Antiproliferative Effects of Enediynes on AIDS-derived Kaposi’s Sarcoma Cells

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Abstract

We have investigated the antiproliferative and cytotoxic effects of selected enediynes against three Kaposi’s sarcoma (KS) cell lines. The enediynes tested were found to be very potent in inhibiting the growth of KS cells. Treatment with concentrations of $10^{-10}$ M or less were capable of producing 50% inhibition of growth. Furthermore, treatment of KS cells with enediynes induced apoptosis in up to 80% of the cells. This unique class of antitumor agents has therapeutic potential for the treatment of KS.

Introduction

KS is the most common HIV-associated malignancy (1). KS eventually develops in approximately 20% of patients with AIDS. No fully satisfactory therapeutic modalities have emerged for treating this condition. A number of agents, such as α-interferon, Adriamycin, etoposide, vinblastine, and radiotherapy, have met with some success; however, all have displayed unfavorable side effects and limitations in tumor response (2-5).

The recently described enediynes (reviewed in Refs. 6 and 7) are promising antitumor molecules with potent biological activity against malignant cells. These compounds directly damage the phosphodiester backbone of DNA in a target cell, resulting in DNA cleavage (8, 9). Enediynes have also been demonstrated to trigger programmed cell death (apoptosis; Refs. 10 and 11). The objective of this study was to examine the action of enediynes on KS sarcoma cell cultures and compare the susceptibility of KS cells to that of normal mesenchymal cells and lymphoblastoid cells lines.

Materials and Methods

Cell Lines and Cultures. KSC1 and KSC3 were derived from explants of cutaneous biopsies of KS lesions from HIV-1-infected individuals with typical AIDS-associated Kaposi’s sarcoma. KSC2 was derived from an explant of a cutaneous biopsy of a KS lesion from a HIV-1-negative homosexual male. Explants were selected and maintained in high glucose Dulbecco’s modified medium, devoid of L-valine and containing D-valine, to inhibit fibroblast growth.

HUVeC were obtained at passage 2 from Clonetics (San Diego, CA). The smooth muscle cell line SKLSMS-1, the fibroblast cell line CCD344a, and the T-lymphoblastoid cell line Jurkat were obtained from the American Tissue Culture Collection (Rockville, MD). The T-lymphoblastoid cell lines, WE11/10, was obtained from NIH AIDS research and reference reagent program; the SupT1 line was generously provided by Dr. D. Littman (University of California at San Francisco); and the CEM line was donated by Dr. D. Carson (University of California at San Diego). For drug testing, the cells were passaged using an enzymatic preparation capable of releasing the cells from the substratum (Passage-ease; Vectec, Schenectady, NY) and grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Compounds. Enediynes 5 and 19 and calicheamicin α1 were solubilized in ethanol (Refs. 6 and 11; see Fig. 1 for structures). Stocks ($10^{-3}$ M) were maintained at −70°C until used. The compounds were serially diluted in the culture medium before being added to the cells.

[3H]Thymidine Uptake Assay. Cells (KS, fibroblasts, smooth muscle cells, HUVEC, and T-lymphoblastoid cell lines) were plated in a 96-well plate at a density of 3000–5000 cells/well. [3H]Thymidine (specific activity, 25 mCi/ml) was added to a final concentration of 0.5 μCi/well (only 0.2 μCi/ml for the HUVeC). After 2 days, the cells were fixed once with medium M199 (Gibco/BRL), and 200 μl of a solution of trypsin-EDTA (Gibco/BRL) was added to release the adherent cells from the matrix. The cells were harvested using a Skatron cell harvester onto glass wool filters, and the radioactive signal was determined by placing the filters in 3 ml of Ready-Safe cocktail (Beckman, Fullerton, CA, followed by counting in the tritium channel of a scintillation counter (ICN micromedic; ICN, Huntsville, AL). The resulting counts for each experimental condition were the means of triplicate assays. Results are expressed as the percentage of the control condition, where no drug was added.

Detection of Apoptosis-associated Chromatin Degradation by Flow Cytometry. Cells ($\approx 2 \times 10^5$) were washed in phosphate-buffered saline and resuspended in 30% ethanol and kept at 4°C. The cells were stained with PI as described previously (12, 13) with slight modifications. Briefly, the cells were centrifuged and resuspended in phosphate-buffered saline containing 0.1 mM EDTA(Na)$_2$, RNase A at 50 μg/ml (50 units/mg), and PI (50 μg/ml). Cell cycle analysis was carried out using a EPICS V fluorescence-activated cytometer and analyzed using a cell cycle analysis doublet discrimination protocol. PI was excited using a 488-nm line of an argon laser and detected with a 620–700-nm long-pass filter.

Results and Discussion

Enediynes Inhibit KS Cell Growth in Vitro. Three KS cell lines (KS2 was obtained from a HIV-negative individual) were used to assess the action of enediyne 5, enediyne 19, and calicheamicin α1. Cells were treated with concentrations ranging from $10^{-6}$ to $10^{-16}$ M. Enediynes were serially diluted in the culture medium before being added to the cells. All enediynes were potent inhibitors of KS cell growth, as judged by [3H]thymidine incorporation (Fig. 2: KS1, KS2, and KS3). Calicheamicin α1 was the most potent (IC$_{50}$, $10^{-15}$ M); enediyne 5 had an IC$_{50}$ of $10^{-11}$ M, and enediyne 19, the least potent, had an IC$_{50}$ of $10^{-9}$ M. Growth inhibition was evident within 24 h. Most cells had lifted from the substratum within 48 to 72 h at concentrations over $10^{-6}$ M. A culture of KS cells (KS1) treated for 48 h with $10^{-10}$ M of calicheamicin α1 (Fig. 3B) is shown with a control culture for comparison (Fig. 3A). The treated KS cells displayed altered morphology, initially rounding up and then detaching from the substratum. The characteristic morphology of the cells was suggestive of apoptosis in that apoptotic bodies were present and membrane integrity was preserved, as assessed by vital dye exclusion (trypan blue; Refs. 14 and 15).

Enediyne compounds directly damage DNA by interacting with the phosphodiester backbone of the DNA (nucleophilic attack) and indi...
Fig. 1. Chemical structures of calicheamicin \( \theta_1 \), enediyne 5, and enediyne 19. The enediyne core is the functional group capable of benzogenid diradical generation (arenyl or indenyl radical) that reacts with cellular DNA, resulting in the scission of the phosphodiester backbone.

Directly by triggering apoptosis and activating endogenous endonuclease activity which generates DNA strand breaks at the time of cell replication (S phase). The end result is DNA digestion and cell death by apoptosis (16).

In the case of enediynes 5 and 19 at concentrations of less than \( 10^{-12} \) M, the uptake of thymidine was greater than with the untreated controls. This might be attributable to a DNA repair mechanism increasing the amount of \([\text{H}]\text{thymidine incorporation into the DNA of the treated cells over and above the normal replication. The inhibition of thymidine uptake by calicheamicin } \theta_1 \text{ exceeds the inhibition obtained by the two other enediynes by about 50% over the dilution range. Although the mechanism responsible for the greater potency of calicheamicin } \theta_1 \text{ is unknown at this time, it is possible that calicheamicin } \theta_1 \text{ delivers a lethal number of DNA cuts at much lower concentrations, or alternatively may preferentially cleave DNA, leaving blunt-end double-stranded cuts which are not easily repairable by the mammalian DNA repair machinery (17–19).}

Previous experiments using the 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyl-tetrazolium bromide dye assay (a measure of viable cells; Ref. 20) also demonstrated that extremely high IC\(_{50}\)'s (exceeding \( 10^{-11} \) M) were obtained for this agent (results not shown). This assay also corroborated the IC\(_{50}\) obtained with the thymidine uptake assay. At greater dilutions, a desorption effect may occur, making the interpretation of these results difficult. Every effort was made to avoid this potential problem by solubilizing the compounds in ethyl alcohol and either serially diluting the compounds in culture medium or ethyl alcohol, changing tips for every dilution, and then by directly adding the diluted compounds to the wells containing the cells. The tritiated thymidine uptake results obtained from serial dilution in culture medium or ethyl alcohol were similar.

Fig. 2. Growth inhibitory effect of enediynes on KS cells. Growth in the presence of enediyne 5, enediyne 19 and calicheamicin \( \theta_1 \) of three KS cell lines. The results are expressed as a percentage of control (no drug added). The cells were cultured in the continuous presence of the drug (0.5 \( \mu\text{Ci/well} \) of \([\text{H}]\text{thymidine}) and harvested 48 h after the addition of the drug. Each condition was tested in triplicate.
Enediyne 5 was not found to be cell specific in that all cell types showed growth inhibition, although different cell types exhibited different susceptibilities to the compound. The IC50 of enediyne 5 was found to be two logs less than enediyne 19, and the IC50 of calicheamicin 0j was greater than 10^{-12} m for all the cell types tested.

**Enediyne Induced Programmed Cell Death (Apoptosis) in Treated Cells.** Treatment with enediynes has been shown to induce apoptosis in other cell types (10, 11). The DNA content of KS cells

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**Table 1** IC50 of enediyne 5 on selected cell types

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Enediyne 5 (M)</th>
</tr>
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<tbody>
<tr>
<td>KS 1</td>
<td>10^{-11}</td>
</tr>
<tr>
<td>KS 2</td>
<td>10^{-11}</td>
</tr>
<tr>
<td>KS 3</td>
<td>10^{-10}</td>
</tr>
<tr>
<td>SKLMS-1</td>
<td>10^{-12}</td>
</tr>
<tr>
<td>CCD344u</td>
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<td>HUVEC</td>
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</tr>
<tr>
<td>Jurkat</td>
<td>10^{-8}</td>
</tr>
<tr>
<td>CEM</td>
<td>10^{-6}</td>
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</tbody>
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**Enediyne Inhibited the Growth of Normal Mesenchymal Cells and Transformed Lymphocytes.** We also evaluated the specificity of inhibition of enediynes on a number of normal mesenchymal cell cultures and T-cell lines. We chose endothelial cells, fibroblasts, and smooth muscle cells because the cell of origin for Kaposi sarcoma remains controversial. Vascular endothelial and smooth muscle cell origins are both favored, and fibroblast morphology is reminiscent of the so-called spindle cells seen in KS lesions (21, 22). We tested the T-cell lines to further evaluate the specificity of the enediynes, especially because these cells have been reported to be exquisitively sensitive to this class of compound (6). Table 1 shows the IC50 of enediyne 5 when tested (range from 10^{-5} to 10^{-12} M) against a number of cells of mesenchymal origin and T-cell lines. The action of enediyne 5 was not found to be cell specific in that all cell types showed growth inhibition, although different cell types exhibited different susceptibilities to the compound. The IC50 of enediyne 19 was found to be two logs less than enediyne 5, and the IC50 of calicheamicin 0j was greater than 10^{-12} m for all the cell types tested.
treated with enediyne was assayed by PI staining and flow cytometric cell cycle analysis to determine if treated cells displayed a pattern of reduced DNA content (less than in G1 of the cell cycle), characteristic of cells undergoing apoptosis. Representative flow cytometric profiles of KS cells which have been treated with calicheamicin G1 (10^{-10} M) and control untreated cells are shown in Fig. 4. Fig. 4A represents the untreated cells; Fig. 4B depicts KS cells treated with calicheamicin G1 that, however, remain attached to the substrate; Fig. 4C represents the cells that have been released from the matrix in this same culture. A large proportion (~75% at 48 h after treatment in three independent experiments) of cells are detected in the pre-G1 region, as compared to the profile of untreated cells, consistent with the induction of apoptosis in the treated cell culture population. Similar results were obtained for enediyne 5, although higher concentrations were required to generate the same percentage of apoptotic cells present in the treated cultures. The enediyne compounds also directly contribute to DNA fragmentation, and the reduced DNA content profiles obtained may reflect the cleaved DNA.

Potential Therapeutic Role of Eneidyens in the Treatment of AIDS-associated KS. Eneidyens are intriguing antitumor agents due to the potent cytotoxic effects of these agents. Calicheamicin G1 is one of the most cytotoxic compounds known. At a concentration of 10^{-15} M (the IC_{50}, it corresponds to approximately 20 molecules/cell (in a volume of 200 μl with 5000 cells/well). ImmunoToxins containing different ribosome-inactivating proteins coupled to antibodies typically have IC_{50} in the 10^{-15} M range (23). Other antitumor agents generate apoptosis in treated cells, but resistance to these agents has been reported (24). Eneidyens might also be used in combination with other agents inducing apoptosis, such as retinoids, in the treatment of KS (25). The lack of specificity for tumor cells may limit the usefulness of unmodified enediyens as systemic chemotherapeutic agents for the treatment of KS. However, the panel tested represents only a small fraction of available compounds. Preliminary animal studies with enediyen 1 in athymic nude mice (10 mg/kg) have demonstrated mild toxicity, with moderate follicular reactions in the spleen and little effect on other organs (26). In addition, the potential exists for targeting these cytotoxic agents to KS cells or other tumor cell targets by conjugating enediyens to antibody (Fab fragments) or oligonucleotides without interfering with the enediyen reactive core, and this is an area of active investigation. Selective delivery of agents to lesions could also be achieved by topical application or intralesional injection, and a number of patients afflicted with painful, disfiguring KS lesions might still benefit from treatment with locally administered enediyens. Since enediyens induce apoptotic death, permitting clearance of dead cells by the normal phagocytic pathways, these agents might be expected to induce minimal inflammation, compared to agents producing necrotic tumor cell death (27). The use of dimethyl sulfoxide as a solvent, which might enhance percutaneous delivery, also enhanced the cytotoxic potency of these compounds (not shown), probably because of increased entry into target cells. This study warrants the investigation of enediyens as antitumor agents in animal models of carcinoma and other malignancies (28). If the results of such studies are encouraging, local administration (topical or intralesional) of enediyens should be considered for human clinical evaluation.

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References
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