Proceedings of the First International Conference on PEP005

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The sap of *Euphorbia peplus*, commonly know as 'petty spurge', 'radium weed' or 'milkweed' has been used for centuries as a traditional treatment for skin conditions, including warts, corns and cancers of the skin. Documentation of its use by medical professionals to treat basal cell carcinoma (BCC) dates from the early 19th century. Individuals who participated in a 1988 survey of home treatments for cancer indicated the sap of *E. peplus* was an effective cure for actinic lesions leading the investigators to suggest that this potential utility should be further explored in controlled clinical trials. The fractionation of the sap *E. peplus* using solvents of varying polarity yielded several macrocyclic diterpenes, many of which were found to have cytotoxic activity or the ability to influence cellular differentiation. Ultimately, ingenol 3-angelate (I3A) of PEP005, emerged as a promising potential new anti-cancer treatment. Here we report the proceedings from the First International Conference on PEP005, covering the exciting potential of PEP005 as the therapeutic agent for the treatment of skin cancer, leukemia and bladder cancer. *Anti-Cancer Drugs* 18:357–362 © 2007 Lippincott Williams & Wilkins.

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The First International Conference on PEP005 was held in Manchester, UK, on 4–5 January 2006. The conference was organized to bring together researchers working on the development of PEP005, with the primary aim of providing an opportunity for the worldwide research team to present experimental data and to exchange ideas. The conference combined scientific presentations on various aspects of the development of PEP005 as an anti-cancer drug with periods of open discussion.

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Introduction

The sap of *Euphorbia peplus*, commonly known as 'petty spurge', 'radium weed' or 'milkweed' (Fig. 1), has been used for centuries as a traditional treatment for skin conditions, including warts, corns and cancers of the skin. Reports exist of its use as a treatment for asthma and catarrh as well as a purgative. Documentation of its use by medical professionals to treat basal cell carcinoma (BCC) dates from the early 19th century [1]. A case of biopsy-proven cure of a BCC after the application of the sap of *E. peplus* has also been reported [2]. Individuals who participated in a 1988 survey of home treatments for cancer indicated the sap of *E. peplus* was an effective cure for actinic lesions, leading the investigators to suggest that this potential utility should be further explored in a controlled clinical trial [3].

The fractionation of the sap of *E. peplus* using solvents of varying polarity yielded several macrocyclic diterpenes, many of which were found to have cytotoxic activity or the ability to influence cellular differentiation. A high-performance liquid chromatographic technique was subsequently developed that enabled the separation and concentration of these compounds in quantities sufficient to study their therapeutic potential [4]. From these studies, ingenol 3-angelate or PEP005 as it is now known, emerged as a promising potential new anticancer treatment (Fig. 2).

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Use of ingenol 3-angelate as a topical chemotherapeutic agent for skin cancer

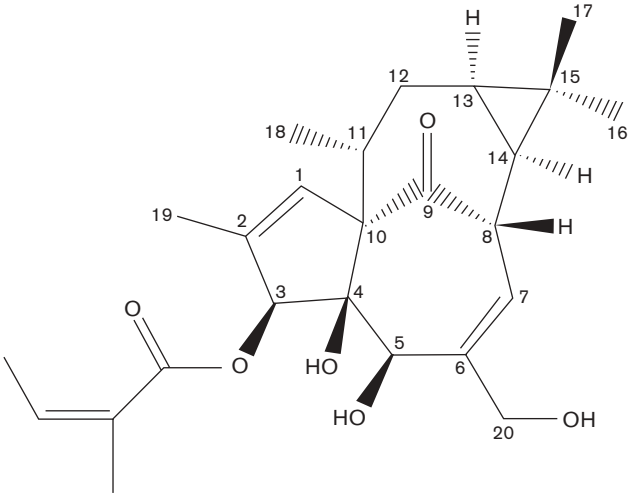
PEP005 is being actively developed as a topical treatment for both actinic keratoses (AK) and non-melanoma skin cancer (NMSC). AK is characterized by rough, scaly patches or crusts on the skin and has been shown to progress to squamous cell carcinoma (SCC). It is the most common precancerous lesion worldwide, affecting an estimated 50% of Caucasians over 40 years of age. Skin cancer (both melanoma and NMSC) is the most common form of cancer with around 1 million new cases diagnosed in the US each year. NMSC includes BCC (approximately 80% of NMSC cases) and SCC (approximately 20%) [5].

The current first-line treatment for AK, BCC and SCC is surgery. Guidelines and recommendations advocate electrodesiccation and curettage or excisional surgery with a 4-mm margin for low-risk SCC and BCC [6,7]. Excisional surgery with a wider margin (6 mm) or micrographic surgery is recommended for larger ($\geq$2 cm diameter) lesions or those in high-risk locations (such as the eyelid, brow, nose or ear) [7]. Cryosurgery is generally used for smaller, clearly demarcated lesions and is frequently used to treat AK [6,8]. Although effective, surgery does have limitations and is not suitable for many patients. Radiotherapy is a useful option for elderly or debilitated patients who are unable to undergo surgery,

Fig. 1

Euphorbia peplus.

Fig. 2



Chemical structure of ingenol 3-angelate.

but its use is limited by cost and the requirement for several treatment sessions [6].

Alternative treatments including topical fluorouracil, topical imiquimod, intralesional interferon, systemic retinoids, carbon dioxide laser surgery and topical photodynamic therapy have shown some promise but are not widely used [6].

Thus, there is considerable scope for a convenient and effective therapy for AK and NMSC.

Preclinical evaluation of ingenol 3-angelate for topical treatment of skin cancer

Initial *in vitro* experiments indicated PEP005 had an LD_{90} (lethal dose for 90% of cells) of 180–220 μmol/l against a range of mouse and human cancer cell lines [9]. To investigate the topical activity of PEP005 *in vivo*, the drug was applied to mouse (B16 melanoma, LK2 ultraviolet-induced SCC and Lewis lung carcinoma) and human (DO4 melanoma, HeLa cervical carcinoma and PC3 and DU145 prostate carcinoma) tumors established subcutaneously in C57BL/6 or *Foxn1*^{nu} mice. The tumors were allowed to grow to approximately 10–15 mm^3 before PEP005, formulated as an isopropanol-based gel, was applied. Of the various regimens tested, daily application for 3 days at a dose of 42 nmol was the most effective, achieving a 100% cure rate [9]. PEP005 (42 nmol) induced an acute erythema, which peaked at 2–3 days posttreatment onset, and then rapidly resolved over the next 10–20 days. This erythematous response was associated with (1) upregulation of macrophage inflammatory protein-2 [the murine counterpart of interleukin (IL)-8], tumor necrosis factor (TNF)-α and IL-1β, and (2) a substantial infiltration of neutrophils [10].

Mechanism of action

The initial cytotoxic activity of PEP005 is characterized by rapid disruption of the plasma membrane, swelling of mitochondria and cell death via primary necrosis. This occurs at concentrations of approximately 100 μg/ml (230 μmol/l) [9]. It is postulated that PEP005 dissolves in the plasma membrane and in an attempt to clear the drug, the cell endocytoses the membrane. The endocytosed vesicles are destabilized by the presence of PEP005, which has membranologic properties, resulting in endosome disruption and release of calcium into the cytoplasm. The rapid rise in calcium levels results in rapid mitochondrial swelling, collapse in ATP production and finally, primary necrosis.

Neutrophils have been shown to mediate anticancer activity [11], and removal of neutrophil activity (using anti-Ly-6G antibody or CD18 hypomorphic mice) resulted in substantial increases in the relapse rate of tumors (from 0–8 to 83–100%) after topical treatment with PEP005. Similar relapse rates were also found in SCID, but not nude mice, suggesting a role for B cells. In intact mice, PEP005 treatment also resulted in the induction of anticancer antibodies. Neutrophils have been shown to mediate anticancer activity via antibody-dependent cellular cytotoxicity [12,13]. These data therefore suggest that, following the initial chemo-ablation of the tumor by PEP005, relapse is prevented by neutrophil-mediated antibody-dependent cellular cytotoxicity of residual tumor cells [10]. On the basis of the findings of these studies, a two-stage mechanism of action for topical PEP005 treatment has been proposed: initial chemo-ablation, followed by neutrophil-dependent eradication of residual tumor cells.

Cosmesis

PEP005 has a very favorable cosmetic effect following topical administration to subcutaneous tumors in mice. Eschar formation occurs within 3–4 days of treatment and resolves within a week. After 3 weeks, the skin at tumor sites was similar to untreated skin and had normal elasticity. Slight scarring and erythema were evident, but resolved over 2–3 months [9]. It has been suggested that both the activation of protein kinase C (PKC) and stimulation of neutrophils contribute to the good cosmesis seen after PEP005 treatment via the scavenging of cell debris and secretion of mediators that promote wound healing. Various studies have shown PEP005 upregulates key cytokines and chemokines and increases mRNA and protein levels of key molecules involved in wound-healing responses in several cell types. These include IL-8 and TNF-α in human keratinocytes, IL-8 and IL-6 in human fibroblasts, IL-1β, IL-2, IL-6, IL-8 and TNF-α in human peripheral blood mononuclear cells, and IL-8 in human tumor cells and neutrophils. Projects have been initiated to further investigate and define the promotion of wound healing by PEP005.

Potential in bladder cancer

The potential of PEP005 as an intravesicular treatment for bladder cancer is also under investigation. PEP005 was shown to be antiproliferative to both human and rat bladder tumor cells *in vitro*. Although the mechanism of the antiproliferative activity in bladder cells is not yet determined, initial *in vivo* studies have indicated that concentrations of PEP005 utilized in topical studies could be contemplated for use in the bladder and hence, similar mechanisms to those discussed above may be relevant. A potential therapeutic window is implied by the observation that normal human urothelial cells are less sensitive to PEP005 than human bladder urothelial carcinoma cells. Research is ongoing to investigate the activity of PEP005 in rat bladder tumor models.

Tolerance studies

Tolerance to dermally applied PEP005 has been studied in rats and mini-pigs. Topical PEP005 was associated with dermal irritation – evidenced by erythema, edema, desquamation, acanthosis and scabbing – across all species that was dose-dependent and frequency-dependent. Symptoms generally resolved within 14 days but, at higher dose levels and greater dosing frequencies, resolution was delayed. Importantly, in all studies, systemic exposure to PEP005 was limited or did not occur and no systemic toxicity was observed.

Clinical program for topical use

The topical drug product is an isopropyl alcohol-based gel that is stable at 2–8°C. Stable cream formulations have also been developed and preliminary pharmacological testing indicates a similar clinical response to that obtained with the gel product.

The clinical trial program for topical PEP005 is summarized in Table 1. Phase I trials are complete and the phase

Table 1 Overview of the clinical trial program for topical PEP005

Study	Phase	Status	Indication	Concentration of PEP005 (%)	Topical regimen
Peplin *E. peplus* sap	I/II	Complete	Actinic keratosis or nonmelanoma skin cancer	Unknown	100–300 μl of sap, once daily for 3 days
PEP005	I	Complete	Actinic keratosis	0.01	Single application
PEP005-001	IIa	Complete	Actinic keratosis	0.0025 0.01 0.05	Once daily for 2 days (either day 1 and 2 or day 1 and 8)
PEP005-002	IIa	Ongoing	Nodular basal cell carcinoma	0.0025 0.01 0.05	Once daily for 2 days (either day 1 and 2 or day 1 and 8)
PEP005-003	IIa	Ongoing	Superficial basal cell carcinoma	0.0025 0.01 0.05	Once daily for 2 days (either day 1 and 2 or day 1 and 8)
PEP005-004	II	Ongoing	Actinic keratosis	0.01 + dose escalation to identify maximum tolerated dose	Once daily for two consecutive days

II trial programs for AK, nodular BCC and superficial BCC are well advanced. The phase IIa program currently comprises four separate studies, which are designed to evaluate the potential of PEP005 topical gel in AK, superficial BCC and nodular BCC.

No serious adverse events related to drug therapy were reported in the phase IIa study in patients with AK treated with placebo, 0.0025% PEP005, 0.01% PEP005 or 0.05% PEP005 topical gel. As was expected, the most common local reactions were erythema, scabbing/crusting and flaking/scaling/dryness, most of which were classified as mild or moderate in severity, with a dose–response relationship evident. On a per lesion basis, 0.05% PEP005 completely cleared 71% of lesions compared with 27% for placebo treatment; this was statistically significant ($P < 0.0001$). A more recently completed open-label, dose-escalation trial confirmed that PEP005 topical gel 0.05% is well tolerated. Further phase IIa studies investigating the safety of topical PEP005 applied to superficial BCC or nodular BCC are underway. Preliminary results for superficial BCC were similar to those reported in the AK study; two applications of PEP005 0.05% gel cleared 71% of lesions. The gel had a favorable safety profile and was well tolerated. Results for the nodular BCC study are expected towards the end of 2006.

The phase III clinical trials program for topical PEP005 is planned to commence in 2008.

Systemic ingenol 3-angelate

Potential against leukemia cells *in vitro*

Screening of PEP005 against human tumor cell lines included in the US National Cancer Institute Developmental Therapeutics Program tumor cell line screen indicated the drug had significant activity against leukemia cell lines. Subsequent investigations revealed that PEP005 was active against a broad range of leukemia cells *in vitro* as shown in Table 2.

Table 2 Concentration of PEP005 causing 50% growth inhibition (GI_{50}) in various leukemia cell lines *in vitro*

Cell line	Concentration of PEP005 causing GI_{50} (nmol/l)
HL60	40
U937	25
NB4	25
K562	10
CCRF-CEM	10
Jurkat	1
MyLa/10	2
Raji	3
RPMI	7
SR	2
MOLT-4	300

PEP005 was added to tumor cells growing in culture. After 5 days, the effect of the drug was determined by comparing total protein levels or cellular activity with control treated cells via protein staining with sulfur rhodamine blue (SRB) or assay with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide). PEP005, ingenol 3-angelate.

Molecular mechanism of action in leukemia cells

In the presence of PEP005, HL60, NB4 and U937 myeloid leukemia cell lines were observed to enter apoptosis, as well as differentiate according to CD11b expression, with the drug having an optimal effect at a concentration of 20 nmol/l. K562 cells entered apoptosis at a much lower PEP005 concentration, but did not express CD11b. After analysis of the effects of PEP005 in several other leukemia cell lines, the investigators concluded that the induction of apoptosis was the predominant effect of the drug. PEP005 also induced apoptosis in blasts isolated from the bone marrow of patients with acute myeloid leukemia (AML) at concentrations of 2–10 nmol/l, but did not in normal $CD34^+$ myeloblasts isolated from cord blood, even at up to 2-log concentrations higher than those required to induce apoptosis in AML blasts [14].

Subsequent experiments revealed that the relative sensitivity of HL60, U937 and NB4 cell lines to PEP005 was correlated with expression of PKC-δ. The probable role of PKC-δ in mediating the proapoptotic effects of PEP005 was lent further support by the finding that the broad-range PKC inhibitor, bisindolylmaleimide 1, inhibited PEP005-induced apoptosis in HL60 cells, whereas Gö6976, which is a specific inhibitor of PKC-α and PKC-β, did not. In contrast to most leukemia cell lines, KG1a cells did not enter apoptosis or express markers of differentiation and, in final support of the involvement of PKC-δ in the antileukemia activity of PEP005, KG1a cells transiently transfected with PKC-δ were rendered sensitive to PEP005.

Further investigation into the effects of PEP005 on PKC-δ found it induced a different intracellular PKC-δ translocation pattern to the typical phorbol ester, phorbol 12-myristate 13-acetate (PMA, also known as TPA). Low concentrations of PEP005 (10 nmol/l) were associated with rapid translocation of PKC-δ to the internal membranes and the nuclear membrane, with some presence at the plasma membrane evident at later time-points. In contrast, PMA caused PKC-δ to translocate first to the plasma membrane and then to the nuclear membrane [15]. To determine whether this different PKC-δ translocation pattern might influence biological activity, levels of secreted IL-6 in WEHI-231 cells were analyzed after PMA or PEP005 treatment. PEP005, but not PMA, produced a biphasic PEP005 concentration versus IL-6 secretion curve [15]. As cytokine secretion is generally regulated at the transcriptional level, the effects of PEP005 and PMA on the activation of various transcription factors, including nuclear factor (NF)-κB, were studied in nuclear extracts of WEHI-231 cells. The response of the NF-κB family members to the presence of PEP005 or PMA was found to be complex and changed with time. Up to 6 h after exposure to PEP005, p65 and, to a lesser extent, c-Rel

showed a biphasic pattern of activation. On the basis of these findings, the effects of PEP005 on the DNA binding of p65-containing NF-κB could be responsible for the biphasic IL-6 secretion observed. Further experiments showing that IκB-α levels negatively correlate with p65 levels support this supposition [16].

Clinical trials of PEP005 in patients with AML have not yet been initiated, but preliminary *in vitro* studies aimed at selecting patients who may benefit from treatment with PEP005 have been commenced. Markers being studied include patient age, sex, disease history (e.g. myelodysplastic syndrome, relapse), French–American–British subtype, lineage/differentiation markers (CD4, CD13, CD14, CD15, HLA-DR, CD33 and CD34), cytogenetics and Flt3.

Molecular mechanism of action in melanoma cells

PEP005-induced apoptosis in certain melanoma cell lines has been linked to the effects of the drug on PKC [17]. The results of a more recent study indicate that the effects of PEP005 on PKC in melanoma cells may lead to senescence [18].

PEP005 and PMA induced a similar pattern of G_1 and G_2/M cell cycle arrest in human melanoma cell lines that were sensitive to the two drugs, but not in resistant cell lines. Both drugs strongly induced acidic β-galactosidase – a marker for senescence – in sensitive, but not resistant, cells. Loss of telomerase activity was also observed in sensitive, but not resistant, cells following exposure to either drug [18].

Despite the downregulation of PKC-α, PKC-δ and PKC-ε being evident in both sensitive and resistant cells, inhibitors of the PKC (bisindolylmaleimide 1) and mitogen-activated protein kinase (MAPK) (PD98059) pathways were found to prevent the irreversible growth arrest associated with PEP005 or PMA in a sensitive cell line [18].

Transcriptional profiling of cells treated with PEP005 or PMA for 6, 24 or 72 h revealed selective downregulation of transcription factors and cell-cycle genes in sensitive cells. PEP005 or PMA-induced senescence was associated with the loss of E2F-1 and PCNA proteins within 24 h after treatment in sensitive, but not resistant, cells. Conversely, p21 expression increased in sensitive cells. Further investigations showed that PKC and MAPK activation drive the molecular changes in senescent cells, suggesting PKC-dependent MAPK activation and subsequent downregulation of E2F-1 may account for the growth arrest in sensitive cells treated with PEP005 [18].

The mechanism underlying resistance has not yet been determined; however, cluster analysis of constitutive gene expression identified at least one candidate gene, HRev107 that is consistently overexpressed in resistant cells. HRev107 has a dampening effect on the MAPK pathway. Although activation of MAPK is normally viewed as a negative feature that promotes tumor cell growth, it appears that exposure to PEP005 or PMA results in overstimulation, which leads to senescence via an unknown mechanism. It is hypothesized that the downregulating effect of HRev107 on the MAPK pathway somehow protects resistant cells from senescence.

Conclusion

There is a clear need for more effective and efficient treatments for NMSC. While surgery – the current first-line therapy – is effective, it is painful, potentially disfiguring, expensive, often unsuitable for certain patients or lesion locations and ultimately, a less than ideal therapy. Currently available topical therapies, however, are not as effective as surgery. Topical 5-fluorouracil is associated with recurrence rates of between 14 and 30% depending on the type of cancer and requires prolonged follow-up. Topical imiquimod has been reported to be effective for BCC, but requires a prolonged administration regimen [19]. Preliminary evidence suggests that topical PEP005 is a convenient, effective and well-tolerated treatment for AK and superficial BCC, and it is anticipated to be equally as effective and well tolerated in nodular BCC and SCC *in situ*. If this is confirmed in large-scale clinical trials, PEP005 may represent a significant advance in the management of AK and NMSC.

In contrast to the necrotic effect seen with topical application, the potent antileukemic effects of PEP005 at low concentrations *in vitro* are due to apoptosis and correlate to the expression of PKC-δ. Importantly, cells not expressing PKC-δ are resistant to the drug. A detailed understanding of the mechanism of action of a systemic anticancer therapy is critical for the successful progression of an experimental therapy through clinical development. This understanding allows for the selection and enrolment of patients who are most likely to benefit from the therapy. Clinical trials of PEP005 in patients with AML have not yet been initiated, but preclinical studies are ongoing, and not only indicate the potential of a 10-fold therapeutic window between the sensitivity of leukemic blasts and normal blasts to PEP005, but will also provide a suitable system to select patients who may benefit from treatment with PEP005. A phase I clinical trial to determine the safety of systemically administered PEP005 is planned.

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