Advances in Brief

Enhancing the Efficacy of Photodynamic Cancer Therapy by Radicals from Plant Auxin (Indole-3-Acetic Acid)¹

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Abstract

Indole-3-acetic acid (plant auxin) has low toxicity but dramatically enhances the killing of mammalian cells on illuminating phenothiazinium dyes with red light. Suitable dyes include toluidine blue, used in cancer diagnosis because of localization in tumors, and methylene blue, used in experimental photodynamic therapy of cancer. The photosensitized oxidation of indole acetic acid forms a free radical that fragments in microseconds, forming reactive cytotoxins. Unlike conventional photodynamic therapy, requiring excitation of oxygen to the reactive singlet state, the treatment is effective even at the low oxygen levels common in tumors and with much lower light doses than normally used.

Introduction

Treatment of cancer by PDT¹ usually involves the generation of reactive, excited oxygen molecules (1). A photosensitizing dye absorbs light, exciting it to an energetic triplet state capable of transferring its energy efficiently to ground state, triplet molecular oxygen to form excited, singlet oxygen. The latter is the damaging species, but its efficiency of production is much reduced at low oxygen tensions. Tumors are often poorly vascularized and have hypoxic regions; furthermore, oxygen depletion in the treatment area during PDT is a problem (2). Because red light penetrates tissue much deeper than that of shorter wavelength, photosensitizers absorbing in the red are preferred. Phenothiazinium dyes such as MB⁺ or TB⁺ absorb strongly at approximately 600–670 nm, and have been identified as having potential for cancer diagnosis or treatment. TB⁺ has been widely evaluated as a diagnostic agent for oral cancers because of localization in tumors, and methylene blue, used in experimental photodynamic therapy of cancer. The photosensitized oxidation of indole acetic acid forms a free radical that fragments in microseconds, forming reactive cytotoxins. Unlike conventional photodynamic therapy, requiring excitation of oxygen to the reactive singlet state, the treatment is effective even at the low oxygen levels common in tumors and with much lower light doses than normally used.

Materials and Methods

Reagents. IAA, MB⁺ (recrystallized from methanol/benzene), and TB⁺ were from Sigma. PBS [PBS, 0.14 M NaCl, 3 mM KCl, and 10 mM phosphate (pH 7.4)] was from Oxoid Ltd. (Basingstoke, Hampshire, United Kingdom). Sources of other chemicals and media have been described (11–13).

Cells. Chinese hamster V79 lung fibroblast cells and MCF7 human breast carcinoma cells were obtained from the European Collection of Cell Cultures and cultured as described previously (13).

Phototoxicity Measurements. Cells (200–20,000) were plated into standard six-well plates and allowed to attach for 1 h (V79) or 4 h (MCF7) before replacing the medium with IAA (100 μM) and photosensitizer (2 μM) in PBS (2 ml, −2.1 mm solution depth). The light source for 630-nm illumination was an 8 × 7 cm matrix of 200 LEDs (Agileent HLMP-ED18-TW000; Farnell Electronic Components, Leeds, United Kingdom) positioned ~13 cm above the six-well plate, which was placed over a mirror to reflect light. For 660-nm illumination, four adjacent 5-cm diameter circular clusters of 50 LEDs (Kingbright BL0307-50-44; RS Components Ltd., Corby, Northamptonshire, United Kingdom) were placed ~11 cm above the six-well plate. The illumination intensities were estimated using a DUO power meter (Gentec Electro-Optics; Quebec City, Canada) with PS-310 detector. Cells with MB⁺ were illuminated at ~660 nm, intensity −4.6 mW/cm²; cells with TB⁺, −630 nm, intensity −13.3 mW/cm². Illumination began immediately after adding IAA and photosensitizer, except for the experiments involving hypoxia, which involved 15 min of preincubation with frequent, gentle agitation for gas equilibration with oxygen was achieved.

The hypothesis was tested using mammalian cells in vitro; chemical products were measured in cell-free systems. Light doses of 1–6 J/cm² (a few minutes of illumination with a matrix of red LEDs) were sufficient to kill >99% of either Chinese hamster V79 lung fibroblast cells or MCF7 human breast carcinoma cells, using a concentration of...
IAA likely to be achievable in humans (see below). Fig. 2A shows that MB/H11001 without IAA was hardly phototoxic at these very low light doses, but the presence of IAA enhanced cell kill dramatically. The dyes alone, without illumination, or IAA alone (11, 13) have no detectable toxicity at these concentrations and exposure times. Similar experiments using TB/H11001 as photosensitizer required about twice the illumination for similar cell kill (Fig. 2B). However, from the measured absorbances of the solutions, with two light passes through 2.1 mm of medium, it is calculated that ~12% of the light was absorbed with 2 \mu M MB and ~6% with TB+. Hence, the efficiencies of the two dyes were broadly similar. (Thus, the absorbed light doses in the present experiments are of the order of one hundredth of the doses commonly used in PDT, which are up to ~100 J/cm²; Refs. 1, 14).

Fig. 2, C and D, shows that human breast carcinoma MCF7 cells were more sensitive to the treatment than V79 cells, with about half the illumination times needed for comparable cell kill.

The laboratory environment is not representative of tumors; in particular, the oxygen concentration in air-equilibrated medium is ~20-fold higher than many tumor cells. Experiments were extended to V79 cells at 37°C equilibrated with 1% oxygen, a level common in tumors. Fig. 2E shows that phototoxicity of TB+ with IAA in PBS at 37°C equilibrated with air required shorter illumination times for toxicity comparable with the experiments at room temperature (Fig. 2B), probably resulting in part from increased intracellular uptake of the photosensitizer during the 15-min preillumination incubation used. Fig. 2F shows that reducing the oxygen level from 21% (air; Fig. 2E) to 1% v/v did not have a marked effect on the phototoxicity of the TB+/IAA combination. However, cells subjected to illumination in hypoxia grew into very small, diffuse colonies, suggestive of different or additional mechanism(s) of cytotoxicity compared with aerobic treatment.

Illumination of cell-free phosphate-buffered solutions of IAA and Fig. 3. Measurement of products from photosensitized decomposition of IAA. HPLC chromatograms after illumination (12 min) of 100 \mu M IAA with 2 \mu M MB+ (A) or TB+ (B) in PBS, using the same light sources, geometry, vessels, and volume of solutions as in the cell work. Peak labels correspond to the structures labeled in Fig. 1; those asterisked (*) may result from reaction of IAA with singlet oxygen.

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MB+ or TB+ showed that MOI (Fig. 1, 9) and a number of precursors were produced (Fig. 3). Table 1 shows that the yield of MOI increased considerably on standing at 37°C for 1 h, together with complete loss of the hydroperoxide precursor. Air (Fig. 1, 7) and an increase in the amount of the intermediate (Fig. 1, 8). The concentration of the aldehyde (Fig. 1, 6) also decreased on standing at 37°C. Bubbling the solutions with 1% oxygen reduced both the yield of MOI and the loss of IAA. Lower IAA destruction probably arises from much-reduced singlet oxygen production, which degrades IAA to other products; the HPLC peaks thought to result from the attack of singlet oxygen on the indole ring were reduced ~50-fold in 1% oxygen compared with air.

To help assess whether the concentrations of IAA (100 μM) used in these studies in vitro might be achieved in tumors, tissue samples in mice bearing the mammary carcinoma NT were analyzed 30 min after administration of IAA (50 mg/kg i.p., 5 mg/ml in 50 mM NaHCO3/2% v/v ethanol/water). The concentrations of IAA in tumor, plasma, and liver were 306 ± 64, 885 ± 93, and 675 ± 46 μM, respectively (mean ± SE; n = 3). No evidence of toxicity was observed.

Discussion

The results validate the hypothesis that photosensitized oxidation of IAA by phenothiazinium dyes and red light offers the potential to kill and hypoxic tumor cells using low light doses. Much relevant data already exists to facilitate rational and rapid development of this concept. IAA is a catabolite of tryptophan, with micromolar levels in human plasma. Doses of IAA of 100 mg/kg p.o. were given to 18 humans in an early study for another purpose (15), with some reduction in blood glucose levels but no other effects reported. In another clinical investigation (16), doses of IAA corresponding to approximately 40–150 mg/kg p.o. were given, also with apparently no untoward effects. Only 50 mg/kg IAA administered to mice in the present work was sufficient to achieve tumor concentrations of IAA three times those used in the in vitro experiments. The phenothiazinium dyes have already been used clinically in the context of cancer therapy with topical (3) or oral administration (8), infusion into the bladder (8), and intravenous (17) or intratumor (9) injection. MB+ is used as a treatment of methemoglobinemia at 1–2 mg/kg i.v., and its pharmacokinetics in blood and urine in humans after i.v. and oral dosage (100 mg: approximately 4–5 μmol/kg) have been studied, as well as organ distribution in rats (18). There is interest in optimizing the biodistribution properties of similar dyes (6). An increasingly important medical use of MB+ exploiting its photodynamic activity is to reduce viral contamination of blood plasma by MB+ and light. Other clinical uses of MB+ include treatment of ifosfamide-induced encephalopathy (17) and methemoglobinemia. The recent study of PDT with TB+ in vitro (4) noted the safety data available on the use of TB+ in humans. Thus, whereas a full toxicological investigation is required, there is every expectation that tumor levels of IAA and of the photosensitizer comparable with those used in the present study could be achieved in humans.

Broadly similar effects in both V79 fibroblasts and MCF7 tumor cells were expected: earlier work involving peroxidase-catalyzed oxidation of IAA or a fluorinated analogue had shown that the treatment was effective in a variety of tumor cells (13, 19), although the cytotoxic response might vary with the levels of nucleophiles competing with the critical target(s) for the reactive methylene oxindole. The present experiments cannot replicate quantitatively the potential application of photosensitized formation of cytotoxins from IAA in vivo. The majority of the incident light was absorbed by the extracellular medium rather than the cell monolayers of attached cells. Other studies (4, 6) involving MB+ or TB+ in vitro have used loading of the cells with the dyes (which accumulate intracellularly) for up to several hours followed by removal of the extracellular dye before illumination. The calculation of absorbed light doses is difficult in such experiments. In the present work, limited measurements were made where attached V79 cells were incubated with TB+ (10 μM) with or without IAA (100 μM) for 30 min, followed by washing with PBS and illuminating in fresh PBS containing neither dye nor IAA. As expected, cell kill required light doses considerably higher compared with the experiments when extracellular dye absorbed much more light. However, the surviving fraction of the cells that had been pretreated with IAA was ~5-fold lower than those treated with TB+ alone. The intracellular concentration of IAA is difficult to assess in such experiments and is likely to be much reduced after washing.

A problem with conventional PDT is the absolute requirement for oxygen and reduced efficacy at low oxygen tension (2, 14). The IAA photo-oxidation can use oxygen, but the addition of oxygen to the skatolyl radical (Fig. 1, structure 3) is extremely fast, so that only very low levels would be needed. The very low light doses used in the present study suggest that a high light dose rate (intensity) would be unnecessary, reducing the possibility of local oxygen depletion, and low oxygen concentrations should be sufficient to ensure dye redox cycling. Furthermore, experiments using anaerobic peroxidase-catalyzed oxidation of IAA via gene transfection had shown cytotoxicity, although reduced compared with aerobic experiments (19). Cytotoxicity in anaerobic or hypoxia may arise via reactions of the skatolyl radical. The cytotoxicity of methylene oxindole and reactivity toward thiols as illustrative nucleophiles were reported recently (12). Methylen oxindole is produced on illumination of IAA in the presence of riboflavin or Rose Bengal (20). It is possible that the cytotoxicity of photosensitizers can be enhanced by the presence of IAA at concentrations likely to be achievable in humans. The use of IAA, or analogues with modified redox properties, with these or other suitable photosensitizers is attractive because of the opportunity for rational selection of both photosensitizer and IAA analogue, based on thermodynamic (redox) and kinetic arguments. In principle, a requirement is that the reduction potential of the photosensitizer triplet state [e.g.,
of the couple ‘MB⁺/MB’, where MB is the radical (semiquinone)] is higher than that of the indolyl radical cation (IAA⁻/IAA); ideally, the reduced dye should also be efficiently “redox cycled” to the parent dye by oxygen. These requirements appear to be fulfilled for MB⁺ and IAA. Rational consideration of alternative photosensitizers should be possible from kinetic studies and measurements of IAA-derived cytotoxins in cell-free systems.

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References

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