Celecoxib and NS-398 Enhance Photodynamic Therapy by Increasing In vitro Apoptosis and Decreasing In vivo Inflammatory and Angiogenic Factors

Angela Ferrario, Anita M. Fisher, Natalie Rucker, and Charles J. Gomer

The Saban Research Institute, Children's Hospital Los Angeles and Departments of Pediatrics and Radiation Oncology, Keck School of Medicine, University of Southern California, Los Angeles, California

Abstract
Photodynamic therapy (PDT) elicits both apoptotic and necrotic responses within treated tumors and produces microvascular injury leading to inflammation and hypoxia. PDT also induces expression of angiogenic and survival molecules including vascular endothelial growth factor, cyclooxygenase-2 (COX-2), and matrix metalloproteinases. Adjunctive administration of inhibitors to these molecules improves PDT responsiveness. In the current study, we examined how the combination of PDT and COX-2 inhibitors improve treatment responsiveness. Photofrin-mediated PDT combined with either celecoxib or NS-398 increased cytotoxicity and apoptosis in mouse BA mammary carcinoma cells. Immunoblot analysis of protein extracts from PDT-treated cells also showed poly(ADP-ribose) polymerase cleavage and Bel-2 degradation, which were further enhanced following combined therapy. Tumor-bearing mice treated with PDT and either celecoxib or NS-398 exhibited significant improvement in long-term tumor-free survival when compared with PDT or COX-2 inhibitor treatments alone. The combined procedures did not increase in vivo tumor-associated apoptosis. Administration of celecoxib or NS-398 attenuated tissue levels of prostaglandin E 2 and vascular endothelial growth factor induced by PDT to treated tumors and also decreased the expression of proinflammatory mediators interleukin-1β and tumor necrosis factor-α. Increased tumor levels of the antiinflammatory cytokine, interleukin 10, were also observed following combined treatment. This study documents for the first time that adjunctive use of celecoxib enhances PDT-mediated tumoricidal action in an in vivo tumor model. Our results also show that administration of COX-2 inhibitors enhance in vitro photosensitization by increasing apoptosis and improve in vivo PDT responsiveness by decreasing expression of angiogenic and inflammatory molecules. (Cancer Res 2005; 65(20): 9473-8)

Introduction
Photodynamic therapy (PDT) is used to treat tumors and the non-oncologic disorder age-related macular degeneration (1–3). This therapy involves administration of a photosensitizer followed by localized exposure of the target tissue to laser-generated light. Absorption of light by the photosensitizer initiates the photochemical generation of cytotoxic singlet oxygen within the irradiated field (4). The porphyrin-based photosensitizer, Photofrin, is administered systemically for the clinical treatment of esophageal and bronchial carcinomas as well as for treating Barrett’s esophagus. An increasing number of new photosensitizers with enhanced tissue localization properties, clearance kinetics, and/or superior light absorption characteristics are also being examined in clinical trials (1, 4).

PDT induces both necrosis and apoptosis in treated cells and tumors (5, 6). Caspase activation, poly(ADP-ribose) polymerase (PARP) cleavage, and Bel-2 degradation are observed following PDT. A plethora of signal transduction pathways, gene activation, and immunologic responses are also associated with PDT-mediated oxidative stress (1, 5, 6). The impact of most of these events to treatment efficacy is unknown. Direct tumor cell damage, microvascular injury, hypoxia, and inflammation are produced by PDT and correlate with increased expression of angiogenic growth factors, matrix metalloproteinases (MMP), cytokines, and prostaglandins (7–9). Elevated levels of these molecules within PDT target tissue can adversely affect tumor response. Therefore, experimental protocols combining PDT with procedures targeting these molecules are being examined in an effort to improve treatment efficacy.

Cyclooxygenase is a key enzyme involved in the conversion of arachidonic acid to bioactive lipids including prostaglandins and thromboxanes (10). The two isoforms of cyclooxygenase, COX-1 and COX-2, are encoded by separate genes and play different physiologic and pathologic roles. COX-1 is constitutively expressed in most tissues and is involved in homeostatic functions, whereas COX-2 is an inducible early response gene involved with inflammation and mitogenesis. A number of selective COX-2 inhibitors, including celecoxib, have been clinically approved and are highly effective in the treatment of osteoarthritis, rheumatoid arthritis, and postsurgical pain (11). However, there are significant concerns regarding long-term use of COX-2 inhibitors and cardiovascular safety (12). At the same time, growing evidence indicates that COX-2 activity is involved in the development and progression of a variety of cancers (13). COX-2 inhibitors can attenuate tumor growth and this finding has led to the clinical evaluation of selective COX-2 inhibitors, including celecoxib, in chemoprevention and as an adjuvant to radiation or chemotherapy for treating solid tumors (14).

We recently documented that PDT induces prolonged expression of COX-2 in a mouse fibrosarcoma tumor model and that combining PDT with the COX-2 inhibitor, NS-398, enhanced tumor response without increasing normal tissue photosensitization (8). In the current study, we examined treatment efficacy when PDT was combined with celecoxib and observed that this combination increased long-term survival in a mouse mammary carcinoma.

References

Requests for reprints: Charles J. Gomer, Children’s Hospital Los Angeles, Mail Stop 67, 4650 Sunset Boulevard, Los Angeles, CA 90027. Phone: 323-669-2335; Fax: 323-669-0742; E-mail: cgomer@chla.usc.edu.
model. Our results also showed that COX-2 inhibition enhanced PDT-mediated apoptosis in cultured tumor cells and decreased the \textit{in vivo} expression of angiogenic and inflammatory factors in treated tumors.

### Materials and Methods

#### Drugs

The photosensitizer Photofrin porfimer sodium was a gift from Axcan Scandipharm, Inc., Birmingham, AL and was dissolved in 5% dextrose in water to make a 2.5 mg/mL stock solution. The COX-2 inhibitor NS-398 [N-(2-cyclohexyloxy-4-nitrophenoxy)-methane sulfonamide] was purchased from Cayman Chemical Co., Ann Arbor, MI, and was dissolved in DMSO to make an 8.0 mg/mL stock solution. Celecoxib was purchased by Pfizer Inc., New York, NY and was dissolved in DMSO to make a 4.0 mg/mL stock solution. Working solutions of each COX-2 inhibitor were obtained by diluting the stock solutions either in saline for \textit{in vivo} experiments or in RPMI medium for \textit{in vitro} experiments.

#### Cell culture and tumor model

Mouse mammary carcinoma (BA) cells were grown in monolayer cultures in RPMI 1640 supplemented with 10% FCS and antibiotics. BA tumors were generated by s.c. trochar injection of 1 mm² pieces of tumor to the right flank of 8- to 12-week-old female C3H/HeJ mice (9).

**In vitro and \textit{in vivo} treatment protocols.** For \textit{in vitro} treatments, BA cells were seeded in Petri dishes and incubated overnight in complete growth medium to allow for cell attachment. Photosensitization experiments were done as previously reported (15). Briefly, attached cells were incubated in the dark at 37°C with Photofrin (25 μg/mL) for 16 hours in RPMI 1640 supplemented with 5% FCS. Cells were then incubated for 30 minutes in fresh growth medium, rinsed in medium without serum, and exposed at room temperature to broad spectrum red light (570-650 nm) generated by a parallel series of Mylar filtered 30 W fluorescent bulbs delivered at a dose rate of 0.35 mW/cm². Light exposures ranged from 0 to 525 J/m² with exposure times of 0 to 150 seconds. Treated cells were re-fed with complete growth medium and incubated for 7 to 10 days to determine clonogenic survival. In selected experiments, COX-2 inhibitors (50 μmol/L) were added to culture dishes immediately following light treatment and kept in the incubation medium for the remainder of the experiment.

For \textit{in vivo} PDT treatments included an i.v. injection of Photofrin (5 mg/kg) followed 24 hours later with nonthermal tumor irradiation using an argon-pumped dye laser (Coherent, Palo Alto, CA) emitting red light at 630 nm. Light was delivered via a quartz fiber micro-lens delivery system and the light dose rate was measured with a power meter. A light dose rate of 75 mW/cm² and total light doses ranging from 0 to 200 J/cm² were used for \textit{in vivo} PDT treatments. Tumors measuring 6 to 7 mm at the largest diameter were treated with PDT and then monitored thrice per week for detection of tumor recurrence. Tumor cures were defined as treated mice being disease-free for at least 90 days following PDT. NS-398 and celecoxib were given by i.p. injection (10 mg/kg) starting immediately after light exposure (time 0), and then at 4, 24, 48 hours after treatment and then every other day for 20 days post-PDT treatment.

#### Apoptosis measurements

Quantitative analysis of \textit{in vitro} apoptosis was done using the Cell Death Apoptosis Detection ELISA Plus kit (Boehringer Mannheim, Indianapolis, IN). This kit quantifies mono- and oligonucleosomes from cell lysates using mouse monoclonal antibodies directed against DNA and histones in a quantitative photometric sandwich enzyme immunoassay (15). Cells were plated in 60 mm dishes and treated as described above. Five or 24 hours after light treatment cells were lysed and analyzed for apoptosis. Lysates were centrifuged and cytoplasmic fractions were placed in streptavidin-coated microtiter plates with an immunoreagent containing a biotinylated anti-DNA and anti-histone. The solution was incubated at room temperature for 2 hours and then the substrate 2,2'-azino-di-(3-ethylbenzthiazoline) sulfonate was added for 20 minutes. Absorbance at 405 nm was measured for control and treated samples. Readings for treated cells versus control cells were used to determine the apoptotic enrichment factor. Results were normalized for protein concentrations.

### In vivo apoptosis

\textit{In vivo} apoptosis was measured in BA tumors before and after PDT treatments using an ApopTag \textit{In situ} Apoptosis terminal deoxynucleotidyl transferase–mediated DUTP-biotin nick-end labeling (TUNEL) fluorescein detection kit (Intergen, Purchase, NY) for experiments involving NS-398 and using a colorimetric TUNEL Assay Kit (Promega, Madison, WI) for experiments involving celecoxib. The assays used TUNEL methodology and were applied to formalin-fixed tissue sections collected 6 hours after PDT. COX-2 inhibitor treatments consisted of three i.p. injections of either NS-398 or celecoxib (20 mg/kg/injection) given immediately after PDT as well as 2 and 4 hours after PDT. For the fluorescent ApopTag assay, slides were stained with terminal deoxynucleotidyl transferase and then double-stained with propidium iodide and digoxigenin/antidigoxigenin conjugated to fluorescein for \textit{in situ} detection of apoptotic cells. Under a fluorescent microscope, all cell nuclei appeared red (propidium iodide–stained), whereas only apoptotic cells appeared green (fluorescein stained). Five or six randomly selected (110 × 140 μm) areas were photographed on each slide and slides from five tumors per treatment conditions were each examined. Photographs were coded and two independent examiners blinded to treatment conditions scored apoptotic nuclei and total cell number in each region of interest. The apoptotic index was determined as the number of green stained cells per field versus the total number of cells per field. For the colorimetric assay, biotinylated nucleotide was incorporated at the 5'-OH DNA ends using terminal deoxyribonucleotidyl transferase and then the sections were incubated with horseradish peroxidase–labeled streptavidin and detected using hydrogen peroxide and diaminobenzidine.

### Western immunoblot analysis

Constitutive and inducible expression of PARP, Bcl-2, and MMP-9 was determined by Western immunoblot analysis (8, 9). Cells were collected 24 hours after PDT and placed in an SDS lysing buffer [4% SDS, 0.125 mol/L Tris base, 10% glycerol, 4% mercaptoethanol, and 0.02% bromophenol blue (pH 6.8)]. Tissue samples were collected 24 hours after PDT and homogenized with a Polytron in 1× reporter lysis buffer (Promega). Protein samples were size-separated on discontinuous polyacrylamide gels (7.5-12.5%) and transferred overnight to nitrocellulose membranes. Filters were blocked for 2 hours with 5% nonfat milk and then incubated for 3 hours with either mouse monoclonal anti-PARP (clone C2101), mouse monoclonal anti-Bcl-2 (C-2) or goat polyclonal anti-MMP-9 (sc-66841; Santa Cruz Biotechnology Inc., Santa Cruz, CA). Filters were then incubated with either an anti-mouse or an anti-goat peroxidase conjugate (Sigma, St. Louis, MO), and the resulting complexes were visualized by enhanced chemiluminescence autoradiography (Amersham Life Science, Chicago, IL). Protein loading was evaluated by incubating the same filters with a mouse monoclonal anti-actin antibody (clone C-4; ICN, Aurora, OH). Autoradiographs were quantified by scanning densitometry.

#### ELA and ELISA assays

Enzyme immunoassay kits and Quantikine M mouse ELISA kits were used to quantify prostaglandin E₂ (PGE₂), vascular endothelial growth factor (VEGF), interleukin-1α (IL-1α), IL-10, and tumor necrosis factor-α (TNF-α) levels in control and treated tumor lysates (Cayman Chemical and R&D Systems, Minneapolis, MN). Tumors were homogenized in 1× reporter lysis buffer (Promega). Supernatants were assayed according to the manufacturer's instructions and results were then normalized for protein concentrations.

#### Statistics

The Wilcoxon rank-log test was used to compare long-term Kaplan-Meier cure rate curves. A two-way ANOVA followed by an unpaired Bonferroni multiple comparison t test analysis of differences between two groups was used to determine statistical differences for apoptotic indexes, ELA and ELISA values, and MMP-9 densitometry. Differences with \( P < 0.05 \) were regarded as significant.

### Results

Cytochrome-c-2 inhibitors increase photodynamic therapy–induced cytotoxicity and apoptosis in BA mammary carcinoma cells. Cytotoxicity of BA cells treated with PDT and COX-2 inhibitors was determined using a clonogenic assay. Plating...
efficiencies for nontreated controls or for cells incubated with Photofrin, celecoxib, or NS-398 alone were all comparable, averaging 58% to 60%. This indicates that the COX-2 levels used in this in vitro experiment were not cytotoxic. These incubation conditions inhibit PDT-mediated expression of PGE$_2$ (data not shown). The survival curve in (Fig. 1A) shows that both NS-398 and celecoxib increased cellular photosensitization in a dose-dependent manner. Cytotoxicity correlated with activation of caspase-3-like proteases as indicated by PARP cleavage from the native 116 kDa enzyme to an 85 kDa fragment as shown in Fig. 1B. Combining PDT with COX-2 inhibitors resulted in increased PARP cleavage at 24 hours following treatment when compared with PDT alone. Degradation of the antiapoptotic Bcl-2 was also observed within this same time frame (Fig. 1B). Apoptosis was quantified for control and treated cells at either 5 hours (Fig. 1C) or 24 hours (Fig. 1D) after treatment. COX-2 inhibitors alone had minimal effect on cell apoptosis during this timeframe, whereas PDT induced a dose-dependent increase in apoptosis. Combining PDT with COX-2 inhibitors resulted in further increases in apoptosis at both 5 and 24 hours posttreatment.

Combining photodynamic therapy with cyclooxygenase-2 inhibitors increases in vivo tumoricidal activity without a concomitant increase in apoptosis. We previously reported that NS-398 potentiates the tumoricidal activity of PDT in a murine fibrosarcoma tumor model (8). In the current study, we examined if treatment potentiation also occurs in the BA carcinoma tumor model using either the clinically approved COX-2 inhibitor celecoxib or NS-398. Figure 2A shows the percentage of mice without tumor recurrence as a function of days after treatment. A single 200 J/cm$^2$ PDT dose resulted in a 22% cure rate, whereas combining this same PDT dose with multiple injections of either celecoxib or NS-398 (10 mg/kg starting immediately after PDT, 4, 24, and 48 hours after PDT and then once every other day up to day 20) resulted in a 90% cure rate. These results show for the first time that celecoxib extensively enhanced the tumoricidal action of PDT. We did not observe any increased photodamage to the

![Figure 1](image-url)
overlying normal skin for the combined procedures when compared with PDT alone. This agrees with our previous study where no significant differences in the magnitude or repair of skin damage were observed following PDT plus NS-398 versus PDT alone (8).

We next examined apoptosis within treated tumors using quantitative TUNEL assays. Figure 2B shows apoptotic cells in control and PDT-treated BA tumors collected 6 hours after a 100 J/cm² light dose. Double-stained tumor sections are shown with all cell nuclei appearing red from propidium iodide staining and apoptotic cells appearing green from fluorescein TUNEL staining. These results show that in vivo apoptosis can be readily documented following PDT in BA tumors. An apoptotic index was calculated from the ratio of apoptotic cells (green) versus the total number of cells (red) in each field. Figure 3C shows apoptotic index values for control, single agent, and combined modality treatments. Our results show that administration of NS-398 (20 mg/kg, immediately after PDT and 2 and 4 hours after PDT) decreased the level of apoptosis at 6 hours when compared with PDT treatments alone. Comparable results were also obtained when apoptosis was determined using a colorimetric TUNEL assay following PDT and celecoxib treatments. In this case, the average number of apoptotic cells per observation field was 50 for tumors treated with PDT alone and 38 for tumors treated with PDT and celecoxib.

Cyclooxygenase-2 inhibitors decrease photodynamic therapy-mediated expression of inflammatory and proangiogenic factors in treated tumors. Overexpression of angiogenic and inflammatory molecules within the tumor microenvironment play a significant role in tumor recurrence, growth, and invasion (13, 14). We examined expression profiles of proangiogenic factors and inflammatory cytokines in tumor tissue 24 hours following PDT or combination treatments involving PDT and either celecoxib or NS-398. PDT treatments induced increased levels of TNF-α, IL-1β (Fig. 3A), PGE₂, VEGF (Fig. 3B), and MMP-9 (Fig. 3C). Combination procedures employing either celecoxib or NS-398 together with PDT resulted in down-regulation of the expression of each of these angiogenic and inflammatory factors. Conversely, PDT treatment alone resulted in the decreased expression of the antiinflammatory cytokine, IL-10, whereas combination treatments using PDT and either of the COX-2 inhibitors reversed this effect (Fig. 3A).

Discussion

The goals of the current study were to determine if celecoxib improved the in vivo tumoricidal responsiveness of PDT and to examine mechanisms associated with the potentiation of PDT when combined with selective COX-2 inhibitors. We previously reported that PDT induces expression of biologically active COX-2 in tumor tissue and that a combination procedure using PDT with the nonclinical COX-2 inhibitor NS-398 is more effective than either treatment alone at producing long-term cures in a mouse fibrosarcoma tumor model (8). These results have been confirmed by other laboratories and support the premise that protocols designed to combine PDT with a COX-2 inhibitor may be clinically beneficial (16–18). A primary finding of our current study is that...
the Food and Drug Administration–approved COX-2 inhibitor, celecoxib, enhances the long-term tumoricidal actions of PDT in a mouse mammary carcinoma model. This documents, for the first time, the efficacy of a clinically relevant COX-2 inhibitor, celecoxib, as an in vivo adjuvant for PDT and extends the types of cancers that are responsive to this combined modality approach.

Experiments were also performed to examine why combining PDT with specific COX-2 inhibitors improves tumor treatment responsiveness. COX-2 inhibitors induce a variety of cellular responses when used in chemoprevention or cancer therapy studies including modifications in apoptosis, cell cycle progression, invasion, and angiogenesis (19). PDT also alters these same physiologic and pathologic processes (3–6). Oxidative stress generated by PDT produces mitochondrial damage leading to rapid induction of apoptosis in cancer cells growing either in vitro or in vivo (1, 5). We observed that celecoxib and NS-398 produced a modest increase in both cytotoxicity and apoptosis in PDT-treated BA mammary carcinoma cells. PDT induced PARP cleavage, Bcl-2 degradation, and DNA fragmentation (variables associated with apoptosis) and all of these responses were further enhanced when PDT was combined with either celecoxib or NS-398. These results agree with in vitro studies examining cellular responses when COX-2 inhibitors are combined with radiation or chemotherapy (20–22). However, in a previous report, cell survival was not altered when NS-398, rofecoxib, or nimesulide were combined with Photofrin-mediated PDT in a mouse colon carcinoma cell line (17). In that study, a crystal violet vital staining procedure was used 24 hours after PDT to monitor cytotoxicity, whereas we employed a clonogenic assay in our current experiments. Differences in cell types as well as reported variations in the time-dependent efficiency of dye exclusion assays to detect cytotoxic and apoptotic responses following exposure to COX-2 inhibitors may partially explain these differences (23). Our apoptosis results are comparable to recent work where NS-398 induces a small increase in apoptosis in human HeLa cells treated with hypericin-mediated PDT (24).

Reduction in tumor progression following administration of COX-2 inhibitors may be caused in part by an apoptotic mechanism (13, 19, 22). Interestingly, COX-2 inhibitors with similar IC50 values can differ significantly in their ability to induce apoptosis (23). This suggests that cyclooxygenase-independent pathways may be implicated in the apoptotic responses observed with COX-2 inhibitors (19, 25). Our results show that PDT at a suboptimal dose of 100 J/cm2 induced apoptosis in tumors when measured 6 hours after treatment. The extensive and rapid tissue destruction associated with in vivo PDT precluded us from examining in vivo apoptosis at high PDT doses or at extended time intervals. Combining PDT with either celecoxib or NS-398 decreased the levels of detectable apoptosis from levels observed for PDT alone. These results may be due to the difficulty in measuring differences in apoptosis when much of the tumor tissue is rapidly destroyed or that COX-2 inhibitors do not modulate in vivo PDT-mediated apoptosis. In addition, PDT-induced inflammation can lead to the rapid accumulation of host inflammatory cells within treated tumor tissue (26). Early on, these inflammatory cells are primarily neutrophils, which undergo constitutive apoptosis in the presence of TNF-α and could show up as TUNEL-positive cells at 6 hours post-PDT. The higher level of apoptosis found with PDT alone could be due in part to the decrease in inflammation, leukocyte infiltration, and TNF-α within tumors when PDT is combined with a COX-2 inhibitor.

COX-2-mediated expression of PGE2 plays a significant role in tumor angiogenesis by inducing expression of angiogenic regulatory proteins such as VEGF (27). Interestingly, VEGF is also associated with the up-regulation of COX-2 expression in endothelial cells and this involves a GATA cis-acting element in the COX-2 gene (28). This suggests that these two genes may be

Figure 3. COX-2 inhibitors modulate in vivo expression patterns of cytokines and proangiogenic molecules in PDT treatments in BA tumors. Mice were treated with PDT at a dose of 200 J/cm2. NS-398 and celecoxib were given i.p. at a dose of 20 mg/kg immediately after light treatment and again 2, 4, and 23 hours later. Tumor lysates were collected 24 hours after PDT and assayed for levels of TNF-α, IL-1β, and IL-10 using ELISA kits (A), and for PGE2 and VEGF levels using EIA kits (B). Western analysis was used to document MMP-9 expression. Densitometry ratios from MMP-9 versus actin were calculated and plotted (C). Columns, mean; bars, ± SE for three to five mice. *, P < 0.01 (PDT versus control); **, P < 0.01 (PDT versus + Inhibitor).
mutually regulated. PDT induces a proinflammatory response within treated tumors associated with concomitant expression of cytokines including IL-1β and TNF-α (26). Growing evidence indicates that these molecules play a significant role in angiogenesis (29, 30). Our results show that COX-2 inhibitors, at doses that block PGE2 production, attenuate IL-1β and TNF-α expression within PDT-treated tumors. This suggests a direct interaction between COX-2 inhibitor–mediated down-regulation of PGE2 and decreased angiogenesis when PDT is combined with celecoxib or NS-398. Microvessel density measurements could often be used as an indicator of angiogenesis within tumor tissue but the rapid and extensive tumor vessel damage induced by PDT precluded the use of microvessel density analysis to examine the effects of COX-2 inhibitors on PDT responses (27). However, the extensive attenuation of angiogenic growth factor expression following the combined modality provides evidence for blockage of angiogenesis as a major basis for improved tumor responses.

The applicability of long-term use of selective COX-2 inhibitors, including celecoxib, is currently under serious review due to increases in cardiovascular toxicity (12). Nevertheless, preclinical studies and initial clinical trials indicate that celecoxib is effective as both a chemopreventive agent and as an adjuvant to radiation and chemotherapy in treating solid tumors (19). PDT efficiently and rapidly reduces tumor burden, which would leave only minimal disease needing to be targeted with COX-2 inhibitors (3, 8). This suggests that the COX-2 inhibitor doses and treatment schedules may be significantly less than those needed with standard radiation therapy or chemotherapy. Treatment optimization experiments will be required in order to determine appropriate dosing and scheduling variables. Normal skin photosensitization is not increased when PDT and COX-2 inhibitors are combined to treat solid tumors, which suggests that this combination may produce a significant therapeutic gain (8). PDT is approved for the treatment of Barrett’s esophagus and clinical studies continue to document the efficacy of PDT in treating this disorder (31). There is also growing evidence suggesting that COX-2 levels are elevated in Barrett’s esophagus and that COX-2 inhibitors may be beneficial as an adjuvant for treating this premalignant lesion (32). Likewise, recent reports suggest that PDT combined with COX-2-selective inhibitors may be useful in the treatment of carcinomas of the skin and oral cavity (18). Well-designed clinical trials are required to determine if the use of COX-2 inhibitors for limited time periods following PDT have a role in the clinical armamentaria against solid tumors.

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