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Regulation of Apoptosis in Leukemic Cells by Analogs of Dynemicin A

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Abstract—The naturally occurring enediyne antibiotics, which include calicheamicin, esperamicin, neocarzinostatin, kedarcidin and dynemicin, are a unique class of reactive compounds which can undergo aromatization to produce cytotoxic biradicals and can result in phosphodiester bond breakage of DNA. Synthetic enediynes designed with low molecular complexity are also highly cytotoxic, specifically to human leukemic cells, by a mechanism involving the induction of apoptosis. We have used a variety of biological assays to evaluate the cytotoxic properties of synthetic dynemicin analogs which contain a spectrum of structural modifications and reactivities. It was found that the induction of apoptosis and nuclear degradation by the synthetic compounds did not require an ability to bind or cleave DNA. Prevention of apoptosis was observed in analogs which were electronically stabilized to inhibit aromatic rearrangement and generation of diradicals. The preventive capability of the stabilized analogs was observed against a wide variety of toxic agents including topoisomerase I and II inhibitors, anti-mitotic and DNA anti-metabolite drugs, as well as alkylating agents. The structural determinants involved in inhibiting the induction of apoptosis are described. The significance of these results with respect to relevant mechanism of tumor regression are discussed.

The enediyne family of antitumor antibiotics consists of the neocarzinostatin chromophore,[1–4] the esperamicins,[5,6] calicheamicins,[7,8] kedarcidin,[9] and dynemicins.[10] In spite of the extensive structural diversity of these compounds, they appear to function by a common mechanism involving aromatization of the enediyne.[11,12] It is generally accepted that DNA is the target of each of these compounds since they all contain functionalities conferring DNA affinity (reviewed in Reference 13). *In vitro*, these compounds are capable of DNA cleavage via the inducible generation of an arenyl or indenyl diradical.[14–17] The diradical is thought to be the causative agent of cytotoxicity by abstraction of hydrogen atoms from the sugar–phosphate backbone of DNA resulting in strand scission. The affinity and targeting to DNA is accomplished by a variety of strategies including apoprotein delivery systems,[18] minor groove binding functionalities,[15, 19–21] and intercalation moieties or combinations of these motifs.[17]

Dynemicin A, a potent antibiotic derived from *Micromonospora chersina*, is an unusual hybrid molecule in which the reactive enediyne group is coupled with the intercalative capability of a substituted anthraquinone.[17] The anthraquinone is structurally similar to anthracycline antibiotics, such as daunomycin, which readily intercalates into DNA, possibly at specific nucleotide sequences.[22] *In vitro*, DNA cleavage experiments with dynemicin have demonstrated some sequence specificity to plasmid DNA strand scission.[17]

In order to investigate the contributions of DNA intercalative functions and enediyne reactivity to the cytotoxic properties of the dynemicin enediynes, analogs have been evaluated in which the anthraquinone group is absent.[13] These synthetic enediynes were tested against a variety of mammalian cell lines to assess their cytotoxic potential.[23] Whereas natural dynemicin was generally cytotoxic to all of the cell lines tested ($IC_{50} = 10^{-8}$–10^{-10} M), some of the synthetic enediynes were found to be selectively cytotoxic to human leukemic cells ($IC_{50} \sim 10^{-14}$) due to a potent induction of a program of apoptosis.[24] It was found that the reactivity of the enediynes was a prerequisite for cytotoxicity. Analogs with a limited capacity to undergo the Bergman rearrangement were far less cytotoxic than their reactive counterparts .

Apoptosis is seen in regressing tumors,[25] in drug-induced differentiation of leukemia cells[26] and is the mechanism by which cytotoxic T-lymphocytes and tumor necrosis factor induce target cell death.[27] It also appears to be the mechanism by which a wide variety of cancer chemotherapeutic agents including cisplatin,[28] vincristine,[29] camptothecin and many others[30] induce cell death. Although many cytotoxic compounds can induce apoptosis in Molt-4 leukemic cells, little is known about the mechanisms involved in the regulation of this process or the cellular factors which make these cells prone to apoptotic death.

We have evaluated dynemicin-like analogs containing a spectrum of structural modifications and reactive capabilities. These include analogs containing structural modifications which influence the triggering or progression of the Bergman aromatization. We have identified a variety of novel compounds whose biological activities profoundly affect apoptotic cell death in leukemic cells.

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Abbreviations used are EDTA (ethylene diamine tetraacetic acid), Me_2SO (dimethyl sulfoxide).

Materials and Methods

Synthesis of enediynes

Synthesis of the reactive dynemicin analogs **1** and **2** as well as the stabilized analogs **6** and **8** have previously been described[31] as have the syntheses of the stabilized analogs **7**,[32] **3** and **9**,[33] **4**,[34] **5** and **10**,[35] and **11** and **12**.[36] Dynemicin A, purified from *Micromonospora chersina* was supplied by Dr M. Konishi, Bristol–Meyers–Squibb, Japan, and calicheamicin γ^{I}_{1}, purified from *Micromonospora echinospora*, was donated by Dr G. Ellestad and Dr D. Borders of Lederle Laboratories, U.S.A. The chemical reactivity of synthetic analogs, referred to in the text, was measured by incubation in 1 mM ATP, 200 mM phosphate using DMSO to vary the pH to 11.[24] Reactions were done in a total volume of 20 µL for 10 min and were immediately analyzed by HPLC. The percentage of enediyne remaining after the reaction was used as the parameter of instability. Enediynes referred to as being reactive invariably suffered a complete rearrangement of the enediyne moiety under these conditions.

Plasmid DNA cleavage assay

20 µL reaction mixtures contained enediyne and supercoiled pUC18 (50 µg/mL) in a Tris buffer (50 mM Tris-Cl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.5) containing no more than 10 % Me_2SO. Samples were incubated at 37 °C for 30 min; loading buffer was added and the sample was loaded onto 0.8 % agarose gels.[37]

Nuclear DNA extraction

DNA was isolated from 10^6 Molt-4 cells using 10 mM Tris-Cl, 1 mM EDTA, pH 8.0 (TE) containing 0.2 % Triton-X-100. DNA was precipitated from the lysate by the addition of 1/10th volume 5 M sodium acetate, pH 5.0 and 3 volumes 95 % ethanol. After centrifugation, the DNA pellet was resuspended in 25 µL TE containing 1 mg/mL RNAse A, incubated for 10 min at 60° C followed by agarose gel electrophoresis in Tris–borate buffer.[37]

Cells and culture conditions

The Molt-4 cell line was obtained from American Type Culture Collection. To visualize the apoptotic morphology cells were attached to glass slides by low speed centrifugation and stained with Diff-Quik (Baxter Healthcare Corp., Miami, FL). Viability assays were performed in microtiter plates using the XTT vital staining method.[38] The apoptotic index is the percentage of cells that display the apoptotic morphology. This value was determined in three separate assays for each drug where a minimum of 300 cells were visually scored by microscopy at 50x magnification per assay.

Results

1. Biological activity of reactive dynemicin analogs

Compound **1** (Figure 1) possesses a reactive capability, similar to the natural products, for production of diradicals through aromatization of the enediyne. Cellular DNA extracted from Molt-4 human leukemic cells that had been incubated with 1 x 10^{-7} M of analog **1** for 4 h contained fragments with repeat units of ~180 base pairs, characteristic of nucleosomal ladder formation (Figure 2A), indicating an effective induction of apoptosis. Cells visualized by centrifugation and staining confirmed an apoptotic morphology at 4 h in ~40 % of the population (Figure 2B). Calicheamicin and dynemicin also induced apoptosis at 10^{-7} M. The enantiomer of **1**, compound **2**, was a poor inducer of apoptosis at 10^{-6} M, demonstrating that the target of the dynemicin analogs could, to a certain extent, distinguish between the enantiomeric forms. Although significant levels of site specific cleavage of plasmid DNA was observed using 10^{-7} M synthetic calicheamicin or 10^{-4} M dynemicin, we detected no plasmid DNA cutting activity from either of the synthetic compounds up to 10^{-3} M (Figure 3). The cytotoxicity of these compounds, measured by the XTT assay,[38] reflected their relative intensity of inducing apoptosis (Table 1).

2. Biological activity of stabilized dynemicin analogs

A series of compounds was synthesized where the inherent capability to undergo the Bergman rearrangement was compromised by ligands which stabilized the epoxide group.

To measure the regulation of apoptotic DNA degradation, Molt-4 cells (10^5/mL) were induced to undergo apoptosis using 10^{-7} M calicheamicin. The stabilized analogs were tested for their ability to inhibit the calicheamicin-induced DNA degradation by co-incubating the analog at 10^{-4} M in the reactions.

i. The enediyne bridge. Compound **3**, containing only one of the triple bond components of the enediyne is effective in inhibiting both DNA degradation and the apoptotic cell morphology induced by calicheamicin (Figure 4, Table 1). Adducts which substitute for the double bond of the enediyne, such as compound **4**, do not inhibit apoptosis and have no biological activity in these assays.

ii. Adducts attached to the ring nitrogen. Phenyl carbamate (**5**), phenyl sulfoxide carbamate (**6**), and methylated phenyl carbamate sulfone ligands of the ring nitrogen (**7**), are effective inhibitors of apoptosis. These compounds are blocked from undergoing Bergman aromatization due to the absence of an appropriate triggering mechanism yielding the free amine intermediate. An alternate triggering mechanism involving a *t*-$BuCO_2$- group attached to the *para* position of the backbone phenyl ring,[31,39] has been demonstrated.[40] Inhibition of DNA degradation however is highly effective when the *t*-$BuCO_2$- ligand is attached to the *meta* position of the aromatic ring (**8**).

iii. Bergman product analogs

Opening of the epoxide to produce an hydroxyl is a key step enabling the subsequent aromatization of the enediyne and generation of a Bergman rearrangement product. Bergman

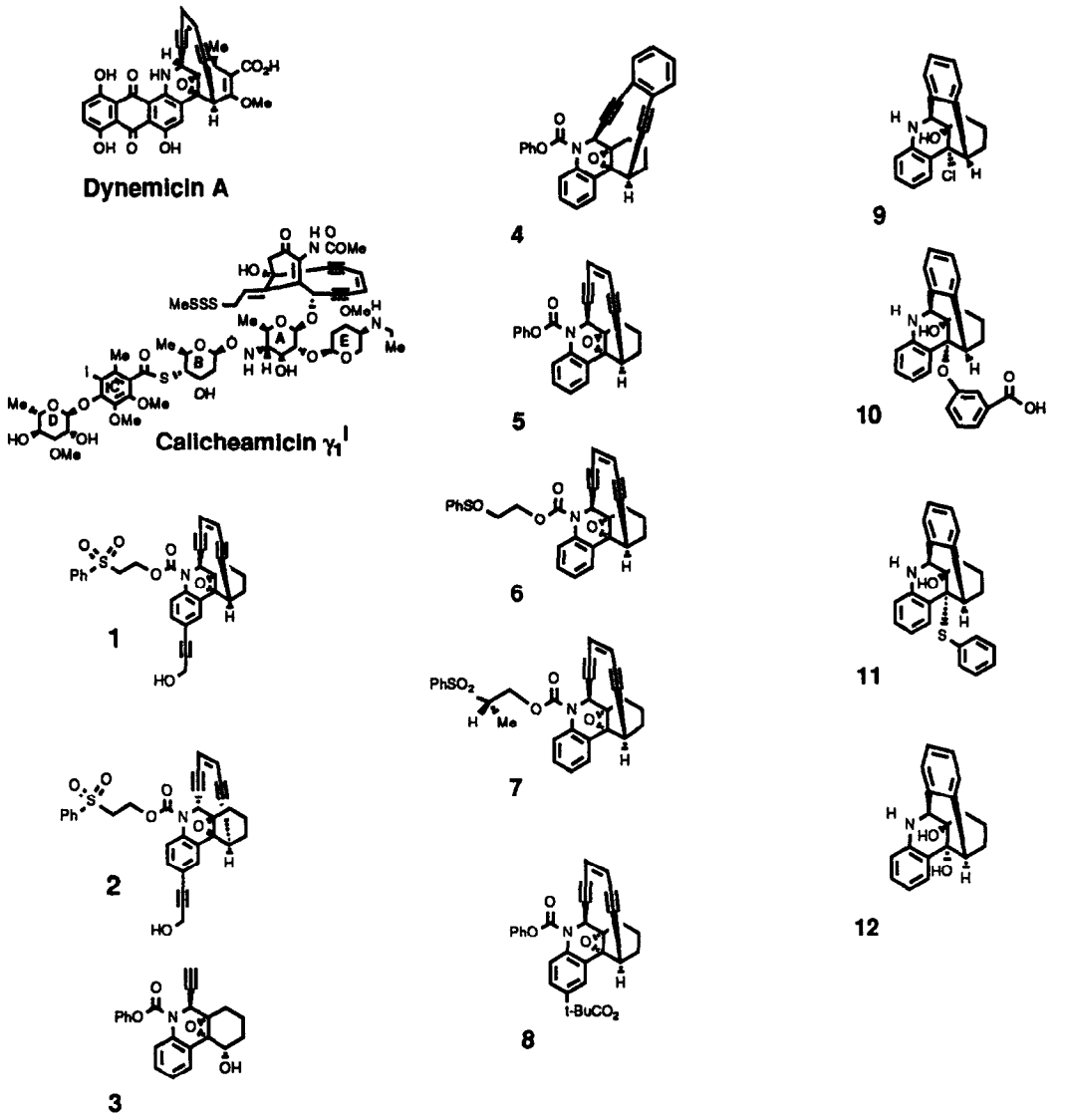

Figure 1. Chemical structures and biological activities of dynemicin analogs.

product analogs containing other functionalities substituting the epoxide (**9**), as well as substituted analogs of the Bergman product, such as **10**, are inhibitors of apoptosis. Substitution at this position can also result in inert compounds having no biological activity (**11**). The Bergman product containing two hydroxyls, **12**, exhibited no biological activity in these assays.

3. Biological activity of tlte stabilized enediynes against a variety of cytotoxic agents

We used a variety of compounds representative of different cytotoxic mechanisms, to initiate death of Molt-4 cells in the presence and absence of stabilized enediynes. Most of the compounds, in the absence of enediyne, induced apoptotic cell death (Table 2). However, we also tested cytotoxic agents which resulted in distinctly different cell death morphologies. In common among all of the cytotoxic agents employed was the induction of massive chromatin rearrangement. In most cases, this rearrangement involved degradation of DNA into nucleosomal fragments whereas in other cases, such as taxol, tubercidin, vinblastine, and morpholinodoxorubicin no such degradation was observed. Compound **3** was tested for its ability to inhibit the drug-induced DNA degradation and apoptotic morphology by co-incubation in the reactions at 10^{-4} M. The cytotoxic drugs employed were cyanomorpholinodoxorubicin (alkylating agent), camptothecin (topoisomerase I inhibitor), morpholinodoxorubicin (topoisomerase II inhibitor), ara-C, tubercidin (DNA antimetabolite), taxol and vinblastine (antimitotic agents).[41]

We found that the particular morphology of chromatin rearrangements induced by each of these compounds was prevented by the stabilized enediyne (Table 2). In cases where nucleosomal DNA degradation was induced by the cytotoxic compound, this was also inhibited by the stabilized enediyne.

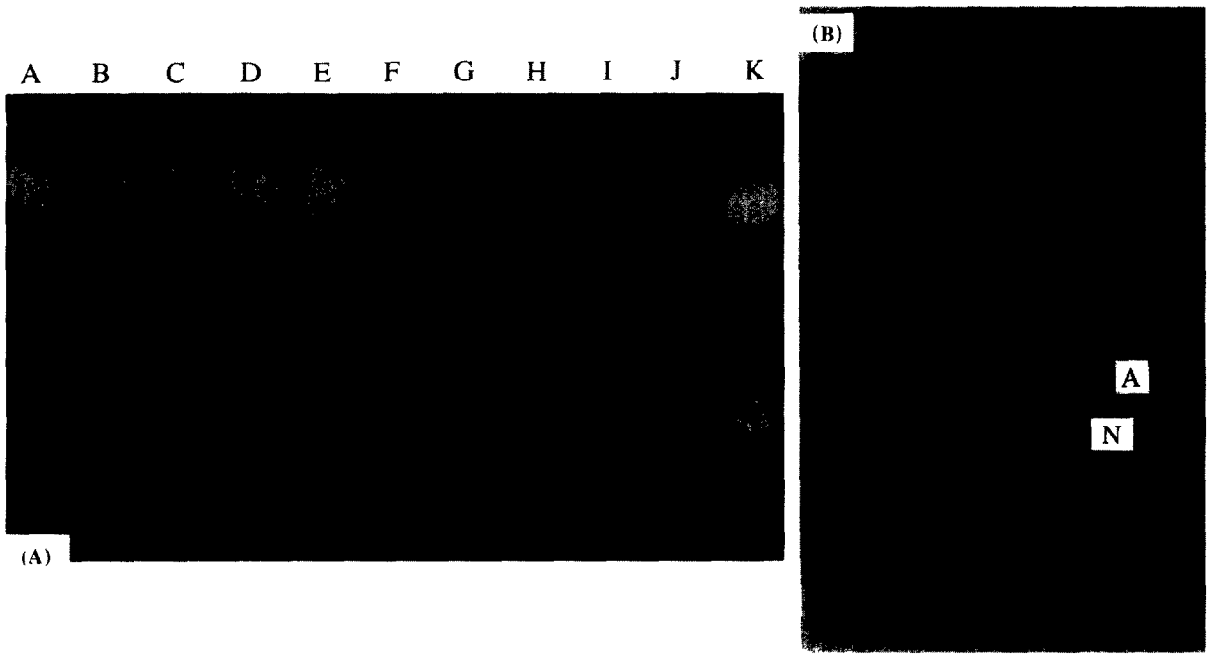

Figure 2A. DNA degradation into nucleosomal fragments induced by dynemicin analogs. Molt-4 cells were incubated with enediyne as described in the Materials and Methods, and extracted DNA was evaluated on agarose gels. **A**, 10^{-7} M calicheamicin γ^I_1; **B**, 10^{-7} M synthetic calicheamicin γ^I_1; **C**, 10^{-7} M dynemicin A; **D**, 10^{-7} M compound **1**; **E**, 10^{-4} M compound **2**; **F–J**, were the same as **A–E** but also contained 80 mM $ZnCl_2$, an inhibitor of apoptotic DNA degradation;[24] **K**, molecular weight markers consisting of multiples of a 123 base pair fragment (Gibco BRL); **2B**, the apoptotic morphology of Molt-4 cells. "A" indicates an apoptotic cell and "N" indicates a normal cell.

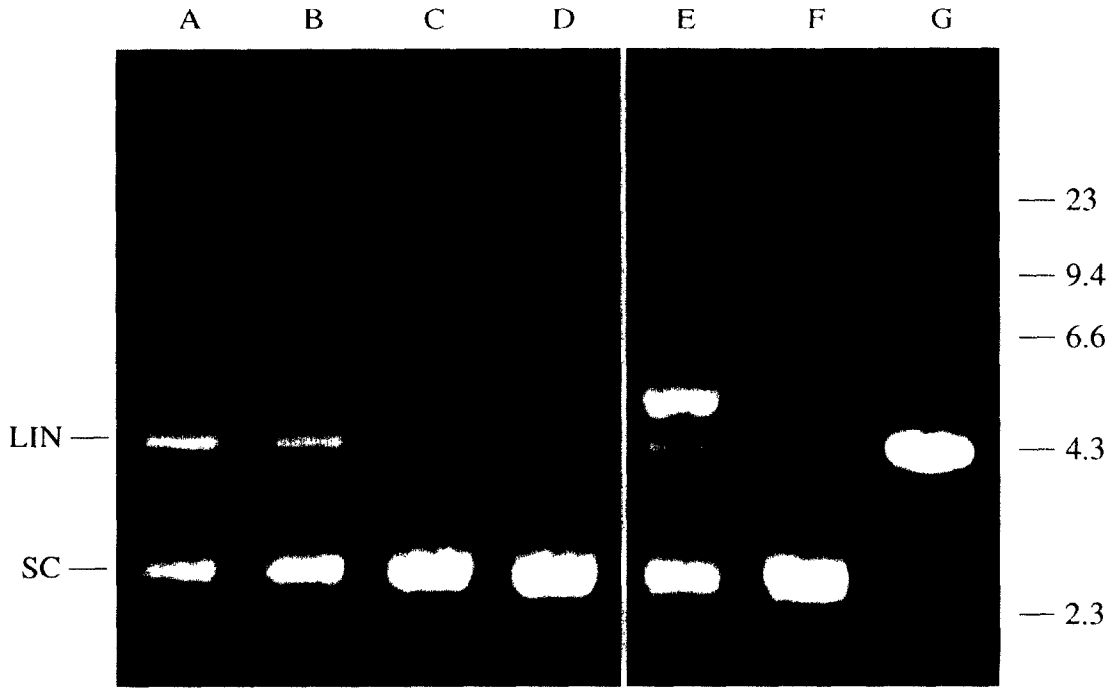

Figure 3. DNA cleavage assays using naturally occurring and synthetic enediynes. Co-incubations of enediyne analogs and plasmid DNA were performed as described in the Materials and Methods and evaluated by agarose gel electrophoresis. **A**, 10^{-7} M calicheamicin γ^I_1; **B**, 10^{-7} M synthetic calicheamicin γ^I_1; **C**, 10^{-6} M enediyne **1**; **D**, 10^{-4} M enediyne **2**; **E**, 10^{-4} M dynemicin A; **F**, control pBR322 plasmid DNA; **G**, EcoR1 restriction enzyme digested pBR322 (linearized). "SC" refers to supercoiled plasmid DNA and "LIN" refers to linearized plasmid DNA. The molecular weight markers, denoted in kilobase pairs on the right, are derived from Hind 3 digested λ DNA.

Table 1. Biological activity of dynemicin analogs

Compound	Cytotoxicity[1]	Apoptotic Index	% Apoptosis Inhibition
dynemicin	10^{-12}	34	0
calicheamicin	10^{-14}	46	0
1	10^{-12}	39	0
2	10^{-9}	10	0
3	$>10^{-6}$	0	95
4	$>10^{-6}$	0	0
5	$>10^{-6}$	0	94
6	$>10^{-6}$	0	96
7	$>10^{-6}$	0	98
8	$>10^{-6}$	0	90
9	$>10^{-6}$	0	91
10	$>10^{-6}$	0	93
11	$>10^{-6}$	0	0
12	$>10^{-6}$	0	0

[1]Viability was assayed using the XTT staining method (38). The apoptotic index is the percentage of cells that display the apoptotic morphology. This value was determined in three separate assays for each drug where a minimum of 300 cell were visually scored by microscopy at 50X magnification per assay. Apoptotic inhibition measured the reduction in the number of cells that were apoptotic resulting from co-incubation with the indicated compound, expressed as a percentage.

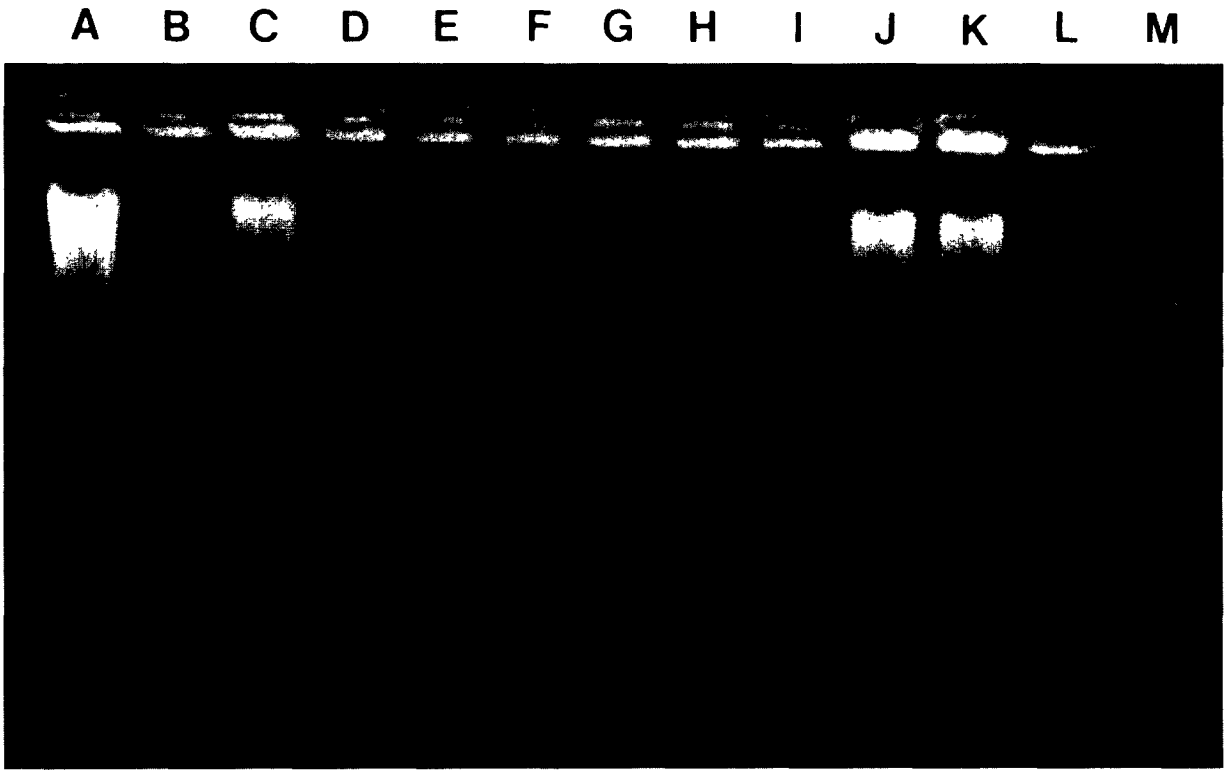

Figure 4. Inhibition of apoptotic DNA degradation by stabilized dynemicin analogs. Molt-4 cells were co-incubated with calicheamicin γ^I_1 (10^{-7} M) and 10^{-4} M of the indicated synthetic analog for 4 h. Cellular DNA was extracted as described in the Materials and Methods, and evaluated on agarose gels. **A**, 10^{-7} M calicheamicin γ^I_1 alone; **B–O**, 10^{-7} M calicheamicin γ^I_1 co-incubated with 10^{-4} M of the following compounds: **B**, compound **3**; **C**, compound **4**; **D**, compound **5**; **E**, compound **6**; **F**, compound **7**; **G**, compound **8**; **H**, compound **9**; **I**, compound **10**; **J**, compound **11**; **K**, compound **12**; **L**, no additions; **M**, molecular weight markers consisting of multiples of a 123 base pair fragment (Gibco BRL).

Table 2. Biological activity of stabilized enediyne 3 against a variety of cytotoxic agents

Compound	Chromatin[1] morphology	Nucleosomal ladder induction	% Inhibition of morphology by 3	Inhibition of ladder by 3
cyanomorpholino-doxorubicin	apoptotic	yes	90	yes
camptothecin	apoptotic	yes	93	yes
morpholino-doxorubicin	apoptotic	no	92	-
ara-C	apoptotic	yes	96	yes
tubercidin	apoptotic	no	90	-
taxol	non-apoptotic	no	98	-
vinblastine	non-apoptotic	no	93	-
compound 3	normal	no	-	-

[1]Cells were visualized and evaluated as described in Table 1.

Discussion

There are many inhibitors of apoptosis, some of which have defined and characterized target molecules. The most commonly used inhibitor, zinc, is thought to inhibit a Ca^{2+}-Mg^{2+}-dependent endonuclease essential for the formation of nucleosomal fragments.[27,42,43] Similarly, aurintricarboxylic acid is thought to act by a similar mechanism of inhibition although this reagent is known to have effects on other cellular enzymes.[44] Both of these reagents inhibit all of the morphological characteristics of apoptosis. Consequently, it is thought that the fragmentation of DNA must somehow control the subsequent process of cell breakup. The mechanism by which this occurs remains to be elucidated. However, these results serve to divide apoptosis into two distinct phases, the first involving nuclease digestion and the second involving the changes in nuclear morphology and cell shape during the formation of apoptotic bodies. Support for this conception of the two phases of apoptosis in Molt-4 cells has been provided by inhibition studies using the fungal metabolite cytochalasin B.[45] This drug inhibits cell fragmentation and the formation of apoptotic bodies probably by its ability to interfere with actin polymerization. This effect was seen when HL-60 cells were pretreated with cytochalasin B and then exposed to one of a number of apoptosis-inducing agents, including UV irradiation, camptothecin, aphidocholin, or the phorbol ester, PMA plus ionomycin. Cytochalasin had no effect on the DNA fragmentation or on nuclear condensation and fragmentation. Similarly, inhibitors of protein kinase C (PKC), such as staurosporin, were observed to inhibit the latter stages of apoptosis in Molt-4 cells. This is thought to be due to the role that PKC plays as a potentiator of microtubule assembly. Although the involvement of PKC in the apoptosis of other cell types is a matter of some controversy, in Molt-4 cells PKC appears to be primarily involved in the microtubule-dependent formation of apoptotic bodies. In view of the various inconclusive findings[46–49] it is currently unclear what role is played by protein kinase C in either the early or the later stages of apoptosis. Whether inhibitors of the later stages of apoptosis also influence the early stages is also a matter of controversy. The question of how inhibition of the early phase of apoptosis can prevent the entire apoptotic program is crucial to understanding the role of DNA structure in the maintenance of normal cell morphology.

In summary, there are many factors which can modulate the propensity of a cell to undergo apoptotic death. Understanding those factors which are directly involved in the initiation of cell death is crucially important to understanding the regulation of the entire apoptotic pathway. Either by control of nucleases or by control of DNA substrate availability, the regulation of early apoptosis dictates the triggering and subsequent progression of the entire program. Although specific enzymes have been isolated which are involved in early apoptosis,[50] the involvement of additional key enzymes which can control the triggering of the apoptosis cascade is probable. These as yet uncharacterized enzymes may define the earliest events in the induction and regulation of the apoptotic pathway. Our approach has been to synthesize useful derivatives of the dynemicin analogs since these compounds appear to target a crucial process involved in apoptosis initiation.

The enediynes are an unusual group of compounds which are generally capable of inducing apoptosis in susceptible cell lines by a mechanism involving the generation of cytotoxic diradicals.[23,24] The stabilized analogs are invariably inhibitors of the early events of apoptosis.[24] Our results suggest that there are key aspects of the structure of these compounds which contribute to their biological activity. In particular, it appears that the epoxide ring is located within a region of the molecule in which addition of polar or charged ligands cari eliminate biological activity.

Our results suggest that the cytotoxic intermediates involved in the rearrangement of dynemicin analogs may be initiating a chain of metabolic events which involve chromatin rearrangement and degradation, and the ultimate disintegration of the cell into apoptotic bodies. The non-reactive analogs, incapacitated from undergoing rearrangement, may initiate an alternative regulatory response involving the suppression of both the nucleolytic activity and the chromatin rearrangement involved in apoptosis. The nature of the cellular targets is unknown.

Although Molt-4 cells are prone to undergo apoptosis, the cellular factors which are responsible for making these cells uniquely susceptible to apoptotic death have not been characterized. Since the enediyne analogs are selectively cytotoxic to Molt-4 cells compared to the parent compound dynemicin, and are relatively non-toxic to melanoma, carcinoma, and normal cell lines, they are affecting factors involved in the propensity of Molt-4 cells to undergo apoptotic death.

The naturally occurring enediynes, such as calicheamicin, are thought to exert their cytotoxic effects by reactivity directed specifically at the phosphodiester backbone of DNA. Calicheamicin is clearly a potent DNA cleaving compound.[21] Nuclear DNA strand scission is one of many triggers that can initiate DNA degradation and apoptotic cell death. However, our data suggests that local strand scission may not be sufficient in itself to initiate the massive DNA degradation characteristic of apoptotic cell death. Initiation of apoptotic DNA degradation may require further interactions with cellular constituents participating in the overall process of chromatin rearrangement and degradation. The synthetic dynemicin analogs may identify one such cellular factor.

Apoptotic death is distinct from other forms of cell death, particularly necrosis, by a variety of morphological features whereby selected cells undergo drastic morphological changes.[51] The goal of apoptosis is to attain orderly disintegration of cells and their chromatin into structures suitable for phagocytosis.[52–54] This primarily involves chromatin condensation and degradation of genomic DNA into nucleosomal fragments. Apoptosis is known to be involved in developmental and tissue specific processes which require removal of cell populations,[55,56] in immunological processes of cell selection,[57–59] during *Shigella* invasion of colonic mucosa,[60] in T cell death resulting from HIV-1 infection,[61,62] and during tumor regression.[63–66] Identification of the cellular factors involved in the progression and control of this type of cell death will be an important first step towards understanding how the commitment to cell death is regulated.

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