The Skin Cancer Chemotherapeutic Agent Ingenol-3-Angelate (PEP005) Is a Substrate for the Epidermal Multidrug Transporter (ABCB1) and Targets Tumor Vasculature

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Abstract

Ingenol-3-angelate (Ing3A), extracted from Euphorbia peplus, is currently in clinical trials for eradicating basal cell carcinoma, actinic keratosis, and squamous cell carcinoma (SCC) in situ by topical application. Although structurally related to phorbol esters and a protein kinase C activator, topical Ing3A, but not phorbol 12-myristate 13-acetate (PMA), inhibited the growth of subcutaneous tumors derived from PAM212 (mouse SCC) and B16 (mouse melanoma). Ing3A and PMA both induced acute neutrophilic inflammation on mouse skin, but only Ing3A caused subcutaneous hemorrhage and vascular damage. Both Ing3A and PMA activated extracellular signal-regulated kinase 1/2 (ERK1/2) in epidermis, but Ing3A also activated ERK1/2 in skin dermal fibroblasts and endothelial cells. Pretreatment with topical cyclosporin A (CsA), verapamil, or XR9576, modulators of P-glycoprotein (P-gp), prevented Ing3A-induced hemorrhage but not neutrophil infiltration. CsA also impaired the anticancer activity of Ing3A, whereas the anti-inflammatory dexamethasone did not. Ing3A, but not PMA, blocked photoaffinity labeling of human P-gp with [125I]iodoaryazidoprazosin and inhibited P-gp–mediated drug resistance to HCT-15 cells. The intracellular levels of Ing3A were significantly lower in P-gp–expressing cells, and treatment with XR9576 increased the levels to those of cells that do not express P-gp, showing that Ing3A binds to and is transported by P-gp. Taken together, our results suggest that P-gp–mediated absorptive transport, dermal penetration, and vascular damage contribute to the anticancer activity of Ing3A in vivo. Cancer Res; 70(11); 4509-19. ©2010 AACR.

Introduction

Nonmelanoma skin cancer (NMSC), primarily basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), is the most prevalent cancer in the Caucasian population. Although rarely lethal in the northern hemisphere, in warmer climates or in immunosuppressed patients it can metastasize and cause death (1). Under all circumstances, its treatment constitutes an important component of health care costs. Traditionally, treatment for NMSC involves surgical intervention and consequent esthetic deformity due to the common location on exposed surfaces on which these sun-induced lesions are most frequently found. Treatments for the much more prevalent precancerous actinic keratosis are often scarifying as well, adding to the personal and financial expense associated with skin cancer diagnosis and therapy. Thus, the discovery of nondeforming but curative medical therapies is a high priority for these conditions.

With advances in cutaneous biology research, understanding the molecular pathology of skin cancer development has expanded. The cause of the great majority of BCC involves alterations in components of the sonic hedgehog pathway (2), and targeted drugs are in clinical trials (3). The mechanism for the less common but more aggressive SCC has been more difficult to delineate, although considerable insight has been achieved (4).

The discovery that protein kinase C (PKC) δ is a proximal regulator of keratinocyte viability (5) and was catalytically inactivated or downregulated in cutaneous squamous cell tumors presented a druggable target for tumor therapy (6–9). Ingenol-3-angelate (Ing3A), an active component extracted from Euphorbia peplus, is a PKCδ agonist with selectivity for PKCδ (10–12). This molecule had been reported to be a strong irritant but has weak tumor-promoting activity for mouse skin (13). Ing3A has antitumor activity when applied topically on mouse skin overlying subcutaneous mouse and human tumor xenografts (14). A mechanism whereby topical skin treatment could inhibit subcutaneous tumor growth was unclear, but the compound has been pursued...
as a potential skin cancer treatment by Peplin, Inc. (drug name, PEP005) and is now in clinical trials with promising results (15). Topical Ing3A elicits a strong inflammatory response subcutaneously, and the subsequent tumor killing was considered a necrotic response to a marked microenvironmental inflammation (16).

Ing3A is structurally similar to phorbol 12-myristate 13-acetate (PMA), the classic mouse skin tumor promoter, and elicits similar responses as PMA in cultured human cancer cells and isolated cell membranes or lipid vesicles (12). Because the two compounds have not been tested side by side in vivo, and PMA is equally proinflammatory, we focused on defining a unique activity of Ing3A that may contribute to its antitumor properties. We now show that Ing3A, like PMA, engages the epidermis but then traverses through the epidermis by absorptive transport as a P-glycoprotein (P-gp) substrate and reaches the subepidermal and subcutaneous environment to engage and damage the tumor vasculature. This property is essential to its antitumor activity and provides a novel paradigm for cutaneous drug therapy.

Materials and Methods

Chemicals and antibodies

Ing3A was kindly provided by Peplin. PMA and cyclosporin A (CsA) were purchased from LC Laboratories. Dexamethasone, indomethacin, and verapamil were purchased from Sigma. XR9576 (tariquidar) was a gift of Xenova Research. Anti-PKCα, PKCδ, PKCε, PKC-pan, and mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase (MEK) 1/2 antibodies were from Santa Cruz Biotechnology. Anti-phospho–PKC-pan (P–PKC-pan), phospho-PKCδ (P–PKCδ), phospho-ERK1/2 (P–ERK1/2), and phospho-MEK1/2 (P–MEK1/2) antibodies were purchased from Cell Signaling, Inc. Anti–P-gp (clone C219) monoclonal antibody was from Fujirebio Diagnostics.

Cell culture

Mouse squamous carcinoma cell line PAM212 (17) was cultured as described previously (18). Mouse melanoma B16 cell line was kindly provided by Dr. Glenn Merlino (National Cancer Institute, Bethesda, MD) and cultured in DMEM (Lonza). Hifive cells overexpressing human P-gp (19), KB-31 and KB-V1 cells (20), and HCT-15 and HCT-15 cells overexpressing human P-gp (21) were cultured as previously described. Immortalized mouse endothelial cell line 3B11 was purchased from the American Type Culture Collection and cultured according to the instruction. The endothelial tube formation assay was carried out by plating and culturing 3B11 cells on BD Matrigel (BD Biosciences). The preformed endothelial tubes were then treated with Ing3A, PMA, or CsA and photographed using an inverted phase-contrast microscope (Nikon). EpiDerm Full Thickness 400 human skin equivalent was purchased from MatTek Corp. and cultured on rafts. Cultures were incubated in serum-free DMEM for 24 hours, and Ing3A or PMA was applied directly on the air surface.

Tumor grafts and topical treatment of mice

Athymic NCr-nu/nu and BALB/cAnNCr-nu/nu mice were obtained from the National Cancer Institute (NCI)–Frederick (Frederick, MD), and SKH1 mice were purchased from Charles River Laboratory. PAM212 tumors were established by s.c. injection of 5 million cells on the flank of the BALB/cAnNCr-nu/nu mice. B16 tumors were obtained by s.c. injection of 250,000 cells on the flank of athymic NCr-nu/nu mice. For topical treatment of the mice, Ing3A, PMA, or other reagents were dissolved in acetone. All mouse experiments were performed under a protocol approved by the NCI Animal Care and Use Committee.

Immunoblotting

Cell lysates were collected in lysis buffer from Cell Signaling and then processed for immunoblotting. For detection of mdr1 in mouse skin and liver and human skin equivalents, the frozen tissues were first mechanically disrupted, placed into a buffer containing 25 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 250 mmol/L sucrose, 1 mmol/L EDTA, 1 mmol/L EGTA, and 100 μmol/L phenylmethylsulfonyl fluoride; and then sonicated. The particulate fractions of the tissue extracts were isolated by ultracentrifugation at 100,000 × g for 1 hour and then subjected to immunoblotting using anti–P-gp antibody. Horseradish peroxidase–conjugated goat antirabbit or anti-mouse IgG antibodies were used as secondary antibodies (Cell Signaling). The immunoblots were visualized by enhanced chemiluminescence (Pierce).

Multiplex bead array assay for cytokines/chemokines

Skin samples were collected from mice after treatment and then homogenized in PBS. The supernatants were collected after centrifugation at 14,000 rpm for 30 minutes. The level of multiple cytokines/chemokines in the skin extracts was detected using a multiplex kit (Mouse Cytokine/ Chemokine 22 Plex kit, Linco) according to the manufacturer’s instruction and carried out using BioPlex (Bio-Rad).

Reverse transcription-PCR

The expression of mouse tumor necrosis factor α (TNFα) RNA by PAM212 cells was detected by reverse transcription followed by PCR using primers described previously (22). The PCR products were resolved by 2% agarose gel. The expression of the mouse mdr1 transcript Abcb1a (primer set from SuperArray) from the skin and liver of nude mice and human ABCB1 from skin equivalents was detected by reverse transcription followed by quantitative competitive reverse transcription-PCR (Bio-Rad) and normalized to the level of glyceraldehyde-3-phosphate dehydrogenase in each sample.

Histology and immunohistochemistry

Tissue samples were fixed in 4% formalin (Fishier, Inc.). Tissue sections were stained with H&E. Immunohistochemistry for myeloperoxidase (Dako), CD3 (PECAM-1; Santa Cruz Biotechnology), CD3 (Dako), F4/80 (Caltag), and CD45R/B220 (BD) was performed to detect neutrophils, endothelial cells, T lymphocytes, macrophages, and B lymphocytes, respectively.
Labeling and detection of vasculature in mouse skin

To reveal the blood vessels in mouse skin, control or treated mice were anesthetized and then injected with FITC-lectin (Vector Laboratories) via tail vein. After 2 minutes, the mice were perfused with 4% paraformaldehyde via the left ventricle (23). Skin sections (100 μm) were mounted with mounting medium containing 4′,6-diamidino-2-phenylindole (Vector Laboratories) for visualization by confocal microscopy (Zeiss NLO510). A series of images were taken and projected in three dimensions using Zeiss LSM Image Browser.

Photolabeling of P-gp with [125I]iodoaryazidoprazosin

The interaction of Ing3A with P-gp was assessed by an in vitro photo labeling assay as previously described (24). Briefly, crude membranes from Hifive cells overexpressing human P-gp were incubated with Ing3A, PMA, or CsA for 5 minutes, after which 3 to 5 nmol/L [125I]iodoaryazidoprazosin (IAAP; 2,200 Ci/mmol; Perkin-Elmer) in 50 mmol/L Tris-HCl (pH 7.5) was added. After exposure to UV light for 10 minutes at room temperature, the samples were separated by electrophoresis, followed by drying and exposing to Bio-Max MR film (Eastman-Kodak). Alternatively, the dried gel was scanned by STORM 860 phosphor-imager system (Molecular Dynamics) and then quantified by the ImageQuant software.

Efflux assay for P-gp–mediated transport

Inhibition of P-gp–mediated transport by Ing3A or PMA was determined by flow cytometry using P-gp–expressing
KB-V1 cells and the P-gp–specific substrate calcein AM, as described previously (24). To evaluate P-gp–mediated transport of Ing3A, cell-associated Ing3A was determined using cultured KB-V1 and KB-31 cells incubated with Ing3A or Ing3A plus XR9576. The ethanol extracts of cells were collected and subjected to mass spectrum analysis for quantification of Ing3A.

Results

**Ing3A activates PKC in mouse keratinocytes and mouse skin**

Ing3A directly binds and activates PKC in vitro (12). A single dose of 25 nmol of Ing3A or PMA applied topically onto the back skin of nude mice induced phosphorylation of PKC isoforms and MEK1/2 (Fig. 1A and B), suggesting that Ing3A, like PMA, activates PKC in mouse skin. Figure 1C shows that the induction of TNFα by both Ing3A and PMA was similar in mouse skin. SCC in situ is one target for Ing3A therapy, and Ing3A, similar to PMA, induced a rapid and dose-dependent phosphorylation of ERK1/2, PKC-pan, and PKCδ; downregulated PKCα, PKCδ, and PKCζ; and elevated TNFα transcripts in cultures of the mouse squamous cancer cell line PAM212 (Supplementary Fig. S1A–C). Although Ing3A and PMA activate PKC similarly in PAM212 cells, Ing3A is more potent in eliciting those responses. Nevertheless, Ing3A and PMA inhibited the growth of PAM212 cells at equivalent doses (Supplementary Fig. S1D). These results indicate that Ing3A, similar to PMA, activates a common PKC pathway in both mouse skin and cultured mouse squamous cancer cells, and both agents have similar direct effects on the cancer cells.

**Topical application of Ing3A induces cutaneous inflammation and hemorrhage**

The effects of Ing3A and PMA on the skin of nude mice, the strain that has been the recipient of tumor xenografts in preclinical studies, were compared. Three hours after initial treatment, topical administration of Ing3A in either the clinical gel formulation or dissolved in acetone caused skin hemorrhage without actual breakage of the skin, but PMA did not cause visible change in the treated area (Fig. 2A). H&E staining showed that Ing3A and PMA induced some common inflammatory changes, including edema and inflammatory cell infiltration, by 3 to 6 hours, but only Ing3A induced red cell extravasation in the dermis and subcutis of the skin (Fig. 2A). Myeloperoxidase staining revealed that the predominant infiltrating cells are neutrophils in both treatment groups.
Ing3A also induced subepidermal hemorrhage when applied to immunocompetent mice of the hairless SKH1 strain, indicating that immunodeficiency was not responsible for the unique response. Ing3A did not increase B lymphocyte, T lymphocyte, or macrophage infiltration in the skin of SKH1 mice when examined at 5 hours after treatment (Supplementary Fig. S2); however, both Ing3A and PMA activated mast cells similarly (Supplementary Fig. S3). To investigate the pathways that mediate Ing3A-induced inflammatory responses including the hemorrhage, various pathway specific inhibitors were tested by applying the inhibitors 15 to 30 minutes before the application of Ing3A and examining the nude mice 5 hours later. As shown in Fig. 2C, dexamethasone and indomethacin successfully blocked Ing3A-induced neutrophil infiltration but not hemorrhage. Both dexamethasone and indomethacin are known to inhibit PMA-induced inflammation and inflammatory mediators (25, 26). In contrast, CsA blocked hemorrhage but not neutrophil infiltration in Ing3A-treated mice. These results suggest that Ing3A-induced hemorrhage and inflammatory cell infiltration are two independent events that are mediated by distinct pathways. In contrast, Ing3A- and PMA-induced inflammatory cell infiltration is similar. A multiplex assay was used to

![Figure 3](attachment:image3.png)

Figure 3. Ing3A damages blood vessels in vitro and in vivo. A, the preformed capillary network from 3B11 cells with or without CsA (20 μmol/L) for 30 min was treated with Ing3A or PMA (50 nmol/L). Photos were taken 1 h later. Scale bar, 50 μm. B, FITC-lectin–labeled blood vessels in mouse skin from mice treated with acetone (Control), Ing3A, or PMA. Blood vessels in 100-μm-thick skin sections were visualized using confocal microscopy. The pictures (top or side view) are three-dimensional projections from a series of z-sections. Scale bar, 50 μm. Damaged areas are outlined in Ing3A–treated skin samples. Blue, nuclei. C, CD31 immunohistochemistry detects blood vessels in skin from mice treated with acetone (Control) or Ing3A. Scale bar, 25 μm. Bottom, vessel ultrastructure images. Scale bar, 2 μm.
determine the level of 22 cytokines/chemokines in skin extracts collected from mice treated with either Ing3A or PMA. Eighteen of 21 detected cytokines/chemokines were induced by both Ing3A and PMA treatment, and the induction of each cytokine/chemokine was similar by either inducer (Supplementary Table S1). These results indicate that a unique pattern of Ing3A-induced cytokine/chemokine secretion in skin is not observed and could not be responsible for Ing3A-induced hemorrhage.

**Ing3A penetrates beyond the epidermis in skin and damages blood vessels *in vivo***

We hypothesized that Ing3A-induced hemorrhage may be caused by Ing3A directly targeting the blood vessels in the skin. It has been reported previously that activation of PKC in monolayer cultured endothelial cells induces endothelial cell activation but not cell death (27). To test Ing3A in three-dimensional culture conditions, mouse 3B11 endothelial cells were plated on Matrigel, where they formed a network of capillary-like structures composed of tubes and branches (Fig. 3A). Addition of Ing3A or PMA caused disaggregation of the endothelial tube and outward migration of single endothelial cells. Pretreatment with CsA did not block the effect of Ing3A or PMA *in vitro*, suggesting that CsA does not directly protect endothelial cells when Ing3A is applied after CsA *in vivo*. Figure 3B shows FITC-lectin–labeled blood vessels from control- and PMA-treated mice displayed continuous green fluorescence. However, loss of continuous FITC-lectin staining in Ing3A-treated mice is detected 40 minutes after initial treatment, well before the appearance of hemorrhage on the treated skin. To better visualize the three-dimensional images of FITC-lectin–labeled blood vessels, a surface rendering technique (Imaris 6.1 software) was used. The reconstructed FITC-lectin–labeled vessels are markedly damaged by Ing3A treatment (Supplementary Fig. S4), leading to discontinuous patterns appearing as “holes.” Damage was detected well into the deep dermis in the large- or medium-sized blood vessels in the skin. Immunohistochemistry for CD31 expression and electron microscopy also revealed the loss of the structural integrity of the cutaneous blood vessels after topical Ing3A treatment (Fig. 3C). Disruption of basement membrane of venules, endothelial cell activation in arterioles, and loss of junctions between endothelial cells were documented by electron microscopy (Fig. 3C; data not shown). Disrupted extracellular matrix, RBC extravasation, and hemolysis in the blood vessels were also observed in skin from Ing3A-treated mice. Because Ing3A and PMA had similar effects on endothelial cell tubes *in vitro*, we asked if PMA would mimic Ing3A if it were delivered more directly to endothelial cells in skin. Hemorrhage was observed in mice injected s.c. with either Ing3A or PMA as early as 1 to 3 hours (Supplementary Fig. S5). These results indicate that skin hemorrhage could also be induced by PMA if it is placed closer to the cutaneous blood vessels. To test if topical Ing3A penetrates more deeply in skin than PMA to reach the blood vessels in the dermis and subcutis, P-ERK1/2 immunohistochemistry was performed on skin sections obtained from nude mice treated topically with either Ing3A or PMA. Figure 4A shows that phosphorylation of ERK1/2 induced by Ing3A and PMA in epidermis occurs as
early as 30 minutes. In the dermis and subcutis of skin, Ing3A induced P-ERK1/2 in fibroblasts and endothelial cells to a much greater extent than PMA. Pretreatment with CsA reduced Ing3A-induced ERK1/2 phosphorylation in the fibroblasts and endothelial cells in the dermis and subcutis of skin but not in epidermis. These results suggest that, via a CsA-dependent mechanism, Ing3A penetrates into the subepidermal compartment of skin rapidly and activates PKC in multiple compartments, whereas PMA elicits most responses in the epidermis. Because mouse epidermis is relatively thin, we repeated these experiments by topically treating human skin organotypic raft cultures that reproduce the morphology of human skin, including a stratum corneum (Fig. 4B). As seen for mouse skin in vivo, P-ERK1/2 was stimulated extensively in the reconstructed epidermis by both PMA and Ing3A, but widespread and deep dermal fibroblast stimulation was seen only with Ing3A.

**Ing3A, but not PMA, exhibits anticancer activity for squamous carcinoma and melanoma**

PAM212 or B16 cells were s.c. injected on the dorsum of nude mice at four sites per mouse following the protocol used for the preclinical studies (14). When tumors were visible, the skin over the tumor sites was treated with vehicle

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**Figure 5.** Topical treatment with Ing3A for 3 d reduces tumor size. A, skin overlying subcutaneous tumors derived from PAM212 cells was treated with Ing3A or PMA (25 nmol each). Tumor volume was measured on days 13 and 23 after the initial treatment. Each tumor was normalized to its size at day 0 and expressed as percentage of the initial size. Eight tumors from each group are shown. The dashed line in each panel indicates 100%. B, skin overlying tumors derived from B16 cells was treated with Ing3A or PMA. Tumor volume was measured 10 d after the initial treatment. The mean value in each group is shown. **, P ≤ 0.05. C, H&E staining of the tissue sections from acetone- or Ing3A-treated B16 tumors. Tumors were collected 1 or 5 d after the initial treatment. Magnified areas are indicated by boxes. Scale bars, 500 μm (a–c) or 25 μm (insets A–E).
(acetone), Ing3A, or PMA for 3 consecutive days (the clinical protocol) and the tumor size was measured before and after treatment. As shown in Fig. 5A for PAM212 treated with Ing3A, five tumors were either eliminated or reduced, whereas two tumors were initially reduced and then had resurgent growth between the two measurements. In contrast, the majority of acetone control–treated and PMA–treated PAM212 tumors maintained their size or showed limited growth during the two measurement intervals on days 13 and 23, whereas several showed enhanced growth without treatment. Mice grafted with B16 cells were treated 3 days after injection of tumor cells, and tumor measurements were taken 10 days later due to the rapid growth of B16 tumors. Ing3A treatment eliminated the B16 tumor at five of the eight treated sites (Fig. 5B). In contrast, PMA-treated tumors grew even larger than the controls. Blocking neutrophil infiltration by dexamethasone had no effect on Ing3A-induced tumor reduction. However, blocking hemorrhage by CsA partially reversed Ing3A-mediated anticancer activity (Supplementary Table S2). B16 tumors were also collected 1 or 5 days after initial Ing3A treatment. As shown in Fig. 5C, 1 day after Ing3A treatment, severe hemorrhage is observed at the tumor periphery. Tumor cells in the upper part of the tumor are necrotic and blood vessels are engorged with cells (Fig. 5C, b and insets B and C), but cells in the deeper tumor mass (Fig. 5C, inset D) seem viable. Five days after initial treatment, the entire tumor mass was necrotic (Fig. 5C, c and inset E). These results suggest that topical Ing3A treatment penetrates through the epidermis into the dermis and subcutaneous

**Figure 6.** Ing3A binds to and is transported by P-gp. A, detection of P-gp protein (immunoblotting) and mRNA in mouse skin and two independent samples of human skin reconstructions. B, P-gp inhibitors prevent Ing3A-induced hemorrhage in nude mice. CsA (160 nmol/mouse), verapamil (90 nmol/mouse), and XR9576 (250 nmol/mouse) were applied topically 30 min before Ing3A (25 nmol) and photographed 5 h later. C, Ing3A reverses resistance to doxorubicin (Dox). HCT-15 and P-gp–overexpressing HCT-15 cells were treated with doxorubicin in the presence of either Ing3A or PMA (5 μmol/L). Cell growth was determined 2 d later using the MTT assay. D, Ing3A directly binds to P-gp, blocks P-gp–mediated efflux, and is a substrate for P-gp. Top, plasma membranes from Hfivive cells overexpressing human P-gp were incubated with various amounts of Ing3A, PMA, or CsA and then [125I]IAAP. The incorporation of [125I]IAAP into P-gp was shown by autoradiograph and quantified. Middle, human P-gp–overexpressing KB-V1 cells were loaded with calcein AM, incubated with 5 μmol/L Ing3A or PMA, and analyzed by flow cytometry. Bottom, Ing3A (500 nmol/L), alone or in combination with XR9576 (2.5 μmol/L), was added to KB-V1 and KB-31 cells. After 15 min, ethanol extracts were subjected to mass spectrum analysis for quantification of intracellular Ing3A. Amount of Ing3A in each sample was normalized by total protein. Columns, mean (n = 3); bars, SE.
region and initially targets blood vessel in the tumor periphery, eventually damaging the blood supply to the tumor and causing tumor necrosis.

**Ing3A, but not PMA, binds to and inhibits P-gp function**

The inhibitory effect of CsA on Ing3A-mediated anticancer activity is not likely to result from the involvement of the calcineurin/NFAT pathway because both Ing3A and PMA induced similar levels of interleukin (IL)-2 (Supplementary Table S1). In addition, other phosphatase inhibitors did not block Ing3A-induced hemorrhage (data not shown). CsA was known to target and inhibit the drug transport activities of P-gp, which is linked to the development of multidrug resistance in cancer cells (28). The expression of mdr1/P-gp has been reported in keratinocytes of neonatal and adult mouse skin (29, 30). We also detected the expression of mouse mdr1/P-gp protein and transcript (Abcb1a) in skin extracts from adult nude mice and from extracts of human skin organotypic raft cultures (Fig. 6A). To confirm the involvement of P-gp in Ing3A-induced hemorrhage, other modulators of P-gp, verapamil (31) and XR9576 (32), were applied to the back of nude mice before the application of Ing3A. As shown in Fig. 6B, all three compounds successfully blocked Ing3A-induced hemorrhage. Because CsA, verapamil, and XR9576 are specific but structurally unrelated modulators for P-gp, our results suggested that P-gp contributed to Ing3A-induced hemorrhage in skin. In addition, Ing3A, but not PMA, successfully competed with radiolabeled photoaffinity substrate IAAP for direct binding to P-gp (Fig. 6D, top). At nontoxic concentration, Ing3A, but not PMA, also reversed the resistance to doxorubicin in human P-gp–expressing HCT-15 cells (Fig. 6C). Furthermore, Ing3A was more potent than PMA in blocking calcein AM efflux in human P-gp–expressing KB-V1 cells (Fig. 6D, middle). These results show that Ing3A binds to and inhibits the function of human P-gp. The cell-associated Ing3A was significantly less in P-gp–overexpressing KB-V1 cells than its parental line KB-31, and this was reversed by P-gp inhibitor XR9576 (Fig. 6D, bottom), showing that Ing3A is a transport substrate for P-gp. Based on these findings and the other data presented, we suggest that, as a substrate for P-gp, topically applied Ing3A is transported from the epidermis to the subepidermal cutaneous tissue and causes direct damage on the blood vessels in the dermis and subcutis; in contrast, topically applied PMA is more restricted to the epidermis of skin and does not directly damage the vasculature in the subepidermal compartments.

**Discussion**

Inhibition of both tumor blood flow and neoangiogenesis has been considered a promising approach to cancer therapy. Bevacizumab, a humanized monoclonal antibody to vascular endothelial growth factor, is currently approved as monotherapy or in conjunction with a traditional chemotherapy regimen for colorectal and lung cancers (33). This approach has not been considered for common skin cancers, as the cost is prohibitive and surgical therapies are available. The sensitivity of endothelial cells and endothelial tubes to disruption by PKC activation has been documented in the past (27), and Ing3A may provide a topical approach to penetrate into invading tumor tissue with an agent that targets intrinsic tumor blood vessels through the PKC pathway.

A previous study of topical Ing3A and placebo on mouse skin clearly showed the importance of the neutrophilic infiltrate to mediate the necrotic response of the tumor and prevent tumor recurrence (16). PKC-mediated neutrophilic cutaneous infiltrates are the response to the upregulation of specific chemokines from activated keratinocytes (34, 35), and both topical Ing3A and PMA induced similar chemokine/cytokine profiles in treated skin and the inflammatory response was inhibited by dexamethasone and indomethacin, the same anti-inflammatory agents shown previously to block PKC-mediated inflammation (26, 36, 37). Inhibitor studies indicated that blocking the Ing3A-induced inflammation was not by itself sufficient to alter the antitumor response, whereas blocking the associated hemorrhage reduced antitumor activity. Irritant activity of Ing3A likely accounts for the weak tumor-promoting response on mouse skin (13), but experiments show that irritant activity predominates in eradicating tumors (13). By restricting topical exposure to lesional areas in the therapeutic setting, the irritant activity should not impede efficacy.

The unique finding that CsA inhibited Ing3A-mediated hemorrhage and reduced the antitumor activity without altering the inflammatory response suggested that disruption of tumor vasculature and induction of skin hemorrhage are principal actions for Ing3A to be an effective anticancer drug on skin. Both Ing3A and PMA exerted the identical disruptive effect on cultured endothelial tubes that would be consistent with *in vivo* vascular leakage, but *in vivo*, activated endothelial cells were only detected in the dermis and subcutis after Ing3A treatment of intact skin. Previous studies on *ex vivo* endothelial cells confirmed that Ing3A treatment activated endothelial cells in a PKC-dependent response, upregulating E-selectin, ICAM1, and IL-8 and leading to neutrophil adhesion and migration (38). In our *in vivo* study, electron microscopy, light microscopy, immunohistochemistry, and *in vivo* lectin labeling indicated that only Ing3A disrupted the vasculature below the epidermis and in grafted tumors. However, PMA could also induce hemorrhage if it was delivered to bypass the epidermis. Together, these data indicate that a unique property of Ing3A is its ability to penetrate beyond the epidermis and into the dermis and subcutis where the vasculature resides. Our results indicate that Ing3A, but not PMA, binds to and is a transport substrate for P-gp, that P-gp is expressed in the skin, that multiple disparate modulators of P-gp prevent Ing3A-induced hemorrhage, and that CsA reduces the antitumor activity of Ing3A. Together, we conclude that active transport of Ing3A through the ABC transporter, ABCB1, provides the unique activity of Ing3A, once it has penetrated into the epidermal layer, to be actively transported to the subepidermal compartments. The antitumor benefit of Ing3A thus is provided through an action on deeply embedded tumor cells where it may be directly cytotoxic or growth inhibitory (Supplementary Fig. S1D; ref. 39), inflammation...
provoking, and disruptive of the tumor vasculature through PKC activation of endothelial cells.

P-gp has traditionally been associated with resistance to a variety of natural product anticancer drugs due to its function as the drug efflux pump and decreased drug accumulation in cancer cells. However, P-gp is present in the membranes of many normal cell types, including epithelial cells, where it may function to transport environmental toxins and toxic metabolites out of cells to protect the host. Skin is largely protected from environmental toxins through the formation of the lipid barrier encompassed in the stratum corneum (40). Drug penetration through this barrier and the consequent complex metabolic enzymes of the epidermis complicate this site as a therapeutic portal (41). Mouse and human epidermis and keratinocytes express the mdr1a and mdr1b/MDR1 (ABCB1) gene, and the functional activity of human cutaneous P-gp has been shown by studies of rhodamine 123 transport and inhibition by verapamil and other P-gp inhibitors (30, 42). Furthermore, the expression and activity of P-gp in human skin keratinocytes is increased by inflammatory mediators that would be found in a tumor microenvironment (43). The functioning of cutaneous P-gp in “absorptive” transport, carrying drugs from the skin surface to the dermis and subcutis, was aptly shown by the elegant studies of Ito and colleagues (29). Using genetic approaches, these authors showed that P-gp is essential for the efficient delivery of drugs across the epidermis and into the dermis and hypodermis of mouse skin. Further, they showed by pharmacologic experiments that inhibition of P-gp prevented absorptive drug transport. Our studies now indicate that the biological effectiveness of Ing3A, an anticancer agent that can pass the stratum corneum barrier and traffic to and functions in the dermis and hypodermis, is through P-gp–mediated absorptive drug transport. Whereas our biological conclusions are drawn from mouse preclinical models and known P-gp inhibitors, our studies shown in Fig. 4 indicate that Ing3A penetrates into the dermis of human skin organotypic reconstructions and in Fig. 6 indicate that Ing3A is a substrate for human P-gp. Given this mechanism of action for Ing3A and potentially other topical therapeutics, the documented polymorphisms in the human MDR1 (ABCB1) gene (28, 44) could be another determinant of therapeutic efficacy for individual patients. The skin is increasingly considered an advantageous site for drug delivery (45). The prospect of individual variation in absorptive drug transport may need to be investigated to predict therapeutic potential through this portal.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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The Skin Cancer Chemotherapeutic Agent Ingenol-3-Angelate (PEP005) Is a Substrate for the Epidermal Multidrug Transporter (ABCB1) and Targets Tumor Vasculature

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