Antitumor Activity of 3-Ingenyl Angelate: Plasma Membrane and Mitochondrial Disruption and Necrotic Cell Death

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ABSTRACT

Options for skin cancer treatment currently include surgery, radiotherapy, topical chemotherapy, cryosurgery, curettage, and electrodesication. Although effective, surgery is costly and unsuitable for certain patients. Radiotherapy can leave a poor cosmetic effect, and current chemotherapy is limited by low cure rates and extended treatment schedules. Here, we describe the preclinical activity of a novel topical chemotherapeutic agent for the treatment of skin cancer, 3-ingenyl angelate (PEP005), a hydrophobic diterpene ester isolated from the plant Euphorbia peplus. Three daily topical applications of 42 nmol (18 μg) of PEP005 cured a series of s.c. mouse tumors (B16 melanoma, LK2 UV-induced squamous cell carcinoma, and Lewis lung carcinoma; n = >14 tumors/group) and human tumors (DO4 melanoma, HeLa cervical carcinoma, and PC3 and DU145 prostate carcinoma; n = >4 tumors/group) previously established (5–10 mm 3) on C57BL/6 or Foxn 1 1 mice. The treatment produced a mild, short-term erythema and eschar formation but, ultimately, resulted in excellent skin cosmesis. The L D 09 for PEP005 for a panel of tumor cell lines was 180–220 μmol. Electron microscopy showed that treatment with PEP005 both in vitro (230 μm) and in vivo (42 nmol) rapidly caused swelling of mitochondria and cell death by primary necrosis. 31 P NMR analysis of cell suspensions demonstrated a significant increase in the cell nucleotide pool (14). Cytosolic calcium release, uptake of propidium iodide, and staining with the mitochondria dye JC1, revealed that PEP005 (230 μmol) treatment of tumor cells in vitro resulted in a rapid plasma membrane perturbation and loss of mitochondrial membrane potential. PEP005 thus emerges as a new topical anti-skin cancer agent that has a novel mode of action involving plasma membrane and mitochondrial disruption and primary necrosis, ultimately resulting in an excellent cosmetic outcome.

INTRODUCTION

Skin cancer is the most common form of cancer. Approximately 1 million new cases of skin cancer are predicted to be diagnosed in the United States in 2003, with 9800 of these likely to result in death. The majority of skin cancers are basal cell carcinomas (BCCs) and squamous cell carcinomas (SCCs; Ref. 1). During the mid-1990s, skin cancer cost the American medicare system close to 1 billion US$/year, a figure that is rapidly increasing (2). The overall cost to United States society has been estimated at closer to US$ 2.5 billion/year (2).

Surgical excision is the most common and most successful treatment for localized primary skin lesions. A review of 10 studies showed a 5-year recurrence rate of 10.1% for BCC lesions after surgery (3), although recurrence rates are significantly higher for lesions >2 cm in diameter, for lesions in the head, neck, and face regions and for nodular rather than superficial lesions (4, 5). The 5-year recurrence rates for previously untreated primary SCC lesions after surgery are reported to be 8.1%, but this rises to 23.3% if the lesions had previously failed other treatments (6). Surgery is expensive (2), may not be the treatment of choice in cosmetically sensitive locations (7), and is limited by the proximity of essential anatomical structures (8). In these settings and when primary closure is difficult (large lesions or low skin redundancy), skin grafting or other reconstructive surgery may be required. Surgery may also be complicated for the significant number of patients with multiple lesions (9).

Radiotherapy is often chosen for primary lesions that would normally require extensive or difficult surgery (such as lesions near eyelids and nose), is generally restricted to a single course of treatment in any one field, and cannot be used for multiple lesions in scattered locations. The 5-year recurrence rates for radiotherapy have been reported at 8.7% for BCCs (3) and 10% for SCCs (5). Poor cosmetic results are common after radiotherapy and recurrence often develops in scattered lesions. Radiation therapy is expensive and protracted, usually requiring multiple daily hospital presentations over several weeks. Some wound healing problems can occur (13), and there is an associated carcinogenic risk after treatment (14).

Topical chemotherapy with 5-fluorouracil has shown recurrence rates of 20–30% for intraepithelial carcinomas (15), 13.7% 5-year recurrence for BCCs, and 20% for SCCs (16). 5-Fluorouracil is not effective for invasive lesions (17) and requires prolonged follow-up treatment, and more aggressive lesions may develop after treatment (18). Recently Imiquimod has been reported as an effective topical treatment for superficial BCCs (surface area <15 mm 2) showing initial response rates of 70–100%, depending on treatment regimen (19). However, the treatment required up to two daily topical applications for 6 weeks and was associated with local and some systemic toxicity (19, 20).

Euphorbia peplus, commonly known as “petty spurge” in England or “radium weed” in Australia, has a long history of traditional use for a variety of conditions, including topical self-treatment of basal cell carcinomas with E. peplus sap (21). In a survey of home remedies for skin cancer and solar keratoses, topical administration of the sap was unanimously considered by the users to be effective (21, 22). The sap of E. peplus contains several macrocyclic diterpenes, including 3-ingenyl angelate (PEP005; Fig. 1). Here, we demonstrate the potential of PEP005 as a novel topical chemotherapeutic agent for the treatment of skin cancer. PEP005 causes rapid mitochondrial disruption and cell death by primary necrosis, and ultimately results in a favorable cosmetic outcome.
MATERIALS AND METHODS

Cell Cultures and Reagents. SKMel28 (human melanoma; ATCC HTB-72), B16 (mouse melanoma; ATCC CRL-6322), PC-3 (human prostate carcinoma; ATCC CRL-1435), HeLa S3 (human cervical adenocarcinoma; ATCC CCL-2), MCF7 (human breast adenocarcinoma; ATCC HTB-22), Jurkat (human acute T cell leukemia; ATCC TIB-152) and DU145 (human prostate carcinoma; ATCC HTB-81) were obtained from the ATCC (American Type Culture Collection). The human melanoma cell line MM96L has been described previously (23). The mouse SCC cell line LK2 has been described previously (25). The mouse SCC cell line LK2 has been described previously (25). The human melanoma cell line MM96L has been described previously (23).

In Vivo Acute Cytotoxicity Assay. Cells were seeded (10,000/well) into 96-well tissue culture plates (CSL Biosciences) and were grown for 24 h before treatment. PEP005 was dissolved in 100% acetone and diluted in complete medium; the acetone concentration in the medium did not exceed 1%. The cells were washed twice in PBS and fixed in ethanol, and total protein was determined using sulforhodamine B.

In Vivo Tumor Growth. Cells (5 × 10^5; B16; 1 × 10^6; LK2) were injected (s.c.) into the flanks of female 6–10-week-old FVB/cnu/cnu mice (four tumor sites/mouse) or the shaved rear flanks of female 10-week-old C57BL/6 mice (two tumor sites/mouse; Animal Resource Centre, Perth, Western Australia, Australia). PEP005 was dissolved in an isopropanol-based gel composed of 25% w/w isopropyl alcohol and 25% w/w propyl alcohol in water (pH 4–6). The gel (10 µl) was applied topically onto established tumors and was spread over the tumor site with a small sterile spatula. Tumor size was measured using calipers. Mice were euthanized by CO2 inhalation when the cumulative tumor burden per mouse exceeded 1000 mm^3.

Electron Microscopic Analysis. C57BL/6 mice were inoculated with B16 cells as described above. C57BL6 mice were euthanized by CO2 inhalation at the indicated times after a single topical application of PEP005 (42 nmol; 18 µg) in isopropyl alcohol (10 µl) or vehicle alone to the established B16 tumors. The treated tumors were excised, cut into 1 mm^3 pieces and fixed in 3% glutaraldehyde in 0.1 M sodium-phosphate buffer (pH 7.4). The samples were treated with 1% osmium tetroxide, dehydrated with acetone and embedded in Epon 612 resin. Sections collected on grids were stained with uranyl acetate and lead citrate and examined on a JEOL 1010 transmission electron microscope at 80 kV.

Cultured cells were washed once in PBS and fixed for 30 min in 1% glutaraldehyde in 0.1 M sodium-phosphate buffer (pH 7.4) and the samples were processed as above.

Fig. 1. Structure of the diterpene ester 3-ingenyl angelate (PEP005).

Fig. 2. Dose response for acute cell cytotoxicity of 3-ingenyl angelate (PEP005). Table 1. LD_{50} of PEP005 (3-ingenyl angelate) in vitro

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<table>
<thead>
<tr>
<th>Tumor cell line</th>
<th>Species</th>
<th>LD_{50} (µM)</th>
<th>Tumor type</th>
</tr>
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<tbody>
<tr>
<td>LK2</td>
<td>Mouse</td>
<td>190 ± 4</td>
<td>UV-induced SCC^a</td>
</tr>
<tr>
<td>B16</td>
<td>Mouse</td>
<td>190 ± 0.5</td>
<td>Melanoma</td>
</tr>
<tr>
<td>MM96L</td>
<td>Human</td>
<td>187 ± 4</td>
<td>Melanoma</td>
</tr>
<tr>
<td>SKMel28</td>
<td>Human</td>
<td>191 ± 21</td>
<td>Melanoma</td>
</tr>
<tr>
<td>MCF7</td>
<td>Human</td>
<td>182 ± 4</td>
<td>Breast carcinoma</td>
</tr>
<tr>
<td>DU145</td>
<td>Human</td>
<td>189 ± 1</td>
<td>Prostate carcinoma</td>
</tr>
<tr>
<td>Jurkat</td>
<td>Human</td>
<td>221 ± 8</td>
<td>T-cell leukemia</td>
</tr>
</tbody>
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^a Mean ± SE of two or more experiments.

^b SCC, squamous cell carcinoma.
removed from the two tubes and were diluted in 2 ml of PBS. The cells were analyzed by fluorescence-activated cell sorting using a FacsCalibur instrument and CellQuest 3.3 software (Becton Dickinson, Palo Alto, CA). The percentage of cells that had clearly exceeded the fluorescence intensity threshold that distinguished live versus dead cells was determined.

RESULTS

Anticancer Activity of PEP005 in Vitro. The acute cytotoxic activity of PEP005 was determined by the loss of adherent cells from culture after 2 days of treatment. The relationship between the dose of PEP005 and cytotoxicity is shown for five tumor lines (the human melanoma cell line SKMel28 and the mouse melanoma cell line B16) in Fig. 2. The 90% lethal dose (LD90) is shown for these lines and an additional five tumor lines (Table 1). The dose response curves (Fig. 2) and the LD90 values (Table 1) were broadly similar for all of the tumor lines tested, with the LD90 dose ranging from 180 to 220 μM (Table 1). Similar results were obtained when more sparsely seeded cultures were treated for 5 days (data not shown). Treatment for 24 h at the LD90 dose followed by removal of PEP005 was sufficient to prevent any outgrowth of remaining cells (data not shown).

Topical Treatment of Tumors Grown on C57BL/6 and Foxn1nu Mice. PEP005 dose response (Fig. 3A) and treatment frequency (Fig. 3B) was investigated in vivo using the mouse UV-induced SCC cell line, LK2, grown in Foxn1nu mice. LK2 tumors grown s.c. to 17–20 mm3 (14 days after inoculation) were treated three times daily with 14, 42, or 126 nmol of PEP005. Although the lowest dose produced a significant delay in tumor growth (P < 0.001), doses over 42 nmol were required to effect a 100% cure rate (Fig. 3A). Comparison of different treatment frequencies (with the total dose remaining constant) showed that three daily applications gave a significantly better cure rate that one or seven daily applications (P < 0.001 and P = 0.04), respectively (Fig. 3B). Untreated tumors grew rapidly, whereas tumors treated daily for three days with 42 nmol of PEP005 were cured. One-day (high dose) or 7-day (low dose) regimens failed to cure several of the 16 treated tumors. The growth of tumors treated with vehicle alone was no different from that of untreated tumors (data not shown).

The efficacy of three daily applications of 42 nmol of PEP005 was further demonstrated in the B16 tumor model using C57BL/6 mice. Topical treatment of established B16 tumors with PEP005 resulted in 100% regression of tumors, with animals remaining tumor-free for 94 days after the initiation of treatment (14 tumors/group, 2 tumors/mouse; Fig. 3C). Control tumors that were treated with the isopropanol gel vehicle alone grew rapidly, reaching ~200 mm3 within 17 days after treatment onset.

Treatment with PEP005 also cured a panel of tumor lines grown s.c. on Foxn1nu or C57BL/6 mice (Table 2). The effective treatment dose did not vary significantly for the different tumor lines (data not shown).

Irrespective of the tumor type being treated or the mouse strain used, an acute erythema was apparent after completion of the three daily treatments with 42 nmol of PEP005, which lasted for 2–3
days, with mild erythema visible for 2–3 weeks. Eschar formation occurred within 3–4 days after completion of treatment and resolved within a week. The cosmetic effect after treatment was very favorable and is illustrated for Foxn1nu mice, which had B16 tumors treated 3 weeks previously (Fig. 3D, panel 2). The skin at this time was similar to untreated skin and had normal elasticity. Although slight scarring and erythema remained (Fig. 3D, panel 2), both continued to resolve over 2–3 months (data not shown).

**Mitochondrial Swelling and Necrosis after Treatment with PEP005 in Vitro and in Vivo.** To further investigate the nature of PEP005-mediated cytotoxicity, B16 mouse melanoma cells, treated with PEP005 in vivo and in vitro, were analyzed by transmission electron microscopy. The first identifiable morphological change after PEP005 treatment was the pronounced swelling of mitochondria seen within 3.5 h of treatment of cells in vitro (230 μm; Fig. 4A, panel 2), and within 6 h of a single topical application of 42 nmol of PEP005 to a B16 melanoma established s.c. on C57BL/6 mice (Fig. 4B, panel 3). Mitochondria in control cells treated with vehicle alone remain unaffected (Fig. 4A, panel 1, and Fig. 4B, panel 2). By 6 h, the majority of cells treated in vitro exhibited clear signs of primary necrosis, with disintegration of the plasma membranes and disruption of cytoplasmic organelles (Fig. 4A, panel 3). A similar picture emerged 24 h after PEP005 treatment of B16 tumors in vivo (Fig. 4B, panel 4). Control cells treated with vehicle remained unaffected (Fig. 4A, panel 1, and Fig. 4B, panel 1). Electron microscopy analysis of Lewis lung cells treated with PEP005 in vitro showed essentially identical results (data not shown). These electron micrographs illustrate that at cytotoxic doses, PEP005 caused rapid mitochondrial swelling and cell death by primary necrosis.

That PEP005 cytotoxicity involves primary necrosis was supported by the observation that overexpression of the apoptosis resistance gene Bcl2 in B16 cells did not convey resistance to PEP005 treatment (data not shown). Furthermore, treatment of B16 cells with 230 μM PEP005 failed to show any DNA laddering or PI-independent Annexin V staining, which are often characteristics of apoptosis (data not shown).

**Loss of Mitochondrial Membrane Potential after Treatment with PEP005 in Vitro.** Mitochondrial swelling is often associated with the loss of mitochondrial membrane potential (ΔΨm), a phenomenon readily measured using the mitochondrial dye JC1 and fluorescence-activated cell sorting analysis (28). Under normal circumstances, JC1 accumulates in the inner mitochondrial membrane in which it oligomerizes and fluoresces red. A reduction in ΔΨm results in diffusion of the dye from the mitochondria and a subsequent reduction in the mean red fluorescence intensity. As expected, control tumor cells showed a high mean red fluorescence (set to 100%) after staining with JC1 (Fig. 5). After treatment with 200 μM carbonyl cyanide m-chlorophenylhydrazone, a known mitochondrial protonophore, the mean red fluorescence of the mitochondria dropped rapidly within 30 min, indicating that the ΔΨm had collapsed (Fig. 5A). Tumor cells treated with 230 μM PEP005 also showed a rapid reduction in the mean red fluorescence intensity, which, in repeated experiments, was always slightly slower than that seen for carbonyl cyanide m-chlorophenylhydrazone (Fig. 5A, and data not shown). Thus, treatment of tumor cells with cytotoxic doses of PEP005 causes a rapid loss of ΔΨm.

An analysis of the relationship between ΔΨm and PEP005 concentration revealed that subcytolytic doses of PEP005 also decreased ΔΨm, albeit less efficiently than a cytolytic dose of PEP005 (230 μM). This result does not mirror the data presented in Fig. 2, indicating that loss of mitochondrial membrane potential may not be sufficient for PEP005-mediated killing. Furthermore, treatment of cells with reactive oxygen species inhibitors (e.g., DTT) did not prevent PEP005-induced cell death (data not shown).

**Plasma Membrane Perturbations after Treatment with PEP005 in Vitro.** To further investigate the mechanism of PEP005-mediated killing, the plasma membrane integrity of PEP005-treated tumor cells was investigated using 51Cr release and PI uptake. Within 10 min of PEP005 (230 μM) addition to B16 or LK2 cells in vitro, ~30% of the total amount of 51Cr loaded into the cells was already released, with nearly 80% released after 1 h (Fig. 6, A and B). Within 30 min, uptake of PI was seen in 15% of cells and in 80% after 2 h (Fig. 6C; the scale on the X axis is different in A and B). These studies indicate that PEP005 mediates rapid plasma membrane damage resulting in rapid efflux of 51Cr and in entry of PI (uptake of PI, a high molecular-weight, charged compound, is a sensitive indicator of membrane integrity).
test for membrane integrity and indicates primary necrosis when observed in the absence of evidence for apoptosis (29).

Fluorescence-activated cell sorting analysis using anti-Annexin V staining showed that PEP005 (230 μM)-treated cells did not become Annexin V positive unless they became PI positive (data not shown), further illustrating that apoptosis was not a feature of PEP005-mediated killing at this dose.

**DISCUSSION**

This report describes the preclinical activity of a new topical anticancer agent, PEP005 that was capable of regressing a wide range of established s.c. tumors in mice after three daily treatments, yet achieving a favorable cosmetic effect. PEP005 also demonstrated a novel mode of action for a chemotherapeutic agent, causing plasma membrane disruption and rapid loss of the mitochondrial membrane potential and subsequent mitochondrial swelling, followed by cell death by primary necrosis within 1 h.

The morphological characteristics of PEP005-mediated killing, the rapid loss of plasma membrane integrity, and the lack of DNA laddering, PI-independent Annexin V staining, and Bcl2 sensitivity provide compelling evidence for induction of primary necrosis by PEP005 rather than apoptosis. Mitochondria are well known as targets for chemotherapeutic agents, because they have a central role in the induction and regulation of apoptotic cell death (30, 31). However, it has recently become evident that mitochondria can also play a role in primary necrosis (32, 33), although few current chemotherapeutic agents induce this form of cell death. The early mitochondrial swelling seen after PEP005 treatment and the necrotic outcome is reminiscent of ischemia reperfusion injury in which plasma membrane disruption (34) and mitochondrial dysfunction and rapid depletion of ATP are central to the induction of cell death (35). Cryotherapy also induces primary necrosis and, like PEP005, is associated with an inflammatory reaction at the treatment site (36). Plasma membrane disruption is likely to cause changes in cytoplasmic ionic concentrations, which can lead to mitochondrial swelling and loss of mitochondrial membrane po-

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**Fig. 5.** Mitochondrial membrane depolarization after treatment with 3-ingenyl angelate (PEP005). A, tumor cells (SKMel28 melanoma) were treated with 200 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP; □) or with 230 μM PEP005 (■) and were stained with JC1, a dye that oligomerizes in normal mitochondria and fluoresces red. Loss of red fluorescence indicates loss of mitochondrial membrane potential (ΔΨm). The mean red fluorescence of drug-treated cells was measured at 0.5, 1, 2, and 4 h after treatment and are presented as a percentage of the mean red fluorescence of controls (±SE). The insets, the fluorescence-activated cell sorting profiles of control cells and cells treated for 2 h with PEP005 (Y axis, red fluorescence; X axis, green fluorescence). B, tumor cells (SKMel28 melanoma) were treated with 0, 2.3, 23, 58, 116, 174, or 230 μM PEP005 and were stained with JC1. The mean red fluorescence of drug-treated cells was measured at 4 h after treatment and are presented as a percentage of the mean red fluorescence of untreated controls (±SE).

**Fig. 6.** Release of $^{31}$Cr and uptake of propidium iodide (PI) after 3-ingenyl angelate (PEP005) treatment. A, tumor cells (LK2 SCC) were labeled with $^{31}$Cr, washed and treated with 230 μM PEP005 (■) or diluents (□). The accumulated $^{31}$Cr released into the supernatant at the indicated times is presented as a percentage of total cell $^{31}$Cr (±SD). B, as for A, but using B16 melanoma cells. C, B16 cells were treated with 230 μM PEP005 (■) or 1% acetone (v:v; □) and then with PI. The percentage of drug or acetone-treated cells (±SD) taking up PI at the indicated time points is shown. The scale on the X axis is different for C and A/B.
tential (32–34). The immediate plasma membrane effects seen after PEP005 might, therefore, support this sequence of events for the PEP005 mechanism of action. PEP005, thus, emerges as having a mode of action distinct from most anticancer agents, inducing cell death in a manner more akin to that seen after injury, but leading to rapid healing, resolution of the lesion, and swift regeneration of normal skin architecture.

Several ingenol esters are known to activate protein kinase C (PKC; Ref. 37), and PEP005 is also a potent activator of PKC enzymes. There are a number of anticancer agents currently in development that modulate PKC enzymes (38, 39), and certain PKC isoenzymes have been associated with mitochondrial disruption (40–42). However, we have thus far been unable to demonstrate that PKC modulation by PEP005 is responsible for its cytotoxic activity, because the PKC inhibitors bisindolyl maleimide and GO6976 failed to block the acute cytotoxicity of PEP005 in vitro (data not shown). However, such results may simply reflect an inability of the inhibitors to prevent PEP005-mediated translocation of specific PKC isoenzymes, or mismatched bioavailability profiles for PEP005 and the inhibitors (38, 39, 43, 44). A deoxy derivative of PEP005 [3-(20-deoxyingenyl) angelate] has similar acute cytotoxicity but activates PKC very poorly and is inactive as a topical chemotherapeutic agent in mice (data not shown). PKC activation may thus contribute to, but may not be sufficient for, achieving the efficacy of PEP005. The inflammatory response seen after topical PEP005 treatment is likely to be a consequence of PKC activation (45), and one might speculate that PKC activation and stimulation of inflammatory cells contribute to the effective wound healing seen after PEP005 treatment (46, 47).

At the high local concentration achievable by local application, PEP005 is an acutely cytotoxic drug that rapidly kills tumor cells and shows little specificity for different tumor cell types. Only three topical applications of PEP005 were required for tumor cure in mouse models, and to date, the only significant finding from formal toxicology studies evaluating topical treatment with PEP005 was local inflammation (data not shown). PEP005, thus, represents a novel topical chemotherapeutic agent, which induces primary necrosis and is, therefore, unlikely to have its activity compromised by the development of apoptosis resistance in tumor cells (48, 49). Despite this mode of action, PEP005 treatment is associated with a very favorable cosmetic outcome (Fig. 3 D), a feature that was also noted after the use of sap to treat patients with nonmelanoma skin cancer (48, 49). Despite this mode of action, PEP005 treatment is likely to be a consequence of PKC activation (45), and one might speculate that PKC activation and stimulation of inflammatory cells contribute to the effective wound healing seen after PEP005 treatment (46, 47).

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