Amiloride Intercalates into DNA and Inhibits DNA Topoisomerase II*

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Jeffrey M. Besterman‡‡, Lynn P. Elwell†, Steven G. Blanchard‡‡, and Michael Cory**

From the Departments of Molecular Biology, Microbiology, and Organic Chemistry, Wellcome Research Laboratories, Research Triangle Park, North Carolina 27709

Amiloride is capable of inhibiting DNA synthesis in mammalian cells in culture. Recent evidence indicates that the enzyme, DNA topoisomerase II, is probably required for DNA synthesis to occur in situ. In experiments to determine the mechanism of inhibition of DNA synthesis by amiloride, we observed that amiloride inhibited both the catalytic activity of purified DNA topoisomerase II in vitro and DNA topoisomerase II-dependent cell functions in vitro. Many compounds capable of inhibiting DNA topoisomerase II are DNA intercalators. Thus, we performed studies to determine if and how amiloride bound to DNA. Results indicated that amiloride 1) shifted the thermal denaturation profile of DNA, 2) increased the viscosity of linear DNA, and 3) unwound circular DNA, all behavior consistent with a DNA intercalation mechanism. Furthermore, quantitative and qualitative measurements of amiloride fluorescence indicated that amiloride (a) bound reversibly to purified DNA under conditions of physiologic ionic strength, and (b) bound to purified nuclei in a highly cooperative manner. Lastly, amiloride did not promote the cleavage of DNA in the presence of DNA topoisomerase II, indicating that the mechanism by which amiloride inhibited DNA topoisomerase II was not through the stabilization of a "cleavable complex" formed between DNA topoisomerase II, DNA, and amiloride. The ability of amiloride to intercalate with DNA and inhibit topoisomerase II is consistent with the proposed planar, hydrogen-bonded, tricyclic nature of amiloride's most stable conformation. Thus, DNA and DNA topoisomerase II must be considered as new cellular targets of amiloride action.

MATERIALS AND METHODS

Enzymes, Nucleic Acids, and Antitumor Drugs—Calf thymus DNA topoisomerase II was purified to greater than 95% homogeneity according to the procedure of Hahlin et al. (12). Plasmid DNA was purified from P1 tailless capsids as previously described (13). Plasmid pBR322 was purified using a discontinuous, two-step CsCl-ethidium bromide gradient technique (14). 4'-(9-Acridinylamino)methane sulfon-m-anisidide (m-AMSA, 1 NSC 249992) and its isomer o-AMSA (NSC 156506) were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institutes. All drugs were dissolved in dimethyl sulfoxide (Me2SO) at 1 mg/ml. Amiloride HCl was a gift from Merck Sharp and Dohme.

Assays for DNA Topoisomerase II Function—End-labeled pBR322 DNA was prepared as published (15). The cleavable-complex assay, the topoisomerase II unknotting-inhibition assay, and the topoisomerase II relaxation-inhibition assay were performed as described (9, 16, 17). The final concentration of Me2SO in all assays did not exceed 1% (v/v). This was especially important for the cleavable-complex assay in which Me2SO at concentrations greater than 1% stimulates topoisomerase II-mediated DNA cleavage.

DNA—Calf thymus DNA (Warington) was dissolved in 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0, to a final concentration of 3 mg/ml, and sonicated as described by Davidson et al. (18). DNA concentrations were determined using an extinction coefficient of 6600 M at 260 nm and are expressed in terms of nucleotide equivalents per liter. The purified sonicated DNA samples displayed an A260/A280 ratio of between 1.85 and 1.9 and a total hyperchromicity at 260 nm of 30%. These spectral properties are consistent with published values (19). The DNA contained less than 0.5% residual RNA as determined by the method of Savitsky and Stand (20).

Thermal Denaturation—Thermal denaturation experiments were performed by the method of Cory et al. (21, 22) on a Varian CARY 2290-UV-VIS recording spectrophotometer with a five cell temperature-controlled turret. The average value of the Tm for calf thymus DNA was 88.1 °C (n = 40, S.D. = 1.5 °C).

Viscometric Titrations—The viscometric titrations were done by the procedure of Cory et al. (21). The buffer used was 2 mM MES at pH 6.3, containing 1 mM EDTA. Ammonium fluoride was added to the MES buffer to adjust the ionic strength. Reduced specific viscosity was calculated by the method of Cohen and Eisenberg (23). Data

1 The abbreviations used are: m-AMSA, 4'-(9-acridinylamino)-methylene sulfon-m-anisidine; o-AMSA, 4'-(9-acridinylamino)methane sulfon-o-anisidine; Tm, melting temperature; MES, 2-(N-morpholino)ethanesulfonic acid; RPMI, Roswell Park Memorial Institute; DMEM, Dulbecco's modified Eagle's medium; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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† To whom all correspondence should be addressed.

‡‡ Present address: Dept. of Chemotherapy, Glaxo Research Laboratories, Research Triangle Park, NC 27709.
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analysis was done using the FIT LINE procedure on the PROPHET (24) computer.

Unwinding Angle Determination—The unwinding angle determination was done using the viscometric titration technique of Revet et al. (25). The high copy number 6.9-kilobase plasmid pOPlA6 (26) was isolated by the method of Garger et al. (14) and shown to be supercoiled DNA by gel electrophoresis.

Cell Culture and Synchronization—The human promyelocytic cell line, HL-60, was grown in spinner culture in RPMI 1640 medium containing 10% (v/v) fetal bovine serum. BALB/c 3T3 fibroblasts were isolated by the method of Garger et al. (14) and shown to be quiescent in response to tumor-promoting phorbol dibutyrate and other addenda as specified. After 4 h, the number of adherent cells was determined as described previously (27).

Assay for DNA Synthesis in Aphidicolin-synchronized HL-60 Cells—Aphidicolin-synchronized HL-60 cells were released from the block and allowed to traverse S phase by washing the cells once in the absence or presence of amiloride or DNA topoisomerase II inhibitors, as indicated. After 1 h of incubation at 37 °C, [3H]thymidine was added to a final concentration of 1 μCi/ml and incubation continued at 37 °C for 4 h. Incorporation of [3H] thymidine was halted and measured by precipitation with trichloroacetic acid, passing the sample over a GF/C filter, washing the filter with 1% NaOH, and counting the filter using Aquasol-2.

Fibroblist Mitogenesis Assay—Monolayers of BALB/c 3T3 fibroblasts were rendered quiescent by culture for 24 h in DMEM, 0.5% fetal bovine serum. All monolayers received the same number of washes and reincubation in fresh DMEM, 0.5% fetal bovine serum. The high copy number 6.9-kilobase plasmid pOPlA6 (26) containing 10% (v/v) fetal bovine serum. BALB/c 3T3 fibroblasts were rendered quiescent by culture for 24 h in DMEM, 0.5% fetal bovine serum. Cells-Aphidicolin-synchronized HL-60 Cells—Aphidicolin-synchronized HL-60 cells were released from the block and allowed to traverse S phase by washing the cells once in the absence or presence of amiloride or DNA topoisomerase II inhibitors, as indicated. After 1 h of incubation at 37 °C, [3H]thymidine was added to a final concentration of 1 μCi/ml and incubation continued at 37 °C for 4 h. Incorporation of [3H] thymidine was halted and measured by precipitation with trichloroacetic acid, passing the sample over a GF/C filter, washing the filter with 1% NaOH, and counting the filter using Aquasol-2.

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Amiloride Inhibits Topoisomerase II-dependent Cell Functions in Vivo—Although mammalian DNA topoisomerase II has been suggested to play a role in numerous cellular (nuclear) processes (i.e. DNA replication, chromosome sorting, and transcription; for review see Ref. 29), there does not exist an assay of intact mammalian cell function as a standard barometer of DNA topoisomerase II activity in vivo. However, using three cellular assay systems in which DNA topoisomerase II activity appears to be required, we determined the likelihood that amiloride inhibited DNA topoisomerase II in intact cells.

Amiloride Inhibits Phorbol Dibutyrate-induced Adhesion of HL-60 Cells—HL-60 cells, in human promyelocytic cell line, differentiates in response to tumor-promoting phorbol diesters, such as phorbol dibutyrate (30, 31). Protein kinase C, a calcium- and phospholipid-dependent protein kinase, is activated by phorbol dibutyrate (reviewed in Ref. 32). Previous work from our laboratory indicated that protein kinase C phosphorylated purified DNA topoisomerase II in vitro, resulting in the activation of the topoisomerase (33). These findings indicated the possibility that DNA topoisomerase II played a role in phorbol diester induced-differentiation of HL-60 cells. Consistent with this hypothesis, we have reported previously that the phorbol diester-induced differentiation of HL-60 cells (as assessed by induction of cell adhesion) was blocked by the topoisomerase II inhibitors novobiocin and m-AMSA, but not by the inactive o-AMSA (33). Using an improved version of this assay, Fig. 3 indicates that amiloride, like novobiocin and m-AMSA, inhibited phorbol diester-induced adhesion of synchronized HL-60 cells in a dose-dependent manner, with an apparent Ki of 200 μM. These results

RESULTS

Amiloride Inhibits the Catalytic Activities of Purified Mammalian DNA Topoisomerase II in Vitro—Two different measures of catalytic activity of purified calf thymus DNA topoisomerase II were assayed in vitro. The ability to strand-pass was measured using the bacteriophage P2 unknotting assay and the ability to relax supercoiled DNA was measured using pBR322 plasmid DNA. Amiloride, like the DNA topoisomerase II inhibitor novobiocin, inhibited in a dose-dependent manner the topoisomerase II-mediated conversion of P4 DNA from the knotted to the unknotted form (Fig. 1). Inhibition of DNA topoisomerase II catalysis by amiloride occurred with an apparent Ki of 0.15 mM (Fig. 2). Although amiloride is a weak inhibitor by comparison to most other known DNA topoisomerase II inhibitors (e.g. m-AMSA, Ki = 25 μM, Ref. 9; ellipticine, Ki = 4 μM, Refs. 10 and 11; epipodophyllotoxin VM-26, Ki = 5 μM, Ref. 16), amiloride is commonly used in experiments with cultured cells at concentrations of 1 mM or greater. Thus, it was necessary to determine whether amiloride inhibited functions associated with DNA topoisomerase II in intact cells.

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![Fig. 1. Inhibition of the DNA unknotting activity of calf thymus DNA topoisomerase II by amiloride and novobiocin.](https://example.com/figure1.png)

The strand-passing activity of calf thymus DNA topoisomerase II was monitored by the P2 unknotting assay (13). The positions of unknotted (U), knotted (K), and linear (L) P2 DNA are indicated. Lane M was the knotted DNA control (no enzyme and no inhibitor). Lanes A—L contained DNA, topoisomerase II, and inhibitors as indicated. Lanes A—F contained amiloride (5.0, 2.5, 1.25, 0.62, 0.31, and 0.15 mM, respectively); lanes G—L contained novobiocin (10, 5.0, 2.5, 1.25, 0.62, and 0.31 mM, respectively).
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FIG. 2. Inhibition of the DNA relaxation activity of calf thymus DNA topoisomerase II by amiloride and novobiocin. The strand-passing activity of calf thymus DNA topoisomerase II was monitored by the ability to relax supercoiled pBR322 plasmid DNA. The positions of relaxed (REL.) and supercoiled (SC) pBR322 DNA are indicated. Lane SC was the supercoiled DNA control (no enzyme and no inhibitor). Lane (+) was the enzyme control (topoisomerase II plus DNA; no inhibitor). Lanes A–L contained DNA, topoisomerase II, and inhibitors as indicated. Lanes A–F contained amiloride (5.0, 2.5, 0.5, 0.25, 0.125, and 0.06 mM, respectively); lanes G–L contained novobiocin (5.0, 2.5, 0.5, 0.25, 0.125, and 0.06 mM, respectively).

FIG. 3. Amiloride inhibits phorbol dibutyrate-induced adhesion of HL-60 cells. Assays were performed as described under “Materials and Methods.” Amiloride was added from 500-fold concentrated stock solutions in dimethyl sulfoxide, and the final concentration of dimethyl sulfoxide never exceeded 0.5% (v/v). Amiloride was added to the cells 15 min prior to the addition of 200 nM phorbol dibutyrate. Each value represents the mean of three independent determinations. All S.E. were ±5%.

suggest that amiloride may have the ability to inhibit topoisomerase II in intact HL-60 cells. However, we have previously shown that amiloride, unlike novobiocin and m-AMSA, can inhibit protein kinase C in vitro and in vivo with a $K_i$ of $\approx 1$ mM (27). Thus, additional assays of topoisomerase II function in intact cells were explored.

Amiloride Inhibits DNA Synthesis in Synchronized HL-60 Cells—DNA topoisomerase II may be required for DNA synthesis (6, 7), and the enzyme has been shown to be associated with newly replicated DNA (8). Aphidicolin is a reversible, potent, and specific inhibitor of DNA polymerase $\alpha$ and is often used to synchronize mammalian cells at $G_1/S$ (34). Removal of aphidicolin provides a synchronized wave of cell traversal through $S$ phase (DNA synthesis). In this model of cellular DNA synthesis, the DNA topoisomerase II inhibitors novobiocin and m-AMSA, but not $o$-AMSA, blocked incorporation of $[^3H]$thymidine by synchronized HL-60 cells. Novobiocin (275 $\mu$M), m-AMSA (2.5 $\mu$M), and $o$-AMSA (5 $\mu$M) inhibited $[^3H]$thymidine incorporation by 70%, 67%, and 70% (mean ± S.E.), respectively. Likewise, amiloride inhibited the incorporation of $[^3H]$thymidine by aphidicolin-synchronized HL-60 cells in a dose-dependent manner, with an apparent $K_i$ of 300 $\mu$M (data not shown). The inhibition of $[^3H]$thymidine incorporation by amiloride was not due to an effect on $[^3H]$thymidine pools, as has already been ruled out previously for experiments in 3T3 fibroblasts (4).

Amiloride Inhibits Serum-induced DNA Synthesis by Fibroblasts in the Same Time Frame as Topoisomerase II Inhibitors—Amiloride can inhibit mitogen-induced DNA synthesis by quiescent fibroblasts (4, 35). The ability of amiloride to inhibit mitogenesis was ascribed initially to the ability of amiloride to inhibit growth factor induced Na+/H+ exchange (35, 36). However, work by ourselves (4, 27) and others (37, 38) indicated that amiloride can inhibit many cellular processes including RNA synthesis, protein synthesis, and protein kinase activity. In addition, we observed that inhibition by amiloride required only that the drug be present in the hours immediately preceding $S$ phase, a time period incompatible with a requirement for Na+/H+ exchange (4).

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| Treatment | Time of treatment | $[^3H]$Thymidine incorporation (% inhibition) |
|-----------|-----------------|-------------------------------------------|
| $o$-AMSA  | +               | 6 ± 2                                     |
|           | –               | 4 ± 2                                     |
| m-AMSA    | +               | 19 ± 4                                    |
|           | –               | 54 ± 5                                    |
| Novobiocin| +               | 18 ± 6                                    |
|           | –               | 59 ± 7                                    |
| Amiloride | +               | 10 ± 5                                    |
|           | –               | 51 ± 5                                    |
that inhibition of DNA synthesis by amiloride may, at least in part, result from inhibition of topoisomerase II.

**Elucidating the Mechanism by Which Amiloride Inhibits DNA Topoisomerase II** — To determine the mechanism by which amiloride inhibits the catalytic activity of DNA topoisomerase II in vitro and in vivo, studies were performed to assess if and how amiloride might interact directly with DNA.

**Thermal Denaturation of DNA** — The ability of an agent to alter the thermal denaturation profile of DNA (in excess of ionic strength effects alone) is used as an indication of the ability of the compound to bind to DNA (22). Table II shows the increase in thermal denaturation temperature of amiloride-DNA complexes (ΔTm), measured at fixed DNA concentration and varying amiloride concentration. Comparison with the standard, ethidium bromide, indicates that amiloride interacts with DNA with a lower affinity. Control experiments indicated that the increased ionic strength due to the addition of the amiloride HCl salt could not account for the ΔTm generated by amiloride. These results indicate that amiloride binds to DNA, although these observations alone cannot distinguish between intercalation into DNA and other modes of DNA binding.

**Viscometric Titrations of Linear DNA** — One characteristic of intercalative binding to DNA is the increase in DNA length that results when a ligand stacks between the base pairs. Sonicated DNA in solution exhibits hydrodynamic behavior similar to that of a rigid rod. The helical extension afforded to rod-like DNA by intercalators can be measured by viscometric titration, and the relative increase in DNA contour length (L/L0) versus the ligand/nucleotide ratio can be determined. Fig. 4 shows a comparison of the viscometric titrations with amiloride and ethidium bromide at low ionic strength, 1 mM ammonium fluoride buffer. The ethidium bromide data are consistent with the neighbor exclusion principle in that saturation occurs at 0.25 DNA phosphates, indicating binding of one ethidium molecule per two base pairs. While the initial slope of the amiloride titration is the same as that for ethidium bromide, the titration saturated at a drug/phosphate ratio of approximately 0.1 to 0.15, indicating binding of one amiloride molecule per four base pairs at saturation.

In general, high concentrations of salt weaken the binding of compounds to DNA. In analogy to Fig. 4, viscometric titration of DNA with amiloride and ethidium bromide were performed in high ionic strength buffer, 500 mM ammonium fluoride. The tightly binding intercalator, ethidium bromide, again showed neighbor-excluded intercalation (saturation at 0.25 DNA phosphates per drug molecule), with a viscosity slope only slightly lower than that in low salt. The weaker binding amiloride, however, showed no intercalation (zero slope) in the presence of 500 mM ammonium fluoride (data not shown).

**Viscometric Titrations of Circular DNA: Determining the Unwinding Angle** — Although the increase of viscosity of linear DNA which occurs in the presence of amiloride is consistent with intercalative binding, binding to the DNA surface could stiffen the helix and result in an increase in DNA viscosity. However, a property exclusive to intercalative DNA binding is the resulting left-handed unwinding of the DNA helix. This unwinding is necessary for accommodation of the intercalating chromophore without breakage of the sugar-phosphate backbone (39). To determine if amiloride could unwind DNA, a series of viscometric titrations were performed with varying concentrations of closed, circular, supercoiled plasmid DNA and amiloride or ethidium bromide, and the data expressed as a Vinograd plot (25). By comparing the results obtained with amiloride with those obtained with ethidium bromide (a standard of known unwinding angle, Ref. 50), we calculated that amiloride generated a DNA-unwinding angle of 5.9° (data not shown). Thus, amiloride binds to DNA via intercalation.

**Amiloride Fluorescence Reflects Reversible Binding to DNA** — To demonstrate directly the ability of amiloride to bind to DNA under conditions of physiological ionic strength, the fluorescent properties of amiloride were used. The excitation spectra of amiloride alone, DNA alone, and amiloride and DNA together in 25 mM Hepes, pH 7.3, 5 mM MgCl2 are shown in Fig. 5. Amiloride alone fluoresced with an intensity centered at approximately 350 nm. No appreciable fluorescence was observed for DNA alone (Fig. 5A). Addition of DNA to amiloride resulted in a decrease (quench) of the fluorescence intensity of amiloride. Furthermore, the shape of the spectra changed from a poorly resolved doublet to a single broad peak centered at approximately 370 nm. The difference spectrum (amiloride alone minus amiloride in the presence of DNA) is shown in Fig. 5B. The magnitude of the fluorescence decrease was dependent on the ionic strength. As the ionic strength was increased (by adding increasing amounts of NaCl), the magnitude of the fluorescence quenching was decreased, suggesting that increasing ionic strength promoted dissociation of bound amiloride from DNA. Ionic strength did not significantly alter the excitation spectrum of amiloride alone. At physiologic ionic strength (in the presence of 100 mM NaCl), significant binding of amiloride to DNA was still observed as evidenced by fluorescence quenching.

**Amiloride Binds to Purified Nuclei in a Highly Cooperative Manner** — To determine if amiloride could bind to native DNA (chromatin), amiloride fluorescence was used to measure directly binding to purified, intact nuclei isolated from HL-60

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**Table II**

| Compound          | Concentration (μM) | Compound/DNA ratio (mol/mol phosphate) | ΔTm °C |
|-------------------|--------------------|---------------------------------------|--------|
| Amiloride         | 1                  | 1:10                                  | 1.3 ± 1.7 (4) |
|                   | 5                  | 1:2                                   | 2.0 ± 0.4 (5) |
|                   | 10                 | 1:1                                   | 2.8 ± 1.5 (4) |
|                   | 50                 | 5:1                                   | 5.0 ± 1.1 (9) |
|                   | 100                | 10:1                                  | 8.6 ± 0.9 (5) |
| Ethidium bromide  | 1                  | 1:10                                  | 11.5 ± 0.4 (2) |

**Fig. 4.** Viscometric titration of calf thymus DNA with amiloride and ethidium bromide in 1 mM NH₄F. Experiments were performed as described under "Materials and Methods." □, amiloride; Δ, ethidium bromide.
cells. Mixtures of amiloride and nuclei were incubated for 30 min at 37°C followed by very rapid separation of nuclear-bound amiloride from free amiloride. Fig. 6 indicates that amiloride bound to nuclei in a specific and saturable manner with an apparent \(K_d\) of 1.3 mM. Transformation of the binding isotherm data by Hill plot (Fig. 7) reveals that the binding of amiloride to nuclei was highly cooperative, exhibiting a Hill coefficient of 3.8. Such highly cooperative binding is characteristic of the interaction of some DNA intercalators with DNA (40–42) and with chromatin (43).

**Kinetics of DNA Topoisomerase II Catalysis**—Although amiloride interacts with DNA in a manner consistent with that of a DNA intercalator, not all DNA intercalators inhibit the action of DNA topoisomerase II (9, 11). Amiloride has been shown to inhibit numerous protein kinases (27, 38), and for protein kinases the apparent mechanism of inhibition is competition with ATP (27, 38). Since DNA topoisomerase II also utilizes ATP, we used a classic enzyme kinetic approach to determine whether amiloride inhibited topoisomerase II by competing with ATP. Measuring initial rates of reaction, and at fixed concentrations of amiloride (0.3, 1.0, or 3.0 mM), neither varying the concentration of DNA over an 8-fold range (from approximately one-half of the \(K_m\) to \(\approx 4\) times the

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**Fig. 5. Amiloride fluorescence in the presence of purified DNA.** Excitation spectra were measured on a Perkin-Elmer 512 fluorimeter at an emission wavelength of 420 nm. Excitation and emission slit-widths were 3 and 10 nm, respectively. Data were digitized at 0.2-nm intervals using a microcomputer (Digital Specialities, Chapel Hill, NC). A, quenching of amiloride fluorescence in the presence of DNA. The excitation spectrum of 10 \(\mu\)M amiloride in buffer (see "Materials and Methods") showed a poorly resolved doublet with maxima at \(\approx 345\) and 380 nm. In the presence of purified calf thymus DNA \((13 \times 10^{-8} \text{ M DNA phosphate})\), there was a decreased fluorescence intensity at the maxima with a change in shape of the excitation curve to an asymmetrical singlet at \(\approx 370\) nm. The flat line at zero fluorescence intensity was the signal observed at the same concentration of DNA in the absence of amiloride. B, effect of ionic strength on the DNA-induced fluorescence quench. Difference spectra were obtained by subtraction of the digitized spectra and are plotted as Fluorescence (amiloride alone) minus Fluorescence (amiloride + DNA) at the same salt concentration. Concentrations of amiloride and DNA were as in A. Concentrations of NaCl of 0, 50, and 100 mM are shown. Increasing concentrations of salt caused a decrease in the magnitude of the quench observed at 330 nm. There was no obvious trend to the effects of increased ionic strength on the magnitude of the (minor) peak at 380 nm.

**Fig. 6. Association of amiloride with purified nuclei from HL-60 cells.** Nuclei were prepared from HL-60 cells as described under "Materials and Methods." 0.3-ml aliquots of nuclei were incubated with the indicated concentrations of amiloride for 30 min at 37°C, and the nuclei were pelleted by centrifugation for 30 s in a microcentrifuge. The pellets were washed once in ice-cold buffer, repelleted, and suspended in 0.35 ml of buffer. The fluorescence remaining associated with the nuclei was determined by fluorimetry. Excitation was at 340 nm and emission was at 420 nm. The excitation and emission slits were both set at 10 nm.

**Fig. 7. Hill plot of amiloride binding to nuclei from HL-60 cells.** The data in Fig. 6 are shown in the form of a Hill plot. The maximum fluorescence, \(F_{max}\), was estimated to be 16 units from the data shown in Fig. 6. The linear region of the plot shows a slope (Hill coefficient) of \(\approx 3.8\), indicative of a highly cooperative interaction between amiloride and the nuclei. The \(x\)-intercept of the plot gave an estimate of the \(K_d\) of 1.3 mM under these conditions.
topoisomerase I but not via stabilization of a ternary DNA-topoisomerase-amiloride intermediate, the cleavable complex. Using three model assay systems, we have demonstrated that amiloride inhibits intact cellular functions sensitive to inhibition by classic topoisomerase II inhibitors. These findings could be used to suggest that inhibition of topoisomerase II is the primary mechanism by which amiloride inhibits cellular replication. However, because amiloride is known to be capable of inhibiting so many processes we can only add topoisomerase II to the list of potential cellular targets of amiloride action.

Although amiloride is a DNA intercalator, its affinity for DNA is substantially less than other DNA intercalators (9-11). Amiloride consists of a substituted pyrazine ring with an acyguanidino group attached to the ring at position 2, and this structure is usually depicted as shown in Fig. 8A. At first glance the mononuclear structure of amiloride would not lead one to expect that it might intercalate with DNA. However, studies by Smith et al. (3) using natural abundance $^{13}C$, and $^{15}N$ NMR in conjunction with quantum mechanics computations suggest that the ground-state tautomer of amiloride hydrochloride is the planar, hydrogen-bonded, tricyclic acyl-amino tautomer (Fig. 8B). This conformation of amiloride has been observed in crystal structures (3) and could easily intercalate into DNA; it provides a planar ring system approximately 10 Å across with a set of hydrogen bond-donating groups on either end of the system. These hydrogen bond groups, the 5-amino and the terminal-nitrogen atoms of the guanidino moiety, could interact with the phosphodiester backbone of B-DNA since the backbone P atoms are approx-

DISCUSSION

Previous work by our laboratory and others had indicated that amiloride was most effective in inhibiting DNA synthesis in cultured cells when given during the prereplicative period (4, 9, 49). While examining the role of DNA topoisomerase II in DNA synthesis we noticed that DNA topoisomerase II inhibitors also were most effective in inhibiting DNA synthesis if given during the prereplicative period. These observations led us to determine whether amiloride was also a DNA topoisomerase II inhibitor. The data presented here clearly indicate that amiloride can bind to purified nuclei, intercalate into DNA, and inhibit the catalytic activity of topoisomerase II but not via stabilization of a ternary DNA-topoisomerase-amiloride intermediate, the cleavable complex. The unwinding angle determined for amiloride (5.9°) is low compared to most intercalating compounds. However, given that amiloride binds only weakly to DNA it is possible that all of the amiloride was not bound during the determination of the unwinding angle. Unbound drug would result in a lower estimate of the unwinding angle value.

At present we do not understand the mechanism by which amiloride inhibits the catalytic activity of DNA topoisomerase II. The data indicate that the mechanism of inhibition does not involve competition with ATP or with DNA, or occur via generation of the cleavable complex. Possession of such properties by a DNA intercalator is not without precedent, however. Like amiloride, ethidium bromide intercalates into DNA, inhibits the catalytic activity of topoisomerase II, but does not generate the cleavable complex (11). Clearly, definition of the mechanism of inhibition of topoisomerase II by amiloride awaits further work.

Amiloride Does Not Generate the Cleavable Complex—Many DNA intercalators have been shown to induce protein-linked DNA breaks in cultured cells and in isolated nuclei, the protein being linked to the 5' ends of the broken DNA strands (9, 10, 15, 45-47). The DNA breakage induced by the intercalative drugs has been demonstrated to be mediated by DNA topoisomerase II (9, 10). The intercalators interfere with the breakage reunion reaction of the DNA topoisomerase II by stabilizing a drug-topoisomerase II-DNA ternary intermediate, giving rise to a "cleavable complex." Treatment of this cleavable complex with protein denaturants results in both single and double strand DNA breaks and the covalent linking of one topoisomerase II subunit to each 5' phosphate end of the broken DNA. Since many DNA intercalators stimulate the formation of the cleavable complex using purified DNA and purified mammalian DNA topoisomerase II (9-11), we have used this assay system to determine if the mechanism of inhibition of DNA topoisomerase II by amiloride is via stabilization of the cleavable complex. In the presence of purified DNA topoisomerase II, amiloride failed to stimulate the generation of cleaved DNA fragments, whereas m-AMSA, the established intercalative topoisomerase II inhibitor, strongly promoted topoisomerase II-mediated DNA cleavage (data not shown). Amiloride, however, is not the only example of a DNA intercalator which does not generate the cleavable complex. Ethidium bromide, a far more potent DNA intercalator than amiloride, does not cause formation of the cleavable complex (Ref. 11 and data not shown). Furthermore, novobiocin, a nonintercalating antibiotic which inhibits topoisomerase II via competition with ATP (Ref. 44 and data not shown) does not promote topoisomerase II-mediated DNA cleavage. Clearly, only some topoisomerase II inhibitors generate the cleavable complex. Amiloride, ethidium bromide, and novobiocin do not.

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FIG. 8. Structural depictions of amiloride.
imately 14 Å apart. Studies are currently underway using a series of amiloride congeners to determine those structural features which enable amiloride to both bind to DNA and to inhibit DNA topoisomerase II.

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