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Inhibition of Histidine Ammonia Lyase by 8-Methoxypsoralen and Psoralen-oxidized Photoproducts

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ABSTRACT

The effect of 8-methoxypsoralen-UVA therapy on the catalysis of histidine to *trans*-urocanic acid by histidine ammonia lyase (HAL, EC 4.3.1.3) was examined using an enzymatic assay from Sigma-Aldrich where the growth of the *trans*-urocanic acid peak at 277 nm was monitored. A Rayonet Photochemical Mini Reactor (Model RMR-600) equipped with eight, 3500 Å light sources and a custom UVA filter (Model S-BAL3 2.9 mm), from the Solar Light Company, were used to expose various reaction mixtures to broadband UVA light and UVA/UVB light. A UV-Vis spectrophotometer (Model Shimadzu UV 2540) with a temperature-controlled cell holder (Model TCC240) was used to monitor the growth of the *trans*-urocanic peak. Results of dark-binding experiments of 8-methoxypsoralen in denatured ethanol indicate no inhibition of enzyme activity due to ethanol but noncompetitive inhibition due to 8-methoxypsoralen. The effects of preirradiated 8-methoxypsoralen, with both broadband UVA and UVA/UVB, indicate that inhibition was due to psoralen-oxidized photoproducts. Inhibition of HAL was found when exposed to broadband UVA/UVB and to a lesser extent when exposed to broadband UVA.

INTRODUCTION

In the decades since 1974, when Harvard Medical School dermatologists first successfully treated patients with psoriasis using 8-methoxypsoralen (8-MOP, see Fig. 1) and UVA, photochemotherapy has become an important therapeutic tool in treating numerous dermatological ailments (1). At present, there is strong evidence that photochemotherapy acts *via* modulation of the immune system. For example, it is known to affect antigen-presenting cells, to modulate cytokine production of various cells, to damage DNA, to increase chromosomal aberrations in lymphocytes and to increase the risk of squamous cell carcinoma and melanoma (2–4). 8-Methoxypsoralen-UVA (PUVA) therapy is coupled with application of dihydroxyacetone to protect patients from the adverse effects of photochemotherapy (5,6). In addition, psoralen is known to react with proteins, RNA, lipids and also affect enzyme activity (7–11). However, even with the advances in immunology and molecular biology, we are far from understanding the mechanisms, and adverse effects, underlying photochemotherapy and what role photochemotherapy plays in photoimmunosuppression. *Trans*-urocanic acid (*trans*-UCA), which is produced by the histidine ammonia lyase (HAL) in the skin (Fig. 2), was considered to be skin's natural sunscreen because its UV absorption spectrum overlaps with that of DNA (12). *Trans*-UCA is now considered as a photoreceptor for photoimmunosuppression. *Trans*-UCA undergoes isomerization to *cis*-urocanic acid (*cis*-UCA) when exposed to UVB light (13–15). *Cis*-UCA has received a great deal of attention, as Mohammed *et al*. (16) point out in their review article, since De Fabo and Noonan (17) first proposed *cis*-UCA is responsible for photoinduced immunosuppression. Since the seminal article by De Fabo and Noonan (17), numerous studies have investigated the immunomodulatory role of *cis*-UCA (18–20) and others have examined the role of *cis*-UCA in the course of UV therapy (3,21–23). Results of these and other studies (24–26), suggest that PUVA therapy

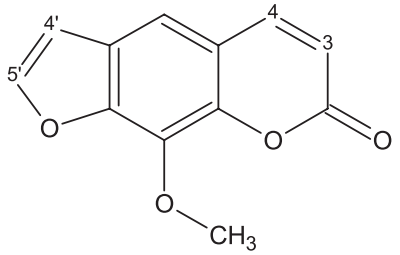

Figure 1. Lewis structure of 8-methoxypsoralen.

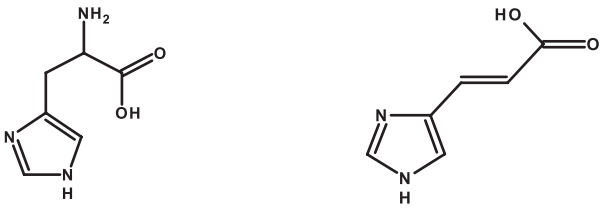

Figure 2. Reaction scheme of enzyme catalysis.

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and *cis*-UCA are two modes of immunosuppression that are not causally linked. Most studies of the photosensitizing effects of psoralens have investigated 8-MOP, 5-methoxypsoralen, psoralen, angelicin, *etc*., and found each to have different photosensitizing properties (7–11). None of these are known to increase the activity of an enzyme; in fact, the activity is hindered. However, Mandel *et al*. (26) found that an increase in HAL activity occurred with the prolongation of laser radiation and the photosensitizers, brilliant green and ammifurin. Ammifurin is a mixture of two compounds that are similar in structure to 8-MOP. If PUVA therapy is shown to inhibit HAL activity, theoretically less *trans*-UCA will be produced and, in turn, less *cis*-UCA. How does the suppression of *trans*-UCA acting as a natural photoreceptor, and *cis*-UCA, acting as an immunosuppressant, affect the immunosuppression of patients undergoing PUVA therapy?

Specific aims

The long-range scope of this research is to determine the effect that PUVA therapy has on the enzymatic catalysis of histidine to *trans*-UCA by HAL in skin and its role in photoimmunosuppression. Within this scope are two paths of study that are related. First, to determine the type of enzymatic inhibition that is occurring, *in vitro*, and second, the type of photochemical reaction that causes inhibition. In the process of applying PUVA therapy, is inhibition due solely to the irradiation, a psoralen-oxidized photoproduct (POP) or a photoadduct of 8-MOP and HAL? These are difficult questions to answer. If the inhibition is not due to direct irradiation or to a photoadduct of HAL-8-MOP, then the POPs present when 8-MOP is irradiated must be isolated, purified, and the enzyme kinetics determined individually ([30] and references therein). It is the isolation and purification that proves difficult; few POPs, due to their instability, have been isolated and purified. The present scope of this research is to show the type and cause of the inhibition due to 8-MOP and if POPs inhibit HAL. The specific type of photochemical reaction will be investigated in the future.

MATERIALS AND METHODS

The activity of HAL was determined using an enzymatic assay from Sigma-Aldrich in which the growth of the characteristic peak of *trans*-UCA at 277 nm was monitored. A Shimadzu UV-Visible spectrophotometer (Model UV2540) equipped with a Shimadzu temperature-controlled cell holder (Model TCC 240) was used to monitor the growth of the *trans*-UCA peak. All components of the assay (buffers, enzyme, 8-MOP) shown in Table 1 are added together prior to adding the histidine. Denatured ethanol (anhydrous, denatured 94%–96%) was obtained from EMD Chemicals, TRIS hydrochloride (molecular biology grade) was obtained from VWR International, magnesium chloride (ACS reagent grade) from Fisher Scientific, glutathione (97% Grade) from Alfa Aesar, L-histidine (reagent plus grade ≥99%) from Sigma-Aldrich and, 8-methoxypsoralen (≥98% [GC]) from Sigma-Aldrich. The enzyme solution was kept at 25°C prior to adding the histidine and during the collection of the absorbance data. All experiments were compared to a baseline of the HAL activity rate which is HAL without exposure to UVA light or any inhibitor. The effect of PUVA treatment on HAL was examined after using a Rayonet Photochemical Mini Reactor (Model RMR-600) equipped with eight, 3500 Å light sources. The lamp wavelength range is from 300 nm to 400 nm with 90% in the 350 nm range. A filter (Model S-BAL3 2.9 mm) from the Solar Light Company was used to keep the broadband UVA radiation between 322 nm and 400 nm.

Table 1. Components of enzymatic assay.

	Baseline	Test	Blank
Tris HCl buffer pH = 9.0	0.50	0.50	0.50
$MgCl_2$ (10 mM)	1.00	1.00	1.00
Glutathione (100 mM)	0.10	0.10	0.10
Enzyme	0.05	0.05	—
Enzyme diluent	0.05	—	0.05
8-MOP in ethanol (100.0 μM)	—	0.03	0.03
Deionized H_2O (Incubate 30 min)	0.90	0.92	0.92
Histidine	0.40	0.40	0.40
Total volume	3.00	3.00	3.00

Modified *Escherichia coli* BL21 (DE3) were obtained from Professor Janos Retey and his colleagues at the University of Karlsruhe in Germany. HAL was harvested and purified according to Langer *et al*. (27). A Bradford assay was used to determine histidase concentration and to calculate turnover values. Experiments were run in triplicate for each concentration of histidine and the error bars found in the figures represent one standard deviation from the average. The parameters modified were the concentration of histidine, concentration of 8-MOP and the type of radiation administered. Experiments included dark binding, where no radiation is administered, broadband UVA (322–400 nm) and broadband UVA/UVB (300–400 nm). Single wavelength monitoring was used to obtain the rate of change of the 277 nm peak of *trans*-UCA. Data are presented as Lineweaver–Burke plots since the Lineweaver–Burke equation is both the reciprocal of the Michaelis–Menten equation and a linear equation and, as such, presents a direct method of comparison of the data. The kinetic parameters K_m and V_{max} were obtained from linear regression of the data.

RESULTS AND DISCUSSION

8-MOP is not soluble in water and had to be added as solution in denatured ethanol. It was not apparent if denatured ethanol may cause inhibition. The enzyme activity of HAL, with 0.3 mL of denatured ethanol added, compared to a baseline activity of HAL (rate of activity of HAL without exposure to UVA or any inhibitor), indicate HAL was not inhibited.

Dark binding refers to the reaction in the absence of radiation whether it is 8-MOP or a solution of 8-MOP and HAL. This is an important step and the first series of experiments involve 8-MOP in denatured ethanol. The final concentration of 8-MOP was 10 μM.

A Lineweaver–Burke plot of solutions of 8-MOP in ethanol added to HAL is shown in Fig. 3 and the kinetic parameters are listed in Table 2. From the difference in structures of histidine and 8-MOP (see Figs. 1 and 2), competitive inhibition due to 8-MOP was not expected. However, it was not clear if 8-MOP would cause uncompetitive or noncompetitive inhibition. As shown in Fig. 3, 8-MOP inhibits HAL and the inhibition increases as the concentration of 8-MOP increases. The K_m values suggest that the inhibition is noncompetitive. There are significant differences ($P < 0.05$) between all of the date points with respect to the baseline data except for the data points of the lowest concentrations (0.67 mM) of histidine.

As Potapenko (28) has shown, POPs, from preirradiated psoralen, are also effective in treating skin ailments, suggesting that POPs also play a role in the mechanism of photochemotherapy. From Caffieri's ([29] and references therein) review article on the photochemistry of psoralens, it is not clear what type of inhibition is expected if psoralen is irradiated first and then added to HAL. Numerous products are formed when

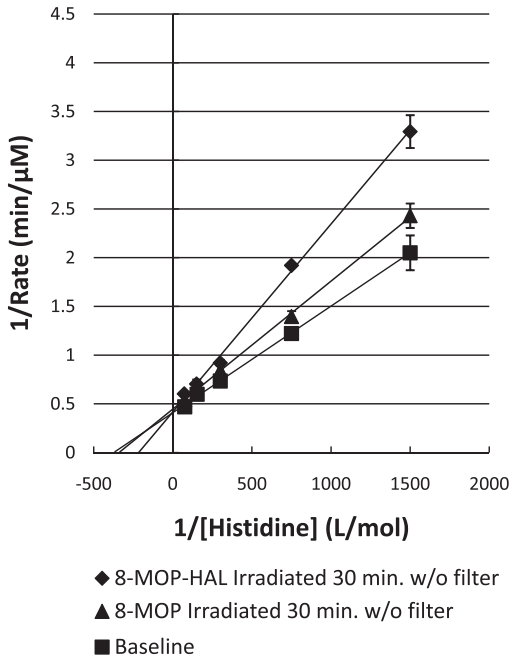

Figure 3. Lineweaver–Burke plot of 8-MOP inhibition of HAL.

psoralen is irradiated, and only a few of the structures of the stable products have been elucidated (Caffieri [29] and references therein). The determination of the specific POP(s) responsible for inhibition is beyond the scope of this present research.

As mentioned above, the UV lamps used have a small percentage of wavelengths below 320 nm. A filter (Model S-BAL3 2.9 mm) from the Solar Light Company was used to keep the broadband UVA radiation between 322 nm and 400 nm. Solutions of 8-MOP in ethanol irradiated, in air, for 30 min were examined which resulted in a dosage of 148 J cm^{-2}.

Presented in Fig. 4 are Lineweaver–Burke plots of HAL with 8-MOP in ethanol that has been preirradiated. The 8-MOP was irradiated with broadband UVA and broadband UVA/UVB, separately. A comparison of the baseline and preirradiated sample of 8-MOP with UVA, along with the kinetic parameters listed in Table 2, suggest uncompetitive inhibition. There are significant differences ($P < 0.01$) between the data points of the baseline and of those irradiated with UVA. A comparison of the baseline and preirradiated sample of 8-MOP with UVA/UVB, along with the kinetic parameters, suggest mixed inhibition. There are significant differences ($P < 0.05$) between the data points of the baseline and of those irradiated with UVA/UVB except for those of the lowest concentration of histidine (0.67 mM) and those of 6.67 mM histidine. As mentioned, Potapenko (28) has shown POPs, from preirradiated psoralen, are effective in treating skin ailments; however, most of the products have not been identified. This is the first time that POPs are shown to inhibit HAL. A comparison of the broadband UVA *versus* broadband UVA/UVB shows that there is more inhibition of HAL with the broadband UVA.

It has been well established that 8-MOP reacts with DNA, RNA, lipids and proteins ([7] and references therein). The work of Sastry (30) has indicated that 8-MOP reacted with tyrosine in a protein and formed a photoadduct. However, Sastry did not propose a structure after isolating the tyrosine-psoralen photoadduct. Direct irradiation of mixtures of 8-MOP and HAL is an important step in determining the cause of the inhibition. Comparisons of POPs *versus* 8-MOP–HAL mixtures irradiated with broadband UVA are shown in Fig. 5. There are significant differences ($P < 0.05$) between three of the five data points of the baseline and of those of 8-MOP-HAL irradiated with UVA; significant differences were not found for the data points of histidine concentrations of 3.33 mM and 1.33 mM. When HAL is mixed with 8-MOP and irradiated with broadband UVA we find a decrease in inhibition when compared to 8-MOP irradiated directly with broadband UVA. The inhibition from 8-MOP-HAL irradiated together comes from another source. It can be either a POP that is formed in the system, a photoadduct of 8-MOP and HAL, from singlet oxygen due to 8-MOP being a photo sensitizer or directly from UVA. In any case, the inhibition is significantly less when compared to the POPs formed in broadband UVA. A comparison of POPs *versus* 8-MOP-HAL mixtures irradiated with broadband UVA/UVB is shown in Fig. 6. There are significant differences ($P < 0.05$) for all of the data between the baseline and 8-MOP-HAL irradiated with UVA/UVB. Interestingly, when HAL is mixed with 8-MOP and irradiated with broadband UVA/UVB we find an increase in the inhibition when compared with 8-MOP irradiated directly. Similar to what was stated above, this can be due to several different sources but, additionally, could be due to the UVB radiation present.

It has been shown that amino acids are susceptible to photo-oxidation (31,32). As mentioned, one question raised was whether HAL is inhibited solely by irradiation. In turn, direct irradiation of HAL was examined to determine whether or not it is inhibited by broadband UVA or broadband UVA/UVB.

Table 2. Kinetic parameters of HAL experiments.

Sample	V_{max} (μM min^{-1})	K_m (mM)	K_{cat} (min^{-1})
Baseline	2.61 ± 0.100	0.0026 ± 0.0001	965442
8-MOP (10 μM) unirradiated	1.09 ± 0.033	0.00241 ± 0.0001	403192
8-MOP (100 μM) unirradiated	1.98 ± 0.059	0.00237 ± 0.0003	732404
8-MOP irradiated 30 min w/o filter	2.45 ± 0.096	0.00318 ± 0.0001	906258
8-MOP irradiated 30 min w/filter	1.39 ± 0.086	0.00222 ± 0.0002	514162
8-MOP + assay irradiated 30 min w/o filter	1.87 ± 0.009	0.002867 ± 0.004	691715
8-MOP + assay irradiated 30 min w/filter	2.00 ± 0.002	0.00264 ± 0.002	739802

HAL = histidine ammonia lyase; MOP = methoxypsoralen.

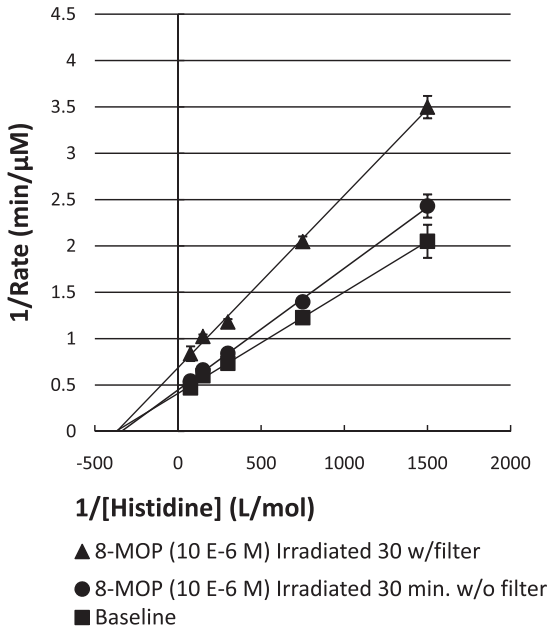

Figure 4. Lineweaver–Burke plot of HAL with 8-MOP irradiated 30 min using broadband UVA and broadband UVA/UVB.

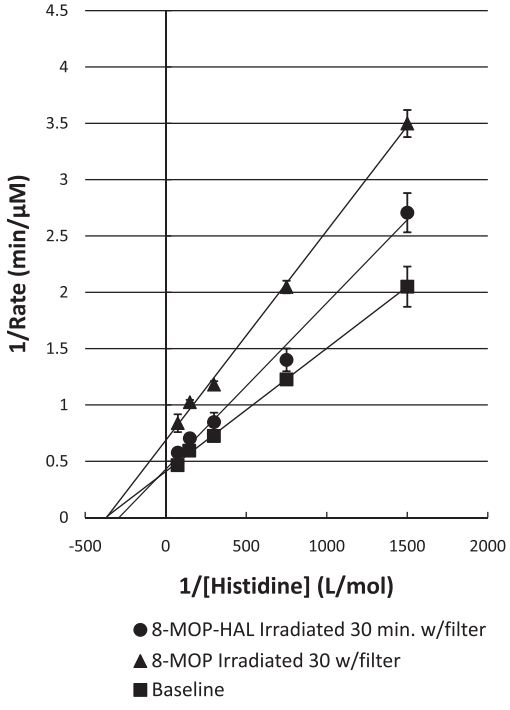

Figure 5. Lineweaver–Burke plot of HAL-8-MOP irradiated 30 min with broadband UVA.

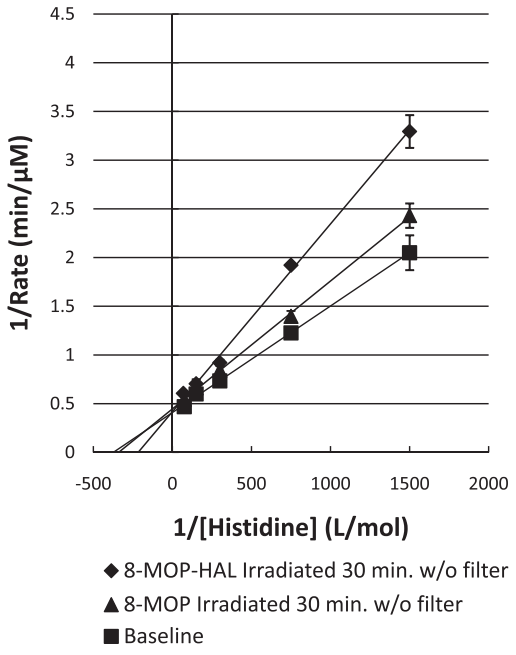

Figure 6. Lineweaver–Burke plot of HAL-8-MOP irradiated 30 min with broadband UVA/UVB.

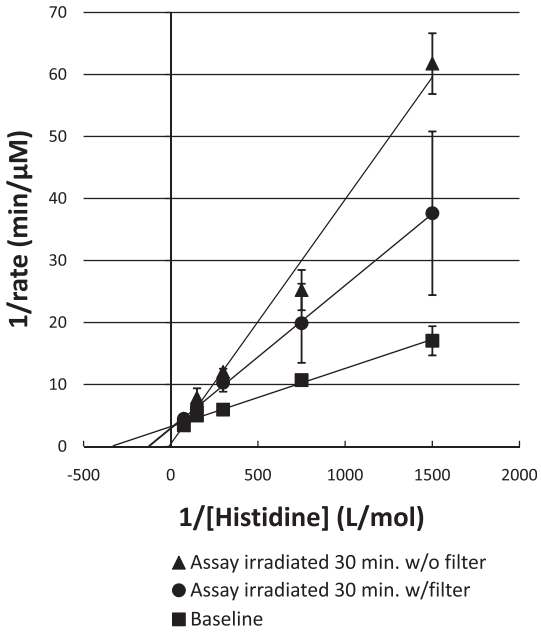

Figure 7. Lineweaver–Burke plot of HAL irradiated 30 min with broadband UVA and broadband UVA/UVB.

Shown in Fig. 7 are the Lineweaver–Burke plots of HAL irradiated directly for 30 min with broadband UVA and with broadband UVA/UVB. The enzyme concentration of the data shown in Fig. 7 was higher than enzyme concentration shown in other figures as it was a different batch of enzyme used. This set of experiments was performed last but, due to the limited lifetime of the original enzyme stock solution the results were erroneous. In turn, a new batch of enzyme was used. No attempt was made to match the level of enzyme since the sole purpose of the experiments was only to show the effect of UVB on enzyme activity. As such, there is a change in activity of HAL when it is exposed to UVA radiation. However, when broadband UVA/UVB is applied there is an increase in inhibition due to the amount of UVB present. Although there is a large variance in the data, all the data points are significantly different ($P < 0.05$), with respect to the baseline, except for the two highest concentrations of histidine (6.67 mM and 13.3 mM).

The photochemical reactions under conditions in which oxygen is present and absent from the sample being irradiated will have to be investigated in the future. It is generally accepted that there are three types of reactions of photosensitization (33,34). In Type I reactions, the excited state of psoralen can generates radicals in the substrate as shown:

$$^3\text{Psoralen} + \text{Substrate} \rightarrow \text{Psoralen} + \text{Substrate}^{\bullet}$$

In turn, the substrate may take up oxygen. In Type II reactions, the excited state of psoralen transfers its energy to molecular oxygen generating singlet oxygen; in turn, singlet oxygen can react with the histidase, psoralen or other species present as shown:

$$^3\text{Psoralen} + \text{O}_2 \rightarrow {}^1\text{O}_2 + \text{Psoralen}$$

In Type III reactions the excited state of psoralen reacts directly with the substrate forming a covalently bonded photoadduct as shown:

$$^3\text{Psoralen} + \text{Substrate} \rightarrow \text{Substrate} - \text{Psoralen}$$

When a solution is deoxygenated, Type I and II reactions can be distinguished from Type III reactions. If we eliminate oxygen, different POPs are obtained and we would expect different inhibition. If Type I and II reactions are eliminated as the source of inhibition, this research takes on a new dimension. Similar to what Sastry (30) accomplished, an examination of the possible photoproducts would have to be undertaken. If Type III is eliminated we will have to investigate the role that oxygen and singlet oxygen play in the inhibition of HAL. The specific type of photochemical reaction will be investigated in the future.

As mentioned above, the long-range goal of this research is to determine the role that PUVA therapy has on the enzyme HAL and its role in photoimmunosuppression. If PUVA therapy is shown to inhibit HAL activity *in vivo*, theoretically less *trans*-UCA will be produced and, in turn, less *cis*-UCA. The question then becomes, does the suppression of *trans*-UCA acting as a natural photoreceptor, and *cis*-UCA, acting as an immunosuppressant, affect the immuno-suppression of patients undergoing PUVA therapy? Vestey and Norval (35) showed that there was an anti-psoriatic effect in subjects with a topical application of *cis*-UCA which, again, suggest that these two modes (PUVA therapy and *cis*-UCA) of photoimmunosuppression are not causally linked. In addition, what role, if any, does this have on the more serious side effects of PUVA therapy, the increased risk of squamous cell carcinoma and melanoma (2–4)? DeFabo *et al.* (25) addresses part of this issue in their paper on levels of *trans*-UCA in mice regulated by dietary levels of l-histidine and their relevance to cancer. Their roles are still under investigation.

We have shown that 8-MOP and its oxidized photoproducts inhibit HAL *in vitro*. This work is at the crossroads of enzyme kinetics, photochemistry, photochemotherapy and photoimmunosuppression. In order to advance the understanding of the role PUVA therapy plays in photoimmunosuppression, we advocate a multi-prong approach. First, as Weichenthal and Schwarz (36,37) suggest, further investigation into the mechanisms of UV therapy are warranted. Second, some work has been done on urocanic acid levels *in vivo* (12,15,38,39) but a comprehensive, *in vivo*, study on the levels of histidine, *trans*-UCA, *cis*-UCA and HAL activity, comparing subjects undergoing PUVA therapy with those that are not, is needed. Third, a study to determine the type of photochemical reaction responsible for the inhibition of HAL, *in vitro*, is necessary.

CONCLUSIONS

Presented in this paper were the effects that 8-methoxypsoralen and POPs have on the catalysis of histidine to *trans*-urocanic acid by HAL (EC 4.3.1.3) using an enzymatic assay that monitored the growth of the *trans*-urocanic acid peak at 277 nm. The dark binding experiments of HAL have shown there is no inhibition due to denatured ethanol and the type of inhibition caused by 8-methoxypsoralen was determined to be of a noncompetitive type. POPs, formed when irradiated by both broadband UVA and broadband UVA/UVB, were found to inhibit HAL. HAL, when irradiated with filtered radiation (322–400 nm), was not destroyed due to photo-oxidation of the amino acids but did show inhibition when compared to the baseline. When unfiltered irradiation (300–400 nm) was used, the enzyme was found to be inhibited more than when irradiated with UVA radiation alone.

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