# Identification of new scavengers for hydroxyl radicals and superoxide dismutase by utilising ultraviolet A photoreaction of 8-methoxypsoralen and a variety of mutants of *Escherichia coli*: Implications on certain diseases of DNA repair deficiency

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8-Methoxypsoralen + UVA (ultraviolet light of 320–400 nm) known as PUVA has been in use for a number of years for the treatment of psoriasis and vitiligo. The treatment possibly works on the basis of UVA photoactivated 8-methoxypsoralen binding to DNA forming both single strand and double strand type damage.

We have used *Escherichia coli* as model system in studying PUVA induced DNA damage and repair. It has been known for some time that the photoactivated 8-methoxypsoralen, besides intercalating with DNA, generates at least two reactive oxygen species (ROS): hydroxyl radicals and superoxide anions, and also singlet oxygen. In this study it has been found that, in *E. coli*, malate dehydrogenase, succinate dehydrogenase and NADH:ubiquinone oxidoreductase can protect cells from PUVA killing presumably by scavenging these ROS. Possible mechanisms have been proposed for these enzymes as cell protectors. Studies also suggest the potential for the use of PUVA in the treatment of a large number of human diseases.

This study also finds that, unlike 8-methoxypsoralen, trioxsalen (4,5′,8-trimethylpsoralen, another derivative of psoralens) does not generate ROS by UVA photoactivation; and hence the mode of action of trioxsalen and PUVA overlaps only in the binding of these molecules to DNA in the presence of UVA.

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## 1. Introduction

8-Methoxypsoralen (8-MOP) plus near ultraviolet A light of 320–400 nm (PUVA) has been in use for a long time for the treatment of psoriasis and vitiligo [1,2]. In recent years the use of PUVA has been extended to a variety of other skin ailments such as localised morphoea, cutaneous T-cell lymphoma, palmoplantar psoriasis, anetodermic mastocytosis, systemic sclerosis, Schamberg's disease, multiple nonmelanoma skin cancer (especially squamous cell carcinoma), Woringer-Kolopp disease, folliculotropic mycosis fungoides and mycosis fungoides [3–12]. In fact in one study in Spain, 41 skin diseases were attempted to be treated with PUVA [13]. Also in one study the effects of PUVA on apoptosis of HL-60 leukaemia cells was tested [14]. From these studies the importance of PUVA in the treatment of a variety of human ailments is apparent.

8-MOP is an angular coumarin having a furan at one end and pyrone at other. When photoactivated by UVA, 8-MOP binds to DNA in three stages; in stage 1 the 8-MOP molecule intercalate in the groove of the DNA. In stage 2 this molecule is excited by UVA photons leading to a cyclobutane addition product at the 5, 6 double bond of an adjacent pyrimidine (usually thymine) and the 4, 5 double bond in the furan ring or the 3, 4 double bond in the pyrone ring. In stage 3, if a monoadduct is positioned properly opposite to a pyrimidine, the excitation of the molecule by additional photons forms a second cyclobutane ring between the positions of the pyrone double bond and the 5, 6 double bond in the adjacent pyrimidine forming a covalent cross-link, i.e. only a specific type of monoadduct can enter to form a diadduct [15].

Alternative mechanisms proposed for the damage to cellular systems by PUVA are based on the findings that this agent also produces hydroxyl radicals ($^\cdot$OH), superoxide anions ($O_2^{\cdot-}$) and singlet oxygen ($^1O_2$) [16–18] and that these agents may also be responsible for cell death. In certain cases the formation of $^\cdot$OH by PUVA was determined by the formation of 8-hydroxy-2′-deoxyguanosine 8-OHdG in DNA [18,19].

For a number of years we have been carrying out studies on PUVA using *Escherichia coli* as a model system. In this organism we isolated two specific mutants, SA270 (*puv*R) as hyper resistant and JH110 (*puv*A) as sensitive to PUVA [20,21]. Genetic locations of these mutations have been determined and it has been proposed

that SA270 is able to repair PUVA induced DNA damage more efficiently than its parent strain, Hfr KL16 [21]. A 1-D PAGE analysis of this mutant identified a protein of 55 kDal in higher concentration than its parent strain [20]. Next, by introducing Tn10 (tet-r) transposon, the PUVA-sensitive mutant was obtained and in this mutant it was shown that the 55 kDal protein was missing [21].

In subsequent studies 2-D PAGE analysis of SA270 was carried out together with its parent strain. A heavier spot on the gel was discovered for the extract from SA270 when compared with the spot obtained from the parent strain. N-terminal sequencing of the amino acids of this spot identified it as malate dehydrogenase (our unpublished result). It was highly surprising hence we extended our studies and here we show that not only malate dehydrogenase mutants of E. coli but a variety of other mutants are also sensitive to PUVA. Possible reasons for their sensitivity are proposed.

## 2. Materials and methods

### 2.1. E. coli strains

All the E. coli strains tabled below were kindly provided by the E. coli Genetic Stock Centre, Yale University, USA.

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Hfr KL16: Prototroph

SA270: PUVA-resistant derivative of KL16

JH110: PUVA-sensitive derivative SA270, Tn10(tet-r) inserted in puvA

CGSC 5732: mdh⁻, str-r

CGSC 7445: zhd⁻, tet-r, puv-s

CGSC 7538: sdh⁻, mdh⁻, tet-r

CGSC 6300: parent strain of CGSC 5732, CGSC7445, CGSC7538

CGSC 6573: E. coli B/r, parent strain of CGSC 7737, F⁻, lon-11, sul1, λ-R, Mal(ts)

CGSC7636: Parent strain of nuo and sod mutants, F⁻, Δ(araD-araB567, ΔlacZ 4787(::rrnB-3) λ⁻, rph-1, Δ(rhaD-rhaB)568, hsdR514

CGSC 7737: F⁻, lon-11, sulA1, trpE65(Oᶜ), mutM102::kan (mutM:: kan was formerly called fpg-1:: kan)

CGSC 8301: F⁻, Δ(araD-araB)567, ΔlacZ 4787(::rrnB-3) λ⁻,ΔsdhC771:: kan, rph-1, Δ(rhaD-rhaB)568, hsdR514

CGSC 8302: F⁻, Δ(araD-araB)567, ΔlacZ 4787(::rrnB-3) λ⁻,ΔsdhA773:: kan, rph-1, Δ(rhaD-rhaB)568, hsdR514

CGSA 8303: F⁻, Δ(araD-araB)567, ΔlacZ 4787(::rrnB-3) λ⁻,ΔsdhB774:: kan, rph-1, Δ(rhaD-rhaB)568, hsdR514

CGSC 8304: F⁻, Δ(araD-araB)567, ΔlacZ 4787(::rrnB-3) λ⁻, F⁻,ΔnuoK761:: kan, rph-1, Δ(rhaD-rhaB)568, hsdR514

CGSC 8305: F⁻, Δ(araD-araB)567, ΔlacZ 4787(::rrnB-3) λ⁻, F⁻,ΔnuoA770:: kan, rph-1, Δ(rhaD-rhaB)568, hsdR514

CGSC 8315: F⁻, Δ(araD-araB)567, ΔlacZ 4787(::rrnB-3) λ⁻, F⁻,Δqor-745:: kan, rph-1, Δ(rhaD-rhaB)568, hsdR514

CGSC 9395: F⁻, Δ(araD-araB)567, ΔlacZ 4787(::rrnB-3) λ⁻,ΔsodC724:: kan, rph-1, Δ(rhaD-rhaB)568, hsdR514

CGSC 9402: F⁻, Δ(araD-araB)567, ΔlacZ 4787(::rrnB-3) λ⁻,ΔsodB734:: kan, rph-1, Δ(rhaD-rhaB)568, hsdR514

CGSC 10798: F⁻, Δ(araD-araB)567, ΔlacZ 4787(::rrnB-3) λ⁻,ΔsodA768:: kan, rph-1, Δ(rhaD-rhaB)568, hsdR514

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### 2.2. Growth media

#### 2.2.1. Luria Broth and LB agar plates

These were prepared by dissolving 2.0 g of Lenox Broth Base (from Invitrogen, USA) in 100 ml distilled water. For the LB agar plates, to the 100 ml Lenox Broth were added 1.5 g of Bacto agar (Bacto Dickinson & Co., USA), sterilised and plates prepared.

### 2.3. Phosphate buffer

200 mM solutions of $K_2HPO_4$ and $KH_2PO_4$ were prepared and sterilised. 30.75 ml of $K_2HPO_4$ and 19.25 ml of $KH_2PO_4$ were added to 450 ml of sterile water to give pH 7.0 and 20 mM final concentration.

### 2.4. Reagents

8-MOP, Nitroblue tetrazolium (NBT) and trioxalene (4,5′,8-trimethylpsoralen) were purchased from Sigma, Japan and mannitol, ethyl alcohol, $K_2HPO_4$ and $KH_2PO_4$ from BDH, Japan.

### 2.5. 8-Methoxypsoralen and trioxsalen solutions

9.25 mmole $l^{-1}$ stock solutions of 8-MOP and 21.9 mmole $l^{-1}$ trioxsalen were prepared by dissolving them in absolute ethyl alcohol. This was the maximum concentration achievable. Its sterilization was considered non-essential as they were dissolved in absolute alcohol. For experiment the stock solution was added to the reaction mixture to get a final concentration of 92.5 μmole $l^{-1}$ of 8-MOP and 219 μmole $l^{-1}$ of trioxsalen.

### 2.6. Near UV lamp

The near UV (NUV) light source used was lamp FL15BL-B from National Panasonic Co., Japan. The lamp was located in a black box and the height of the lamp from the samples was adjusted by moving the platform up and down. Two platform settings were usually employed, the upper one (receiving 3900–4000 μwatt radiation/$cm^2$) and the lower one (900–990 μwatt radiation/$cm^2$). The lamp was always warmed up for 30 min for stabilization of energy emission prior to use.

### 2.7. UV metre

The UV metre employed was a UVR-2 radiometre equipped with UD-36 (TOPCON Corp., Japan).

### 2.8. Growth of bacterial cultures

The E. coli strains employed in these experiments were grown on Luria agar (LA) plates and maintained at 4 °C. For experiments an isolated colony from the LA plate was transferred to 10 ml Luria broth (LB) and grown overnight at 37 °C. The culture was then diluted (1 in 20 ml) in fresh LB and grown with aeration at 37 °C until the turbidity reached the desired level.

### 2.9. Qualitative and quantitative sensitivity tests of various strains for PUVA

These were carried out as previously described [22,23].

## 3. Results

### 3.1. Sensitivity of various mutants of E. coli for PUVA

The identification of a heavy spot of corresponding to malate dehydrogenase from an SA270 extract on 2D-PAGE analysis suggested that the high level synthesis of this enzyme may be responsible for conferring hyper-resistance of the strain to PUVA. If so, it was anticipated that the mutant of E. coli lacking malate dehydrogenase ($mdh^-$) should be sensitive to PUVA. To find this out the malate dehydrogenase deficient mutant of E. coli (CGSC 5732) was tested for PUVA sensitivity in two stages: firstly, by employing