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Mechanism of action of psoralens: isobologram analysis reveals that ultraviolet light potentiation of psoralen action is not additive but synergistic*

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Summary. The combination of psoralens and ultraviolet light (UVA, 320–400 nm), referred to as PUVA, inhibits proliferation of a variety of cell types. In the present studies, we used S-180 cells to investigate the mechanism underlying the antiproliferative actions of PUVA. We found that inhibition of growth of S-180 cells by PUVA was dependent on the concentration of psoralen as well as the dose of UVA light. Neither the psoralens nor UVA light by themselves inhibited cell growth. Several clinically important psoralen analogs inhibited cell growth. The potent phototoxin 4,5′,8-trimethylpsoralen was the most active psoralen analog tested, followed by 5-methoxypsoralen and 8-methoxypsoralen. The angular furocoumarin, 5-methylangelicin, was the least active inhibitor of growth. Multivariate (isobologram) analysis of the growth-inhibition curves revealed that combinations of psoralens and UVA light were not simply additive but synergistic. Similar results were observed when inhibition of DNA synthesis was used as an endpoint for the biological effects of PUVA. These studies are the first to demonstrate that psoralens and UVA light act synergistically. Our results suggest that the synergism between the psoralens and UVA light may be an important property of PUVA that contributes to its therapeutic efficacy in proliferative diseases.

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Abbreviations: 8-MOP, 8-methoxypsoralen; 5-MOP, 5-methoxypsoralen; TMP, 4,5′,8-trimethylpsoralen; 5-MA, 5-methylangelicin; UVA, ultraviolet light of 320–400 nm wavelength; PUVA, psoralens used in combination with UVA light; DMEM, Dulbecco's Modified Eagle's Medium; PBS, phosphate-buffered saline; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulfonic acid; IC_{50}, level of PUVA treatment that inhibits measurable cellular parameters by 50%; [^{3}H]-TdR, [methyl-^{3}H]-thymidine; TCA, trichloroacetic acid; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; CHO, Chinese hamster ovary; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor

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Introduction

Psoralens are clinically important drugs used in the treatment of a number of epidermal proliferative disorders including psoriasis, vitiligo, eczema, and mycosis fungoides [14, 16, 18, 19]. The therapeutic effectiveness of these compounds is dependent on their activation by ultraviolet light in the 320–400 nm (UVA) range [22]. Several naturally occurring psoralen analogs and synthetic derivatives have been used in photochemotherapy (see Fig. 1 for representative structures).

Following absorption of UVA light energy, the psoralen molecule is converted to an electronically excited state that can react directly with cellular macromolecules [20]. Photoactivated psoralens can also transfer energy to molecular oxygen, generating reactive oxygen species such as superoxide anion and singlet oxygen [9]. The formation of reactive oxygen in cells as well as psoralen-protein and psoralen-nucleic acid adducts have been implicated in the biological and therapeutic effects of psoralens and UVA light [21]. For example, treatment of cells with psoralens followed by UVA light results in the formation of both mono- and bifunctional DNA adducts [20]. It is thought that DNA damage resulting from psoralen binding can lead to mutations [1] and decreased cell survival [26].

In the present studies, we analyzed the growth-inhibitory properties of several clinically useful psoralens and UVA light on mouse S-180 sarcoma cells. The biological properties of these psoralen analogs were compared with those of 5-methylangelicin (5-MA, Fig. 1), an angular furocoumarin that cannot cross-link DNA and has little or no activity in cutaneous photosensitization assays [23, 29]. We report that for each of the compounds tested, growth inhibition was dependent on the dose of UVA light as well as the concentration of psoralen. Isobologram analysis revealed that with increasing concentrations of the psoralens, less UVA light was required to inhibit cell growth than would be expected from an additive relationship. Similarly, with increasing UVA light doses, less psoralen was required to inhibit cell growth than expected.

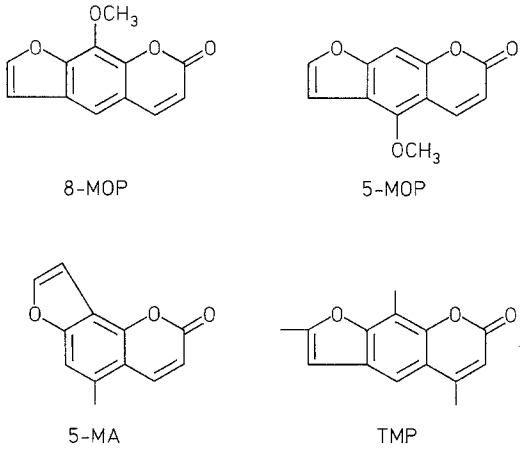

Fig. 1. Structures of psoralens

Table 1. Effect of 8-MOP and UVA light on cell growth and [^{3}H]-TdR incorporation into DNA in S-180 cells[a]

	Cell growth (cells/dish)	[^{3}H]-TdR incorporation (cpm/10^6 cells)
Control (untreated)	5.6×10^5 (100)[b]	4138 (100)
8-MOP (330 nM)	5.5×10^5 (99)	4091 (99)
UVA light (0.69 J/cm^2)	5.4×10^5 (97)	3998 (97)
8-MOP+UVA light	0.7×10^4 (12)*	231 (6)*

[a] Cell growth and [^{3}H]-TdR uptake experiments were performed as described in Materials and methods. Each point represents the average of triplicate plates (SEM, ≤ 0.05)

[b] Growth as a percentage of control values

* Statistically significant ($P \leq 0.01$) differences between control and treated samples

These data demonstrate that at biologically active concentrations, psoralens and UVA light act synergistically to inhibit cell growth. Our data suggest that the doses of UVA light and the concentration of psoralen used are important in determining the optimal therapeutic efficacy of this drug regimen.

Materials and methods

Chemicals. 8-Methoxypsoralen (8-MOP), 5-methoxypsoralen (5-MOP), and 4,5′ 8-trimethylpsoralen (TMP) were obtained from Elder Pharmaceuticals (Bryant, Ohio); 5-MA was provided by Dr. M. A. Pathak, Harvard Medical School (Boston, Mass). [methyl-^{3}H]-thymidine ([^{3}H]-TdR, 2 Ci/mol) was purchased from New England Nuclear (Boston, Mass.).

Cells and culture conditions. The murine sarcoma (S-180) cell line was obtained from Dr. M. T. Hakala, Roswell Park Memorial Institute (Buffalo, N.Y.) and maintained as previously described [11]. Cells were grown in monolayer culture in medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 0.09% sodium bicarbonate, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37° C in an atmosphere containing 5% CO_2. Cells were routinely detached from the culture plates for passaging with 0.2% ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS).

Growth-inhibition assays. For growth-inhibition assays, S-180 cells were inoculated into 3.5-cm plastic cell-culture plates at a density of 5×10^3 cells/cm^2. After incubation for 24 h at 37° C, the growth medium was removed from the plates and the cells were incubated for 30 min at 37° C in 1 ml incubation buffer (25 mM HEPES in PBS, pH 7.4) containing increasing concentrations of the appropriate psoralen analog. Cells were then exposed to UVA light emitted from a bank of four fluorescent light tubes (F40 BL, Sylvania) placed approximately 10 cm above the culture plates. The incident light on the culture plates was 1.6 mW/cm^2 as measured using a model IL 442A International Light UV-radiometer (International Light Inc., Newburyport, Mass.). Control cultures were exposed to drug-free incubation buffer with and without UVA light exposure. Following PUVA treatment, the incubation buffer was removed and the cells were refed with fresh growth medium and incubated at 37° C. After 5 days, the cells were detached from the culture plates and then quantified using a Coulter counter.

[^{3}H]-TdR uptake studies. DNA precursor uptake into exponentially growing S-180 cells was monitored as previously described [11]. Briefly, 5×10^5 S-180 cells were inoculated into 6-cm culture dishes. After 48 h, the cells were treated with PUVA as described for the growth-inhibition assays. Immediately following PUVA treatment, the incubation medium was removed and the cells were refed with serum-free DMEM supplemented with [^{3}H]-TdR (0.2 µCi/ml). After incubation for 1 h at 37° C, the labeling medium was decanted and the cells were washed five times with ice-cold PBS, following which 5 ml ice-cold 5% trichloroacetic acid (TCA) was added to each dish. After 10 min on ice, the TCA was removed from the cells and the monolayer was washed with 5% TCA (5×5 ml) and solubilized with 2 ml 0.2 *N* NaOH for scintillation counting. Protein content was assayed by the method of Bradford [4] using bovine serum albumin as the standard.

Isobologram analysis. Isobologram analysis of the growth-inhibition data was performed as described by Grindey et al. [7] and Elion et al. [5]. In this type of analysis, the dose-response of PUVA is presented graphically with two independent variables ($\times$ = psoralen concentration, z = UVA light dose) and one dependent variable (y = cell growth or DNA synthesis). (For review, application, and interpretation of isobolograms, see Roos et al. [24], Steel and Peckham [28] and Berenbaum [2]. For simplicity, the data for S-180 cells are presented as IC_{50} values for cell growth or DNA synthesis inhibition.

Results

In initial studies, we analyzed the effects of various concentrations of psoralen analogs and doses of UVA light on cell growth. Monolayers of S-180 cells (5×10^3 cells/cm^3) were pretreated for 30 min with increasing concentrations of psoralens and then exposed to a measured dose of UVA light. Cell growth was quantified 5 days later.

Table 1 shows that neither UVA light (0.69 J/cm^2) nor the psoralen analog 8-MOP (330 nM) alone were growth inhibitory for S-180 cells. Increasing the dose of UVA light to 2.6 J/cm^2 or the concentration of 8-MOP to 4.4 µM also had no effect on cell growth (not shown). In contrast, when cells were pretreated with 330 nM 8-MOP and then exposed to UVA light, cell growth was inhibited by >85% (Table 1). Similar results were obtained using TMP, 5-MOP, and 5-MA (Fig. 2). For each of the psoralens tested, growth inhibition was dependent on the concentration of psoralen used and the presence of UVA light. TMP was the most effective inhibitor of cell growth (IC_{50},

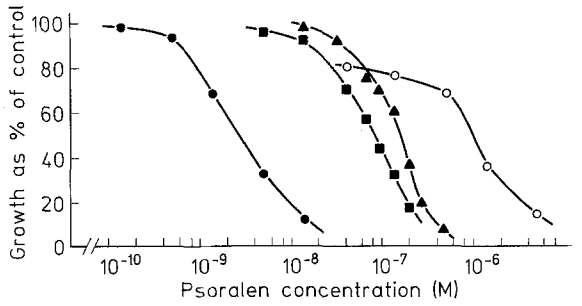

Fig. 2. Inhibition of S-180 cell growth by various psoralen analogs and UVA light. S-180 cells were treated with increasing concentrations of the different psoralen analogs and then exposed to a measured dose of UVA light (0.69 J/cm^2) as described in Materials and methods. Growth inhibition was quantified 5 days later. Data represent a percentage of control values; each point represents the mean of three plates (SE, $\leq$0.06%). The psoralens used were: TMP (●——●), 5-MOP (■——■), 8-MOP (▲——▲), and 5-MA (○——○)

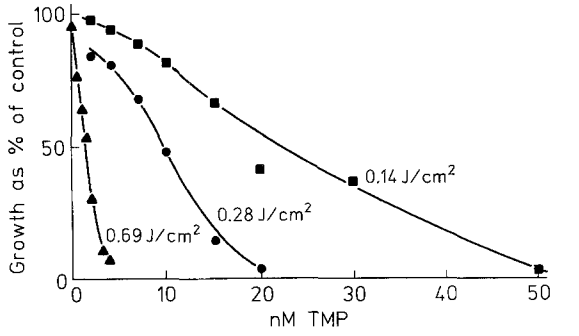

Fig. 3. Effect of UVA light on psoralen-induced growth inhibition. S-180 cells were treated with TMP and 0.69 J/cm^2 (▲——▲), 0.28 J/cm^2 (●——●), or 0.14 J/cm^2 (■——■) UVA light. Cell growth was measured 5 days later. Each point represents the mean of triplicate plates (SE, $\leq$0.06%). Note that the ability of psoralen to inhibit cell growth is dependent on both the psoralen concentration and the UVA light dose. In the absence of UVA light, TMP did not inhibit cell growth (not shown)

2.5 nM), followed by 5-MOP (IC_{50}, 80 nM) and 8-MOP (IC_{50}, 150 nM). The angular furocoumarin, 5-MA, was the least effective inhibitor of cell growth (IC_{50}, 950 nM).

We also found that the ability of the psoralens to inhibit cell growth was dependent on the dose of UVA light. Figure 3 shows the effect of TMP on the growth of S-180 cells in the presence of increasing doses of UVA light. We found that as UVA light exposure was increased, lower concentrations of TMP were required to inhibit cell growth. Unexpectedly, the relationship between the dose of UVA light used and the concentration of TMP required to inhibit cell growth did not appear to be entirely additive. For example, decreasing the UVA light dose from 0.69 to 0.28 J/cm^2, which represents a 2.5-fold reduction, resulted in a 10-fold increase in the concentration of TMP required to inhibit cell growth. Thus, the IC_{50} of TMP was 0.9 nM at 0.69 J/cm^2 UVA light but amounted to 10 nM at 0.28 J/cm^2 UVA light. When the UVA light was further decreased to 0.14 J/cm^2, the IC_{50} of TMP increased only by approximately 2-fold to 22 nM.

To further characterize this response, we analyzed PUVA-induced growth inhibition in S-180 cells using

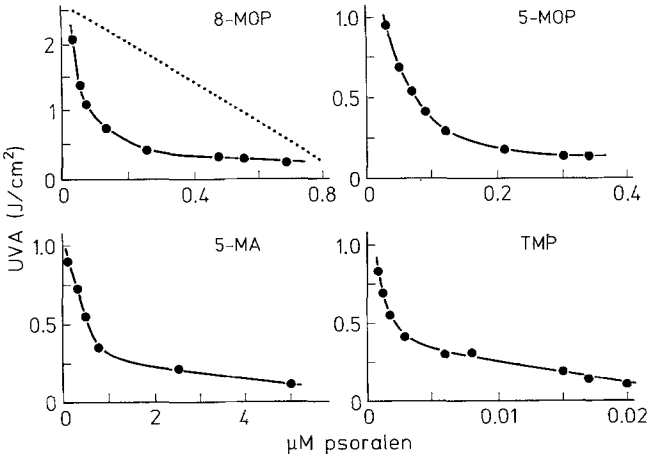

Fig. 4. Isobologram analysis of growth inhibition of S-180 cells by PUVA treatment. From the dose-response curves for the combination of psoralens and UVA light, the IC_{50}s for growth inhibition were determined and an isobologram was constructed [5]. An additive relationship between psoralen and UVA light would produce a straight line as indicated by the *dotted line* in the upper left panel. Curves to the right of the line indicate an antagonistic relationship, whereas curves to the left of the line indicate a synergistic relationship. Note that all of the psoralens tested interacted synergistically with UVA light

isobolograms. In these studies, the concentrations of psoralen and doses of UVA light that produced 50% inhibition of cell growth were plotted against one another. These values are shown in Fig. 4 for each of the psoralen analogs. The dashed line in Fig. 4 (upper left panel) represents the theoretical curve of IC_{50}s, assuming a completely additive relationship between psoralen and UVA light.

It is apparent from the isobologram that the interaction of 8-MOP and UVA light is not additive but synergistic (Fig. 4). With increasing doses of UVA light, lower concentrations of 8-MOP were required to inhibit growth than would be predicted for an additive relationship. Similarly, with increasing concentrations of 8-MOP, lower doses of UVA light were required for growth inhibition than would be predicted by additivity. Similar results were observed using 5-MOP, 5-MA, and TMP. Thus, the observed synergism was independent of the potency of the psoralen analog and of its relative lipophilicity. Furthermore, the fact that 5-MA interacted synergistically with UVA light to inhibit cell growth indicates that DNA cross-link formation is not required for this biological effect.

To determine whether the synergism also occurred at the level of DNA synthesis, we next examined the ability of the psoralens and UVA light to inhibit [^{3}H]-TdR incorporation into S-180 cells. As observed for growth inhibition, neither 8-MOP nor UVA light alone inhibited DNA synthesis in the cells (Table 1). In contrast, the combination of 8-MOP and UVA light produced a 95% inhibition of DNA synthesis. Inhibition of DNA synthesis was also found to be dependent on the concentration of 8-MOP and the dose of UVA light used (not shown). An isobologram of the inhibition of DNA synthesis in S-180 cells by 8-MOP and UVA light is shown in Fig. 5. As with growth inhibition, 8-MOP and UVA light were found to inhibit DNA synthesis in the cells in a synergistic manner.

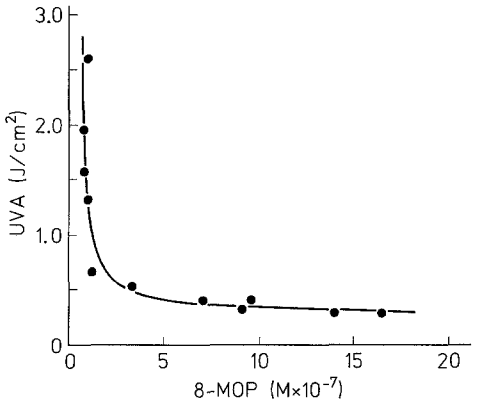

Fig. 5. Isobologram analysis of inhibition of DNA synthesis in S-180 cells following PUVA treatment. Inhibition of DNA synthesis by PUVA was measured as described in Materials and methods. IC_{50}s from dose-response curves for combinations of TMP and UVA light were quantified and used to construct the isobologram. Note that the curve shows upward concavity, a finding consistent with synergism between the psoralens and UVA light

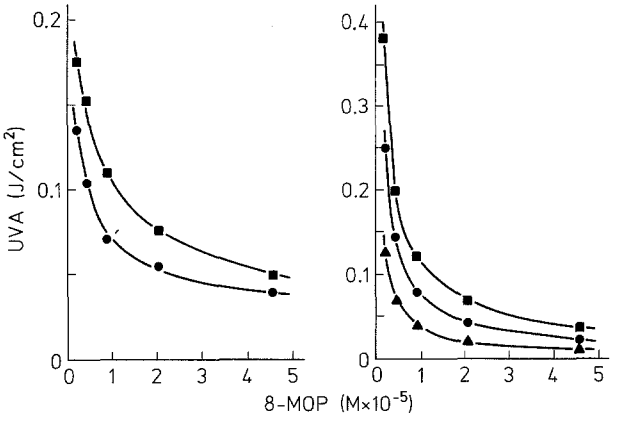

Fig. 6. Synergistic interaction between 8-MOP and UVA light on cell survival and mutation induction in CHO cells. Data on cell survival and mutation induction in CHO cells used to construct these isobolograms were extrapolated from 8-MOP and UVA light dose-response curves reported by Schenley and Hsie [26]. From the dose-response curves for cell survival presented by these authors, the concentrations of 8-MOP and doses of UVA light resulting in either 10% (*squares*) or 50% (*circles*) relative survival of the CHO cells were determined and used to construct the isobologram (*left panel*). The isobologram for mutation induction (*right panel*) was constructed from the dose-response curves of 8-MOP or UVA light generating either 100 (*triangles*), 200 (*circles*), or 300 (*squares*) mutants/10^6 clonable cells. Note that the interaction of 8-MOP and UVA light was synergistic with respect to both cell survival and mutation induction

Since we found that the action of psoralens and UVA light was synergistic, it was of interest to determine whether there was a similar relationship with PUVA for other properties known to be associated with psoralen phototoxicity, including the induction of mutations and cytotoxicity. For these studies, we used the data reported by Schenley and Hsie [26]. These investigators analyzed the ability of 8-MOP and UVA light to inhibit the survival of Chinese hamster ovary (CHO) cells as determined in colony-formation assays and to induce somatic mutations as measured using the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) marker. In this system, inactivation of the HGPRT gene by mutation enables the cells to survive in the presence of 6-thioguanine [17].

Schenley and Hsie [26] demonstrated that cell survival and mutation frequency were dependent on the concentration of 8-MOP and the dose of UVA light. To generate isobolograms from their curves, we arbitrarily chose 10% or 50% relative survival and 100, 200, or 300 mutations/10^6 cells following PUVA treatment as the endpoints for cell survival and mutation frequency, respectively. Data from both experiments show that, as with inhibition of growth and DNA synthesis in S-180 cells, the interaction between the psoralens and UVA light in eliciting biological activity in CHO cells is synergistic (Fig. 6).

Discussion

Our results demonstrate that combinations of psoralens and UVA light inhibit the growth of S-180 sarcoma cells in culture. Growth inhibition was dependent on the dose of UVA light and the concentration of psoralens used. A number of different psoralen analogs were tested and each was found to inhibit S-180 cell growth. TMP, a highly active lipophilic psoralen analog, was the most effective inhibitor, followed by 5-MOP and 8-MOP, two analogs that are frequently used in PUVA photochemotherapy. 5-MA was also found to be growth inhibitory for S-180 cells, but only at concentrations 3- to 4-fold higher than those of the other psoralen analogs. The lower activity of 5-MA in this and other bioassays for psoralens, including cutaneous photosensitization [23, 29], may have been due to the angular nature of this furocoumarin derivative (Fig. 1). In the absence of UVA light, linear furocoumarins such as TMP, 8-MOP, and 5-MOP readily intercalate into cellular DNA, a well-characterized target for the psoralens [20]. Steric considerations limit the ability of 5-MA to associate with DNA and form DNA cross-links [29], and this may decrease its growth-inhibitory potency.

It is well known that the biological activity of the psoralens is dependent on exposure to UV light. However, the resulting effects of PUVA at different doses of psoralen or UV light have not previously been analyzed using isobolograms. Our results show that within the range of biologically active combinations, the psoralens and UVA light interact in a synergistic manner. This was observed in growth inhibition as well as DNA-synthesis inhibition assays. The observation of this synergism in growth-inhibition assays for different psoralens demonstrates that this effect is independent of the relative potency of each analog. Furthermore, our findings with 5-MA and UVA light indicate that this interaction is also independent of the ability of the psoralens to cross-link DNA.

At this time, the mechanism underlying the synergism between the psoralens and UVA light is not known. Although it may reflect physicochemical properties of the drugs during irradiation with UVA light, it may also be due to biological effects on the target cells. Several models based on the inhibition of multiple metabolic targets have

been suggested to explain the interaction of two or more chemotherapeutic agents used in combination [3, 6, 8, 25]. These agents are distinct from PUVA in that each has biological activity of its own. However, the application of these models to PUVA dose-response data suggests that there are multiple sites of action for the photoactivated psoralens.

Consistent with this model is our recent characterization of a site of psoralen action distinct from DNA. In particular, we have identified specific saturable receptor sites for psoralens on sensitive cell types, including S-180 cells [12]. This receptor has been identified as a 22,000 Da protein that is present in membrane and cytoplasmic fractions of cells [30]. We have hypothesized that psoralen receptor binding and UVA light activation initiates some of the biological actions of the psoralens, including growth inhibition [10, 30]. One specific membrane target that may be linked to psoralen receptor activation is the epidermal growth factor (EGF) receptor. PUVA is a potent inhibitor of EGF receptor binding [13, 15]. EGF is a growth-regulatory peptide [27], and cell-growth inhibition following PUVA treatment may, at least in part, be the result of alterations in the response of cells to growth factors. Thus, the biological activity of PUVA may be due to the ability of the photoactivated psoralens to interact with the psoralen receptor as well as DNA. The concentrations of psoralen and the doses of UVA light used may dictate the sites of action of PUVA, and appropriate inhibition of each site could lead to the observed synergism.

The synergism betwen psoralen and UVA light was not limited to growth- and DNA-synthesis inhibition, but was also observed in mutation induction and cell survival. These data indicate that this response is potentially important in toxic as well as therapeutic actions of the psoralens. Since toxic responses limit the use of PUVA, it is important that the interaction of the psoralens and UVA light be examined in these as well as in therapeutic responses to enable a better definition of the role of this synergism in the action of PUVA.

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