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Comparison of the DNA Damage Photoinduced by Fenofibrate and Ketoprofen, Two Phototoxic Drugs of Parent Structure

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ABSTRACT

Fenofibrate and ketoprofen (KP) are two drugs of similar structure derived from that of benzophenone. Both are photoallergic and promote cross reactions in patients. However, the cutaneous photosensitizing properties of KP also include phototoxic effects and are more frequently mentioned. To account for this difference in their *in vivo* properties, their *in vitro* photosensitizing properties on DNA were compared. First, it was shown that under irradiation at 313 nm, fenofibric acid (FB), the main metabolite of fenofibrate, photosensitized DNA cleavage by a radical mechanism similar to that proposed for KP but with a 50 times lower efficiency. Furthermore, FB did not photosensitize the formation of pyrimidine dimers into DNA in contrast to KP, which did promote this type of DNA damage. Their difference in efficiency as DNA breakers was compared to their relative photochemical reactivity and the quantum yield of FB photolysis was found to be eightfold lower than that of KP. The reactivity of these drugs cannot explain alone the difference in their photosensitizing properties. Other factors such as the magnitude of the ionic character of the photodecarboxylation pathway of these benzophenone-like drugs are considered in the discussion.

INTRODUCTION

Fenofibrate is the most frequently prescribed agent in the treatment of hyperlipidemia, with an estimated 6 million patients per year in the world (1). In Europe there are more than 6.5 million patients who have been treated by fenofibrate since 1975 (2). However, fenofibrate promotes adverse reactions. The most frequent side effects of this drug are gastrointestinal, but skin eruptions may occur in 2% of patients (3). These cutaneous effects include pruritus, dry skin, brittle hair, oral lesions, maculopapular eruptions, urticaria, erythema multiforme, a lupus-like syndrome and photosensitivity. Some cases of photoallergic reactions to fenofibrate (3–6) have been reported in humans. This can be correlated to the fact that fenofibrate is phototoxic *in vitro* (7,8) and fenofibric acid (FB)† (Fig. 1) the major metabolite of fenofibrate (9,10), is photolabile (8). The irradiation of FB in aqueous solution induces a photodecarboxylation reaction leading to two photoproducts: 4-chloro-4′-isopropoxybenzophenone and 4-choro-4′-(1-hydroxy-1-methylethyl) benzophenone.

The radicals formed during this photodegradation are very likely at the origin of the photosensitization observed *in vivo, via* the formation of oxidative stress. The behavior is similar to that of ketoprofen (KP) that also has a benzophenone-like structure; however, their therapeutic actions are different because KP is essentially used in the treatment of rheumatoid and osteoarthritis. Ketoprofen is phototoxic after oral absorption and photoallergic after local application (11–13). Upon irradiation, it undergoes a photodegradation reaction (14–16) similar to that of fenofibrate. This cross photoreaction between fenofibrate and ketoprofen was shown in some patients by photopatch tests (4). This observation led us to compare the *in vitro* photosensitizing action with regard to a biological target, of FB, which is water soluble, to that of KP previously reported (17–19). For this purpose, both compounds were irradiated at 313 nm in the presence of DNA. Their relative effectiveness to induce DNA photodamage have been correlated to their rate of photodegradation estimated by HPLC.

MATERIALS AND METHODS

Chemicals. Ketoprofen was purchased from Specia. Fenofibrate, monosodium and disodium phosphate, Tris, mannitol, *tert*-butanol, sodium benzoate, 5,5-methyl-1-pyrroline-1-oxide (DMPO) and ethidium bromide, superoxide dismutase (SOD), and catalase were purchased from Sigma. Fenofibric acid was obtained by saponification of fenofibrate by NaOH followed by precipitation in the presence of H_2SO_4. Supercoiled ΦX174 DNA (molecular weight 3.6 ×

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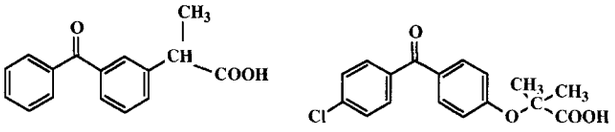
†*Abbreviations:* D, number of pyrimidine dimers per mole of DNA; DMPO, 5,5-dimethyl-1-pyrroline-1-oxide; ESR, electron spin resonance; FB, fenofibric acid, 2-[4′-(4-chlorobenzoyl)phenoxy]-2-methyl-propanoic acid; KP, ketoprofen, 2-(3-benzoylphenyl) propionic acid; NSAID, nonsteroidal antiinflammatory drug; S, number of single-strand breaks per mole of DNA; SOD, superoxide dismutase; SSB, single-strand breaks.



Ketoprofen (KP) **Fenofibric acid (FB)**

Figure 1. Chemical structures of FB and KP.

10^6 Da, 5386 base pairs) form I was purchased from Pharmacia. The DNA was used after dilution in 5 m*M* phosphate buffer, pH 7.4, containing 10 m*M* NaCl so that the concentration of the solution was 14 n*M* in DNA molecules or 75.4 μ*M* in base pairs. The amount of contaminant form II was checked by agarose gel electrophoresis followed by microdensitometry and was less than 10%. No form III was detected in the starting material. Electrophoresis-grade agarose was obtained from Touzart et Matignon (Vitry sur Seine, France). The DNA concentration (mg) was determined by UV absorption spectroscopy using a conversion factor of 21 absorbance units/mg of DNA. The DNA concentrations in base pairs were determined spectrophotometrically at 260 nm using an extinction coefficient of 13 200 M^{-1} cm^{-1} (20). Phage T4 endonuclease V was prepared according to the procedure of Lommel and Hanawalt (21). Endonuclease activity checked before use was 4.3×10^{12} sites/μL/min. All solutions were prepared in phosphate buffer (5 m*M*, 10 m*M* NaCl, pH 7.4) using bidistilled water.

Irradiation procedure. Samples were prepared by mixing 5 μL of 14 n*M* DNA (75.4 μ*M* bp), 5 μL of drug solution at a fixed concentration in phosphate buffer and 10 μL of phosphate buffer. The sample containing DNA alone was prepared from 5 μL of 14 n*M* DNA and 15 μL phosphate buffer. The mixture was placed in 3 mm internal diameter glass tubes and incubated for 20 min in the dark. In the quenching experiments, the scavengers were dissolved in phosphate buffer, pH 7.4 (5 m*M*, 10 m*M* NaCl). Five microliters of the solution of scavengers replaced 5 μL of phosphate buffer in the samples so that the final concentration of the scavenger was 50 m*M* for mannitol, 100 m*M* for sodium benzoate, 4 vol% for *tert*-butanol, 22 U/mL for SOD and 20 U/mL for catalase. For the experiments in deaerated conditions, the starting solutions were first bubbled with argon and the tubes containing the mixture were then flushed with argon and capped. All concentrations given in the text are the final concentrations in the tubes. The FB experiments used drug concentrations varying from 0.001 to 1.25 m*M*; for KP only a drug concentration equal to 0.2 m*M* was used. The solutions were irradiated at 313 nm for various periods of time with a Muller reactor device equipped with a 200 W high-pressure mercury lamp (Osram), a water-cooling filter and an interference filter (Oriel 313FS 10–50 12% transmission at 313 nm, bandwidth 10 nm). The energy was monitored with an EGG gamma radiometer–photometer system. The power received by the samples was $\sim 8 \times 10^{-4}$ W/cm^2. The molar extinction coefficient at 314 nm in phosphate buffer was found to be 900 M^{-1} cm^{-1} for KP and 10 700 M^{-1} cm^{-1} for FB.

Photosensitized DNA cleavage experiments. After irradiation, 5 μL of a mixture containing 250 m*M* HEPES, pH 7.45, 75% glycerol and 0.05% bromophenol blue were added to the irradiated solution. The sample was then analyzed by electrophoresis on a 0.8% agarose horizontal slab gel in Tris borate buffer, and quantification of the various forms of DNA (I, II, III) was performed as described by Artuso *et al.* (18) The number of single-strand breaks (SSB) per mole of DNA (S) generated by photosensitization was calculated from the relative percentage of forms I and II, assuming a Poisson distribution and using the formula $S = \text{Ln } C/C_o$, where C_o is the initial concentration of DNA in form I and C the concentration of form I after irradiation. A coefficient of 1.66 was used to correct the lower efficiency of ethidium bromide binding to DNA to form I with respect to forms II and III. The quantum yield of the photosensitized formation of SSB was calculated from the total number of DNA breaks per second and the number of photons absorbed during the same time by the drug in the sample. The rate of SSB formation was evaluated using only the linear part of the curve S = f(t) to minimize the possible perturbation induced by the photodegradation of the drug and the occurrence of inefficient cleavage or dimerization reactions on form II. The values of quantum yields of cleavage obtained by this method were slightly underestimated because this reaction, occurring on form II and not leading to form III, was not taken into account.

Photosensitized dimerization experiments. At the end of the irradiation, DNA was precipitated by the addition of 100 μL of cold ethanol and 2 μL of 3 *M* sodium acetate buffer (pH 5.5) to the irradiated mixture. The samples were stored at −20°C for an hour and centrifuged for 45 min (10 000 rpm). The residue was washed in 70% ethanol, centrifuged and dried under vacuum. Twenty microliters of diluted phage T4 endonuclease V in tris-acetate buffer (pH 8) were added to the residue, and the solution was incubated for 30 min at 37°C. Proteins were removed from the mixture by washing with chloroform–phenol–isoamyl alcohol. The samples were further treated by the same procedure as used for the cleavage experiments. Controls used the same procedure without endonuclease and the residue was dissolved in 20 μ*M* phosphate buffer. The number of dimers per mole of DNA (D) in presence of FB could be evaluated from the number of SSB detected after treatment by phage T4 endonuclease V, after subtraction of the number of SSB obtained before treatment.

Photolysis experiments. One hundred microliters of FB or KP (1 m*M*) phosphate-buffered solutions were irradiated under aerobic conditions at room temperature, at 313 nm in glass tubes (3 mm diameter) with the same irradiation device as that described above (200 W high-pressure mercury lamp, Oriel 313FS interference filter) for irradiation times varying from 7 to 60 s. Each sample was then analyzed by HPLC using a Waters-Millipore 510 apparatus equipped with an analytical Waters μBondapack column (125 Å, 10 μ*M*). The eluent was a water/acetonitrile 57/43 mixture; the flow rate was 1.5 mL/min. The eluate was monitored by following the absorbance at 260 nm with a Waters UV detector. An external calibration was performed for each compound in the same concentration range (10^{-3}–10^{-4} *M*) and used for the determination of the amount of nonphotolyzed KP or FB. The percentage of conversion given in the text corresponds to the mean value of three determinations.

Electron spin resonance (ESR) spectra were obtained with a Bruker EFP 300e spectrometer (band X at 9 Ghz). An ESR pyrex capillary (50 μL) filled with a phosphate-buffered solution of an FB (10^{-1} *M*) DMPO (10^{-3} *M*) mixture was introduced into the cavity and irradiated at room temperature with an Oriel apparatus equipped with a 200 W high-pressure mercury lamp and a quartz optic fiber.

RESULTS

FB photosensitization of SSB

Phage ΦX174 DNA was irradiated at 313 nm in the presence or absence of FB (0.2 m*M*) in phosphate-buffered solution. The formation of SSB was followed by gel electrophoresis and quantified by densitometry. The results are shown in Fig. 2. The number (S) of SSB is not significant regardless of irradiation time when DNA is irradiated alone. The value of S increases with irradiation time when the DNA is photosensitized by FB. The quantum yield of SSB (ΦSSB) induced by FB at 313 nm is 2×10^{-6}. The influence of different parameters on the number of photoinduced breaks were studied: absence or presence of oxygen, FB concentration and addition of specific scavengers. The irradiation induces more SSB in deaerated solution than in aerated solution (Fig. 2). The rate of cleavage in the absence of oxygen is 3.5-fold higher than in the presence of oxygen at the beginning and only 1.4-fold higher when the irradiation time increases.

The efficacy of DNA photocleavage in aerobic conditions was found to be FB concentration dependent: The number of SSB obtained for an irradiation time of 90 s increases when the concentration varies from 0.001 to 0.2 m*M*. The

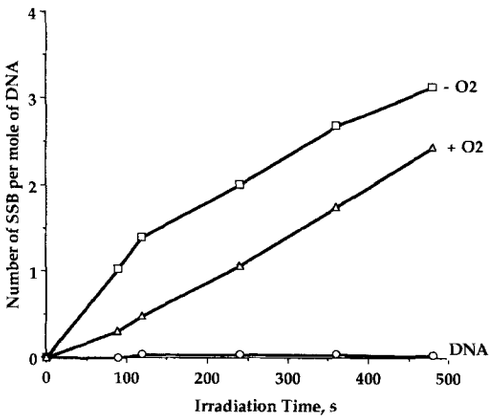


Figure 2. Variation in the number of SSB per mole of DNA (S) photoinduced by FB (0.2 m*M*) irradiated at 313 nm in aerated (—△—) or deaerated (—□—) phosphate-buffered solution as a function of the irradiation time.

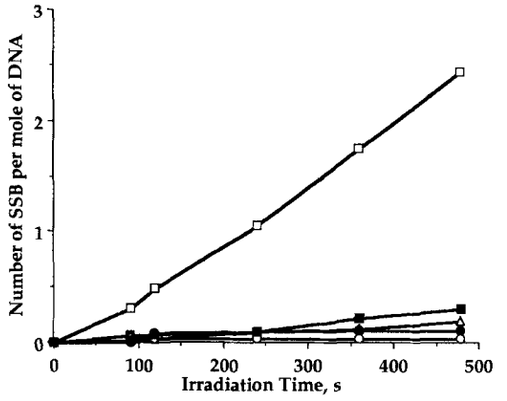


Figure 4. Influence of mannitol (50 m*M*), *tert*-butanol (4 vol%) and sodium benzoate (100 m*M*) on the number of SSB induced at 313 nm by FB (0.2 m*M*): FB alone (—□—), FB + mannitol (—■—), FB + *tert*-butanol (—△—), FB + sodium benzoate (—●—). Control sample: DNA alone (—○—).

number of SSB reaches a maximum at 0.2 m*M* concentration and then decreases when the concentration increases up to 1.25 m*M*. Because FB at 0.2 m*M* absorbs 97% of the light at 313 nm, this decrease may be explained, in the absence of stirring, by an inner filter effect. As can be seen in Fig. 3 the quantum yield of SSB decreases drastically from 6.2 $\times 10^{-5}$ to 6.6 $\times 10^{-7}$ when the concentration increases from 0.001 to 0.2 m*M*.

Different radical scavengers were added to a mixture of FB (0.2 m*M*)/DNA: mannitol (50 m*M*), *tert*-butanol (4 vol %), sodium benzoate (100 m*M*), SOD (22 U/mL) and catalase (20 U/mL). These various scavengers decreased the number of SSB induced by FB (Fig. 4). DNA cleavage was almost completely inhibited by mannitol, *tert*-butanol or sodium benzoate but was poorly modified by catalase or SOD. These results show that the mechanism of DNA cleavage photosensitized by FB may involve mainly hydroxyl radicals. The roles of superoxide anion or H_2O_2 are negligible.

The presence of hydroxyl radicals in the medium was confirmed by ESR studies. These ESR experiments, using DMPO as spin trap, were performed during FB photolysis in the absence of DNA. The characteristic spectrum of the DMPO-OH adduct (a_N = 14.9 G, a_H = 14.9 G) was obtained with a low intensity. The 5,5-dimethyl-2-pyrrolidone 1-oxyl radical that may result from the oxidation of DMPO by singlet oxygen (a_H = 4.1 G, a_N = 7.2 G) was not detected.

FB photosensitization of pyrimidine dimers

In order to detect the formation of pyrimidine dimers, the irradiated solution was treated by the phage T4 endonuclease V that cuts DNA at dimer sites. The number of SSB formed on DNA in the presence of FB (0.2 m*M*) was increased by this treatment. The number of SSB corresponding to pyrimidine dimers formed in the presence of FB was obtained by subtracting the SSB observed before treatment (Fig. 5). The DNA alone that was not broken under irradiation at 313 nm underwent a dimerization reaction on the pyrimidine sites. The number of dimers formed on the DNA was lower in the presence of FB than in its absence indicating that FB does not photosensitize the formation of pyrimidine dimers and

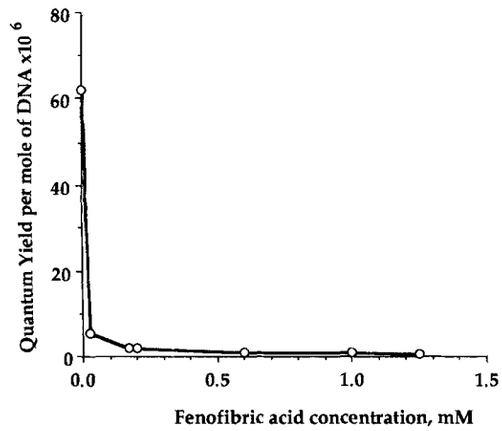


Figure 3. Dependence the quantum yield of SSB per mole of DNA on the FB concentration.

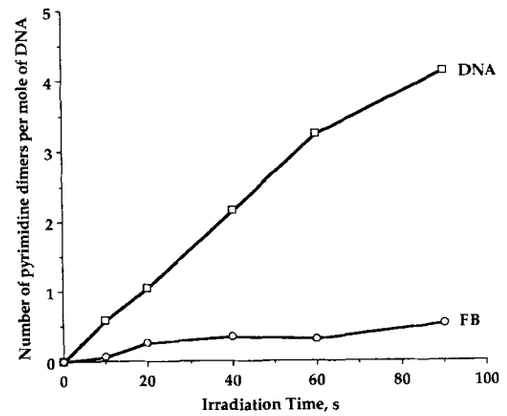


Figure 5. Variation of the number of pyrimidine dimers per mole of DNA photoinduced by FB (0.2 m*M*) as a function of the irradiation time.

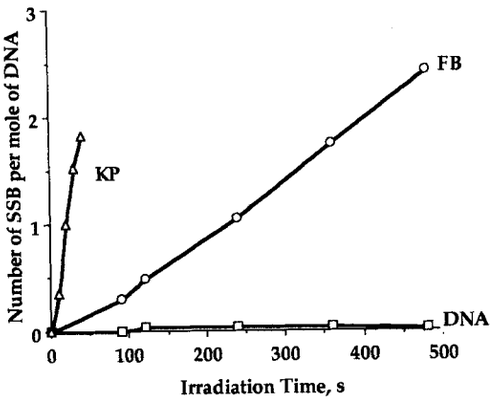


Figure 6. Comparison of the DNA cleavage photosentitized respectively by KP (—△—) and FB (—○—) in phosphate-buffered solution (0.2 m*M*). Control sample: DNA alone (—□—).

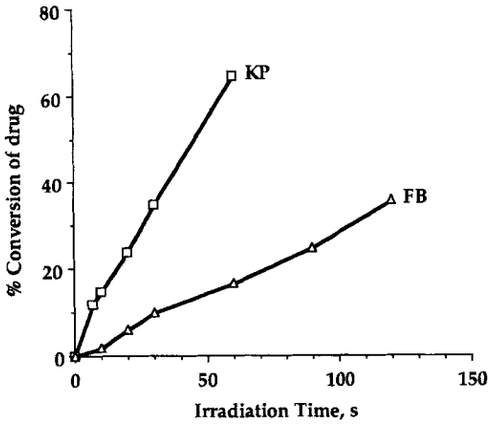


Figure 7. Comparison of the rate of photolysis of KP (—□—) and FB (—△—) at 313 nm for solutions of same concentration (1 m*M*). Yields are relative to photodegraded drug and constitute mean values of at least three experiments.

even has a photoprotective effect with regard to this DNA damage.

Pyrimidine dimer formation was no longer in evidence for higher FB concentrations (1 m*M* and 1.25 m*M*). The inner filter effect, mentioned previously to explain the decrease in SSB for FB concentrations up to 0.2 m*M*, may also be at the origin of this decrease dimer formation.

Comparative study of DNA photosensitization by FB and KP

The DNA cleavage was then photosensitized simultaneously by FB and KP (0.2 m*M*) in the same conditions (Fig. 6). The FB appears significantly less efficient than KP in inducing SSB under these conditions: the quantum yield of SSB (evaluated from the initial rate) at 313 nm is 50 times more elevated for KP (1×10^{-4}) than for FB (2×10^{-6}).

Comparative study of FB and KP photolysis

The mechanism of photosensitization of SSB by KP as well as FB is essentially a radical mechanism. Thus, it was of interest to compare the quantum yield of DNA cleavage to the quantum yield of photodecarboxylation of these two drugs under aerobic conditions at the same wavelength. Phosphate-buffered solutions of FB and KP (1 m*M*) were irradiated at 313 nm in the absence of DNA and their photodegradation followed by HPLC. The photodegradation of FB gave rise to two photoproducts, 4-chloro-4′-isopropoxybenzophenone and 4-choro-4′-(1-hydroxy-1-methylethyl)benzophenone, the structures of which were determined from their UVA spectrum previously described by Miranda *et al.* (8). The percentage of photodegradation varies linearly with the irradiation time (Fig. 7). The comparison between FB and KP shows that for the same concentration, the rate of photodegradation of KP is 3.5 times greater than that of FB, which corresponds in our conditions to a quantum yield of photodegradation 8 times higher for KP than for FB.

Here, we found that the photoreactivity of KP was still significantly higher than that of FB but in far lower proportions than that previously observed in the case of DNA photosensitization.

DISCUSSION

These findings show that FB irradiated at 313 nm photosensitizes the formation of SSB that are not observed when DNA is irradiated alone at this wavelength. The number of SSB, S, increases with irradiation time and with concentration as long as the concentration is weaker than approximately 0.2 m*M*, where nearly all the irradiation light is absorbed by the FB. The decrease of S for upper FB concentration may be attributed very likely to an inner filter effect. The quantum yield of cleavage, ΦSSB, decreases drastically when FB concentration increases. A comparison between the efficiency of FB and KP as DNA breakers shows that FB photosensitizing efficiency is far weaker than that of KP. The quantum yield of SSB initiated by KP (10^{-4}) at 313 nm is 50-fold stronger than that induced by FB (2×10^{-6}) for the same concentration (0.2 m*M*) and for same irradiation conditions.

The DNA cleavage photosensitized by FB in the presence of air was shown to be due mainly to the involvement of hydroxyl radicals. The ESR experiments confirmed that such radicals are formed during the irradiation of FB. Hydroxyl radicals are known to be efficient DNA breaker agents (22) as opposed to superoxide anion or singlet oxygen. Their participation in DNA cleavage is in concordance with the significant value of the quantum yield but does not exclude the possibility that other pathways may have occurred either from FB or from their photoproducts in their excited state. The same mechanism involving $OH^{\bullet}$ radicals has been proposed for DNA cleavage mediated by KP (18) and various other nonsteroidal antiinflammatory drugs (NSAID) (18,23,24).

Hydroxyl radicals must be formed from a radical process participating in FB and KP photolysis. Usually photodecarboxylation reactions go through a homolytic process. However, in the case of KP, the participation of an ionic mechanism was postulated to explain the formation of certain photoproducts (16) and recently confirmed by Monti *et al.* (25) and Martinez and Scaiano (26) on the basis of flash photolysis experiments. The unusual participation of a pho-

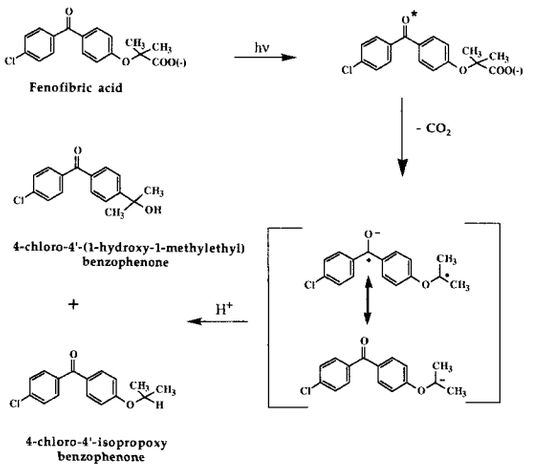


Figure 8. Postulated mechanism of the photodecarboxylation of FB.

tochemical ionic mechanism seems to be favored by the benzophenone-like structure of these compounds (27). A scheme similar to that established by Monti and Scaiano for the photodegradation of KP may be proposed for FB, with different steps that are mainly (1) an intramolecular electron transfer between the excited carboxylate and the carbonyl of the benzophenone moiety that is known for its electroaffinity, (2) a decarboxylation reaction that leads to a biradical more stable in its carbanionic form and (3) a protonation of this carbanion giving rise directly then to a reduced product and to a carbinol after rearrangement (Fig. 8). The participation of a carbanion, shown by flash photolysis experiments in the case of KP, is strongly supported in the case of FB by the formation of a carbinol resulting from a Wittig rearrangement (8).

The relative ratio of the ionic and radical pathways in KP and FB photolysis may explain the difference in their photosensitizing efficiency. The radicals responsible for DNA photocleavage may be formed from different minor radical processes. Free radical species have also been invoked to account for FB-photoinduced peroxidation of linoleic acid or FB-photosensitized lysis of erythrocyte membranes.

The difference between the maximum absorption wavelength of both compounds indicates that FB (λ_{max} = 292 nm) has a more conjugated structure than KP (λ_{max} = 260 nm). This might be in connection with the lower efficiency of FB photodecarboxylation and consequently the lower efficiency of the FB-induced DNA cleavage. The energy of the triplet states of both compounds can be assumed to follow the same relative order as their singlet states. The energy transfer between KP and the DNA pyrimidines, which is responsible for the formation of thymine dimers (28), is not very efficient due to the low energy of the triplet state of KP (69 kcal/mol) (19). The absence of thymine dimers by FB photosensitization may result from a lower energy or the short lifetime of its triplet state.

In conclusion, it is clear from the results reported here that fenofibrate is a less efficient *in vitro* photosensitizer than KP. This correlates with the clinical differences in the photosensitized reactions observed in patients because fenofibrate *via* FB promotes only photoallergic reactions, whereas KP induces phototoxic and photoallergic reactions.

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