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Benzophenone and Padimate-O Protect *Saccharomyces cerevisiae* From UV Radiation and Cause Little Harm From UV-Induced Reactive Chemical Species

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It has been shown that some of the compounds used in sunscreens form harmful reactive species upon exposure to UV radiation, which may counteract protective benefits the sunscreen may provide. The protection provided for *Saccharomyces cerevisiae* by two sunscreen chemicals, padimate-O and benzophenone, during a UV light survival assay outweighed potential negative effects that the chemicals may have had. No effect on *S. cerevisiae* was observed when the yeast were subjected to chemicals exposed to UVA light, suggesting that the parameters in this experiment were insufficient to generate free radicals in these chemicals, or were unable to maintain the chemicals in an excited state long enough to produce any effect on cells. Further, the effects of intracellular free radical generation on the yeast cells were minimal and were obscured by cell death caused by direct UV radiation.

In today's health conscious world, the use of sunscreen is encouraged year-round for those who venture outdoors. However, it has been shown that some of the compounds used in sunscreens form harmful reactive species upon exposure to ultraviolet radiation (UV) radiation, which may counteract protective benefits the sunscreen may provide (3-6, 8, 12).

Ultraviolet radiation is categorized according to wavelength, the shortest of which is Ultraviolet-C at 100 to 280 nm. Since this type of UV radiation does not reach the earth's surface due its absorbance by the ozone and atmosphere, it is of little interest here. Ultraviolet-B radiation ranges from 280 to 320 nm, and while the shorter wavelengths may also be absorbed by the stratospheric ozone, the higher wavelengths are capable of causing structural and cellular skin damage, erythema and skin cancers (5). Finally, Ultraviolet-A radiation has the longest wavelengths at 320 to 400 nm, and is responsible for wrinkling, photoaging of the skin, and melanoma induction (5). Ultraviolet radiation exerts its damaging effects on nucleic acid by creating cyclobutane-type dimers, pyrimidine adducts, pyrimidine photohydrates and DNA-protein cross-linking (13). It also has effects on certain amino acids, such as by splitting disulfide bonds, and negatively affects the lipid membranes of cells (13). These effects cause cell death and mutations which can lead to the above mentioned skin abnormalities.

Commercial sunscreens consist of a variety of compounds that absorb or block UVA and/or UVB radiation. They contain both chemical filters, which are generally organic molecules which absorb mainly UVB radiation, and physical filters, which scatter and reflect UVA and UVB. Most sunscreens contain these filters in varying combinations since no single agent currently provides acceptable Sun Protection Factor (SPF) levels on a broad enough spectrum (11). Some of the most common chemical filters are derivatives from PABA (p-aminobenzoic acid) and benzophenone (11). These parent compounds, once widely used in sunscreens, have declined in use and have even been banned in Europe due to elucidation of their toxic effects (1, 11).

Padimate-O (2-ethylhexyl-4-dimethylaminobenzoate, see Figure 1) is a PABA derivative that adsorbs UVB (7). However, upon illumination in aqueous media, padimate-O generates singlet molecular oxygen as well as carbon-centered free radicals, which could be potentially damaging to cells (1, 7). The dimethylamino group in padimate-O (electron-donating) is conjugated to a carbonyl-group (electron withdrawing) by an aromatic ring, which allows for free radical formation (7). Padimate-O has been found to be mutagenic to yeast to the same degree as benzophenone through DNA sequencing techniques (6). The DNA damage inflicted by illuminated padimate-O results directly from free radical generation, in particular hydroxyl formation, which creates strand breaks by attacking GC base-pairs (8). As well, padimate-O has been shown to be absorbed by human skin cells, thus it may have intracellular effects that can have greater damaging potential (6, 11).

Benzophenone (see Figure 1) is not currently in use as a sunscreen agent for human application, however several of its derivatives are. Benzophenone has been called 'the most potent free radical generator known to man' and is commonly used in industry as a photoinitiator to manufacture agricultural chemicals and pharmaceuticals (9). Upon excitation with UV light, benzophenone forms ketyl radicals and triplet state molecules, which are able to create cyclobutane-type dimers and pyrimidine adducts within DNA (2). Since the carbonyl group involved in free radical

formation is constant among all of the benzophenone derivatives used in sunscreen products, the DNA damage inflicted by them is expected to be comparable to their benzophenone parent compound.

This experiment examined whether the benefits of using two common UV absorbers for UV protection is annulled by the potentially damaging reactive species formed by these compounds upon UV exposure. Padimate-O and benzophenone were chosen for their abilities to both protect cells from UV damage and to form reactive species upon activation by UV light. *Saccharoymyces cerevisiae* was chosen as the model organism to represent human skin cells on which sunscreens are applied.

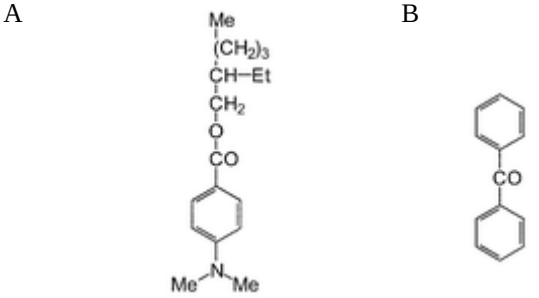

Figure 1. Chemical structures of (A) padimate-O and (B) benzophenone (modified from 6)

MATERIALS AND METHODS

Yeast strains and culture conditions. Experiments were carried out with two wild-type wine strains of *Saccharomyces cerevisiae*, Vin7 and ST (commercially available strains supplied by the Wine Research Centre at UBC). Unless specified otherwise, cultures were grown at 30ºC with aeration in YPD broth (10).

Preparation of media and stock solutions. Yeast peptone dextrose (YPD) broth and agar (2%) were prepared as previously described (10). Benzophenone and padimate-O (both from Sigma-Aldrich) stock solutions were made in either acetone at 50% w/v and 50% v/v, respectively, or in 95% ethanol at 10% w/v and 10% v/v, respectively. Stock solutions were stored in the dark at 4ºC.

Protective effects of benzophenone and padimate-O for *S.cerevisiae* from UV radiation. Overnight cultures of each yeast strain were diluted 1/5 with distilled H_20 in a total volume of 5 mL, to which 50 uL of 50% benzophenone, 50 uL of 50% padimate-O, 50 uL of acetone or nothing was added. Samples were poured into 100 mm polystyrene Petri dishes and exposed to UV doses from 0 to 5000 J/m^2 in a Stratalinker 2400 Crosslinker (Stratagene, 254nm) or kept in the dark without UV exposure. After each UV dose, dishes were shaken slightly and 100 uL of each sample was collected and plated in duplicate on YPD agar at appropriate dilutions. Plates were incubated at 30°C for 48 hours.

Damaging effects on *S.cerevisiae* by reactive species of UV-activated benzophenone and padimate-O. One hundred mm polystyrene Petri dishes were coated with either benzophenone or padimate-O by adding 1 ml of 10% benzophenone or 10% padimate-O to the dishes. The ethanol was evaporated off by placing the plates at 37ºC for 2 hours with the lids off. Overnight cultures of both yeast stains were washed twice and re-suspended in PBS (spin cells at 9050 g for 1 min). Cells were diluted 1/100 with PBS. The coated Petri dishes were exposed to 0, 2, and 4 min of UV radiation in a plate illuminator box with a 6 watt UV lamp (Cole-Parmer 9815-series) set at 364 nm. Ten ml of the diluted culture was immediately added to each plate and then incubated at room temperature on a rotating platform for 10 min. Appropriate dilutions of each sample were plated in duplicate on YPD agar and incubated at 30ºC for 48 hours.

Intracellular Effects of benzophenone and padimate-O on *S.cerevisiae*. Two ml of overnight culture of each yeast strain was added to aluminium foil wrapped 10 ml culture tubes, to which 100 ul of 50% benzophenone, 100 uL of 50% padimate-O, or 100 uL of acetone was added. Samples were incubated at 30ºC in a horizontal tube rotator for 2 hours. One ml of each sample was removed and rinsed twice with PBS before being re-suspended in 1ml of PBS (spin cells at 9050 g for 1 min). 100 ul of each sample was collected and set aside for plating. Remaining samples were poured into 35 mm polystyrene Petri dishes and exposed to 2 min of UV radiation in a plate illuminator box. One hundred ul of each sample was removed for plating. Samples for plating were plated in duplicate on YPD agar at appropriate dilutions. Plates were incubated at 30ºC for 48 hours.

RESULTS

Protective effects of benzophenone and padimate-O for *S.cerevisiae* from UV radiation. It was important to determine whether the UV protective qualities of benzophenone and padimate-O were greater than the harmful qualities of their reactive species. In order to explore this, a basic UV light survival assay was set up employing *S. cerevisiae*, with or without benzophenone and padimate-O. Incubation of yeast cells in the dark in the presence of un-activated benzophenone or padimate-O resulted in no apparent cell death (data not shown). Therefore, cell death in control samples (Fig. 2 and 3) was due to exposure to UV radiation and cell death in treated samples was due to direct effects from the UV radiation and possibly in part by free radical formation. Figures 2 and 3 indicate less cell death occurs after UV radiation when either padimate-O or benzophenone is present, compared to the control. Also, there appeared to be little significant difference between the protective abilities of benzophenone in comparison with padimate-O.

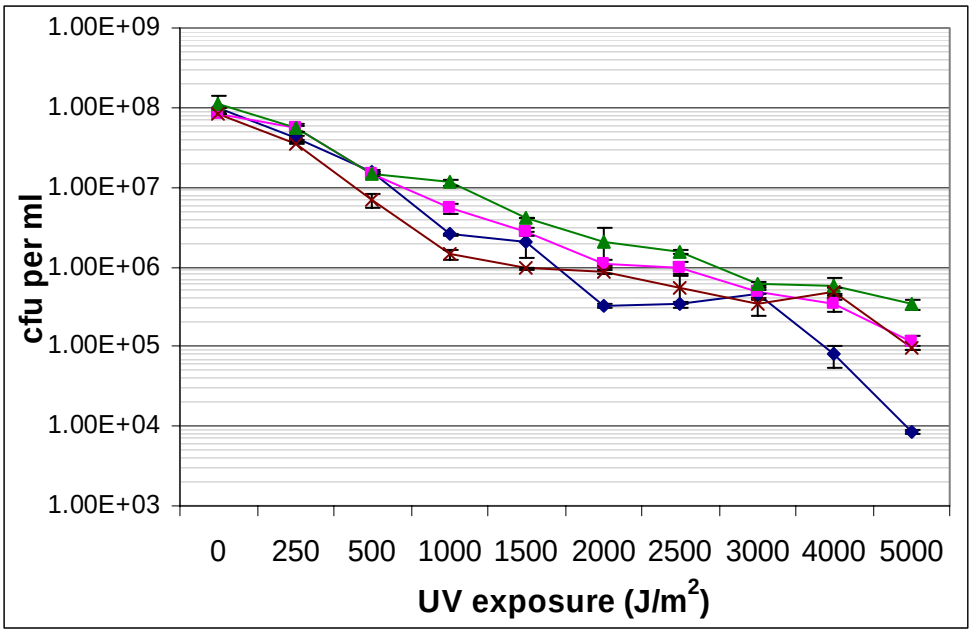

Figure 2. Protective effects of benzophenone (—■—) and padimate-O (—▲—) on *S. cerevisiae* Vin7 compared to an untreated control (—◆—) and an acetone control (—×—). Colony forming units per ml for benzophenone and padimate-O treatments from 500 to 5000 J/m^2 were derived from estimated standard plate counts, as were the control and the acetone treatments from 3000 to 5000 J/m^2.

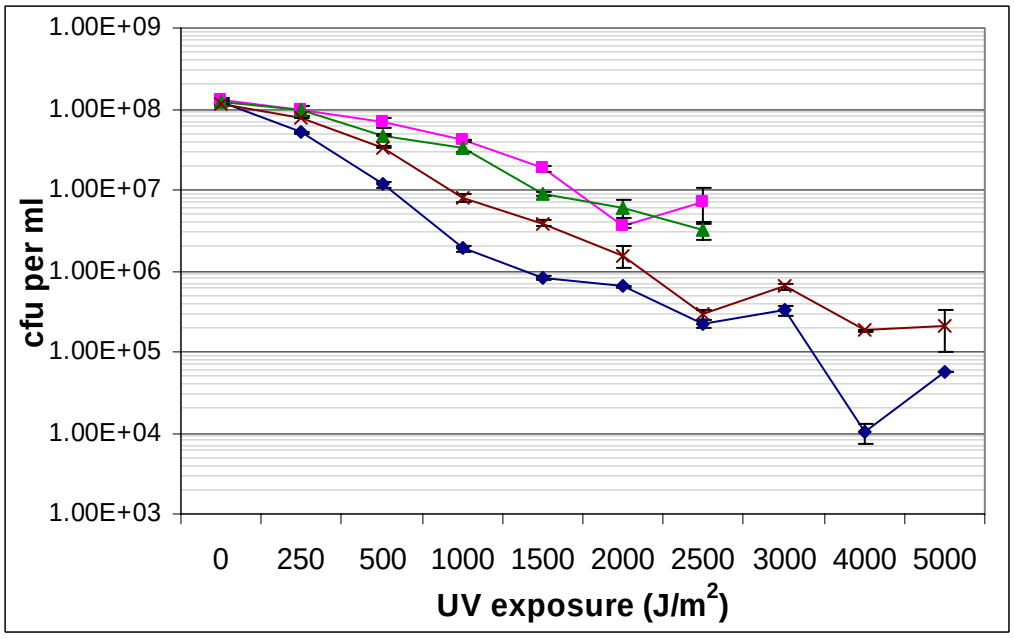

Figure 3. Protective effects of benzophenone (—■—) and padimate-O (—▲—)on *S. cerevisiae* ST compared to an untreated control (—◆—) and an acetone control (—×—). Colony forming units per ml for benzophenone and padimate-O treatments from 500 to 2500 J/m^2 were derived from estimated standard plate counts, as were the control and the acetone treatments from 3000 to 5000 J/m^2. More than one half of the surface of each plate for the benzophenone and padimate-O treatments from 3000 to 5000 J/m2 were covered with a confluent lawn of growth.

Damaging effects on *S.cerevisiae* by reactive species of UV-activated benzophenone and padimate-O. Whether or not benzophenone or padimate-O could form reactive species upon exposure to UVA light that could irreversibly damage *S. cerevisiae* cells was tested. Both chemicals were exposed to UV light before being exposed to yeast cells. This exposure appeared to have no adverse effect over exposure to the non-activated compounds (Fig. 4). All cell counts were similar in comparison with the control (no chemical, data not shown). Observable variation in cell counts is likely due to the flocculative abilities of the Vin7 strain. Indeed, although flocculation was partly broken up during mixing, some cells maybe have remained clustered. These results indicate that under the experimental conditions evaluated, insufficient or no damage was rendered to the cells.

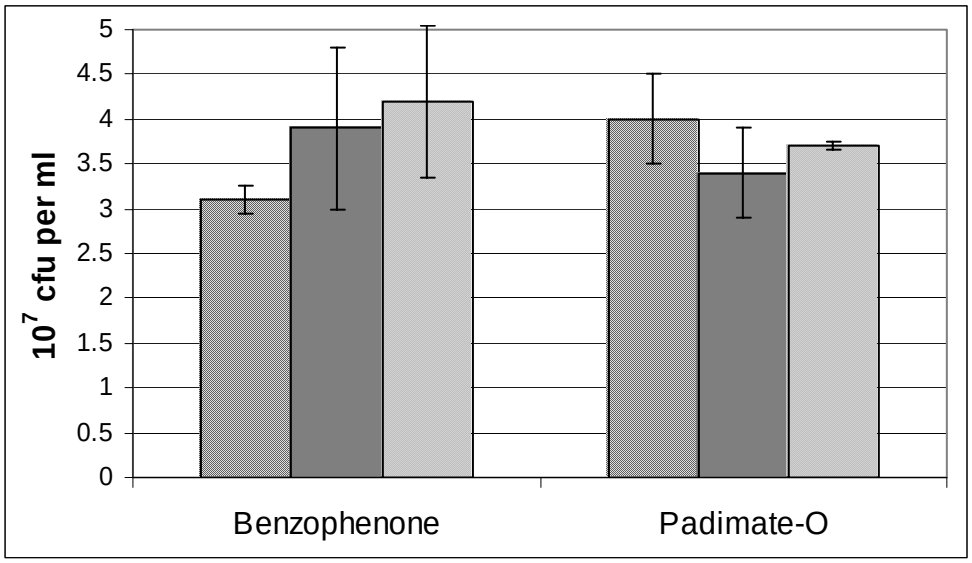

Figure 4. Survival of the Vin7 strain after incubation with benzophenone and padimate-O immediately after the chemicals were exposed to 0 min (diagonal hatching), 2 min (vertical hatching), and 4 min (crossed hatching) of UVA light. All values were derived from estimated standard plate counts.

Intracellular effects of benzophenone and padimate-O on *S.cerevisiae*. Cells were pre-incubated with benzophenone or padimate-O to allow intracellular uptake of either chemical, followed by exposure to UVA light for short periods of time. There was very little decrease in cell numbers during the two hours of pre-incubation with the chemicals (data not shown). Upon exposure to UVA light cell death occurred in both strains (Fig. 5). The ST strain control sample with acetone alone had very similar drops in cell numbers confounding the observation. In contrast, there was a significant difference between the acetone control and treated samples in the Vin7 strain (Fig. 6), as benzophenone and padimate-O treated cells have lower cell counts after UV exposure than the acetone control.

DISCUSSION

It was found that both benzophenone and padimate-O are able to protect *S.cerevisiase* from UV-induced death, with greater protective effects observed for some yeast strains compared to others. Any negative effects of the chemicals such as free radical formation through UV-induced photoactivation are minimal in comparison to the protective effects they provide. No intracellular effects were observed after incubation with both chemicals.

As greater cell survival was observed in UV irradiated cultures containing benzophenone or padimate-O, the net protective qualities of these chemicals appeared to be greater than the damaging qualities observed under photoactivation. It is probable that as these chemicals are known to absorb the UV light into their structure, they are decreasing the amount of UV available to damage the cells.

Padimate-O and benzophenone had nearly equal protective qualities on cells, despite differences in chemical composition. For example, it might have been expected that padimate-O would have greater protective capacity because some of the chemical was found layered on top of the cell medium, covering approximately 10% of the surface area of the culture in the Petri dish, and would absorb the incoming UV light before it reaches the cells. In contrast, benzophenone was found as a precipitate amongst the cells, and would not absorb UV before it reached the cells. However, neither padimate-O nor benzophenone completely covered the cultures, and therefore the

difference in cell death between treated and control samples may have been due to localized protection of the chemicals.

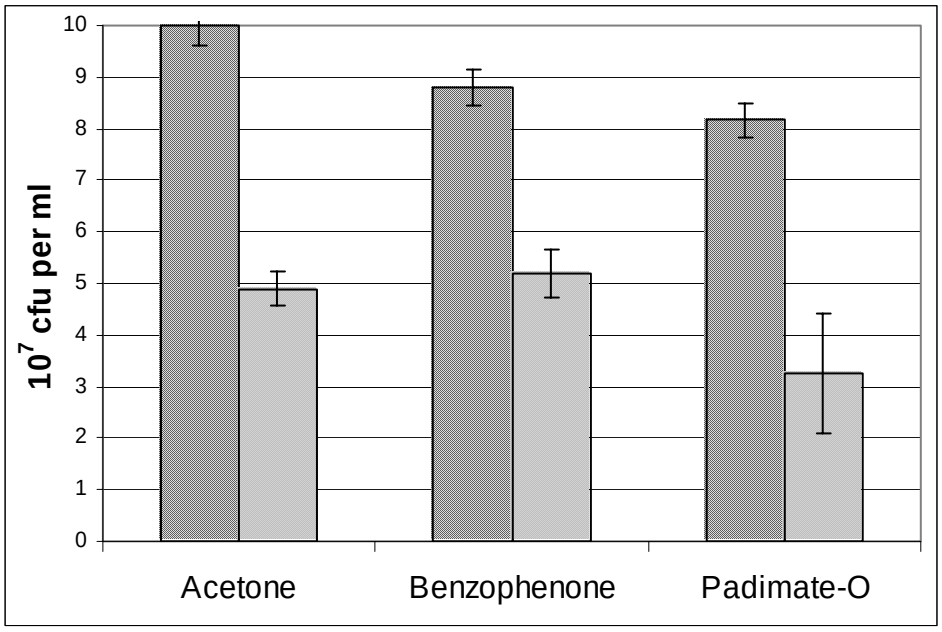

Figure 5. Intracellular effects of benzophenone and padimate-O on *S.cerevisiae* ST before (diagonal hatching) and after (crossed hatching) UVA exposure. All values were derived from estimated standard plate counts.

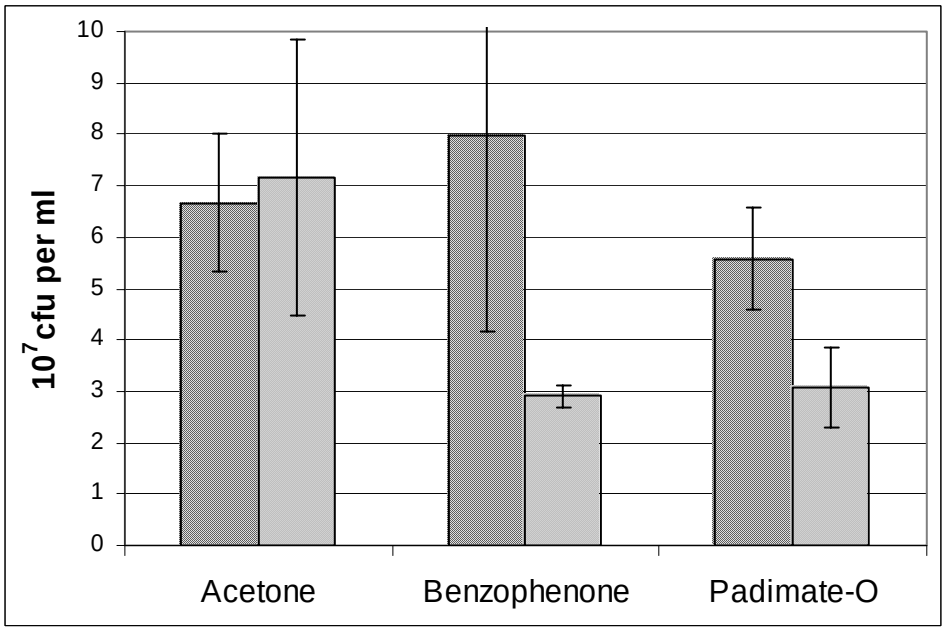

Figure 6. Intracellular effects of benzophenone and padimate-O on *S.cerevisiae* Vin7 before (diagonal hatching) and after (crossed hatching) UVA exposure. All values were derived from estimated standard plate counts.

The protective effects of benzophenone and padimate-O may have been greater if the chemicals were not present in the cell culture itself but rather spread as a thin layer above the cells (i.e. on saran wrap or even the Petri dish lids). However, this would allow for the chemicals to physically block the UV light along with absorbing it, making their effects seem greater. It would also not account for any negative effects inflicted upon the cells through free radical generation, which is an important factor to consider as sunscreen is applied directly onto human

skin cells, and absorbs into the skin. As well, the insolubility of both benzophenone and padimate-O may have decreased their abilities to thoroughly protect the yeast in liquid culture from UV damage, as a heterogeneous mixture of the chemicals and culture occurred. This could mitigate the negative effects of the activated chemicals on the yeast cells due to reduced contact with the chemical.

There were no observable negative effects to yeast cells upon incubation with UVA photoactivated benzophenone or padimate-O. This suggests that reactive species did not form or the cells did not come into contact with reactive species to cause irreversible damage. This result probably occurred because the reactive species formed by exposure to UV were too ephemeral to survive until addition of the yeast cells. Considering that the half-life of free radicals can be as short as 65 microseconds for the benzophenone molecule (2), too much deterioration may have occurred before the cells were added (after approximately 5 seconds) to give a noticeable effect. The range of light emitted from the 364 nm bulb in the plate illuminator box may not have been sufficiently energetic at the correct wavelengths to photoactivate the padimate-O or the benzophenone (both activated by UVB). As well, reactive oxygen species are known to be formed by padimate-O upon exposure to UVA light in aqueous solution and the lack of an aqueous component during irradiation with UVA would have abrogated this effect. Lastly, singlet-oxygen has been shown to cross-link proteins and other macromolecules, and the effect of cross-linking proteins may not cause noticeable cell death rates.

Intracellular benzophenone and padimate-O appeared to cause cell death upon illumination of the cells. Since the mere presence of the unexcited chemicals in the yeast cultures did not cause any of the differences in cells numbers observed in figures 5 and 6, any decrease in cell numbers between the cultures before and after UV exposure was due to either UV-induced effects or free radical formation from benzophenone and padimate-O. However, as a longer wavelength of light at a lower intensity was used (364 nm at 6 watts instead of 254 nm at 75 watts) the germicidal effects of the UV light alone should have been negligible. Therefore, any significant cell death observed could be attributed to reactive species of padimate-O or benzophenone forming and causing irreparable cell damage. However, this was not the case with both strains, as seen by the cell death occurring after UV exposure in the acetone control for the ST strain (Fig. 5). That cell death occurred in one of the controls, and not the other suggested an error may have been made in treating one of the controls since the earlier studies showed similar sensitivity of each strain to acetone (Fig. 2 and 3). The model that has been proposed here strongly suggests that such an error may have been made with the ST strain acetone control.

In comparison with experiments regarding extracellular damaging effects of benzophenone and padimate-O, these results seem to agree that there appears to be insignificant cell death due to the reactive species of padimate-O and benzophenone under the conditions explored in this study. The protective benefits seem to surpass potential detrimental effects of the chemicals.

FUTURE EXPERIMENTS

A number of experiments should be undertaken to further investigate and expand on the issues studied here. It would be important to examine the toxic effects of the chemicals on cells, without any exposure to UV radiation. This would further confirm the effects observed are not due to some chemical effect unrelated to UV damage or reactive species. It would be useful to observe if the ability of the chemicals to protect cells from UV is depleted by exposure to UV. This could be investigated by exposing benzophenone and padimate-O to varying amounts of UV exposure without yeast, then adding the chemicals to yeast cultures and assessing yeast UV survival rates. In a similar experiment, the longevity of protection could be assessed by allowing the cells to grow with the chemicals overnight before assessing UV survival rates and comparing to 'fresh' chemicals. This would determine whether the cells are able to break down the chemicals or whether the chemicals themselves breakdown over time and become less effective. This procedure might also be useful to further study intracellular effects of the chemicals on the yeast, as longer incubation times may allow for greater penetration of the chemicals into the cells, and thus larger effects may be observed upon UV exposure.

In regards to further examining the formation of potential reactive species by benzophenone and padimate-O, plates could be coated as was performed in this experiment, but before activation, the chemicals should be covered in aerated buffer lacking any organic compounds. This will potentially allow free radicals to generate reactive oxygen species, which might survive longer than the free radicals themselves.

Lastly, flocculation of yeast may have been an issue in some experiments, preventing accurate measurement of yeast cell numbers. Use of a non-flocculating strain of *S. cerevisiae* in future investigations is recommended.

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REFERENCES

1. **Allen, J.M., and C.J. Gossett.** 1996. Photochemical formation of singlet molecular oxygen in illuminated aqueous solutions of several commercially available sunscreen active ingredients. Chem. Res. Toxicol. **9**: 605-609.
2. **Charlier, M., and C. Helene.** 1972. Photochemical reactions of aromatic ketones with nucleic acids and their components I-. Purine and pyrimidine bases and nucleosides. Photochem. Photobiol. **15**: 71-87.
3. **Chetelat, A., S. Albertini, J.H. Dresp, R. Strobel, and E. Gocke.** 1993. Photomutagenesis test development: I. 8-Methoxypsoralen, chlorpromazine and sunscreen compounds in bacterial and yeast assays. Mutat. Res. **292**: 241-250.
4. **Dean, S.W., R.H. Dunmore, S.P. Ruddock, J.C. Dean, C.N. Martin and D.J. Kirkland.** 1992. Development of assays for the detection of photomutagenicity of chemicals during exposure to UV light. II. Results of testing three sunscreen ingredients. Mutagenesis. **7**: 179-182.
5. **Gasperro, F.P.** 1993. The molecular basis of UV-induced mutagenicity of sunscreens. FEBS Lett. **336**: 184-185.
6. **Knowland, J., E.A. McKenzie, P.J. McHugh, and N.A. Cridland.** 1993. Sunlight-induced mutagenicity of a common sunscreen ingredient. FEBS Lett. **324**: 309-313.
7. **Knowland, J., P.McHugh, and R. Dunford.** 1997. The photochemical potential of some sunscreens to damage DNA, p.47-61. *In* F. P. Gasperro (ed), Sunscreen photobiology: molecular, cellular, and physiological aspects. Springer, Texas.
8. **McHugh, P.J., and J. Knowland.** 1997. Characterization of DNA damage inflicted by free radicals from a mutagenic sunscreen ingredient and its location using an *in vitro* genetic reversion assay. Photochem. Photobiol. **66**: 276-281.
9. **U.S. Department of Heath and Human Services, National Institute of Health.** 2000. National toxicology program technical report on the toxicity studies of benzophenone. Toxicity Report Series. **61**.
10**. Rose, M., F. Winston and P. Hieter.** 1990. Methods in yeast genetics; A Laboratory Course Manual. Cold Spring Harbor Laboratory Press, New York.
11. **Serpone, N., A. Salinaro, A.V. Emeline, S. Horikoshi, H. Hidaka, and J. Zhao.** 2002. An *in vitro* systematic spectroscopic examination of the photostabilities of a random set of commercial sunscreen lotions and their chemical UVB/UVA active agents. Photochem. Photobiol. Sci. **1**: 970-981.
12. **Sun, J.S., K.M. Shieh, H.C. Chiang, S.Y. Sheu, Y.S. Hang, F.J. Lu, and Y.H. Tsuang.** 1999. Scavenging effect of benzophenones on the oxidative stress of skeletal muscle cells. Free Radic. Biol. Med. **26**: 1100-1107.
13. **Tavini, M.** 1993. UV-B radiation and ozone depletion; effects on humans, animals, plants, microorganisms, and materials. Lewis Publishers, Florida.