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5-(3-Phenylpropoxy)psoralen and 5-(4-phenylbutoxy)psoralen: mechanistic studies on phototoxicity

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5-(3-Phenylpropoxy)psoralen and 5-(4-phenylbutoxy)psoralen are blockers of the lymphocyte potassium channel Kv1.3 with EC_{50} values < 10 nM and may be used as new potential immunosuppressive drugs. As reported for many furocoumarins, these compounds show phototoxic properties and react under UV radiation with different targets in the cell, e. g. DNA or proteins and lipids in membranes. The photoreactions with these targets were investigated and compared to the well-known derivatives psoralen, 5-methoxypsoralen, 8-methoxypsoralen and 4,5′,8-trimethylpsoralen. Moreover, absorption properties and the capability to photoproduce singlet oxygen ($^1O_2$) and reactive oxygen species (ROS) were studied. It was found that 5-(3-phenylpropoxy)psoralen and 5-(4-phenylbutoxy)psoralen are similar or less phototoxic *in vitro* as 5-methoxypsoralen which is the furocoumarin with the weakest phototoxicity *in vivo* compared to psoralen, 8-methoxypsoralen and 4,5′,8-trimethylpsoralen.

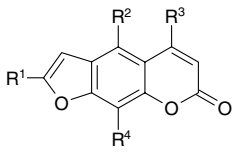
1. Introduction

5-(3-Phenylpropoxy)psoralen (**1**) and 5-(4-phenylbutoxy)psoralen (**2**) are low nanomolar blockers of the lymphocyte potassium channel Kv1.3 (Vennekamp et al. 2004), which is expressed in T-lymphocytes and plays an important part in activation of the immune system (Wulff et al. 2003a). Blockers of Kv1.3 may, therefore, be potential drugs for autoimmune disorders (Wulff et al. 2003b). Systemical therapy with **1** and **2** could implicate phototoxic reactions which are known for other furocoumarins (Averbeck 1989). In order to investigate the possibility of phototoxic reactions, mechanistic studies can be performed regarding targets like DNA, proteins and lipids (Spielmann et al. 1994). This is important because furocoumarins reach nucleus, cytoplasm and membrane lipids of cells (Moreno et al. 1982). In general the extent to photoproduce singlet oxygen ($^1O_2$) and other reactive oxygen species (e. g. $O_2^{\bullet -}$) must be considered, too, since these oxygen derivatives can damage DNA, proteins and lipids (Potapenko 1991).

All tests were carried out in comparison to psoralen (**3**), 5-methoxypsoralen (5-MOP, bergapten, **4**), 8-methoxypsoralen (8-MOP, xanthotoxin, **5**) and 4,5′,8-trimethylpsoralen (TMP, trioxsalen, **6**). These compounds are the most frequently used furocoumarins in PUVA therapy (Njoo and Westerhof 2001) and, therefore, their phototoxic behaviour is well documented, which facilitates an estimation regarding the *in vivo* phototoxicity of **1** and **2** (for structures cf. Table 1).

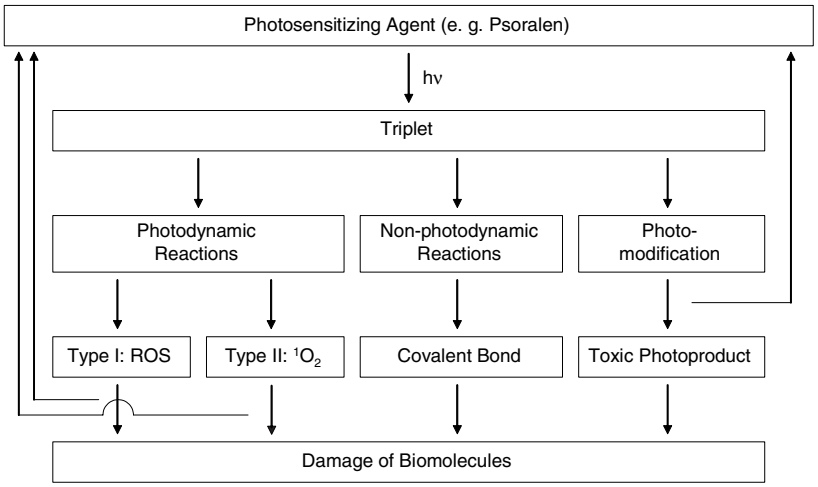
Photosensitization (phototoxicity) can be divided into different clinical categories (Spielmann et al. 2000). Furocoumarins applied topically or systemically in combination with UV radiation can cause photoirritative (acute photo-

Table 1: Chemical structures of furocoumarins



No.	Compound	R^1	R^2	R^3	R^4
1	5-(3-Phenylpropoxy)psoralen	−H	$-O(CH_2)_3Ph$	−H	−H
2	5-(4-Phenylbutoxy)psoralen	−H	$-O(CH_2)_4Ph$	−H	−H
3	Psoralen	−H	−H	−H	−H
4	5-Methoxypsoralen	−H	$-OCH_3$	−H	−H
5	8-Methoxypsoralen	−H	−H	−H	$-OCH_3$
6	4,5′,8-Trimethylpsoralen	$-CH_3$	−H	$-CH_3$	$-CH_3$

Scheme



toxic), photocarcinogenic (Averbeck 1989) and photoallergic reactions in human skin. Nevertheless, photoallergies provocated by furocoumarins seem to be very rare (Ravenscroft et al. 2001).

A common first step is absorption of radiation by a photosensitising agent which results in the excited singlet state and after intersystem crossing in the triplet state. Due to the longer lifetime of the triplet state in comparison to the singlet state most photoreactions take place from the triplet state (Quintero and Miranda 2000). UVA (320–400 nm) is the most frequent radiation responsible for photosensitization, but there are also some compounds that are activated by UVB (280–320 nm) or visible light (400–800 nm). Generally, UVA radiation is required for furocoumarin photosensitization with a maximum activity in the range of 335 nm to 365 nm (Schlatter 1988). On the molecular level, different photochemical reactions occur which damage endogenous biomolecules (Scheme). Depending on site and intensity of damage acute phototoxic, photocarcinogenic or photoallergic reactions appear.

Furocoumarins exhibit the following photoreactions (Potapenko 1991):

- Production of singlet oxygen ($^1O_2$) concomitant with oxidation of unsaturated fatty acids, cholesterol, amino acids, nucleic bases and furocoumarins themselves (Type II reactions).
- Generation of reactive oxygen radicals ($O_2^{\bullet -}$, $HO_2^{\bullet}$, $HO^{\bullet}$) and electron transfer reactions followed by oxidation of unsaturated fatty acids, cholesterol, amino acids and nucleic bases (Type I reactions).
- Formation of covalent bonds with DNA, lipids, proteins and of furocoumarins with each other (Non-photodynamic reactions).

Nowadays, these reactions engaging DNA, lipids and proteins with and without the participation of oxygen are linked to photobiological effects in skin (Zarębska et al. 2000).

2. Investigations, results and discussion

The absorption properties of **1**–**6** are summarized in Table 2. All these furocoumarins show a log $\varepsilon > 1$ in the region above 280 nm, which is an essential requirement for photoactivity according to OECD Testguideline 432. Interestingly **1** and **2** show the same log ε as **4** above 230 nm, in spite of the phenylalkoxy substitution. This fact suggests similar photophysical properties.

All furocoumarins show quite different levels of singlet oxygen production. The chosen method is not influenced by ROS (Pathak and Joshi 1984) and represents an often used standard method (e.g. Körner 2003). In Table 3 the relative singlet oxygen production is shown as decrease in absorption after 5 h of irradiation compared to **3**. Our sequence **3 > 6 > 5 > 4** corresponds to the results published by Pathak and Joshi (1984). For **1**, **2** and **4**, a relatively low degree of singlet oxygen production was found. This may be explained by almost equal substituents at C-5 (alkoxy residues), since the substitution pattern of the furocoumarin skeleton has an important influence (Körner 2002).

Superoxide radical anions were identified by the reduction of the scavenger nitro blue tetrazolium (NBT) to a formazan. The selectivity of this method was proven by quenching studies using superoxide dismutase. Again, many furocoumarins have been measured by this method (e.g. Vedaldi et al. 1995). In Table 3 the relative $O_2^{\bullet -}$-photopro-

Table 2: Wavelength and molar absorption coefficient in ethanol of three absorption maxima at highest wavelengths

Comp.	$\lambda_{max,\,1}$ (nm)	log $\varepsilon_{max,\,1}$	$\lambda_{max,\,2}$ (nm)	log $\varepsilon_{max,\,2}$	$\lambda_{max,\,3}$ (nm)	log $\varepsilon_{max,\,3}$
1	260	4.22	268	4.25	310	4.17
2	260	4.23	268	4.25	310	4.18
3	246	4.40	290	4.02	328	3.79
4	260	4.23	268	4.27	310	4.19
5	218	4.39	250	4.37	300	4.08
6	250	4.48	296	4.02	336	3.80

Table 3: Photoproduction of $^1O_2$ and $O_2^{\bullet -}$ and ΔT_m-values (N.D. = not determined)

Compound	Relative $^1O_2$-Photoproduction	Relative $O_2^{\bullet -}$-Photoproduction	ΔT_m (°C)
1	0.17 ± 0.04	0.06 ± 0.00	0.3 ± 0.5
2	0.25 ± 0.07	0.06 ± 0.00	0.1 ± 0.1
3	1.00 ± 0.03	0.08 ± 0.00	7.9 ± 0.4
4	0.10 ± 0.04	0.01 ± 0.00	11.9 ± 0.9
5	0.39 ± 0.04	0.01 ± 0.00	7.0 ± 0.1
6	0.83 ± 0.02	0.05 ± 0.01	$> 28.4 \pm 0.1$
Riboflavin	N.D.	1.00 ± 0.03	N.D.

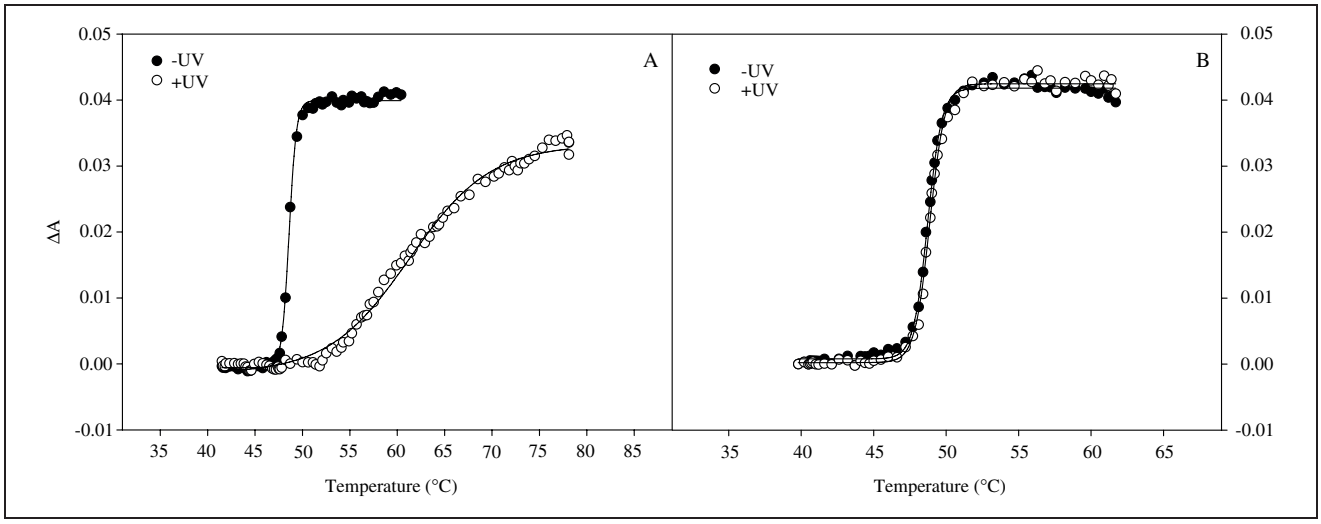


Fig.: Melting profiles of DNA treated with **4** (A) and **2** (B) in the dark and under irradiation

duction is represented as increase in absorption after 30 min of irradiation in comparison to riboflavin. The sequence riboflavin $> \mathbf{3} > \mathbf{6} > \mathbf{4} \approx \mathbf{5}$ according to Pathak and Joshi (1984) was confirmed but at a lower degree due to application of phosphate buffer with an almost physiological pH of 7.8 as proposed by Beauchamp and Fridovich (1971) instead of carbonate buffer pH 10. Our lower pH value may better correlate with the physiological situation, because generation of radicals could not be observed in some studies (Gasparro et al. 1997). In this context photoproduction of $O_2^{\bullet -}$ seems to be of little importance concerning the phototoxicity of **1** and **2**.

Furocoumarins crosslink DNA by means of UVA radiation. Photoreactions between DNA and furocoumarins occur predominantly in DNA regions rich of adenine and thymine (Sage and Moustacchi 1987). Therefore, utilization of DNA containing only adenine and thymine enhances sensitivity of the reaction. Crosslinks were measured as differences in melting points (ΔT_m) between DNA exposed to furocoumarins in the dark and crosslinked DNA after exposure to furocoumarins and UVA radiation. Melting points (T_m) are defined as 50% of thermal helix to coil transition, which is observed photometrically by an increase of absorption (Dall'Acqua et al. 1971). Crosslinks stabilize the helix leading to an increased melting point.

The differences in melting points (ΔT_m) between samples containing the related furocoumarin with and without irradiation are summarized in Table 3. The Figure exemplifies melting profiles of DNA treated with **2** and **4** in the dark and under irradiation. Untreated DNA (no furocoumarin, no irradiation) shows a melting point of 48.5 ± 0.7 °C. Irradiation of DNA in the absence of furocoumarins results in a melting point of 49.2 ± 0.5 °C. All furocoumarins investigated only induced low shifts (+0.1 °C up to +1.0 °C) of the melting point in the dark compared to the completely untreated DNA. Therefore, strong binding to DNA in the dark can be excluded for all substances. Compounds **4, 5** and **6** showed ΔT_m-values already reported in the literature (Körner 2002, Wulff et al. 1998). Compounds **1** and **2** provoke no crosslinking of DNA after irradiation as there were no differences. Thus both can be regarded as monofunctional furocoumarins (no formation of crosslinks). This may be very important, because some studies attest a greater relevance for photoirritative and photocarcinogenic processes to crosslinks than to monoadducts (e.g. Alcalay et al. 1989). Crosslinks were not developed by **1** and **2** probably due to the bulky phenylalkoxy-substitution, inhibiting intercalation between two DNA strands in the dark.

Erythrocytes represent a very suitable model for the studies of photoeffects of furocoumarins on membranes because they have no nucleus and DNA damage cannot contribute to the lysis of the cells. Furthermore, functional disorders of the erythrocyte membrane can be quantified by measurement of extracellular haemoglobin (Potapenko 1991). Furocoumarins disturb the structure and fluidity of the erythrocyte membrane by the photoreactions discussed above.

Compounds **1**, **2** and **4** reveal small photohaemolysis factors and, therefore, only little membrane photodamage can be attributed (Table 4). Due to the fact that membranes are susceptible to $^1O_2$ (Pape et al. 2001), the low photoreactivity can be explained by small $^1O_2$ production of these compounds. Compounds **3**, **5** and **6** show both greater photohaemolysis factors and $^1O_2$ production. Haemolysis experiments were conducted at very low erythrocyte concentrations (5×10^6 cells per ml) in order to enhance sensitivity towards membrane photoreactions. At increased concentrations ($\geq 1 \times 10^7$ cells per ml) haemolysis was not observed for all studied furocoumarins both in the dark and along with irradiation.

We summarize that **1** and **2** exhibit less or similar *in vitro* phototoxic properties in comparison with **4**. Therefore, we assume that **1**, **2** and other furocoumarins with a bulky alkoxy substitution at C-5 show this minor phototoxicity as a result of the same photophysical and photodynamic properties as **4** and no occurrence of DNA crosslinks. Therefore, a lower phototoxicity than **4** may be expected

Table 4: Haemolysis caused by furocoumarins and standard compounds

Comp.	$H_{50,\,-UV}$ (mol/l)	$H_{50,\,+UV}$ (mol/l)	PHF
1	$(6.5 \pm 0.2) \times 10^{-6}$	$(2.5 \pm 0.1) \times 10^{-6}$	2.6 ± 0.2
2	$(6.6 \pm 0.2) \times 10^{-7}$	$(2.0 \pm 0.2) \times 10^{-7}$	3.3 ± 0.3
3	1.6×10^{-3} (c_{max})	$(5.8 \pm 0.2) \times 10^{-5}$	$> 28 \pm 1.2$
4	$(1.1 \pm 0.1) \times 10^{-3}$	$(3.4 \pm 0.3) \times 10^{-4}$	3.1 ± 0.4
5	1.4×10^{-3} (c_{max})	$(4.3 \pm 0.4) \times 10^{-5}$	$> 32 \pm 3.0$
6	4.4×10^{-4} (c_{max})	$(4.2 \pm 0.9) \times 10^{-5}$	$> 10 \pm 2.3$
SDS	$(9.8 \pm 0.8) \times 10^{-5}$	$(9.8 \pm 3.4) \times 10^{-5}$	1.0 ± 0.4
Ketoprofen	$(1.0 \pm 0.1) \times 10^{-2}$	$(1.9 \pm 0.2) \times 10^{-5}$	544 ± 64

in vivo. In connection with the 10^4-fold higher Kv1.3 blocking potency of **1** and **2** as compared to **4** (Vennekamp et al. 2004) an important step forward was done in designing furocoumarin-derived Kv1.3 blockers with less adverse effects as phototoxicity.

3. Experimental

Methionine was purchased from Aldrich, Ketoprofen was obtained from Bayer, 8-MOP, psoralen, riboflavin and SDS were supplied by Fluka, DMSO Uvasol®, EtOH Uvasol®, MeOH Uvasol®, KH_2PO_4, K_2HPO_4, NaCl, glucose, *N,N*-dimethyl-4-nitrosoaniline and histidine were bought from Merck, Na_2HPO_4 was acquired from Riedel-de Haën, 5-MOP was delivered from Roth, 3-(*N*-morpholino)propanesulfonic acid (MOPS), nitro blue tetrazolium (NBT), poly(deoxyadenylic-thymidylic) acid sodium salt (Poly(dA-dT)-Poly(dA-dT)) and 4,5′,8-trimethylpsoralen were bought from Sigma. **1** and **2** were synthesized according to Vennekamp et al. (2004). Swine blood was gained from a local slaughterhouse. A HP 8845A diode-array photometer fitted with a Julabo F20-C temp. controller was used for photometric determinations. Irradiation was performed with a 150 W Heraeus Fluotest mercury-vapor lamp emitting 365 nm radiation or a 30 W Philips TL 36 D25/09 N sunlamp emitting 320–420 nm with the highest output at 355 nm. Intensities were measured by a Holtkamp UVA meter 360. Buffer solutions were prepared using bidistilled water and filtered with a 0.2 μm sterile filter prior to use. All experiments were performed in triplicate. Results are specified as mean values with standard deviation.

3.1. Absorption spectra

Absorption spectra were recorded using solutions containing 20 μM of the investigated furocoumarin in ethanol.

3.2. Photoproduction of $^1O_2$

$^1O_2$ was determined according to Kraljic and El Moshni (1978). Incubations were prepared in O_2-saturated phosphate buffer (0.01 M, pH 7.0) containing 10 μM of the investigated furocoumarin (solvent 1% methanol), 4 μM *N,N*-dimethyl-4-nitrosoaniline (dye) and 10 mM histidine ($^1O_2$-specific sensitizer). Irradiation was performed for 5 h using a Heraeus Fluotest lamp. The intensity was 3.5 mW/cm^2 at 20 cm distance. Photometric observation at 440 nm began directly after preparation of the solutions and was continued every 30 min during irradiation. Control experiments without furocoumarins were performed.

3.3. Photoproduction of $O_2^{\bullet -}$

The photoproduction of $O_2^{\bullet -}$ was measured according to Beauchamp and Fridovich (1971) and Kirby and Fridovich (1982). Experiments were conducted in phosphate buffer (0.01 M, pH 7.8) consisting of 10 μM examined furocoumarin (solvent 1% methanol), 150 μM NBT (dye) and 500 μM methionine (sensitizer). Irradiation was carried out for 30 min with a Heraeus Fluotest lamp at 37 cm distance resulting in 1.2 mW/cm^2. Absorption at 560 nm was measured immediately after preparation of solutions and after 5, 10, 20 and 30 min of irradiation. Control experiments without furocoumarins were measured as well. Riboflavin was additionally measured as a very strong producer of $O_2^{\bullet -}$ (Pathak and Joshi 1984).

3.4. Crosslinking of poly(dA-dT)-poly(dA-dT)-DNA

The level of crosslinking was quantified by the method of Dall'Acqua et al. (1971). 0.1 U Poly(dA-dT)-Poly(dA-dT) and 10 μM of the examined furocoumarin (solvent 1% DMSO) were dissolved in MOPS buffer (0.1 M, pH 7.2). Subsequently the solutions were irradiated 30 min using a Heraeus Fluotest lamp emitting 3.5 mW/cm^2 at 20 cm distance or kept in the dark for the same time. Heating was applied next at a rate of ca. 1 °C min^{-1} with absorbance (260 nm) and temperature data sampling. Heating was stopped when maximum helix-coil transition was exceeded or when a temperature of ca. 80 °C was reached. If no melting point was achieved due to strong furocoumarin crosslinking by irradiation, the highest temperature was used for calculation of a minimal melting point difference ($>\Delta T_m$).

3.5. Haemolysis of porcine erythrocytes

Photohaemolysis was determined according to the method proposed by Pape et al. (2001), slightly modified. Isolated porcine erythrocytes were suspended in PBS buffer containing glucose (pH 7.4) resulting in a low final test concentration of 5×10^6 cells per ml according to Vedaldi et al. (1988). The compounds were added in different concentrations (solvent 5% DMSO). All incubations were performed in 24-well microtiter plates with a final volume of 1 ml and kept thermostated at 25 °C by a Julabo temp. controller. One microtiter plate was irradiated for 60 min using a Philips Sunlamp emitting 3.0 mW/cm^2 at a distance of 5 cm, followed by a past-irradiation phase of 30 min in the dark. Another microtiter plate was kept for 90 min in the dark. Suspensions without compounds (0% haemolysis) and erythrocytes in bidistilled water (100% haemolysis) were both irradiated and incubated in the dark. Immediately after incubation the suspensions were centrifugated for 5 min in 1.5 ml centrifugation tubes using a Heraeus Sepatech Biofuge 17 S at 3000 rpm (805 × g). In the supernatant, haemolysis was measured photometrically at 416 nm as release of haemoglobin (Vedaldi et al. 1988). H_{50}-values (concentration of 50% haemolysis) for both dark and irradiation incubation were determined of concentration-effect-relationships, and photohaemolysis factors (PHF) were calculated using the ratio of H_{50} in the dark and H_{50} with irradiation. If H_{50} was not determinable for the dark incubation, the highest employed concentration (c_{max}) was applied for calculation of a minimal photohaemolysis factor (> PHF). Photohaemolysis factors can be regarded as a measure of membrane photodamage. SDS (sodium dodecyl sulfate) and ketoprofen were used as negative and positive controls, respectively (Pape et al. 2001).

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