Vitamin D3 enhances the apoptotic response of epithelial tumors to aminolevulinate-based photodynamic therapy

Sanjay Anand¹,², Clara Wilson², Tayyaba Hasan³, and Edward V. Maytin¹,²,³,⁴

¹Departments of Dermatology; and ²Biomedical Engineering, Lerner Research Institute, Cleveland Clinic, Cleveland, OH 44195; and ³Wellman Center for Photomedicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114.

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To whom correspondence should be addressed, at:
Edward Maytin, M.D. Ph.D.
Mailstop ND-20, Biomedical Engineering,
Cleveland Clinic,
9500 Euclid Avenue, Cleveland, OH 44195
E-mail: maytine@ccf.org

Abbreviations used:
ALA, δ-aminolevulinic acid; ALAD, ALA dehydratase; calcitriol, 1α,25-dihydroxy-vitamin D3; CPO, coproporphyrinogen oxidase; E-cad, E-cadherin; EdU, 5-ethynyl-2'-deoxyuridine; FC, ferrochelatase; PBGD, phorphobilinogen deaminase; PDT, photodynamic therapy; PpIX, protoporphyrin IX; TNFα, tumor necrosis factor alpha; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; Vit D, vitamin D.

Running Title: Calcitriol and photodynamic therapy of squamous carcinoma
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ABSTRACT

Photodynamic therapy, mediated by exogenously administered aminolevulinic acid (ALA-PDT) followed by exposure to a laser or broadband light source, is a promising modality for treatment of many types of cancers, but it remains inadequate to treat large, deep solid tumors. Here we report that calcitriol, the active form of Vitamin D3, can be administered prior to ALA as a non-toxic preconditioning regimen to markedly increase the efficacy of ALA-PDT. Using mouse models of squamous skin cancer for preclinical proof of concept, we showed that calcitriol delivered topically or intraperitoneally increased tumoral accumulation of the PDT-activated ALA product protoporphyrin-IX (PpIX) up to 10-fold, mainly by altering expression of the porphyrin synthesis enzymes coproporphyrinogen oxidase (increased) and ferrochelatase (decreased). Calcitriol-pretreated tumors underwent enhanced apoptotic cell death following ALA-based PDT. Mechanistic studies documented activation of the extrinsic apoptotic pathway, with specific cleavage of caspase-8 and increased production of TNFα in tumors preconditioned by calcitriol treatment before ALA-PDT. Very low doses of calcitriol (0.1-1 μg/kg body weight) were sufficient to elicit tumor-selective enhancement to ALA-PDT efficacy, rendering toxicity concerns negligible. Our findings define a simple, non-toxic and highly effective preconditioning regimen to enhance the response of epithelial tumors to ALA-PDT, possibly broadening its clinical applications by selectively enhancing accumulation of photosensitizer PpIX along with TNFα in tumors.

Précis (approved by the Editor-in-Chief):

This important study shows how vitamin D3 can be used as a simple, nontoxic and highly effective pre-conditioning regimen to enhance the response of epithelial tumors to ALA-PDT, possibly broadening its clinical applications.
INTRODUCTION

Photodynamic therapy (PDT) is a cancer treatment that combines a photosensitizer (PS), light, and oxygen to achieve selective cell killing (1, 2). PDT offers the advantage of dual targeting, since both the PS and the visible light can be effectively localized to the tumor (1). Excellent selectivity can be achieved with 5-aminolevulinic acid (5-ALA; ALA), a small molecule precursor for the synthesis of endogenous porphyrins, most notably protoporphyrin IX (PpIX) within cells (3). Because PpIX is the actual photosensitizer, not ALA, and because pathways that regulate ALA uptake and conversion into PpIX are generally more active in tumor cells than in normal cells, tumor-selective PpIX accumulation to very high levels can be obtained (4). ALA-PDT has proven clinically useful for wide field treatment of carcinoma in-situ of the skin (4-7) and cervix (8). ALA-PDT is also useful for image-guided surgical resection of urinary tract dysplasia (9) and high-grade gliomas in the brain (10, 11). However when employed as a monotherapy, ALA-PDT remains unsatisfactory for large and deep tumors, with incomplete response rates and recurrence rates that are often less than ideal (reviewed in (4)).

To improve the efficacy of ALA-PDT, our goal has been to develop new combination regimens based upon the observation that certain pharmacologic agents, which we call "differentiating agents," can enhance PpIX accumulation in a variety of epithelial cancer cells (12-14). For example, we recently reported that methotrexate, administered systemically as a 3-day preconditioning regimen prior to ALA-PDT, significantly enhances the accumulation of PpIX within squamous tumor cell lines in vitro and in vivo (14, 15). Remarkably, MTX exerts these effects at subnanomolar concentrations (i.e., 100- to 1000-fold lower than those typically used in the clinic (14, 15), clearly too low to be tumoricidal yet sufficient to trigger differentiation in choriocarcinoma cells (16, 17), normal keratinocytes (18), and A431 carcinoma cells (15). At these same low levels, methotrexate was shown to enhance PpIX accumulation through a mechanism involving increased expression of coproporphyrinogen oxidase (CPO), one of the porphyrin-synthetic enzymes (15).
In the current paper, another class of molecule, Vitamin D, is shown to selectively enhance PDT by preferentially increasing PpIX accumulation in preneoplastic and neoplastic cells in vivo, while causing very little damage to normal tissue. Vitamin D comprises a family of steroid hormones that regulate bone and mineral metabolism, calcium homeostasis, and cellular differentiation; reviewed in (19-21). While pathways for synthesis and processing of the various isoforms of Vit D are rather complex, it is clear that the major isoform which interacts with nuclear receptors and mediates hormone effects at the end-organs is the tri-hydroxylated molecule 1α, 25-dihydroxy-vitamin D3 (or calcitriol) (20). The first indication that Vit D might be useful for enhancing PDT was our finding that calcitriol and synthetic Vit D analogs can stimulate increases in PpIX levels in LNCaP prostate cancer cells (13). In other cells, however, this phenomenon did not seem entirely consistent. For example, when Vit D was added to cultures of skin keratinocytes, no PpIX-elevating effect was observed in monolayer cell culture (22). However, when the same cells were exposed to calcitriol in a 3-D (organotypic) culture system, they responded by accumulating significantly higher levels of PpIX (22). The remarkable potency of calcitriol (in the picomolar range) for stimulating PpIX accumulation in 3-D cultures, coupled with indications that such effects require a native tissue environment, encouraged us to examine Vit D preconditioning in vivo.

Here, we describe an investigation of the ability of Vit D to affect PpIX accumulation in fully-formed epithelial tumors in vivo. Two different tumor models were used to represent premalignant dysplasia and malignant carcinoma, respectively. We confirm that Vit D confers a tumor-specific increase in photosensitizer (protoporphyrin) levels, and demonstrate the underlying mechanism, namely, a change in the expression of two rate-limiting porphyrin-synthetic enzymes. Further, we show that Vit D preconditioning selectively stimulates increased tumor cell killing after ALA-PDT, relative to unconditioned controls. Finally, we explore the mechanism of increased cell death and show that Vit D encourages activation of the extrinsic apoptotic pathway after ALA-PDT, accompanied by massive increases in TNFα production.
MATERIALS AND METHODS

Chemicals.
Vit D ointments Dovonex® (calcipotriene, 0.005%) and Vectical® (calcitriol, 3 μg/g) were obtained from Warner Chilcott Inc. (Rockway, NJ) and Galderma Laboratories (Fort Worth, TX), respectively. Calcitriol (1α, 25-dihydroxyvitamin D₃) was from Sigma-Aldrich (Saint Louis, MO).

Generation of tumor models in vivo.
Superficial tumors in SKH-1 mice were generated by topical application of DMBA and TPA as described (15). To generate deep subcutaneous tumors, immunocompromised nude mice (Charles River Laboratories) were injected with 2 x 10⁶ A431 cells in the flanks, as described (15). The A431 human squamous cell carcinoma cell line was purchased from ATCC (American Type Culture Collection; Manassas, VA) where the line was characterized by morphological appearance during growth and recovery, cytochrome C oxidase I PCR for species specificity, and human origin by short tandem repeat (STR) profiling.

Preconditioning regimen using Vitamin D.
Superficial tumors were preconditioned with topical Vectical, Dovonex, or petrolatum (control) once daily for 3 days followed by topical application of ALA (Levulan Kerastick, DUSA Pharmaceuticals, Wilmington, MA) for 4 hours on the fourth day. Deep tumors were preconditioned via systemic Vit D in PBS (1 μg/kg) or PBS alone, delivered daily for 3 days by intraperitoneal route. ALA was administered as described (15).

Analysis of PpIX fluorescence and histological markers in tumors.
For analysis of PpIX levels, tumors were harvested, embedded for frozen sectioning, and analyzed on a Leica confocal microscope as described (15). Standard hematoxylin-and-eosin staining was done on formalin-fixed/paraffin-embedded tissues, and immunohistochemical staining was performed as described (23). Sources and dilutions of antibodies were: Active caspase-3 (BioVision, Mountain View CA; 1:50), ferrochelatase FC (1:100), E-Cad (Santa Cruz...
Biotechnology, 1:100), Ki67 (NeoMarkers, Fremont, CA; 1:250), GAPDH (Santa Cruz Biotechnology; 1:100), TNFα (BioXcell, 1:100), CY3 or FITC-conjugated Donkey anti-rabbit (Jackson ImmunoResearch; 1:1500). To assess cell proliferation in vivo, EdU (5-ethynyl-2′-deoxyuridine) was injected (100 μg/mouse, i.p.) 1 hour prior to sacrifice. Formalin fixed tissues were sectioned and incubated with the Click-iT® reaction cocktail per manufacturer’s instructions (Invitrogen, Carlsbad, CA). To estimate cell death, the TUNEL assay was performed on paraffin sections as directed by the manufacturer (Roche Applied Science, Indianapolis, IN).

Light exposures for PDT in vivo.

Tumors were irradiated at a fluence of 100 J/cm² using a 633 nm non-coherent light source (LumaCare Products, Newport Beach, CA). The source was calibrated using a FieldMate laser power meter (Coherent, Portland OR).

Western blot analyses.

Frozen tumors were powdered in a tissue pulverizer (Fisher Scientific, Waltham, MA), dissolved in urea lysis buffer, and analyzed by the western technique as described (15). Primary antibodies were directed against: ALAD (Abnova, Walnut CA; 1:1000), Active/Cleaved caspase-8 (Novus Biologicals, Littleton CO; 1:5000), Cleaved caspase-9 (Cell Signaling Technology, Danvers MA; 1:1000), Caspase-12 (Santa Cruz Biotechnology, Santa Cruz CA; 1:1000), ferrochelatase FC (a gift of Dr. Harry Dailey; 1:5000), GAPDH (Santa Cruz Biotechnology; 1:5000), PBGD (Abnova; 1:1000), TNFα (BioXcell, West Lebanon NH; 1:1000) and α-tubulin (Sigma-Aldrich; 1:10000). Secondary peroxidase-conjugated goat anti-rabbit IgG or donkey anti-rabbit IgG, or goat anti-rat IgG were used (Jackson ImmunoResearch, West Grove, PA; 1:20,000). Anti-CPO, a custom-made antibody (15), was used at 1:5000 dilution.

Measurements of serum Vit D (calcitriol) levels.

Serum levels of Vit D were determined in SKH-1 mice treated with systemic or topical calcitriol, or vehicle controls, as described in Supplemental Table 1. Mice were sacrificed by cardiac puncture.
Serum was isolated from the blood after coagulation and stored frozen at -80 °C, then shipped to Heartland Assays Inc. (Ames, IA) on dry ice for determination of serum calcitriol levels by radioimmunoassay (RIA).
RESULTS

To test the hypothesis that Vitamin D, delivered as a preconditioning regimen, may enhance the production of intracellular protoporphyrin and subsequent responses to ALA-PDT, two in vivo epithelial tumor models were employed: (i) superficial, low-grade squamous carcinomas (papillomas) generated by chemical carcinogenesis in hairless mice (Fig. 1A), and (ii) a malignant carcinoma model in which human squamous carcinoma cells (A431) were implanted subcutaneously into immunocompromised nude mice (Fig. 1B).

Topical preconditioning of superficial tumors with Vitamin D analogs enhances the accumulation of PpIX photosensitizer.

Superficial skin tumors were pretreated topically with inert vehicle (petrolatum), or with an ointment containing one of two different active Vitamin D analogs (calcitriol or calcipotriene), daily for 3 days. The mice were then treated with topical ALA for 4 h, and tumors were harvested 4 days after the initiation of Vit D treatment. Frozen tumor sections were examined by confocal microscopy under conditions that selectively excite the PpIX molecule and allow detection of PpIX-specific fluorescence (Fig. 1C). This technique was shown previously to correlate closely with biochemical measurements of PpIX in cells and tissues (15). In Vit D preconditioned tumors, PpIX was more abundant (as documented by fluorescent intensity measurements averaged over the entire tumor, Fig. 1D) and was also qualitatively present at greater depths within the tumor, as compared to non-conditioned (control) tumors. This was true whether calcitriol (Fig. 1C) or calcipotriene (Fig. 1D) was used. With either Vit D analog, the ~3-fold increase in PpIX observed within tumors was statistically significant (Fig. 1D), whereas no differential effect upon PpIX was observed in normal skin (data not shown).

Systemically-delivered Vitamin D enhances PpIX accumulation in deep squamous (A431) tumors.

To examine effects of Vit D in an invasive tumor model, squamous carcinoma cells (A431) were implanted in nude mice. Animals with visible tumors received systemic preconditioning with
Calcitriol or vehicle alone (saline) over a 3-day period. On the fourth day ALA was given intramuscularly, and 24 hr later tumors were harvested and analyzed for PpIX levels (Fig. 1E). Calcitriol pretreatment caused a marked increase in PpIX accumulation throughout the tumor (Fig. 1E, compare upper vs. lower panels). A dose-response study (Fig. 1F) revealed a significant increase in PpIX (4-fold) at a dose of 0.1 μg/kg calcitriol, and PpIX levels reached maximal levels (10-fold) at 0.5 μg/kg calcitriol and above (Fig. 1F). Local (perilesional) injection of calcitriol into the skin was also effective for delivery (Fig. 1F, bar 7). Interestingly, intracutaneous injection of calcitriol at a site distant from the tumor also exerted a PpIX-elevating effect, although less pronounced (Fig. 1F, bar 8). Measurements of the serum calcitriol concentration at 1 hr after injection or topical application revealed calcitriol levels between 6 - 9 ng/ml; residual levels at day 4 (after three preconditioning doses) were indistinguishable from normal physiological levels of calcitriol (Supplemental Table 1).

Changes in porphyrin synthetic enzyme expression contribute to the increase in PpIX accumulation in Vit D pretreated tumors.

To explore the mechanism of increased PpIX accumulation after calcitriol pretreatment, A431 tumor lysates from mice preconditioned with calcitriol (1 μg/kg) or saline alone were examined for changes in levels of the four porphyrin-synthetic enzymes (ALAD, PBGD, CPO, and FC) that have been described as potentially rate-limiting under various circumstances in different biological systems (24-29). Two enzymes were substantially altered after Vit D pretreatment (Fig. 2A). Copropopyrinogen oxidase (CPO) was increased ~ 5-fold, and ferrochelatase (FC) was decreased ~4-fold (Fig. 2B). Each of these changes (higher CPO levels, and lower FC levels) favors an increase in accumulation of PpIX because the enzymes lie immediately upstream and downstream of PpIX, respectively.
Vitamin D pretreatment elicits preferential changes in cellular differentiation and proliferation within tumors in vivo.

In our previous studies in various epithelial cancer cells in vitro (12-14) and in vivo (15), we had shown that differentiation-promoting agents such as methotrexate cause an increase in PpIX accumulation that is tightly correlated with the enhancement of terminal differentiation. Methotrexate always caused growth arrest, but in the case of Vit D, the effects upon growth were less clear. For example, in an organotypic keratinocyte model, calcitriol caused enhanced differentiation while having no apparent effect upon proliferation (22).

To examine the effects of Vit D upon epithelial tumors in vivo, mice bearing superficial papillomas or deep A431 tumors were preconditioned with Vit D using topical or systemic delivery, respectively, and markers of cellular differentiation (E-cadherin) and proliferation (Ki67) were examined histologically (Fig. 3). After Vit D pretreatment, E-cadherin expression was increased 4-6 fold in both types of tumors, compared to an insignificant increase (< 1.5-fold) in normal skin (Fig. 3A). Vit D pretreatment also increased expression of the proliferative marker (Ki67) in both tumor types, as compared to no significant increase in normal skin (Fig. 3B). To confirm that Vit D stimulates tumor cell proliferation in vivo, a second assay was performed in which EdU was injected into mice to measure incorporation into newly-synthesized DNA. The latter experiments confirmed that DNA synthesis is stimulated in tumors by pretreatment with Vit D (Fig. 3C).

To determine which tumor cell populations express the highest levels of PpIX following Vit D pretreatment, colocalization experiments were performed in which adjacent histological sections were examined for PpIX (Supplemental Fig. 1A), E-cadherin (Supplemental Fig. 1B), and Ki67 expression (Supplemental Fig. 1C). The data show that the highest levels of PpIX in the A431 tumors correspond to differentiating areas, and these are almost completely distinct from regions that express the proliferation marker, Ki-67 (Supplemental Fig. 1A-C). The differentiating tumor areas are also the site of TNFα production following light exposure (Supplemental Fig. 1D), to be described in more detail below.
**PDT-mediated cell death is enhanced in Vitamin D preconditioned tumors.**

Tumors were preconditioned with calcitriol for 3 days, treated with ALA and exposed to light, and then harvested at various time points for analysis of cell death (Fig. 4). Using the TUNEL assay on A431 tumors, a large increase in cell killing was observed in Vit D pretreated tumors (relative to vehicle alone) at 1, 6 and 24 h, representing an ~6-fold difference between the two conditions at all time points (Fig. 4A, B). In the squamous papilloma model, a similar preferential enhancement of cell death in Vit D preconditioned tumors was observed (Fig. 4C). To better examine the microanatomical extent of destruction, tumors were also examined using hematoxylin and eosin (H&E) staining and scored by morphological criteria, primarily the complete loss of cells (Fig. 4D and Supplemental Fig. 2A,B). Although evidence of cell death was observable in control tumors by 6 h after ALA-PDT, significantly more areas of cell death were seen by 1 h in Vit D-preconditioned tumors, rising to a significantly larger fraction of the total tumor area (~250% larger) by 24 hr (Fig. 4E). Enhancement of cell killing occurred preferentially in Vit D-preconditioned tumor tissue as compared to adjacent normal skin, indicating tumor selectivity of the Vit D effect (Supplemental Fig. 2C).

**Enhancement of PDT-mediated cell death by Vit D involves cleavage of caspase-3 and caspase-8.**

Because positive TUNEL labeling indicates (but does not prove) that an apoptotic pathway is activated after ALA-PDT, histological sections from papillomas (Fig. 5A) or A431 tumors (Fig. 5B) were probed with an antibody specific for the activated (cleaved) form of caspase-3. The results show that caspase-3 is activated by ALA-PDT by 6 h in both systems, and Vit D appears to cause a preferential increase in caspase-3 activation. To learn which upstream proteolytic pathway is responsible for caspase-3 activation (30), tumor lysates were evaluated by western analysis; no activation of caspase-9 (Supplemental Fig. 3A; associated with the mitochondrial pathway) (30), nor of caspase 12 (Supplemental Fig. 3B; associated with the endoplasmic reticulum pathway) (31) could be demonstrated. In contrast, a plasma membrane-associated caspase (caspase-8) was significantly affected by Vit D pretreatment (Fig. 5C). Expression of the
precursor form (Fig. 5D) and levels of the cleaved forms, p41 and p43 (Fig. 5E), were all increased significantly. Together, these results indicate that caspase-8 mediated apoptosis plays a major role in Vit D enhancement of tumor cell death after ALA-PDT.

The combination of Vit D preconditioning and ALA-PDT significantly increases production of TNFα within tumors in vivo.

The fact that caspase-8 is preferentially cleaved in Vit D-preconditioned tumors after ALA-PDT (Fig. 5) suggests that a membrane-associated death receptor, such as TNFR, might be activated in this process. To test this possibility, levels of TNFα in calcitriol- pre-conditioned tumors were compared to TNFα levels in non-conditioned tumors; a small, < 2-fold increase in the baseline level was observed in the Vit D subset (Fig. 6A). Following ALA-PDT alone (in the absence of Vit D), a robust increase in TNFα levels of ~10-15 fold was seen at 1 h and remained nearly constant out to 24 h (Fig. 6B and 6C, left side). In contrast, ALA-PDT delivered after calcitriol preconditioning led to a dramatic and significant increase in TNFα levels, reaching a ~70-fold elevation by 24 h (Fig. 6B and 6C, right side). Immunostaining of PDT-treated tumor tissue revealed that the highest TNFα expression was found in linear, isolated regions (pockets) near the central portions of the tumors, which appear to represent vascular structures (Fig. 6D; Supplemental Fig. 1D). However, the majority of TNFα staining colocalized within tumor cells that also expressed E-cadherin, i.e., in the differentiated regions of Vit-D preconditioned tumors (Supplemental Fig.1D).
DISCUSSION

Our data show that epithelial skin tumors in vivo, whether premalignant or malignant, accumulate higher intracellular levels of PpIX when preconditioned with Vit D (calcitriol). These PpIX increases occur preferentially in neoplastic versus normal tissues. The underlying biochemical basis for these observations is an altered expression of two enzymes in the porphyrin synthesis pathway after Vit D preconditioning. CPO is increased while FC is decreased; the net effect is a greater accumulation of PpIX. As a consequence, Vit D-preconditioned tumors show an enhanced cytotoxic response to ALA-PDT.

Our data suggest that in addition to a selective increase in PpIX, other mechanisms contribute to Vit D-mediated enhancement of tumor killing. In the Vit D-pretreated tumors prior to ALA-PDT, a small (~2-fold) increase in TNFα can be detected. However, following exposure to ALA-PDT, TNFα levels become preferentially elevated in the Vit D preconditioned tumors (~70-fold above baseline), mostly within the differentiated tumor cells but also within vascular structures, compared to unconditioned tumors (~10 fold above baseline). (Of note, expression of TNFα within blood vessels and endothelial cells in inflamed tissues has been reported before (32, 33). High TNFα levels are often associated with activation of the extrinsic apoptotic pathway, in which pro-caspase-8 cleavage occurs after ligation of death receptors such as TNF-R and TRAIL-R1 on the plasma membrane of cells (34, 35). The extrinsic pathway is involved after PDT, given that caspase-8 and caspase-3 are preferentially activated, whereas caspase-9 and caspase-12 are not. In the few studies in the literature that examined mechanisms of cell death following aminolevulinate-based PDT, caspase activation in leukemia and lymphoma cells was either undetectable (36) or involved caspase-9 (37). However, human oral (epithelial) cancer cells showed activation of both caspase-8 and caspase-9 after PDT using 5-ALA (38). The latter report is consistent with our observation of ALA-PDT mediated activation of caspase-8 in Vit D-pretreated A431 epithelial tumors in vivo.
Although the ability of Vit D to elevate PpIX levels in cultured cells was described previously (13, 22, 39), our new data are significant because they establish a tumor-selective PpIX-inducing effect of Vit D in vivo. The magnitude of PpIX enhancement achievable in tumors in vivo equals or exceeds the effects obtainable in vitro, an observation that may indicate a role for paracrine signaling between epithelial and stromal cells. The 3- to 10-fold rise in PpIX accumulation in benign papillomas and A431 subcutaneous tumors compares very favorably to calcitriol-induced elevations in PpIX reported previously in cultured cell lines. For example, PpIX was elevated 1.5-fold in cultured A431 cell monolayers (39), 2.3-fold in organotypic REK keratinocytes (22), and 10-fold in LNCaP prostate carcinoma cells (13), when those cultures were preincubated with calcitriol (pM to nM concentration range, 24-96 hr incubation) followed by ALA or hexyl-ALA.

The finding that Vit D leads to activation of the extrinsic pathway, and increased TNFα production is particularly compelling because the literature suggests a beneficial link between high TNFα levels and increased PDT efficacy. Bellnier showed that for subcutaneously-implanted adenocarcinomas, injection of a single dose of recombinant TNFα at 3 hr prior to PDT (using Photofrin as the photosensitizer) led to an improved tumor response, with no deleterious effects upon normal skin photosensitization (40). The same group demonstrated that pre-administration of 5,6-dimethylxanthenone-4 acetic acid (DMXAA), a drug that elevates TNFα levels, causes significant enhancement in tumor responsiveness to Photofrin-mediated PDT in mice with subcutaneous RIF-1 fibrosarcomas (41). Also, DMXAA displayed a similar PDT-enhancing effect using a different tumor model (colon carcinoma cells) and photosensitizer (Photochlor; HPPH) (42). Although the PS used in those studies was not 5-ALA, the results suggest that any agent capable of increasing intratumoral TNFα levels at the time PDT is administered might help to increase overall cell killing. Preconditioning with Vit D represents a novel way to achieve this end.
Vit D now joins methotrexate (MTX) in a growing arsenal of agents that effectively promote PpIX accumulation. However, MTX stimulates tumor cell differentiation and inhibits proliferation (15), whereas Vit D appears to stimulate both differentiation and proliferation (Fig. 3, and Supplem Fig 1). This brings up two important questions: (i) what is the cellular basis for Vit D-stimulated proliferation in these areas, and (ii) what are the possible consequences for the therapeutic response to PDT? The fact that Vit D causes increased proliferation in the normal epidermis of mice (see Fig. 3C) is actually a well-reported phenomenon (43, 44) whose molecular basis remains controversial. A common misconception is that Vit D always causes epithelial growth-arrest, but Gniadecki showed that in human keratinocytes, exposure to calcitriol (at pM or nM concentrations) can cause either growth arrest or hyperproliferation, depending upon whether cells are undifferentiated or committed to terminal differentiation (45). In breast cancer cell lines (46), calcitriol stimulated cell growth at low concentrations (0.1 nM). In that situation, the balance between proliferation and growth arrest appeared to depend upon levels of a pro-proliferative molecule (a degradation product of calcitriol) that accumulates through the action of an oxidative enzyme, CYP24 (46). Taking everything above into account, the observation that some regions of epithelial tumors in vivo exhibit a pro-proliferative response to calcitriol whereas other regions exhibit a differentiation response appears to make sense.

The more pressing question, of course, is how the differentiated versus proliferative regions of Vit D-pretreated tumors respond to ALA-PDT, and whether the regions with low PpIX could be a problem if surviving cells were to continue to proliferate after treatment. Our data showed a robust cell kill in the central zone of nodular tumors (Supplemental Fig 2), consistent with a combined effect of ALA-PDT and hypoxia in these regions. However, the surviving rim of cells observed at the tumor periphery is a reminder that this report is only an initial description of Vit D and ALA-PDT; no judgment about preclinical efficacy can be made until detailed animal studies are performed using higher fluences of light, multiple PDT treatments, and longer-term analyses of tumor regression and regrowth. The literature, however, offers indications that hyperproliferation in
response to Vit D could represent an advantage rather than a disadvantage. Tumor cells may become more susceptible to PDT when forced to actively proliferate; this was shown in studies (with either hematoporphyrin or PpIX) examining relationships between cell cycle stage and PDT responsiveness, which showed that cells are usually most sensitive in S-phase, i.e., undergoing active DNA synthesis (47-49). Also, as suggested by Gniadecki's work (45), epithelial cells with a hyperproliferative response to Vit D may actually represent a population already committed to terminal differentiation, thereby having a relatively low growth potential. A final point is that TNFα released within Vit D preconditioned tumors may produce a bystander effect, promoting apoptosis in cells which manage to escape direct photocytotoxicity.

Thinking ahead to the clinic, it will be important to address the advantages and disadvantages of vitamin D preconditioning compared to preconditioning with methotrexate (MTX), another clinically used drug that has demonstrated efficacy in increasing levels of ALA-induced PpIX (15). The potential for undesirable side effects, e.g. liver toxicity (MTX) and hypercalcemia (Vit D), may be relatively small due to the short duration and low doses used to achieve transient PpIX-enhancement prior to PDT. However, only clinical trials can establish this for sure. In a pilot study to address the question of hypercalcemia in mice, peak and trough serum levels of calcitriol were measured (Supplemental Table1) and compared to values from the literature (50). Calcitriol serum levels were measured 1 hr after the following conditions: (i) injection of a 1 μg/kg dose; (ii) injection of an 8 μg/kg dose, replicating the study of Muindi et al. (50); (iii) a single application of calcitriol ointment. We found that peak levels were similar for all conditions (6 - 9 ng/ml), within the range reported by Muindi et al. (50). No evidence for a residual calcitriol elevation was found, i.e., the serum trough levels after 3 days of preconditioning were indistinguishable from physiological background levels in mice (50). This suggests a reasonable safety margin, given that Muindi et al. (50) employed calcitriol doses at least 8 times higher than our study, and the doses used were sufficient to exert antitumor effects without any detectable side effects in mice (50). In humans, whether or not calcitriol can offer a safe and effective adjuvant approach for PDT remains an open
question. Alternatively, could one use another form of Vitamin D, such as 25-OH Vit D3 (calcidiol, the major isoform of Vit D found in health food supplements) that requires subsequent conversion to calcitriol? These will be interesting questions for future studies.

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FIGURE LEGENDS

Figure 1. Illustration of the tumor models in this study. (A) Superficial carcinogen-induced papillomas. (B) Subcutaneous A431 human squamous cell carcinoma at 10 days after subdermal injection of 2 million A431 cells. (C) PpIX levels in superficial tumors, after 3 days of daily pretreatment with topical calcitriol (3 μg/g) or inert vehicle (petrolatum), followed by administration of 5-ALA on day 4 to stimulate PpIX synthesis. Confocal micrographs of frozen tumor sections are shown. (D) PpIX in deep A431 tumors after 3 d of pretreatment with intraperitoneal calcitriol (1 μg/kg) or with saline, followed by 5-ALA on day 4. (E) Digital quantification of PpIX-specific fluorescence in papillomas pretreated with either topical calcipotriene (0.005%) or calcitriol. Mean ± SEM from 3-6 tumors/condition; fold-increases relative to inert vehicle. (F) Digital quantification of PpIX-specific fluorescence in A431 tumors subjected to increasing doses of calcitriol (μg/kg body weight), delivered either by an intraperitoneal route or by injection into the skin at the site of the lesion (Lesion). In the latter case, another tumor located ~4 cm away from the first was analyzed to examine the systemic effect of intracutaneous injection (Distal). Mean ± SEM from 6 tumors/condition The p-values from unpaired two-sided t-tests are indicated above the brackets. Scale bar, 50 microns.

Figure 2. Expression of key enzymes in the porphyrin synthetic pathway, with or without Vitamin D pretreatment. (A), Examples of western blots from different A431 tumor lysates, visualized using antisera to ALA dehydratase (ALAD), porphobilinogen deaminase (PBGD), coproporphyrinogen oxidase (CPO), and ferrochelatase (FC); GAPDH was the invariant control. (B), Densitometric quantitation of western blots from 6 individual tumors (3 control, 3 calcitriol-treated). Asterisks, p values from unpaired two-tailed t-tests.

Figure 3. Vitamin D-induced responses in tumor cell proliferation and differentiation due to in vivo preconditioning with calcitriol. Images of paraffin sections from papillomas or A431 tumors, immunostained with antisera to the following molecules: (A) E-cadherin; (B) Ki-67; (C) EdU (in
mice injected with EdU 1 hr prior to sacrifice). (D) Positive and negative controls for immunostaining. Quantitation of relative intensity of staining (measured with a thresholding technique described in Methods) from multiple histological specimens from papillomas or A431 tumors. A minimum of 3 tumors was analyzed for every data point. Numbers above each bracket are the p value of unpaired, 2-sided t-tests. Scale bars, 50 microns.

**Figure 4.** Photodynamic destruction of tumor cells *in vivo* is selectively enhanced as a result of Vitamin D preconditioning. (A) Quantitation of TUNEL-stained cells in subcutaneous A431 tumors. P-values from unpaired 2-sided t-tests are shown. *nd*, not done; *None*, no PDT performed. (B) Examples of TUNEL stained sections from A431 tumors. Scale bar, 100 μm. (C) Quantitation of TUNEL-stained cells in papillomas (D) Low power images of hematoxylin and eosin stained A431 tumors, to illustrating the enhanced cell death in the central regions of Vit D-preconditioned A431 tumors. Scale bar, 250 μm. (E) Quantification of cell death as measured by loss of H&E-stained cells within the A431 tumors. In all graphs, data points represent the mean ± SEM of 9 histological sections (3 tumors, 3 sections per tumor).

**Figure 5.** Changes in expression of caspase-3 and caspase-8, in Vitamin D-pretreated (+Vit D) versus non-pretreated (-Vit D) tumors, as a function of time after ALA-PDT. Immunodetection of activated caspase-3 (*insets*) and quantitative counts of activated caspase-3 (*graphs*; mean ± SD of 2 tumors, 3 sections/tumor) in histological specimens from (A) squamous papillomas and (B) A431 tumors. *White bars*, no preconditioning; *Gray bars*, Vit D preconditioned. (C) Western analysis of pro-caspase-8 expression (top half; 6 μg protein/lane), and cleaved forms of caspase-8 (bottom half; 25 μg protein/lane) from A431 tumors. (D) Densitometric scans of the pro-caspase blot in panel C, mean ± range. (E) Densitometric analysis of two western blotting experiments that analyzed cleaved caspase-8; mean ± SEM, n= 4 tumors per point. Asterisks, significantly higher than the corresponding time point in non-pretreated tumors, *, p< 0.05; **, p< 0.005.
**Figure 6.** TNFα expression is increased in PDT-damaged A431 tumors as a result of Vitamin D preconditioning (+Vit D). (A) In the absence of PDT, TNFα levels in tumors are increased ~2-fold as a result of preconditioning with Vit D; western blot of individual tumor lysates. (B) TNFα levels are elevated at various times after ALA-PDT, and even more when PDT is combined with Vit D preconditioning. (C) Quantitation of the relative changes in TNFα as determined by densitometry of two pooled experiments (n=4 tumors per point), mean ± SEM. ***, significantly higher than corresponding time point in non-preconditioned tumors, p< 0.0005. (D) Immunochemical demonstration of elevated TNFα in Vit D-preconditioned tumors, vs. non-conditioned tumors, at 6 hr post-PDT. Inset: Control, no primary antibody. Scale bar, 50 μm.
**FIGURE 2**

A

![Image of protein expression for ALAD, PBGD, CPO, and FC with and without Vitamin D pretreatment](image)

B

**Porphyrin-synthetic enzymes in A431 tumors with or without Vitamin D pretreatment in vivo**

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<td>CPO</td>
<td>1.8 (No Vit D) 2.0 (+Vit D)</td>
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<td>FC</td>
<td>1.1 (No Vit D) 1.0 (+Vit D)</td>
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<td>GAPDH</td>
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* p < 0.05

** p < 0.005
FIGURE 6

A

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C

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D

TNFα immunostaining

No Vit D

+ Vit D
Vitamin D3 enhances the apoptotic response of epithelial tumors to aminolevulinate-based photodynamic therapy

Sanjay Anand, Clara Wilson, Tayyaba Hasan, et al.

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