Low-Dose Methotrexate Enhances Aminolevulinate-Based Photodynamic Therapy in Skin Carcinoma Cells In vitro and In vivo

Sanjay Anand, Golara Honari, Tayyaba Hasan, Paul Elson, and Edward V. Maytin

Abstract

Purpose: To improve treatment efficacy and tumor cell selectivity of δ-aminolevulinic acid (ALA)-based photodynamic therapy (PDT) via pretreatment of cells and tumors with methotrexate to enhance intracellular photosensitizer levels.

Experimental Design: Skin carcinoma cells, in vitro and in vivo, served as the model system. Cultured human SCC13 and HEK1 cells, normal keratinocytes, and in vivo skin tumor models were preconditioned with methotrexate for 72 h and then incubated with ALA for 4 h. Changes in protoporphyrin IX (PpIX) levels and cell survival after light exposure were assessed.

Results: Methotrexate preconditioning of monolayer cultures preferentially increased intracellular PpIX levels 2- to 4-fold in carcinoma cells versus normal keratinocytes. Photodynamic killing was synergistically enhanced by the combined therapy compared with PDT alone. Methotrexate enhancement of PpIX levels was achieved over a broad methotrexate concentration range (0.0003-1.0 mg/L; 0.6 nmol/L-2 mmol/L). PpIX enhancement correlated with changes in protein expression of key porphyrin pathway enzymes, ~4-fold increase in coproporphyrinogen oxidase and stable or slightly decreased expression of ferrochelatase. Differentiation markers (E-cadherin, involucrin, and filaggrin) were also selectively induced by methotrexate in carcinoma cells. In vivo relevance was established by showing that methotrexate preconditioning enhances PpIX accumulation in three models: (a) organotypic cultures of immortalized keratinocytes, (b) chemically induced skin tumors in mice; and (c) human A431 squamous cell tumors implanted subcutaneously in mice.

Conclusion: Combination therapy using short-term exposure to low-dose methotrexate followed by ALA-PDT should be further investigated as a new combination modality to enhance efficacy and selectivity of PDT for epithelial carcinomas.

Photodynamic therapy (PDT) is a cancer treatment modality increasingly used for precancerous lesions of the skin and also for thin nonmelanoma skin cancers and for palliative therapy of advanced internal malignancies. A popular type of PDT uses δ-aminolevulinic acid (ALA), or its methyl ester (methyl-ALA), small-molecule precursors that are taken up as a prodrug by cancer cells and then converted into an intracellular photosensitizer [protoporphyrin IX (PpIX)] that is activated by visible light (reviewed in refs. 1–3). ALA-PDT offers an opportunity to selectively target tumors through several mechanisms, including preferential uptake and accumulation of ALA in tumor versus normal tissues, preferential synthesis of PpIX in cancer cells versus normal cells, and targeted illumination to selectively encompass the lesion and spare normal tissue (4–6). Unfortunately, current ALA-PDT protocols have proven insufficient for the treatment of large or biologically aggressive tumors. In cutaneous oncology, ALA-PDT is successfully used for the treatment of sun-induced precancers (actinic keratoses; refs. 7, 8) and squamous carcinomas in situ (7), but the success rate for nodular basal cell carcinoma and invasive squamous cell carcinoma remains inadequate (9). The reasons for incomplete success are not entirely clear. Topical delivery of ALA and its more lipophilic esters are now largely optimized (10, 11) so that depth of penetration of the prodrg is thought to be adequate for effective therapy (12, 13). Similarly, modern light sources including lasers (14) and intense pulsed light (15) generate high energies at long wavelengths and should therefore be expected to penetrate well into the dermis. However, one factor that is not yet optimized is the intracellular production of the photosensitizer. Inadequate production of PpIX, and a nonhomogeneous distribution of PpIX within tumors, could scuttle any attempt to provide reliable photodynamic killing of all cancer cells within a population.

Authors’ Affiliations: Departments of 1Dermatology, 2Biomedical Engineering, and 3Quantitative Health Sciences, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio and 4Wellman Center for Photomedicine, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts.

Received 11/22/08; revised 1/20/09; accepted 2/5/09; published online 5/15/09.

Grant support: NIH grant CA84203.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Requests for reprints: Edward V. Maytin, Department of Biomedical Engineering, Cleveland Clinic, Mailstop ND-20, 9500 Euclid Avenue, Cleveland, OH 44195. Phone: 216-445-6676; Fax 216-444-9198; E-mail: maytine@ccf.org.

© 2009 American Association for Cancer Research.


Downloaded from clincancerres.aacrjournals.org on July 15, 2017. © 2009 American Association for Cancer Research.
levels in LNCaP prostate carcinoma cells (17, 18). Likewise, androgens stimulate a large increase in PpIX and at the same time increase PpIX in epithelial cancers. For example, androgens increase PpIX in keratinocytes (16), we found several other agents that can induce cellular differentiation and at the same time increase PpIX in epithelial cancers. For example, androgens stimulate a large increase in PpIX levels in LNCaP prostate carcinoma cells (17, 18). Likewise, vitamin D or 9-cis-retinoic acid increase PpIX levels significantly in those cells (18). Why most agents capable of elevating PpIX levels also promote cellular differentiation remains an interesting but yet unexplained association. The term “differentiation therapy” has been used before in oncology (retinoic acid, for example, as used for promyelocytic leukemia to differentiate immature cancer cells to a more mature and differentiated state; refs. 19–21), but the notion of using a differentiating agent in combination with ALA-PDT to enhance therapy has not been explored.

Methotrexate is a familiar chemotherapeutic agent that inhibits cell proliferation due to its potent inhibition of dihydrofolate reductase and thymidylate synthesis (22). Methotrexate also triggers cellular differentiation (23). Methotrexate induces differentiation in normal human keratinocytes (24) and in cancer cell lines including human HL-60 promyelocytic cells (25), neuroblastoma LA-N1 cells (26), and choriocarcinoma cells (27, 28); it is probably through this prodifferentiating mechanism that methotrexate can successfully control aggressive human choriocarcinoma tumors (29). Because of the possible association between prodifferentiating properties and an ability to promote PpIX accumulation (see above), we tested methotrexate as a preconditioning agent during ALA-PDT of prostate tumor (LNCaP) cells and found that such preconditioning led to enhanced PpIX levels and enhanced photodynamic killing in monolayer cultures (30).

Despite our earlier in vitro work, the question of whether PpIX-enhancing effects of methotrexate may occur selectively in tumor cells relative to normal cells had not been addressed. Also, it was unknown whether methotrexate preconditioning combined with ALA-PDT might provide benefit for actual tumors in vivo. In this article, we address these two questions in cell culture models and animal models of skin cancer. First, we show that methotrexate exerts a highly selective up-regulatory effect on PpIX production in skin carcinoma cells compared with normal keratinocytes. The mechanism underlying selective PpIX up-regulation involves methotrexate-inducible enhancement of a key PpIX synthetic enzyme, coproporphyrinogen oxidase (CPO). Extending these studies into an organotypic skin model, we show that methotrexate can enhance PpIX accumulation in a three-dimensional tissue. Taking this to the in vivo level, studies in two different models, carcinogen-induced skin tumors in mice and subcutaneous human tumors (A431 cells) in nude mice, confirm that methotrexate pretreatment can significantly and selectively enhance PpIX accumulation in squamous skin tumors in vivo.

Materials and Methods

Culture of primary keratinocytes and carcinoma lines. Normal human epidermal keratinoctyes (NHEK) from Cascade Biologies were cultured at 37°C in a humidified CO2 incubator in EpiLife medium with human keratinocyte growth supplement (Cascade Biologies), 0.06 mmol/L calcium chloride, and 100 units/mL penicillin-100 μg/mL streptomycin. For passaging, subconfluent cells were released with 0.05% trypsin-53 mmol/L EDTA solution, and when cells had just begun to detach, an equal volume of trypsin neutralizer (Cascade Biologies) was added. Detached cells were centrifuged at 180 × g in a conical tube, the supernatant was aspirated, and the cell pellet was replated in serum-free NHEK medium.

SCC13 cells (31) were kindly provided by Dr. Jonathan Garlic (Tufts University) and cultured at 37°C in SCC13 growth medium consisting of keratinocyte serum-free medium (Invitrogen) supplemented with 0.33 ng/mL epidermal growth factor, 2.5 μL/mL bovine pituitary extract, 0.3 mmol/L calcium chloride, and penicillin-streptomycin.

HEK1 cells (32), also called HEK001, were obtained from the American Type Culture Collection and cultured at 37°C in HEK1 growth medium consisting of keratinocyte serum-free medium (Invitrogen) supplemented with epidermal growth factor (8.4 ng/mL) and penicillin-streptomycin.

Immortalized rat epidermal keratinocytes (REK cells) were maintained as described previously (33) for use in organotypic three-dimensional cultures (see below).

A431, a human squamous cell carcinoma cell line, was obtained from the American Type Culture Collection and cultured at 37°C in high-glucose DMEM (American Type Culture Collection) supplemented with 10% fetal bovine serum (Biowhittaker) and penicillin-streptomycin. They were used in the subcutaneous nude mouse model (see above).

Pretreatment with methotrexate and measurement of PpIX in cell lysates from monolayer cultures. For each experimental condition, four 25 cm² flasks were plated at 200,000 cells per flask. On the second day, the medium was replaced with medium containing different doses of methotrexate (Sigma-Aldrich) for an additional 72 h. The medium was aspirated and replaced with medium containing ALA (Sigma-Aldrich; 1 mmol/L for NHEK and SCC13 and 0.5 mmol/L for HEK1) for an additional 4 h. Medium without ALA was added to control flasks. One flask from each set of treatments was observed and photographed on a phase-contrast microscope and cells were counted from five random fields. Cells were lysed and vortexed in 1 mL Solvable (Perkin-Elmer...
Life and Analytical Sciences) and centrifuged at 10,000 \( \times g \) at 4°C. PpIX content of a 100 \( \mu L \) aliquot of the supernatant was measured in triplicate in clear plastic 96-well plates (Corning) using a SpectraMAX GeminiXS spectrophotofluorimeter (Molecular Devices) at the excitation and emission wavelengths of 395 and 633 nm, respectively. In every experiment, four 25 cm\(^2\) flasks were plated with 200,000 cells and processed as described in Materials and Methods.

**Fluorescence analysis of PpIX in living cells and frozen tissue sections.** PpIX-specific fluorescence was analyzed by fluorescence microscopy on a confocal laser scanning microscope (Leica Microscopy Systems). Cells, plated on sterile microscope coverslips in 35 mm dishes at 50,000 per dish, were conditioned with methotrexate or control medium as above. Confocal images at higher magnification to illustrate localization of PpIX in normal NHEK (j) versus carcinoma SCC13 (k) cells; both had received methotrexate preconditioning. PpIX signal is observable in plasma membranes (arrows) and cytoplasm (Cy) but not in the nucleus (Nu). Bar, 50 \( \mu m \).

**Light exposure and fluorescein diacetate-ethidium bromide survival assay.** Cells were plated 50,000 per 35 mm dish and after 24 h were incubated with or without methotrexate for an additional 72 h. Fresh ALA-containing medium was added for 4 h. Cells were irradiated using a custom-built monochromatic light source consisting of four light-emitting diodes with a peak output at 395 nm and bandwidth (FWHM) of 3335

![Image](https://www.aacrjournals.org/content/cclinca/15/10/3335.full)

**Fig. 1.** Intracellular PpIX selectively accumulates in skin carcinoma cell lines (SCC13 and HEK1) following preconditioning with methotrexate relative to normal human keratinocytes (NHEK). A, confocal microscopic imaging of three cell types: normal keratinocytes NHEK, SCC13 cells, or HEK1 cells that received either no preconditioning (a–c) or preconditioning with 0.01 mg/L methotrexate for 72 h (d–f) followed by incubation with ALA (4 h) and visualization with confocal microscopy. Phase-contrast images (g–i) correspond to PpIX images directly above. White-boxed insets, dishes that did not receive ALA (negative controls; -ALA). Note that PpIX levels are preferentially elevated in the carcinoma cells pretreated with methotrexate (e and f) relative to NHEK (d). Bar, 50 \( \mu m \). B, confocal images at higher magnification to illustrate localization of PpIX in normal NHEK (j) versus carcinoma SCC13 (k) cells; both had received methotrexate preconditioning. PpIX signal is observable in plasma membranes (arrows) and cytoplasm (Cy) but not in the nucleus (Nu). Bar, 50 \( \mu m \).
Fig. 2. Cell type specificity and dose dependence of PpIX accumulation following methotrexate preconditioning and correlation with changes in PpIX metabolic enzymes. A, quantification of PpIX signal in the different cell lines using spectrofluorimetry. Cells were seeded in replicate 25 cm² flasks at ~60% confluence and induced with methotrexate (0.01 mg/L) for 72 h. Following incubation with ALA, cell numbers were determined by photography and manual counting, and cells were lysed and the lysates were measured in a spectrofluorimeter (see Materials and Methods). PpIX levels per cell are shown relative to untreated controls. Columns, mean of three separate flasks; bars, SD. **, P < 0.005, significant differences (methotrexate versus no methotrexate). B, quantification of PpIX signal by analysis of digital confocal images. Living cells on coverslips were analyzed with confocal microscopy and image processing as described in Materials and Methods. Integrated fluorescence intensities (arbitrary fluorescence units) were calculated from four images/condition, corrected for cell number, and expressed relative to zero-methotrexate controls. *, P < 0.05; **, P < 0.005, significant differences at the level. C, methotrexate dose-ranging experiments to examine induction of PpIX in normal human keratinocytes, SCC13 cells, and HEK1 cells. Experiments in which the confocal image processing method was used to determine relative PpIX concentration per cell were done after 72 h incubation in methotrexate (concentrations shown on the X axis as a logarithmic scale). Points, mean of three or more independent experiments (actual number in parentheses), with at least three confocal fields per experiment; bars, SE. All PpIX values were normalized to the zero-methotrexate NHEK control, which was arbitrarily set at 1.0. D, methotrexate preconditioning selectively increases the expression of CPO in carcinoma cells while not affecting ferrochelatase. Cells were incubated for 72 h in the presence of the following concentrations of methotrexate: lane 1, 0 mg/L; lane 2, 0.001 mg/L; lane 3, 0.01 mg/L; lane 4, 0.1 mg/L; lane 5, 1.0 mg/L. Cells were then harvested and the lysates were analyzed on Western blots using antisera specific to CPO, ferrochelatase, or GAPDH (with bands detected at the expected sizes of ~38, ~40, and ~37 kDa, respectively). Relative changes in band intensity at each methotrexate concentration, relative to the no-methotrexate control for each given cell type, as measured by gel densitometry.
of 14 nm. Dishes received 4 ml/cm² (power density 0.1 mW/cm² at 5 cm distance as measured with an IL1700 radiometer; International light). At 24 h after light exposure, the cells were PBS-rinsed, 50 μg/mL fluorescein diacetate and 4 μg/mL ethidium bromide were added, and the cells were observed under epifluorescence using a FITC/TRITC filter (34).

**Western blot analyses.** Cells were lysed in lysis buffer (7 mol/L urea, 2% Igepal, and 5% β-mercaptoethanol) supplemented with protease inhibitor cocktail (Sigma-Aldrich). Samples with equal quantities of protein, as determined by Bradford’s method (Bio-Rad), were denatured in sample buffer and sample reducing agent (Invitrogen) for 10 min at 70°C and then resolved on a 4% to 12% bis-Tris acrylamide gel (Invitrogen) along with molecular size markers (MagicMark, Invitrogen). Electrophoresis was done at constant voltage (200 V) at room temperature. Proteins were electrophoretically transferred to Immobilon polyvinyldene fluoride membrane (Millipore) at constant voltage (100 V) for 1 h at 4°C. Following transfer, the blot was stained with Ponceau red S to check transfer efficiency. Blots were then blocked with 10% nonfat dry milk and incubated with the following primary antibodies. A rabbit polyclonal antisera against a COOH-terminal peptide of murine CPO, consisting of the amino acid sequence CLEVRHHPDWH, which is 83% homologous to human, was produced as described previously (30). Incubation with anti-CPO (1:5,000) or with antibodies against ferrochelatase (gift of H. Dailey; 1:5,000), GAPDH (Santa Cruz Biotechnology; 1:5,000), p27 (Santa Cruz Biotechnology; 1:2,000), PCNA (Santa Cruz Biotechnology; 1:2,000), E-cadherin (Santa Cruz Biotechnology; 1:2,000), filaggrin (Covance; 1:2,000), involucrin (Santa Cruz Biotechnology; 1:2,000), or keratin-10 (Covance; 1:2,000) was followed by peroxidase-conjugated goat anti-rabbit IgG or donkey anti-goat IgG (Jackson Immunolaboratory; 1:20,000), and the blot was developed using enhanced chemiluminescence reagents (ECL kit; Amersham Biosciences).

**Organotypic three-dimensional epidermal cultures.** Rat epidermal keratinocytes (REK) were cultured at the air-medium interface to induce stratification (lift cultures) as described previously (33). At 48 h after lifting, REK medium was replaced with medium containing different doses of methotrexate and the cultures were preconditioned for 72 h. The medium was aspirated, medium containing 1 mmol/L ALA was added for another 4 h, and the cultures were frozen-embedded for cryosectioning and confocal analysis of PpIX.

**Chemically induced tumors in mouse skin.** Tumors in SKH-1 mice (Charles River Laboratories) were generated by topical application of DMBA (50 μg/100 μL in acetone, one time application) and TPA (5 μg/200 μL acetone, twice a week for up to 20 weeks) on the flanks. These papillomas were preconditioned with methotrexate treatment (2 mg/kg, intramuscularly, daily for 1 or 3 days), or with saline for 3 days (no-methotrexate control). ALA (Levulan Kerastick, DUSA Pharmaceuticals) was topically applied for 4 h. The amount of PpIX in the tumors was estimated by noninvasive dosimetry (see below). Mice were then euthanized and tumors were harvested. Skin biopsies were embedded in OCT compound, and 10 μm frozen sections were analyzed for PpIX levels by confocal microscopy as described earlier.

**In vivo measurement of PpIX in papillomas by Aurora fluorescence dosimetry.** Noninvasive, real-time measurements of PpIX fluorescence in normal skin and tumors on the skin surface were done at 0, 2, and 4 h post-ALA application using an Aurora dosimeter (Aurora Optics). This device employs a optical fiber-based probe in which a small 2 mm area receives excitation from a diode laser at ~ 400 nm, and PpIX emission in the red range is collected (35). Five readings were averaged for each tumor (see Fig. 5B).

**Generation of subcutaneous tumors by dermal injection of human squamous cell carcinoma cells in nude mice.** Immune-compromised nude mice (Charles River Laboratories) received intradermal injections of 2 x 10⁶ A431 cells (a squamous cell carcinoma line originally obtained from a human cutaneous squamous carcinoma) in each flank. After 6 to 10 days, visible nodules were observed. For the preconditioning therapy, mice received systemic methotrexate (2 mg/kg, intramuscularly, daily for 1 or 3 days) for half of the animals; all others received sham injections of PBS. Subsequently, ALA was administered in PBS (75 mg/kg, intramuscularly) for 24 h, and the mice were euthanized for tumor harvest. Frozen tumor sections were analyzed by confocal microscopy as described above. Some sections were postfixed in 4% paraformaldehyde for 1 h at room temperature and stained with H&E to visualize the tumor morphology.

**Statistics.** Two-sample t tests were used to compare differences in PpIX accumulation, or survival after PDT, between treated and untreated controls. P ≤ 0.05 was considered statistically significant. To determine whether the cytotoxic effect of combining methotrexate and PDT was additive or synergistic (see data in Fig. 3B), the fractional product of PpIX was used.

**Results**

**Preincubation with methotrexate selectively enhances the production of PpIX in skin carcinoma cell lines relative to normal keratinocytes.** For therapeutic success, ALA-PDT requires selective accumulation of high amounts of PpIX within tumor cells. NHEK, when incubated with ALA and visualized by confocal microscopy, accumulated negligible amounts of PpIX (near the level of background autofluorescence; Fig. 1A, a). Carcinoma cells, on the other hand, developed relatively high PpIX signals when incubated in ALA (Fig. 1A, b and c). Although higher baseline levels of PpIX might be expected to confer some selective therapeutic advantage in carcinoma cells, we wished to ask a different question: Can pretreatment with methotrexate cause an even more pronounced, selective increase in PpIX levels in the carcinoma cells? Accordingly, SCC13 cells, HEK1 cells, and normal keratinocytes were pretreated with methotrexate for 72 h before receiving ALA. As shown in Fig. 1A (d-f, row 2), methotrexate-pretreated carcinoma cells produced higher cytoplasmic amounts of PpIX relative to the ALA-alone controls (Fig. 1A, row 1). Almost no induction of PpIX was seen in primary keratinocytes (d).

Following methotrexate pretreatment, a qualitative difference in the intracellular location of PpIX was observed between normal cells and carcinoma cells. In primary keratinocytes, bright PpIX signals were found only in cell membranes (Fig. 1B, j). In the carcinoma lines, however, strong PpIX signal was found principally in cytoplasmic/perinuclear regions that contain mitochondria (Fig. 1B, k). Therefore, methotrexate appears to induce tumor cell-selective accumulation of PpIX in intracellular organelles that represent traditional targets for PDT.

To quantify cellular increases in PpIX, two different methods were employed (Fig. 2A and B). In the first, flasks of cells were pretreated with methotrexate or vehicle alone followed by measurement of PpIX in cell lysates using spectrophotometry (Fig. 2A). Methotrexate pretreatment increased PpIX levels by 3- to 4-fold. In the second method, confocal fluorescent images of cells grown on coverslips were digitally analyzed over the entire microscopic field, with correction for autofluorescent background and for cell number (Fig. 2B). Although estimates of PpIX with this method are inherently less linear than are measurements done after cell solubilization (to break up porphyrin aggregates that may cause quenching), the confocal method still permits the detection of large and significant relative changes in PpIX levels even if PpIX amounts are potentially
underestimated. Importantly, direct microscopic observation of PpIX in individual, living cells is essential to normalize PpIX measurements for changes in cell number. As documented in Supplementary Table S1, cell density declined significantly with methotrexate concentration by up to 50% at the highest methotrexate dose. Therefore, all subsequent experiments used the confocal method to normalize PpIX levels to the number of cells per dish.

PpIX production is enhanced by very low concentrations of methotrexate in squamous carcinoma cells. To examine the methotrexate dose dependence of methotrexate-induced enhancement of PpIX levels, a large number experiments were done as summarized in Fig. 2C. Methotrexate had only a slight effect on normal human keratinocytes (NHEK), leading to no more than a 2.5-fold PpIX increase at methotrexate concentrations of ≥0.003 mg/L (Fig. 2C, dotted line). For the two carcinoma cell lines, however, PpIX levels were 3-fold higher than NHEK even at baseline and accumulated to very high relative levels (10- to 12-fold higher than NHEK at a methotrexate concentration of 0.01 mg/L). Above 0.01 mg/L methotrexate, levels of PpIX trended downward probably due to increasing cytotoxicity of the methotrexate. We should note that the range of methotrexate concentrations that appear optimally effective here, between 0.001 and 0.1 mg/L (2-200 nmol/L), is lower...
Methotrexate-induced accumulation of PpIX, in a combined photodynamic regimen, leads to selective killing of squamous carcinoma cells in vitro. To ask whether methotrexate-induced elevation of PpIX translates into enhanced cell killing, we employed an in situ survival assay (Fig. 3; ref. 34). Living cells were tagged with fluorescein (green), dead cells were tagged with ethidium (orange), and survival was expressed as the proportion of total cells with green fluorescence. When methotrexate and ALA-PDT were administered to normal keratinocytes, either singly or in combination, very little toxicity was observed (Fig. 3A, row 1). However, in HEK1 cancer cells, greater cytotoxicity was observed with the combination treatment than with either agent alone (Fig. 3A, row 2). To test the hypothesis that the combination of methotrexate and PDT selectively enhances treatment efficacy in the cancer cells, experiments were done in which all cell lines were preconditioned with methotrexate at various doses followed by administration of ALA-PDT. Cell survival was assessed 24 h later (Fig. 3B). With methotrexate treatment alone, a methotrexate dose-dependent loss of cell viability was observed that appeared to be similar in all cell lines (Fig. 3B, black circles). PDT alone (without methotrexate) had relatively little effect on any of the cell lines at the sublethal (LD20) light dose employed in the experiment (Fig. 3B, white circles, far left). However, after combination therapy (methotrexate followed by ALA-PDT), the SCC13 and HEK1 cells were markedly more sensitive than normal keratinocytes (Fig. 3B, white circles). Negative controls exposed only to light in the absence of ALA (gray circles) showed minimal cell death.

To determine whether the combination of methotrexate and ALA-PDT provides synergistic (as opposed to an additive) enhancement of cytotoxicity, a formal analysis of synergy was done (Supplementary Table S2). The results showed that the enhancement of cytotoxicity with the combination therapy was synergistic for SCC13 and for HEK1 carcinoma cells but not for normal keratinocytes.

Mechanism of methotrexate-enhanced PpIX accumulation involves changes in expression of heme enzymes in the setting of increased cellular differentiation. Based on evidence that suggested a possible link between cellular differentiation, increased synthesis of PpIX, and elevated levels of the porphyrin synthetic enzyme CPO (16, 18, 30), we examined the expression of two enzymes (CPO and ferrochelatase) located immediately upstream and downstream from PpIX in the heme synthesis pathway and that therefore might be rate-limiting. In methotrexate-treated cells, both carcinoma lines showed a robust increase in CPO protein levels, whereas the CPO level in normal cells remained essentially unchanged (Fig. 2D). Ferrochelatase, immediately downstream of PpIX, was either slightly decreased or unchanged in cells incubated with methotrexate (Fig. 2D).

To evaluate the growth and differentiation status of the cells in response to methotrexate, some general markers of cell cycle progression (PCNA), growth arrest (p27kip1), and cell differentiation (E-cadherin) were also examined. PCNA, expressed in proliferating cells in S phase (37), was slightly induced at low methotrexate concentrations (0.001 mg/L) in all cell types, reflected by a slight increase in cell number (Supplementary Table S1). However, at higher methotrexate doses, PCNA was selectively decreased in carcinoma cell lines relative to normal keratinocytes, suggesting growth arrest in the carcinoma cells (Supplementary Fig. S1A). Two general markers of growth arrest and differentiation, p27 and E-cadherin, respectively, were selectively induced by methotrexate in the carcinoma lines.
particularly at low methotrexate doses (Supplementary Fig. S1B and C). Specific epidermal differentiation markers, such as involucrin, K10, and filaggrin (38–40), were reduced in squamous carcinoma cells relative to normal keratinocytes, reflecting neoplasia-associated loss of differentiation. However, those markers were reexpressed after incubation with methotrexate (Supplementary Fig. S1D-F). Collectively, these methotrexate-related changes in differentiation markers confirmed an interesting association between methotrexate inducibility of CPO and induction of cellular differentiation, occurring selectively in squamous carcinoma cells and not in normal epidermal keratinocytes.

Methotrexate pretreatment enhances photosensitizer levels in an organotypic, three-dimensional epithelial model tissue. Monolayer cell cultures do not always mimic the physiologic behavior of living cells in an intact organ such as the skin. To ask whether effects of methotrexate observed in monolayer cultures are relevant in a three-dimensional tissue, experiments were done in an organotypic model. In these three-dimensional cultures of immortalized keratinocytes that have lost their dependence on exogenous paracrine support, we have shown previously that pretreatment with another differentiation-promoting agent (vitamin D) supports PpIX accumulation and improves ALA-PDT efficacy (33). As seen in Fig. 4A, preincubation of the lift cultures with methotrexate followed by incubation with ALA and then euthanized and the tumors were harvested and PpIX was analyzed by confocal microscopy of frozen sections. a and b, typical PpIX signals; c and d, corresponding tumor morphology. D, quantitation of the PpIX signal from digital confocal images, from tumors preconditioned with no methotrexate, 1 d of methotrexate, or 3 d of methotrexate before harvest. Mean ± SD of images from at least three independent tumors (numbers in parentheses).

Chemically induced skin tumors, when preconditioned with methotrexate, produce relatively high amounts of PpIX. Tumors were generated by chemical carcinogenesis on the dorsum of SKH-1 hairless mice and then pretreated with systemic methotrexate (or saline vehicle) for 1 or 3 d followed by application of ALA for 2 or 4 h before measurement of PpIX by noninvasive dosimetry (A and B) or by tissue biopsy (C and D). A, schematic diagram of the Aurora noninvasive fluorescence dosimeter. B, fluorescence signal is significantly increased in tumors from mice pretreated with methotrexate for 3 d followed by ALA for the times indicated compared with normal skin (NL Skin) on the same mouse. Columns, mean of three tumors (five readings per tumor); bars, SE. C, mice were treated with or without methotrexate and with ALA and then euthanized and the tumors were harvested and PpIX was analyzed by confocal microscopy of frozen sections. a and b, typical PpIX signals; c and d, corresponding tumor morphology. D, quantitation of the PpIX signal from digital confocal images, from tumors preconditioned with no methotrexate, 1 d of methotrexate, or 3 d of methotrexate before harvest. Mean ± SD of images from at least three independent tumors (numbers in parentheses).
SKH-1 hairless mice. Mice were then pretreated systemically with either methotrexate or saline vehicle for 1 or 3 days followed by topical ALA for up to 4 h to allow production of PpIX. PpIX levels were analyzed in two ways. First, a noninvasive surface dosimetry technique was used in the living animals (Fig. 5A), and enhancement of PpIX within tumors was observed in the methotrexate-preconditioned mice (Fig. 5B). In the second technique, tumors were harvested and the amount of PpIX in frozen tissue sections was analyzed by confocal microscopy (Fig. 5C). Again, a higher level of PpIX was seen in tumors preconditioned with methotrexate than in saline-only controls (Fig. 5D). These increases in PpIX were statistically significant for both 1- and 3-day pretreatments.

**Human squamous tumors (subcutaneous A431 cells in mice) accumulate high amounts of PpIX as a result of methotrexate preconditioning.** Subcutaneous tumors were generated by intradermal injection of human A431 cells. Mice with palpable tumors were preconditioned with intramuscular methotrexate or saline alone. PpIX production was initiated by systemic ALA injection, and tumors were harvested for analysis of PpIX by confocal microscopy as described in Materials and Methods. In tissue cryosections, a bright PpIX signal was observed preferentially in methotrexate-treated tumors compared with saline-treated controls (Fig. 6A). Quantitatively, an increase in PpIX was observed following 1 day (single mouse examined) or 3 days (statistically significant increase) of daily methotrexate injections (Fig. 6B).

**Discussion**

Data presented in this article show that the efficacy and cancer selectivity of ALA-based PDT can be enhanced by using methotrexate as a preconditioning agent. Methotrexate, when used at concentrations too low to provide any significant tumoricidal activity on its own, significantly and selectively enhances the production of PpIX within carcinoma cells. PpIX then serves as the target for light. Although the skin carcinoma cell lines intrinsically produced ∼3-fold higher levels of PpIX than did normal epithelial keratinocytes, this difference did not appear to offer any selective advantage for photodynamic killing in the absence of methotrexate (see Fig. 3). However, when cells were preconditioned with methotrexate, a significant induction of PpIX levels occurred only in the cancer lines (Fig. 2), and this induction translated into selective and efficient photodynamic killing of the carcinoma cells (Fig. 3). Cytotoxic enhancement due to the combination of methotrexate and ALA-PDT was more than additive (synergistic) in the monolayer cultures (Table 2). Importantly, very low methotrexate concentrations (∼2 nmol/L) proved to be quite effective at enhancing ALA-PDT.

In a whole-tissue setting, the effects of methotrexate preconditioning were explored in three models that, together, indicate the existence of a significant PpIX-enhancing effect in vivo. First, methotrexate was shown to enhance PpIX levels ∼2-fold in a differentiating (organotypic) epidermal model. Second, PpIX enhancement of ∼6-fold was shown in skin tumors produced by chemical carcinogenesis in mice. Third, PpIX enhancement of ∼3-fold was shown in subcutaneous human A431 tumors in nude mice. Although tumor survival/regression experiments in animals were beyond the scope of this study, previous work on vitamin D as an adjuvant for PDT, in the same organotypic model as that used here, showed an enhancement in photodynamic killing that ran parallel to the increase in PpIX, with a similar magnitude of effect (2.5-fold increase in PpIX and...
One of the most notable features of our study is the finding that PpIX levels are enhanced at very low, nontoxic concentrations of methotrexate. This has important clinical implications. Based on current clinical protocols, concentrations of methotrexate measured in the serum and tissues of patients undergoing treatment for psoriasis or rheumatoid arthritis using oral methotrexate are similar to concentrations found to be effective in our study. For example, plasma drug concentrations achieved when treating rheumatoid arthritis (41) and psoriasis (42) with low-dose oral methotrexate fall within the range of 10 and 100 nmol/L (41, 42). In a psoriasis study using 15 mg/wk, the methotrexate peak was 105 to 150 nmol/L, and the steady-state concentration was ∼10 to 20 nmol/L at 12 h (42). Thus, tissue levels of methotrexate that fall within the broad range of methotrexate concentrations (2-2,000 nmol/L) effective for inducing PpIX seem readily obtainable. Although more preclinical and clinical work will be required, a combination of oral methotrexate and ALA-PDT for skin carcinoma could lead to a safe and effective new treatment regimen.

In considering possible mechanisms for methotrexate-mediated accumulation of PpIX, we felt that an increase in prodrug (ALA) uptake into cells was unlikely to be important because (a) no differential effect of methotrexate on ALA uptake was found in our previous work with prostate carcinoma cells (30) and (b) no significant differences in ALA uptake between several colon carcinoma lines was observed despite significant differences in PpIX production (43). Instead, we focused on evidence that changes in PpIX synthesis play a crucial role. CPO, a major enzyme for protoporphyrin synthesis, was shown previously to be induced in methotrexate-treated prostate cancer cells; however, no comparison with normal cells was available at that time (30). In the current study, two lines of cutaneous carcinoma were compared with normal keratinocytes. Methotrexate-mediated increases in CPO expression, PpIX levels, and photodynamic killing were all shown to occur selectively in the carcinoma cells.

Because CPO is increased by methotrexate, it is important to consider another heme metabolic enzyme, ferrochelatase, which lies downstream from PpIX and might be relatively rate-limiting. Ferrochelatase is unequivocally rate-limiting after chelation of iron with desferoximine (44). In other studies, reduced activity of ferrochelatase was invoked to explain higher levels of methotrexate that fall within the broad range of methotrexate concentrations (2-2,000 nmol/L) effective for inducing PpIX. This has important clinical implications. Because methotrexate levels fall within the range used here, whereas we found NHEK to be largely unresponsive to methotrexate. This apparent discrepancy may be due to the fact that thymidine-free culture medium was used in their experiments to amplify the methotrexate blockade on thymidylate synthesis and to enhance subsequent DNA synthesis inhibition and growth arrest (24). In our work, higher methotrexate sensitivity of the skin-derived squamous cell carcinoma cells relative to normal keratinocytes may be due to a loss of thymidine salvage pathways in the carcinoma cells. Other researchers have shown that, in the presence of methotrexate, NHEK are able to obtain thymidylate from alternative sources and thus maintain their DNA synthesis, whereas skin-derived carcinoma cells cannot do this (52, 53).

In summary, this study reports the following important observations: (a) PpIX levels can be induced in carcinoma cells using low, nontoxic doses of methotrexate. (b) Inducible changes in mitochondrial CPO expression and subsequent accumulation of PpIX appear to underlie this response. (c) The cytotoxic (therapeutic) effect achieved by combining methotrexate and ALA-PDT is synergistic in cell culture and is also significant in organotypic and in vivo animal models. (d) The beneficial effect of methotrexate is tumor-selective, occurring preferentially in carcinoma cells and not in normal epithelial cells. Futures studies will be required to translate methotrexate-PDT combination therapy into a useful modality for human skin carcinomas.

Disclosure of Potential Conflicts of Interest

No conflicts of interest were disclosed.

Acknowledgments

We thank Harry A. Dailey and Tammy Dailey (University of Georgia-Athens) for a kind gift of anti-ferrochelatase antibody, Ellen Lee for assistance with the initial methotrexate titration studies, Anatoly Prokovitch for help with the spectrophotometric PpIX assays, Christine Baran for statistical support, and Judy Draza for help with confocal microscopy.
References


Low-Dose Methotrexate Enhances Aminolevulinate-Based Photodynamic Therapy in Skin Carcinoma Cells  *In vitro* and *In vivo*

Sanjay Anand, Golara Honari, Tayyaba Hasan, et al.


Updated version  Access the most recent version of this article at: http://clincancerres.aacrjournals.org/content/15/10/3333

Supplementary Material  Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2009/05/20/1078-0432.CCR-08-3054.DC1

Cited articles  This article cites 50 articles, 5 of which you can access for free at: http://clincancerres.aacrjournals.org/content/15/10/3333.full#ref-list-1

Citing articles  This article has been cited by 8 HighWire-hosted articles. Access the articles at: http://clincancerres.aacrjournals.org/content/15/10/3333.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.