **3** (1.1 g, 3 mmol) in 5 ml of anhydrous DMF in an ice bath was added DCI (0.49 g, 3 mmol). The solution was stirred for 30 min and freshly distilled ethylenediamine (0.18 g, 3 mmol) was added. It was stirred at 0 °C for 2 h, then the temperature was allowed to rise to the ambient and stirring was continued for 8 h. The volume of the reaction mixture was reduced to 1 ml by evaporation and the remaining mixture was diluted with acetone. The resulting solid was filtered, washed with acetone and dried under vacuum at room temperature. The crude product was basified with 1 M LiOH to pH above 9. A gummy solid was filtered, washed with water and dried under vacuum. The free base was converted into the salt by taking up in 3 ml of methanol, treated with 2 ml of 2 M HCl and stirred for 30 min. Acetone was added and the final product was filtered, washed with acetone and recrystallized from absolute ethanol to give 0.75 g of **5.** Yield 62%; m.p. 246–248 °C; ^{1}H NMR (DMSO-d_6) δ 10.13 (br, 1H), 8.03 (d, 2H), 7.90 (d, 2H), 7.37 (d, 1H), 7.35 (d, 1H), 3.51 (t, 2H), 3.49 (t, 4H), 2.91 (t, 2H), 2.01 (m, 2H); ^{13}C NMR (DMSO-d_6) δ 158.0, 157.6, 154.4, 144.1, 132.0, 128.4, 128.2, 124.3, 119.6, 110.2, 43.7, 38.6, 38.5, 17.6. Anal. (C, H, N): $C_{17}H_{20}N_4O_2$•2HCl•H_2O (403.0).

2.1.4. N-*(2-aminoethyl)-5-[4-(4,5-dihydro-*1H-*imidazol-2-yl)phenyl]-2-furancarboxamide dihydrochloride (**6**)*

This compound was synthesized using a similar procedure as for **5.**

Yield 64%; m.p. 237–239 °C; ^{1}H NMR (DMSO-d_6) δ 10.66 (br, 1H), 8.07 (dd, 4H), 7.42 (d, 1H), 7.40 (d, 1H), 4.02 (s, 4H), 3.51 (t, 2H), 2.91 (t, 2H); ^{13}C NMR (DMSO-d_6) δ 163.2, 157.6, 154.2, 145.4, 134.1, 129.5, 124.6, 121.7, 119.7, 111.0, 44.5, 43.7, 38.5. Anal. (C, H, N): $C_{16}H_{18}N_4O_2$•2HCl•H_2O (389.0).

2.1.5. N-*(2-(4-(4-*bis*(2-chloroethyl)aminophenyl)butyryl)-aminoethyl)-5-[4-(3,4,5,6-tetrahydro-*1H-*pyrimidine-2-yl)-phenyl]-2-furancarboxamide hydrochloride (**8**)*

Compound **5** (0.75 g, 2 mmol) was dissolved in 10 ml of anhydrous DMF. To this stirring solution triethylamine (0.28 ml, 2 mmol) was added. The solution was cooled to 0 °C before adding chlorambucil (0.61 g, 2 mmol). This provided a clear solution into which DCC (0.42 g, 2 mmol) was added all at once. It was stirred at 0 °C for 5 h, and then warmed to room temperature where it was kept for 24 h. The precipitate of dicyclohexylurea was removed by filtration. Concentration under vacuum gave a colorless solid which crystallized from methanol to give **8** (0.70 g). Yield 60%; m.p. 246–248 °C; ^{1}H NMR (DMSO-d_6) δ 10.13 (br, 1H), 8.03 (d, 2H), 7.90 (d, 2H), 7.37 (d, 1H), 7.35 (d, 1H), 7.12 (m, 2H), 6.65 (m, 2H), 3.64 (t, 4H), 3.56 (m, 4H), 3.54 (m, 2H), 3.51 (m, 4H), 3.40 (t, 2H), 2.92 (m, 2H), 2.64 (t, 2H), 2.21 (q, 2H), 2.01 (m, 2H); ^{13}C NMR (DMSO-d_6) δ 174.7, 158.0, 157.6, 154.4, 144.1, 136.9, 136.3, 132.0, 129.3, 128.5, 128.4, 128.2, 124.3, 119.6, 110.2, 48.7, 43.7, 41.9, 38.6, 38.5, 35.0, 28.9, 27.4, 17.6. Anal. (C, H, N): $C_{31}H_{36}N_5O_3Cl_2$•HCl•H_2O (652.5).

2.1.6. N-*(2-(4-(4-*bis*(2-chloroethyl)aminophenyl)butyryl)-aminoethyl)-5-[4-(4,5-dihydro-*1H-*imidazol-2-yl)phenyl]-2-furancarboxamide hydrochloride (**9**)*

This compound was synthesized using a similar procedure as for **8.**

Yield 64%; m.p. 232–234 °C; ^{1}H NMR (DMSO-d_6) δ 10.60 (br, 1H), 8.07 (dd, 4H), 7.44 (d, 1H), 7.40 (d, 1H), 7.12 (m, 2H), 6.65 (m, 2H), 4.02 (s, 4H), 3.64 (t, 4H), 3.56 (t, 4H), 3.51 (t, 2H), 3.40 (t, 2H), 2.92 (m, 2H), 2.64 (t, 2H), 2.21 (q, 2H); ^{13}C NMR (DMSO-d_6) δ 174.7, 163.2, 157.6, 154.2, 145.4, 136.9, 136.3, 134.1, 129.5, 129.3, 128.5, 124.6, 121.7, 119.7, 111.0, 44.5, 48.7, 43.7, 41.9, 38.5, 35.0, 28.9, 27.4. Anal. (C, H, N): $C_{30}H_{35}N_5O_3Cl_2$•HCl•H_2O (638.5).

2.2. Pharmacology

2.2.1. Materials

Ethidium bromide, netropsin, distamycin, calf thymus DNA, T4 coliphage DNA, homopolymers poly(dA–dT)poly-(dA–dT) and poly(dG–dC)poly(dG–dC), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (USA). Topoisomerase II was purchased from Amersham Pharmacia Biotech. Stock cultures of breast cancer, MCF-7 and MDA-MB-231, were purchased from the American Type Culture Collection, Rockville, MD. Dulbecco's minimal essential medium (DMEM) and fetal bovine serum (FBS) used in celI culture were products of Gibco (USA). Glutamine, penicillin and streptomycin were obtained from Quality Biologicals Inc. (USA). [^{3}H]-thymidine (6.7 Ci/mmol) was the product of NEN (USA).

2.2.2. Cell culture

Human breast cancer MDA-MB-231 and MCF-7 cells maintained in DMEM supplemented with 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin at 37 °C. Cells were cultured in Costar flasks and subconfluent cells were detached with 0.05% trypsin and 0.02% EDTA in calcium-free phosphate-buffered saline (PBS), counted in hemocytometers and plated at 5×10^5 cells per well of six-well plates (Nunc) in 2 ml of growth medium (DMEM without phenol red with 10% CPSR1). Cells reached about 80% of confluency at day 3 and in most cases such cells were used for the assays.

2.2.3. DNA synthesis assay

To examine the effect of studied compounds on cells proliferation MCF-7 and MDA-MB-231cells were seeded in six-well plates and grown as described above. Cells culture were incubated with varying concentrations of compounds **7–9,** chlorambucil and 0.5 μCi of [^{3}H]-thymidine for 24 h at 37 °C. The cells were then harvested by trypsinization and washed (with cold PBS) with centrifugation for 10 min at 1500 g several times (4–5) until the desintegration per minute (dpm) in the washes were similar to the reagent control. Radioactivity was determined by liquid scintillation counting. [^{3}H]-thymidine uptake was expressed as dpm/well.

2.2.4. Cell viability assay

The assay was performed according to the method of Carmichael using MTT [11]. Confluent cells, cultured for 24 h with various concentrations of studied compounds in six-well plates were washed three times with PBS and then incubated for 4 h in 1 ml of MTT solution (0.5 mg/ml of PBS) at 37 °C in 5% CO_2 in an incubator. The medium was removed and 1 ml of 0.1 mol/l HCl in absolute isopropanol was added to attached cells. Absorbance of converted dye in living cells was measured at a wavelength of 570 nm. Cell viability of breast cancer MCF-7 cells cultured in the presence of ligands was calculated as a percent of control cells.

2.2.5. Relaxation assay of topoisomerases I and II

PBR322 plasmid DNA (0.083 μg) was incubated with 1 unit of human topoisomerase I (reaction buffer: 50 mM Tris–HCl (pH 7.9), 1 mM EDTA, 0.5 M NaCl and 1 mM dithiothreitol) or human topoisomerase II (reaction buffer: 10 mM Tris–HCl (pH 7.9), 1 mM ATP, 50 mM KCl, 5 mM $MgCl_2$, 50 mM NaCl, 0.1 mM EDTA and 15 μg/ml bovine

Table 1
Viability of MCF-7 and MDA-MB-231 cells treated for 24 h with different concentrations of compounds **7–9** and chlorambucil

Concentration (μM)	Viability of cells (% of control)[a]							
	7		**8**		**9**		Chlorambucil	
	MCF-7	MDA-MB-231	MCF-7	MDA-MB-231	MCF-7	MDA-MB-231	MCF-7	MDA-MB-231
0	100	100	100	100	100	100	100	100 ± 2
10	65 ± 2	66 ± 2	78 ± 2	74 ± 2	62 ± 2	55 ± 2	82 ± 2	80 ± 2
25	58 ± 2	56 ± 2	68 ± 2	64 ± 2	48 ± 2	44 ± 2	73 ± 3	70 ± 2
50	40 ± 2	42 ± 2	60 ± 2	56 ± 2	30 ± 2	26 ± 2	65 ± 2	62 ± 2
75	31 ± 1	28 ± 2	52 ± 2	49 ± 2	23 ± 2	18 ± 2	56 ± 2	54 ± 2
100	25 ± 1	20 ± 2	44 ± 2	40 ± 2	16 ± 2	8 ± 2	48 ± 2	48 ± 2

[a] Mean values ± S.D. from three independent experiments done in duplicate are presented.

serum albumin) in the presence of varying concentrations of the test compound. The mixture was incubated at 37 °C for 1 h and the reaction was terminated by addition of 2 μl of 10% SDS and 2 μl of proteinase K (1 mg/ml). The reaction mixture was subjected to electrophoresis through a 0.8% agarose gel containing 0.5 mg/ml ethidium bromide in TBE buffer (90 mM Tris–borate and 2 mM EDTA). The gels were stained with ethidium bromide and photographed under UV light. For the quantitative determination of topoisomerase concentration activity, photographic negatives were scanned and the area representing supercoiled DNA, migrating as a single band at the bottom of the gel was measured using UVI-KS4000i gel documentation and analysis system (SyngenBiotech, San Carlos, CA, USA). The concentrations of the inhibitor that prevented 50% of the supercoiled DNA from being converted into relaxed DNA (IC_{50} values) were determined by averaging the data from at least three experiments.

2.2.6. Ethidium displacement assay

Fluorescence was measured using a Hitachi spectrophotometer F-2500 FL (Tokyo, Japan) at room temperature. The DNA–ethidium complex was excited at 546 nm and the fluorescence was measured at 595 nm. To 2 ml of ethidium bromide (5.0×10^{-6} M) in 10 mM Tris–HCl (pH 7.4), 75 mM NaCl buffer solution, containing 25 μl of DNA solution (A_{260} = 2) was added, and the maximum fluorescence was measured. Aliquots of a 10 mM stock the test compound solution were then added to the DNA–ethidium solution, and the fluorescence was measured after each addition until a 50% reduction of fluorescence had occurred. Theoretical curves were fit to the fluorescence intensity data points with non-linear least-squares computer routines. The apparent binding constant was calculated from K_{EtBr}[EtBr] =K_{app}-[drug], where [drug] = the concentration of the test compound at a 50% reduction of fluorescence and K_{EtBr} is known [12]. The compounds **4–9** and their complexes with DNA showed neither optical absorption nor fluorescence at 595 nm and did not interfere with the fluorescence of an unbound ethidium bromide.

2.2.7. Statistical analysis

In all experiments, the mean values for three assays ± standard deviations (S.D.) were calculated. The results were submitted to statistical analysis using the Student's *t*-test. Differences were considered significant when $P < 0.05$. Mean values, the S.D. and the number of measurements in the group (n) are presented in the figures.

3. Results

Synthesis of compounds **7–9** was accomplished starting with ethyl 5-(4-cyanophenyl)-2-furancarboxylate (Fig. 1). This compound was treated under Pinner reaction conditions to generate amidine hydrochloride terminal groups by reaction with HCl in ethanol, followed by treatment with ammonia or aliphatic diamine in ethanol [9,10]. The amidine esters were isolated by precipitation from ethanol with ether and were used without additional purification. The esters were subjected to saponification with lithium hydroxide in THF/H_2O (1:1) solution. The sodium salts were acidified to pH 3 with 2 M HCl and diluted with acetone. The obtained acids **1–3** were conjugated with ethylenediamine in the presence of DCI as condensing agent in DMF at 0 °C to give the key intermediates for the synthesis of amidine analogs of chlorambucil compounds **4–6.** The final incorporation of the alkylating unit into the DNA-binding moieties was achieved using the classical coupling procedure mediated by DCC in the present triethylamine. These new amidine analogs of chlorambucil (**7–9**) differ by the nature of their terminal basic side chains were isolated as the hydrochloride salts.

Cell viability of breast cancer cells was measured by the method of Carmichael et al. [11] using tetrazolium salt (Table 1). No significant reduction of viability of both MCF-7 and MDA-MB-231 cells growth was observed for compounds **4–6** utilizing the same concentration range (not shown). Although growth inhibition was concentration dependent in either cell line, it was more pronounced at shorter times, in MDA-MB-231 than MCF-7 (Table 1). In terms of reduction in cell viability, the compounds rank in both MCF-7 and MDA-MB-231 cells in the order **9** >**7** >**8** > chlorambucil. The values of IC_{50} were relatively higher for **9** and **7** which possess a cationic 4,5-dihydro-1*H*-imidazol and amidine function, respectively. Among the derivatives, compound **8** in both MDA-MB-231 and MCF-7 proved to be only slightly more potent than chlorambucil,

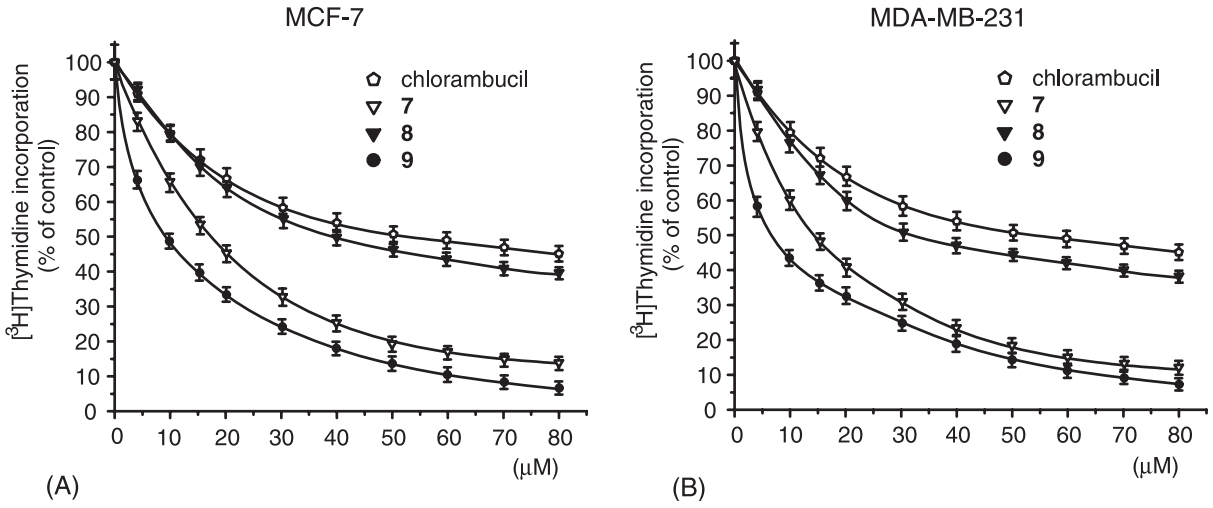

Fig. 2. Cytotoxic effects of chlorambucil and compounds **7–9** on the cultured breast cancer MCF-7 (A) and MDA-MB-231 (B) cells as measured by inhibition of [^{3}H]-thymidine incorporation into DNA. Mean values ± S.D. of three independent experiments (n = 4) done in duplicates are presented.

with IC_{50} values of 70 ± 2 and 76 ± 2 μM, respectively, compared to 92 ± 2 and 97 ± 2 μM for chlorambucil. In contrast, compound **9,** which contains the 4,5-dihydro-1*H*-imidazol moiety is clearly much more active and showed a high level of cytotoxic potency, IC_{50} 22 ± 2 and 18 ± 2 μM in MCF-7 and MDA-MB-231, respectively. Compound **9,** the most active of the series, is approximately five times more potent than chlorambucil.

To analyze if the inhibition in cell viability was due to decreased cell proliferation, we measured DNA synthesis in presence of compounds **7–9** and chlorambucil (Fig. 2). All of the tested compounds showed concentration-dependent activity, yet with different potency. Furthermore, the profiles of DNA synthesis obtained were similar between MCF-7 and MDA-MB-231 (Fig. 2). The concentrations of compounds **7–9** needed to inhibit [^{3}H]-thymidine incorporation into DNA by 50% (IC_{50}) in MDA-MB-231 was found to be 12 ± 2, 33 ± 3 and 6 ± 2 μM, respectively, suggesting higher cytotoxic potency compared to chlorambucil (IC_{50} = 49 ± 2 μM). The concentrations of **7, 8, 9** and chlorambucil needed to 50% reduction in [^{3}H]-thymidine incorporation into DNA in breast cancer MCF-7 (IC_{50}) was found to be 16 ± 2, 40 ± 2, 9 ± 2 and 54 ± 2 μM, respectively.

To test whether cytotoxic properties were related to DNA-binding and topoisomerase I/II inhibition, the cyclic amidine analogs of chlorambucil (**8** and **9**) were evaluated in a cell-free system. The binding affinities of compounds **7–9,** netropsin and distamycin to calf thymus DNA, T4 coliphage DNA, and synthetic polymers poly(dA–dT)$_2$ and poly(dG–dC)$_2$ were compared by using the ethidium displacement assay [12,13]. Table 2 summarizes the results for those ligands that did affect the fluorescence due to the intercalated ethidium at pH 7.4. Compounds **7–9** showed base specificity and groove-binding characteristics to DNA similar to that of the aromatic amidines **4–6.** The large apparent binding constants for T4 coliphage DNA for **4–9** gave evidence of their minor groove selectivity, because the major groove of T4 coliphage DNA is blocked by α-glycosylation of the 5-(hydroxymethyl)cytidine residues [14]. The homopolymer DNA-binding data reported in Table 2 characterizes the affinity of the compounds **4–9** for a more limited set of DNA-binding sites and can give an indication of base-sequence specificity for DNA-binding molecules. Compounds **4–9** were found to interact with a GC base pair though the binding affinity were weak compared with that for an AT base pair (Table 2). The binding constant obtained here for binding of **9** to poly(dC–dG)$_2$ polymer is almost 12 smaller than the association constant for binding of **9** to poly(dA–dT)$_2$. The compounds **4–9** bind to DNA in ethidium bromide displace-

Table 2
DNA binding and topoisomerase II inhibitory effect of netropsin, distamycin and compounds **4–9**

Ligand	Calf thymus DNA[a] ($K_{app} \times 10^5$/M)	T4 DNA[a] ($K_{app} \times 10^5$/M)	poly(dA–dT)$_2$[a] ($K_{app} \times 10^5$/M)	poly(dG–dC)$_2$[a] ($K_{app} \times 105$/M)	Inhibition of topo II (μM)
Netropsin	8.7	8.3	875	2.5	5
Distamycin	7.5	6.4	340	2.0	5
4	2.0	2.2	3.6	1.2	80
5	0.8	0.6	1.2	0.4	110
6	2.6	2.4	4.2	1.9	50
7	1.8	1.4	3.0	0.3	10
8	0.7	0.6	0.8	0.3	70
9	2.6	2.2	3.8	0.3	5

[a] The error for netropsin, distamycin and compounds **4–9** is ±0.2 × 10^5 M^{-1}.

ment assays more weakly than the extensively studied minor groove binders such as netropsin and distamycin (Table 2).

To test whether cytotoxic properties were related to DNA-binding and topoisomerase I/II inhibition, the cyclic amidine analogs of chlorambucil (**8** and **9**) were evaluated in a cell-free system. The binding affinities of compounds **7–9,** netropsin and distamycin to calf thymus DNA, T4 coliphage DNA, and synthetic polymers poly(dA–dT)$_2$ and poly(dG–dC)$_2$ were compared by using the ethidium displacement assay [12,13]. Table 2 summarizes the results for those ligands that did affect the fluorescence due to the intercalated ethidium at pH 7.4. Compounds **7–9** showed base specificity and groove-binding characteristics to DNA similar to that of the aromatic amidines **4–6.** The large apparent binding constants for T4 coliphage DNA for **4–9** gave evidence of their minor groove selectivity, because the major groove of T4 coliphage DNA is blocked by α-glycosylation of the 5-(hydroxymethyl)cytidine residues [14]. The homopolymer DNA-binding data reported in Table 2 characterizes the affinity of the compounds **4–9** for a more limited set of DNA-binding sites and can give an indication of base-sequence specificity for DNA-binding molecules. Compounds **4–9** were found to interact with a GC base pair though the binding affinity were weak compared with that for an AT base pair (Table 2). The binding constant obtained here for binding of **9** to poly(dC–dG)$_2$ polymer is almost 12 smaller than the association constant for binding of **9** to poly(dA–dT)$_2$. The compounds **4–9** bind to DNA in ethidium bromide displacement assays more weakly than the extensively studied minor groove binders such as netropsin and distamycin (Table 2).

The ability of compounds **4–9** to inhibit topoisomerases I and II activity was quantified by measuring the action on supercoiled pBR322 DNA substrate as a function of increasing concentration of the ligands by the use of agarose gel electrophoresis. Chlorambucil as a control was, as expected, ineffective in this assay. These results demonstrated that **4–6** have topoisomerase II inhibitory activity with 50% inhibitory concentrations (IC_{50}) ranging from 5 to 70 μM (Table 2). None of the compounds **4–9** inhibited the topoisomerase I-mediated relaxation of supercoiled DNA at a concentration of 150 μM (data not shown). Compound **9** was the most potent topoisomerase II inhibitors, with 50% inhibitory concentration (IC_{50}) 5 μM. Table 2 also shows that compound **9** is more active than **4–8** as a topoisomerase II inhibitor. The result of DNA-binding studies, reveal that **9** does have a greater DNA-binding affinity, which correlates with its greater potency relative to **7** and **8** as a topoisomerase II inhibitor.

The topoisomerase II-targeting drugs can be classified as either topo II poisons, which act by stabilizing enzyme–DNA cleavable complexes leading to DNA breaks, or topo II catalytic inhibitors, which act at stages in the catalytic cycle of the enzyme where both DNA strands remain intact and no DNA strand breaks occur [15,16]. Treatment with the classical topo II poison, e.g. etoposide, results in the production of linear DNA which demonstrates that these compounds stabilize DNA–topo II covalent complexes and hence stimulate a double-strand cleavage by the enzyme. Conversely, with **7–9** no band corresponding to linear DNA was detected in the presence of enzyme (data not shown), implying that these compounds do not act as topo poisons. It is likely that the ability of compounds **7–9** to inhibit the activity of topo II that we have observed (Table 2) is simply due to blockade of the binding of these enzymes to DNA. It is possible that compounds **7–9** acts through inducing a conformational change in the DNA and hindering the formation of the cleavable complex.

4. Discussion

In breast cancer studies, estrogen responsive and non-responsive breast cancer cell lines have been extensively used for elucidating the factors responsible for cell growth and for developing new strategies to inhibit cell growth. Our experimental studies have demonstrated that compounds **7–9** treatment prevented the exponential growth and decreased the number of viable cells in both estrogen receptor-positive and -negative breast cancer cells. As the antiproliferative effect of compounds **7–9** is independent of the estrogen receptor status of the breast cancer cells, these potent inhibitors are a potential pharmacological agents for the treatment of both hormone responsive and non-responsive breast cancer cells.

The compound **9,** which possess a 4,5-dihydro-1*H*-imidazol function, is the most cytotoxic amidine analogs of chlorambucil among a series of derivatives we have synthesized to date. The increase in potency for this compound may be related, in part, to an enhanced binding to DNA and topoisomerase II inhibition. These data suggest that steric factors associated with substituents at *N*-terminal position may substantially influence the activity of the cyclic amidine analogs of chlorambucil.

We have shown in the present report that compounds **7–9** are potent catalytic inhibitors of topoisomerase II. This topological enzyme binds at least in part to AT rich sequences in the minor groove [17,18]. Topoisomerase II which has been identified as a major scaffold protein of mitotic chromosomes being present in the interphase nuclear matrix, is a homodimeric nuclear enzyme essential for DNA functioning, in particular negative supercoiling of DNA organized as superhelix, a process necessary for replication, recombination and transcription of nuclear DNA [15,18]. It is probable that deregulation of DNA replication and transcription by inhibition of topoisomerase II activity contribute significantly to the cytotoxicity of alkylators in addition to primary drug–DNA reaction products [19]. This suggests that DNA binding may be implicated in the cytotoxicity of compounds **7–9** possibly by inhibiting interactions between topoisomerases and their DNA targets.

It should be noted that other factors such as low penetration into cell, cellular distribution and metabolic deactivation

may also influence the cytotoxicity results, but they are not assessed in the present study. Further biological evaluation is underway and these results, cell uptake studies and sequence selective protection of restriction enzyme recognition sites by described compounds will be described in due course.

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