Preclinical Evaluation of Illudins as Anticancer Agents

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ABSTRACT

Illudins are low molecular weight natural products which were previously evaluated as anticancer drugs using rodent tumor models. In the present studies, we used in vitro cultures of human cancer cells to reevaluate their potential as anticancer agents. Using continuous exposure, Illudins S and M were cytotoxic to human leukemia cells at concentrations of 6–100 nM, but dihydroidillumin M was 3 orders of magnitude less toxic, thus identifying a ketone site as a structural feature critical for cytotoxicity. Cytokinetic studies showed that illudin S caused a complete block at the G1-S phase interface of the cell cycle. Kinetics of inhibition of radiolabeled thymidine, uridine, and leucine incorporation suggested a primary effect on DNA synthesis. In colony and liquid culture assays, cell killing was time dependent but near maximal with a 2-h exposure. Myeloid and T-lymphocyte leukemia cells were most sensitive (50% inhibitory concentration, 6–11 nM), but B-cell leukemia/lymphoma, melanoma, and ovarian carcinoma cells were at least 10 times more resistant. Bone marrow granulocyte/macrophage progenitors showed intermediate sensitivity. Illudin S was equally effective against CEM T-lymphocyte leukemia cells expressing the multidrug resistance phenotype associated with M, 180,000 glycoprotein and the parental cell line, CEM cells resistant to doxorubicin, epipodophyllotoxins, and 1-P-D-arabinofuranosycytidine showed only a 2-fold increased resistance to illudin S. Illudins are novel and potent cytotoxins which may be preferentially active on human myeloid and T-cell leukemias, including cells resistant to more conventional chemotherapeutic agents. The present studies illustrate the breadth of information which can be obtained on a new agent using present in vitro screening procedures and human cells.

INTRODUCTION

During the past 3 decades, many new, effective chemotherapeutic agents have been identified and introduced into clinical practice. Although some are active against nonhemopoietic tumors, such as testicular and ovarian cancers, their greatest impact has been on treatment of hemopoietic malignancies. A few were rationally synthesized, but most were selected by empirically screening large numbers of compounds in rodent tumor models (1–4). Despite successful application to treatment of leukemias and lymphomas, drugs with potentially curative impact on common solid tumors have not been identified.

Improved culture techniques have revived interest (5, 6) in in vitro screening using primary human tumors and cell lines (7, 8). As reviewed several months ago (17), in vitro screens have advantages over rodent tumor models in speed, accuracy, and quantities of new drugs required. The availability of multiple tumor lines and primary culture techniques for human tumors makes it possible to test agents against specific tumor types and may permit identification of compounds with antitumor efficacy restricted to certain types of human cancer (5–8). Recent studies suggest that in vitro testing using primary human tumors identifies a different set of chemicals than rodent tumor screens (6). Using in vitro techniques we have identified illudins as potential chemotherapeutic agents.

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Illudins are novel natural products isolated from certain mushrooms (9). They were previously evaluated by the NCI in a variety of rodent tumor models and were found to have a narrow therapeutic index. The present studies show that illudins are potent in vitro antiproliferative agents effective against human tumor cells resistant to known anticancer drugs and may be preferentially active against leukemias. The use of a variety of in vitro culture methods and specialized drug resistant human cell lines illustrates the broad range of preclinical data which can be obtained using available in vitro systems.

MATERIALS AND METHODS

Preparation of Illudin Compounds. Illudins S and M were isolated from cultures of Clitocybe illudins as described previously (9). Dihydroidillumin M was prepared by reduction of the parent compounds with sodium borohydride (10). The structures of the compounds are shown in Fig. 1. All compounds were stable in distilled water at room temperature or 4°C and were diluted in medium just prior to addition to cultures. Cell Lines. Myeloid leukemia cell lines HL60 and KG-1, B-cell derived leukemia/lymphoma lines Namalva and 8392, and T-cell acute lymphoblastic leukemia lines 8402 and CEM were maintained in RPMI 1640 (11). Ovarian carcinoma line 547 was established and maintained in 10% FBS in a similar manner. The AHH-1 B-cell lymphoma cell line was obtained from Gentest, Woburn, MA. Tumor cell colony forming and liquid cultures were performed as described previously (12). CCRF-CEM human lymphoblasts and their drug resistant variants were maintained as described (13). The growth inhibiting effects of illudins on these variant lines were also detected as described previously (14). Human bone marrow was obtained in heparin from the posterior iliac crest of normal volunteers using procedures approved by the Committee on Human Subjects, University of California, San Diego. Granulocyte/macrophage colony forming units or liquid cultures were grown using placenta conditioned medium as a source of colony stimulating factor (15).

Assessment of Illudin Cytotoxicity. To assess toxic effects of illudins, various concentrations were added to cultures of HL60 cells, and cell growth was monitored by daily cell counts. To assess cytotoxicity, cells were incubated in media containing illudins for 1–24 h, washed 3 times, and plated in semisolid, colony forming assays (12, 16). As an alternative, cells were plated in liquid culture, pulsed with [3H]thymidine for 4 h, and harvested as described previously (12, 15). To assess effects on DNA or RNA synthesis, HL60 cells were incubated with 1.0 to 1000 ng/ml (3.8 to 3800 nm) illudin S for 2 h and cultured for 14–16 h, and tritiated aliquots were pulsed with triitated thymidine (2 μCi/ml) or uridine (2 μCi/ml) (Amersham, Arlington Heights, IL) for 4 h. Protein synthesis inhibition was determined in a similar manner by incubating illudin treated cells with [3H]leucine (2 μCi/ml) for 4 h in leucine free medium (16). Kinetic effects were examined by incubating HL60 cells for 1 to 24 h in 38 nmol/l of illudin S and determining triitated thymidine, uridine, and leucine incorporation.

Cell cycle status of illudin-treated HL60 cells was determined after incubating cells with 100 and 1000 ng/ml (380 to 3800 nm) of illudin S for 2–24 h and staining the cells for DNA content using propidium
PRECLINICAL EVALUATION OF ILLUDINS

Fig. 1. Chemical structures of illudin S, illudin M, and dihydroilludin M.

Fig. 2. Effect of illudin S (• •), illudin M (• •), and dihydroilludin M (* •) on HL60 cells as measured by a 48-h growth inhibition assay. The standard error for each data point was 6% or less, N = 3. One ng/ml is equivalent to 3.8 nmol/liter.

Fig. 3. Colony formation of HL60 cells after exposure to illudin S for 1 (•), 2 (A), 4 (•), and 24 (x) h. The standard error for each data point was 8% or less, N = 3. One ng/ml is equivalent to 3.8 nmol/liter.

Fig. 4. Macromolecule synthesis as measured by radiolabeled thymidine (A), uridine (•), or leucine (•) incorporation in HL60 cells after a 2-h exposure to illudin S. The standard error was 8% or less for thymidine, 10% or less for uridine, and 12% or less for leucine, N = 3. One ng/ml is equivalent to 3.8 nmol/liter.

iodine (15). Cell cycle status was determined by computer analysis of DNA histograms using an Ortho 50H cytofluorograph (15). Statistical analysis of experimental mean values was performed using the t test for unpaired observations.

RESULTS

The effects of illudins S and M and of dihydroilludin M were initially assessed on HL60 cells (Fig. 2). Various concentrations of each compound were added at initiation of the culture, and cell counts were assessed for 5 days. Results represent the means of 3–4 experiments. At concentrations greater than 15 nM, illudin S or M (Fig. 2) completely inhibited HL60 cell growth. By comparison, dihydroilludin M (Fig. 2) was 10,000 times less toxic than either of the parent compounds. These screening studies showed that illudins were highly toxic to hemopoietic cells. Differences between illudin and dihydroilludin toxicity indicate the ketone site is critical to the molecule's toxicity.

To assess cytotoxicity, HL60 cells were exposed to various concentrations of illudin S, washed, and plated in a colony forming assay (Fig. 3). Results shown represent the means of 3 experiments. Illudin cytotoxicity was both time and concentration dependent. Increasing the exposure time resulted in a further increase in cell death and the dose-response curves remained log-linear. The IC50 for a 2-h exposure to illudin S was estimated at 2.9 ng/ml (11 nM). Thus, illudin S was cytotoxic to human leukemia cells, and linear dose-response relationships suggested activity against cells in all phases of the cell cycle.

In similar studies with both colony forming assays and [3H]thymidine incorporation, illudin S toxicity was compared in a variety of malignant human cells and normal marrow myeloid...
preliminary studies of this novel compound suggest that it inhibits, or prevents entry into, DNA synthesis.

The discovery of new anticancer agents requires identification of novel structures non-cross-resistant with known drugs. To determine whether resistance to illudins occurred in common with other anticancer drugs, toxicities of illudin S for malignant blood cells expressing drug resistant phenotypes were assessed. When tested against CEM leukemia cells with the multidrug resistant phenotype associated with gp180 (13), illudin S was 2-fold more toxic to the multiple drug resistant than the parent cell line (Table 2). Illudin S was slightly less effective against an "atypical" multidrug resistant cell line, CEM/VM-1. \(^5\) This cell line is resistant to the epidophyllotoxin teniposide and does not express gp180 or its mRNA. \(^6\) The VLB100 variant cells were significantly more sensitive (\(P < 0.01\)) and the VM-1 cell line was significantly more resistant (\(P < 0.05\)) than parent CEM cells. Illudin S showed similar effects against another gp180 positive line, CEM/DOX, and a non-gp180 resistant cell line, CEM/ara C. The variations in illudin S IC\(_{50}\)s between the parent CEM cell line and the various drug-resistant daughter lines were always less than 0.3 log. Thus, in malignant cells, the novel structure of illudin conferred relative non-cross-resistance to known chemotherapeutic agents.

**DISCUSSION**

Recent advances in culture of malignant human cell lines and primary tumors have revived interest in *in vitro* drug screening. The advantages of *in vitro* drug screens have been reviewed (17, 18) and include low cost, reproducibility, rapid assay performance, and requirements for small amounts of new drugs. All but two of the most important antitumor agents originating from plants were discovered by *in vitro* cytotoxicity tests (19). Using tumor colony forming assays, a recent multicenter trial screened new and established chemotherapeutic agents and identified potentially active drugs previously rejected using murine leukemia models (6). In the present studies, we examined the *in vitro* activity of illudins, compounds previously tested in murine tumor screens, against human tumor cell lines.

Our studies of illudins illustrate the broad data base which

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**Table 2. Illudin S IC\(_{50}\) for parent CCRF-CEM cell line and various drug resistant variant cell lines**

The 48-h IC\(_{50}\)s were assessed using cell counts in liquid culture (14). Results are the means ± SE of 2–7 experiments.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC(_{50}) growth inhibition assay (nmol/liter)</th>
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<tbody>
<tr>
<td>CCRF-CEM</td>
<td>8.3 ± -2.6 (5)*</td>
</tr>
<tr>
<td>CEM-VMB*</td>
<td>3.7 ± -0.7 (7)</td>
</tr>
<tr>
<td>CEM/DOX*</td>
<td>16.2 ± -6.4 (7)</td>
</tr>
<tr>
<td>CEM/ara C</td>
<td>14 (2)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, number of studies.

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can now be obtained using human cell lines. We demonstrated the extraordinary toxicity of the compounds by dose-response testing. On a molar basis, illudin S is as toxic to CEM, 8392, and HL60 cells as the plant toxin ricin. Multiple cell lines span, but increasing the dosage of illudin S resulted in a higher mortality (Table 3). Further investigation using other in vivo systems will be required to confirm these findings.

The ability of in vitro screens to survey large numbers of compounds across broad concentration ranges also provided insight into structure-function relationships and mechanisms of illudin toxicity. Linear dose-response relationships and time dependent cell killing suggested that the actions of the compound were not limited to one phase of the cell cycle. Testing of dihydroilludin M identified a critical active site for further study; the compound inhibits DNA synthesis. Finally, testing against well characterized, drug resistant lines identified illudins as novel structures which do not share cross-resistance with known anticancer drugs.

We are currently studying illudin inhibition of DNA synthesis in greater detail and intend to study illudin S activity in leukemia xenograft models. Because of the potency of illudin, we are also synthesizing analogues suitable for conjugation to monoclonal antibodies as immunotoxins.

REFERENCES


*PRECLINICAL EVALUATION OF ILLUDINS*

Table 3 Screening data from the Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD

<table>
<thead>
<tr>
<th>Dosage (mg/kg)</th>
<th>Survival (no. of survivors/total)</th>
<th>% of treated vs. control</th>
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<tbody>
<tr>
<td>0.30</td>
<td>Death pattern not given</td>
<td>375</td>
</tr>
<tr>
<td>0.15</td>
<td>Death pattern not given</td>
<td>375</td>
</tr>
<tr>
<td>0.08</td>
<td>Death pattern not given</td>
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Friend virus leukemia in mice, dosage of illudin S given daily for 11 days

<table>
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<td>0.120</td>
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<td>*</td>
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<tr>
<td>0.060</td>
<td>0/10</td>
<td>*</td>
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<tr>
<td>0.030</td>
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<tr>
<td>0.007</td>
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P388 leukemia in mice, dosage of illudin S given daily for 10 days

<table>
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<th>Dosage</th>
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<tbody>
<tr>
<td>1.60</td>
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<tr>
<td>0.20</td>
<td>6/6</td>
<td>135</td>
</tr>
</tbody>
</table>

* Data not given.
* Survival system for P388 leukemia considered successful when % of treated versus control greater than 125.

7 J. Leonard, and R. Taetle, unpublished observations.
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