# Antitumor AHMA Linked to DNA Minor Groove Binding Agents: Synthesis and Biological Evaluation

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*Received February 12, 2002*

DNA minor groove binder hybrid molecules, netropsin derivatives such as *N*-[2-(dimethylamino)-ethyl]-1-methyl-4-aminopyrrolo-2-carboxamide (MePy) or its derivatives containing two units of *N*-methylpyrrolecarboxamide (diMePy) and bisbenzimidazole (Ho33258), were linked to the $NH_2$ function of AHMA or to the $CH_2OH$ group of AHMA-ethylcarbamate to form AHMA−*N*-netropsins (**13**−**16**) and AHMA−ethylcarbamate-*O*-netropsins (**19**−**22**), and AHMA−bisbenzimidazole (AHMA−Ho33258, **25**), respectively. These conjugates' in vitro antitumor activity, inhibition of a variety of human tumor cell growth, revealed that AHMA−ethylcarbamate-*O*-netropsin derivatives were more cytotoxic than AHMA−*N*-netropsin compounds. In the same studies, all compounds bearing MePy were more potent than those compounds linked with diMePy. Moreover, AHMA−netropsin derivatives bearing a succinyl chain as the linking spacer were more potent than those compounds having a glutaryl bridge. Among these hybrid molecules, AHMA−ethylcarbamate-*O*-succinyl-MePy (**19**) was 2- to 6-fold more cytotoxic than the parent compound AHMA (**5**) in various cell lines, whereas compound **25** had very poor solubility and was inactive. Studies on the inhibitory effect against topoisomerase II (Topo II) and DNA interaction of these conjugates showed no correlation between the potency of DNA binding and inhibitory activity against Topo II.

## Introduction

The macromolecular DNA embodies the genetic specification of an organism, directs cellular proliferation and differentiation, and is a potential cellular target for drug action. Natural antitumor antibiotics such as actinomycin D,[1] doxorubicin,[2] daunomycin,[3] and quinoxaline derivatives (echinomycin, a bifunctional intercalator)[4] are examples of drugs that bind to short DNA sequences via two processes, intercalation and groove binding. Both processes contribute to the affinity and sequence-specific recognition to the DNA. The antitumor antibiotic dynemicin A[5] and neocarzinostatin[6] bind to DNA by intercalating their chromophore (anthraquinone for dynemicin and naphthoate for neocarzinostatin). A free radical mechanism enables their active enediyne core to cleave the DNA at a specific sequence. The mode of interaction, sequence-specificity of binding, uptake, and pharmacokinetics of the drug affect these agents' antitumor selectivity.

Footprinting techniques have aided in better understanding of the gene's molecular biology and established various binding properties for DNA interacting antitumor agents. As a result, many research efforts have focused on the agent's ability to target specific sequences on the DNA and improve its selectivity and pharmacological activities. A variety of natural products and synthetic ligands have been developed and used as molecular probes for studying the drug−DNA binding. These agents selectively inhibit gene transcription and can be applied for designing ligand−drug conjugate as DNA specific targeting antitumor agents. The oligopeptide, *N*-methylpyrrolecarboxamide derivatives [distamycin A (**1**), and netropsin (**2**)],[9−11] and synthetic bisbenzimidazole dye (Ho33258 and Ho33342, **3** and **4**, respectively, Chart 1)[7,8] are known to bind to the minor groove of DNA with A−T specificity and cause widening of the minor groove.[12,13] Ho33342 induces protein−DNA cross-links and DNA strand break in cultured mammalian cells.[14] G2-phase and chromosome endoreduplication are also prominent effects of Ho33342 treatment.[15,16] In addition, these minor groove binders are able to impede the catalytic activity of Topo I[17,18] and Topo II.[19,20]

In view of these minor groove binding characteristics, these hybrid molecules possess several properties for rational drug design including (i) enhanced DNA-binding strength and selectivity, (ii) interference with topoisomerases, and (iii) facilitation of the cellular transport of the drugs, thus promoting their potential use in cancer chemotherapy.[21] Consequently, the minor groove binding ligands are applied widely for the design and synthesis of DNA specific targeting antitumor agents. DNA-alkylating agent (i.e., *N*-mustard derivatives and anthramycin) linked with Ho33258 or distamycin A[22−28] and photoactivable isoalloxazine (flavin) or bioreductive mitomycin C linked with distamycin or netropsin were synthesized to improve the selectivity

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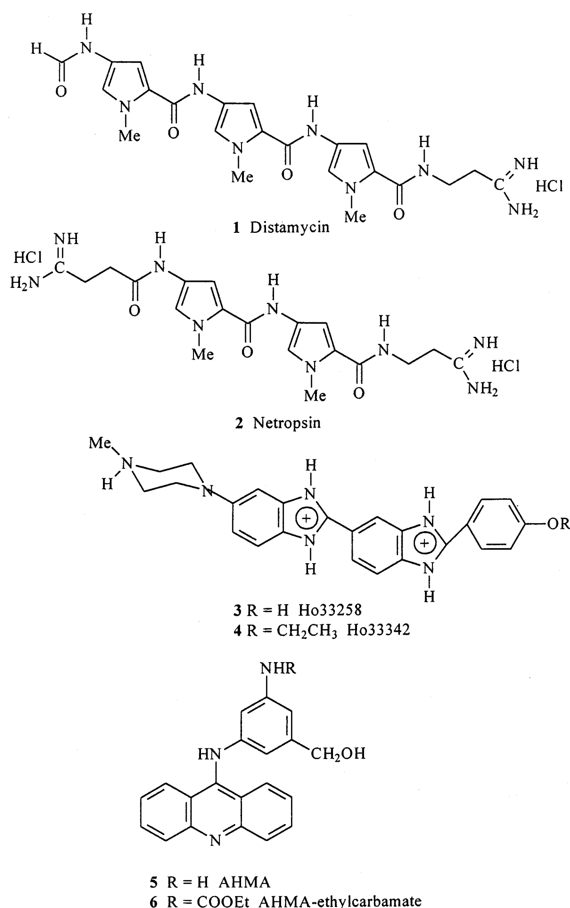
10.1021/jm0200714 CCC: $22.00 © 2002 American Chemical Society
Published on Web 08/21/2002

**Chart 1**




of the anticancer agents.[29,30] Several DNA-intercalators linked to DNA minor groove binding agents, such as ellipticine–distamycin,[31,32] anthracycline–distamycin,[33] and acridine–netropsin[34–37] hybrid molecules, were also designed to improve their DNA sequence-specific binding and antitumor activities.

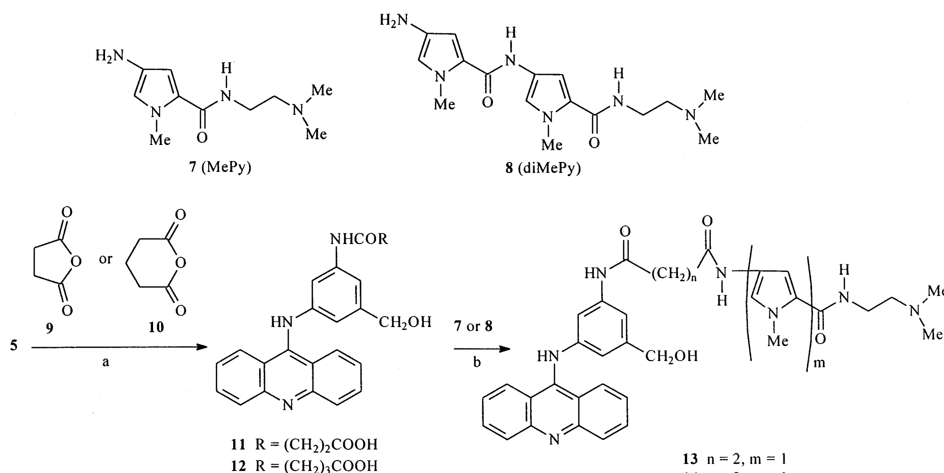
Recently, we synthesized a series of DNA topoisomerase II (Topo II)-mediated anticancer 9-anilinoacridine derivatives, namely, 3-(9-acridinylamino)-5-hydroxymethylaniline (AHMA, **5**) derivatives and their AHMA-alkylcarbamates (**6**).[38,39] AHMA (**5**) is one of the representative compounds in the new 9-anilinoacridines generation. This agent possesses an intriguing chemical structure, where the substituents on the anilino ring are in the positions meta to each other. Unlike *m*-amsacrine (*m*-AMSA), AHMA can avoid bio-oxidation to form quinonediimine, thus having a longer half-life in human plasma than *m*-AMSA. AHMA is a potent Topo II inhibitor and has antitumor efficacy superior to that of *m*-AMSA and VP-16 in mice bearing E0771 mammary adenocarcinoma or B-16 melanoma.[38] Similarly, AHMA-alkylcarbamates are more potent than their parent compounds.[39] Both AHMA-ethylcarbamate and AHMA-*tert*-butylcarbamate have antitumor efficacy better than that of either *m*-AMSA or adriamycin in nude mice bearing human breast tumor MX-1 and its resistant tumor MCF-7/Ad xenografts with less toxicity toward the host.[40] Our structure–activity relationship (SAR) study indicates that modification of the $NH_2$ or $CH_2OH$ function(s) with long side chains does not greatly alter the antitumor activity of the parent compound (AHMA).

Our computer modeling study of AHMA–DNA binding reveals that the $NH_2$ function, which lies outside the drug–DNA complex, may be favorable for interaction with Topo II at the active binding site. DNA cleavage assay has been used to show that AHMA inhibits Topo II-mediated relaxation of supercoiled DNA and promotes DNA breaks at a subset of the cleavage sites of *m*-AMSA.[41] Both drugs cleave DNA at sites that are different from those of the other Topo II inhibitors. Although AHMA and *m*-AMSA have similar mechanisms of action, there is a greater selectivity of DNA cleavage sites by AHMA. The substituent(s) on the anilino ring alter the strength of the DNA interaction and the sequence-specific binding to poly(dA-dT)$_2$ and poly(dG-dC)$_2$ of the drug. The intrinsic equilibrium binding data show a larger binding constant for the more cytotoxic derivatives for binding to poly(dA-dT)$_2$ compared to that for binding to poly(dG-dC)$_2$. However, the circular dichroism (CD) assays demonstrate that the $NH_2$-substituted derivatives have a better intercalated binding geometry to the poly(dG-dC)$_2$, suggesting that specific intercalations with GC-rich areas in natural DNA for AHMA and derivatives with a free $NH_2$ group may stabilize the drug–DNA complex. Although the DNA–drug sequence-specific binding of AHMA derivatives may be affected by the substituent(s) on the anilino ring, it is still unclear which substituent ($NH_2$ or $CH_2$-OH) on the anilino ring of AHMA is the critical element for Topo II inhibition and cytotoxicity. Therefore, linking a DNA minor groove binding ligand (i.e., netropsin derivatives or Ho33258) to the $NH_2$ or $CH_2OH$ function of AHMA may reveal the role of enzyme–drug interaction and DNA–drug sequence-specific binding. In this paper we describe the syntheses, antitumor effects, Topo II inhibition, and DNA interaction of AHMA derivatives linked to DNA minor groove binder. The results show that the AHMA derivatives linked with one or two unit(s) of *N*-methylpyrrolecarboxamide ligand (MePy or diMePy, respectively) to the $CH_2OH$ function of AHMA-ethylcarbamate resulted in an increase in cytotoxicity, whereas these two ligands linked to the $NH_2$ function of AHMA decreased the cytotoxicity of the parent compound.

## Chemistry

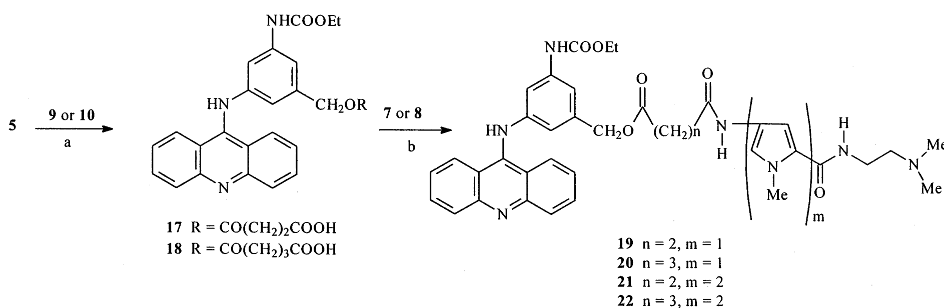
The minor groove binder netropsin derivatives, *N*-[2-(dimethylamino)ethyl]-1-methyl-4-aminopyrrole-2-carboxamide (**7**, MePy) or compound containing two units of *N*-methylpyrrolecarboxamide (**8**, diMePy) were linked to AHMA (**5**). The AHMA–netropsin molecules were constructed using a spacer, either a 4-carbon unit (succinyl) or a 5-carbon unit (glutaryl group), to link the intercalator AHMA and the minor groove binder. The known MePy (**7**) and diMePy (**8**) were linked to the $NH_2$ function of AHMA (**5**) or the $CH_2OH$ group of AHMA–ethylcarbamate (**6**) by following the method described previously.[31] Thus, treating AHMA (**5**) with succinic anhydride (**9**) or glutaric anhydride (**10**) in the presence of pyridine gave AHMA–*N*-carbonylpropionic acid monocarboxamide (**11**) and AHMA–*N*-carbonylbutyric acid monocarboxamide (**12**) (Chart 1), respectively, in good yield. However, occasionally, thin-layer chromatography (TLC) showed a trace of *N,O*-diacyl byproduct. The *O*-acyl function of the byproduct can be converted into acid **11** or **12** by base hydrolysis. The carboxylic function

**Scheme 1**[a]




[a] Conditions: a, DMPA/pyridine, 85−90 °C, 12 h; b, PyBOP/pyridine, Hunig's base at −5 °C, and then rt 12 h.

**Scheme 2**[a]




[a] Conditions: a, DMPA/pyridine, 85−90 °C, 12 h; b, PyBOP/pyridine, Hunig's base at −5 °C, and then rt 12 h.

of **11** or **12** was condensed with MePy (**7**) or diMePy (**8**) in a mixture of benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) and Hunig's base in dried DMF to afford the desired corresponding AHMA−*N*-succinyl-MePy (**13**), AHMA−*N*-glutaryl-MePy (**14**), AHMA−*N*-succinyl-diMePy (**15**), and AHMA−*N*-glutaryl-diMePy (**16**), in good yield (50−70%).

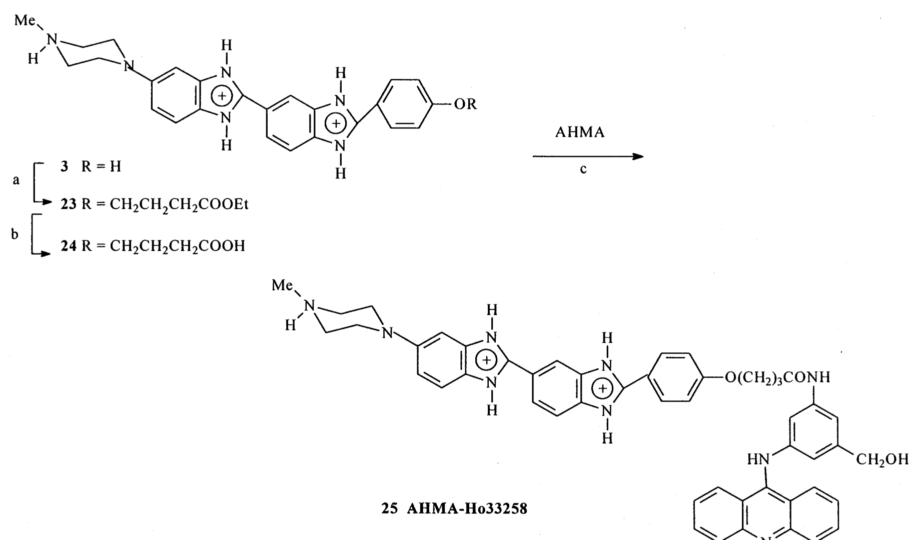
Utilizing the same AHMA−netropsin derivatives synthesis, AHMA−ethylcarbamate−netropsin derivatives (**19**−**22**) were obtained in good yield (85%) by treating AHMA−ethylcarbamate (**6**) with **9** or **10** in dry pyridine in the presence of 4-(dimethylamino)pyridine (DMAP) to give the AHMA−ethylcarbamate−succinylic acid (**17**) or AHMA−ethylcarbamate−glutaric acid monoester (**18**), respectively. Condensation of **17** or **18** with MePy (**7**) or diMePy (**8**) was carried out by the same procedure as described for the synthesis of compounds **13**−**16**. The free products, AHMA−ethylcarbamate-*O*-succinyl−MePy (**19**) and AHMA−ethylcarbamate-*O*-succinyl−diMePy (**21**) were extremely unstable. Purification of these two compounds either by liquid chromatography or preparative thin-layer chromatography ($SiO_2$, in $CHCl_3$/MeOH gradient system) led to the hydrolysis of the ester bond and liberated AHMA−ethylcarbamate (**5**). Although pure **19** and **21** were obtained after chromatography, they decomposed during concentration or standing at room temperature. Therefore, we could not obtain a uniform $^1$H NMR spectrum and elemental analysis. However, we were able to obtain pure **19** after removing the solvent under reduced pressure and washing the residue well with ether or $CHCl_3$ to remove byproducts followed by triturating with EtOH. However, following the same procedure did not yield pure **21**. In contrast with compounds containing a succinyl linking spacer (**19** and **21**), compounds **20** and **22**, which bear a glutaryl spacer, were isolated in good yield by liquid chromatography.

The hybrid molecule (Ho33258, **3**) was also linked to the $NH_2$ function of AHMA (**5**). The synthesis of AHMA−*N*-(Ho33258-*O*-butyl)carboxamide (**25**) is shown in Scheme 3. Treatment of Ho33258 (**3**) with ethyl bromobutyrate ($K_2CO_3$/DMF, 60 °C) gave *O*-alkylated compound **23**, which was saponified in ethanolic NaOH solution to yield acid **24**. Condensation of **24** with AHMA (**5**) in dried DMF in the presence of 2,2′-dithiopyridine/triphenylphosphine[42] gave **25** in 70% yield.

## Biological Results and Discussion

**In Vitro Cytotoxicity.** The cytotoxicity of AHMA derivatives linked with DNA minor groove binder (**13**−**16**, **19**, **20**, **22**, and **25**) against various human tumor cells (colon HT-29, nasopharyngeal carcinoma HONE-1 and BM-1, Hepatoma Hepa-G2, Glioblastoma multifomi DBTRG, breast carcinoma MX-1, and T-cell acute lymphocytic leukemia CCRF−CEM) grown in culture were determined and the $IC_{50}$ values are provided in Table 1. AHMA−*N*-netropsin derivatives (**13**−**16**) displayed less cytotoxicity than either AHMA (**5**) or AHMA−ethylcarbamate (**6**) in inhibiting of cell growth of the

**Scheme 3**[a]




[a] Conditions: a, $K_2CO_3$/DMF, 60 °C, 12 h; b, 1 N NaOH/EtOH, 60 °C, 1 h; c, 2,2′-dithiopyridine/$Ph_3P$/DMF, −5 °C, and then rt 24 h.

**Table 1.** In Vitro Cytotoxicity of AHMA Derivatives Linked with Minor Groove Binder Against a Variety of Human Tumor Cell Growth

| compd | inhibition of cell growth ($IC_{50}$ $\mu$M) | | | | | | | | percentage of protein-linked DNA breaks[a] |
|---|---|---|---|---|---|---|---|---|---|
| | HT-29 | HONE-1 | BM-1 | TSGH | Hepa-G2 | DBTRG | MX-1 | CCRF−CEM | |
| **5** | 0.88 | 0.31 | 0.86 | 0.45 | 1.4 | 3.5 | ND[b] | 0.36 | 10.5 |
| **6** | 0.48 | 0.07 | 0.38 | 0.34 | 0.46 | 5.0 | ND | 0.057 | 15.0 |
| **13** | 1.7 | 0.85 | 7.0 | 4.6 | 4.2 | 8.1 | ND | ND | 3.3 |
| **14** | >100 | 39 | 73.3 | 46.8 | 100 | 73.6 | ND | ND | 0.0 |
| **15** | 4.4 | 2.4 | 8.6 | 4.8 | 7.0 | 20 | ND | ND | 3.3 |
| **16** | >100 | 63.9 | 77.8 | 36.4 | 68 | >100 | ND | ND | 0.0 |
| **19** | 0.23 | 0.06 | 0.08 | 0.08 | 0.09 | 0.80 | 0.039 | 6.85 | 16.5 |
| **20** | 0.75 | 0.80 | 0.08 | 1.2 | 0.08 | 0.40 | 0.085 | 17.6 | 4.6 |
| **22** | 0.64 | 0.13 | 0.49 | 1.2 | 0.63 | 7.5 | 0.156 | 2.04 | 4.2 |
| **25** | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | ND[b] |

[a] The K−SDS coprecipitation assay: the percentage of protein-linked DNA breaks (PLDBs) generated by AHMA-netropsin derivatives at concentration of 10 $\mu$M. [b] Not determined.

tested tumor cell lines in culture. This clearly demonstrates that AHMA−*N*-succinyl-MePy (**13**) and AHMA−*N*-succinyl-diMePy (**15**) are more cytotoxic than the corresponding AHMA−*N*-glutaryl-MePy (**14**) and AHMA−glutaryl-diMePy (**16**). The hybrid molecules bearing a succinyl function (4-carbon chain) as linking spacer were more cytotoxic than those compounds bearing a glutaryl spacer (5-carbon chain) (**13** vs **14** and **15** vs **16**). Depending upon the tumor cell lines tested, it was also revealed that compounds with MePy were either as potent as or more potent than compounds bearing diMePy moiety (i.e., **13** vs **15** and **14** vs **16**). For example, compound **13** was approximately 2.5-fold more potent than **15** in inhibition of HT-29 and HONE-1 tumor cell growth in culture. In the series of AHMA−ethylcarbamate-*O*-netropsin derivatives (**19**, **20**, and **22**), we found that these compounds were significantly more potent than the corresponding AHMA−*N*-netropsin derivatives and, in some cases, were as potent as or more potent than AHMA (**5**) or AHMA−ethylcarbamate (**6**). Similarly, it revealed that compounds with a succinyl linker were more potent than compounds with a glutaryl linker (**19** vs **22**), and compounds linked with MePy were more cytotoxic than those linked with diMePy (**21** vs **22**). Among these compounds, AHMA−ethylcarbamate-*O*-succinyl-MePy (**22**) was about 1−10 times more potent than AHMA (**5**) or AHMA−ethylcarbamate (**6**) depending on the tumor cell lines tested. The in vitro cytotoxicity study showed that AHMA−*N*-Ho33258 (**25**) was inactive at the concentration up to 100 $\mu$M on the inhibition of human tumor cells tested in culture, perhaps because of its poor solubility and inability to penetrate into the cell.

Previously, Rene et al.[37] synthesized acridine-netropsin and demonstrated that the 1′-substituent on the anilino ring of amsacrine was a critical element for Topo II inhibition and cytotoxicity. Additionally, 9-anilinoacridine linked with MePy was more cytotoxic than the corresponding compound linked with diMePy. Similar findings were shown in our present study. Furthermore, our recent work on the synthesis of AHMA−EDTA conjugates revealed that AHMA linked with EDTA, a metal chalator, to the $NH_2$ function was significantly less cytotoxic than compounds bearing an EDTA at $CH_2$-OH function.[43] These results demonstrated that AHMA derivatives bearing a bulky substituent on the $NH_2$ function decreased their cytotoxicty, indicating that the $NH_2$ may have played an important role in drug−Topo II interaction.

**Interaction of AHMA−Netropsin Derivatives with Topo II.** We had previously demonstrated that AHMA was a potent inhibitor of Topo II.[38] To investigate the inhibitory activity of AHMA−netropsin derivatives against Topo II, an in vitro Topo II relaxation

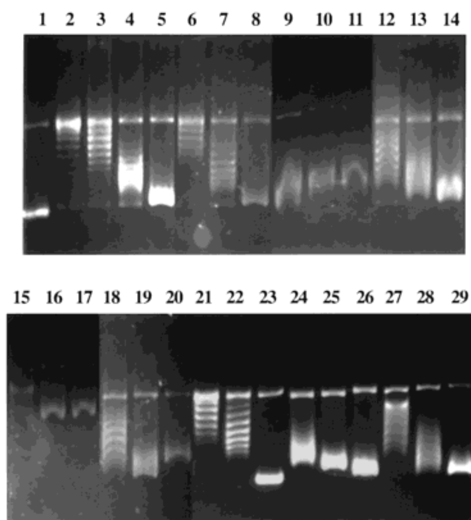



**Figure 1.** Inhibition of the DNA topoisomerase II catalytic activity by AHMA derivatives linked with minor groove binder. The experiment was performed by the method described previously.[48] Different concentrations (5, 10, and 25 $\mu$M) of the following compounds were tested: lane 1, DNA alone; lane 2, DNA plus 2 units of enzyme; lanes 3–5, compound **5**; lanes 6–8, compound **6**; lanes 9–11, compound **13**; lanes 12–14, compound **14**; lanes 15–17, compound **15**; lanes 18–20, compound **16**; lanes 21–23, compound **19**; lanes 24–26, compound **20**; lanes 27–29, compound **22**.

assay using pBR322 supercoiled DNA was performed. As shown in Figure 1, the AHMA netropsin derivatives were tightly bound to the double helical DNA at concentrations between 5 and 25 $\mu$M, making it difficult to compare the relative potency of these compounds on Topo II inhibition. To overcome this problem, an in vitro k-SDS coprecipitation assay was carried out to measure the amount of protein-linked DNA breaks (PLDBs) generated by these compounds. As shown in Table 1, after a 30-min exposure to increasing concentrations of these compounds, steady-state levels of PLDBs were increased in a dose-dependent manner, followed by a plateau of PLDBs levels (data not shown). The order of ability to generate the maximal amount of PLDBs of these compounds was **19**, **6** > **5** > **22**, **20** > **13**, **15** > **14**, **16** (see Table 1). The study demonstrated that AHMA–*N*-netropsin derivatives (**13**–**16**) were poor inhibitors of Topo II and therefore had less cytotoxicity.

**Interaction of AHMA–Netropsin Derivatives with DNA.** To investigate whether enhancement of DNA binding activity of AHMA–netropin derivatives could increase the inhibitory activity against Topo II and subsequently lead to an improvement in their cytotoxicity, a DNA circular-ligation assay with linearized pBR322 DNA and T4 ligase was performed. The assay allowed for the detection of tertiary structure alteration caused by DNA binding of both intercalating and nonintercalating drugs. As shown in Figure 2, compound **5** (lanes 4–7) produced a concentration-dependent band shift, indicating a change in the DNA linking number. Compound **5** produced positive supercoiled DNA at a concentration of greater than 2.5 $\mu$M. Furthermore, in the presence of compounds **13**, **14**, **15**, and **22** at 10 $\mu$M, substrate linear DNA remained unchanged, indicating that strong intercalating activity of these compounds caused inhibition of T4 DNA ligase. The order of DNA intercalating activity was **13**, **15** > **14**, **22** > **18** > **5**, **6**, **19**, and **20**, suggesting that no correlation exists between the potency of DNA binding and inhibitory activity against Topo II. Compounds **5**, **6**, **19**, **20**, and **22** were more cytotoxic toward various human cancer cells than other derivatives (**Table 1**). The present studies revealed that increasing DNA intercalating activity did not correlate with enhancing inhibitory activity against Topo II and cytotoxicity of these compounds.

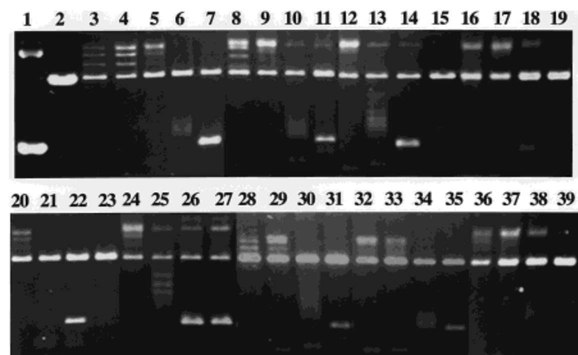



**Figure 2.** Effect of DNA unwinding by AHMA derivatives linked with minor groove binder measured as described previously.[50] Different concentrations (0.1, 0.5, 2.5, and 10 $\mu$M) of the following compounds were tested: lane 1, supercoiled DNA; lane 2, DNA; lane 3, linear DNA plus T4 ligase; lanes 4–7, compound **5**; lanes 8–11, compound **6**; lanes 12–15, compound **13**; lanes 16–19, compound **14**; lanes 20–23, compound **15**; lanes 24–27, compound **18**; lanes 28–31, compound **19**; lanes 32–34, compound **20**; lanes 35–39, compound **22**.

## Conclusion

We have synthesized a series of AHMA-minor groove binder hybrid molecules, AHMA–*N*-netropsins (**13**–**16**), AHMA–ethylcarbamate-*O*-netropsins (**19**, **21**, and **22**), and AHMA–bisbenzimidazole dye (**25**) (AHMA-Ho33258) for evaluation of their in vitro antitumor activity, inhibitory effect against Topo II, and DNA interaction. Our study reveals that AHMA–*N*-netropsin derivatives are less active than AHMA–ethylcarbamate-*O*-netropsin derivatives. In addition, all compounds bearing MePy were found to be more cytotoxic than those compounds linked to diMePy in the same study. Moreover, AHMA–netropsin derivatives using succinyl chain as the linking spacer are more potent than those compounds having a glutaryl bridge. Among these hybrid molecules, compound **19** is 2- to 6-fold more cytotoxic than the parent compound, AHMA (**5**), in various human tumor cell lines. Compound **25** has very poor solubility and is inactive. The Topo II relaxation assay reveals AHMA–*N*-netropsin derivatives (**16**–**19**) as poor inhibitors of Topo II in comparison with the AHMA–ethylcarbamate-*O*-netropsine derivatives. Therefore, the former has weak in vitro cytotoxicity in all tumor cell lines tested. In contrast, we find that there is no correlation between the cytotoxicity of drugs tested and their interaction with double helical DNA.

The high activity of the AHMA–ethylcarbamate-*O*-netropsin derivatives shows that the hydroxymethyl function of AHMA faces the DNA helix, whereas the $NH_2$ function is located outside the helix. Increasing the size of the substituent on the $NH_2$ function may interfere with the drug–Topo II interaction and de-

crease the cytotoxicity. Because drug–DNA binding has no correlation with the drug's cytotoxicity, linking MePy moiety to the $CH_2OH$ group of AHMA–ethylcarbamate may stabilize the formation of DNA–drug–Topo II ternary complexes and, hence, have potent cytotoxicity.

## Experimental Section

Melting points were determined on a Fargo melting points apparatus and are uncorrected. Column chromatography was carried out over silica gel G60 (70–230 mesh, ASTM; Merck). Thin-layer chromatography was performed on silica gel G60 F 254 (Merck) with short wavelength UV light for visualization. Elemental analyses were done on a Heraeus CHN–O rapid instrument. $^1$H NMR spectra were recorded on a Brucker 400 spectrophotometer with $Me_4Si$ as internal standard.

**AHMA–*N*-Succinic Acid Monocarboxamide (11).** A mixture of **5** (1.1 g, 3.48 mmol), succinic anhydride (350 mg, 3.49 mmol), and DMAP (122 mg, 0.1 mmol) in pyridine (15 mL) was heated at 85–90 °C for 12 h. The solvent was evaporated in vacuo to dryness, and the residue was chromatographed over silica gel column (3 × 55 cm). Gradient elution with $CHCl_3$/MeOH/$NH_4OH$ (15:84:1) afforded the compound **11**: 940 mg (65.2%); mp 235–236 °C. $^1$H NMR (DMSO-$d_6$) δ 2.49–2.53 (4H, m, 2 × $COCH_2$), 4.43 (2H, s, $ArCH_2$), 5.19 (1H, brs, OH), 6.44 (1H, s, ArH), 6.91 (2H, m, 2 × ArH), 7.24 (2H, m, 2 × ArH), 7.32 (2H, m, 2 × ArH), 7.54 (3H, m, 3 × ArH), 8.24 (1H, m, ArH), 9.89 (1H, s, CONH), 10.89 (1H, s, NH). Anal. ($C_{24}H_{21}N_3O_4$·$2H_2O$) C, H, N.

Through the use of the same procedure as that used for the synthesis of **11** the following compounds were synthesized.

**AHMA–*N*-Glutaric Acid Monocarboxamide (12).** Compound **12** was prepared from **5** (950 mg, 3.01 mmol), glutaric anhydride (345 mg, 3.02 mmol), and 4-(dimethylamino)-pyridine (DMAP, 122 mg, 1.0 mmol) in pyridine (15 mL): yield 920 mg (71%); mp 179–180 °C. $^1$H NMR (DMSO-$d_6$) δ 1.76 (2H, m, $CH_2$), 2.27 (4H, m, 2 × $COCH_2$), 4.42 (2H, s, $ArCH_2$), 5.0 (1H, brs, OH), 6.40 (1H, s, ArH), 6.91 (2H, m, 2 × ArH), 7.20 (1H, s, ArH), 7.30 (2H, m, 2 × ArH), 7.46 (3H, m, 3 × ArH), 8.16 (2H, m, ArH), 9.77 (1H, s, NHCO), 10.90 (1H, brs, NH). Anal. ($C_{25}H_{23}N_3O_4$·2.5 $H_2O$) C, H, N.

**AHMA–Ethylcarbamate-*O*-Succinic Acid Monoester (17).** Compound **17** was prepared from **5** (950 mg, 2.45 mmol), succinic anhydride (260 mg, 2.59 mmol), and DMAP (100 mg, 0.81 mmol) in pyridine (15 mL): yield 1.0 g (84%); mp 228–229 °C. $^1$H NMR (DMSO-$d_6$) δ 1.21 (3H, t, $J$ = 6.4 Hz, Me), 2.23 and 2.43 (each 2H, m, 2 × $COCH_2$), 4.07 (2H, q, $J$ = 6.4 Hz, $CH_2$), 4.98 (2H, s, $ArCH_2$), 6.38 (1H, m, ArH), 6.83 (2H, m, 2 × ArH), 7.12 (2H, m, 2 × ArH), 7.33 (2H, m, 2 × ArH), 7.46–7.50 (3H, m, 3 × ArH), 8.14 (1H, m, ArH), 9.57 (1H, brs, NHCO), 10.9 (1H, brs, NH). Anal. ($C_{27}H_{25}N_3O_6$) C, H, N.

**AHMA–Ethylcarbamate-*O*-Glutaric Acid Monoester (18).** Compound **18** was prepared from **5** (930 mg, 2.40 mmol), glutaric anhydride (290 mg, 2.54 mmol), and DMAP (100 mg, 0.81 mmol) in pyridine (15 mL): yield 1.0 g (83%); mp 181–182 °C. $^1$H NMR (DMSO-$d_6$) δ 1.21 (3H, t, $J$ = 7.2 Hz, Me), 1.72 (2H, m, $CH_2$), 2.23 (2H, m, $CH_2CO$), 2.35 (2H, m, $COCH_2$), 4.06 (2H, q, $J$ = 7.2 Hz, $CH_2$), 4.98 (2H, m, $ArCH_2$), 6.39 (1H, s, ArH), 6.84 (1H, s, ArH), 6.90 (2H, m, 2 × ArH), 7.37 (2H, m, 2 × ArH), 7.38–7.61 (3H, m, 3 × ArH), 8.11 (2H, m, 2 × ArH), 9.59 (1H, s, NHCO), 11.13 (1H, brs, NH). Anal. ($C_{28}H_{27}N_3O_6$) C, H, N.

***N*-[2-(Dimethylamino)ethyl]-1-methyl-4-[[[3-(9-acridinylamino)-5-hydroxymethylanilino]carbonylpropanoyl]-amino]pyrrole-2-carboxamide (AHMA–*N*-succinyl-MePy) (13).** A mixture of **11** (273 mg, 0.65 mmol) and PyBOP (357 mg, 0.68 mmol) in dry DMF (10 mL) was stirred at −5 °C for 2 h. A solution of freshly prepared **7** (151 mg, 0.72 mmol) in DMF (5 mL) was added slowly into the above reaction mixture, followed by addition of Hunig's base (0.11 mL, 0.63 mmol) at −20 °C under argon atmosphere. The temperature was then allowed to rise to room temperature and the reaction mixture was continuously stirred for an additional 12 h. The solvent was evaporated under reduced pressure to dryness and the residue was chromatographed over a column of silica gel (2 × 30 cm). Compound **13** was eluted from $CHCl_3$/MeOH/$NH_4OH$ (v/v 90:9:1): 226 mg (57%); mp 186–187 °C. $^1$H NMR (DMSO-$d_6$) δ 2.54 (4H, m, 2 × $COCH_2$), 2.77 (6H, s, $NMe_2$), 3.14 (2H, m, $NCH_2$), 3.46 (2H, m, $NHCH_2$), 3.76 (3H, s, NMe), 4.40 (2H, s, $ArCH_2$), 5.17 (1H, brs, OH), 6.54 and 6.72 (each 1H, m, 2 × ArH), 7.08–7.21 (3H, m, 3 × ArH), 7.27 (1H, m, ArH), 7.39–7.64 (4H, m, 4 × ArH), 7.97 (2H, m, 2 × ArH), 8.14 (2H, m, CONH and ArH), 9.84 and 9.91 (each 1H, s, 2 × CONH). Anal. ($C_{34}H_{37}N_7O_4$) C, H, N.

Following the same procedure as that for the synthesis of **16**, the following compounds were synthesized.

***N*-[2-(Dimethylamino)ethyl]-1-methyl-4-[[[3-(9-acridinylamino)-5-hydroxymethylanilino]carbonylbutanoyl]-amino]pyrrole-2-carboxamide (AHMA–*N*-glutaryl-MePy) (14).** Compound **14** was prepared from **12** (230 mg, 0.53 mmol), PyBOP (313 mg, 0.60 mmol), and freshly prepared **7** (124 mg, 0.59 mmol): 182 mg (55%); mp 218–219 °C. $^1$H NMR (DMSO-$d_6$) δ 1.85 (2H, m, $CH_2$), 2.16 (6H, s, $NMe_2$) 2.26 (4H, m, 2 × $COCH_2$), 2.33 (2H, m, $NCH_2$), 3.24 (2H, m, $NHCH_2$), 3.77 (3H, s, NMe), 4.43 (2H, s, $ArCH_2$), 5.15 (1H, brs, OH), 6.46 (1H, m, ArH), 6.85, 6.92, and 7.09 (6H, each 2H, 6 × ArH), 7.23–7.30 (3H, m, 3 × ArH), 7.46 (2H, m, 2 × ArH), 7.86 (2H, m, CONH and ArH), 8.13 (1H, m, ArH), 9.76 and 9.77 (each 1H, s, 2 × CONH), and 10.87 (1H, brs, NH). Anal. ($C_{35}H_{39}N_7O_4$·1.3$H_2O$) C, H, N.

***N*-[2-(Dimethylamino)ethyl]-1-methyl-4-[1-methyl-4-[1-methyl-4-[[[3-(9-acridinylamino)-5-hydroxymethylanilino]carbonylpropanoyl]amino]pyrrole-2-carboxamido]-pyrrole-2-carboxamide (AHMA–*N*-succinyl-diMePy) (15).** Compound **15** was prepared from **11** (264 mg, 0.63 mmol), PyBOP (357 mg, 0.68 mmol), and freshly prepared **8** (246 mg, 0.74 mmol): yield 250 mg (54%); mp 181–182 °C. $^1$H NMR (DMSO-$d_6$) δ 2.50 (4H, m, 2 × $COCH_2$), 2.59 (6H, s, $NMe_2$), 2.89 (2H, m, $NCH_2$), 3.53 (2H, m, $NHCH_2$), 3.81 (6H, s, 2 × NMe), 4.40 (2H, s, $ArCH_2$), 5.16 (1H, brs, OH), 6.37 (1H, m, ArH), 6.86–6.91 (4H, m, 4 × ArH), 7.13–7.22 (3H, m, 3 × ArH), 7.35 (1H, m, ArH), 7.40–7.58 (4H, m, 4 × ArH), 7.85 (1H, m, ArH), 8.08 (1H, s, CONH), 9.88 (3H, brs, 3 × CONH). Anal. ($C_{40}H_{41}N_9O_5$) C, H, N.

***N*-[2-(Dimethylamino)ethyl]-1-methyl-4-[1-methyl-4-[[[3-(9-acridinylamino)-5-hydroxymethylanilino]carbonylbutanoyl]amino]pyrrole-2-carboxamido]-pyrrole-2-carboxamide (AHMA–*N*-glutaryl-diMePy) (16).** Compound **16** was prepared from **12** (304 mg, 0.70 mmol), PyBOP (400 mg, 0.76 mmol), and freshly prepared **8** (233 mg, 0.70 mmol): yield 270 mg (51.8%); mp 178–180 °C. $^1$H NMR (DMSO-$d_6$) δ 1.83 (2H, m, $CH_2$), 2.17 (6H, s, $NMe_2$), 2.24 (4H, m, 2 × $COCH_2$), 2.41 (2H, m, $J$ = 2.4 Hz, $NCH_2$), 3.28 (2H, m, $J$ = 2.4 Hz, $NHCH_2$), 3.79 and 3.81 (each 3H, s, 2 × NMe), 4.42 (2H, s, $ArCH_2$), 5.14 (1H, brs, OH), 6.39 (1H, s, ArH), 6.82–6.92 (4H, m, 4 × ArH), 7.17–7.30 (5H, m, 5 × ArH), 7.66 (3H, m, 3 × ArH), 7.86 (2H, m, CONH and ArH), 8.15 (2H, m, 2 × ArH), 9.81, 9.83, and 9.84 (each 1H, s, 3 × CONH), 10.90 (1H, s, NH). Anal. ($C_{41}H_{45}N_9O_5$·2.5$H_2O$) C, H, N.

***N*-[2-(Dimethylamino)ethyl]-1-methyl-4-[[[3-(9-acridinylamino)-5-(ethoxycarbonylamino)benzyloxy]carbonyl-propanoyl]amino]pyrrole-2-carboxamide (AHMA–Ethylcarbamate-*O*-succinyl-MePy) (19).** Compound **19** was prepared from **17** (307 mg, 0.72 mmol), PyBOP (391 mg, 0.75 mmol), and freshly prepared **7** (151 mg, 0.72 mmol). The reaction mixture was evaporated in vacuo to dryness and the residue was washed well with ether (20 mL × 4), followed with $CHCl_3$ (20 mL × 3). The solid residue was then triturated with EtOH and the resulted solid product **19** was collected by filtration and dried: 260 mg (53%); mp 159–161 °C. $^1$H NMR (DMSO-$d_6$) δ 1.20 (3H, t, $J$ = 7.3 Hz, Me), 2.20 (6H, s, $NMe_2$), 2.41 and 2.53 (each 2H, m, 2 × $COCH_2$), 2.59 and 3.36 (each 2H, m, $NCH_2$ and $NHCH_2$), 3.76 (3H, s, NMe), 4.08 (2H, q, $J$ = 7.3 Hz, $CH_2$), 4.98 (2H, s, $ArCH_2$), 6.41, 6.65, and 6.83 (each 1H, s, 3 × ArH), 7.10 (2H, m, 2 × ArH), 7.21–7.61 (5H, m, 5 × ArH), 7.88 (2H, m, CONH and ArH), 8.05 (2H, m, 2 × ArH), 9.57 and 9.83 (each 1H, s, 2 × CONH), 10.93 (1H, s, NH). MS: 679 ($M^+$). Anal. ($C_{37}H_{41}N_7O_6$·$H_2O$) C, H, N.

***N*-[2-(Dimethylamino)ethyl]-1-methyl-4-[[[3-(9-acridinylamino)-5-(ethoxycarbonylamino)benzyloxy]carbonylbutanoyl]amino]pyrrole-2-carboxamide (AHMA−Ethylcarbamate-*O*-glutaryl-MePy) (20).** Compound **20** was prepared from **18** (300 mg, 0.59 mmol), PyBOP (358 mg, 0.69 mmol), and fresh prepared **7** (143 mg, 0.68 mmol). The product **20** was purified by column chromatography ($SiO_2$, $CHCl_3$/MeOH, 10:1, v/v): 310 mg (78%); mp 147−148 °C. $^1H$ NMR (DMSO-$d_6$) δ 1.20 (3H, t, $J$ = 7.2 Hz, Me), 1.80 (2H, m, $CH_2$), 2.18 (6H, s, $NMe_2$), 2.26 (2H, m, $CH_2CONH$), 2.37 (4H, m, $COCH_2$ and $NCH_2$), 3.25 (2H, m, $NHCH_2$), 3.76 (3H, s, NMe), 4.07 (2H, q, $J$ = 7.2 Hz, $CH_2$), 5.0 (2H, s, $ArCH_2$), 6.40 (1H, m, ArH), 6.66 and 6.84 (each 2H, m, 4 × ArH), 7.03 (3H, m, 3 × ArH), 7.10 and 7.32 (each 2H, m, 4 × ArH), 7.46 (3H, m, 3 × ArH), 7.90 (2H, m, CONH and ArH), 9.61 and 9.78 (each 1H, s, 2 × CONH), 10.95 (1H, s, NH). Anal. ($C_{38}H_{43}N_7O_6{\cdot}2H_2O$) C, H, N.

***N*-[2-(Dimethylamino)ethyl]-1-methyl-4-[1-methyl-4-[[[3-(9-acridinylamino)benzyloxy]carbonylpropanoyl]amino]pyrrole-2-carboxamido]pyrrole-2-carboxamide (AHMA−Ethylcarbamate-*O*-succinyl-diMePy) (21).** Compound **21** was prepared from **17** (300 mg, 0.61 mmol), PyBOP (358 mg, 0.69 mmol), and freshly prepared **8** (229 mg, 0.69 mmol). The reaction mixture was evaporated to dryness, and the residue was washed well with $CHCl_3$ to remove the major byproduct. The residue was triturated with EtOH to give **24**, yield 240 mg (49%), which was ca. 85% pure detected by its $^1H$ NMR spectrum. $^1H$ NMR (DMSO-$d_6$) δ 1.20 (3H, t, $J$ = 7.3 Hz, Me), 2.55 and 2.60 (each 2H, m, 2 × $COCH_2$), 2.73 (6H, s, $NMe_2$), 3.06 and 3.51 (each 2H, $NCH_2$ and $NHCH_2$), 3.80 and 3.81 (each 3H, s, 2 × NMe), 4.09 (2H, q, $J$ = 7.3 Hz, $CH_2$), 4.99 (2H, s, $ArCH_2$), 6.42, 6.83, and 6.89 (each 1H, s, 3 × ArH), 6.94 (2H, m, 2 × ArH), 7.12 and 7.18 (each 2H, m, 4 × ArH), 7.38−7.52 (5H, m, 5 × ArH),8.11 (2H, m, CONH and ArH), 9.57, 9.88, and 9.90 (each 1H, s, 3 × CONH).

***N*-[2-(Dimethylamino)ethyl]-1-methyl-4-[1-methyl-4-[[[3-(9-acridinylamino)-5-(ethoxycarbonylamino)benzyloxy]carbonylbutanoyl]amino]pyrrole-2-carboxamido]pyrrole-2-carboxamide (AHMA−Ethylcarbamate-*O*-glutaryl-diMePy) (22).** Compound **22** was prepared from **18** (213 mg, 0.42 mmol), PyBOP (325 mg, 0.62 mmol), and freshly prepared **8** (150 mg, 0.47 mmol): yield 174 mg (50.8%); mp 166−168 °C. $^1H$ NMR (DMSO-$d_6$) δ 1.20 (3H, t, $J$ = 7.0 Hz, Me), 1.81 (2H, m, $CH_2$), 2.20 (6H, s, $NMe_2$), 2.29 (4H, m, 2 × $COCH_2$), 2.40 (2H, m, $NCH_2$), 3.16 (2H, m, $NHCH_2$), 3.79 and 3.81 (each 3H, s, 2 × NMe), 4.07 (2H, q, $J$ = 7.0 Hz, $OCH_2$), 4.99 (2H, s, $ArCH_2$), 6.40 (1H, m, ArH), 6.84 and 7.15 (each 2H, m, 4 × ArH), 7.32−7.48 (5H, m, 5 × ArH), 7.94 (2H, m, CONH and ArH), 8.10 (2H, m, 2 × ArH), 9.58, 9.81, and 9.84 (each 1H, s, 3 × CONH), 10.94 (1H, brs, NH). Anal. ($C_{44}H_{49}N_9O_7{\cdot}1.5H_2O$) C, H, N.

**Ethyl Ho33258-*O*-Butyrate (23).** To a stirred suspension of Ho33258 (**3**, 1.8 g, 3.91 mmol) and $K_2CO_3$ (1.48 g, 10.75 mmol) in DMF (20 mL) was added ethyl-4-bromobutyrate (0.8 mL, 5.55 mmol), and the reaction mixture was heated at 60 °C for 12 h. After cooling, the mixture was filtered and the filtrate was evaporated in vacuo to dryness. The residue was dissolved in MeOH. Silica gel (3 g) was added to the methanolic solution and the solution was evaporated under reduced pressure. The dry silica gel residue was moved to the top of a silica gel column (4 × 30 cm) and chromatographed using $CHCl_3$ as the eluant. The product **23** was eluted from $CHCl_3$/MeOH (10:1, v/v) and recrystallized from EtOH: 1.4 g (68%), mp 230−231 °C. $^1H$ NMR (DMSO-$d_6$) δ1.19 (3H, t, $J$ = 5.6 Hz, Me), 2.03 (2H, m, $CH_2$), 2.45 (2H, m, $COCH_2$), 2.90 (3H, s, NMe), 3.08 and 3.16 (4H, m, 2 × $NCH_2$), 3.90 (4H, m, 2 × $NCH_2$), 4.08−4.12 (4H, m, 2 × $OCH_2$), 7.21 (3H, m, 3 × ArH), 7.34 (1H, m, ArH), 7.73 (1H, d, $J$ = 9.0 Hz, ArH), 7.94 (1H, d, $J$ = 8.3 Hz, ArH), 8.14 (1H, m, ArH), 8.24 (2H, m, 2 × ArH), 8.57 (1H, s, ArH). Anal. ($C_{31}H_{37}N_5O_3$) C, H, N.

**Ho33258-*O*-Butyric acid (24).** A suspension of **23** (1.30 g, 2.46 mmol) in a mixture of 1 N NaOH (5 mL) and ethanol (5 mL) was heated at 60 °C for 1 h. The reaction mixture was cooled and acidified with 10% HCl to pH 4, and the resulting precipitate was collected by filtration. The solid cake was washed sequentially with water, acetone, and ether, and dried to afford **24**: 1.10 g (90%); mp 261−262 °C. $^1H$ NMR (DMSO-$d_6$) δ 1.99 (2H, m, $CH_2$), 2.42 (2H, t, $COCH_2$), 2.45 (3H, s, NMe), 2.84 (4H, m, 2 × $NCH_2$), 3.44 (4H, s, 2 × $NCH_2$), 4.10 (2H, t, $OCH_2$), 6.99 (1H, d, $J$ = 8.6 Hz, ArH), 7.11 (3H, m, 3 × ArH), 7.50 (1H, d, $J$ = 8.8 Hz, ArH), 7.66 (1H, m, ArH), 8.06 (1H, d, $J$ = 7.8 Hz, ArH), 8.24 (2H, d, $J$ = 8.8 Hz, 2 × ArH), 8.40 (1H, s, ArH). Anal. ($C_{29}H_{33}N_5O_3$) C, H, N.

**2′-[4-[3-(9-Acridinylamino)-5-hydroxymethylanilino]carbonylpropoxyphenyl]-5-(4-methyl-1-piperazinyl)-2,5′-bi-1*H*-benzimidazole (AHMA−Ho33258) (25).** To a mixture of **24** (350 mg, 0.68 mmol), 2,2′-thiopyridine (151 mg, 0.68 mmol), and $Ph_3P$ (178 mg, 0.68 mmol), DMF (5 mL) was added in argon atmosphere, and the mixture was stirred for 0.5 h. A solution of AHMA (214 mg, 0.68 mmol) in 5 mL of DMF was added at −5 °C, and the reaction mixture was stirred for 24 h. The solvent was removed under reduced pressure. The residue was diluted with water and ethyl acetate, and the organic phase was washed with sodium bicarbonate solution, dried over $Na_2SO_4$, and crystallized from ethanol to afford **25** (400 mg, 72%); mp >280 °C. $^1H$ NMR (DMSO-$d_6$) δ 2.13 (2H, t, $CH_2$), 2.55 (3H, s, NMe), 2.58 (2H, t, $COCH_2$), 2.90 (4H, m, 2 × $NCH_2$), 3.96 (4H, m, 2 × $NCH_2$), 4.20 (2H, t, $OCH_2$), 4.52 (2H, s, $ArCH_2$), 7.07 (1H, s, ArH), 7.23−7.26 (3H, m, 3 × ArH), 7.39 (1H, d, ArH), 7.50−7.54 (2H, m, 2 × ArH), 7.63 (1H, s, ArH), 7.77 (2H, m, 2 × ArH), 7.97 (1H, d, ArH), 8.04−8.08 (2H, m, 2 × ArH), 8.13 (2H, m, 2 × ArH), 8.29−8.34 (5H, m, 5 × ArH), 8.72 (1H, s, ArH), 10.37 (1H, s, CONH), 10.98 (1H, s, NH). Anal. ($C_{49}H_{48}N_9O_3$) C, H, N.

## Biological Assays

**Cytotoxicity Assays.** The effects of the compounds on cell growth were determined in all human tumor cells (i.e., colon HT-29, nasopharyngeal carcinoma HONE-1 and BM-1, hepatoma Hepa-G2, breast carcinoma MX-1, gastric carcinoma TSGH, brain tumor DBTRG, and T-cell acute lymphocytic leukemia CCRF−CEM), in a 72-h incubation, by XTT-tetrazolium assay, as described by Scudiero et al.[44] After the addition of phenazine methosulfate-XTT solution at 37 °C for 6 h, absorbance at 450 and 630 nm was detected on a microplate reader (EL 340; Bio-Tek Instruments Inc., Winooski, VT). Six to seven concentrations of each compound were used. The $IC_{50}$ and dose−effect relationships of the compounds for antitumor activity were calculated by a median-effect plot,[45,46] using a computer program on an IBM-PC workstation.[47]

**Inhibition of Topoisomerase II Catalytic Activity by Drugs.** Topoisomerase II catalytic activity was assayed by the ATP-dependent relaxation of pBR322 supercoiled DNA.[48] Various concentrations of the drugs were incubated with 2 units of DNA topoisomerase II (Topogen) and 0.25 μg of pBR322 DNA. The inhibition of the relaxation activity is determined by comparison with an untreated control. AHMA was used as a positive control.

**Measurement of Protein-Linked DNA Breaks.** Cells in log phase growth were labeled with [$^{14}C$]-thymidine for 24 h. After the cells were labeled, they were trypsinized, resuspended in fresh medium at the density of 5 × $10^5$ cells/mL, and shaken gently in a 37 °C water bath for 1 h in suspension. Various concentrations of drugs were added and incubation was continued for an additional 0.5 h. The cells were collected and analyzed for protein-linked DNA breaks by the potassium−sodium dodecyl sulfate (K−SDS) precipitation method, as described previously.[49]

**DNA Unwinding Measurement.** The DNA unwinding effect of drugs was assayed using a method described previously.[50] Briefly, plasmid DNA was linearized with *HindIII* restriction endonuclease and recovered by phenol extraction and ethanol precipitation. Reaction mixture containing 66 mM Tris−HCl (pH 7.6), 6 mM $MgCl_2$, 10 mM dithiotreitol, 0.7 mM ATP, and 0.6 $\mu$g of DNA, and drugs were equilibrated at 15 °C for 10 min and then incubated with an excess amount of T4 DNA ligase at 15 °C for 60 min. The reaction was stopped by addition of 20 mM EDTA. DNA was analyzed by agarose gel electrophoresis after removal of drugs from the reaction mixture: extraction with phenol and ether, and precipitation with ethanol. The DNA circle-ligation assay, using nicked DNA as substrate, was performed by a method based on that of Montecucco et al.[51]

**Acknowledgment.** This work was supported by the National Science Council, Taiwan (Grant NSC90-2320-B-001-032).

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JM0200714