The photomutagenicity of fluoroquinolones in tests for gene mutation, chromosomal aberration, gene conversion and DNA breakage (Comet assay)

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The ability of fluoroquinolones to cause light-induced adverse effects has been established in experimental studies and clinical observations. The formation of active oxygen species appears to be responsible for this activity. Photomutagenicity tests with bacterial, lower eukaryotic and mammalian cells were performed with three fluoroquinolones (Fleroxacin, Ciprofloxacin and Lomefloxacin). After concomitant irradiation with simulated solar light (with a reduced UVB component), weak increases in the number of revertants were observed in Salmonella typhimurium TA104 and TA100. No photomutagenic activity was detected in Saccharomyces cerevisiae D7. In the chromosomal aberration (CA) test with Chinese hamster V79 cells the number of aberrant metaphases was markedly increased. In the Comet assay with mouse lymphoma cells, evidence of extensive DNA breakage was obtained. All three compounds showed similar potencies in the Comet and Ames assays while there was a clear gradation of potencies in the CA assay (Lomefloxacin > Fleroxacin > Ciprofloxacin), which conformed with reports on the relative potencies regarding phototoxicity. The oxygen radical scavengers catalase, superoxide dismutase and N,N'-dimethylurea modulated the photoclastogenicity and phototoxicity but had no influence on the effects in the Comet and Ames tests. It thus appears that different kinds of mechanism are responsible for toxicity and clastogenicity on the one side and DNA breakage and gene mutation on the other side. Pre-irradiation of the test articles did not lead to enhanced genotoxicity, indicating the involvement of very short lived genotoxic agents. The results endorse the advice to avoid excessive light exposure during antibiotic therapy with fluoroquinolones.

Introduction

The fluoroquinolones are second-generation antibiotics derived from nalidixic acid. They are employed very successfully in the treatment of a broad spectrum of infectious diseases. Fluoroquinolones are potent mutagens in bacteria. Their activity depends on interaction with the bacterial gyrase (topoisomerase II) enzyme. Effects in mammalian cells are generally much reduced or absent. Several reviews on the mutagenicity and carcinogenicity of fluoroquinolones and mammalian topoisomerase-interactive agents have been published recently (Holden et al., 1989; Fort, 1992; Anderson and Berger, 1994; Ferguson and Baguley, 1994; Albertini et al., 1995).

In the early 1970s, nalidixic acid was reported to cause phototoxic reactions in clinical practice. More recently the phototoxicity of the fluoroquinolones has been established in a number of experimental studies and in some clinical case reports (Wagai and Tawara, 1992; G.Klecak et al., unpublished data). Cutaneous photoreactions are evoked when a photosensitizing drug, usually absorbing in the UVA range, is present in sufficient amounts in the skin and exposure to light from the sun or from artificial sources induces the formation of reactive molecules, causing damage to cellular components. Exaggerated sunburn, scaling and blisters may be experienced.

The photochemistry of the fluoroquinolones indicates that, upon irradiation with UVA, active oxygen species (AOS) are formed. These molecules (-O2-, H2O2, OH and singlet oxygen) are generated in a variety of endogenous or exogenous processes. They are known to be capable of damaging cellular components, including DNA (Levin et al., 1982; Ames et al., 1993). In particular, these molecules have been shown to produce clastogenic effects (Philips et al., 1989; Cantoni et al., 1994; Youssef et al., 1994; Duell et al., 1995). Therefore it was of interest to investigate the photomutagenic potential of the fluoroquinolone antibiotics. Apart from providing evidence for the clastogenic potential, we report on the ability of the test articles to induce mitotic conversion in yeast, mutations in the Ames test and DNA breakage in mouse lymphoma cells under concurrent irradiation with UV light. An assessment of risk regarding photocarcinogenicity is attempted by comparing the findings to results obtained with the exemplary photomutagen 8-methoxypsoralen.

Material and methods

Chemicals and irradiation source

The chemical structures of the three fluoroquinolones tested, together with their absorption spectra, are shown in Table 1. The scavengers superoxide dismutase and N,N'-dimethylurea were obtained from Fluka (Switzerland) and catalase from Boehringer Mannheim (Germany).

The SUNTEST CPS accelerated exposure machine (Heraeus, Germany) has an emission spectrum between 250 and 800 nm. As the radiation source the machine contains a xenon arc with special filter devices so that the spectral composition simulates solar radiation. In order to reduce the intrinsic mutagenic action of the irradiation we passed the light through a 3 mm glass window pane (Ames test) or through the covers of the plastic cell containers [yeast, chromosome aberration (CA) and Comet tests], which completely or partially removes the UVB component. For detailed emission spectra and UV dose determinations, see Chételet et al. (1993a). The exposure rates (UVA) were set to 0.5 mW/cm2 for the Ames and Comet assays, 0.4 mW/cm2 for the CA assay and 0.65 mW/cm2 for the yeast assay, as measured with an RM2 UV-meter (Dr Gröbel GmbH, Germany).

Photomutagenicity tests

Ames test. The Salmonella tester strains TA100, TA102 and TA104 were obtained from B.N.Ames (University of California, Berkeley, CA) and are described elsewhere (Ames et al., 1975; Levin et al., 1982; Maron and Ames, 1983). Overnight cultures (20 ml) were inoculated from frozen aliquots containing 10% dimethylsulphoxide. Strain TA102 was grown with 0.3 μg tetracycline per ml nutrient broth (NB) medium to ensure an adequate copy number of the pAQ1 plasmid (Albertini and Gocke, 1988). The overnight cultures were centrifuged and resuspended in an equal volume of cold phosphate buffer. For irradiation 1 ml of the cell suspension was pipetted into plastic Petri dishes (diameter 5 cm). Phosphate buffer, test compound and...
Scavenger solutions (where appropriate) were added to give a final volume of 5 ml. During irradiation the suspensions were stirred continuously. After irradiation the suspensions were kept at room temperature (22°C) in the dark to reach a total incubation time of the cells with the fluoroquinolones of ~60 min. Thereafter 1.5 ml of the irradiation mix was transferred to Eppendorf incubation tubes centrifuged in a Sigma 2MK centrifuge at 10 000 r.p.m. for 5 min, washed once and resuspended in 1 ml phosphate buffer. Next, 300 µl of cell suspension, 200 µl of his/bio solution and 100 µl of NB medium were pipetted onto each plate. The further handling of the plates was as for the standard plate incorporation method (Maron and Ames, 1983). Positive controls were sodium azide (TA100), MMC (TA102) or methylglyoxal (TA104) in the absence of irradiation, and 8-methoxypsoralen (8-MOP) (TA102) in its presence.

Saccharomyces cerevisiae D7 test. Cells were obtained from F.K. Zimmermann (TU. Darmstadt. Germany). Photomutagenicity tests were performed as described elsewhere (Chetelat et al., 1993a). Briefly, yeast cells were irradiated in tissue culture clusters (24 wells from COSTAR, Cambridge, MA). Incubation samples were prepared with 980 µl of cell suspension in potassium phosphate buffer) and 20 µl of test substance, reference substance, solvent or placebo. The cells were immediately exposed to irradiation and stirred continuously. After treatment, aliquots of the cell suspensions were pipetted into soft agar.
The spontaneous level of mutant colonies of strain TA104 is ~470 colonies per plate. Control experiments showed that exposure to solar simulated light, filtered through a glass pane to cut off the UVB component, caused at most a marginal increase, if any. Exposure to Floxacin and Lomefloxacin in the absence of irradiation had a more definite but still weak effect (maximal increase to ~700 colonies per plate). Ciprofloxacin was more toxic, which did not allow for the recognition of this ‘dark’ effect at the concentrations shown here.

The cells were exposed to the three fluoroquinolones at concentrations of 10, 31.6 and 100 μg/ml and concomitantly irradiated with UVA doses of 90, 300 and 600 mJ/cm² (3, 10 and 20 min of irradiation). Under these conditions all three compounds caused increasing numbers of mutant colonies compared with the respective controls without test compound and/or without irradiation (Figure 1). Concomitant irradiation increased the mutant values to maximally 927 for Floxacin (100 μg/ml, 600 mJ/cm²), 1159 for Lomefloxacin (100 μg/ml, 600 mJ/cm²) and 1063 for Ciprofloxacin (10 μg/ml, 300 mJ/cm²); toxicity reduced the mutant values at higher Ciprofloxacin and UV doses.

The three fluoroquinolones caused marginal photomutagenetic effects in strain TA100 (data not shown). The mutant values were increased by a factor of <2. Ciprofloxacin was also studied in strain TA102. Because of the intrinsic genotoxicity of the gyrase inhibitors in this strain only concentrations of up to 0.32 μg/ml were usable. No additional increase of the mutant numbers was induced by the concomitant light exposure (data not shown), which provides evidence that UV exposure does not enhance the gyrase-mediated genotoxicity (by enhancing the formation or the stability of the cleavable complex).

We evaluated whether the mutagenic action of the fluoroquinolone photoproducts in strain TA104 could be modulated by addition of scavengers (catalase, which inactivates H₂O₂, 1,4-di- 
N, N'-dimethylthiourea, which acts predominantly as an scavenger, and superoxide dismutase, which converts \(O_2^-\) to \(H_2O_2\)). The experiments were performed with Ciprofloxacin and Lomefloxacin because they had caused more definite photomutagenetic effects in the Ames assay. However, as seen in Figure 2, there was no difference in the effect when the three scavengers were used.

**Lower eukaryotic system (Saccharomyces cerevisiae D7)**

For the evaluation of the photomutagenetic potential of the three fluoroquinolones, gene conversion was selected as the genetic endpoint. Earlier investigations with chlorpromazine and 8-MOP had shown that conversion is an appropriate endpoint for measuring photomutagenic effects (Chetelat et al., 1993a). Cells were treated with different concentrations of the three fluoroquinolones (50, 200 and 400 μg/ml) and UV doses of 3800/75 and 7600/150 mJ/cm² (UVA/UVB component). The data indicate induction of gene conversion at the trp5 locus by UV light alone (a factor of 3–4 above non-irradiated control). Addition of the fluoroquinolones did not increase the frequencies of convertants above the corresponding irradiated controls (Table II).

**CA test**

Exposure to the fluoroquinolones and concomitant irradiation with UVA doses of up to 500 mJ/cm² produced a clear concentration-related increase in the frequency of aberrant cells as well as of the number of aberrations per cell in the dose range evaluated (6–200 μg/ml at a UVA dose of 500 mJ/cm²; Figure 3). Statistically significant increases in the frequency of aberrant cells were seen at concentrations of 6.5 μg/ml for Lomefloxacin and 13 μg/ml for the other two
compounds. The concentrations at which more than one aberration per cell was observed were 25 μg/ml for Lomefloxacin, 100 μg/ml for Fleroxacin and 200 μg/ml for Ciprofloxacin. For Fleroxacin and Lomefloxacin the frequency of aberrant metaphases decreased at high concentrations probably due to cell cycle delay.

We evaluated whether the mutagenic action of the fluoroquinolone photoproducts could be modulated by addition of scavenger compounds such as catalase, DMTU or SOD. The experiments were performed at concentrations of 13 and 25 μg/ml of each of the fluoroquinolones. All three scavengers, at all concentrations, significantly reduced the frequency of aberrant metaphases as well as the number of aberrations per cell. The effect was more apparent for Ciprofloxacin and Fleroxacin at relatively lower aberration frequencies than for Lomefloxacin at a high level of damage where multiple aberrations are more common (Figure 4).

Comet assay
In the absence of UV irradiation, the fluoroquinolones did not induce DNA damage in mouse lymphoma cells as visible in the Comet assay (Figure 5A). When the cells were concurrently irradiated, a dose-dependent shift to cells with more and more extensive comets was apparent (Figure 5B shows a representative experiment with Fleroxacin). Comet length was not a good parameter for quantification of the damage, so we classified the cells into the four categories described in Materials and methods. UV irradiation alone caused only a marginal shift from class 1 to class 2; at Fleroxacin concentrations of 3 and 10 μg/ml most cells were in class 2, at 30 μg/ml most cells were in class 3 and at 100 μg/ml most cells were in class 4. Data on the relative potencies of the three test compounds in this test did not conform with the results obtained in the CA assay, but rather with the results in the Ames test. In Figure 6, the fraction of heavily damaged cells (classes 3 and 4) is plotted as a function of dose. Fleroxacin seems to produce slightly less photo-damage than either Ciprofloxacin or Lomefloxacin.

The scavengers CAT, SOD and DMTU did not modulate the effects in the Comet assay, as shown in Figure 7. No obvious change in the fraction of heavily damaged cells compared with the level in absence of scavengers was seen.

Pre-irradiation of the fluoroquinolones
Solutions of the three fluoroquinolones were irradiated with a UVA dose of 600 mJ/cm² (for the Ames test) or 200 mJ/cm² (for the CA test) and immediately afterwards added to the bacteria (strain TA104) or the V79 cells. No increases in the number of revertant colonies or chromosomal aberrations were apparent (data not shown) when compared with the non-irradiated samples. This observation indicates that the genotoxic agents are very short lived, as expected for AOS.
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Discussion

The phototoxic action of fluoroquinolones has been attributed to the UVA-induced production of AOS (Wagai and Tawara, 1992). These molecules (O$_2^-$, H$_2$O$_2$, OH and singlet oxygen) are known to be damaging to cellular components, including DNA (e.g. Ames et al., 1993). Of the standard Ames tester strain set, TA100 is known to be weakly responsive to the oxidative agent is much lower when compared, for example, with the clastogenicity tests: Kaneko et al., 1994). The sensitivity of _S.cerevisiae_ (Brennan et al., 1988). More recently Brennan and co-workers reported that AOS significantly increase the frequency of interchromosomal recombination in _S.cerevisiae_ (Brennan et al., 1994). The sensitivity of the assay towards the oxidative agent is much lower when compared, for example, with the clastogenicity tests: Kaneko et al. reported a sizable increase in gene conversion at 50 mM

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Percentage of survival on synthetic complete medium and frequency of colonies grown on trp-less medium per 10$^6$ cells (convertants) calculated from the values shown in the Appendix Tables. Abbreviations: surv.: survival; Conv.: convertant; Conc.: concentration; Chlorpro.: chlorpromazine; Plac.: placebo.
reduced the photoclastogenic effect of irradiated fluoroquinolones. In our experiments, we also observed a protective effect by superoxide dismutase (SOD), which converts $\text{O}_2$ to $\text{H}_2\text{O}_2$. This is in contrast to the study on phototoxicity of the fluoroquinolones, where SOD was reported to increase toxicity (Wagai and Tawara, 1992), and the findings of Duell et al. (1995), who report an enhancement of the clastogenicity of the xanthine/xanthine oxidase system by SOD. It is, however, in line with the findings of Emerit and others (see Emerit, 1994), who observed protection by SOD against the clastogenic action of a variety of oxidative agents, including, for example, the xanthine/xanthine oxidase system. Apparently, some as yet undefined culture conditions are responsible for the divergent effects of SOD, and additional factors such as adaptive response of the cell lines and pre-existing resistance to, for example, $\text{H}_2\text{O}_2$ oxidative stress in V79 variants may also play a role (Cantoni et al., 1994). The observation that the exogenously added enzymes CAT and SOD, which do not enter the cells, are protective against the photoclastogenic effects of the fluoroquinolones suggests that the reactive AOS responsible for the effects are either long lived enough to travel from the medium to the nucleus or that indirect effects, e.g. damage to the membranes, are mediating the clastogenicity. Formation of secondary clastogenic material of cellular origin, called clastogenic factors (CF), has been implicated in the clastogenicity of a wide variety of processes causing oxidative stress (Emerit, 1994). It appears that CFs are chemically very heterogeneous. Degradation products of arachidonic acid may be candidates, but at present the whole process is still rather mysterious.

Our observation that the scavengers were not able to protect against the photomutagenicity of the fluoroquinolones in the Ames and Comet assays is most easily explained by attributing the effects observed in these two test systems to reactive products generated in the immediate vicinity of the DNA so that there is no chance for the scavengers to intercept the molecules. More puzzling is the observation that in the Ames and Comet assays the three fluoroquinolones appear to be about equipotent (with Fleroxacin possibly slightly less active), while in the CA test and in the phototoxicity tests there is a clear gradation, with Lomefloxacin being the most potent, followed by Fleroxacin and then Ciprofloxacin. The photochemical reactivity of Lomefloxacin is clearly higher than that of Ciprofloxacin, with Fleroxacin somewhere in the middle (K.H.Pfoertner, unpublished data). Thus, one would expect a clear gradation of activity independent of whether the responsible AOS were generated close to the DNA or at a greater distance. The discrepancy might be explained by the assumption that gene mutations and DNA breakage are caused by a subtype of reaction products which are generated in equal amounts from the three compounds or, alternatively, it might be that the three quinolones are present within the cells at different concentrations, counterbalancing the different photo-reactivities.

While the underlying mechanisms are not yet clear, the observations might suggest that the type of damage measured in the Comet assay is not responsible for the toxic and clastogenic effects, but could be responsible for the gene-mutation effect.

Several studies on the photocarcinogenesis of fluoroquinolones have been reported. UV doses which, in combination with the drugs, produced severe, dose-dependent toxicity led to development of skin tumours in Swiss and Skh-1 mice (Johnson et al., 1989). A comprehensive study with
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Fig. 5. Results of the Comet assay with Fleroxacin in mouse lymphoma cells without (A) and with concurrent irradiation with 500 mJ/cm² (B). Classification: class 1: cells without any or with only a few (<10) trailing fluorescing particles; class 2: cells with a thin trailing streak of particles; class 3: cells with strong comet; and class 4: comets with decomposed nucleus.

Fig. 6. Results of the Comet assay with Fleroxcin, Lomefloxacin and Ciprofloxacin at a UVA dose of 500 mJ/cm². The fraction of cells in classes 3 plus 4 is given.

Subphototoxic dose levels was performed (Urbach et al., 1995; G.Klecak, unpublished data) with 8-MOP and a number of fluoroquinolones, including those studied in the experiments presented here. All fluoroquinolones studied were capable of enhancing UVA-induced phototumorigenesis. Almost all tumours were benign except in the 8-MOP-positive controls (which received markedly lower UV doses) and the Lomefloxacin-treated mice. It is notable that median latent periods increased in the order Lomefloxacin→Fleroxacin→Ciprofloxacin. Thus, relative potencies parallel the relative phototoxic/photoclastogenic potencies rather than the relative DNA-damaging and gene mutation-inducing potencies, as observed in the Comet and the Ames test. It appears that the fluoroquinolones are compounds which might be useful to investigate the relative contributions of phototoxicity—with subsequent enhanced cell proliferation—and DNA damage and mutation to the processes of skin tumour formation.

Conclusion

With the prior knowledge of the phototoxic mechanisms the positive responses in the photomutagenicity tests were to be expected. AOS are produced in many exogenous cellular processes as well as by exogenous agents. Cells have developed a multitude of defence mechanisms against oxidative damage, including accumulation of glutathione, ascorbate, tocopherols and carotinoids (Ames et al., 1993). These defences are partly missing in established cell lines cultured in vitro. But even in the whole animal, antioxidant defences are not perfect. It is estimated that the genetic material of a human cell experiences up to 10 000 oxidative hits per day. DNA repair enzymes remove most but not all of the lesions formed. Apoptosis of skin cells (i.e. sunburn)—as would potentially be induced by the phototoxic action of the fluoroquinolones—represents a further level of defence.

Nevertheless, whether human antibiotic therapy with the fluoroquinolones could represent a significant additional factor in the formation of skin cancer has to be discussed. Clearly, it is advisable that humans treated with these antibiotics should avoid extensive exposure to sunlight or artificial UVA light.

In the context of risk assessment, it is of interest to consider the health risk associated with PUVa treatment, which consists of exposure to 8-MOP in combination with extensive UVA irradiation. 8-MOP is the photomutagenic compound par excellence: the genetic lesions involve cross-links as well as oxidative damage. Photoclastogenicity of 8-MOP plus UVA appears to be largely due to AOS (Youssefi et al., 1994). In
the toxicological comparison with the fluoroquinolones the PUVA case would, therefore, represent a very extreme ‘worst case’ scenario.

Despite the strong experimental evidence for the photomutagenicity of 8-MOP, no unequivocal indication for an increased risk of human skin cancer was apparent in a multitude of epidemiological studies. Those patients who developed skin cancer were exposed to large doses of UVA, administered over many years, and other risk factors also existed (Stern, 1989; Studniberg and Weller, 1993). In those cases where tumours developed, the patients had received at least 5 years of PUVA treatment. On this basis, treatment with fluoroquinolone antibiotics is not expected to pose an appreciable risk, even when simple precautions against excessive light exposure are not taken, in which case the patient will experience repeatedly the uncomfortable immediate reaction of sunburn. But light-induced adverse effects will not occur in the first place if simple precautions are taken during the relatively short periods of antibiotic therapy with the fluoroquinolones (at most a few weeks).

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References


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