Cell-specific regulation of apoptosis by designed enediynes


ABSTRACT

The naturally occurring enediyne antibiotics are a unique class of antitumor drugs that combine reactive enediynes with additional structural features conferring affinity for DNA. Dynemicin A, in which an enediyne core is attached to an anthraquinone group capable of DNA intercalation, readily cleaves double-stranded DNA. This activity is thought to be the basis of its potent antitumor cytotoxicity. To investigate cell-specific mechanisms of cytotoxicity in the absence of DNA affinity, we have synthesized a variety of dynemicin-like enediynes that lack the anthraquinone moiety. We have found that the cytotoxicity of these compounds is dependent on their chemical instability and their enantiomeric form. Their selective toxicity results from a potent induction of apoptosis primarily in human leukemic cells. A group of synthetic enediynes were designed to be highly stable. These compounds were found to inhibit apoptotic cell death. This inhibition was observed in competition with the chemically unstable enediynes, including dynemicin and calicheamicin. The stable synthetic enediynes could also block the apoptotic morphology induced by unrelated cytotoxic agents such as cycloheximide, actinomycin D, and ultraviolet radiation. The results suggest that the cellular target(s) of synthetic enediynes may play a central role in regulating programmed cell death; a specific receptor–ligand interaction is proposed.

Recently, considerable effort has been made to identify compounds that can regulate programmed cell death (apoptosis) in specific cell types. The control of apoptosis in specific cells has crucial relevance to developmental biology (1, 2), treatments for viral (3, 4) and bacterial (5) pathogens, and cancer chemotherapy (6). Apoptosis represents an active process whereby selected cells undergo drastic morphological changes involving chromatin condensation and degradation of genomic DNA into nucleosomal fragments (7) prior to disintegration into structures suitable for phagocytosis (8–10). Apoptosis is known to be involved in developmental and tissue-specific processes that require removal of cell populations (11, 12), in immunological processes of cell selection (13–15), during Shigella invasion of colonic mucosa (5), in T-cell death resulting from human immunodeficiency virus 1 infection (3, 4), and during tumor regression (16–19).

We have investigated a class of synthetic compounds, the designed enediynes, which are potential regulators of programmed cell death and may be useful as chemotherapeutic agents (20–22). Naturally occurring enediynes contain either DNA intercalating groups (such as dynemicin A) (23) or DNA minor groove binding functions [such as calicheamicin γ1 (24, 25) and esperamycin A1 (26, 27)] and are potent DNA-damaging agents due to their ability to generate benzenoid diradicals (28). Other enediynes were synthesized lacking intercalating or minor groove binding functionalities (20–22). Structural modifications were incorporated to modulate the inherent chemical reactivity. The cytotoxicity of these compounds against certain types of normal cells was reduced (IC50 ∼10 μM) compared to dynemicin (IC50 ∼10 nM). However, some of the enediynes were exceptionally cytotoxic toward human T-cell leukemia lines (IC50 ∼10 fM for enediyne 1 on the MOLT-4 cell line; structures shown in Fig. 2). Dynemicin A generally displays low cell type specificity for killing (W.W., unpublished results).

We have synthesized a variety of additional enediyne analogs to begin investigating parameters of structure and reactivity that may be involved in the cell-specific toxicity of these compounds.

MATERIALS AND METHODS

Drugs and Chemicals. Dynemicin A was supplied by M. Konishi (Bristol-Meyers Squibb, Syracuse, NY), and calicheamicin γ1 was donated by G. Eileston and D. Borders (Lederle Laboratories, Pearl River, NY). Actinomycin D and cycloheximide were from Sigma. The synthesis of enediynes 1 and 2 has been described (21) as have enediynes 3, 4, 5, 8, and 9 (29, 30) and enediynes 6 and 7 (31).

Cells and Culture Conditions. MOLT-4 and SK-Mel-28 cell lines were obtained from American Type Culture Collection. Viability assays were performed in microtiter plates using the XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt) vital staining method (32). To visualize the apoptotic morphology, cells were attached to glass slides by low-speed centrifugation and stained with Diff-Quik (Baxter Healthcare, Miami). The apoptotic index is the percentage of cells that display the apoptotic morphology. This value was determined in three assays for each drug where a minimum of 300 cells was visually scored by microscopy at ×50 magnification per assay.

DNA Extraction and Flow Cytometry. DNA extractions were from 10⁶ MOLT-4 cells in 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 0.1 vol of 5 M sodium acetate (pH 5.0) and 3 vol of 95% ethanol. After centrifugation, the DNA pellet was resuspended in 25 μL of TE containing RNase A (1 mg/ml), incubated for 10 min at 60°C, followed by agarose gel electrophoresis in Tris borate buffer (45 mM Tris borate/1 mM EDTA, pH 8.0).

Flow cytometry was performed on 10⁴ cells by using a FACSscan cell sorter (Becton Dickinson).

Chemical Stability of Enediynes. The chemical stability was measured by incubating enediyne in 1 mM ATP/200 mM sodium phosphate using dimethyl sulfoxide to vary the pH to either 10.5 or 11. 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, where 100 indicates a complete loss of enediyne and zero is a completely stable enediyne.

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analysis of MOLT-4 cells at 4 h of exposure to enediyne 1 corroborated the morphological observations (Fig. 1D).

Exposure of SK-Mel-28 melanoma cells to the same concentrations of enediyne 1 at any time interval up to 24 h did not result in any morphological abnormalities.

DNA isolated from 10^6 MOLT-4 cells after 4 h of exposure to 0.1 μM enediyne 1 clearly demonstrated the characteristic pattern of DNA degradation into nucleosomal fragments (Fig. 1E) (35). In all cases where apoptotic cell death was apparent, we observed nucleosomal fragments. ZnCl2 (0.5 mM) added to MOLT-4 cells with 0.1 μM enediyne 1 inhibited both the nucleosome ladder (Fig. 1E) and all of the morphological characteristics of apoptosis (36–38).

**Structural Requirements for Synthetic Enediylene Cytotoxicity.** Although enediyne 1 is extremely cytotoxic to MOLT-4 cells, a variety of other structural analogs have reduced cytotoxicities that may be due to differences in chemical stability (20). We quantified the chemical stability of the analogs in Fig. 2 by incubation for 10 min at pH 10.5 or 11, followed by measurement of reaction products by HPLC. The ratio of peak areas of the enediyne before and after the reaction was used as the index of stability. The scheme at the bottom of Fig. 2 depicts a possible rearrangement intermediate (diradical) resulting from nucleophilic attack (Nu) and chemical instability of the synthetic enediynes. With the exception of enantiomers of structure 9, the compounds shown in Fig. 2 are not enantiomerically pure. The chemical stability values were compared to the apoptotic index (39) and the cytotoxicity as measured by dye-exclusion assays (32).

Various strategies were used to modulate the chemical stability of these enediynes. A key element in the generation of benzenoid radicals is the ability of the enediyne ring to collapse due to the locking mechanism involving opening of the epoxide ring by electron donation. Electron-withdrawing groups at the indicated bridgehead position, such as methoxy (enediyne 2), incapacitate the molecule from entering the Bergman rearrangement (at the bottom of Fig. 2). Enediyne 2 is not cytotoxic at 1 μM and does not induce apoptosis at that concentration.

**RESULTS**

**Apoptotic Cell Death Induced by Enediyne 1.** Although synthetic enediynes are capable of DNA cleavage (20), the concentrations required for this activity are far in excess of the physiologically relevant levels. Enediyne 1 kills MOLT-4 cells at 10 μM; however, the concentrations required to cleave supercoiled DNA were found to be in the range of 1 mM (20). It is well established that dynemicin and calicheamicin can cleave plasmid DNAs at 10 μM and 10 nM, respectively (33, 34). Our cytotoxicity results suggested that enediyne 1, and possibly other enediynes, may accumulate in cells due to an affinity for a target site other than DNA. A 20-min exposure of MOLT-4 cells to 0.1 μM enediyne 1, followed by low-speed centrifugation and resuspension in medium without drug, is sufficient for nearly complete cell killing after 22 h (Table 1). This suggested that enediyne 1 was taken up rapidly by MOLT-4 cells and that a program of cell death was being initiated by the pulse.

Examination of cell morphology after a 4-h exposure of MOLT-4 cells to 0.1 μM enediyne 1 revealed the typical characteristics of apoptotic cell death (Fig. 1A) compared to normal MOLT-4 cells (Fig. 1C). Fragmentation of the nucleus occurred as well as disintegration of the affected cells (20–40% of the total) into apoptotic bodies. Flow cytometry

<table>
<thead>
<tr>
<th>Time of incubation, h</th>
<th>% viability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control 10^-10 M</td>
</tr>
<tr>
<td>1</td>
<td>90</td>
</tr>
<tr>
<td>4</td>
<td>87</td>
</tr>
<tr>
<td>8</td>
<td>84</td>
</tr>
<tr>
<td>22</td>
<td>81</td>
</tr>
</tbody>
</table>

Approximately 5 × 10^6 MOLT-4 cells were incubated with various concentrations of enediyne 1 for 20 min followed by low-speed centrifugation and resuspension in fresh medium containing no enediyne. Serial dilutions were performed to estimate cell viability at various times by using the XTT vital staining method. Data are expressed as the percent of total cells that were viable.

**Fig. 1.** Apoptosis and nucleosomal fragments induced by enediynes. Cells were exposed to enediyne for 4 h after which they were visualized by staining. DNA was extracted, or flow cytometry was performed. (A) MOLT-4 cells after 4 h of 0.1 μM enediyne 1 (a, apoptotic; n, normal). (B) MOLT-4 cells after 4 h of 0.1 μM enediyne 1 plus 0.1 mM enediyne 2. (C) Normal MOLT-4 cells. (D) Distribution of cells after enediyne 1 treatment: M1 (apoptotic), 23%; M2 (G0/G1 phase), 45%; M3 (S phase), 27%; M4 (G2/M phase), 7%. The graph plots cell number (x axis) versus relative DNA content (y axis). (E) DNA isolated from 10^6 MOLT-4 cells after 4 h of exposure to the following conditions: Lanes: 1, actinomycin (1 μg/ml); 2, actinomycin (1 μg/ml)/0.1 mM enediyne 2; 3, cycloheximide (100 μg/ml); 4, cycloheximide (100 μg/ml)/0.1 mM enediyne 2; 5, 0.1 μM enediyne 1; 6, 0.1 μM enediyne 1/0.1 mM enediyne 2; 7, 0.1 μM enediyne 1/500 μM ZnCl2; 8, 0.1 mM enediyne 2; 9, no additions; 10, UV radiation (302 nm, 15 min)/0.1 mM enediyne 2; 11, UV radiation (15 min). The lanes at each end of the gel are molecular mass standards.
\[ \text{Activity} \]

\begin{tabular}{|c|c|c|c|c|c|}
\hline
\text{Compound} & \text{Activity} & \text{A} & \text{B} & \text{C} & \text{D} & \text{E} \\
\hline
1 & & \text{>10}^{-14} & 100 & 89 & 40 & 73 \\
2 & & \text{10}^{-6} & 0 & 0 & 0 & 0 \\
3: \text{R}_1=\text{CH}_3, \text{R}_2=\text{H}, \text{X=SO}_2 & & \text{10}^{-6} & 24 & 12 & 0 & 6 \\
4: \text{R}_1=\text{H}, \text{R}_2=\text{CH}_3, \text{X=SO}_2 & & \text{10}^{-9} & 24 & 12 & 0 & 16 \\
5: \text{R}_1,\text{R}_2=\text{CH}_3, \text{X=SO}_2 & & \text{10}^{-4} & 0 & 0 & 0 & 0 \\
6: \text{R}_1,\text{R}_2=\text{H}, \text{X=S} & & \text{10}^{-5} & 0 & 0 & 0 & 0 \\
7: \text{R}_1,\text{R}_2=\text{H}, \text{X=SO} & & \text{10}^{-5} & 0 & 0 & 0 & 0 \\
8 & & \text{10}^{-11} & 57 & 5 & 4 & 11 \\
(\text{+})-9 & & \text{10}^{-13} & 100 & 100 & 14 & 41 \\
(\text{-})-9 [\text{enantiomer of (+)-9}] & & \text{10}^{-7} & 100 & 100 & 2 & 30 \\
(\pm)-9 [\text{racemic 9}] & & \text{10}^{-11} & 100 & 100 & 6 & 24 \\
\hline
\end{tabular}

\text{\textbf{FIG. 2.} Chemical instability and biological activity of enediyne structural analogs. The inherent stability of various enediynes was measured by HPLC. The cytotoxicity against MOLT-4 cells was measured after 3 days of exposure to serial dilutions starting at 0.1 mM of each compound. Apoptosis was quantified by microscopic inspection of cells. Columns: A, the IC}_{50} (M) values for each enediyne determined using a starting concentration of 0.1 mM; B, the chemical instability index at pH 11; C, the chemical instability at pH 10.5; D, the apoptotic index using 0.1 \text{\mu}M enediyne; E, the apoptotic index using 1 \text{\mu}M enediyne.}

$\beta$-Elimination involving the phenyl sulfone carbamate attached to the ring nitrogen is required for triggering the cycloaromatization of some of these enediynes leading to benzenoid diradicals (compounds 6 and 7 do not degrade under these conditions, whereas compound 8 suffers a different type of activation involving ester hydrolysis). Chemical modifications of the trigger would be expected to modulate the reactivity and cytotoxicity of the enediyne. In addition, the ability of any particular cell line to activate the trigger may be an important factor determining the cell-type specificity of these compounds. Methylation of this moiety at the $\alpha$-position (enediynes 3–5) modulates both the chemical stability and cytotoxicity (30, 31). The mechanism of this effect is not clear. The inability of the molecule to undergo a $\beta$-elimination in the case of the thiophenyl carbamate (enediyne 6) or the phenyl sulfoxide carbamate (enediyne 7) completely deoids the molecule from its ability to cause apoptosis and diminishes the cytotoxicity by a factor of $10^4$.

An alternate triggering mechanism involving a $t$-BuCO$_2$-group attached to the ring backbone (enediyne 8) (21, 22) suggests additional possibilities for modulating the reactivity of these compounds. The results demonstrate that chemical instability of these compounds is a prerequisite for induction of apoptosis and cytotoxicity. Bergman cycloaromatization may be the primary mechanism of cytotoxicity since structures that cannot undergo chemical rearrangement are generally not cytotoxic below 1 \text{\mu}M and do not induce apoptosis.

In addition to the contribution of chemical instability, it is apparent that other structural determinants are contributing to apoptosis and cytotoxicity. The enantiomeric enediynes...
Table 2. Inhibition of cytotoxicity and apoptosis in MOLT-4 cells by enediyne 2

<table>
<thead>
<tr>
<th>Addition(s)</th>
<th>% apoptotic cells</th>
<th>Ladder</th>
<th>IC_{50}, M</th>
</tr>
</thead>
<tbody>
<tr>
<td>No additions</td>
<td>No</td>
<td></td>
<td>—</td>
</tr>
<tr>
<td>Enediyne 2 (0.1 mM)</td>
<td>1</td>
<td>No</td>
<td>5 x 10^{-5}</td>
</tr>
<tr>
<td>Enediyne 1 (0.1 uM)</td>
<td>40</td>
<td>Yes</td>
<td>1 x 10^{-12}</td>
</tr>
<tr>
<td>Enediyne 1 + 2</td>
<td>2</td>
<td>No</td>
<td>6 x 10^{-7}</td>
</tr>
<tr>
<td>Calicheamicin (0.1 uM)</td>
<td>43</td>
<td>Yes</td>
<td>3 x 10^{-12}</td>
</tr>
<tr>
<td>Calicheamicin + 2</td>
<td>1</td>
<td>No</td>
<td>2 x 10^{-8}</td>
</tr>
<tr>
<td>Dynemicin (0.1 uM)</td>
<td>36</td>
<td>Yes</td>
<td>4 x 10^{-11}</td>
</tr>
<tr>
<td>Dynemicin + 2</td>
<td>2</td>
<td>No</td>
<td>5 x 10^{-9}</td>
</tr>
<tr>
<td>Cycloheximide (100 μg/ml)</td>
<td>20</td>
<td>Yes</td>
<td>2 x 10^{-7}</td>
</tr>
<tr>
<td>Cycloheximide + 2</td>
<td>2</td>
<td>No</td>
<td>5 x 10^{-7}</td>
</tr>
<tr>
<td>Actinomycin D (1 μg/ml)</td>
<td>37</td>
<td>Yes</td>
<td>3 x 10^{-8}</td>
</tr>
<tr>
<td>Actinomycin D + 2</td>
<td>2</td>
<td>No</td>
<td>4 x 10^{-8}</td>
</tr>
<tr>
<td>UV irradiation (15 min)</td>
<td>85 (10% necrosis)</td>
<td>Yes</td>
<td>—</td>
</tr>
<tr>
<td>UV irradiation (15 min) + 2</td>
<td>Necrosis</td>
<td>No</td>
<td>—</td>
</tr>
</tbody>
</table>

In each case, the initial starting concentration of cytotoxic drug was 0.1 μM and the initial starting concentration of enediyne 2 (2) was 0.1 mM. Incubation of cells under the various conditions to determine cytotoxicity was for 3 days at a starting concentration of 10^5 cells per ml. The IC_{50} molarity refers to the molarity of the cytotoxic drug and was measured using UTT staining. "Ladder" refers to the presence or absence of a nucleosomal ladder derived from DNA extracted from 10^5 MOLT-4 cells after the 4-h exposure to drugs or UV.

(−)-9 and (+)-9 readily undergo the Bergman rearrangement reaction in vitro (29, 30). Enediyne (−)-9, however, is 10^6-fold less cytotoxic and also has a significantly lower apoptotic index than (+)-9. The differential cytotoxicity of these stereoisomers suggests that cellular factors that can distinguish enantiomeric forms are mediating their biological effects.

Inhibition of Cytotoxicity and Apoptotic Morphology by Stable Enediynes. We performed competition experiments using enediyines of relatively low cytotoxicity such as compound 2. The experiments were designed to determine whether enediyines with low chemical reactivity could block the cytotoxic effects of enediyines with high chemical reactivity. MOLT-4 cells were preincubated at an initial concentration of 0.1 mM enediyne 2 for 1 h. The medium was then made 0.1 μM enediyne 1 followed by serial dilution to determine the IC_{50} value. These dilutions were compared to the cytotoxic drug alone at the equivalent initial concentration. The results (Table 2 and Fig. 1E) clearly demonstrate a reduction in the cytotoxicity of enediyne 1 when enediyne 2 is present. Similar experiments were performed in which dynemicin or calicheamicin was used to initiate apoptosis. In each case, the cytotoxicity was reduced by a factor of 10^2-10^6.

The reduction in cytotoxicity was also reflected in an absence of the apoptotic morphology of the cells (Fig. 1B). This was quantified by assigning an apoptotic index to the cell population (Table 2). In these assays, MOLT-4 cells (10^5 cells per ml) were preincubated with 0.1 mM enediyne 2 for 1 h followed by 0.1 μM enediyne 1, calicheamicin, or dynemicin for 4 h. In this time frame, the enediynes alone produce a classic apoptotic morphology in 20–40% of the cells. None of the cytotoxic enediyines produce an apoptotic cell morphology in combination with enediyne 2 (Fig. 1B).

To extend these observations, we coinubcated enediyne 2 with initiators of apoptosis that do not contain enediyne functional groups as well as with nonchemical initiators of apoptosis. In many cell lines (40, 41), including MOLT-4, actinomycin D and cycloheximide are powerful inducers of apoptosis. At concentrations of 1 and 100 μg/ml, respectively, apoptosis was observed in 30% of the cells after 4 h. In each case, no apoptosis was observed at this time interval when enediyne 2 was present at 0.1 mM (Table 2). Exposure of many cell lines to UV radiation frequently results in extensive apoptosis. Using 302-nm UV irradiation, apoptosis was observed in nearly all MOLT-4 cells. Addition of enediyne 2 immediately after the UV treatment appeared to subvert the apoptotic pathway into necrosis since 75% of the cells were fully lysed. Of the remaining intact cells, about half had condensed nuclei and half appeared unaffected. Although the cytotoxicity of these treatments is not reduced by the presence of enediyne 2, it is apparent from both the morphological appearance of the cells and from the absence of a nucleosomal ladder (Fig. 1E) that an apoptotic program is not the mechanism of cell death. Similar results were obtained using the stable enediyne 7 as an inhibitor of apoptosis.

DISCUSSION

Calicheamicin and dynemicin, as well as other naturally occurring enediyines (42, 43), are potent antitumor antibiotics that are thought to be cytotoxic due to radical generation occurring at the phosphate backbone of nuclear DNA. Calicheamicin in particular has received considerable attention because its plasmid DNA cleavage has been shown to be sequence-selective (34). If indeed the cytotoxicity of calicheamicin is due to DNA damage, our results demonstrate that this damage is not sufficient to commit MOLT-4 cells to a cell death pathway.

We have found that all of the characteristics of apoptotic morphology induced by a variety of initiators are prevented when MOLT-4 cells are exposed to stable synthetic enediyines. The increased viability of these cells when stable enediyines are coincubated with chemically unstable enediyines demonstrates that the stable enediyines are functional at low concentrations, in the range of 0.01 to 1 nM. The results suggest that these enediyne target sites control the ability of MOLT-4 cells to undergo apoptotic cell death.

Whereas the stable enediyines clearly control the morphology of cell death, they do not result in increased viability when cycloheximide or actinomycin D are used to initiate cell death. For UV irradiation, cell death clearly occurs by necrosis in the presence of stable enediyines, suggesting that necrosis may be an alternate program of cell death when apoptosis is blocked.

The enantiomeric specificity of enediyne cytotoxicity as well as the high cell specificity at subnanomolar concentrations is strongly suggestive of a protein-mediated mechanism of action. Candidate mechanisms of enediyne action would have to include classic receptor–ligand interactions, such as the steroid–hormone receptor interaction, although numerous other models are consistent with the results. Direct measurement of cellular uptake and ligand binding in cell extracts will be necessary to define these mediating events. The ability of the chemically stable enediyne 2 to prevent apoptosis in the absence of a dependence on RNA or protein synthesis suggests an interaction with a preexisting population of cellular factors that may regulate the mechanism of programmed cell death. We speculate that the apparent affinity of enediyne 2 for a key component of the apoptotic pathway enhances the ability of this pathway to prevent apoptosis. Enediyne 1, on the other hand, may have an affinity for the same component but acts to interfere with its function by generation of highly reactive free radicals. This hypothesis further suggests that this affinity may be a principal determinant in generating the rearrangement reaction and that the stable enediyines may be molecular mimics of biomolecules that serve to regulate the progression of cell death pathways in MOLT-4 cells. Further definition of the apparent agonist–antagonist relationship of stable and unsta-
nable enediynes will require the characterization of their target site interactions.

The thousandfold greater susceptibility of the human leukemic MOLT-4 line compared to all other cell lines tested may be a reflection of the propensity of human leukemic cells to undergo apoptotic death. The target of the synthetic enediynes in MOLT-4 cells may help to define cellular junctions through which apoptotic cell death programs must pass. Conceivably, the synthetic enediynes could act by a mechanism from which a generalized strategy for the control of cell death programs in various cell types might emerge.

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