

Identification of Singlet Oxygen as the Cytotoxic Agent in Photo-inactivation of a Murine Tumor¹

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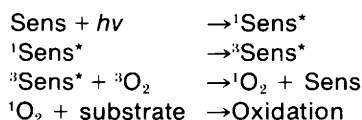
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SUMMARY

Singlet oxygen, a metastable state of normal triplet oxygen, has been identified as the cytotoxic agent that is probably responsible for *in vitro* inactivation of TA-3 mouse mammary carcinoma cells following incorporation of hematoporphyrin and exposure to red light. This photodynamic inactivation can be completely inhibited by intracellular 1,3-diphenylisobenzofuran. This very efficient singlet oxygen trap is not toxic to the cells nor does it absorb the light responsible for hematoporphyrin activation. We have found that the singlet oxygen-trapping product, o-dibenzoylbenzene, is formed nearly quantitatively intracellularly when both the furan and hematoporphyrin are present during illumination but not when only the furan is present during illumination. The protective effect against photodynamic inactivation of the TA-3 cells afforded by 1,3-diphenylisobenzofuran coupled with the nearly quantitative formation of the singlet oxygen-trapping product indicates that singlet oxygen is the probable agent responsible for toxicity in this system.

INTRODUCTION

Singlet oxygen (¹O₂), the metastable excited state of triplet molecular oxygen, in which all electrons are paired (¹Σ_g⁺ or ¹Δ_g) is becoming increasingly recognized as an important chemical and biological agent. Numerous chemical and biological oxidative processes appear to involve this reactive intermediate. For example, the specific type of oxidation of *cis*-1,3-dienes brought about by the action of oxygen in the presence of certain photosensitizing dyes and light is now known to proceed through the formation of singlet oxygen that is formed in an energy transfer process from the excited triplet state of the dye (sensitizer; Ref. 7).



where Sens is sensitizer, ¹Sens* is excited singlet state of sensitizer, ³Sens* is excited triplet state of sensitizer, ³O₂ is

ground state triplet oxygen, and ¹O₂ is excited singlet state of oxygen.

In the biological area the well-studied photodynamic reaction [known since 1900 (21)] that involves modification or destruction of biological molecules and systems under these same oxidative conditions (dye + light + O₂) in many instances may also proceed via singlet oxygen pathways. Thus, Ackerman *et al.* (1) have shown that identical product mixtures are obtained from various olefins when they react with singlet oxygen generated in a microwave discharge or under classical photodynamic conditions with various sensitizers. Krinsky (13) has recently demonstrated the possible role of singlet oxygen as a mediator of the antibacterial action of human leukocytes. It has also been suggested that singlet oxygen may be formed from the superoxide radical (O₂⁻) in the course of pyrimidine nucleotide oxidation (2). Other biological systems thought to involve singlet oxygen include xanthine oxidase (3), linoleate-lipoxygenase (20), and NADPH-dependent microsome lipid peroxidation (19).

In most instances, the involvement of singlet oxygen in biological systems is inferred from the protection effect against inactivation imparted by naturally occurring or added carotenoids that are well established as efficient physical quenchers of singlet oxygen (6). However, in these complex biological systems, carotenoid protection alone is not sufficient to establish singlet oxygen involvement, since the carotenoids have low-lying triplet states that may quench the triplet states of the sensitizers and/or act as free radical scavengers. In some of the simpler systems, the presence of such reactions frequently can be detected kinetically (7).

One approach to overcoming these difficulties of identification of singlet oxygen in biological systems is to incorporate an innocuous chemical quencher that yields a unique product with singlet oxygen and to determine a material balance between quencher consumed and product formed while monitoring a biological end point. We have succeeded in this approach, using 1,3-diphenylisobenzofuran as quencher of photoinactivation of TA-3 mouse mammary carcinoma cells containing hematoporphyrin, and have shown that singlet oxygen is the likely cytotoxic agent in this process.

MATERIALS AND METHODS

Tumors and Chemicals. Ascitic TA-3 mouse mammary carcinoma cells were carried in syngeneic A/St mice (11). The tumor-doubling time is approximately 11 hr.

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HPD³ was prepared by the method of Lipson *et al.* (14) by dissolving the hydrochloride (Roussel Corp., New York, N. Y.) in acetic acid:sulfuric acid (9:1), by filtering, and then precipitating with 3% sodium acetate. The sodium salt used throughout was prepared by dissolving 200 mg of HPD in 0.1 N sodium hydroxide (10 ml) and 10 ml 0.9% NaCl solution. This stock solution was then diluted with PBS to give 0.5 mg/ml.

1,3-Diphenylisobenzofuran was used as obtained from Aldrich Chemical Company, Milwaukee, Wis.

Red Light Source. A 500-watt slide projector was modified by inserting a 600-nm cut-off filter (Corning 2418) before the condenser. This lamp emitted 1.3 milliwatts/sq cm between 610 and 630 nm (range of hematoporphyrin absorption) where the cells were placed during exposures. Intensity was determined with a calibrated Oriel Model 7010 radiometer.

PROCEDURES

Approximately 15 ml of 8×10^6 /ml of freshly harvested TA-3 cells in exponential growth (approximately 3 to 5×10^7 cells/mouse) were suspended in PBS (pH 7.2) to which was added HPD to a concentration of 4.5×10^{-5} M. After 2 hr in the dark, with occasional resuspension the cells were thoroughly washed (PBS) to remove all excess dye and were kept in the dark. These cells were brightly fluorescent (red) and did not lose hematoporphyrin. Intracellular HPD was determined in several ways. A direct spectrophotometric measurement on the cell suspension at 500 nm could be used if the reference side of the spectrophotometer was balanced with an equal number of cells without HPD. For such measurements a molar extinction coefficient of 4500, determined in PBS, was used. Alternatively, cells were exposed to tritiated hematoporphyrin prepared by New England Nuclear, Boston, Mass., from the free base that was subsequently treated according to the method of Lipson *et al.* (14). Isotope counting was carried out on a Searle Mark III liquid scintillation counter. A 3rd method involved dissolving of the HPD-containing cells in Protosol (New England Nuclear tissue solubilizer) and determining HPD absorbance at 500 nm for comparison with a standard curve established by adding known amounts of HPD to Protosol. The 3 methods agreed within $\pm 30\%$. Cell volume was taken as 5×10^{-10} ml for these calculations (i.e., average diameter, 10 μ m.)

Diphenylisobenzofuran accumulation was carried out by exposing about 10^8 cells (with or without HPD) in 15 ml PBS to a furan concentration of 1.5×10^{-5} M prepared by adding a small volume of a concentrate of the furan in ethanol to the PBS. Cells were kept in the dark for 1 hr and washed several times (PBS) to remove excess furan. Intracellular furan concentration was determined by suspending a known number of cells in acetone which quantitatively removed all of the furan (but not hematoporphyrin). The acetone was evaporated and replaced by ethanol for quantita-

tive determination of the furan from its absorbance at 400 nm. The furan oxidation product, *o*-dibenzoylbenzene, was determined on an aliquot of this solution by gas chromatography following calibration (10% Carbowax 6000 on Chromosorb at 220°).

For exposure to the red light, 5 ml of a cell suspension in PBS were placed in a 30-ml plastic T-flask at a concentration to give a monolayer or less of cells (approximately 4×10^6 cells/ml or less). Cells were exposed for various times and viability was assessed by i.p. inoculation of about 10^6 cells into A/St mice (3 to 6/group). After 3 days or more, while still in exponential growth, the cells were quantitatively recovered from the peritoneal cavity by repeated washings and were compared with control groups for survival. We previously have shown this to be a reliable assay for cell survival (5). Controls were cells with HPD or HPD plus diphenylisobenzofuran and were kept in the dark.

The triplet state energy of 1,3-diphenylisobenzofuran was kindly determined for us by Dr. William Herkstroeter of Eastman Kodak Company, Rochester, N. Y. Its phosphorescence spectrum obtained at 77° in ethanol:ether (2:1) showed a O,O band at 458 nm (62.5 kcal/mole). The phosphorescence yield was less than 1% and was unchanged for material as received and that recrystallized twice in methanol, under nitrogen. The phosphorescence did not correspond to that of the oxidation product *o*-dibenzoylbenzene. Because of the low phosphorescence yield, however, we cannot be certain that an impurity is not responsible for the observed spectrum.

RESULTS

The TA-3 cells exposed for 2 hr to 4.5×10^{-5} M HPD contained 6×10^{-4} M intracellular dye. The survival curve for these cells upon exposure to red light (1.3 milliwatts/sq cm between 610 to 630 nm) is shown in Chart 1.

The HPD absorption at 610 to 630 nm was used for activation, rather than the other visible wavelengths, in order to correlate results with our *in vivo* work where the increased penetration of red light is important (5). We find that 23 min of exposure reduces viability to 10%, compared with that of controls. Cells exposed only to the light for similar times or containing HPD and kept in the dark show no reduced viability. Cells containing 1,3-diphenylisobenzofuran (1.5×10^{-3} M intracellular) in addition to HPD are protected from the lethal effect of the red light until the intracellular furan concentration drops below about 10^{-3} M (or 10 min), at which point cellular destruction commences (Chart 1). At an intracellular furan concentration of 4×10^{-3} M, survival of light-exposed cells is extended to about 20 min. 1,3-Diphenylisobenzofuran exhibits no toxicity to the cells even in high concentration (10^{-2} M intracellular), provided the cells are protected from visible light absorbed by the furan (400 nm). Also, cells containing only the furan remain 100% viable upon exposure to the red light (620 to 640 nm) for 1 hr or more. The furan does not absorb the red light necessary for HPD activation.

Intracellular furan consumption follows a pseudo first-order relationship (Chart 2). To ensure that the furan consumption was consistent with singlet oxygen trapping,

³ The abbreviations used are: HPD, hematoporphyrin derivative; PBS, phosphate-buffered saline (16 g NaCl, 0.4 g KCl, 2.3 g Na₂HPO₄, and 0.4 g KH₂PO₄ in 2 liters distilled water, pH 7.2).

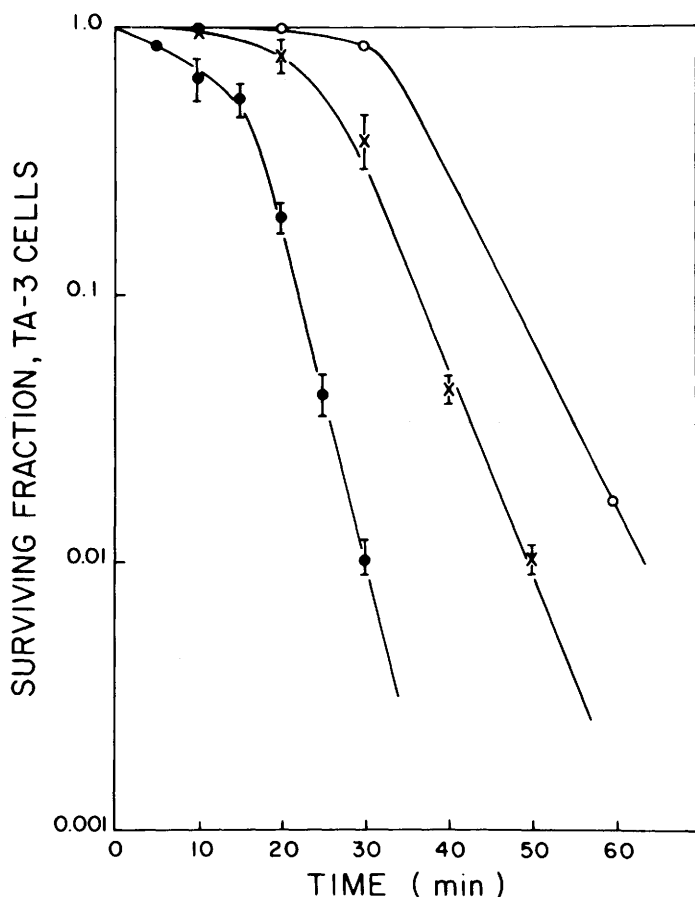


Chart 1. Surviving fraction versus illumination time (1.3 milliwatts/sq cm between 610 to 630 nm) for TA-3 cells containing 6×10^{-4} M intracellular HPD and various initial amounts of 1,3-diphenylisobenzofuran. ●, no added furan; ×, 1.5×10^{-3} M furan; ○, 4×10^{-3} M furan. Vertical bars, S.D. of 3 or more experiments.

large numbers of doubly labeled cells containing 10^{-3} M HPD plus 10^{-2} M furan were exposed for 60 min to the red light, lysed in acetone, and the extracted products were analyzed by gas chromatography. When compared with similarly labeled cells not exposed to red light, more than 90% of the consumed 1,3-diphenylisobenzofuran could be accounted for as *o*-dibenzoylbenzene, the singlet oxygen-trapping product (8, 18).

DISCUSSION

The protective effect against photodynamic inactivation of the TA-3 cells afforded by 1,3-diphenylisobenzofuran coupled with the nearly quantitative recovery of the singlet oxygen-trapping product that is formed intracellularly only when hematoporphyrin is also present, indicates that singlet oxygen is probably formed within the cells, following excitation of hematoporphyrin, and is the agent responsible for cellular inactivation.

In order to rule out the possibility of quenching of the hematoporphyrin triplets ($^3\text{Sens}^*$) by 1,3-diphenylisobenzofuran, we attempted to measure the triplet state energy of the furan from its phosphorescence spectrum. A weak phosphorescence (less than 1%) was observed at 458 nm

(62.5 kcal/mole). Although the low phosphorescence yield makes definite identification somewhat uncertain, the furan is unlikely to have a triplet state of sufficiently low energy to effectively quench the very low-lying hematoporphyrin triplets that must have energy below the excitation energy of 45 kcal/mol (620 nm).

The initial rate of consumption of furan in the cells follows pseudo first-order kinetics, which is consistent with its competing with other rapid processes for the singlet oxygen. In alcohol solution, this trap reacts with singlet oxygen at a rate of $8 \times 10^8 \text{ sec}^{-1}$ (17, 21). If one ignores possible furan complexing and other factors and assumes a similar rate constant, an intracellular concentration of about 10^{-3} M would be required for high efficiency in trapping, since singlet oxygen reacts with histidine and tryptophan residues in proteins (10^{-2} M intracellularly) at about $10^8 \text{ liter mole}^{-1} \text{ sec}^{-1}$ (16). Our data are within the correct range predicted from these values, since protection of the cells decreases rapidly below 10^{-3} M intracellular furan.

Our interest in this system stems from the demonstration by us (5) and others (4, 9, 12) that hematoporphyrin, which is known to accumulate specifically in malignant tissues (10, 14), can be photochemically activated *in vivo* to cure spontaneous and transplanted mouse and rat tumors. We have preliminary evidence to be reported elsewhere that parenterally administered 1,3-diphenylisobenzofuran also

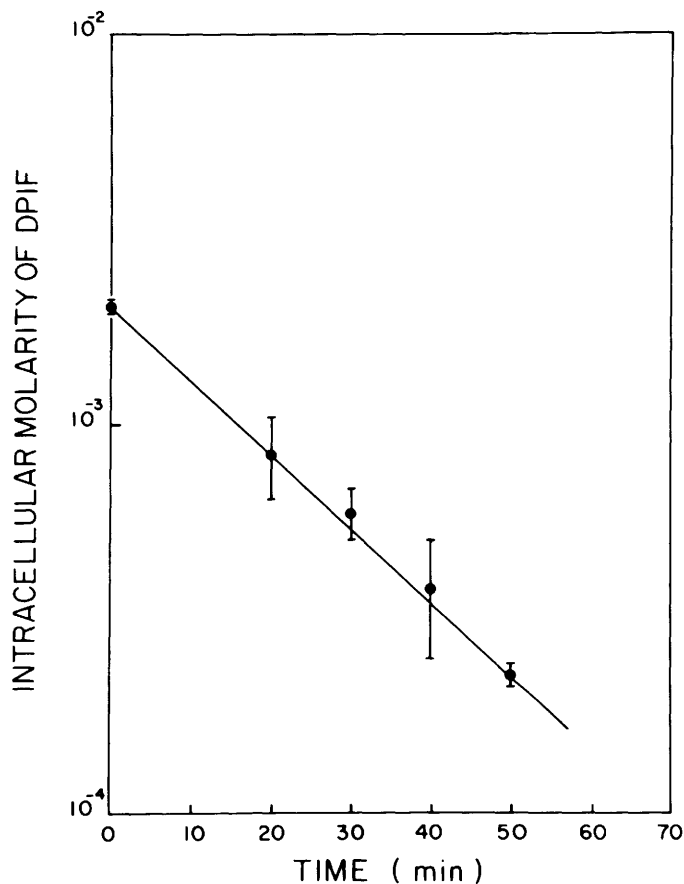


Chart 2. Rate of consumption of 1,3-diphenylisobenzofuran (DPIF) contained within TA-3 cells (intracellular HPD, 6×10^{-4} M) versus illumination time (1.3 milliwatts/sq cm between 610 to 630 nm). Vertical bars, S.D. of 3 or more experiments.

affords protection *in vivo* to such tumors treated with hematoporphyrin and red light, possibly implicating singlet oxygen in these processes as well as the *in vitro* cases.

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