Antitumor Activity of Magainin Analogues against Human Lung Cancer Cell Lines

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ABSTRACT

Magainin 1 and magainin 2, originally isolated from African clawed frog Xenopus laevis skin, inhibit the growth of bacteria and fungi. Synthetic magainin A (MAG A) and magainin G (MAG G) are more potent against bacteria and protozoa. In order to determine the antitumor activity of these analogues, we have tested these two analogues against six small cell lung cancer (SCLC) cell lines NCI-H82, NCI-H526, NCI-H678, NCI-H735, NCI-H841, and NCI-H889, which were known to differ by more than 10-fold in their sensitivity to different chemotherapeutic agents, and four normal human fibroblast cell lines. Semi-automated 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays of six SCLC cell lines revealed average concentrations producing 50% inhibition (IC50) values of 2.6 µM (range, 0.49-9.30 µM) for cisplatin, 2.5 µM (range, 0.39-6.00 µM) for etoposide, and 138.8 nM (range, 55.0-450.0 nM) for doxorubicin. The average IC50 of MAG A was 8.64 µM (range, 6.23-11.7 µM) and that of MAG G was 8.82 µM (range, 4.44-12.5 µM) against the SCLC cell lines. Despite a 10-fold difference in sensitivity to standard chemotherapeutic agents, the IC50s of MAG A and MAG G differ by less than 3-fold. The average IC50 against four normal human fibroblast cell lines was 21.1 µM (range, 12.7-25.6 µM) for MAG A and 29.2 µM (range, 21.3-34.8 µM) for MAG G. Combined exposure to the IC50 concentration of MAG A or MAG G plus IC50 of etoposide or cisplatin decreased the percentage of surviving SCLC cells to 29.0% (range, 26.1-31.7%). MAG A or MAG G had an additive effect when used with standard chemotherapeutic agents. These data suggest that MAG A and MAG G have in vitro antitumor activity against SCLC cell lines.

INTRODUCTION

Patients with SCLC1 respond to standard combinations of chemotherapeutic agents with a partial or complete response in approximately two-thirds of the patients (1). However, <15% of SCLC patients survive >2 years (2-4). De novo or acquired resistance to standard chemotherapeutic agents is recognized as a cause for failure of cancer chemotherapy (5). Thus, the development of new chemotherapeutic agents which have a different mechanism of action than currently available drugs are needed to improve the therapy for patients with SCLC (6).

The frog skin is very rich in biologically active peptides. Many of them bind to specific receptors and mediate biological responses in mammalian tissues. These peptides include angiotensin, bombesin, bradykinins, caeruleins, dermorphins, sauvagine, spasmolysins, tachykinsins, thyrotropin-releasing peptide, and xenopins (7). It was only recently that magainins (8, 9), pGLA, and xenopin precursor fragment (10) were found to have antimicrobial activity. The absence of local inflammation during the healing of surgical wounds in the African clawed frog Xenopus laevis was observed and attributed to locally acting antimicrobial peptides (8). Notably, magainins exhibited in vitro antibiotic activity on both Gram-positive and Gram-negative strains of bacteria, fungi, and protozoa (9, 11). Although the mechanism of magainins has not been clearly elucidated, the amphiphilic nature of these peptides and the close correlation of enhanced α-helical conformation to increased antibacterial activity suggest that the peptides act on the phospholipid of the plasma membrane to elicit antimicrobial activity (12, 13).

Based on the increase of the α-helical potential, numerous synthetic analogues have been synthesized and are available for in vitro testing. Magainin A and magainin G were the most promising peptides because they have one to two orders of magnitude more potency than the naturally occurring magainins against bacteria (12). Juretic et al. (14) reported that these magainin analogues, magainin A and magainin G, also had greater activity against bacteria than naturally occurring magainin 1 and magainin 2. Although magainin A has detectable but low hemolytic activity, magainin G, like magainin 1 and magainin 2, is void of such activity. Thus, magainin G is likely to have the most favorable therapeutic index (15). We therefore examined the antitumor activity of magainin A and magainin G against human small cell lung cancer cell lines using a semiautomated tetrazolium dye (MTT) assay.

MATERIALS AND METHODS

Cell Lines. Six human small cell lung cancer cell lines and four normal human fibroblast cell lines were tested for their sensitivity to different chemotherapeutic agents. All SCLC cell lines were established in the NCI-Navy Medical Oncology Branch laboratory from patients with histologically proven small cell lung cancer (16). The SCLC cell lines from previously treated and untreated patients were grown in RPMI-1640 supplemented with HITES (17) (medium for NCI-H526, NCI-H678, NCI-H735, NCI-H841, NCI-H889 cell lines) or RPMI-1640 medium (for NCI-H82 cell line) supplemented with 5% heat-inactivated FBS, 100 units/ml penicillin G sodium, and 100 µg/ml streptomycin sulfate. NCI-H889 and NCI-H841 cell lines were also able to grow in HITES serum-free medium. The cells were grown in an incubator at 37°C in an atmosphere of 5% CO2 and 95% air. The administration of chemotherapy to the patients prior to obtaining tumor tissue for establishment of the cell lines and number of cells per well used in the MTT assays are listed in Table 1.

Four normal human fibroblast cell lines were purchased from American Type Culture Collection (Rockville, MD). CCD-27Sk was tested in passage 15, Fe Sin and CCD-39Sk were tested in passage 9, and CCD-43Sk was tested in passage 10. CCD-27Sk was incubated in Dulbecco’s essential medium supplemented with 10% FBS, 100 units/ml penicillin G sodium, and 100 µg/ml streptomycin sulfate. The other cell lines were maintained in Eagle’s essential medium supple-
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Drugs and Chemicals. Magainin A (Me, 2450; formula weight, 3020) and magainin G (Me, 2473; formula weight, 3043) were synthesized by the standard Merrifield solid-phase method with benzhydrylamine resin and coupling procedures using symmetric anhydrides or active esters of t-butyloxycarbonyl amino acids as described previously (12). All other drugs were obtained from commercial sources. DDP and VP-16 were purchased from Bristol Laboratories, Inc. (Syracuse, NY), and DOX was purchased from Adria Laboratories, Inc. (Columbus, OH). DOX was dissolved in distilled water and diluted with phosphate-buffered saline; the other drugs were dissolved and diluted with phosphate-buffered saline immediately before use. MTT and dimethyl sulfoxide were purchased from Sigma Chemical Co. (St. Louis, MO). Media and supplements for cell culture were obtained from Gibco (Grand Island, NY).

MTT Assay. Single-cell suspensions were prepared by mechanical disaggregation of SCLC cell lines. For normal fibroblast cell lines, single-cell suspensions were prepared by treatment with trypsin-EDTA solution. The MTT assay was performed as previously reported (18, 19). Briefly, after the cells were counted with a hemacytometer, they were incubated in 180 µl medium for 1 day using 96-well multilouples (Costar 3596, Cambridge, MA) prior to the drug addition. Seedings densities of the cells were determined for each cell line, ensuring that cultures did not become confluent before conducting the assay and absorbance reached 0.3-0.6. Then, 20 µl of a 10-fold drug solution was added to each well. Following a 4-day incubation, 100 µM of MTT was added to each well and incubated for 4 h. The plates were then centrifuged at 1000 x g for 5 min. The supernatant was removed by aspiration, and 150 µl of 100% dimethyl sulfoxide was added to each well to resolublize the MTT formazan crystal. The spectrophotometric absorbance at 540 nm was determined using a scanning multiwell spectrophotometer (Biotek Instruments Inc., Burlington, VT). Dose-response curves were plotted for all drugs with the IC50 for each cell line determined graphically as the dose of drug causing a 50% reduction in absorbance compared to control values. The combination effect of magainin analogues with DDP or VP-16 were evaluated in the NCI-H841 (2). However, NCI-H841 was highly sensitive to VP-16, and therefore, determination of an IC50 was not achievable at the concentrations of VP-16 tested. Although NCI-H889 was reported to be sensitive to DOX and VP-16 in the previous report (19), this cell line showed resistance to DOX and VP-16 in the present study.

Magainin A and magainin G inhibited the growth of SCLC cell lines in a dose-dependent fashion (Fig. 1, a and b). The average IC50 of magainin A and magainin G against 6 SCLC cell lines was 8.64 (range, 6.23-11.7 µM) and 8.82 µM (range, 4.44-12.5 µM), respectively. Despite the wide variation of IC50 values for DDP, VP-16, and DOX, the IC50 of the magainin analogues varied <3-fold. NCI-H889 and NCI-H841 were sensitive to the magainin analogues but relatively resistant to DOX, DDP, and/or VP-16 (Table 2). These data suggest that magainin A and magainin G have similar antitumor activity against chemotherapy-sensitive and -resistant SCLC cells (Fig. 2).

The phospholipids and peptides present in fetal bovine serum may interact with magainins, thereby reducing the amount of free magainin in the media. In order to determine whether fetal bovine serum in the media can cause reduction of antitumor activity, magainin analogues were tested in both serum-free and serum-containing medium. The IC50 of magainin A against NCI-H841 in serum-free medium was 9.11 compared to 9.70 µM in HITES medium containing 5% fetal bovine serum using the cell lines resistant to chemotherapeutic agents. The IC50 of magainin G against NCI-H841 in serum-free medium was 9.11 compared to 9.70 µM in HITES medium containing 5% fetal bovine serum using the cell lines resistant to chemotherapeutic agents. The IC50 of magainin A against NCI-H841 in serum-free medium was 9.11 compared to 9.70 µM in HITES medium containing 5% fetal bovine serum using the cell lines resistant to chemotherapeutic agents.

RESULTS

Anticancer Activity of Magainin Analogues and the Other Drugs against Human Small Cell Lung Cancer Cell Lines. The average absorbance observed for all wells with no added drug or peptide was 0.51 ± 0.2 (mean ± SD). Antitumor activity was determined for 5 chemotherapeutic agents at the following ranges of concentration: doxorubicin, 1–500 nM; cisplatin, 0.1–100 µM; VP-16, 0.1–100 µM; magainin A, 0.83–66.2 µM (2.5–200 µg/ml); magainin G, 0.82–65.7 µM (2.5–200 µg/ml). These concentration ranges gave 95–99% or greater reduction in the production of formazan product for 6 cell lines, allowing the determination of an IC50 for each drug with each cell line (Table 2).

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Fig. 1. a, cell survival curves measured by the MTT assay for magainin A against six different small cell lung cancer cell lines. The data represent the average for 3 experiments performed in 8 replicates. b, cell survival curves for magainin G. The data represent the average for 8 replicates. c, cell survival curves of normal fibroblast cell lines against magainin A. d, cell survival curves of normal fibroblast cell lines against magainin G. The data for CCD-27Sk represent an average for 3 experiments performed in 8 replicates. Control value was set at 1.0.

Growth Inhibition of Normal Human Fibroblast Cells by Magainin Analogues. The magainin analogues also inhibited the growth of normal human fibroblast cells in a dose-dependent fashion (Fig. 1, c and d). The IC50 values of magainin A against normal fibroblast cell lines are listed in Table 3. These values are nearly 2-fold greater than those of SCLC cell lines. Comparisons of the shape of dose-response curve of the magainin analogues against SCLC cell lines and normal fibroblast cell lines suggest that normal fibroblast cell lines are more resistant to magainin analogues than SCLC cell lines, particularly at the higher magainin concentrations.

Combination Effect of Magainin Analogues with DDP or VP-16. In order to determine the potential combination effect of magainin analogues with DDP or VP-16, NCI-H678 cells were exposed to the drugs at the IC50 concentrations. This preliminary experiment was performed using IC50 concentrations of each drug against NCI-H678. Magainin A decreased the surviving fraction to 44.8%, magainin G to 51.5%, DDP to 59.2%, and VP-16 to 49.0% at the previously determined IC50 concentrations. Magainin A plus DDP decreased the surviving fraction to 26.1% and magainin A plus VP-16 decreased the surviving fraction to 26.4%. Magainin G plus DDP decreased the surviving fraction to 31.6%, and magainin A plus VP-16 decreased the surviving fraction to 31.7% (Fig. 3).

DISCUSSION

In this report, we describe the antitumor activity of magainin A and magainin G against six SCLC cell lines which differed in sensitivity to DDP, VP-16 and DOX. Magainin A and magainin G showed consistent growth inhibition against all six SCLC cell lines with IC50 values within a 3-fold range. The variation of IC50 values of magainin analogues had no obvious relation to the variation of IC50 values of the other chemotherapeutic agents tested. We also evaluated the combination effect of magainin analogues with DDP or VP-16. Magainin analogues showed additive antitumor effects when combined with

with 5% FBS. The IC50 of magainin A against NCI-H889 in serum-free medium was 5.79 compared to 6.56 μM in HITES medium with 5% FBS. The IC50 of magainin G against NCI-H889 in serum-free medium was 11.8 compared to 6.57 μM in HITES medium with 5% FBS. The addition of 5% FBS to the medium did not alter the potency of the two magainin analogues tested against the tumor cell lines.

Table 3 Average IC50 values for magainin analogues against 4 normal human fibroblast cell lines obtained by MTT assay

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Magainin A (μM)</th>
<th>Magainin G (μM)</th>
<th>DOX (μM)</th>
<th>VP-16 (μM)</th>
<th>DDP (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCD-27Sk</td>
<td>12.7</td>
<td>21.3</td>
<td>105</td>
<td>0.8</td>
<td>0.42</td>
</tr>
<tr>
<td>CCD-39Sk</td>
<td>25.6</td>
<td>34.8</td>
<td>90</td>
<td>2.4</td>
<td>0.75</td>
</tr>
<tr>
<td>CCD-43Sk</td>
<td>22.4</td>
<td>32.9</td>
<td>170</td>
<td>0.75</td>
<td>0.18</td>
</tr>
<tr>
<td>Fe Sin</td>
<td>23.6</td>
<td>27.9</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Mean</td>
<td>21.1</td>
<td>29.2</td>
<td>121</td>
<td>1.32</td>
<td>0.45</td>
</tr>
</tbody>
</table>

* NT, not tested.
standard chemotherapeutic agents.

The mechanism of cell killing by the magainin peptide is not completely understood. The dissipation of the electric potential across the membranes and uncoupling of respiration is considered to be a possible mechanism of the effect which kills the bacteria (14, 20). Furthermore, the formation of voltage-dependent ion channels which alters membrane potential and function is also considered to be a potential mechanism of antimicrobial and cytotoxic activity of magainin peptides (15). The formation of an amphiphilic α-helix with subsequent interaction with membranes and shielding from proteases are thought to be important properties of these antimicrobial peptides (14). Magainin A and magainin G were designed to have an enhanced α-helical formation compared to the naturally occurring magainin 1 and magainin 2. Furthermore, magainin A has a greater propensity for α-helical formation than magainin G. Magainin G has greater sensitivity to a bacterial pro tease than magainin A (14), so magainin A has greater stability when incubated with bacteria, and the prolonged stability may be responsible for the increased potency. In the present experiment, SCLC cell lines showed similar sensitivity to magainin A and magainin G. Magainin G is less toxic against normal cells than magainin A and may be a more appropriate candidate as an antitumor agent.

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