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INVESTIGATIVE REPORT

Photosensitizing Properties of Compounds Related to Benzophenone

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Benzophenone is a phototoxic compound with absorption maxima in the ultraviolet A (UVA) and ultraviolet B (UVB) range. Many benzophenone derivatives are known to be photosensitizing. On the other hand, 2-hydroxy-4-methoxybenzophenone is used as a photoprotective agent. The aim of the present study was to analyse a range of benzophenone derivatives and thus examine the effects of molecular changes in the benzophenone molecule on phototoxic behaviour. Phototoxicity was tested by an *in vitro* photohaemolysis test. The tested compounds were benzophenone itself and the derivatives 2-hydroxybenzophenone, 2-aminobenzophenone, 2-benzoylbenzoic acid, 3-hydroxybenzophenone, and 4-hydroxybenzophenone, as well as the structurally similar compounds 9-fluorenone, 9-fluorenone-2-carboxylic acid, cyclohexyl phenyl ketone, and 1,4-naphtho-quinone. It was shown that minor changes in molecular structure can result in highly different phototoxic characteristics. *Key words: benzophenone; phototoxicity; photohaemolysis test.*

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Chemical substances related to the aromatic ketone benzophenone are known for their photosensitizing properties. Typical derivatives are, for example, ketoprofen, tiaprofenic acid, and fenofibrate, which induce phototoxic reactions (1, 2) as well as DNA damage (3, 4). On the other hand, benzophenone-derived compounds, e.g. 2-hydroxybenzophenone, are used as sunscreening agents with photoprotective effects (5). Benzophenone itself is known to be a strongly phototoxic substance, with an action spectrum in the ultraviolet B (UVB) and ultraviolet A (UVA) region. In the present study we assessed the *in vitro* phototoxic potential of several benzophenone derivatives using a photohaemolysis test. This assay is a commonly used method, using isolated erythrocytes *in vitro*, for measurement of possible phototoxic effects. The aim of this study was to determine to what extent changes in the structure of benzophenone influence phototoxicity.

MATERIALS AND METHODS

Test substances

Tests were performed with the following compounds (see Fig. S1 for chemical structure; available from: http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-1421): benzophenone, 2-hydroxybenzophenone, 2-aminobenzophenone, 2-benzoylbenzoic acid, 3-hydroxybenzophenone, 4-hydroxybenzophenone, 9-fluorenone, 9-fluorenone-2-carboxylic acid, cyclohexyl phenyl ketone, 1,4-naphtho-quinone.

Benzophenone and 2-benzoylbenzoic acid were purchased from Merck (Darmstadt, Germany), all other compounds were obtained from Sigma-Aldrich (Schnelldorf, Germany).

The test substances were dissolved in methanol and further diluted in TCM buffer (NaCl 35.0 g; Tris 15.0 g; KCl 1.5 g; $MgCl_2 \times 6\ H_2O$ 1.0 g; $CaCl_2 \times 2\ H_2O$ 0.75 g; aqua dest. ad 5,000 ml; pH 7.4; 280 mOsm/kg).

UV sources and dosimetry

Irradiations were performed with the following (*i*) UVA-rich or (*ii*) UVB-rich lamps: (*i*) UVASUN 5000 (Mutzhas, München, Germany), emitting in the region of 320–460 nm (maximum approximately 375 nm). Irradiance was 32.5 mW/cm² UVA at a distance of 40 cm (Fig. S2; available from: http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-1421). (*ii*) TL 20 W/12 light bulbs (Philips, Hamburg, Germany) with a main emission range of 275–365 nm (maximum approximately 315 nm). Irradiance was 0.69 mW/cm² UVB, and 0.34 mW/cm² UVA at a distance of 40 cm (Fig. S3; available from http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-1421). UVA or UVB energy fluencies were measured by an integrating instrument (Centra UV-Meter, Osram, Germany), equipped with 2 filtered photodiodes.

Photohaemolysis test

The photohaemolysis test was performed as described previously (6, 7). Briefly, 3 times washed fresh human erythrocytes from healthy donors (with no history of photodermatoses, and no intake of drugs or vitamins) were suspended at a dilution of 1:200 in TCM buffer containing 0.03% human albumin. A 0.4 ml volume of this erythrocyte suspension and a correspondingly prepared erythrocyte-free sample were incubated with 0.1 ml of the test substance preparations at a concentration of 1.0 mmol/l for 30 min at 37ºC in 24-well plates. Both substance-free erythrocyte samples (blanks), as well as samples containing the test substances (including erythrocyte-free controls), were placed 40 cm below the irradiation sources and exposed to 0, 10, 20, 30, 40, 50 or 75 J/cm² UVA (UVASUN 5000) or to 0 (0), 400 (0.20), 800 (0.40), 1600 (0.80) or 2400 (1.2) mJ/cm² UVB (J/cm² UVA) from TL 20 W/12 light bulbs. During irradiation, samples were kept in a shaking bath at 37ºC. Control plates and the plates with shorter irradiation time were wrapped or covered with aluminium foil, but otherwise exposed to the

same conditions. To obtain total haemolysis (100%) erythrocytes were exposed to distilled water. After an incubation period of 30 min in the dark, supernatants were recovered by centrifugation. The released haemoglobin in the supernatants was determined as cyan-methaemoglobin after incubating the samples for 15 min with Drabkin's solution (Sigma-Aldrich, Schnelldorf, Germany). Haemolysis was determined by reading the absorbance at 550 nm with a Microplate reader (Dynatech Photometer MR 700, Dynatech, Denkendorf, Germany) and calculated on the basis of the absorbance data according to the following formula:

$$\text{Haemolysis (\%)} = 100 \times \frac{\text{test sample} - \text{blank} - \text{erythrocyte-free sample}}{\text{total haemolysis} - \text{blank}}$$

In order to exclude equivocal results, only haemolysis >5% was regarded as a meaningful positive finding. Results are given as means of 3 independent experiments with erythrocytes from 3 different donors for all tested compounds, except for benzophenone (2 donors) and 4-hydroxybenzophenone (5 donors).

Absorption spectra of the substances were determined with a spectrophotometer (UV-Visible Recording Spectrophotometer UV 2100; Shimadzu, Tokyo, Japan).

RESULTS

UVA-induced photohaemolysis was caused by 6 out of 10 tested componds (Fig. 1a). Benzophenone caused photohaemolysis rates of about 94% and 9-fluorenone of about 87%. As an example, the dose-response curve of 9-fluorenone is shown in Fig. 2.

UVB-induced photohaemolysis was caused by 4 compounds (Fig. 1b). Benzophenone caused photohaemolysis rates of about 97% and 1,4-naphtho-quinone of about 61%.

Absorption spectra of benzophenone and 1,4 naphthoquinone (Figs S4 and S5; available from: http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-1421) were not predictive for the action spectra of phototoxicity (data not shown).

DISCUSSION

This study showed that minor structural changes in the benzophenone molecule profoundly influence its phototoxic properties. It was shown that –OH, $–NH_2$, or –COOH groups at C_2 or C_3 position of the benzophenone molecule abolish phototoxicity, whereas the –OH group at C_4 only can reduce this effect. The other compounds exerted phototoxic effects to a lesser degree than benzophenone.

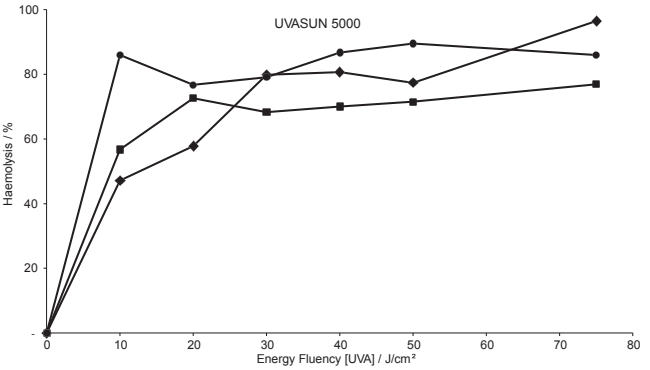

Fig. 2. Haemolysis (percentage of total haemolysis) after incubation with 9-fluorenone (10^{-3} mol/l) after ultraviolet A (UVA)-rich irradiation. Values of experiments with erythrocytes of 3 independent individuals are shown.

During photochemical reactions of benzophenone, an excited state electron of the oxygen atom of the ketone moiety is introduced into an antibonding orbital π*. As a result, the oxygen atom becomes electrophilic and reacts with weak CH groups, leading to elimination of hydrogen. The resulting ketyl radical (BPH•) can react with alkyl radicals and thus benzophenone-2 (2,2',4,4'-tetra hydroxybenzophenone) or benzophenone-3 (oxybenzone) are formed. Conversely, hydrogen transfer benzophenone can also be formed. A second hydrogen elimination leads, via the intermediate BPH•, to the alcohol benzophenone-1. Other current products are the light-absorbing volatile molecules (LAT), which are formed by recombination of 2 BPH• or by reaction of BPH• with alkyl radicals. Under anaerobic conditions the LAT react exclusively with benzophenone, resulting in 2 BPH•. In the presence of oxygen, benzophenone can be formed again from the LAT. When a triplet on a lower level approaches an excited carbonyl an energy transfer is possible. Thus, if the energy acceptor is oxygen, a singlet oxygen can be formed (1).

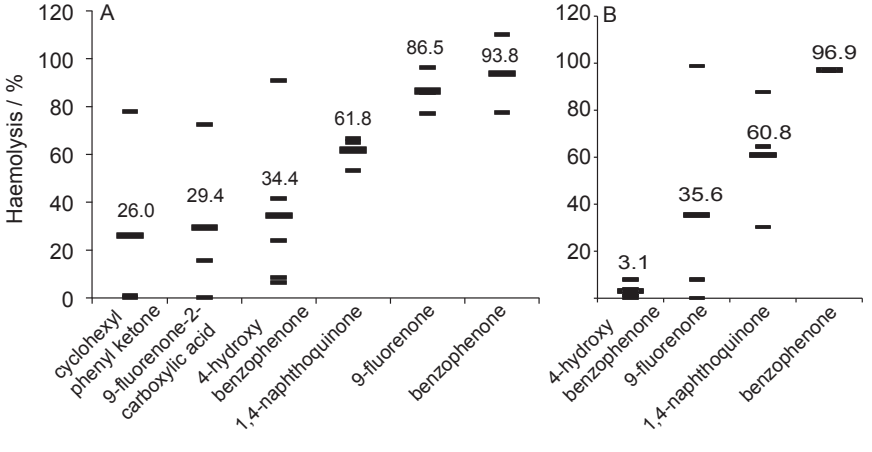

Fig. 1. Means (—) and individual values (—) of photohaemolysis induced by the tested compounds after exposure to (A) ultraviolet A (UVA)-rich irradiation and (B) UVB-rich irradiation. Values from 3 donors are shown, except for benzophenone ($n = 2$) and 4-hydroxybenzophenone ($n = 5$).

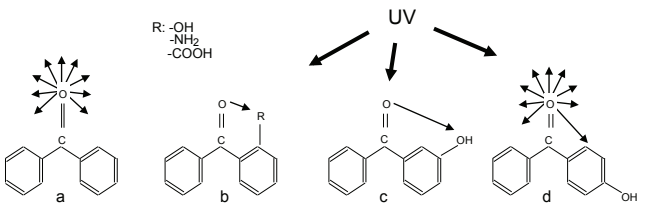

Fig. 3. The oxygen atom in the ketone structure of benzophenone with its radius of action and possible mechanisms for splitting off hydrogen, demonstrated using different tested benzophenone derivatives: (a) benzophenone with the functional radius of the oxygen atom in the ketone structure, in which oxygen can be split off; (b) benzophenone derivatives with different oxygen- or nitrogen-containing functional groups at the C_2 position of the phenol; following activation by ultraviolet (UV) exposure the oxygen atom is able to split off adjoining hydrogen atoms; (c) 3-hydroxybenzophenone, the hydrogen atom of the hydroxyl group is close enough to be split off; and (d) 4-hydroxybenzophenone, the hydrogen atom of the hydroxyl group is outside the functional radius of the oxygen atom, splitting off the hydrogen atom is not possible.

The effect of hydrophilic conditions on the oxidation of linoleic acid, which occurs during photosensitization of benzophenone, was already studied in sodium dodecyl sulphate-micelles (SDS-micelles) (8). The results indicate an involvement of free radicals in this reaction, because it could be inhibited by phenolic antioxidants, such as α-tocopherol, but not by singlet oxygen ($^1O_2$) inhibitors. Therefore the photo-oxidation of linoleic acid by benzophenone was shown to be a type-1 reaction (8), later confirmed by Markovic & Patterson (9). The triplet stage benzophenone molecule eliminates hydrogen atoms from the linoleic acid, resulting in formation of radicals, which react with oxygen.

It is likely that intramolecular transfer of hydrogen from certain substituents of the phenol ring leads to an alteration in the distribution of electrons in the ketone moiety of the benzophenone molecule, which can no longer eliminate hydrogen from components of the cell membrane. This leads to loss of phototoxicity of these substances, through the absence of radical formation and the resulting lack of cell lysis.

If benzophenone loses its phototoxic properties by such intramolecular hydrogen transfer of several groups of the phenol ring to the oxygen atom of the ketone moiety, it is surprising this does not occur in all tested benzophenone derivatives carrying hydrogen-containing residues of the phenyl group. For instance, tests with 2-hydroxybenzophenone and 3-hydroxybenzophenone do not result in photohaemolysis, but the structurally nearly identical 4-hydroxybenzophenone is phototoxic at both tested UV wavelength ranges.

The oxygen atom of the ketone moiety of the benzophenone derivative is only able to eliminate a hydrogen atom from sterically easily accessible groups of the phenol ring. The radius in which the oxygen of the ketone can react with other atoms is 3.1 Å (10) (Fig. 3a). The intramolecular elimination of hydrogen from adjacent OH- and NH- groups at the easily accessible C_2- and C_3-atoms of the phenol ring (Fig. 3b and c) leads to inactivation of the phototoxic effective ketone moiety in 2-hydroxybenzophenone, 2-aminobenzophenone, 2-benzoylbenzoic acid, and 3-hydroxybenzophenone, and, after radiation exposure of these benzophenone derivatives, no haemolysis of erythrocytes is detected. Allergic, but no phototoxic, reactions have been described due to the benzophenone derivatives benzophenone-3 and benzophenone-4, which are also substituted at the C_2 position of the phenyl group. This does not conflict with the hypothesis presented here. The C_4 position of the phenyl group of 4-hydroxybenzophenone is not within a specific radius of the oxygen atom of the ketone moiety. Thus, elimination of hydrogen cannot occur (Fig. 3d). The ketone structure is preserved and the benzophenone derivative can maintain its phototoxic properties.

A limitation of our study is that we used only one *in vitro* phototoxicity test, and that the results of such tests do not always correlate with clinical phototoxicity.

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