PRIMARY MECHANISMS OF PHOTOSENSITIZATION BY FUROCOUMARINS*

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Introduction

The intense current interest in photosensitization of chemical and biological systems by furocoumarin compounds has been motivated by their clinical applications in PUVA therapy. However, the furocoumarins are of intrinsic interest as well, because their photosensitization mechanisms are unusual compared with most dye and drug sensitizers. A key feature is the formation of weak molecular complexes with DNA in the dark, leading to photosensitization by both the free and complexed furocoumarin. As a consequence, several types of initial biochemical lesions can be produced, with potentially different in vivo repair mechanisms and biological endpoints. The current information about these processes is summarized in this paper, emphasizing the possible relationships of the molecular and cellular events to PUVA therapy.

Molecular Mechanisms of Photosensitization

Photosensitization is a process in which the combined action of light plus a sensitizing agent induces effects not observed in the absence of either modality. Typical molecular pathways of photosensitization are shown in Fig.1. The first step involves optical excitation of the sensitizer molecule (S), leading to a short-lived, excited singlet state (S*) that emits its excess energy as heat or light (fluorescence) within about 10^{-8} seconds. A competing process takes place in most important sensitizers, in which S* converts spontaneously to the longer-lived, but still energy rich, triplet state (T). Both S* and T can react with components of the system in close proximity. However, T also may diffuse considerable distances during its longer lifetime. The pathway in which the initial reaction of T takes place with a key component of the system (usually called the "substrate") is described as "type 1". Alternatively, T may react first with molecular oxygen, producing an intermediate product that subsequently reacts with the substrate; these reactions are termed "type 2". The most important intermediates in type-2 reactions are excited singlet oxygen (O_2^*) and the superoxide freed radical anion (O_2^-), which is a precursor of hydrogen peroxide.

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DISCLAIMER

Photosensitization by Furocoumarins

Furocoumarins such as psoralen are transparent at longer wavelengths than 400 nm and therefore they do not act as sensitizers of visible light. However, furocoumarins absorb in the near-ultraviolet ("black light") region, particularly in the UV-A band (320-400 nm) through which most proteins and DNA are very weakly absorbing. The exposure of a chemical or cellular system to UV-A in the presence of low furocoumarin concentrations often leads to significant effects, which are the principal subject of this paper. Furocoumarins were originally described as "non-photodynamic" sensitizers because oxygen is not required for for the photosensitization of DNA. It was deduced many years ago that furocoumarins form weak complexes with DNA in the dark, which are converted to covalent photo-addition products by exposure to UV-A.[10] Monofunctional adducts are formed in the first step, in which either the furan end (4',5' double bond) or pyrone end (3,4 double bond) of the furocoumarin molecule reacts with a double bond of a pyrimidine or purine of the DNA. A certain fraction of the monoadducts can be converted to interstrand cross-links, if the furocoumarin has an unblocked double bond at the other end, the molecular dimensions and monoadduct binding site are correct, and the incident radiation is absorbed by the monoadduct. These conditions are fulfilled by the standard PUVA sensitizer, 8-methoxypsoralen, which forms 2 types of monoadducts and cross-links with DNA. This sequence of events is shown schematically in Fig.2. More recent work has shown that 8-methoxypsoralen[21] and other furocoumarins[7] generate O_2^* when exposed to UV-A, leading to a parallel type-2 photosensitization pathway whose biological implications remain unknown.

Effects at the Cellular Level

Studies on microorganisms and mammalian cell cultures provide ample evidence that furocoumarin photosensitization induces sublethal damage amenable to *in vivo* repair. Cellular repair of furocoumarin lesions was shown by the higher photosensitivity of repair-deficient strains of bacteria[9,16,26] and yeast[3], and recovery during dose fractionation in cultured Chinese hamster cells[5]. The much higher photosensitivity of microorganisms to the cross-linking furocoumarins, such as 8-methoxypsoralen, compared with photosensitization by furocoumarins that presumably form only monoadducts, such as angelicin and 3-carbethoxypsoralen, provides evidence for higher *inherent* lethality of the cross-links[1,3,16]. However, the great preponderance of monoadducts over cross-links with 8-methoxypsoralen at the dose levels at which cells are killed can

lead to a significant contribution of monoadducts to lethality, particularly for the more photosensitive, repair-deficient cells[14]. The mutagenic effects of furocoumarins are of considerable interest in connection with tumorigenesis in laboratory animals and humans. Comparisons of mutation frequencies in E. coli exposed to UV-A in the presence of psoralen and angelicin indicate that cross-links are "more mutagenic" than monoadducts[2]; similar conclusions derive from mutation frequencies in yeast as photosensitized by 8-methoxypsoralen and psoralen compared with angelicin, 3-carbethoxypsoralen and 5,7-dimethoxycoumarin[4]. The mutagenic action of furocoumarin cross-links has been attributed to error-prone repair. The overall mutagenic action of UV-A plus 8-methoxypsoralen probably involves contributions from both monoadducts and cross-links. Mutations and sister chromomatid exchanges have been reported in Chinese hamster cells exposed to UV-A in the presence of 8-methoxypsoralen and 5-methoxypsoralen[2,6]. A recent observation[23] that the mutation frequency in Chinese hamster cells showed "reciprocity" relative to the product of the 8-methoxypsoralen concentration and the UV-A dose is consistent with a major monoadduct contribution to mutations. The involvement of O_2^* has not been evaluated in the previous work. However, there is ample evidence that O_2^* generated by many photosensitizing dyes is a key factor in the inactivation and induction of genetic changes in yeast cells[17], and there is no reason to exclude this possibility with furocoumarins. The diffusion range of O_2^* in water is about 0.2 microns[13], which would make it possible for the O_2^* generated by the excess furocoumarin, not complexed with DNA, to reach DNA, protein and membrane targets[18]. Furthermore, the furocoumarin molecules complexed to DNA and the covalent monoadducts also may generate O_2^*, leading to a high probability of DNA damaged mediated by O_2^*.

Mathematical Modeling of Furocoumarin Photosensitization

It is evident that photosensitization of biological systems to UV-A with furocoumarins is a complex process, involving potential damage related to furocoumarin-DNA monoadducts and cross-links and the generation of singlet oxygen. We have been attempting to develop kinetics models which may assist in data analysis and in the planning of experiments. Since the mathematical details are available in the literature, only the key results are summarized here.

(a) Photosensitization of DNA by Free and Complexed Furocoumarin:

The distribution of free and dark-complexed furocoumarin molecules is determined by the effective concentration of DNA nucleotides, the total concen-

tration of DNA nucleotides, the total concentration of furocoumarin in the cell, and the equilibrium dark binding constants, which have been measured for a number of furocoumarins[11]. The rate of formation of covalent photoadducts can be estimated from data obtained with in vitro model systems[14,22]. The unbound furocoumarin molecules may contribute to damage if the unstable intermediates can diffuse to the DNA surface during their lifetimes. (Each DNA molecule is surrounded by a sheath of active medium, whose width depends on the type of reactive agent.) The detailed analysis for 8-methoxypsoralen shows that each DNA monoadduct is accompanied by about 80 hits of O_2^* at the DNA surface[25]. The probability of O_2^* damage would be even higher if the dark-complexed 8-methoxypsoralen also can generate O_2^*.

(b) Kinetics of DNA-Furocoumarin Photoadduct Production:

The assumption that only the dark-complexed furocoumarin is involved in the formation of covalent photoadducts has been confirmed by model experiments with psoralen and 8-methoxypsoralen[15,25]. Since cross-linking requires two successive photochemical steps, the UV-A dose corresponding to about one cross-link per DNA molecule should depend on $1/f^{\frac{1}{2}}$, where f is the fraction of occupied dark binding sites on the DNA. However, this quantity is quite insensitive to the toal concentration of furocoumarin. Consequently, biological effects induced by furocoumarin cross-links should increase slowly with the total furocoumarin in the system. Deviations from this response can be taken as evidence for physiological factors, such as a strong dependence of furocoumarin uptake on its concentration or metabolic inactivation of the furocoumarin prior to the UV-A exposure. The biological effects of furocoumarin photosensitization have been analyzed in terms of "repair-lethality" parameters, determined by the probability the initial lesion is not repaired and the inherent lethal effect of the unrepaired lesion[14]. (Similar factors may be defined for mutagenesis.) Since these parameters should depend only on the repair capability of the strain, for otherwise constant irradiation conditions, they can be estimated by fitting the experimental survival data to the predictions of the mathematical model. The application to data for inactivation of E. coli K-12 photosensitized by 8-methoxypsoralen showed that the monoadducts account for about one half of the lethal lesions in the wild type strain and are the dominant lethal lesions in uvrB and recA strains.

Molecular Mechanisms in PUVA Therapy

The clinical endpoints in PUVA therapy include clearing of psoriatic lesions,

erythema, hyperpigmentation and tumorigenesis. The efficacy of the treatment might be improved and the attendant risks decreased if it were possible to relate the physiological effects to photosensitization mechanisms at the molecular and cellular levels. Unfortunately, only speculation about these interrelationships is possible at this time. The identification of psoriasis clearing with furocoumarin cross-links appeared to be on firm ground until very recently. This mechanism is consistent with inhibition of scheduled DNA synthesis by cross-links[24] and the reported psoriatic inactivity of angelicin[20]. However, the recent report[12] of psoriasis clearing by topical phototherapy with 3-carbethoxypsoralen, which does not form DNA cross-links in yeast for 365 nm radiation[3], suggests that mono-adducts also may be involved in psoriasis clearing. (The rapid photodecomposition of 3-carbethoxypsoralen at shorter UV-A wavelengths may be involved in the PUVA results[19]. Additional evidence against the requirement for cross-links obtains from measurements of cross-link yields in DNA extracted from guinea pig skin after exposure to UV-A in the presence of 8-methoxypsoralen and 4,5',8-trimethylpsoralen[8]. The extent of detectable cross-linking was insignificant at the UV-A and furocoumarin dose levels corresponding to PUVA therapy in humans. These findings provide a strong case against the cross-link mechanism of psoriasis clearing, assuming that the animal model is applicable quantitatively to humans. The tumorigenic action of furocoumarin photosensitization almost certainly involves DNA damage. Since DNA photoadducts and singlet oxygen initiate mutations in microorganisms, any or all of these entities might be involved in PUVA therapy. A clue in support of singlet oxygen involvement derives from the report that 3-carbethoxypsoralen is nontumorigenic in mice[12]. Since this furocoumarin derivative does not appear to generate any singlet oxygen[18], it is likely that singlet oxygen plays a role in the tumorigenic action of furocoumarins. Similarly, the absence of hyperpigmentation with topical PUVA therapy by 3-carbethoxypsoralen[12] suggests the participation of singlet oxygen in mutagenesis.

Concluding Comments

A proper understanding of the PUVA therapy action mechanism requires the synthesis of concepts developed at the level of molecules, single cells and whole organisms. Although progress has been made in identifying key factors within each level of organization, the interrelationships remain obscure. Important unanswered questions at the molecular and cellular levels include: (1) Which excited states of the furocoumarin in molecule (triplet or excited

singlet) are involved in the formation of DNA monoadducts, and the conversion of monoadducts to cross-links? (2) How does the spectrum of the incident radiation affect the distribution of the initial photochemical products from the PUVA sensitizers? (3) What are the relative contributions of furocoumarin-DNA monoadducts, furocoumarin-DNA cross-links and singlet oxygen to mutagenesis and lethality in cells, at the furocoumarin and UV-A dose levels corresponding to PUVA therapy? Additional information about these key aspects of furocoumarin photosensitization should lead to a more definitive relationship of the cellular level events to the endpoints observed with PUVA therapy, and suggest directions for potential improvements in the current clinical procedures.

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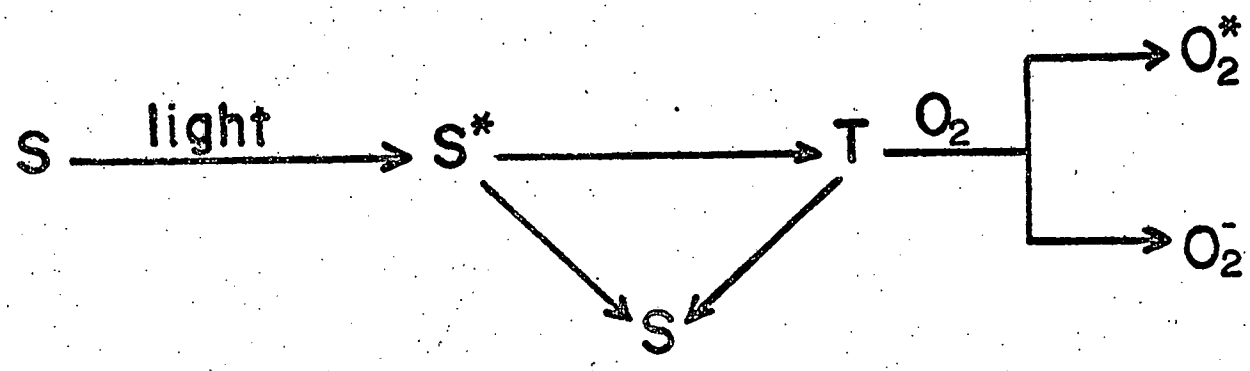
Figure Captions

Figure 1 Typical pathways of photosensitization: Optical excitation of the sensitizer (S) produces an excited singlet state (S*), that may populate the longer-lived triplet state (T) in competition with deactivation, accompanied by the emission of light (fluorescence) or heat. The triplet state may react with molecular oxygen leading to singlet oxygen (O_2^*) or the superoxide radical anion (O_2^-). Reactions in which the initial reaction of the substrate takes place with S* or T are termed type 1. In type-2 reactions, the initial reaction of the sensitizer is with oxygen, leading to the intermediates that react with the substrate.

Figure 2 Photosensitization by furocoumarins: The unbound furocoumarin molecules (F) are similar to other photodynamic sensitizers, in which excitation by near-ultraviolet radiation (UV-A) generates the excited singet state (F*) and the triplet state (F_T), which may sensitize by the type-1 mechanism. The production of singlet oxygen from F_T leads to a type-2 photosensitization pathway. The furocoumarin molecules complexed with DNA in the dark (DNA:F) are converted to covalent monoadducts (DNA-F) by UV-A , a certain fraction of which may be converted to cross-links (DNA=F) in a subsequent photochemical step.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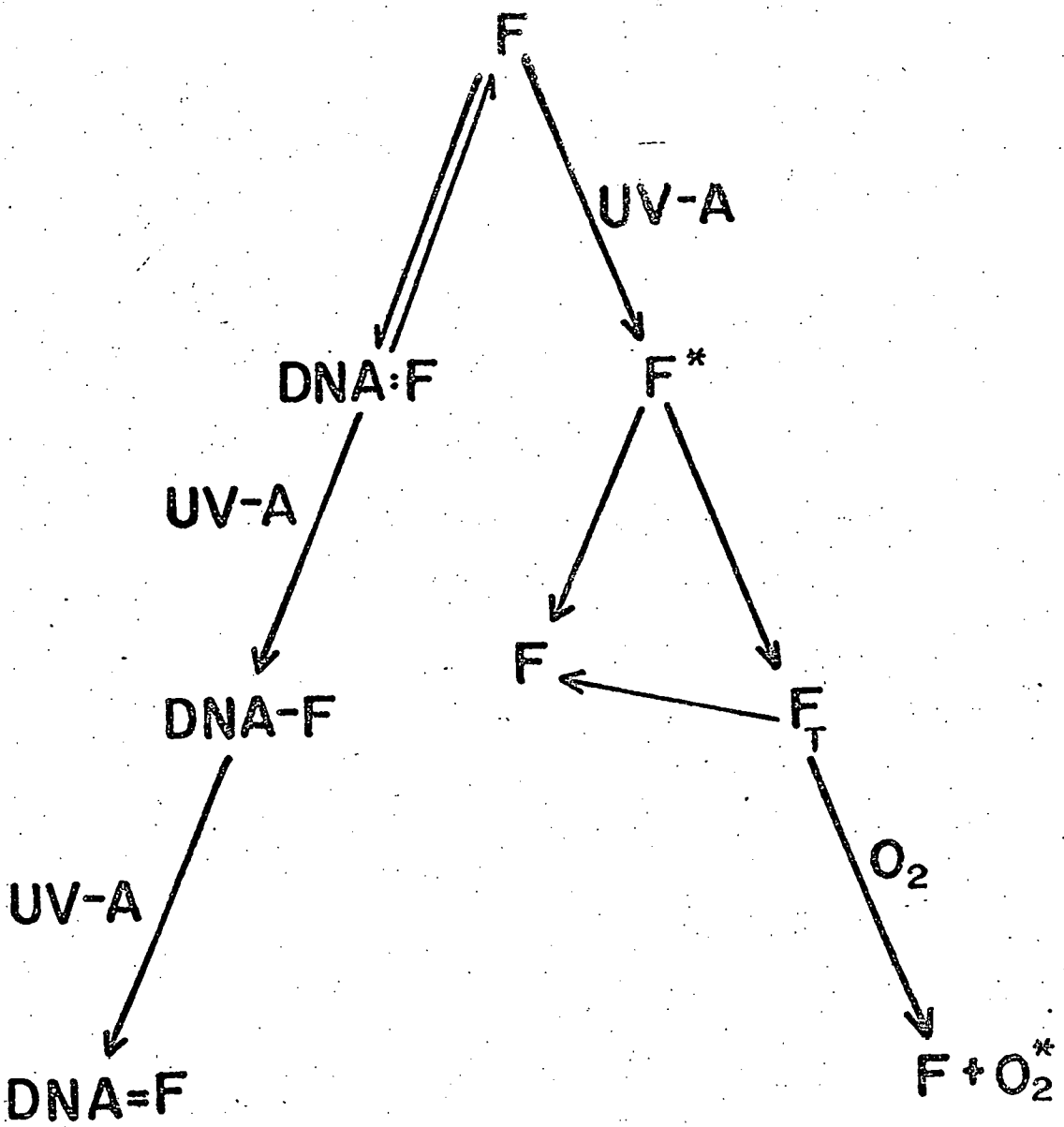

Fig. 2