Photodynamic Therapy Using a Protoporphyrinogen Oxidase Inhibitor

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ABSTRACT

The use of endogenously created porphyrins as an alternative to photosensitizer injection for photodynamic therapy is a rapidly evolving area of study. One common method to induce porphyrin synthesis and accumulation in cells is the topical, oral, or parenteral administration of 5-aminolevulenic acid, a precursor for heme biosynthesis. Porphyrin accumulation may also be elicited by the use of enzyme inhibitors of the heme biosynthetic pathway. Groups of DBA/2 mice bearing SMT-I mammary tumors were placed on a diet containing 0-4000 ppm of a protoporphyrinogen oxidase inhibitor, FP-846. This agent blocks a critical step in porphyrin metabolism and results in elevated intracellular levels of protoporphyrin IX. Light treatment of tumors produced both initial and long-term regression that was dependent on the amount of inhibitor, the duration of inhibitor exposure to animals, and the amount of light used in PDT. Tumor regression occurred without significant destruction of normal tissues in the treatment field and without initial vascular constriction or blood flow stasis. Tumor cure in animals given 4000 ppm FP-846 in feed for 3 days and 300 J/cm² 602-670 nm light (23% cure) was similar to the response in animals given 10 mg/kg Photofrin and the same light dose (20%).

INTRODUCTION

PDT is a new modality for the treatment of solid tumors. Traditionally, PDT involves the administration of a tumor-localizing photosensitizer, followed by excitation of this photosensitizer with light (1). Light excitation results in the generation of toxic species including singlet oxygen (2, 3). Damage to cellular targets by these toxic compounds initiates a multistep cascade that results in selective tumor destruction (4).

An alternative approach to tissue photosensitization for PDT uses specific agents that induce cells to either generate or accumulate endogenous photosensitizing molecules. Tumor photosensitization following modification of the PPIX synthesis pathway in cells is presently under investigation. PPIX is an efficient photosensitizer and is capable of generating toxic species in vivo (5, 6). Two different techniques have been developed to cause PPIX accumulation in cells.

Administration of 5-ALA results in increased biosynthesis and accumulation of PPIX as a result of an overload of the natural biosynthetic pathway for heme (5, 7). Topical administration or injection of ALA has been shown to produce high PPIX levels in tumor compared to surrounding tissues with tumor-surrounding skin ratios that vary between 10:1 and 30:1 (8), depending on the model system investigated. These high ratios of photosensitizer accumulation result in selective destruction of tumor when both tumor and surrounding skin are exposed to light following 5-ALA treatment (6, 9). Clinical application of topical 5-ALA and light has been used for the treatment of basal cell carcinoma, squamous cell carcinoma, carcinoma of the sebaceous gland, and carcinoma of the breast with reports of short-term regression (10-13).

A second approach to induce photosensitizer accumulation in cells involves the use of specific inhibitors of enzymes responsible for heme synthesis. Inhibitors of ferrochelatase have been used in vitro and cause increased PPIX accumulation in both normal and tumor cells both in the presence and absence of exogenous 5-ALA (14, 15). Bacteria with a defect for the ferrochelatase gene show similar accumulation of PPIX (16). A large number of studies have used specific inhibitors of the tetrapterrole synthesis pathway to cause PPIX accumulation in plants and insects (17-19). Although these inhibitors can induce PPIX accumulation alone, the addition of 5-ALA along with these metabolic inhibitors has been shown to increase the amount of PPIX created (20).

Several PPO inhibitors have been recently identified as potential compounds useful in the generation of endogenous PPIX for photodynamic therapy of solid tumors (21, 22). These agents block PPO, a mitochondrial enzyme responsible for the conversion of protoporphyrinogen to PPIX. Paradoxically, this results in increased PPIX because protoporphyrinogen accumulates in the mitochondria and diffuses into the cytoplasm, where it spontaneously oxidizes to form PPIX (22). Because cytoplasmic PPIX is not a substrate for mitochondrial ferrochelatase (which converts PPIX to heme), this PPIX is not metabolized to form heme in the mitochondria, and no feedback inhibition of the synthetic pathway occurs. In this study, we report the use of a PPO inhibitor, FP-846, in mice as a means to elevate PPIX levels in tumors before PDT. The data from the use of other enzyme inhibitors and preliminary data from FP-846 in vitro suggest that this agent may provide sufficient levels of PPIX in tissue to permit tumor destruction after light treatment (22). Mice with SMT-F tumors were exposed to FP-846 in their diet for different regimens, and tumors were exposed to various doses of light in an attempt to optimize therapy using this new compound. These data were compared to results of tumor response after PDT using Photofrin. The relative ability for PDT using FP-846 or Photofrin to induce vascular stasis was also investigated to evaluate the mechanism(s) that lead to tumor destruction.

MATERIALS AND METHODS

Mouse and Tumor System. Female DBA/2 mice (Harlan Laboratories, Indianapolis, IN), 4-6 weeks old (14-22 g), were used in all experiments (23). For tumor implantation into these animals, SMT-F tumor was aseptically removed from donor animals and cut into 1-mm³ fragments. These tumor pieces were placed in HBSS containing penicillin and streptomycin. Tumor pieces were implanted via a trochar into the center of the right flank of shaved mice. Tumors were treated when they reached a diameter of 5-7 mm (8 days after implantation). The skin covering and surrounding the tumor was shaved and depilated 2-3 h before light treatment. Approval for this project was obtained from the Animal Care and Use Committee at the University of Louisville and was in compliance with National Institute of Health guidelines.
FP-846 Administration Protocols. FP-846 was administered to mice in their feed for a period of 3–10 days before light treatment. FP-846 powder was mixed at 2000, 3000, and 4000 ppm with rodent chow and given to animals *ad libitum*. Mixed feed was kept in darkness at 18°C. The average feed intake was measured in animals given FP-846 in their diet and was approximately 4 g feed/mouse/day and did not vary significantly with the dose of FP-846. Body weights of animals given FP-846 were measured daily prior to treatment. For an average animal weight of 17 g, administration of 2000 ppm FP-846 in feed roughly converts to 470 mg/kg FP-846/day, 3000 ppm converts to 700 mg/kg FP-868/day, and 4000 ppm converts to 940 mg/kg FP-846/day.

Photofrin Administration Protocol. Photofrin (porfimer sodium; QLT PhotoTherapeutics, Vancouver, Canada) was resuspended from lyophilized powder in 5% dextrose to a concentration of 2.5 mg/ml. This was further diluted to a concentration of 0.5 mg/ml in normal saline. Solutions containing Photofrin were kept in darkness at 4°C. Animals were injected i.v. (tail vein) with either 5 or 10 mg/kg Photofrin 24 h before light treatment.

Light Treatment Protocols. Tumors on the right flank of animals given FP-846 or controls were illuminated with red light from a 1000-W Xenon arc lamp system (Oriel Corporation) with a condenser and trifurcated fiberoptic bundle. A collimating lens was placed at the end of each fiberoptic to focus the image of the fiberoptic bundle on a treatment area of 1.3-cm diameter (1.33 cm²). The design of the fiberoptic bundle and collimating lens ensured uniform distribution of light over this area. The output spectrum of the arc lamp was tuned to a peak intensity of 630 nm (range, 602–670 nm) using a water filter, a heat-reflecting filter, and a 610-nm long pass filter. Light was delivered at a power density of 135 mW/cm². The possibility of tissue hyperthermia induced by this power density was evaluated by placing thermocouples at selected depths into the tumor during treatment and measuring any temperature rise. No increase in tumor temperature greater than 2.4°C (final temperature of 37.5°C) was observed.

Animals were restrained without anesthesia in specially designed holders. Tumors and surrounding skin were exposed to light from 0 to 450 J/cm² (0–3333 s treatment) at 0, 3, 7, or 10 days after administration of FP-846 in feed or 24 h after injection of Photofrin.

Tumor Response Protocol. Animals were examined daily for tumors regression or regrowth for 14 days after treatment and weekly for up to a total of 91 days after treatment. Mice that remained tumor free at 35 days after treatment were considered cures. No tumor regrowth was noted in any mice between 35 and 91 days after treatment. The number of animals that died before the initiation of light treatment (in animals given 3000 or 4000 ppm FP-846 for 10 days) was excluded in calculations of tumor response and cure.

Fluorescein Dye Exclusion Assay. Animals were given an injection of 150 mg/kg sodium fluorescein i.v. immediately after the completion of light treatment or controls. Animals were sacrificed after 5 min, and a skin flap containing the treated area was raised. Skin flaps were fixed to a glass slide and placed on the stage of a Zeiss model 20 T microscope modified for low magnification fluorescence microscopy. The inner and outer surfaces of the epithelium were examined by fluorescence microscopy to visualize the location of fluorescein dye (450–490 nm excitation wavelength). Microscope images were recorded on videotape using a camera (COHU model 9900; COHU Electronics, San Diego, CA). Camera voltage was standardized at 0.1 V using a 10 ng/ml fluorescein diacetate standard before each experiment.

The concentration of fluorescein dye in specified areas of the skin was examined using digital image analysis. Images were taken from video tape and were digitized at 512 × 512 pixel resolution and 256 gray levels for each video frame and stored in computer memory (PC Vision Plus; Imaging Technology, Inc., Woburn, MA). Spatial averages of tissue fluorescence for sites within the treatment field and adjacent to the treatment site were measured using an image analysis software developed at the University of Louisville, and ratios of fluorescence in tumor compared to skin adjacent to the treatment site were calculated. Three to five measurements of pixel data were taken for each animal and averaged.

Cremaster Muscle Preparation for Microvascular Studies. DBA mice (20–22 g body weight) were anesthetized with sodium pentobarbital (55 mg/kg, i.p.) and placed on their backs on a temperature-controlled heating pad. Rectal temperature was maintained at 37°C, and back temperature was monitored with a thermocouple to avoid local overheating of the skin. The right cremaster muscle was prepared for microvascular observations in the manner reported previously (24). The left carotid artery was cannulated for the measurement of mean arterial blood pressure and heart rate and for the infusion of FITC-labeled albumin. Arteriole and venule pairs in normal cremaster were chosen based on their diameter (20–30 μm) and branching order (fourth order). Vessels in tumor could not be readily classified as arterioles or venules and were chosen based on vessel diameter. The diameter of the column of RBCs in vessels (RBC column diameter) and vessel wall diameter were measured every 5 min for the 37 min of light treatment and every 10 min for a 1-h observation period following light therapy.

RESULTS

Determination of the Optimal Dose of FP-846 for Tumor Destruction. Animals were given 2000, 3000, or 4000 ppm FP-846 in feed daily for 10 days before light treatment of tumor. Tumor implantation was performed on the second day after initiation of FP-846 feeding so that tumors would be at the correct size (5–7-mm diameter) after 10 days total exposure to light. Administration of FP-846 in feed did not alter the time required for implanted tumors to reach a diameter of 5–7 mm, when compared to controls. Animals were exposed to 300 J/cm² red light as described in “Materials and Methods,” and tumor response was evaluated (Fig. 1). For each FP-846 dose studied, response was characterized by necrosis and flattening of the tumor mass and development of an eschar over the tumor site within the first week after light treatment. Slight edema and occasional blanching of tissue was noted in the skin surrounding tumor that was exposed to light during treatment, but this damage did not lead to necrosis. Tumors were cured in 9.1% of the animals given 2000 ppm FP-846 in feed (n = 33) with no apparent systemic toxicity. Animals given 3000 ppm FP-846 in feed for 10 days had tumor cure in 31.6% of surviving animals (n = 19), and those given 4000 ppm FP-846 had tumor cure of 11.8% in surviving animals (n = 17). The animals given 3000 or 4000 ppm FP-846 showed a different timing of tumor regression than animals given 2000 ppm. At the lowest dose of FP-846, tumor regression occurred over 7–10 days as described previously. At either 3000 or 4000 ppm, tumor regression was observed within 24 h of light treatment. Animals given feed without FP-846 (n = 14) and light treatment or animals given FP-846 alone (n = 5) showed no evidence of tumor regression. Long duration of exposure of animals to either 3000 or 4000 ppm FP-846 in feed was accom-
given no FP-846 and 300 J/cm² light showed no short- or long-term tumor response to therapy. Animals given 4000 ppm FP-846 for 3 days (●; n = 22), animals given 4000 ppm FP-846 for 7 days (○; n = 27), and animals given 4000 ppm FP-846 for 10 days (△; n = 17) are shown. Pancreatic tumors were implanted into the right flank 8 days before light treatment and were 5–7 mm in diameter at the time of treatment. Tumors were exposed to 300 J/cm² light at 630 nm (bandwidth, 602–670 nm). The curves represent the percentage of animals with treatment areas free of apparent tumor. Animals given no FP-846 and 300 J/cm² light showed no short- or long-term tumor response to therapy. Animals given 4000 ppm FP-846 for 3 days (●; n = 22), animals given 4000 ppm FP-846 for 7 days (○; n = 27), and animals given 4000 ppm FP-846 for 10 days (△; n = 17) are shown.

Compartmentalized toxicity was unrelated to exposure to ambient lighting because similar results were found in a second group of animals that were kept in complete darkness.

Determination of the Optimal Duration of Exposure of FP-846 in Diet. Mice were exposed to 4000 ppm FP-846 in feed for a total of 0, 3, 7, or 10 days prior to treatment. Mice had SM-T tumors implanted into the right flank 8 days before light treatment and were 5–7 mm in diameter at the time of treatment. Tumors were exposed to 300 J/cm² light at 630 nm (bandwidth, 602–670 nm). The curves represent the percentage of animals with treatment areas free of apparent tumor. Animals given no FP-846 and 300 J/cm² light showed no short- or long-term tumor response to therapy. Animals given 4000 ppm FP-846 for 3 days (●; n = 22), animals given 4000 ppm FP-846 for 7 days (○; n = 27), and animals given 4000 ppm FP-846 for 10 days (△; n = 17) are shown. Pancreatic tumors were implanted into the right flank 8 days before light treatment and were 5–7 mm in diameter at the time of treatment. Tumors were exposed to 300 J/cm² light at 630 nm (bandwidth, 602–670 nm). The curves represent the percentage of animals with treatment areas free of apparent tumor. Animals given no FP-846 and 300 J/cm² light showed no short- or long-term tumor response to therapy. Animals given 4000 ppm FP-846 for 3 days (●; n = 22), animals given 4000 ppm FP-846 for 7 days (○; n = 27), and animals given 4000 ppm FP-846 for 10 days (△; n = 17) are shown. Pancreatic tumors were implanted into the right flank 8 days before light treatment and were 5–7 mm in diameter at the time of treatment. Tumors were exposed to 300 J/cm² light at 630 nm (bandwidth, 602–670 nm). The curves represent the percentage of animals with treatment areas free of apparent tumor. Animals given no FP-846 and 300 J/cm² light showed no short- or long-term tumor response to therapy. Animals given 4000 ppm FP-846 for 3 days (●; n = 22), animals given 4000 ppm FP-846 for 7 days (○; n = 27), and animals given 4000 ppm FP-846 for 10 days (△; n = 17) are shown.
5 mg/kg occurred with only minor damage and necrosis of skin surrounding the tumor. Treatment at the higher Photofrin dose and 300 J/cm² light was not selective; both tumor and surrounding skin were equally damaged. Animals given Photofrin alone (n = 5) or light treatment alone (n = 5) showed no tumor regression and exhibited continued tumor growth.

Effect of PDT Using FP-846 or Photofrin on Blood Flow Stasis. Table 1 presents data for experiments designed to assess global changes in tumor perfusion immediately after PDT using FP-846 or Photofrin. The spatial distribution of fluorescein dye in tumor compared to surrounding untreated skin was determined to provide a relative measure of the degree of vascular stasis. For PDT using 10 mg/kg Photofrin and light, significant reductions in tumor fluorescence were observed which implies that vascular damage and a reduction in blood flow to the tumor had occurred. In mice given 4000 ppm FP-846 for 3 days before light treatment, no significant reductions in tissue fluorescence were observed compared to untreated controls, indicating that PDT using FP-846 does not produce the same magnitude of vascular damage during treatment as seen with Photofrin. The role of vascular damage after PDT using Photofrin and FP-846 was further investigated by intravital microscopy of normal cremaster muscle and SMT-F tumor implanted into cremaster. Animals were injected i.v. with either 10 mg/kg Photofrin or given 4000 ppm FP-846 in feed for 3 days and then were exposed to 300 J/cm² red light. Reductions in the lumen of normal and tumor vessels (20–30 μm diameter) occurred rapidly during PDT using Photofrin and were irreversible (Fig. 5). These changes were completely absent in both normal cremaster and tumor after PDT in animals sensitized with 4000 ppm FP-846 for 3 days.

Effect of Multiple Administrations of Light on PDT Using FP-846. In this series of experiments, animals were given 4000 ppm FP-846 in feed for a total of 3 days followed by treatment of tumor with 450 J/cm² red light. Animals were given FP-846 in feed for 1 additional day after this time, followed by a second treatment with 450 J/cm², or given FP-846 for 3 additional days followed by a second treatment of 450 J/cm² (Fig. 6). Animals given the second light dose the day following treatment had an initial tumor response in 15 of 28 animals (54%) and long-term response in 8 of these animals (29% cure). Animals given the second light dose 3 days after the initial treatment exhibited initial tumor regression in 14 of 25 animals (56%), with 9 of 25 animals tumor-free at day 35 (36% cure). Treatment with either of these multiple light dose protocols produced no significant increase in tumor regression compared to a single light dose protocol.

DISCUSSION

The use of the PPO inhibitor FP-846 was found to successfully induce tumor photosensitivity and highly selective destruction of tumor after light treatment. The basis for this investigation was studies by Halling et al. (22), who showed high accumulated porphyrin levels in tumors in mice given FP-846. Levels in mammary tumors (SMT-F) were 131 μg/g after mice were exposed to 2000 ppm FP-846 in feed for 8 days. These accumulated porphyrin levels are 13–40 times higher than seen in tumors from animals given i.p. or i.v. injected doses of ALA or liposome-encapsulated ALA doses up to 1000 mg/kg and are 100–150 times higher than doses seen after topical administration of 20–40% ALA (4, 8, 13, 25). The studies described in this investigation were performed to investigate the optimal dosage and timing of FP-846 administration and the light dose necessary for successful tumor treatment. We evaluated different treatment and dosage conditions and picked specific combinations that would produce good response to treatment, minimal toxicity, and minimal duration of drug administration.

The optimal concentration of FP-846 in feed was assessed in a dose-response and toxicity study. Increasing the administered dose of
FP-846 from 2000–4000 ppm in feed resulted in greater short-term tumor destruction in animals. The long-term tumor response in animals given 4000 ppm FP-846 was unexpectedly low compared to treatment at lower doses. The reasons for the diminished response are not understood at this time but may be related to the toxicity of the compound when given for a period of 10 days.

Administration of FP-846 for 3 days prior to light treatment produced significant levels of tumor destruction without toxicity to animals. This regimen may be more attractive for clinical use of PPO inhibitors because patients are exposed to the agent for only a short period. Animals submitted to longer feeding periods before light treatment had greater short-term tumor response but no greater tumor cure.

A light dose-response study was initiated to evaluate how tumor destruction could be altered by changing the light dose for FP-846 activation. The results of this study correlate with PDT using exogenous photosensitizers where a threshold dose for response and effective doses were observed (26). Light doses of 150 J/cm² or less did not produce any observable change in the tumors. The two higher light doses used in these experiments, 300 and 450 J/cm², produced good initial response and high levels of tumor cure. The 450 J/cm² light dose caused initial regression in a greater number of animals than the other light doses investigated and slightly higher tumor cure.

The treatment efficacy of PDT after FP-846-induced photosensitization was compared to that of PDT using Photofrin in the same animal tumor model and for identical light doses to place these agents in proper perspective. Animals given 300 J/cm² and 10 mg/kg Photofrin had levels of tumor cure that were indistinguishable from cure in animals given the same light dose and 4000 ppm FP-846 for 3 days. Comparison of response in animals given FP-846 and light compared to published reports of response for PDT using ALA suggest that the use of PPO inhibitors has several advantages: (a) administration of FP-846 produces significantly higher tumor concentrations of photosensitizers compared to ALA. The consequence of this remains unknown, but it implies that greater levels of direct tumor cytotoxicity may occur; and (b) the use of a systemic approach to induce PPIX accumulation may eliminate the observed problems with topical ALA penetration into deep layers of tumors. Systemic administration of PPO inhibitors may allow more homogeneous distribution of PPIX in tumor, particularly at the tumor base where treatment often fails after PDT using topical ALA, and thus offer better long-term tumor control.

An examination of the kinetics of the tumor response curves for PDT using Photofrin compared to PDT using FP-846 illustrates an important difference in early response to therapy. Fewer animals given FP-846 and light showed initial complete tumor response compared to Photofrin, but those tumors that initially responded did not generally reappear over time. This is different from Photofrin, where almost all tumors initially respond to treatment but most recur. The reason why all tumors do not respond equally during the initial days after treatment may be explained by inherent biological differences between animals or more likely by variation in porphyrin accumulation in tumor. Detection of porphyrin fluorescence in tumor before light treatment revealed that animals did not accumulate the same amount of PPIX (data not shown). Animals that had higher initial fluorescence levels tended to respond more favorably to treatment compared to those with lower tumor fluorescence. All animals showed an increase in tumor fluorescence. The lack of complete regression in a significant proportion of treated animals suggests that there may be ways of further optimizing FP-846 administration.

The observation that an initial complete response to PDT using FP-846 was closely predictive of tumor cure (as opposed to PDT using Photofrin) suggests that different mechanisms of damage may be involved with this treatment regimen. Initial tumor destruction after PDT using Photofrin is mediated by blood flow stasis and vascular damage, leading to tissue anoxia and nutrient deprivation (27). At the relatively low Photofrin doses used in treatment, direct damage to tumor cells does not play a significant role in the initial tumor regression (28). The factors that account for the low ratio of tumor cures compared to those tumors that initially responded to PDT using Photofrin remain unknown. In contrast, PDT using FP-846 causes no initial vascular response or change in blood flow during the first hour after the completion of light treatment. These results imply that tumor oxygenation levels remain high during PDT with FP-846, and the treatment is efficient compared to the oxygen limitations of treatment observed during PDT using Photofrin. The combination of high levels of PPIX found in cells in FP-846-treated animals and continued perfusion and oxygenation of tissues may result in sufficient tumor cell death to permit tumor regression and possibly cure. Vascular damage and blood flow stasis presumably occur at some late time after the completion of light treatment, either as a result of direct damage to endothelial cells or breakdown of the entire tumor environment. The contribution of direct cytotoxicity and late vascular damage and stasis for PDT using FP-846 has not yet been addressed.

An absence of a vascular response in tumors or normal tissues in animals sensitized with FP-846 and light is also different from the response observed in animals given PDT using ALA. Although initial reports suggested that direct cytotoxicity was responsible for tumor destruction and was independent of vascular damage (29, 30), recent data find that vascular damage plays a large role in tumor response (6, 31). Treatment of tissues photosensitized by PPIX in response to ALA administration causes endothelial cell damage in vitro (32) and in vivo (33) as well as vessel constriction and reduction or stoppage of blood flow in tumors (6, 34). This response is dependent on the ALA dose and time between drug application and light treatment and is likely related to high circulating levels of PPIX in blood (31).

The lack of initial vascular damage during phototherapy using FP-846 raised the possibility of attempting multiple light treatments to increase the efficacy of tumor treatment. The hypothesis was that tumor cells that were not responsive to the initial treatment may be responsive to a second treatment, provided they could accumulate additional PPIX between treatments and tissue oxygenation remained high. We investigated two different time delays between the initial and second light treatment, but the response of tumors given either of these treatment protocols was disappointing. Further administration of FP-846 in feed and a second light treatment either 1 or 3 days after the initial therapy produced no greater tumor response than the single treatment alone. The reason for this is not clear. This response may result from an inability of additional FP-846 to accumulate in tumor after the initial light treatment due to blood flow stasis or other reasons. Damage to tumor cell mitochondrial enzymes, as seen with PDT using ALA or Photofrin (35), may result in decreased PPIX generation between treatments. Tissue hypoxia resulting from late vessel stasis from the initial treatment or decreased light penetration into tissue because of tissue darkening may also contribute to the lack of additional response.

Photodynamic therapy using FP-846 and red light appears to be a promising alternative to PDT using Photofrin or other exogenous porphyrins. Although a number of basic questions regarding the use of FP-846 still need to be addressed, these data suggest that PPO inhibitors can be successfully used to result in PPIX accumulation in tissue and tumor destruction after light treatment. These studies raise the hope that alternative PPO inhibitors with decreased toxicity and rapid action may be found and raise the possibility that the combined use of PPO inhibitors and exogenous ALA may result in increased tumor response and treatment efficacy.
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