Active oxygen forms in photoreaction between DNA and furanochromones khellin and visnagin

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The two furanochromones khellin and visnagin react with DNA under irradiation by 365 nm light, forming photoadducts. Recently, the use of khellin as therapeutic agent for skin diseases has been proposed. It is well known that during the formation of photoadducts toxic active oxygen forms are produced. We studied therefore the behaviour of the two furanochromones as producers of $^1O_2^-$ and O_2^-. Our results indicate that visnagin is a strong generator of both superoxide radicals and singlet oxygen, while khellin does not exhibit strong production of OO_2^-, which is promptly quenched by superoxide dismutase.

Active oxygen *Khellin* *Visnagin* *Furanochromone* *DNA photoadduct* *Ultraviolet radiation*

1. INTRODUCTION

Oxygen active species are $O_2^{\cdot-}$ (superoxide) and its conjugate acid the hydroperoxyradical $HO_2^{\cdot}$, singlet oxygen $^1O_2$, the hydroxyl radical $^{\cdot}OH$ and hydrogen peroxide H_2O_2. It is well known that the occurrence in cells of these particular forms of oxygen causes serious metabolic disorders leading to such dangerous biological phenomena as chromosomal aberration, mutations, cytotoxicity and carcinogenesis. Very recently, Cerutti [1] made an accurate study on the prooxidant states and tumour promotion. The importance of the active oxygen forms produced during photoreaction with DNA by psoralens has been evaluated by Pathak et al. [2]. The authors confirm in their conclusions that, very probably, the reactive moieties of oxygen play a very important role in understanding the mechanisms of cancinogenesis and damaging reactions to DNA and cell membranes, which usually accompany the therapeutic effects of psoralen derivatives used in PUVA (Psoralen-Ultraviolet-A) treatments of dermatological diseases. Even though some reservations exist, Averbeck et al. [3] consider the relationship between the capacity to generate singlet oxygen and photobiological activities of 3-carbethoxypsoralen and 4'-methylangelicins.

The chemical structure of furanochromones is closely related to that of furanocoumarins and some studies of photoadducts between DNA and visnagin or khellin are reported in the literature [4–7]. These compounds are natural furanochromones, biologically active as spasmolytic and vasodilatatory agents, contained in various parts of *Ammi visnaga* L. (Lam.) [8–10]. Khellin is still used in the therapy of asthma and angina pectoris. Recently, oral administration of khellin followed by sunlight exposure has given good results in the therapy of psoriasis and vitiligo [11,12]. In view of foreseeable developments concerning these interesting therapeutic properties, we considered it very useful to control the behaviour of both compounds visnagin and khellin, concerning the production of active forms of oxygen during the photoreaction between DNA and the two furanochromones.

2. MATERIALS AND METHODS

2.1. *Materials*

The chemicals used in our experiments were obtained as follows: psoralen, nitro blue tetrazolium and riboflavin were from Sigma (St. Louis, MO);

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2′-deoxyguanosine, calf thymus DNA and superoxide dismutase were from Boehringer (Mannheim); khellin and visnagin were kindly furnished by Angelini (Ancona, Italy) and Inverni and Della Beffa (Milano, Italy) respectively. All other chemicals were of analytical grade. For the ultraviolet radiation a 'G and C' lamp 200/250 V, 250 W 3 Pin ME/D Compact Source was used equipped with an Oriel band pass 355 nm filter. The irradiance of emitted light was measured with an Osram UV meter in a quartz cuvette. For spectrophotometric determinations a Beckman DU-8 spectrophotometer was used.

2.2. *Active oxygen forms determination*

The formation of singlet oxygen was detected by measuring the decrease of the absorbance value of 2′-deoxyguanosine at 260 nm [13]. The superoxide radical formation was detected by the method of Korycka-Dahl and Richardson [14]. The irradiation was performed with 3 J/cm^2 and 0.25 J/cm^2 of ultraviolet radiation, respectively.

2.3. *Deletion of DNA heat-induced hyperchromic shift*

260 μg DNA, 5 μg of khellin or visnagin or psoralen (previously dissolved in 50 μl of ethanol) were contained in 10 ml of 0.1 M Tris buffer (pH 7.5). When indicated, 80 U of superoxide dismutase (SOD) were added. After irradiation (2.3 J/cm^2) samples were denaturated in a boiling bath for 8 min and quickly cooled. The absorbance value was monitored at 260 nm.

3. RESULTS AND DISCUSSION

Table 1 reports the ability of khellin and visnagin to form singlet oxygen. Psoralen has been chosen as a well-tested, effective producer of $^1O_2^-$ [2]. As it is possible to observe, visnagin exhibits a rather strong ability to form $^1O_2^-$, almost of the same magnitude as psoralen. Otherwise, even in these severe experimental conditions with a large concentration of chemical and rather strong intensity radiation, khellin produces only negligible amounts of singlet oxygen. Table 2 shows the formation of superoxide radicals by khellin and visnagin. The riboflavin is used as reference compound in the nitro blue tetrazolium reaction. In table 2 it is possible to observe the quenching effect by SOD in the production of these active oxygen forms. From the data reported in table 2, the very strong production of $O_2^{\cdot -}$ by visnagin is evident, while khellin can be considered a rather moderate producer of this active oxygen form. The SOD inhibits almost completely the production of $O_2^{\cdot -}$. In table 3, the effect of deletions of DNA on the heat-induced hyperchromic shift is illustrated. Psoralen, a strong producer of cross links in DNA molecules, has been chosen as a reference compound. It is well known that after heat denaturation native DNA exhibits a hyperchromic shift at 260 nm; this phenomenon disappears when cross links are present in the molecule. Our results indicate that both khellin and visnagin form cross links in the reaction with DNA. The experiments carried out in presence of SOD confirm that, even

Table 1

Photodynamic degradation of 2′-deoxyguanosine by singlet oxygen

Chemicals[a]	% degradation
Psoralen	35.1
Khellin	<1
Visnagin	24.7

[a] 40 μg of chemicals (previously dissolved in 400 μl of ethanol) and 50 μg of 2′-deoxyguanosine were contained in 4 ml of 0.01 M carbonate buffer, pH 10

Table 2

Photoproduction of superoxide radical and its quenching by superoxide dismutase

Chemicals[a]	% relative production of nitro blue formazane (riboflavine = 100%)	Effect of superoxide dismutase (SOD) (% quenching)
Riboflavine	100	87
Khellin	53	98
Visnagin	100	90

[a] 40 μg of chemicals (previously dissolved in 400 μl of ethanol) and 550 μg of nitro blue tetrazolium were contained in 4 ml of 0.01 M carbonate buffer (pH 10)

Table 3
Deletion of DNA heat-induced hyperchromic shift

Reaction mixtures[a]	A_{260nm}		ΔA_{260nm}
	Normal	Denatured	
Native DNA	0.512	0.642	0.130
Native DNA + khellin + light	0.552	0.572	0.020
Native DNA + khellin + light + SOD	0.560	0.590	0.030
Native DNA + visnagin + light	0.562	0.592	0.030
Native DNA + visnagin + light + SOD	0.558	0.593	0.035
Native DNA + psoralen + light	0.625	0.642	0.017
Native DNA + psoralen + light + SOD	0.630	0.638	0.008

[a] When indicated, 80 U of SOD were added to the reaction mixtures

in the presence of this specific quenching agent for the production of $O_2^{\cdot -}$, khellin is able to form cross links in its reaction with DNA.

Our results fit very well with some of the data from the literature. In fact khellin, which generates to a moderate extent only $O_2^{\cdot -}$ (promptly quenched by SOD), has been reported to be a weak phototoxic and genotoxic agent in comparison with the toxic visnagin (a strong producer of $^1O_2$ and $O_2^{\cdot -}$ [7]). Moreover, besides data reported in table 3 other evidence is available that khellin is able to bind DNA, forming a bifunctional photoadduct [15]. The occurrence of cross links in the DNA molecule causes damage which is not easily repairable, but, as reported by Pathak et al. [2] on the basis of a proposal by Hanawalt [16], persistence of cross links in DNA most likely causes cell lethality, whereas the formation of monoadducts, which are not lethal to the cell and lead to error-prone DNA repair, appear to make monofunctional psoralens more carcinogenic than bifunctional photoadducts.

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