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PRODUCTION OF SINGLET OXYGEN AND SUPEROXIDE RADICALS BY PSORALENS AND THEIR BIOLOGICAL SIGNIFICANCE

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We have investigated a series of linear and angular furocoumarins, capable of forming either the monofunctional adducts (single strand) or bifunctional adducts (interstrand cross-links) with DNA with a view to examine the relationship of their skin photosensitizing potency, their ability to produce singlet oxygen ($^1O_2$) or superoxide radicals (O_2^- or HO_2^-), and their carcinogenic activity. The significance of photochemical interactions of psoralens and DNA is well known in skin photosensitization and skin carcinogenesis. Our data suggest that both monofunctional and bifunctional psoralens produce $^1O_2$ and O_2^-, and these reactive forms of oxygen may contribute to the development of skin cancer and membrane-damaging effects of these furocoumarins.

Certain photochemotherapeutic agents commonly referred to as furocoumarins or psoralens (e.g., 8-MOP or methoxsalen) are used extensively in the treatment of various skin disorders such as psoriasis, mycosis fungoides, vitiligo, polymorphous photodermatitis, and eczema (1). The molecular basis for photobiological (skin photosensitization) and photochemotherapeutic effects of psoralens is believed to involve the stable covalent photoconjugation of furocoumarins with DNA of the target cells giving rise to both the monofunctional (single strand) adducts and interstrand cross-link adducts (ICL) between the pyrimidine bases belonging to opposite strands of DNA (2-4). The photochemotherapeutic effectiveness of psoralen + UVA, commonly referred to as PUVA, is believed to be due to: (a) the inhibition of DNA synthesis and subsequent decrease in macromolecular synthesis, (b) the killing of abnormal proliferative

ABBREVIATIONS: $^1O_2$, singlet oxygen; O_2^-, superoxide radical; 8-MOP, 8-methoxypsoralen; ICL, interstrand cross-link; RNO, p-nitrosodimethylaniline; HIS, histidine; NBT, nitro blue tetrazolium; dGus, deoxyguanosine; 5-MOP, 5-methoxypsoralen; TMP, 4,5',8-trimethylpsoralen; DABCO, 1,4-diazabocyclo[2,2,2]-octane; SOD, superoxide dismutase

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cells and the inhibition of recruitment of new cells in the G_0 and G_2 phases, (c) the inhibition of blood vessel cells (angiogenic effect), and (d) the inhibition of leukocytes and subsequent immunosuppression effect on the immune system. The major chemical reaction involving the photoconjugation of psoralen and pyrimidine bases can be classified as a type-I reaction that requires the transfer of hydrogen atoms or electrons but no direct involvement of molecular O_2 (5). We present evidence here to suggest that psoralens also undergo type-II reactions involving the formation of $^1O_2$ and $O_2^{\cdot-}$ (or $HO_2^{\cdot}$) through a sensitized mechanism (photodynamic action) in which the photoexcited psoralen (presumably in its triplet state) reacts with molecular oxygen to form reactive $^1O_2$ or $O_2^{\cdot-}$. These reactive forms of oxygen, we believe, cause cell damage that eventually contributes to skin photosensitization, mutation, error-prone repair, and skin carcinogenesis.

MATERIALS AND METHODS

A horizontal planar array of six 4-foot long UVA-emitting (320 to 400 nm) fluorescent tubes were used in this study. The system produced UVA irradiance of 3.75 mW/cm^2 at 10 cm (measured by IL700, cosine-corrected UVA detector, International Light Radiometer, International Light Company, Newburyport, MA). Psoralen derivatives used in this study were obtained from various sources and purified after recrystallization (Elder Pharmaceuticals, Bryan, OH; Dr. Giovanni Rodighiero, Paduva, Italy; and Dr. June K. Dunnick, Department of Health and Human Services, Research Triangle Park, NC). Other chemicals were obtained from the Sigma Chemical Company, St. Louis, MO. The ICL of psoralens with DNA were examined by hydroxyapatite column chromatography (6) with a linear gradient of 0.05 M to 0.4 M phosphate buffer (pH 7). Calf thymus DNA (4.9 ml), prepared by adding 10 mg DNA in 100 ml 0.05 M phosphate buffer (pH 7), and the test compounds (0.1 ml), obtained from a stock solution of 1 mg/ml in ethanol, were mixed and irradiated in a Petri dish under an UVA-emitting source for a total dose of 8 to 16 J/cm^2. The irradiated DNA was denatured by heating in a boiling water bath (30 minutes) and immediatly cooled in a previously chilled ice bath (15 minutes). The denatured DNA was loaded in a temperature-regulated (60° to 65°C) chromatography column (2.5 x 30 cm) packed with DNA grade Bio-Gel hydroxyapatite (Bio-Rad Laboratories, Richmond, CA) and separated by elution into single-strand (denatured) and double-strand (renatured) DNA containing ICL. The method proposed by Kraljic and Mohsni (7) was used for the detection of $^1O_2$ in aqueous solution. Briefly, the method involved using : 10 ml of 3.75 to 4 x 10^{-5} M solution of RNO prepared in 0.025 M phosphate buffer as the scavenger of $^1O_2$, histidine (10^{-2} M) as the acceptor of $^1O_2$, 0.1 ml test compounds (psoralens) as the sensitizer, and UVA as the generator for $^1O_2$. The formation of $O_2^{\cdot-}$ (or $HO_2^{\cdot}$) was studied by monitoring the photosensitized reduction of NBT (8). The reduction of NBT by $O_2^{\cdot-}$ leads to the formation of a blue-colored compound which can be quantitatively estimated spectrophotometrically at 560 nm. Photooxidation of dGus was measured spectrometrically at 260 nm.

RESULTS

Figure 1 shows the generation of $^1O_2$ by several mono- and bifunctional psoralens using bleaching reaction of RNO at 440 nm induced by the presence of histidine as a selective acceptor. This figure also shows that both the linear psoralens capable of forming ICL in DNA (e.g., 8-MOP, 5-MOP, TMP, and psoralen) and the nonlinear as well as linear psoralens forming only monofunctional adducts in DNA (e.g., angelicin, 5-methylangelicin, and 3-CP) produced $^1O_2$, although at varying degrees. Psoralen and 3-CP were the most effective producers of $^1O_2$. Hematoporphyrin (1 µg/ml) produced a greater amount of $^1O_2$ than many of the psoralens tested except 3-CP. Table 1 provides a summary of chemicals tested

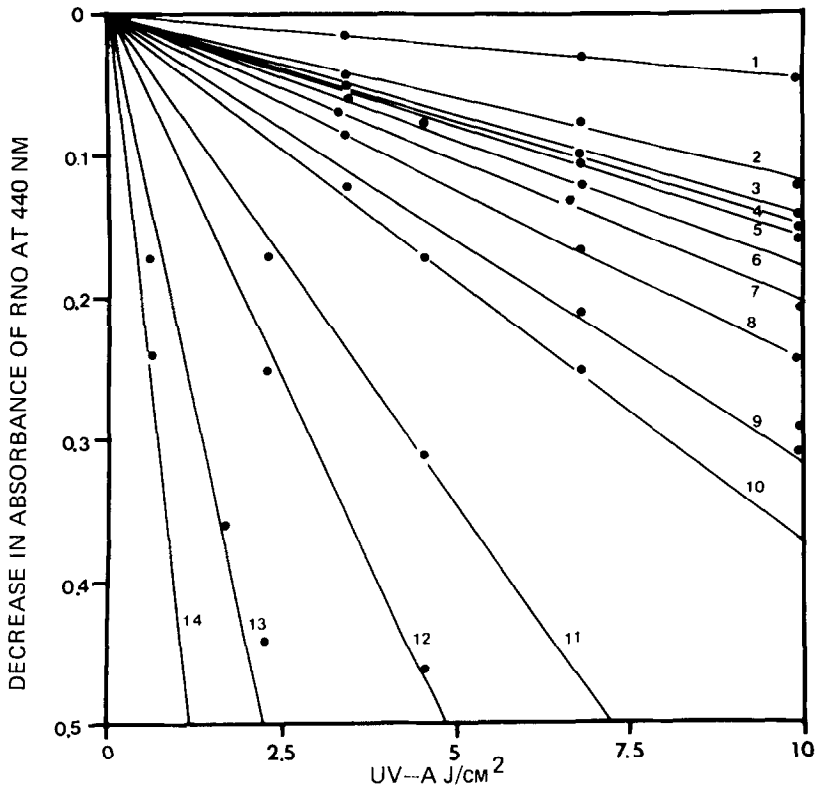

Figure 1. Skin photosensitizing chemicals involved in the formation of $^1O_2$ in an in vitro system. Reaction system: RNO (3.5 to 4.0 x 10^{-5} M) + HIS (10^{-2} M) + sensitizers (10 µg/ml). The sensitizers used were: (1) 5-diethylaminobutoxypsoralen; (2) 5-MOP; (3) 3,4'-dimethyl,8-methoxypsoralen; (4) 8-MOP; (5) 4,5'-dimethylangelicin; (6) TMP; (7) anthracene; (8) 4'-aminomethyl, 4,5',8-trimethylpsoralen; (9) 4,8-dimethyl, 5'-carboxypsoralen; (10) 5-methylangelicin; (11) angelicin; (12) psoralen; (13) hematoporphyrin; and (14) 3-CP.

TABLE: SUMMARY OF CHEMICALS TESTED FOR SKIN PHOTOSENSITIZATION AND FOR $^1O_2$ AND O_2^- PRODUCTION

Compounds Tested	Skin Photosensitizing Activity	Ability to Form Monofunctional Adducts with DNA	Ability to Form Interstrand Cross-Links	$^1O_2$ Production	O_2^- Production
Psoralen	++++	strong	very strong	+++	+++
5-Methoxypsoralen	++	strong	strong	+	+
8-Methoxypsoralen	+++	strong	strong	+	+
4,5',8-Trimethylpsoralen	++++	strong	very strong	+	++
5-Diethylaminobutoxy-psoralen	+	strong	moderate	+/–	++
4'-Aminomethyl, 4,5',8-trimethylpsoralen	++++	strong	very strong	++	not investigated
3,4'-Dimethyl, 8-methoxypsoralen	++	strong	low	+	+
3-Carbethoxypsoralen		strong	absent	++++	+
4,8-Dimethyl, 5'-carboxypsoralen	absent	strong	low	++	++
Angelicin	absent	weak	absent	+++	++
5-Methylangelicin	absent	strong	absent	++	++
4,5'-Dimethylangelicin	absent	moderate	absent	+	+
Hematoporphyrin	+++	absent	absent	++++	++
Methylene blue	absent	absent	absent	++++	+
Anthracene	++	low	trace	++	+++
Riboflavin	uncertain	absent	absent	+++	++++

Key: ++++ = extremely high (very strong)
+++ = moderately high (strong)
++ = moderate
+ = low (weak)
± = trace or uncertain
– = absent

for skin photosensitization reaction and $^1O_2$ production. The table also provides data, albeit qualitative in nature, about the extent of cross-linking in DNA. Quantitative aspects of this ICL reaction will be published elsewhere. The $^1O_2$-producing activity, based on the use of an equivalent concentration of the sensitizer, was found to be in the following order: 3-CP > hematoporphyrin > psoralen > angelicin > 5-methylangelicin > 4,8-dimethyl,5'-carboxypsoralen > 4'-amino-methyl,4',5,8-trimethoxypsoralen > anthracene > TMP > 4,5'-dimethyl-angelicin > 8-MOP > 3,4'-dimethyl,8-methoxypsoralen > 5-MOP > 5-diethylamino-butoxypsoralen. The formation of $^1O_2$ evoked by all the test chemicals studied was found to depend upon: (a) the concentration of the test compound (μg/ml) (b) the dose of UVA radiation (J/cm^2), and (c) the environment (O_2 or N_2 or the solvent used). Reciprocity in the production of $^1O_2$ could be seen by varying either the concentration of the sensitizer or the dose of UVA.

Additional evidence for the production of $^1O_2$ was obtained by examining the bleaching of RNO and concomitantly carrying out $^1O_2$ quenching studies with azide ions (N_3^-, 10^{-2} M) (9), DABCO (2.5×10^{-2} M) (10), β-carotene (10^{-4} to 10^{-3} M) (11), and SOD (3 to 80 units/ml) (12). When N_3^- or DABCO were added, about 70 to 100% inhibition in $^1O_2$ production was observed with all the psoralens tested. Due to the strong absorption band of β-carotene at 440 nm, its quenching effect on $^1O_2$ could not be conclusively interpreted by our spectrophotometric reaction. The generation of $^1O_2$ was also studied by following the reaction kinetics under N_2 and in an O_2-purged system; and also by comparing the rate of $^1O_2$ production in D_2O and water (13). About 75 to 100% inhibition was observed in the rate of $^1O_2$ production when the reaction was carried out under N_2, and about 0 to 10% increase in the rate of $^1O_2$ production was detected when pure O_2 was bubbled through the different photosensitizing systems. When the reaction was carried out in D_2O instead of H_2O, one could observe an increase in the rate of production of $^1O_2$ (range 40 to 50%). 8-MOP, a low generator of $^1O_2$, showed a 40% increase in RNO bleaching when D_2O was used as a solvent (Fig. 2).

Another interesting finding of this study was that practically all the psoralen derivatives were found to generate $O_2^{\cdot-}$ (or $HO_2^{\cdot}$) simultaneously along

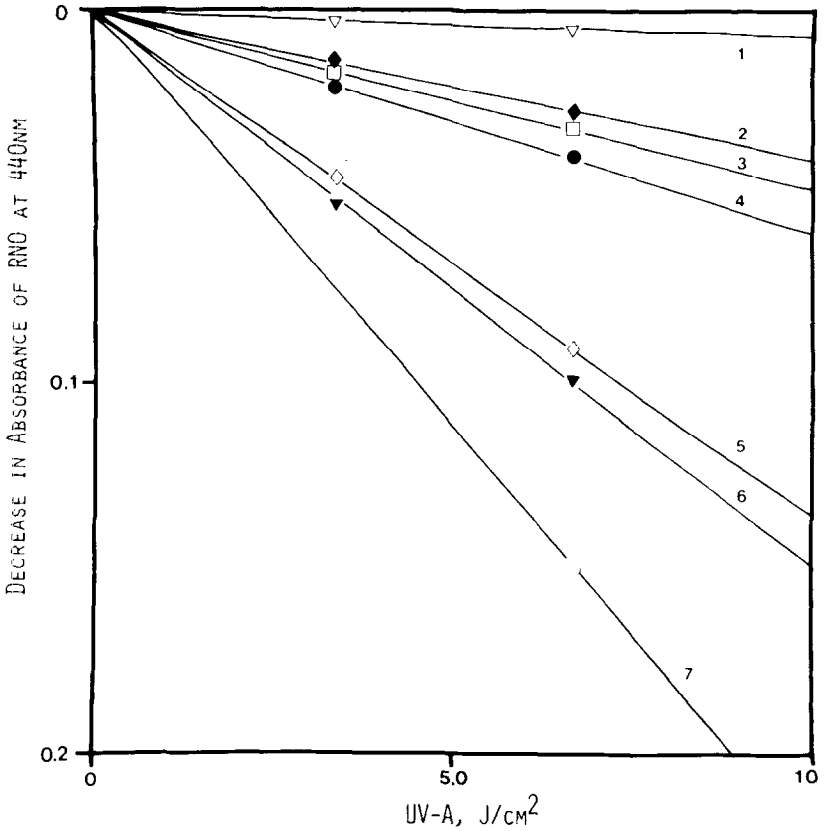

Figure 2. Production of $^1O_2$ by 8-MOP and supporting evidence of its generation by quenching experiments. Reaction system: RNO (3.5 to 4.0 x 10^{-5} M), HIS (10^{-2} M), and 8-MOP (10 µg/ml). (1) RNO + 8-MOP without HIS, (2) azide ions, (3) DABCO, (4) N_2, (5) SOD, (6) 8-MOP only, and (7) D_2O.

with the production of $^1O_2$. Figure 3 provides typical data for the NBT reduction tests indicative of $O_2^{\cdot-}$ formation by monofunctional and bifunctional psoralens. Riboflavin was used as a selective generator for $O_2^{\cdot-}$ (8).

Production of singlet oxygen appears to contribute to the photooxidation of guanine bases of DNA as evidenced by a decrease in the absorbance at 260 nm after exposure of dGus to UVA radiation in the presence of various psoralens. Since $^1O_2$ and $O_2^{\cdot-}$ generated by psoralens can induce photooxidation of lipids (14), it is conceivable that these reactive oxygen moieties cause the membrane-damaging effects. Thus, the production of $^1O_2$ by photoreactive psoralens may contribute to edema and erythema reactions under *in vivo* conditions.

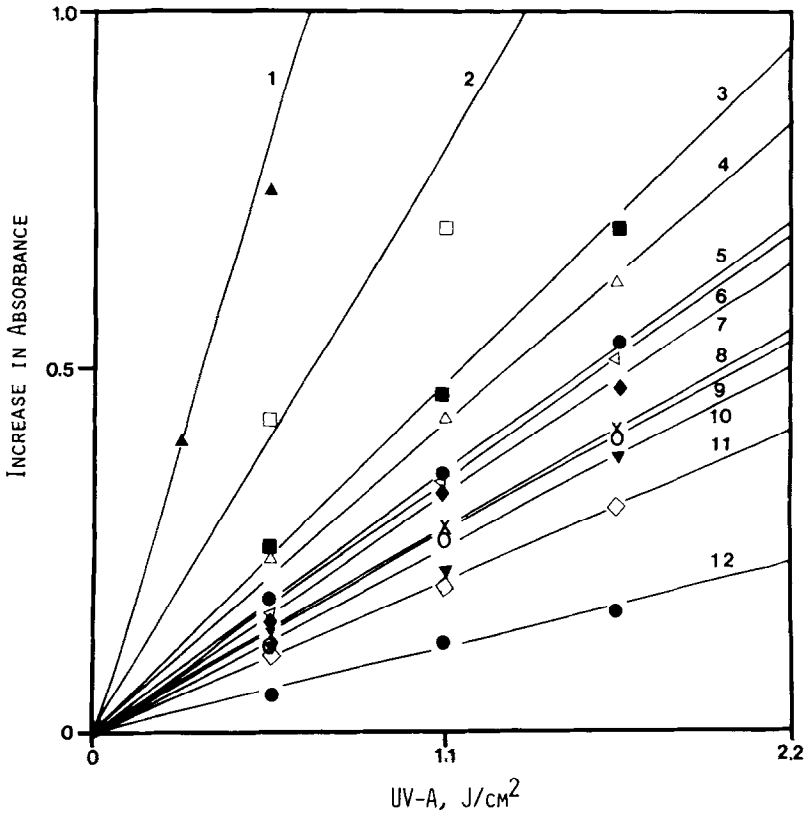

Figure 3. Formation of O_2^- determined by the photosensitized reaction of NBT (1.6×10^{-4} M, pH 10) and increase in absorption at 560 nm due to the formation of nitro blue diformazan in the presence of 10 µg/ml of the following compounds: (1) riboflavin, (2) psoralen, (3) 4,8-dimethyl, 5'-carboxypsoralen; (4) angelicin; (5) TMP, (6) 5-methylangelicin; (7) 5-diethylaminobutoxypsoralen; (8) 4,5'-dimethylangelicin; (9) 3-CP; (10) 8-MOP; (11) 3,4'-dimethyl,8-methoxypsoralen.

DISCUSSION

The production of $^1O_2$ by photoreactive 8-MOP has been reported by Poppe and Grossweiner (15), Singh and Vadsaz (16), and de Mol and Beyersbergan Van Henegouwen (17), but the methods used by these investigators involved either the denaturation of enzymes or the oxidation of 3,4-dihydroxyphenylalanine (dopa). These methods, however, are less specific and indirect for the detection of $^1O_2$. Furthermore, the biological significance of $^1O_2$ and O_2^- production by these investigators has not been stressed in skin photosensitization and skin carcinogenesis reactions evoked by psoralens. In view of the fact that tumor development has been recently implicated in the $^1O_2$- and O_2^--generating capabilities of certain chemicals (18), the question naturally arises to know whether psoralens

contribute to the development of basal cell and squamous cell carcinomas by generating activated forms of oxygen, including $^1O_2$, $O_2^{\cdot-}$, $HO_2^{\cdot}$, and peroxides.

Although our data and observations are generally in agreement with the concept we originally proposed for the role of $^1O_2$ in skin photosensitization, DNA damaging reactions and skin carcinogenesis (19), the major question concerning the molecular basis of psoralen photosensitization reaction remains unanswered since both the monofunctional and bifunctional psoralens produce $^1O_2$ and $O_2^{\cdot-}$ at varying degrees. We do not know which of these chemicals preferentially react through $^1O_2$ or $O_2^{\cdot-}$ production or formation of monofunctional and bifunctional adducts in skin photosensitization reactions and DNA-damaging reactions. Of interest is the fact that until recently monofunctional psoralens were thought to be nonphotosensitizing and noncarcinogenic furocoumarins. The skin photosensitizing and the therapeutic and carcinogenic effectiveness of bifunctional psoralens (e.g., 8-MOP, 5-MOP, TMP, etc.) have been attributed to their relative ability to photoconjugate with DNA to form interstrand cross-links. Recent observations form our laboratory (20), however, indicate that monofunctional psoralens, such as angelicin, 5-methylangelicin and 4,8-dimethylangelicin, are more carcinogenic than the bifunctional psoralens (8-MOP). The fact that monofunctional psoralens produce $^1O_2$ and $O_2^{\cdot-}$ at rates comparable or better than bifunctional psoralens suggests that this reactive moiety of oxygen plays a major role in their carcinogenic property and perhaps in cell membrane damaging (edema) reactions.

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express the transformed phenotype, or specifically, grow in low Ca^{2+} medium. This observation is consistent with the study by Durkin et al. (19), showing that ts cells did not require elevated levels of calmodulin to enter S phase of the cell cycle but calmodulin function was required. This implies that while calmodulin function is vital to cellular regulation, an elevation in calmodulin level is not as critical. Elevated levels of calmodulin cannot explain the loss of Ca^{2+}-regulation that occurs in 6M2 cells at 34°C but a possible explanation may rest with the calmodulin acceptor proteins. It is the CAPs which execute the cell response to a Ca^{2+}-signal for which calmodulin acts as the mediator. The 6M2 as well as NRKLA23 cells have lost CAP_{70} at 34°C (the Ca^{2+}-deregulated temperature) but regain this protein at 39°C. We speculate that in transformed cells the activity or distribution of cellular CAPs are altered and the response of the cell to a regulatory Ca^{2+} signal is modified.

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