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Preface

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近年，化粧品，医薬品，工業原料，環境汚染物質の中には，光照射下の光化学反応によって遺伝毒性が発現する事が注目されるようになり，そのメカニズムの研究，試験法の開発とその評価試験などが行われ，それにともなって，業界や行政による対応等もみられるようになってきました．今回の特集号では，この分野で第一線の研究を進めておられる内外の先生方に，総説，原著論文を含めて多数の寄稿をいただき，極めて内容の濃い特集号を組む事ができました．

海外から寄稿いただきました先生方のうち，Horst Spielmann先生（Germany）は，光毒性試験の分野で，早くからヨーロッパを中心に試験方法の提案とその評価試験を実施され，現在Spielmann先生を中心にOECDの光毒性試験のガイドラインが提案されています．Spielmann先生には光毒性試験法についての総説を寄稿いただきました．Elmar Gocke先生（Switzerland）は，光遺伝毒性を検出するためのAmes試験の条件設定に早くから取り組まれ，先年ワシントンで開催されたIWGT（International Workshop on Genotoxicity Test Procedures）の会議でも，光遺伝毒性試験法の問題点を中心になってまとめられました．Elmar Gocke先生には光Ames試験と光染色体異常試験についての総説をいただきました．また，ドイツにおいて行政の立場で早くから光遺伝毒性の問題に取り組まれているLutz Müller先生（現Switzerland）には，2編の原著論文を寄稿いただきました．

国内からは，製薬企業の立場でフルオロキノロン系抗菌剤の光遺伝毒性について先進的に取り組んでおられる島田弘康先生に，また化粧品の分野で光毒性の検出等に取り組まれている岡本裕子先生には活性酸素種の光毒性発現における役割について，それぞれ寄稿いただきました．一方，中西郁夫先生には光化学的側面から化学物質による光DNA損傷についての総説をいただきました．また原著論文として，高橋和彦先生にはDMBAの光遺伝毒性の発現について，中川ゆづき先生にはplasmid-relaxation assayを用いた光（遺伝）毒性の検出法についての論文をいただきました．さらに，資料・情報として，日本製薬工業協会による日本における医薬品の光遺伝毒性に関する調査結果を掲載いたしました．

光遺伝毒性の検出は，太陽光に暴露された化学物質のphotodynamic actionによる相乗的な生物への反応によって生じると考えられる光発がん物質の検出に極めて重要であります．地球環境の面からもオゾン層破壊による紫外線の増大が懸念されている時期でもあり，特に，環境変異原の面からは，環境中に放出されている多環芳香族炭化水素をはじめとする光遺伝毒性物質の生物への影響が危惧されるところでもあります．本特集号を契機として，この研究分野のより一層の進展のきっかけにもなれば，企画者の1人として大変幸いです．重ねて，寄稿いただきました諸先生に厚く御礼を申し上げます．

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Review

Photochemical genotoxicity testing: experience with the Ames test and the in vitro chromosomal aberration assay

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Summary

Photochemical genotoxicity can be detected using appropriately adapted versions of the bacterial mutagenicty test and the in vitro chromosomal aberration assay. For screening programmes it has been debated whether a photo-clastogenicity assay will be sufficient or whether the photo-Ames test will be necessary for complementation. In order to facilitate this discussion, we present data from in house investigations and refer to pertinent literature data showing that there is no clear precedent of a photochemical genotoxin which is exclusively positive in the bacterial assay. Furthermore, many of the photogenotoxins show only weak activity in the bacterial assay but are potently genotoxic in the clastogenicity assay. On the other hand, it might be prudent to include the bacterial assay because at present there is still a rather limited experience in photochemical mutagenesis testing.

Keywords: photoactivation, phototoxicity, genotoxicity test battery, reactive oxygen species, radicals

Introduction

A bacterial gene mutation assay and an in vitro chromosomal aberration (CA) test are the most fundamental, long established, components of the test battery recommended for the evaluation of the genotoxic potential of environmental chemicals. Thus, it was logical that these two tests were also recommended in the guidelines for the investigation of photochemical mutagenesis screening of cosmetic and sunscreen ingredients issued by the European Scientific Committee for Cosmetology (SCC, Loprieno, 1991). Although a number of pertinent reports on photochemical genotoxicity had been published, little experimental experience with adaptation of the standard test systems for this purpose had been obtained. When developing the guidelines the committee depended largely on the experimental work of Dean et al. (1991). Subsequently several groups in industry tried to establish useful testing protocols (Chételat et al., 1993a, b; Henderson et al., 1994; Utesch and Splittgerber, 1996). In the initial studies two established photochemical mutagens (8-methoxypsoralen (8-MOP), chlorpromazine) were investigated. The strong mutagenic and cytotoxic effect of UVB, specifically in the excision repair deficient strains led to comments that the bacterial tests would not be useful for photochemical mutagenesis screening for compounds which absorb predominantly in the UVB area (280 to 320 nm), such as the most important sunscreen constituents. Alternatively it was proposed to employ S. cerevisiae (Chételat et al., 1993a), which had been used in many photomutagenesis experiments with the psoralens. However, since S. cerevisiae systems are almost completely abandoned in genotoxicity testing, this proposal is not likely to be followed at the present state of development. During the International Workshop for Genotoxicity Test Procedures (IWGTP) discussions (Gocke et al., 2000) there still was no consensus whether a bacterial mutagenicity test was to be recommended for screening experiments in photochemical mutagenesis or whether a test for clastogenicity would be sufficiently informative. In the following we will present in house and published data pertinent to this discussion.

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Materials and Methods

Irradiation conditions

For screening purposes we generally use a solar simulator (suntest CPS, Heraeus, Hanau, Germany) as described in Chételat et al. (1993a). However, if further

information indicate that UVA or visible light is sufficient for photoactivation of the test compound we reduce the UVB component by either using the plastic lid or a glass plate as filter or by employing a halogen lamp (Philips, QVF 416, 1000 W). UV dose rate, or light intensity, were determined using a RM2 UV-meter (Dr Gröbel GmbH, Germany) or a Metrawatt MX4 luxmeter (Gossen, Germany), respectively. Bacteria were irradiated either directly on the Vogel Bonner-minimal agar plates or in phosphate buffer under continuous stirring (Chételat et al, 1993a). The mammalian cells were irradiated in phosphate buffer on glass slides placed in quadriperm culture dishes (Heraeus, Germany) (Chételat et al, 1993b).

All other experimental procedures were according to standard mutagenicity testing praxis (OECD guidelines 471, 473; Maron and Ames, 1983; Savage,1976).

Chemicals

Chlorpromazine, 8-methoxypsoralen (8-MOP), TiO_2, toluidine blue, methylene blue and metyl violet were obtained from commercial sources. The fluoroquinolones and the Roche compounds were synthesized in house.

Results and Discussion

8-MOP

Photochemical genotoxicity of this compound is readily observable in bacterial gene mutation assay. The crosslinking activity of 8-MOP requires the presence of excision repair in the bacterial tester strains for expression of the genotoxic effect. Thus, *S. typhimurium* TA102 and *E. coli* WP2 are responsive (Bridges et al., 1979, Chételat et al., 1993a, Dean et al., 1991). Exposure on plate or in liquid is similarly effective. The effect is easily recognized even with unfiltered solar simulator light (Chételat et al., 1993a, Henderson et al., 1994) or with the Osram Ultra Vitalux lamp, which contains a relatively high UVB component (Dean et al, 1991).

Likewise, there is no problem in detecting the genotoxicity of 8-MOP in mammalian clastogenicity assays (Dean et al, 1991, Chételat et al, 1993b)

Chlorpromazine

Recognition of the photochemical genotoxicity of this tranquilizer is unproblematic in the clastogenicity assays. In the bacterial assay strains TA1537 and TA98 are responsive, but there was a dependence on the irradiation conditions. When unfiltered solar simulator light was used, Chételat et al. (1993a) detected the photomutagenic activity only when the bacteria were irradiated in suspension. Under irradiation with reduced UVB component the photochemical genotoxicity was also recognizable when the cells were irradiated on the plate. Henderson et al. (1994) observed no such dependence on the irradiation conditions. However, they might have employed glass filtered (i.e. UVB-depleted) light.

Fluoroquinolones

Strain TA102 is extremely sensitive to the gyrase mediated genotoxicity of this class of antibiotics. This ‚dark' mutagenicity made it impossible to detect the light dependent genotoxicity of fluoroquinolones in this strain. Therefore, Chételat et al., (1996) employed TA104, which is responsive to the oxidative action of the fluoroquinolones plus light but not responsive to the protein-DNA crosslinks (‚cleavable complex') of the gyrase mediated action. The strong cytotoxicity of the antibiotics made it necessary to use the irradiation in suspension with extensive washing of the cells prior to plating. Furthermore it was necessary to pass the solar simulated light through a glass plate to reduce the UVB genotoxicity. Still, only a rather weak photochemical genotoxicity of, lomefloxacin, fleroxacin and ciprofloxacin was observable (maximally a doubling of the control frequencies). In contrast, the photoclastogenicity assay readily detected the genotoxicity of the fluoroquinolones. Aberration frequencies up to 80% were observed. The absence of a strong cytotoxic, and of a strong clastogenic, activity of the gyrase (topoisomerase II) mediated action of the fluoroquinolone antibiotics further facilitated the investigations with the mammalian system.

Ro 47-7737

This compound, a bis-quinoline derivative of chloroquine, was found to be phototoxic in vitro and in vivo (Ridley et al., 1997). The likelihood of adverse effects was considered to preclude development as an antimalarial, which necessarily has to be used in tropical, sunny climates. Investigations with the CA test showed a clear photogenotoxic effect of the compound (Fig. 1). The attempts to detect photomutagenicity with the Ames test were not successful. The tester strains TA102, TA104, TA1537, TA98, WP2 and WP2 uvrA were used. Irradiation was performed in liquid and on plate. Since the compound possesses a strong absorption at long UVA wavelenghts the UVB component was eliminated by passage through a glass plate, so that the absence of an intrinsic genotoxicity of the irradiation did not preclude the use of high UVA doses. Despite of these variations of the testing methods no indication for an increase of mutant colonies was detectable (Fig. 2).

Ro 19-8022 and structural analogs

Ro 19-8022 was under development as an anxiolyticum (Fischer et al 1990). During early toxicological investigation with the standard Ames protocol a mutagenic activity in strain TA102 was detected. Initial repeats showed an unexplained variability of the activity until it was realized that variations of the ambient light conditions at the laboratory bench (sunny versus cloudy day, morning versus afternoon) played a decisive role. When the agar plates were covered after plating no genotoxicity was apparent while a clear increase of the mutant colony numbers became apparent when the plates were left for 30 minutes or longer on the lab bench (Fig. 3). Further investigations provided evidence that the DNA damage profile induced by Ro 19-8022 plus UVA was most similar to that of known $^1O_2$ producers (Will et al., 1999). The photogenotoxic activity led to discontinuation of the development of Ro 19-8022.

Subsequently, we have tested Ro 19-8022 and a number of close structural analogs in more detail for phototoxic and photogenotoxic properties. Table 1 gives an overview of the results obtained in the Ames and CA assays. A reasonable concordance between the two assays was observed regarding the potencies of photogenotoxicity and of phototoxicity. Ro 12-9786 was the most potent photogenotoxin in both assays, followed by Ro 19-8022. Phototoxic potency was most pronounced for Ro 65-2822, Ro 65-1254 and Ro 17-3271. The one compound (Ro 65-8275) which was negative in both assays for phototoxicity and photogenotoxicity contains a different chromophore than the other 8 compounds and was only marginally photoactive in the 3T3 neutral red uptake test (3T3 neutral red uptake test, data not shown). Two compounds (Ro 65-2996 and Ro 19-5686) were not classified as photomutagenic in the Ames assay but showed weak photoclastogenic effects. One compound (Ro 65-2822) was weakly positive in the Photo-Ames assay but was not detected as positive in the Photo-CA assay, although the compound was clearly phototoxic. It is noteworthy that we tested only a rather few concentrations in the CA assay and it is quite likely that an effect would have been detected with more extensive testing.

Titaniumdioxide (TiO_2)

The photocatalyst TiO_2 (in an ultrafine particulate form) has been reported to be cytotoxic to bacteria and mammalian cells upon irradiation with UVA/visible light. Photogeneration of free radicals is thought to be responsible for this activity. Nakagawa et al. (1997) reported photogenotoxic activity in the CA assay but did not detect photomutagenicity in the Ames test in strains TA100, TA98, TA102. We have also studied TiO_2 for photomuta-

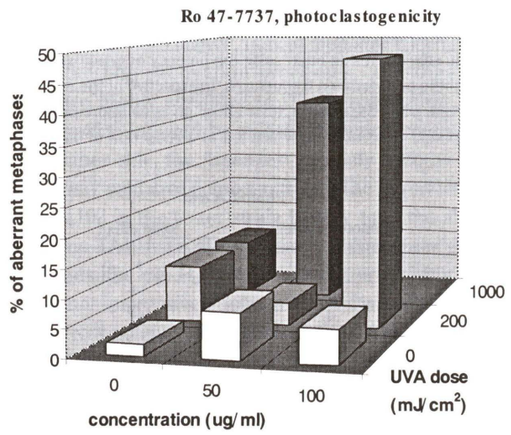

Fig. 1 Photoclastogenicity of Ro 47-7737 in V79 Chinese hamster cells. Cells were irradiated with solar simulated light passing through the plastic lid of the quadriperm culture dish.

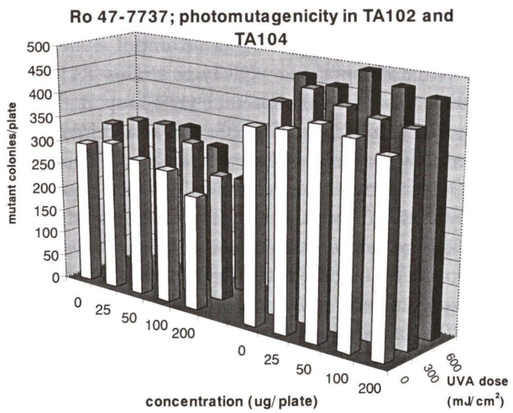

Fig. 2 Absence of a photomutagenic effect of Ro 47-7737 in the Ames test.
Irradiation was on the plates with solar simulated light passing through a glass plate. Exemplary results with TA102 and TA104.

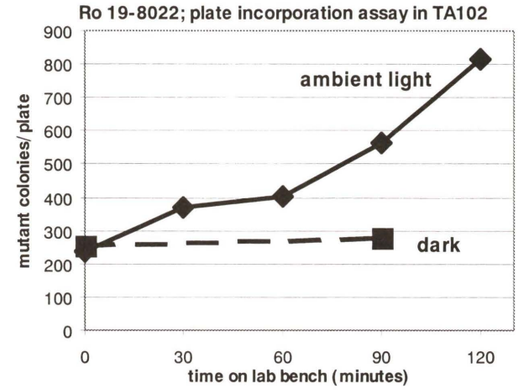

Fig. 3 Testing of Ro 19-8022 in the Ames test (TA102 , without S9). A standard plate incorporation assay was performed. The plates were place on the agar bench at normal ambient light conditions (no direct sunlight) or were covered with a black cloth immediately after plating. After the indicated time periods the plates were transferred to the incubator and kept at 37°C in the dark for 2 days until counting.

Table 1 Photochemical toxicity and genotoxicty of Ro 19-8022 and structural analogs in the Ames test (TA102) and the chromosomal aberration test with V79 Chinese hamster cells. Cells were irradiated with light from a halogen lamp (6300 Lux) for 90 min (Ames test) or 60 min (CA test). The column ‚genotoxic effect' gives an impression the potency of genotoxicity as indicated by the absolute increase of the mutant colony numbers or aberration frequencies, irrespective of the concentration of compound at which the maximal increase was observed, while the column ‚toxic effect' gives an impression of the potency of phototoxicity as indicated by the minimal compound concentration which led to a reduction of background growth (Ames test) or mitotic activity (CA test).

compound	Photo-Ames test		Photo-CA test	
	genotoxic effect	toxic effect	genotoxic effect	toxic effect
Ro 12-9786	+++	+	+++	+
Ro 19-8022	++	+	++	++
Ro 16-8554	+	++	+	++
Ro 65-2822	+	+++	-	+++
Ro 65-1254	-	+++	-	+++
Ro 17-3271	+	+++	+	+++
Ro 65-2996	-	-	+	+
Ro 19-5686	-	+	+	+
Ro 65-8275	-	-	-	-

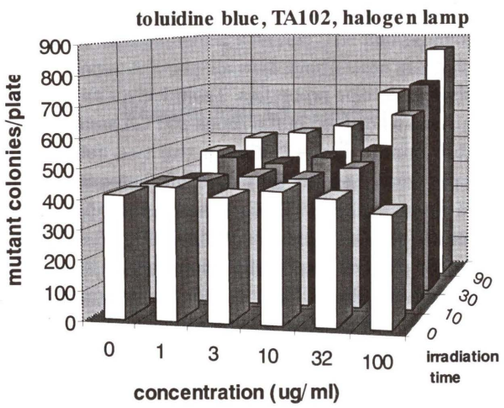

Fig. 4 Photomutagenicity of toluidine blue in the Ames test (TA102, without S9). Cells were irradiated on the plates for the indicated time periods at approximately 6300 Lux with light from a Philips 1000 W halogen lamp.

genicity in the Ames test (strains TA100, TA102, TA104) under irradiation with solar simulated light and could not detect an increase of the mutant colony numbers above the UVB induced levels (data not shown).

Methyl violet, toluidine blue, methylene blue

We evaluated the photomutagenicity of these three dyes in the Ames test. Since the compounds absorb visible light we irradiated the cells either with light from a halogen lamp or simply placed the agar plates on the window sill. All three compounds were clearly photomutagenic. A representative result with toluidine blue is shown in Fig. 4. Methylene blue and methyl violet showed similar activities but proved to be slightly mutagenic already in absence of irradiation (data not shown).

Methylene blue has long been known to cause photogenotoxic effects in bacteria (Brendel, 1968). We observed photomutagenic activity in strains TA102 and TA100 (data not shown). Epe et al. (1989) reported photomutagenicity in TA1535, TA2638, TA100 and TA104 and showed that generation of singlet oxygen is responsible for the effect. Reports on the evaluation of the photogenotoxicity in the CA assay has not reached our attention. However, methylene blue was found to be positive in the in vitro micronucleus test with Chinese hamster cells when concomitantly irradiated with light from a solar simulator (P. Kasper, pers. communication). For the other two compounds we do not know of any photoclastogenicity reports.

Conclusion

Most of the photogenotoxic compounds evaluated in the studies described above were positive in both, the photomutagenicity test with bacteria, and the photoclastogenicity assay with mammalian cells. Discrepant results were obtained for Ro 47-7737 (Ames test negative, CA test clearly positive), TiO_2 and two analogs of Ro 19-8022 (Ames test negative, CA test weakly positive). There was one instance (another analog of Ro 19-8022) which was weakly positive in the Ames test but negative in the CA test. However, as mentioned above, it is considered likely that the CA test would become positive upon more in depth investigation.

When considering the strength of the photogenotoxic effects of the various compounds it is apparent that the clastogenicity assay is usually yielding a stronger response than the Ames test. For instance, the fluoroquinolones are only marginally positive in the Ames assay but very potently clastogenic in V79 cells. The reason for this differential responsiveness is probably attributable to the dominance of oxidative, radical mediated mechanisms among the photoactivation processes. Clastogenicity assays are highly sensitive to the damages (e.g. DNA strand breaks) induced by oxidatively acting compounds. On the other side, the Ames test has only become sensitive to many oxidative compounds with the introduction of strain TA102 in the tester strain battery (and the alternative strains TA104, TA2638). Even with these new strains, compounds like e.g. H_2O_2 or bleomycin, or ionizing radiation are not impressively mutagenic in the bacteria while they are very potently clastogenic in the CA tests.

Crosslinking activity, a second important mode of action in photochemical mutagenicity is sensitively detected with strain TA102, but also here the induction of chromosomal aberrations appears to be at least equally efficient.

Taken together, a clear instance where the Ames test shows a superior sensitivity over the chromosomal aberration assay has not come to our attention. Thus, there is no precedent which indicates a necessity for employment of the Ames test in photogenotoxicity screening programs. Therefore one might conclude that the Ames assay is not complementing the clastogenicity assay for photochemical mutagenicity testing. This conclusion is in stark contrast to our experience in ‚general' genotoxicity screening where we observe that the majority of compounds eliciting genotoxic effects are positive in either the Ames test or the clastogenicity assay, obviously indicating that both assays complement each other.

Under these circumstances it is a matter of opinion, whether the Photo-Ames assay should be added to the photoclastogenicity assay, or not. The IWGTP working group did not reach consensus on this point. Because knowledge in the area of photochemical genotoxicity is still limited, several group members felt that it would be prudent to carry out a photo-gene mutation assay if the initial assessment for photoclastogenicity was negative.

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Acute phototoxicity testing

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Summary

Phototoxicity (=chemical phototoxicity) is an acute reaction which can be induced by a single treatment with a chemical and UV or visible radiation. In vivo, the reaction can be evoked in all subjects, provided that concentration of chemical and dose of light are appropriate. This acute phototoxic reactions in skin can be induced by substances applied topically to the skin or via the systemic route and exposure to UV or visible light. This reaction has to be distinguished from other photosensitized reactions, e.g. photoallergy, photogenotoxicity and photocarcinogenicity. Acute phototoxic reactions can be measured in vivo in humans and laboratory animals and in vitro in a wide spectrum of cell and tissue models. Due to differences in species specificity the acute phototoxicity data obtained in animal models showed a poor correlation to results from human patch testing. Therefore, in 1992 the European Commission represented by the EU validation centre ECVAM decided to develop and validate the most promising in vitro assays for acute phototoxicity in close co-operation with COLIPA, the European Cosmetic, Fragrance and Perfumery Association. From 1992-1998 a series of joint ECVAM/COLIPA prevalidation, formal validation and special studies were conducted in order to achieve regulatory acceptance of the most promising in vitro phototoxicity test both in Europe and at a world wide level. The studies were managed by ZEBET, the national German validation centre, and showed that a photocytotoxicity assay employing the established mouse fibroblast cell line 3T3 provided an almost perfect prediction of the phototoxic potential of chemicals in humans even when testing was conducted under blind conditions in several laboratories. Therefore, in the year 2000 the 3T3 NRU PT test has officially been accepted by the European Commission and all EU member states for classification and labelling of chemicals to assess their acute phototoxic potential. In the present review the potential of the current in vitro and in vivo phototoxicity tests will be described with particular reference to the underlying mechanisms and the results obtained in the European validation study of in vitro phototoxicity tests.

Keywords: chemical phototoxicity, in vitro test, 3T3 NRU PT test, phototoxic potential, acute photocytotoxicity

Introduction

Photosensitization is defined as a process in which reactions to normally ineffective radiation doses are induced in a system by the introduction of a specific, radiation-absorbing substance, the photosensitise, which causes another substance, the substrate, to be changed by radiation. When used to describe the reaction of skin to an exogenous chemical and UV or visible radiation, the term includes phototoxic and photoallergic reactions, as well as photomutagenicity and photocarcinogenicity (Spielmann et al., 1994a).

Phototoxicity (=chemical phototoxicity) is the term used for an acute reaction which can be induced by a single treatment with a chemical and UV or visible radiation. In vivo, the reaction can be evoked in all subjects, provided that concentration of chemical and dose of light are appropriate. The term photoirritation is used to describe phototoxic reactions in skin produced by substances applied topically to the skin or via the systemic route and exposure to UV or visible light. Photoallergy is an acquired immunological reactivity. The skin reaction does not

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occur on first treatment with chemical or light. Rather, an induction period is required before skin reactivity can occur.

The current toxicity assays for "acute dermal phototoxicity" are animal tests using guinea pigs, rabbits, rats or mice. Although standard protocols for phototoxicity testing in animals have recently been published (Nilsson et al., 1993; OECD, 1996), no animal phototoxicity test has yet been accepted by the OECD. Instead, OECD experts recommended a sequential approach for phototoxicity testing, involving the use of in vitro assays prior to testing in animals (OECD, 1995). In 1991 the Direction General (DG) XI of the European Commission and COLIPA agreed to conduct a prevalidation study on in vitro phototoxicity tests. It was the goal of the EU/COLIPA validation project on in vitro phototoxicity tests to determine whether currently available in vitro methods were capable of predicting the phototoxic potential to humans of chemicals applied via the systemic route or topically to the skin.

Among the assays for in vitro phototoxicity testing, two main types can be distinguished, namely cellular assays for screening purposes and mechanistic assays to identify specific mechanisms of phototoxicity (Spielmann et al., 1994a, 2000). The basic mechanism in phototoxicity can be described as an increase in toxicity of a chemical induced by exposure to UV or visible radiation. Therefore, the phototoxic potential of a chemical can be measured as an increase in cytotoxicity after exposure to UV or visible light. A large variety of test systems have been used to screen for phototoxic potential, including mammalian and non-mammalian permanent cell lines and primary cell cultures. The EU/COLIPA in vitro phototoxicity testing program relied on both cellular and mechanistic assays. The 3T3 Neutral Red Uptake phototoxicity (3T3 NRU PT) test, an in vitro cytotoxicity test for chemical phototoxicity, was the most promising in vitro test to identify chemicals with phototoxic potential both in phase I (Spielmann et al., 1994b) and phase II (Spielmann et al., 1998a) of the EU/COLIPA validation study and also in a special study on UV-filter chemcials (Spielmann et al., 1998b). Consequently, the 3T3 NRU PT test was in the year 2000 accepted for regulatory purposes by the European Commission (EC) as the only official test for the evaluation of acute phototoxic potential in Europe (European Commission, 2000).

In the present review the potential of the current in vitro and in vivo phototoxicity tests to assess the acute phototoxic potential of chemcials will be described. This includes the validation of the 3T3 NRU PT test and the testing scheme to assess the phototoxic potential of chemcials, which was recently recommended by the 2nd ECVAM workshop on phototoxicity testing (Spielmann et al., 2000).

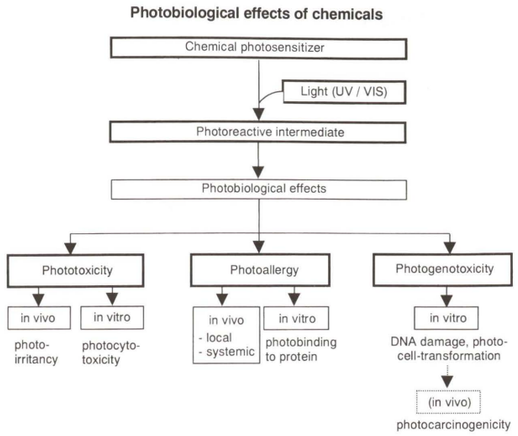

Fig. 1 Flow chart "Photobiological effects of chemicals"

Terminology

Chemical photosensitization as an adverse reaction may be induced by a broad spectrum of industrial or therapeutic agents which may enter the body by being swallowed, injected or topically applied. If phototosensitivity is to occur, absorption and distribution must be efficient in the tissues and detoxification and excretion less so. To clarify technical terms in the field of visible and in particular UV light induced toxicology terminology is essential. Light induced genotoxic effects, as e.g. photomutagenicity and photocarcinogenicity, will not be discussed in this document. The terminology is restricted to defining toxicological and technical terms of light induced acute toxicity (Fig. 1).

Toxicological terms

Photosensitization is defined as a process in which reactions to normally ineffective radiation doses are induced in a system by the introduction of a specific, radiation-absorbing substance (the photosensitizer) that causes another substance (the substrate) to be changed by radiation. When used to describe the reaction of skin to an exogenous chemical and ultraviolet or visible radiation the term includes both phototoxic and photoallergic reactions.

Phototoxicity is an acute reaction which can be caused by a single treatment with a chemical and ultraviolet or visible radiation. In vivo, the reaction can be evoked in all subjects provided that concentration of chemical and dose of light are appropriate. "Acute" includes both immediate and delayed (e.g. 48 hours) reactions.

Photoallergy is an acquired immunologic reactivity. The skin reaction does not occur on first treatment with chemical and light. After repeated application of a chemical an induction period of one or two weeks is required before skin reactivity can be demonstrated.

Photomutagenicity/photogenotoxicity is used to describe mutagenic/genotoxic effects induced by a combined exposure to a chemical (a photosensitizer) and UV or visible light.

Photochemical carcinogenesis is used to describe the induction of a malignant skin tumor, usually a carcinoma, by a combination of systemic (oral) exposure to a chemical (a photosensitizer) and exposure of the skin to UV or visible light.

Technical terms

Dose of light is defined as the quantity of ultraviolet (UV) or visible radiation incident on a surface, measured in Joules per square meter, J/m^2.

irradiance: $J/(s^1m^2) = W/m^2$
wavebands: UVA: 320-400 nm, UVB: 280-320 nm,
UVC: <280 nm, visible spectrum: >400 nm

Two general types of light sources are used in phototoxicity testing. Light sources with a limited emission spectrum, such as the fluorescent tubes for UVA or UVB or mercury arc lamps, and light sources with a solar light simulating emission spectrum. When considering photobiological phenomena, account should be taken of the emission spectrum of the lamp and the action spectrum of the phenomenon in question. Simple summations of radiation within a waveband is often misleading.

Mechanisms of Phototoxic Reactions (Fig. 2)

The chemistry and biology of photosensitized reactions have been reviewed intensively (Johnson, 1987, 1992). Absorption of light in the 300-750 nm range is an essential feature of photosensitizers, which may be degraded by UV radiation into toxic photo-products. The determination of two very simple physico-chemical properties of a new chemical should, therefore, proceed any testing for photosensitizing properties in biological systems in vitro or in vivo:

1. measurement of absorption spectrum, to detect absorption in the UV range, and
2. determination of photostability/photodegradation.

Activated photosensitizers have two systems of electronically excited states, the singlet and the triplet. The singlet is the initial product of light absorption, while the triplet is usually much longer lived. With very few exceptions, photosensitized oxidations proceed by way of the triplet photosensitizer. Therefore, effective photosensitizers are usually those providing a high yield of a long-lived triplet. Stable photodegradation products of some photosensitizers may act as toxins.

The triplet photosensitizer can react in two major oxygen dependent reactions (Johnson, 1987; Fig. 2):

1. by electron or hydrogen transfer (free radical) processes (Type I reaction), which may or may not require oxygen; or
2. by energy transfer to oxygen (Type II reaction) to form excited state singlet oxygen.

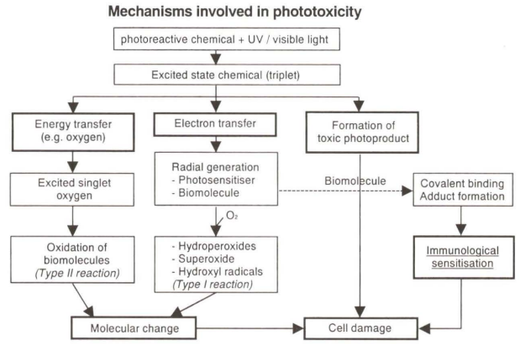

Fig. 2 Flow chart "Mechanisms involved in phototoxicity"

The relative participation of the Type I and Type II processes depends on the chemical nature of the photosensitizer and the substrate, the reaction conditions (solvent, pH, concentrations of photosensitizer, substrate and oxygen), and in some cases on whether the photosensitizer absorbs light into its first or second absorption band. Triplet photosensitizer, singlet oxygen and most free radicals are short lived with sub-millisecond lifetimes and must be produced in the immediate vicinity of substrate if reaction is to occur.

Dark association of photosensitizer and macromolecules may favour covalent photobinding to the macromolecule, e.g. the photoallergen tetrachlorosalicylanilide to protein or the photomutagen/photocarcinogen psoralen to DNA. Stable photodegradation products of some photosensitizers may act as ordinary toxins or as photosensitizers. Metabolites of several systemic drugs have been reported as photosensitizers. Phototoxic effects due to complement activation have only been observed after exposure to UVB (Johnson, 1987, 1992).

Biological targets for photosensitized oxidations include the plasma membrane, cytoplasmic organelles and the nucleus, depending on uptake and localisation of the photosensitizer.

Photogenotoxicity and photocarcinogenicity (Figs. 1 & 2) are side effects of chemical UV sensitization, which have recently come to the attention of regulators and scientists in the drug and cosmetics industry. Standard methods for assessing these endpoints have not been established. Methods for assessing the photogenotoxic potential of chemicals will be covered by other articles of this special issue.

Photoallergy is considered to be a delayed-type hyper-

sensitivity mediated by the formation of a photosensitizer-protein conjugate. In contrast to human photopatch testing (Kligman and Kaidbey, 1982) neither standard in vivo testing in animals nor in vitro tests have achieved general acceptance for photoallergy testing. Therefore, photoallergy testing will not be covered in the present review.

Testing in Animals

Prediction of phototoxic potential of xenobiotics by animal methods has been used for many years. The main problem with animal data is that it is difficult to compare results from different publications and to get a definite idea of the sensitivity of each of the animal models. Differences in the sensitivity of various animal models arise from two major technical problems:

1. There is still a lack of a generally, accepted standardized procedure. This is due to the diversity of species, light sources and UV doses used.
2. Many factors influence the outcome of a test, and not all of the factors have been described sufficiently well in most of the publications on animal methods.

Most frequently used species are mice, guinea pigs and rabbits. The selection of species may also depend on the intended route of exposure, e.g. guinea pigs are generally not used for i.v. injections. Route of administration and pharmacokinetics will influence the appropriate time interval between drug application and irradiation of the animals, which should take place at a time point when bioavailability of the compound is guaranteed, this may depend on the vehicle used.

The two most often applied animal methods to test for acute phototoxic potential of chemicals are the mouse tail swelling method (Ljunggren, 1984) and the mouse ear swelling method (Gerberick and Ryan, 1989). In 1995 the OECD even circulated a draft proposal for an in vivo test entitled "acute dermal photoirritation screening" (OECD, 1995), which was based on a small validation trial, in which a single chemical was tested (Nilsson et al., 1993). For the reasons given above, to date in Europe and at the OECD level no in vivo animal test for acute phototoxicity has been accepted for regulatory purposes. In contrast, since the 3T3 NRU PT in vitro phototoxicity test has been officially accepted by the EU in the year 2000 (European Commission, 2000), both the EU and the OECD have decided not to accept any animal test for acute phototoxicity into their test guidelines.

Photopatch Testing in Humans

The method of photopatch testing has so far not been standardized at the international level. However, the multicenter study from Austria, Germany, and Switzerland appear to provide the most satisfactory sets of data (Hölzle et al., 1991). Recently an update of this study has been published, which is covering 12 years of experience of the German, Austrian and Swiss photopatch test group (Neumann et al., 2000). The following method was employed by the Austrian, German, and Swiss group (Hölzle et al., 1991; Neumann et al., 2000): Test materials are applied via Finn chambers (aluminium chambers) to the back skin for 24 hours. Then, test sites are irradiated with 10 J/cm^2 (320-400 nm, peak 355 nm, Philips TL09). Readings are performed immediately and 24, 48, and 72 hours later. The evaluation score differentiates between erythema, infiltration, papulovesicles, blisters, and erosions. The pattern of phototoxic and photoallergic reactions is different. Basically phototoxic dermal reactions decease in severity over time, while photoallergic responses will increase. Standradization of the time of evaluation allows to distinguish between the two reaction patterns.

Table 1 Rank order of drugs and chemicals according to frequency of phototoxic reactions in human photopatch testing (n = 1129, according to Neumann et al., 2000)

Test Chemicals	Frequency (%)
1. Chlorpromazine	5.63
2. Promethazine	5.15
3. Carprofen	5.15
4. Fenticlor	2.93
5. Hexachlorophene	2.54
6. Balsam of Peru	2.06
7. Triclosan	1.74
8. 2-Hydroxy-4-methoxybenzophenone	1.35
9. 4-Isopropyidibenzoylmethane	1.35
10. Buclosamid	1.11
11. Musk Ambrette	1.11
12. 2-Ethylhexyl-p-methoxycinnamate	1.03
13. Bithionol	0.95
14. 3-(4-Methylbenzyliden)-camphor	0.95
15. 2-Ethylhexyl-p-dimethylaminobenzoat	0.87
16. Tribromsalicylanilide	0.79
17. Sulfanilamide	0.79
18. Quinidine	0.71
19. p-Aminobenzoic acid	0.71
20. Tetrachlorosalicylanilide	0.63
21. Chlorothiazide	0.63
22. Compositas mix	0.56
23. p-Methoxy-isoamyl-cinnamate	0.56
24. 4-tert-Butyl-4'-methoxydibenzoylmethane	0.40
25. Phenylbenzimidazolsulfonic acid	0.40
26. Monobromsalicylchloranilide	0.24

Results of photopatch testing performed according to this procedure are given in Table 1 (Hölzle et al., 1991; Neumann et al., 2000), which is restricted to substances predominantly inducing acute phototoxic reactions on the human skin. The rank order in the table is representing frequency of reactions observed in large representative groups of patients. It is important to note that many of the test chemicals, which show acute phototoxic properties, also show some photoallergic potential in human photopatch testing. Moreover, the two publications of the German, Austrian and Swiss photopatch testing group clearly indicated that the potential of chemicals to induce acute phototoxic reactions in humans is significantly higher than to induce photoallergic reactions (Hölzle et al., 1991; Neumann et al., 2000).

In Vitro Phototoxicity Tests

A considerable number of in vitro methods has been developed to assess phototoxic potential of chemicals. In vitro phototoxicity tests can be assigned to two general groups:

1. general tests on cells and tissues for screening purposes, and
2. mechanistic tests focusing on a specific mechanism of phototoxicity.

The more general tests are used to identify phototoxic potential of chemicals. Table 2 shows that a broad spectrum of cell and tissue culture systems including non-mammalian cells has been developed for this purpose. As described in Section 3 "mechanisms of phototoxic reactions", several specific mechanisms have been identified, each of may induce chemically induced UV phototoxicity. Table 2 demonstrates that these tests have been designed to predominantly assess subcellular and molecular mechanisms.

Table 2 In vitro methods for phototoxicity testing (according to Spielmann et al., 2000 and Gocke et al., 2000)

Type of test	Protocol
A: methods for screening purposes	
A-1: primary cells and cell lines	
3T3 NRU PT test	official EU method B-41 of Annex V
RBC PI test	SOP
Human keratinocytes	SOP
Hepatocytes	SOP
Candida/yeast test	nd
Human lymphocytes	nd
A-2: reconstituted human 3-D skin models	
Skin^2 PI^{TM} test	SOP
EpiDerm^{TM} test	SOP
Episkin^{TM} test	SOP
Skinethic^{TM} test	SOP
B: methods for evaluation of mechanisms	
Histidine photo-oxidation	SOP
RBC haemolysis	SOP
Haemoglobin photo-oxidation	SOP
Photobinding to protein (HSA)	SOP
Linoleic acid peroxidation	nd
Complement photoactivity test	nd
C: photomutagenicity/photogenotoxicity tests	
Bacterial mutation assays	SOP
Clastogenicity & chromos. abberation	SOP
Mammalian gene mutation	SOP
Indicator assays	nd
In vivo assays	nd

SOP: standard protocol (standard operation procedure) available; nd: standard protocol not developed

Methods to screen for phototoxic potential

1) The in vitro 3T3 NRU phototoxicity test (3T3 NRU PT test)

In a recent EU/COLIPA validation study of in vitro methods for phototoxicity testing, the Neutral Red uptake (NRU) growth inhibition assay using mouse Balb/c 3T3 fibroblasts to determine cytotoxicity was adapted for phototoxicity testing in the following manner (Spielmann et al, 1994b): Balb/c 3T3 cells are cultured in 96 well microtiter plates. After 1 hr of incubation with test chemicals plates were exposed to UVA (1.67 mW/cm^2) for 50 minutes (=5 J/cm^2) while a second set of plates with the same chemicals is kept in the dark. After an additional culture period of 24 hrs NRU is determined. A formal validation of the 3T3 NRU PT test was performed in 1994/96 in an international EU/ECVAM/COLIPA validation study (Spielmann et al.,1998a). It was followed by a study on UV filter chemicals (Spielmann et al., 1998b). After critical evaluation of the results of these studies, EU experts of the ECVAM, DG III, DG XI and DG XXIV have recommended the 3T3 NRU PT test for regulatory purposes in the EU.

As described below in section 7, in the year 2000 the 3T3 NRU PT test has been accepted for regulatory purposes as the first in vitro toxicity test in EU member states. Thus, the 3T3 NRU PT test has now been accepted as method B-41 into the Annex V of Directive 67/548/EEC on the classification, packaging and labelling of dangerous substances (European Commission, 2000).

2) Red blood cell phototoxicity test (RBC PT test)

Photohaemolysis is one of the oldest and simplest in vitro techniques for screening of putative photosensitizers (Sacharoff and Sachs, 1905). Many different protocols applying various light sources and sources for erythrocytes have been reported in the literature. Taking into account this information, Hetherington and Johnson (1984) published a photohaemolysis method for determining the phototoxic potential of drugs and other chemicals.

Additional important endpoints of phototoxicity in red blood cells are free-radical production and the oxidation of haemoglobin. Since methaemoglobin formation is often observed in phototoxicity testing with erythrocytes, both phenomena, photohaemolysis and haemoglobin oxidation, can be tested in a combined RBC (red blood cell) photoirritation test (RBC PT test; Pape et al., 1994). This approach allows to screen for photosensitizers and to study phototoxic mechanisms at the cellular level in human material. The combined RBC photohaemolysis

Table 3 Results obtained with the combined photo-RBC test in phase II of the EU/COLIPA in vitro phototoxicity validation project (Pape et al., 2001)

Chem. No.	Chemical	in vivo	Assessment[§] PHF	Δ OD$_{max}$	Final
1	2-hydroxy-4-methoxybenzophenone	-	-	-	-
10	Chlorhexidine dihydrochloride	-	-	-	-
15	Hexachlorophene	-	-	+	+
17	Sodium lauryl sulfate	-	-	-	-
24	Para-Aminobenzoic acid (PABA)	-	-	+	+
25	Penicillin G	-	-	-	-
14	Furosemide	+?	-	-	-
23	Ofloxacin	+	-	-	-
2	5-methoxypsoralene (5-MOP)	+	-	-	-
3	6-methylcoumarin	+	+/-	+	+
4	Acridine - hydrochloride	+	+/-	+	+
5	Acridine - free base	+	+	+	+
6	Amiodarone	+	+/-	-	+/-
7	Anthracene	+	+/-	+	+
8	Bergamot oil	+	-	+	+
9	Bithionol	+	+/-	+	+
11	Chlorpromazine	+	+/-	+	+
12	Demeclocycline	+	-	+	+
13	Fenofibrate	+	+	+	+
16	Ketoprofen	+	+	+	+
18	Musk ambrette	+	+	+	+
19	Nalidixic acid - sodium salt	+	+/-	+	+
20	Nalidixic acid - free acid	+	-	+	+
21	Neutral red	+	+	+	+
22	Norfloxacin	+	-	+	+
26	Promethazine	+	-	+	+
27	Protoporphyrin IX - free acid	+	+	+	+
28	Protoporphyrin IX - disodium	+	+	-	+
29	Rose bengal	+	+	+	+
31	Tiaprofenic acid	+	+	+	+

[§]PHF = photo-haemolysis factor; Δ OD$_{max}$ = difference in optical density at absorption maximum

and photo-haemoglobin oxidation assay is a useful test both for screening and for mechanistic studies. Phototoxic compounds reacting with DNA will provide negative results in the RBC test.

Preliminary data obtained under blind conditions in three laboratories with the RBC PT test in the prevalidation stage of the ECVAM/COLIPA in vitro photoirritation. validation study are shown in Table 3 (Pape et al., 2001). It was concluded from the results that the Photo-RBC test provides a good overall fit with the in vivo endpoints as well as mechanistic information on two different types of photodynamic reactions (met-Hb formation for type I reactions and photo-haemolytic effects as primary type II reactions). In summary, when conducted according to the SOP (Standard Operation Procedures) of the prevalidation study, the combined Photo-RBC test can reliably be performed. Moreover, this test provides relevant mechanistic information on photodynamic reactions, which is useful for the evaluation of the photo-safety of chemicals in a testing strategy that starts with the 3T3 NRU PT test, which does not provide information on photodynamic reactions. An additional advantage of RBC cells is their resistance to the short-waved UVB-part of sun light, which allows to expose RBC cells in the Photo-RBC test to the entire solar spectrum for prolonged periods of exposure.

3) Human keratinocytes

Keratinocytes are in vivo the first target cells exposed to sunlight. The high sensitivity of keratinocytes to sunlight limits their use in evaluating phototoxic effects induced by xenobiotics. Screening of photosensitizers with primary human keratinocyte cultures (Duffy et al., 1987) has not shown any obvious advantage in comparison to fibroblasts such as Balb/c 3T3 mouse fibroblast in the 3T3 NRU PT test in the ECVAM/COLIPA prevalidation trial as shown in Table 4 (Spielmann et al., 1995). The standard test protocol developed for the 3T3 NRU PT test can successfully be used with human keratinocytes, as demonstrated in a study conducted under blind conditions with the chemicals of phase II of the EU/COLIPA validation study and of the UV filter study (Clothier et al., 1999).

Table 4 Summarised data of phase I of the EU/COLIPA validation study of in vitro phototoxicity tests (Spielmann et al., 1994b)

		Mechanistic Assays			Commercial Assays		Growth Inhibition Assays			
		Histidine Photo-oxidation	RBC Photo-hemolysis	RBC Photo-Hb-oxidation	SOLA-TEX PI	Skin[2] ZK 1300 ZK 1350	Yeast growth inhibition	Human lympho-cytes MTT	Human keratino-cytes NRU	COMMON standard 3T3 NRU
Class I	UV-absorbing, phototoxic									
1	Promethazine	+	+	+	+	+	+	+	+	+
2	Chlorpromazine	(+)	+	+	+	+	(+)	+	+	+
3	6-Methylcoumarin	+	+	+	+	+§	+	+	+	+
4	TCSA	+	+	+	+	+	+	-	+	+
5	Doxycycline	+	-	+	+	+	+	+	+	+
6	8-MOP	+	-	+	-	+	+	+	-	+
7	Tetracycline	+	-	+	+	+	-	-	+	+
9	Amiodarone	-	+	+	+	+	+	+	-	+
10	Bithionol	-	+	+	(+)	-	-	-	+	+
11	Neutral Red	+	+	+	+	+	-	+	+	+
12	Rose Bengal	+	+	+	+	+	+	n.t.	+	+
Class II	UV-absorbing non-phototoxic									
8	Piroxicam	-	-	-	-	-	-	-	-	-
13	Cinnamic Ald.	(+)	-	(+)	-	-	-	-	-	-
14	Chlorhexidine	-	+	-	-	-	-	-	-	-
15	Uvinul MS 40	-	-	-	+	-	-	-	-	-
16	PABA	-	-	-	-	-	-	-	-	-
Class III	non UV-absorbing non-phototoxic									
17	Penicillin G	-	-	-	-	-	-	-	-	-
18	L-Histidine	-	-	-	-	-	-	-	-	-
19	Thiourea	-	-	-	n.q.	-	-	-	-	-
20	Lauryl Sulfate	-	-	-	-	-	-	-	-	-

§ positive when applied via the medium; negative when applied to the surface
n.q. = test not qualified; n.t.=not tested

Since the human keratinocyte system is easily available, further improvement and standardization should be encouraged.

4) Candida or yeast phototoxicity test

The yeast test using Candida albicans developed by Daniels (1965) is the most simple in vitro phototoxicity test. The Candida assay works well with linear furocoumarins (psoralens) and provides quantitative and wavelength dependence data. The test provided false negative results with many established photosensitizing chemcials (Knudsen, 1985). Although the Candida yeast phototoxicity assay is well established and supported by a large data base, it cannot be recommended as a screening test, since simple and reproducible mammalian in vitro tests showed a better predictivity in the ECVAM/COLIPA in vitro phototoxicity prevalidation study (Table 4; Spielmann et al. 1995).

Using the yeast assay Averbeck and co-workers (1989, 1990) were among the first to demonstrate the specific property of some bifunctional psoralens, e.g. 8-methoxypsoralen (8-MOP) or 5-methoxypsoralen (5-MOP), to induce genetic damage (mutations and mitotic recombination) in the presence of UVA or simulated solar radiation. These observations were confirmed in cultured mammalian and human cells. Because the exposure to doses of UV- and UV-A radiation could be increased in yeast compared to bacteria and mammalian cells, it has been possible to test combinations of the psoralens with UV filters using conditions which mimic human exposure (Agapakis-Causse et al., 2000).

In summary, in this assay one may not only test the neat compound but also its final galenic form combined with other compounds. Ethanolic or oily solutions of test compounds can be spread on yeast plated on solid growth medium before exposure to UV radiation in order to test for phototoxicity and/or photogenotoxicity. Moreover, the yeast assays can be performed under conditions (e.g., prolonged UV exposures) that are introduced to mimic human exposures but would be too photo-toxic or too photogenotoxic for bacteria and human cells in vitro.

Human 3-D skin models in phototoxicity testing

Reconstituted human skin models (3-D skin models) are available commercially, or from a few experienced laboratories, in three different types: dermal models (containing skin fibroblasts), epidermal models (containing skin keratinocytes and a stratum corneum), and full skin models (containing skin fibroblasts, keratinocytes and a stra-

tum corneum). Since the latter two types contain viable, metabolising primary skin cells and a skin barrier, both are frequently referred to as "3-D skin models". Human skin models have been used quite successfully in routine laboratory investigations, since they are relevant to the organ of interest. For in vitro toxicity testing, standardisation and control of 3-D skin models needs to be defined clearly in order to assure that reliable and reproducible data are obtained.

Human skin models in contrast to normal cell cultures such as 3T3 mouse fibroblasts allow for topical application of various types of chemicals and preparations and seem to have less limitation concerning solubility problems. In 3-D skin models test materials can be applied undiluted, with different extreme pH values or even as 'insoluble' substance as for instance shown by successful usage of such models for testing corrosives.

The first promising data obtained with skin model phototoxicity tests were reported 1994-1995 with a full skin model (Edwards et al., 1994; Liebsch et al., 1995), and an epidermal model (Roguet et al., 1994). Since the commercial production of the full skin model Skin^{2TM} was stopped in 1996, the test protocol was successfully adapted to the use the epidermal model EpiDerm^{TM} (Liebsch et al., 1997) and later evaluated in an ECVAM prevalidation study revealing promising results in three laboratories (Liebsch et al., 1999). The test is currently established in several laboratories of the European cosmetics industry (Jones et al., 1999) and has been successfully adopted to the epidermal model SkinEthic^{TM} (Bernard et al., 1999). Efforts undertaken to optimise the phototoxicity test protocol and prediction model when transferring it from the full skin model to the epidermal model (Liebsch et al., 1997) revealed the basic test protocol and prediction model did not need to be changed. Several studies (Liebsch et al., 1995; Api, 1997) reported that in vivo photoallergens that are not acute photoirritants at the same time (e.g. coumarin, 6-methyl coumarin, musk ambrette), are classified negative by the skin model phototoxicity tests. Finally, dermal models, which do not contain a skin barrier, show a sensitivity to phototoxic chemicals, which is similar to photocytotoxicity tests, as e.g. the 3T3 NRU PT test (Augustin et al., 1997). They are, therefore, not providing any advantage in a phototoxicity testing strategy.

1) Advantages of using human 3-D skin models

Assuming that the 3-D skin models fit to the best available laboratory and scientific standard they will offer the following advantages in comparison to the 3T3 NRU PT test:

· Neat chemicals or complex mixtures thereof can be applied simulating the situation of preparations as applied topically to the skin.

· Test concentrations are closer to real exposure conditions, including dermatological patch techniques.

· Histology can be performed on exposed and control samples.

· Exposure to light can better be adopted to real life situation; e.g. exposure time and spectrum of simulated sun light (a higher dose of short waved light in the range of UVB).

· Depending on the barrier function of the stratum corneum, adsorption and penetration of the original chemicals or molecules created during exposure skin models will provide more relevant results than tests performed on simpler models (less false-positives).

2) Disadvantages of using human 3-D skin models

The following disadvantages also have to be taken into account:

· The number of suppliers is limited (e.g. MatTek^{TM}, Skinethic^{TM}, EPISKIN^{TM}, cellsystems^{TM}).

· Lack of appendices of the skin like hair follicles or sebum and sweat glands, which may be sensitive areas in vivo, could be of relevance, although there is up to now no experimental evidence that this may be a draw back.

· Up to now there is no convincing evidence that photoallergic reaction of chemicals can be predicted in 3-D skin models.

· Since 3-D skin models are still very expensive, currently 25-90 Euro per sample, they are not yet suitable for routine large scale testing.

3) Validation of human skin models

Validation studies have shown the 3T3 NRU PT test will correctly predict the phototoxic potential of chemicals using a monolayer cell culture. Since this may not be relevant when chemicals are applied topically to the skin at lower concentrations in finished products, there is a need for test method development to allow application of complex formulations directly to reconstruct skin models.

In the ECVAM prevalidation study on the EpiDerm phototoxicity test an appropriate test protocol was developed successfully leading to reliable test results in three laboratories testing ten chemicals (Liebsch et al., 1999). From the results obtained in the project there is some evidence that 3-D skin models can be used for the assessment of the potency of a phototoxin applied topically to the skin. Therefore it can be concluded that 3-D skin models can play an essential role as adjunct in a test strategy for the sequential evaluation of phototoxicity.

Experimental Validation of In Vitro Phototoxicity Tests

The principles of validating in vitro toxicity test, that were developed in Europe under the leadership of ECVAM and later accepted world-wide, have been described in detail in the literature (Balls et al., 1995; OECD, 1996).

The ECVAM/COLIPA prevalidation and validation study of in vitro phototoxicity tests

1) Prevalidation study

From 1992-1994 in a joint prevalidation study 6 laboratories representing COLIPA (the European Cosmetic, Toiletry and Perfumery Association) as well as FRAME (Fund for the Replacement of Animals in Medical Experiments, Nottingham, UK) and ZEBET (Berlin, Germany) have evaluated the most promising in vitro methods for phototoxicity testing. 20 chemicals with known photoirritation properties (11 phototoxins, 5 UV-absorbing non-phototoxins and 4 non-UV absorbing non-phototoxins) were tested under identical exposure conditions to UVA in the 3T3 NRU PT test in all laboratories and also in in vitro phototoxicity tests established in laboratories of the European cosmetic industry, e.g. photohaemolysis and haemoglobin oxidation in red blood cells (RBC PT test), histidine oxidation, a Candida albicans assay, a human lymphocyte assay, a human keratinocyte assay, and, in addition, also in two recently developed commercial assays (SOLATEX^{TM} PI, Skin^{2TM} PI). Development and standardization of the 3T3 NRU PT test and of the Skin^{2} PI test were carried out at ZEBET's laboratory.

As shown in Table 4, during prevalidation of the EU/COLIPA study the 3T3 NRU PT test, the combined RBC PT test and the Skin^{2} PT test showed the best overall correlation with in vivo data (Spielmann et al., 1995).

2) Formal validation study of the 3T3 NRU PT test

Subsequently, in 1994-1996 during phase II of the ECVAM/COLIPA study, the 3T3 NRU PT test and the combined RBC PT test underwent formal validation in a blind trial in 11 laboratories in Europe and the USA with 30 carefully selected test chemicals. The data of the formal validation trial were submitted to the Scientific Committee on Cosmetology (SCC) of the Direction General (DG) XXIV of the European Commission in 1996 and the report of the management team has officially been accepted for phototoxicity testing for regulatory purposes in 1997 by the ECVAM Scientific Advisory Committee (ESAC) and in 1998 by the DG XI of the EU Commission and by the DG III, which is responsible for pharmaceutical and cosmetic products. The results of the validation study have been published in detail in 1998 (Spielmann et al., 1998a).

3) Additional study on UV filter chemicals in the 3T3 NRU PT test

At the request of the Scientific Committee on Cosmetology SCC, the expert advisory committee on cosmetics, which reports to the Health and Consumer Protection DG of the EU Commission, a set of the most commonly used UV-filter chemicals, which are not phototoxic in vivo and in most cases poorly soluble in water, was tested in a blind trial in the 3T3 NRU PT test. To obtain information on the optimum test concentration and on the predictivity of the 3T3 NRU PT test, an equivalent set of proven phototoxic chemicals was tested. The results of the study summarised in Table 5 confirm that the 3T3 NRU PT test is able to handle test chemicals irrespective of their solubility (Spielmann et al., 1998b). A thorough biostatistical analysis showed that no false positive results were obtained up to 100 µg/ml, while some false positive results were obtained at higher concentrations. The report also shows that all of the UV-filter chemicals were tested at least up to the highest test concentrations recommended after an independent assessment of solubility.

Table 5 Classification results if the 3T3 NRU PT test in the special study on UV-filter chemicals (Spielmann et al., 1998b)

3T3-NRU PHOTOTOXICITY TEST: STUDY ON UV-FILTERS
4 Laboratories: 20 Chemicals (10 PT/10 NPT)
OVERALL PREDICTIVITY MPE
For test concentrations up to 100 µg/ml

		in vivo classification		
		photo-toxic	non-photo-toxic	total
in vitro classification	phototoxic	40	1	41
	non-phototoxic	0	39	39
	total	40	40	80

Table statistics for the shadowed table

Sensitivity:	100%
Specificity:	98%
Positive predictivity:	98%
Negative predictivity:	100%
Accuracy:	99%
χ^2:	72,25 (>> 3.8)

MPE = Mean Photo Effect, PT = Phototoxin, NPT = Non-phototoxin

Results of the UV-filter study clearly demonstrate that the 3T3 NRU PT test does not provide a yes or no answer, but that the result is clearly dependent on the test concentration applied with an increasing risk of false positive results at high test concentrations. Thus, expert judgement is required, when data obtained in the 3T3 NRU PT test are used for regulatory purposes. It must be stressed that among toxicity tests the 3T3 NRU PT test has a unique position, since it is the only toxicity test predicting the situation in humans due to high quality human in vivo photopatch test data against which it has been experimentally validated. We are not aware of any other toxicity test that is accepted for regulatory purposes, which meet this important criterion.

4) Regulatory acceptance

Early in the year 2000 the 3T3 NRU PT test has been officially been accepted by the EU Commission and the EU member states into Annex V of the EU Directive 86/906/EEC for classification and labelling of hazardous chemicals (European Commission, 2000). In accordance with EU Directive 86/906/EEC, which is regulating the use of experimental animals, the 3T3 NRU PT phototoxicity test must now be used to determine the phototoxic potential of chemicals and animal test are prohibited for this purpose in all EU member states.

Hazard Indentification in Phototoxicity Testing (Fig. 3)

At the first ECVAM workshop on phototoxicity testing (Spielmann et al., 1994a) a tiered testing strategy was recommended to assess the phototoxic potential of test chemicals. According to the tiered testing approach, in the first step validated in vitro phototoxicity tests were recommended, although they did not exist, when the first workshop was held. It was assumed that when no phototoxic potential could be detected in a selected set of validated in vitro phototoxicity tests, one may proceed to clinical testing in humans without any preclinical testing for phototoxicity in animals (Spielmann et al., 1994a).

This tiered testing approach for phototoxic potential of chemicals did not take into account the additional endpoints, photoallergy and photo-genotoxicity/-carcinogenicity. Due to a lack of experimental and clinical experience, in the year 1993 these two new endpoints were not considered at the first ECVAM workshop on phototoxicity. In 1999 the participants of the second ECVAM workshop on phototoxicity testing have, therefore, updated the tiered testing approach and recommended the more complex approach, which is illustrated in Fig. 3 (Spielmann et al., 2000). Fig. 3 is derived from the phototoxicity testing strategy of the official EU test guideline B-41 "phototoxicity - in vitro 3T3 NRU phototoxicity test" of Annex V of the EU Directive 86/906/EEC for classification and labelling of hazardous chemicals (European Commission, 2000).

The following information has to be taken into account, when the phototoxic potential of a chemical is evaluated according to the new sequential testing approach depicted in Fig. 3:

- absorption spectrum
- photodegradation/photostability
- QSAR
- results obtained in the 3T3 NRU PT test

Fig. 3 indicates that according to our current knowledge the 3T3 NRU PT test is providing a positive result with phototoxic chemcials and also with photoallergens and photogenotoxic chemcials. This has been amply shown by the validation studies (Spielmann et al., 1998a, 1998b). Hence a negative result in the 3T3 NRU PT tests,

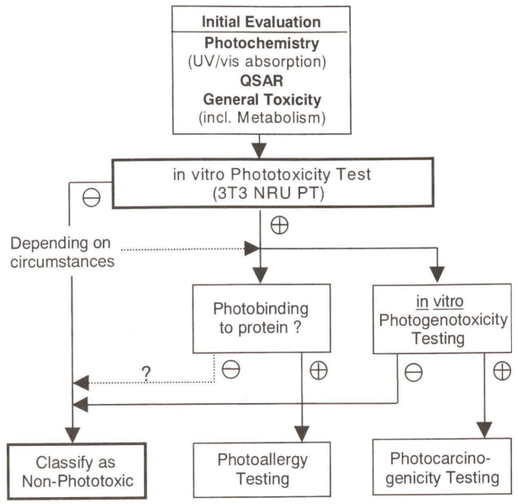

Fig. 3 Flow chart "Phototoxicity: hazard identification"

using a test compound at concentrations up to 100 µg/ml, is good evidence of absence of adverse photobiological effects. However, additional information on the photoallergy and photo-genotoxic/-carcinogenic potential should be obtained, before clinical testing of a new chemical in humans is recommended. Unfortunately, there are to date no validated in vitro or in vivo tests for the evaluation of the photoallergy and the photo-genotoxic/-carcinogenic potential.

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総　説

光増感剤によるDNA損傷の化学

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DNA damage caused by photosensitizers

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Summary

Photoinduced DNA damage is initiated by photoexcitation of photosensitizer (S^*), which results in generation of a variety of reactive species responsible for the DNA damage. When the reduction potential of S^* is more positive than the oxidation potential of guanine (G) in DNA, an electron-transfer oxidation of G by S^* occurs to produce guanine radical cation ($G^{\bullet+}$) and the radical anion of photosensitizer ($S^{\bullet-}$). The $G^{\bullet+}$ thus formed is converted to 7,8-dihydro-8-oxoguanine (8-oxoG) and imidazolone derivative through successive oxidation steps. Electron transfer from $S^{\bullet-}$ to O_2 may also occur to produce superoxide anion, which undergoes the disproportionation to form O_2 and hydrogen peroxide. Hydrogen peroxide further decomposes through a trace-metal-dependent Fenton reaction to produce hydroxyl radical, which causes non-base-selective DNA cleavage via abstraction of hydrogens on the deoxyribose ring or oxidation of nucleobases. An energy transfer from the triplet excited state of photosensitizer ($^3S^*$) to O_2 yields singlet oxygen ($^1O_2$), which can also oxidize G to 8-oxoG via cycloaddition of $^1O_2$ to the imidazole ring of G. Representative examples of photosensitizers having an efficient DNA-cleaving activity are described together with the reactive species involved in the photoinduced DNA-cleavage reactions.

Keywords：photosensitizer, DNA damage, electron transfer, energy transfer, active oxygen species

緒　　言

DNA切断活性を有する化合物は，発がんや老化のメカニズムを解明する上で重要であるばかりでなく，がんの治療薬として応用できる可能性があり非常に興味深い．特に光照射することによりDNA切断活性を発現する光増感剤は，光を照射しなければ生体への毒性を示さない可能性があり，光線力学療法剤として期待できるため，近年，活発に研究されている．光はその強度や波長などの制御が容易で非常に扱いやすく，光増感剤固有の吸収波長の光を照射することで選択的に活性の高い励起状態を作り出すことができる．光増感剤が可視部に吸収をもつ場合には，ほとんどの生体組織はその波長領域の光を吸収しないため，光線力学療法剤として特に有用である．これまでにも光増感剤を用いたDNA切断については非常に数多くの報告例や優れた総説があるが(Armitage, 1998 and references cited therein)，ここでは光DNA切断の反応機構，および特に長波長領域に吸収をもち，すぐれたDNA切断活性を示す光増感剤の代表例と最近の筆者らの成果について概説する．

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1．光DNA切断の反応活性種

光増感剤によるDNA損傷のメカニズムは，主としてFig. 1に示す3つのルート（1．電子移動反応経由，2．ヒドロキシルラジカル（•OH）生成経由，3．一重項酸素（$^1O_2$）生成経由）が明らかにされている（Burrows and Muller, 1998）．

すなわち，光増感剤（S）に光を照射すると，励起状態S^*が生成する．一般に，S^*は基底状態Sよりも励起エネルギーの分だけその還元電位が正側にシフトし，強

力な酸化剤として作用する．S^*の還元電位が核酸塩基の中でもっとも酸化されやすいグアニン（G）の酸化電位よりも高い場合にはGからS^*への電子移動が起こり，すなわち，S^*がGを酸化し，Sのラジカルアニオン（$S^{\bullet-}$）とGのラジカルカチオン（$G^{\bullet+}$）が生成する（タイプI電子移動反応）．後述のように，$G^{\bullet+}$は種々の化学反応を受け，ピペリジンなどの塩基の作用により，DNA鎖の切断が引き起こされる．また，反応系にNADHなどのG以外の還元剤が存在すると，S^*は還元剤により一電子還元を受け，$S^{\bullet-}$が生成する．$S^{\bullet-}$の酸化電位が分子状酸素（O_2）の還元電位より低い場合には，$S^{\bullet-}$からO_2への電子移動が起こり，活性酸素種の一つであるスーパーオキシドアニオン（$O_2^{\bullet-}$）が生成する．生成した$O_2^{\bullet-}$は自発的不均化反応によりO_2と過酸化水素（H_2O_2）に変わった後，反応系中の微量金属によるFenton反応を受け，•OHを生成する．•OHは活性酸素の中でももっとも強力で，DNA糖鎖や核酸塩基と反応し，DNA切断を起こすことが知られている．•OHが活性種の場合には，上述のGの電子移動酸化の場合とは異なり，DNA切断部位の選択性はほとんど観測されない．また，SOD酵素を反応系に添加すると，$O_2^{\bullet-}$の不均化が促進されるため，DNAの切断効率が高くなるという報告もある（Eliot et al., 1984；Nagai and Hecht, 1991；Párraga et al., 1992）．逆に，H_2O_2をO_2とH_2Oに分解するカタラーゼや•OHを消去するアルコール類を加えると，DNA切断が顕著に抑制されるのも•OHを活性種とするDNA切断の特徴である．一方，励起状態S^*が項間交差（ISC：intersystem crossing）を経て三重項励起状態（$^3S^*$）にある場合には，O_2にエネルギーを渡し，$^1O_2$を生成する可能性がある（タイプIIエネルギー移動反応）．この$^1O_2$も後述のように，Gに選択的に付加することによりDNA切断活性を示すことが知られている．$^1O_2$が活性種の場合には，アジ化ナトリウム（NaN_3）などの$^1O_2$消去剤の添加により，DNA切断が顕著に抑制される．また，$^1O_2$の寿命が長くなる重水（D_2O）中では，切断活性が高くなることが特徴的である．

以上のように，光増感剤Sに光照射すると反応活性種として，S^*，•OH，および$^1O_2$が生成するが，次に，これらの反応活性種がどのようにDNA糖鎖や核酸塩基と反応しDNA切断を引き起こすかについて述べる．

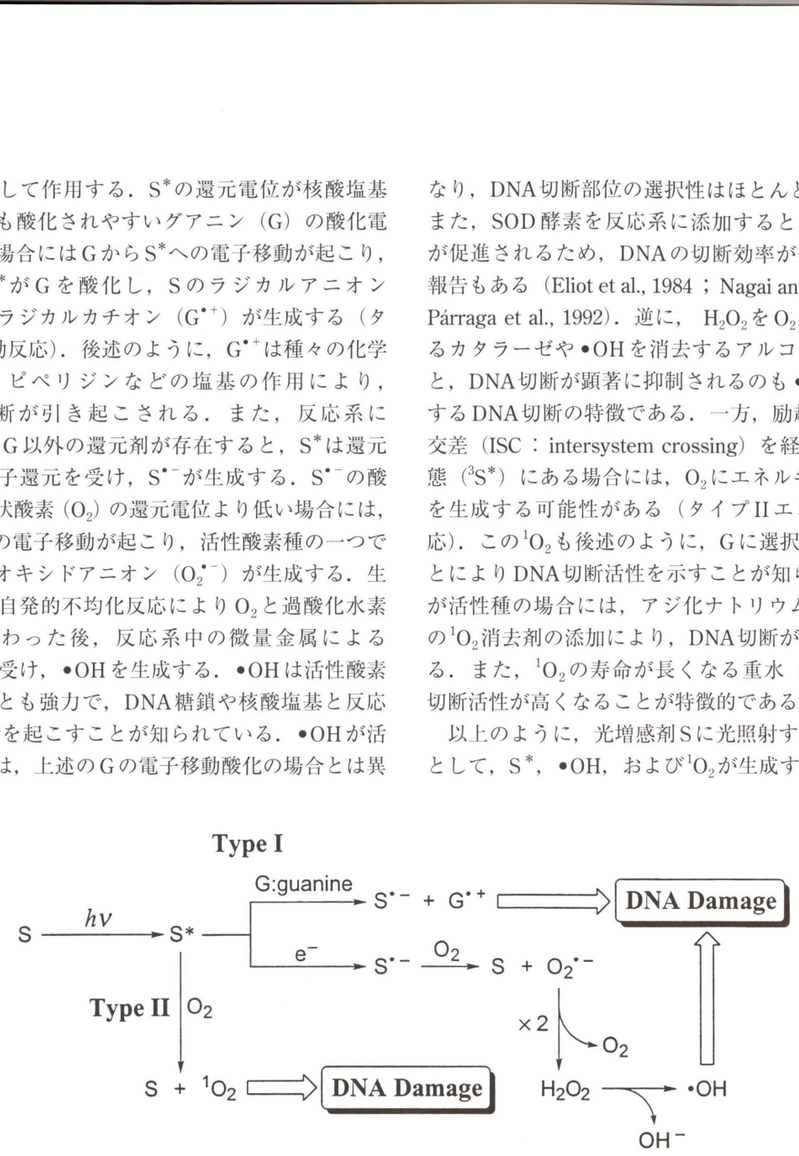

Fig. 1 Mechanism of photoinduced DNA damage

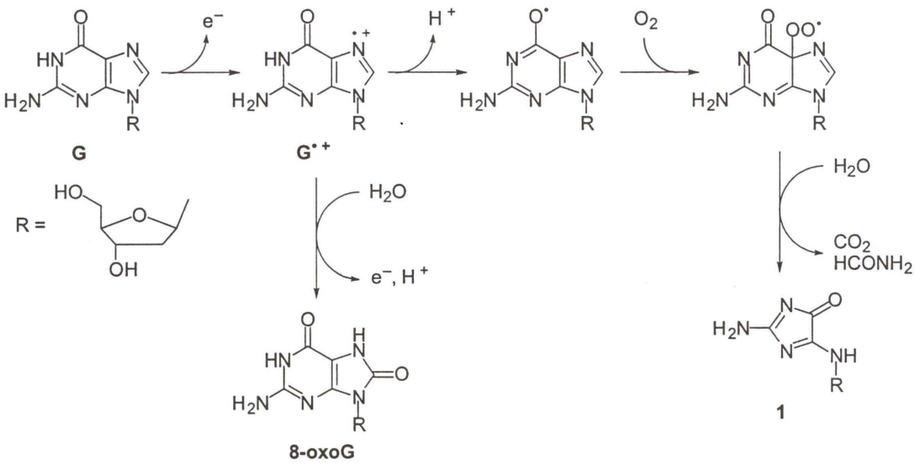

Fig. 2 Electron-transfer oxidation of guanine（G）to form guanine radical cation（$G^{\bullet+}$）and the subsequent reactions

2．反応活性種とDNAとの反応

DNA切断の分子論的な反応機構についてもすでに数多くの優れた研究があり，いくつかの総説が出版されている（Paillous and Vicendo, 1993；Burrows and Muller, 1998；Pogozelski and Tullius, 1998）．ここでは光増感剤の光照射により生成する反応活性種S^*，•OH，および$^1O_2$がDNA糖鎖や核酸塩基にどのように作用するかについて概説する．

上述のように，グアニン（G）は核酸塩基の中でももっとも低い酸化電位（1.29 V *vs.* NHE）をもつため酸化されやすく（Steenken and Jovanovic, 1997），光増感剤の励起状態S^*の還元電位がGの酸化電位よりも高い場合には容易にS^*によって一電子酸化され，ラジカルカチオン$G^{\bullet+}$が生成する．$G^{\bullet+}$はFig. 2に示すように脱プロトン化や水との反応により，それぞれイミダゾロン（**1**）や7,8-ジヒドロ-8-オキソグアニン（8-oxoG）に変換される．これらの化学修飾を受けた核酸塩基は塩基に対して不安定であり，ピペリジン処理により，DNA鎖の切断が引き起こされることが知られている（Burrows and Muller, 1998）．しかし，実際のDNA鎖中のGと単独のGとでは水素結合や溶媒和，そして立体配置などの環境が異なるため，その反応性や反応機構についてはまだ不明な点が残っている．特に，Gの電子移動酸化によるDNA切断の場合には，$^1O_2$の付加場合とは異なり，後述のように特定のG配列に特異的なDNA切断が観測される．

一方，•OHを活性種とするDNA切断は，糖鎖および核酸塩基の両方で非選択的に起こることが知られている．糖にはFig. 3に示すように7つの水素があるが，B型二重鎖DNAの場合には，H-5′とH-4′がDNA鎖の外側に露出しているため，もっとも•OHが近づきやすく，これらの水素が引き抜かれやすいことがわかっている（Pogozelski and Tullius, 1998）．•OHによる水素引き抜きで生成した糖ラジカルはいくつかのステップを経て，DNA鎖の切断へと導かれる（Pogozelski and Tullius, 1998）．DNA糖鎖の水素引き抜きによりDNA切断が起こる場合には，上述のGの電子移動酸化の場合とは異なり，ピペリジンなどの塩基の処理を必要としない．一方，•OHが核酸塩基を攻撃する場合には，非選択的に核酸塩基の芳香環に•OHが付加し，対応する酸化生成物を与える．例として，Gと•OHの反応をFig. 4に示す．•OHはGに付加した後，還元的条件下や酸化的条件下で，それぞれ対応するホルムアルデヒドピリジン体（FAPy-G）や電子移動酸化の場合と同じ生成物である8-oxoGを与え，ピペリジン処理などの塩基の作用でDNA鎖の切断が起こる．

次に，光増感剤の三重項励起状態（$^3S^*$）からO_2へのエネルギー移動により生成した$^1O_2$は，選択的にグアニン塩基（G）に付加することにより8-oxoGを生成し，DNA切断を引き起こすと考えられている（Fig. 5）．

以上のように光増感剤の光照射により生成する活性種

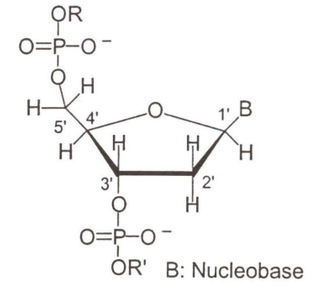

Fig. 3 Seven hydrogens on deoxyribose ring

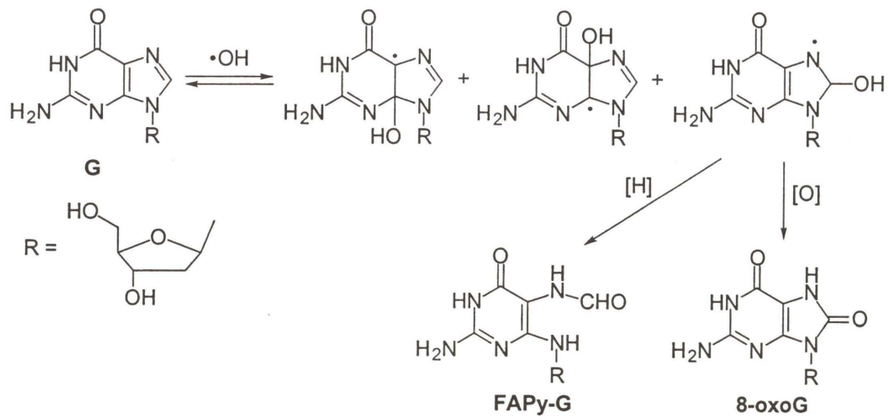

Fig. 4 Addition of hydroxyl radical（•OH）to guanine（G）and the subsequent reactions

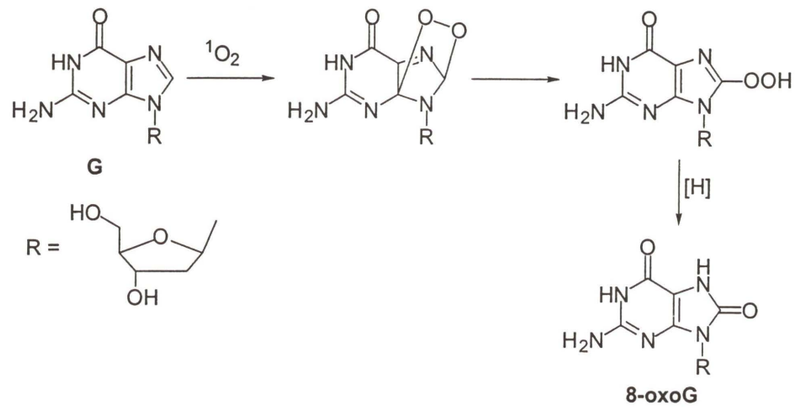

Fig. 5 Addition of sinlet oxygen ($^1O_2$) to guanine (G) to form 8-oxoG

のDNAへの分子論的な作用について述べたが，次に，実際どのような光増感剤がどの活性種を発生してDNAを切断するかについて，代表的な例を示す．

3．DNA切断活性を有する光増感剤

1）電子移動経由

リボフラビンは365 nmの光を照射することでDNA切断活性を示す（Ito et al., 1993）．この場合，5′-GG-3′の5′-Gで選択的に切断が起こること，8-oxoGが生成すること，およびD_2O中で切断活性の増強が観測されないことから，5′-Gからリボフラビンの励起状態への電子移動経由でDNA切断が起こっていると考えられている．

Saitoら（1995）は光DNA切断活性を有するリジン-ナフタルイミド（**2**）を合成し，レーザーフラッシュフォトリシスにより，種々のG配列をもつ2重鎖DNAオリゴマーから**2**の三重項励起状態への電子移動速度を決定し，5′-GGGG＞5′-GGG＞5′-GG-＞5′-AG-, 5′-GA-＞＞-Gの順で電子を放出しやすいことを明らかにしている．また，B型二重鎖DNAオリゴマーのイオン化ポテンシャルを*ab intio*計算によって求めることにより，Gが重なる塩基配列のHOMOは必ず5′側のGに局在化していること，およびもっとも1電子酸化されやすい塩基は，前後をGではさまれたGであることを明らかにしている（Sugiyama and Saito, 1996）．

C_{60}フラーレンも非常にすぐれた光増感剤であり，500 nm以上の可視光照射でG選択的にDNA切断を起こす（Tokuyama et al, 1993；Boutorine et al., 1994；An et al., 1996）．C_{60}は光照射により，一重項励起状態から項間交差により三重項励起状態が生成し，エネルギー移動により効率良く$^1O_2$を生成するため（Terazima et al., 1991；Arbogast et al., 1991），これまでDNA切断の活性種は$^1O_2$であると考えられていた．しかし，Bernsteinら（1999）は酸素非存在下，ベンゾニトリル中で，グアニン誘導体がC_{60}によって光電子移動酸化されることを明らかにし，C_{60}による光DNA切断が電子移動経由で起こり得ることを報告している．

さらに，アントラキノン誘導体（**3**）（Danith et al., 1996），ベンゾトリアゾール誘導体（**4**）（Wender et al., 1996），$[Rh(phi)_2(DMB)]^{3+}$（phi＝フェナントレンキノンジイミン；DMB＝4,4′-ジメチルビピリジン）ロジウム（III）錯体（Hall et al., 1996）などもグアニン残基の光電子移動酸化によりDNA切断を起こすことがわかっている．

2）ヒドロキシルラジカル生成経由

上述のように，$O_2^{\bullet-}$は容易にH_2O_2を経て•OHに変わるため，$O_2^{\bullet-}$を生成する可能性のある化合物のほとんどがDNA切断活性をもつと考えられる．光増感剤Sでは，還元剤による光電子移動還元により生成したラジカルアニオン$S^{\bullet-}$の酸化電位が分子状酸素の還元電位より低い場合に$O_2^{\bullet-}$が生成する．

最近，筆者らはγ-シクロデキストリンに包接させた水溶性C_{60}（C_{60}/γ-CyD）が，O_2と還元剤であるNADH存在下，可視光（532 nm）照射すると，非常に効率良くDNAを切断することを明らかにした（Nakanishi et al., 2001）．また，NADH非存在下ではDNA切断がまったく観測されなかった．レーザーフラッシュフォトリシスによる詳細な速度論的解析やESRスピントラップ実験から，反応中間体として$O_2^{\bullet-}$が生成していることがわかった．すなわち，まずC_{60}は光励起され，項間交差を経て三重項励起状態$^3C_{60}{}^*$に変わる（Fig. 7）．$^3C_{60}{}^*$はNADHに電子移動還元され，ラジカルアニオン$C_{60}^{\bullet-}$を生成する（Fukuzumi et al., 1998；1999）．水溶液中では$C_{60}^{\bullet-}$の酸化電位は分子状酸素の還元電位よりも低いため，$C_{60}^{\bullet-}$からO_2への電子移動が起こり，$O_2^{\bullet-}$が生成する（Miyata et al., 2000）．上述のように$O_2^{\bullet-}$はH_2O_2を経て•OHに変換され，DNA切断を引き起こすと考えられる．実際，このDNA切断はSODで増強され，カタラー

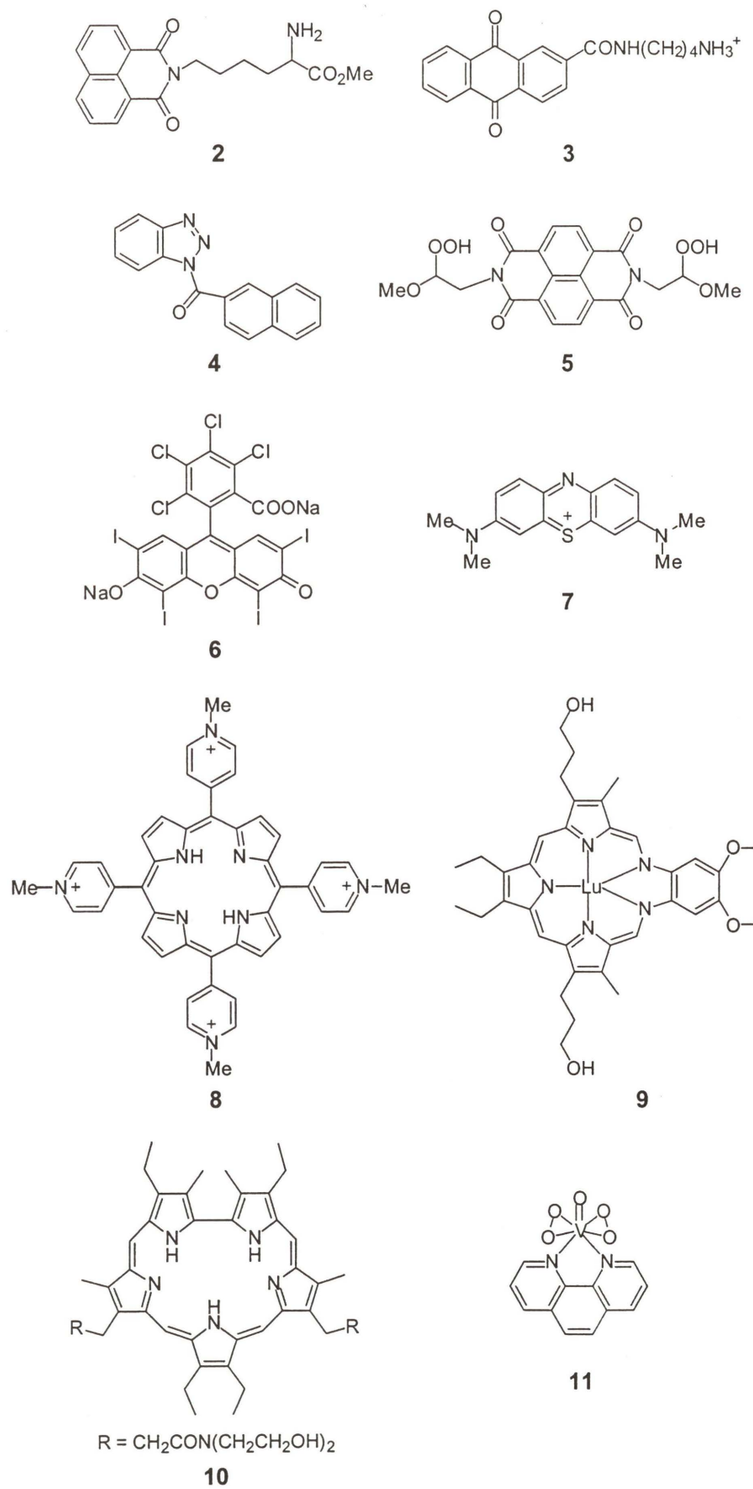

Fig. 6 Photosensitizers which can act as an DNA-cleaving agent

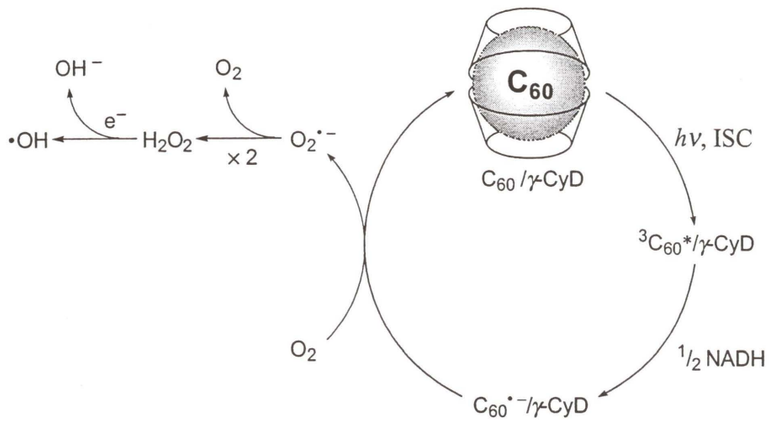

Fig. 7 Mechanism of C_{60}-photosensitized DNA cleavage with NADH and O_2

ゼやアルコール類によって顕著に抑制されることが観測された．この反応系に，$^1O_2$消去剤であるアジ化ナトリウム（NaN_3）を加えてもDNA切断が顕著に抑制されるため，一見，$^1O_2$が活性種であるかのようにみえるが，NaN_3は$^3C_{60}$*自身を効率良く消光することがわかっている．

また，Fig. 1の反応機構とは異なるが，光照射により直接•OHを生成する化合物も知られている．ヒドロペルオキシド誘導体（**5**）は366 nmの光照射により2当量の•OHを生成する（Matsugo et al., 1991）．**5**は光DNA切断活性を示すが，•OHによる非選択的な切断とともに，電子移動経由によるGの酸化も同時に起こっていることが知られている．

3）一重項酸素発生によりDNA切断する化合物

一重項酸素（$^1O_2$）を発生する光増感剤としてもっともよく知られているのは，ローズベンガル（**6**）やメチレンブルー（**7**）などの色素類である．たとえば，メチレンブルーは590 nm以上の可視光を照射することにより非常に効率良く$^1O_2$を発生しG選択的にDNAを切断するが，速度論的解析からその詳細な反応機構が明らかにされている（Buchko et al., 1995）．**8**のような水溶性ポルフィリンも$^1O_2$発生によりDNAを切断する光増感剤である．特に，**9**や**10**のようなポルフィリン類縁体は700 nm以上の可視光照射でも効率良くDNA切断を起こすことから，光線力学療法剤として期待されている（Magda et al., 1995）．$[Ru(bpy)_3]^{2+}$（bpy = 2,2′-ビピリジン）や$[Ru(phen)_3]^{2+}$（phen = 1, 10-フェナントロリン）などのルテニウム（II）錯体は，可視光照射することにより$^1O_2$を発生させ，DNA切断を起こすことが知られている（Kelly et al., 1985；Fleisher et al., 1986）．**11**のようなバナジウム（V）錯体も$^1O_2$発生によりDNA切断を起こすことが報告されているが（Hiort et al., 1996），この場合の$^1O_2$生成は三重項エネルギー移動ではなく，配位子が$^1O_2$として遊離することが知られている（Kwong et al., 1997）．

結　　語

ここで紹介したDNA切断活性をもつ光増感剤はほんの一部であり，これまで非常に数多くの優れた光増感剤が報告されている．中にはポルフィマーナトリウムのようにすでにがんの光線力学療法剤として実用化されているものもある．ヒトゲノムが解読され，今後は特定のDNAの特定の塩基配列を高選択的かつ高効率で切断する試薬の開発がさらに重要な研究課題となるであろう．

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総　説

In vitro 光毒性評価における活性酸素種の影響

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Effects of reactive oxygen species in in vitro phototoxicity assays

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Summary

Three in vitro phototoxicity assays - the photohaemolysis assay, the haemoglobin photo-oxidation assay, and the neutral red uptake assay - were evaluated for use as screening methods in predicting the in vivo phototoxicity of test substances. The photohaemolysis assay evaluates oxygen-dependent membrane damage. The haemoglobin photo-oxidation assay detects haemoglobin oxidation from oxyhaemoglobin to methaemoglobin with UVA irradiation. The neutral red uptake assay evaluates cell survival by assessing the ability of viable cells to take up neutral red dye. Thirty-three test substances were assessed in this study.

The phototoxicity predicted in each assay was compared with that in guinea pigs and in human. The phototoxicity predictions made by the in vitro methods were comparatively good. These results suggest that the three in vitro phototoxicity assays could be used to effectively screen chemicals for phototoxicity.

The effects of reactive oxygen species in two in vitro phototoxicity methods were assessed. Sixteen test substances that indicated positive reaction in the photohaemolysis assay or the 3T3 neutral red uptake assay were evaluated by using scavengers. Of the 16 test substances, 12 indicated the production of singlet oxygen. Nine of these reacted to histidine used as a scavenger of singlet oxygen. In this study we have confirmed that photodynamic mechanisms play a major role in in vitro phototoxicity reactions. These results suggest that the photohaemolysis assay and the 3T3 neutral red uptake assay could be used to evaluate the photodynamic mechanisms of photosensitizing chemicals.

Thus, from the genetic point of view, the production of reactive oxygen species and singlet oxygen responsible for phototoxicity is closely related to the induction of photochemical genotoxicity.

Keywords : in vitro photohaemolysis, 3T3 neutral red uptake phototoxicity assay, reactive oxygen species

緒　　言

光毒性試験は，化学物質に光があたることによって生じる毒性を評価する方法である．生体に対し光毒性を発現する代表的な化学物質には，殺菌剤，抗生物質，色素等がある．また，天然物としては香料，精油等があげられる．局所に対する光毒性評価には従来から，動物の皮膚に化学物質を塗布して光を照射する試験法が用いられている．

光毒性反応は，おもに，化学物質に可視光または紫外線を照射した場合，光照射により化学物質が励起状態となり，そのエネルギーが何らかの形で放出される時に，細胞膜，DNA等を中心に細胞全体を障害することで発現すると考えられている（Moysan et al., 1994）．この反応は，ラジカル反応であるタイプⅠと一重項酸素を発生するタイプⅡ，さらに励起された化学物質が直接作用するタイプⅢの3つに分類される（Laustriat, 1986；Foote, 1991）．タイプⅠでは，反応時に基質（生体膜，脂質等）が存在すると，基質との間で電子，水素原子のやり取りによりフリーラジカルが発生し，これに環境中に存在する酸素が反応し，スーパーオキシドアニオン，ヒドロキシラジカル，H_2O_2等を発生する．これらの活性酸素種により光毒性が発現される．タイプⅡでは，励起状態の物質が基底状態に戻る時に，そこに存在する酸

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Table 1 In vitro methods for phototoxicity testing

1 Method for screening purpose
  1) Methods using monolayer culture cells[a]
    ① Cells
    Mouse fibroblast (Balb/c 3T3 cells clone 31)
    Human kerationcytes (A431 human epidermal cell line)
    Hepatocytes
    Human lymphocytes
    ② End points of assay
    Neutral red uptake assay
    MTT reduction assay
    DNA synthesis
    LDH leakage assay
  2) Methods using micro-organisms[b]
    Candida albicans, Candida utilis, Saccharomyces cerevisiae
  3) Three-dimensional skin models assay[c]
2 Methods for evaluating mechanisms
  1) Methods for evaluation membrane damage[d]
    Red blood cell photohaemolysis
  2) Methods for evaluating the effects to protein[e]
    Haemoglobin photo-oxidation
    Photobinding to protein (human serum albumin)
  3) Others[f]
    Linoleic acid peroxidation
    Histidine photo-oxidation

References :
a : Freeman et al., 1970 ; Lock and Friend, 1986 ; Maier et al., 1991 ; Lasarow et al., 1992 ; Spielmann et al., 1994b
b : Daniels, 1965 ; Sugiyama et al., 1994b
c : Edwards et al., 1994 ; Augustin et al., 1997
d : Khan and Fleischaker, 1971 ; Hetherington and Johnson, 1984 ; Pape et al., 1994a ; Sugiyama et al., 1994a
e : Barratt and Broun, 1985 ; Pendlington and Barratt, 1990 ; Winterbourn, 1985 ; Traynor et al., 1996
f : Lovell and Sanders, 1990 ; Lovell, 1993

素を介して一重項酸素を発生し，光毒性が発現される．一重項酸素の反応性は高く，電子密度の高い部位と反応し，それを酸化する．反応性の高い生体成分として，histidine，tryptophan，不飽和脂肪酸，リン脂質，コレステロール等があげられる（Doleiden et al., 1974 ; Suwa et al., 1978 ; 園田ら，1988）．これらの活性酸素種の中では，一重項酸素，ヒドロキシラジカルの反応性が高い．また，これとは別に光照射により励起された化学物質自体が直接基質と反応し光毒性を示す場合があり，この反応をタイプⅢ反応と呼んでいる（Foote, 1976 ; Ranadive et al., 1993）．

これらの光毒性のメカニズムを踏まえて，最近では，多様なin vitro光毒性評価法が開発されている．In vitro光毒性試験法は，その目的から，1）光毒性物質をスクリーニングすることを目的とした評価法，2）光毒性メカニズムの検討を目的とした評価法に大別される（Spielmann et al., 1994a）（Table 1）．

われわれは，生体膜に対する作用およびタンパク質の光過酸化を指標とした評価法である赤血球溶血試験，ヘモグロビン光過酸化試験と，スクリーニング評価法として開発された培養細胞を用いた試験法であるニュートラルレッド取り込み法について，光源としてUVAを用いてin vivo光毒性結果との対応を評価した．さらに主な活性酸素種のin vitro光毒性に対する影響について，各活性酸素種の消去剤を用いて検討した．また一重項酸素の発生の測定も実施した（Okamoto et al., 1999a ; Okamoto et al., 1999b）．

本稿ではin vitro光毒性評価とin vivo結果との対応性，およびin vitro光毒性に対する活性酸素種の影響を中心に概説する．

1．In vitro光毒性評価とin vivo結果の比較

赤血球光溶血試験法（Hetherington and Johnson, 1984 ; Sugiyama et al., 1994a）は，光による生体膜破壊を指標としたin vitro光毒性試験法である．本検討では，ヒツジ赤血球を用い，被験物質を添加してUVA照射した時に放出されたヘモグロビンの吸光度を測定し，UVA照射と未照射の差から光溶血%を求め，光溶血性を評価した．

ヘモグロビン光酸化試験法（Pape et al., 1994a）は，ヘモグロビンを用い，そのタンパク光過酸化を指標とした試験法である．本検討では，ヒツジ赤血球から得られたヘモグロビン溶液に被験物質を添加し，UVA照射した時に生じたメトヘモグロビンの吸光度を測定し，UVA照射と未照射の差から光過酸化%を算出し評価した．UVA照射量は両試験とも30 J/cm^{2}に設定した（照射装置M-DMR-100：東芝FL. 32S BLB：300-400 nm：UV強度4.3 mW/cm^2）．

培養細胞を用いた試験法は，ニュートラルレッド取り込み法（NRU PT assay）（Spielmann et al., 1994b）を用い，UVA照射と非照射から，それぞれ50%細胞生存率（IC_{50}）を求め，EU/COLIPAの方法（Spielmann et al., 1998b）に従ってphoto irritation factor（PIF値）{(IC_{50}；UV_-)/(IC_{50}；UV_+)} を算出し，カットオフポイントをPIF値5として評価した．本検討では，Balb/3T3 A31-1-1およびNB1RGBの2種の細胞を用いた．UVA照射量は5 J/cm^{2}に設定した（照射装置Bio-Solar simulator Model WXS UVA 320-400 nm：UVカットフィルター使用UV強度：8.3 mW/cm^2）．

一重項酸素の測定はArakaneら（1996）の方法に準じ被験物質をchloroformに溶解し，UVA領域のアルゴンレーザーを照射した時に発生する一重項酸素を1268 nmの特異発光量として検出した．

殺菌剤，香料を含む33種の被験物質に対するin vitro試験法によるモルモットおよびヒト結果に対する対応性は，両者ともに良好であった（Table 2，3）．特に，赤血球光溶血試験法とBalb/3T3 A31-1-1によるNRU PT assayが全体に良好な対応性を示した．また，一重項酸素の測定のみでもin vitro光毒性をある程度予測できることがわかり，一重項酸素の光毒性反応への関与の大きさが示唆された．

Table 2 In vitro and in vivo phototoxicity assay results

Test substances	in vitro Phototoxicity methods: Photocytotoxicy 3T3	NB1RGB	Photohaemolysis	Hb photo-oxidation	Singlet oxygen emission[a]	in vivo data (guinea pig)	in vivo[b] data (human)
Antimicrobials							
3,4,4′-Trichlorocarbanilide	−	−	−	−	−	−	
3,4-, 5′-Tribromosalicylanilide (TBSA)	+	+	−	+	+	−	
5-Bromo-4′-chlorsalicylanilide	+	−	−	−	+	−	
Bithionol	+	+	+	+	−	−	+, A
Chlorhexidine dihydrochloride	−	−	−	−	−	−	−
Hexachlorophene	−	−	+	−	*+	+	(+ −) ?
Fragrances							
5-Methoxypsoralen (5-MOP)	+	−	−	−	+	+	+
6-Methylcoumarin (6-MC)	−	−	−	+	+	−	+, A
8-Methoxypsoralen (8-MOP)	+	−	−	−	+	+	+
Benzyl salicyrate	−	−	−	−	−	−	
Cinnamaldehyde	−	−	−	−	+	−	−, A
Cinnamyl alcohol	−	−	−	−	−	−	
Galaxolide (50% B.B.)	−	−	+	−	**−	+	
Isoeugenol	−	−	+	+	**−	−	
Jasmine Mix	−	−	+	−	**+	−	
Musk ambrette	+	+	−	−	−	−	(+ −), A
Musk ketone	−	−	−	−	−	−	
Oil of Bergamot	−	−	+	+	**+	+	+
Phantolide	−	−	+	+	+	+	
UV Absorbers							
2-Ethyihexyl-p-methoxycinnamate	−	−	−	−	−	−	−
2-Ethylhexyl-p-dimethylaminobenzoate	−	−	−	−	−	−	
2-Hydroxy-4-methoxybenzophenone	−	−	−	−	−	−	(+ −)
4-Isopropylebenzoylmethan	−	−	−	−	−	−	
4-t-Butyl-4-methoxydibenzoylmethane	−	−	−	−	−	−	
p-Amino benzoic acid	−	−	−	−	−	−	(+ −) ?, A
Drugs							
Chlorpromazine HCL	+	+	+	+	+	+	+, A
Piroxicam	−	−	−	−	+	−	−
Other chemicals							
Acridine	+	+	+	+	+	+	+
Anthracene	+	+	+	+	+	+	+
Anthraquinone	−	−	+	+	+	−	
L-Histidine	−	−	−	−	−	−	−
Thiourea	−	−	−	−	−	−	−, A
Sodium lauryl sulfate	−	−	−	−	−	−	−

+ : phototoxic or singlet oxygen emission positive, − : non-phototoxic or singlet oxygen emission negative
(+ −) : positive, but insufficient data, ? = unclear
Hb : haemoglobin, A : photo allergen, B.B : benzyl benzoate solution
a : singlet oxygen emission (output power : 70 mW)
the vehicle used : chloroform, the concentration used : 0.1 mM
* : the test concentration : 1.0%, ** : the test concentration : 0.1%
b : Spielmann et al., 1994a, ; Spielmann et al., 1998a

In vitro光毒性結果によるモルモットin vivo結果の予測性パラメーター（Balls et al., 1990）の解析では，positive predictive valueが全体に低く，false positiveが多いことがわかった．特に3,4,5′-tribromosalicylanilide (TBSA)，bithionol，musk ambrette，6-methylcoumarin (6-MC) は，各試験において，それぞれfalse positiveを示した．これらはすべて光感作性物質として知られている物質である（Kligman, 1966 ; Kaidbey and Kligman, 1978 ; Kaidbey and Kligman, 1980 ; Cronin, 1984）．TBSAは光タンパク結合性を示すことが報告されている（Barratt and Brown, 1985 ; Pendlington and Barratt, 1990）．6-MCでは，光酸化反応のみ認められた．Papeら（1994a）は，6-MCは光細胞毒性を示さないが，明らかなヘモグロビン光酸化，光タンパク結合を示したと報告しており，このことが，6-MCの持つ光感作性に関与している可能性を示唆していると報告している．このよ

Table 3 Prediction of phototoxic effects in the in vivo data by in vitro assays

Method	Sensitivity	Specificity	Predictive parameters[a] (value : %) Positive predictive value	Negative predictive value	Equivalence
The comparison with guinea pig data (n = 33)					
Balb/3T3	56	83	56	83	76
NB1RGB	33	88	50	78	73
Photohaemolysis	78	83	64	91	82
Hb photo-oxidation	56	79	50	83	73
Singlet oxygen emission	89	71	53	94	76
The comparison with human data (n = 19)					
Balb/3T3	58	100	100	58	74
NB1RGB	42	100	100	50	63
Photohaemolysis	50	100	100	54	68
Hb photo-oxidation	50	100	100	54	68
Singlet oxygen emission	67	71	80	56	68

a : Calculations were conformed to the description by Balls et al (1990).
Sensitivity (TP/TP + FN), Specificity (TN/TN + FP)
Positive predictive value (TP/TP + FP), Negative predictive value (TN/TN + FN)
Equivalence (TP + TN/TP + TN + FP + FN)
TN : true negative, TP : true positive, FN : false negative, FP : false positive

うに光感作性物質がin vitro試験法でfalse positiveとなることは，Spielmannら（1998a）も指摘している．これは，in vitro評価法が，その反応メカニズムから，これらの光感作性物質と光毒性物質を識別できないという特徴を持つことを示している．

一方，8-methoxypsoralen（8-MOP）等のpsoralen類は，既知の光毒性発現物質であるが，これらの光毒性を検出できた試験系はBalb/3T3を用いたNRU PT assayのみであり，培養細胞を用いた方法の優位性が示唆された．また，phantolide等3種の香料の光毒性は，培養細胞法では検出できなかったが，光溶血試験法またはヘモグロビン光酸化試験法で検出できた．

したがって，in vitro光毒性試験法の検出力を高めるためには，それぞれのin vitro光毒性試験法が持つ特徴とそのメカニズムを考慮したうえで，その組み合わせ（battery）を検討することが重要であることが示唆された．

2．In vitro光毒性評価に対する活性酸素消去剤の影響とそのメカニズムの考察

In vitro光毒性反応に対する活性酸素種の影響について検討するため，in vitro光毒性を示した被験物質について各活性酸素種の消去剤を添加した時の抑制効果を検討した．

一重項酸素の消去剤は，一重項酸素への特異性が高いhistidine（10 mM）を採用した（Krajic, 1986）．タイプⅠ反応の評価では，反応性の高いラジカルであるヒドロキシラジカルとH_2O_2をその指標とし，消去剤としてmannitol（10 mM），catalase（1400 units/ml）を採用した．mannitolやcatalaseはその他のラジカルや一重項酸素に対する消去能も持つが，ヒドロキシラジカルとH_2O_2への反応性が勝っていることから，消去剤として用いた．さらに，活性酸素種全般に作用するものとしてsuperoxide dismutase（SOD）（250 units/ml）の消去能についても同時に検討した（Kimura, 1991）．

活性酸素種の影響は，各被験物質に対して活性酸素消去剤を添加した時の消去剤無添加時（standard）のin vitro光毒性に対する抑制率として評価した（Table 4, 5）．各活性酸素消去剤の寄与についてTable 6にまとめた．

1）赤血球光溶血試験法による結果

各被験物質の各濃度における光溶血%と活性酸素消去剤の影響をTable 4に示した．

今回の検討では，7種の被験物質でhistidineによる抑制効果が認められた．これらのうち，galaxolideを除く6種に一重項酸素の発生が確認されている．特にacridineでは，histidineによる明らかな光溶血反応の阻止が確認された．また，anthraceneは，SOD以外の消去剤で抑制効果が認められた．bithionolでは，histidineでの抑制効果は認められず，catalaseによる弱い抑制効果が認められた．

2）培養細胞を用いた試験法による結果

各被験物質の各濃度における生存率と活性酸素消去剤の影響をTable 5に示した．

培養細胞による評価では，5種の被験物質がhistidineによる抑制効果を示した．これらのすべてに一重項酸素の発生が確認されている．特にanthracene，acridine，TBSA，5-bromosalicylanilideでは強いhistidineによる効果が観察された．

一方，bithionol，musk ambretteではhistidineの効果は認められず，それ以外の消去剤の効果が認められた．

Table 4 The effect of scavengers in photohaemolysis assay

Test substances	Conc. evaluated (μg/ml)	Photohaemolysis (%) : Standard	Inhibition effects of scavengers[a] Histidine	Mannitol	SOD	Catalase
Antimicrobials						
Bithionol	25	60	−	−	−	+ −
	50	61.7	−	−	−	−
	100	64.4	−	−	−	−
Hexachlorophene	6.25	34.2	−	−	−	+ −
	12.5	10	−	−	−	+ −
	25	9.6	−	−	−	+ +
Fragrances						
Galaxolide (50 % B.B.)	250	14.9	+	−	+ −	+ −
	500	47.1	+ −	+ −	+ −	+ −
	1000	79.5	−	+ −	−	−
Isoeugenol	250	0.0	b	b	b	b
	500	49.6	−	−	−	−
	1000	0.0	b	b	b	b
Jasmine mix	250	12.1	+ −	−	+ −	+ +
	500	37.1	−	−	+ −	+ +
	1000	13.3	−	+ −	+	+
Oil of bergamot	250	22	−	−	+ −	−
	500	40.2	−	−	+ −	−
	1000	35.3	+ −	−	+ −	−
Phantolide	62.5	9.6	+	−	−	−
	125	36.1	+	−	−	−
	250	62.1	+ −	−	−	−
Drugs						
Chlorpromazine HCL	12.5	12.8	+	+ −	−	−
	25	37.1	+	+ −	−	−
	50	63	−	−	−	−
Other chemicals						
Acridine	25	50.6	+ +	−	−	−
	50	54.1	+ +	−	−	−
	100	58.8	+ +	+ −	+ −	−
Anthracene	12.5	17.2	+ −	+ −	−	+
	25	17.5	−	+ −	−	−
	50	19.0	+ −	+ −	−	−
Anthraquinone	1000	20.0	−	−	−	−
	500	21.0	−	−	−	−
	250	19.0	−	−	−	−

The photohaemolysis (%) were indicated as the mean of two experiments.
a : effects of scavengers in photohaemolysis :
+ + : 90% decrease from standard photohaemolysis (%), + : 50% decrease, + − : 25% decrease,
− : less than 25% decrease
b : The photohaemolysis were not obtained.
B.B : benzyl benzoate solution

8-MOP，5-methoxypsoralen（5-MOP）では，一重項酸素を発生していたが，各活性酸素消去剤による抑制効果は認められなかった．

3）In vitro光毒性評価に対する活性酸素消去剤の影響

今回のin vitro光毒性評価で陽性を示した被験物質16種のうち，何らかの活性酸素種消去剤の効果が認められたものは12種であり，in vitro光毒性に対する活性酸素種の関与が大きいことが示唆された（Table 6）．また，タイプⅠの反応を生じたものでは，mannitol，catalaseの両方の効果が認められたものが多かった．これは，catalaseのヒドロキシラジカル消去能が作用したためと考えられる．また，SODが単独で効果を示したものはなかった．これらの結果は，タイプⅠがラジカル反応であり，主反応を生ずる活性酸素種の特定が本試験系では困難であることを示唆している．

一方，活性酸素消去剤の効果が認められなかったものは，8-MOP，5-MOP，isoeugenol，anthraquinoneであった．Isoeugenolはin vivoで皮膚感作性を持つことが知られているが光毒性の報告はない（Koch et al., 1973）．

8-MOPおよび5-MOPはpsoralen類として類似の化学構造を持つものであるが，これらは，in vitro光毒性では細胞試験法のみに陽性であり，光溶血反応，ヘモグロビン光酸化反応は認められなかった．

Table 5 The effects of scavengers in 3T3 NRU assay

Test substances	Conc. evaluated (μg/ml)	Cell survival (% ± SD) : Standard	The effects of scavengers[a] Histidine	Mannitol	SOD	Catalase
Antimicrobials						
3,4-, 5′-Tribromosalicylanilide (TBSA)	2.5	50.6 ± 39.4	++	+	++	++
	5	39.8 ± 40.6	++	+	−	+
	10	18.1 ± 20.6	++	+−	−	+
5-Bromo-4′-chlorsalicylanilide	2.5	44.7 ± 27.9	++	+	+−	++
	5	16.4 ± 10.2	++	−	++	++
	10	5.3 ± 4.1	++	+−	−	++
Bithionol	1.25	67.3 ± 25.4	−	−	−	+−
	2.5	43.3 ± 31.4	−	+	+	++
	5	30.0 ± 27.3	−	++	+−	++
Fragrances						
5-Methoxypsoralen (5-MOP)	2.5	43.0 ± 13.2	−	−	−	−
	5	40.3 ± 11.0	−	−	−	−
	10	37.5 ± 12.4	−	−	−	−
8-Methoxypsoralen (8-MOP)	25	42.9 ± 1.2	−	−	−	−
	50	47.6 ± 12.3	−	−	−	−
	100	39.8 ± 5.2	−	−	−	−
Musk ambrette	6.25	73.1 ± 27.3	−	+−	+−	−
	12.5	58.9 ± 22.0	−	−	−	−
	25	49.1 ± 9.5	−	−	−	−
Drugs						
Chlorpromazine HCL	0.25	71.7 ± 13.2	−	−	−	+−
	0.5	58.3 ± 27.6	+−	+−	−	+−
	1	31.8 ± 23.5	+	+	+	+
Other chemicals						
Acridine	0.25	69.8 ± 18.8	−	−	−	−
	0.5	47.9 ± 20.2	+−	−	−	−
	1	9.2 ± 7.3	++	−	−	−
Anthracene	0.0125	47.6 ± 27.5	++	+	+−	++
	0.025	23.3 ± 10.0	+	++	+	+
	0.05	9.2 ± 7.3	++	++	++	++

The cell survival (%) were indicated as the mean score with SD.
a : the effects of scavengers
++ : 100% increase from standard cell survival (%), + : 50% increase, +− : 25% increase, − : less than 25% increase

8-MOPの光毒性発現には，標的細胞のDNA，膜脂質およびタンパクへの結合能の関与が報告されている（Song and Tapley, 1979 ; Kittler, 1988 ; Averbeck, 1989）．8-MOPの光毒性発現に関する主な反応部位はDNAであるとされており，UVA照射によりDNAのピリミジン塩基と結合しDNA合成阻害を生じると考えられている（松尾，1997）．一方，ケラチノサイトでの検討によると，細胞膜にも8-MOPのレセプタータンパクがあり，UVA照射によりそれと結合する．特にEGFレセプターに作用し，正常な表皮のターンオーバーを障害することが報告されている（Laskin, 1994）．8-MOPが光溶血反応を生じなかったのは，8-MOPの主作用が細胞膜（脂質）損傷ではないためと推察できる．Papeら（1994a）は，8-MOPには，光溶血反応は認められなかったが，ごく弱い光酸化反応が認められたと報告している．さらに，ヒト血清アルブミンに対する光タンパク結合が陽性であったとも報告している（Pape et al., 1994b）．今回の8-MOPの細胞毒性試験結果を詳細に検討すると，その程度は濃度依存的ではなく，高濃度でもほぼ一様に約30～40％の生存率を示し，この点は，Papeら（1994b）の報告とも一致した．これらの特徴は，8-MOPが，細胞の代謝，増殖能等に関連した作用を有していることを示唆している．

われわれの結果では，8-MOPは，一重項酸素は発生しているが，活性酸素消去剤の効果が認められず，そのin vitro光毒性作用に対する活性酸素種の関与は検出されなかった．8-MOPをsqualeneと混合し，UVAを照射した実験において，8-MOPはsqualeneの過酸化を生ぜず，一重項酸素の発光量が検出限界以下であったことから，8-MOPの反応への一重項酸素の関与を否定している報告がある（松尾，1997）．一方，UVAとUVBの混合照射条件において，histidineの酸化能を評価したところ，8-MOPは弱いhistidine酸化能を持っていたことから，一重項酸素が関与しているという報告がある（Pape et al, 1994b）．今回の検討では，UVA照射により，8-MOPは一重項酸素を発生していた．しかし，発生した一重項酸素のin vitro光毒性に対する関与は検出できなかった．

Table 6 The major factor of photodynamic reactions in two in vitro phototoxicity

Test substances	Types of reaction[a] Photohaemolysis Phototoxicity	Photohaemolysis Major factor	3T3 phototoxicity assay Phototoxicity	3T3 phototoxicity assay Major factor
Antimicrobials				
3,4-, 5′-Tribromosalicylanilide (TBSA)	n		p	Ⅰ, Ⅱ
5-Bromo-4′-chlorsalicylanilide	n		p	Ⅰ, Ⅱ
Bithionol	p	(Ⅰ)	p	Ⅰ
Hexachlorophene	p	(Ⅰ)	n	
Fragrances				
5-Methoxypsoralen (5-MOP)	n		p	b
8-Methoxypsoralen (8-MOP)	n		p	b
Galaxolide (50% B.B.)	p	(Ⅰ, Ⅱ)	n	
Isoeugenol	p	b	n	
Jasmine mix	p	Ⅰ, (Ⅱ)	n	
Musk ambrette	n		p	(Ⅰ)
Oil of bergamot	p	(Ⅰ, Ⅱ)	n	
Phantolide	p	Ⅱ	n	
Tar ingredients				
Acridine	p	(Ⅰ), Ⅱ	p	Ⅱ
Anthracene	p	(Ⅰ, Ⅱ)	p	Ⅰ, Ⅱ
Anthraquinone	p	b	n	
Drugs				
Chlorpromazine HCL	p	(Ⅰ), Ⅱ	p	Ⅰ, Ⅱ

a : types of reaction :
type Ⅰ : reactions of superoxide anion, hydrogenperoxide, hydroxyradical
type Ⅱ : reactions of exited singlet oxygen
Parentheses indicate the weak effects.
b : The effects of scavengers couldn't be obtained. p : phototoxic in vitro, n : non-phototoxic in vitro
B.B : benzyl benzoate solution

Anthracene の光溶血は既知であり，それが酸素依存であること，さらに溶血したヘモグロビンが変性することが報告されている（野中ら，1992）．さらにその反応はタイプⅡであると報告されている．今回の結果では，タイプⅠ，タイプⅡの両方の反応を示した．

Chlorpromazineは，タイプⅠ，タイプⅡの両方の反応を示した．Chlorpromazineの反応部位はDNA損傷ではなく，タンパク，脂質であり，脂質への作用には一重項酸素の関与が指摘されている（Kochevar, 1981）．8-MOPと同様のsqualeneによる過酸化実験において，squaleneの過酸化が確認され，さらにその反応は重水中で減少したことから一重項酸素の関与が報告されている（松尾，1997）．今回の結果も，histidineによる抑制効果が顕著であり，chlorpromazineのおもな作用が一重項酸素による可能性が示唆された．

結　　語

In vitro光毒性評価における各活性酸素種の影響について，各活性酸素種消去剤を用いて検討した．その結果，一重項酸素を発生しているものでは，histidineによる抑制効果が大きく，それらの光毒性反応の主体は一重項酸素の関与するタイプⅡの反応であることが示唆された．また，一重項酸素を発生しないものでも，その他の活性酸素種消去剤による反応の抑制が認められ，in vitro光毒性における活性酸素種の影響が大きいことが確認された．

また，培養細胞を用いた方法は，タイプⅠ，タイプⅡの反応の識別に加えて8-MOP，5-MOPのような反応も識別評価できることから，この方法を中心に試験法を組み合わせることで，光毒性発現のメカニズム評価が可能

であることが示唆された．

このように光毒性の発現は，紫外線によって励起された化学物質によって生じた一重項酸素やその他の活性酸素種に起因している可能性が大きいことから，DNAを標的とする遺伝的傷害の可能性もあり，光毒性と光遺伝毒性の密接な関連が示唆される．

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総　説

フルオロキノロン系合成抗菌剤の光遺伝毒性

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Photogenotoxicity of fluoroquinolone antibacterial agents

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Summary

Fluoroquinolone antibacterial agents are widely used for their broad and strong antimicrobial spectrum. Recent reports on their photogenotoxicity indicated that some fluoroquinolones have potent genotoxicity under UV or visible light irradiation. This review paper describes the characteristics of photogenotoxicity of fluoroquinolones, mechanisms, structure-activity relationship and a predictivity of photocarcinogenicity.

Keywords : photogenotoxicity, fluoroquinolone

緒　言

キノロン系合成抗菌剤は，その優れた抗菌作用と安全性から感染症領域で広く使われている．キノロン系合成抗菌剤の開発の歴史は1962年に米国のSterling-Winthrop研究所で発見されたnalidixic acid（NA）に始まる．NAはnaphtyridine環を基本骨格として，主としてグラム陰性菌に抗菌作用を示し，当時よく使われていたサルファ剤や抗生物質の耐性菌に対しても有効性を示した．NAは経口吸収性には優れていたが，体内では大部分が抗菌活性のないグルクロン酸抱合体に代謝されるため，適応が局所感染症に限定されていた．1970年代に入ると代謝面および抗菌活性ともにNAより優れたキノロン剤が開発され，1978年にはquinoline環を基本骨格として，抗菌活性の増強および代謝面での改善を目的として，C6位およびC7位にそれぞれフルオロ基（-F）およびpiperazinyl環を導入したnorfloxacin（NFLX）が開発された．C6位のフルオロ基導入により抗菌活性が約10倍以上も増強したことから，その後C6位にフルオロ基をもつ様々なキノロン剤が開発され，これがフルオロキノロンとして現在のキノロン剤の主流を占めるようになった（Fig. 1）．

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フルオロキノロンの光遺伝毒性が最初に報告されたのは1990年代後半からであるが，それより以前から光毒性phototoxicityがあることが報告されていた．光毒性では生体内組織侵襲による二次反応が加わることになる．ここでは，フルオロキノロン光遺伝毒性の特性について概説する．なお，本文に使用したキノロンの一般名と略称は，Fig. 1およびTable 1を参照されたい．

1．光遺伝毒性発現のメカニズム

光遺伝毒性が発現するメカニズムとしては，①UVB（290-320 nm）照射とDNAの直接作用によるピリミジンダイマーの形成，および②UVB，UVA（320-400 nm）あるいは可視光（400-700 nm）と薬物の光反応生成物による活性酸素・フリーラジカルの生成が主要なものとして考えられている．前者を光遺伝毒性photogenotoxicity，後者を光化学的遺伝毒性photochemical genotoxicityと区別する場合もあるが，ここでは両者を区別せずに光遺伝毒性と呼ぶことにする．

薬物と光照射によるDNA損傷のメカニズムには3つのルートがあることが明らかにされている．すなわち，①電子移動反応経由，②ヒドロキシラジカル生成経由，および③一重項酸素生成経由，である（中西および宮田，2001）．詳細については本特集号の中西および宮田の総説を参照されたい．

フルオロキノロンについて光照射によるDNA損傷のメカニズムを明らかにしようとする研究がいくつか報告

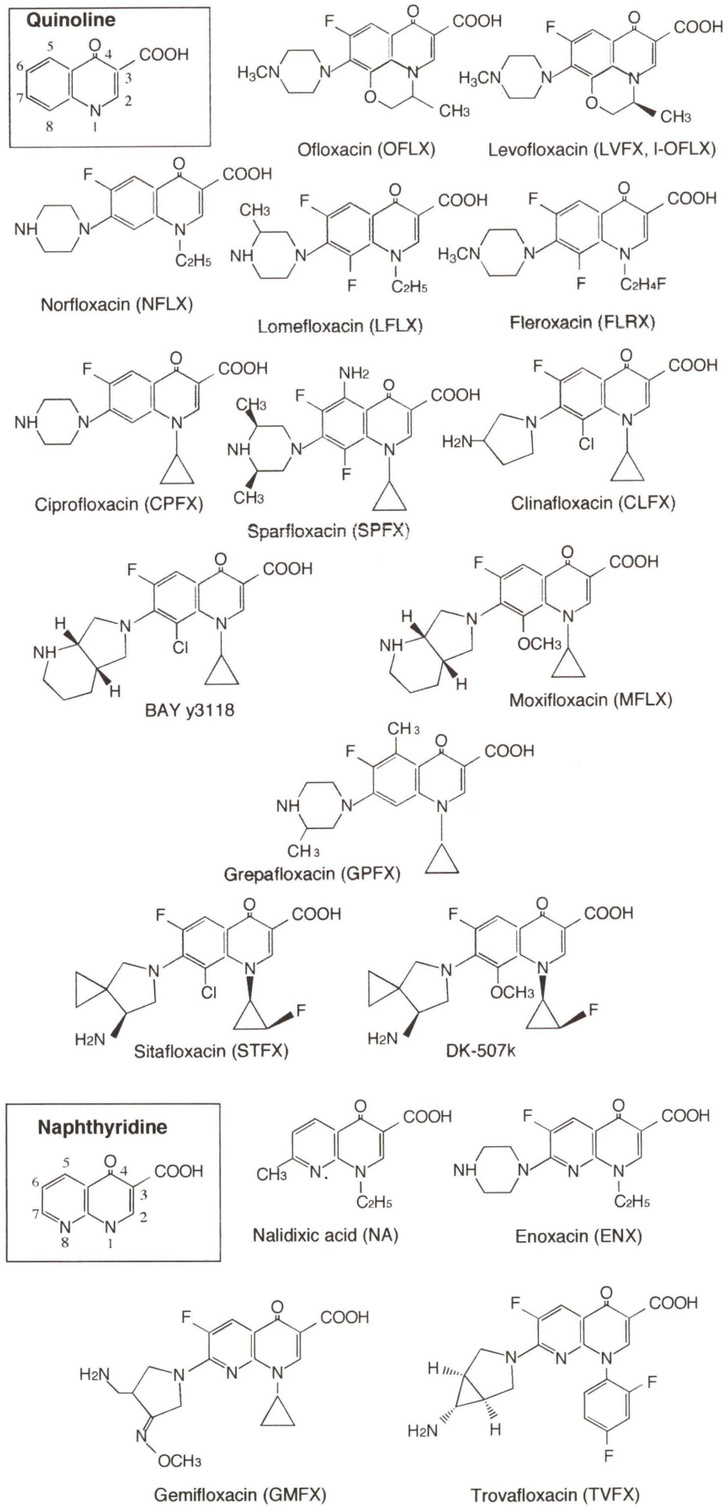

Fig. 1 Structure of quinolones

Table 1 Photogenotoxicity studies of fluoroquinolone antibacterial agents

Quinolones	DNA damage[1] in vitro	DNA damage[1] in vivo	Gene mutation Ames	Gene mutation D7	CA in vitro	References
Clinafloxacin (CLFX)	+	+			+	Bulera et al. (1999)
					+[2]	Snyder & Cooper (1999)
					+	Itoh et al. (2001)
Ciprofloxacin (CPFX)	+		+	−	+	Chételat et al. (1996)
	+					Reavy et al. (1997)
	+	+			−	Bulera et al. (1999)
					+[2]	Snyder & Cooper (1999)
Enoxacin (ENX)	w +[3]					Martinez & Chignell (1998)
					w +[2]	Snyder & Cooper (1999)
					+	Itoh et al. (2001)
Fleroxacin (FLRX)	+		+	−	+	Chételat et al. (1996)
	+					Reavy et al. (1997)
	+[3]					Martinez & Chignell (1998)
					+	Itoh et al. (2001)
Gemifloxacin (GMFX)					+	Itoh et al. (2001)
Grepafloxacin (GPFX)					+[2]	Kersten et al. (1999)
					+	Itoh et al. (2001)
Levofloxacin (LVFX)					+	Itoh et al. (2001)
Lomefloxacin (LFLX)	+		+	−	+	Chételat et al. (1996)
	+					Reavy et al. (1997)
	+[3]					Martinez & Chignell (1998)
	+	+			+	Bulera et al. (1999)
					+[2]	Kersten et al. (1999)
					+[2]	Snyder & Cooper (1999)
	+					Traynor & Gibbs (1999)
					+	Itoh et al. (2001)
	+[4]			+		Marrot et al. (2001)
Moxifloxacin (MFLX)					w +	Itoh et al. (2001)
Nalidixic acid (NA)	−					Reavy et al. (1997)
	+[3]					Martinez & Chignell (1998)
					+[2]	Snyder & Cooper (1999)
Norfloxacin (NFLX)	+					Reavy et al. (1997)
	w +[3]					Martinez & Chignell (1998)
					w +[2]	Snyder & Cooper (1999)
Ofloxacin (OFLX)					w +[2]	Snyder & Cooper (1999)
Sitafloxacin (STFX)					+	Itoh et al. (2001)
Sparfloxacin (SPFX)					+[2]	Snyder & Cooper (1999)
					+	Itoh et al. (2001)
Trovafloxacin (TVFX)					w +[2]	Snyder & Cooper (1999)
					−	Itoh et al. (2001)
BAYy3118	+					Reavy et al. (1997)
					+[2]	Kersten et al. (1999)
	+[4]			+		Marrot et al. (2001)
DK-507k					−	Itoh et al. (2001)

Abbreviations：Ames：Ames test (*Salmonella typhimurium* TA104), D7：*Saccharomyces cerevisiae* D7
CA：Chromosome aberration, +：Positive, w +：Weak positive, −：Negative
[1] Comet assay, [2] in vitro micronucleus test, [3] pBR322, [4] Comet assay and pBR322

されている．

Martinezら（1997）は，C8位にフルオロ基をもつFLRXおよびLFLX，C8位にフルオロ基をもたないCPFXおよびNFLXについて量子反応論的な解析を行い，光照射によりフルオロ基が遊離することによりC8位の原子が活性化し，DNAと直接反応して遺伝毒性が発現するとし，FLRXおよびLFLXがフルオロ基を1つしかもたないCPFXやNFLXに比べて光遺伝毒性が強く発現することを説明した．また，彼らはENX，FLRX，LFLX，NAおよびNFLXについて，プラスミドpBR322を用いてDNA損傷を定量的に解析し，C6およびC8位にフルオロ基をもつFLRXおよびLFLXは，C6位のみにフルオロ基をもつNFLXに比べDNA損傷作用が約10倍強くなることを明らかにし，光遺伝毒性発現には活性酸素種は必ずしも必要ではなく，C8位原子の活性化が重要な役割を果たしているとした（Martinez and Chignell, 1998）．

一方，活性酸素・フリーラジカルがフルオロキノロン

の光遺伝毒性の原因とする研究報告も多い．Vernaら（1998）は，ラット肝細胞（ARL-18）を用い，LFLXの光照射により生じた8-oxo-dGが，一重項酸素消去剤存在下では減少したもののヒドロキシラジカル消去剤存在下では変化しなかったことから，光遺伝毒性の発現には一重項酸素が関与しているとした．また，Chételatら（1996）は，CPFX，FLRXおよびLFLXの光照射による染色体異常誘発作用がオキシラジカル消去剤では減少したものの，Ames試験およびComet assayの結果には影響しなかったことから，光照射による染色体異常と遺伝子突然変異／DNA損傷は，それぞれ異なる発現メカニズムが関与している可能性を示唆した．

またTraynor and Gibbs（1999）は，ヒトケラチノサイトを用い，光照射下ではLFLXの低濃度からピリミジンダイマーが生成することを明らかにし，光発がん性への関与を示唆した．

2．フルオロキノロンの光遺伝毒性の特性

キノロン剤のうちC6位にフルオロ基がついたものをフルオロキノロンfluoroquinoloneと呼ぶが，さらにC8位の置換基が作用の増強に関係しており，ハロゲン基がつくと光遺伝毒性が増強することが報告されている．各種フルオロキノロン剤の光遺伝毒性試験に関するこれまでの報告をTable 1にまとめた．

光遺伝毒性を検出する試験系としては，その発現メカニズムから考えてDNA損傷を検出する試験系が適している．一方，多くのキノロン剤はtopoisomerase II阻害作用に関連して染色体異常を誘発することが報告されている（Takayama et al., 1995；Shimada and Itoh, 1996）．これらのことから，フルオロキノロンの光遺伝毒性試験としてはDNA損傷および染色体異常を検出する試験系での報告が多い．DNA損傷を検出する試験系としては，プラスミドpBR322を用いた検出系やin vitro系でのComet assayが多いが，一部in vivo/in vitro系でのComet assayも試みられている．染色体異常を検出する試験系としては，株化細胞を用いた染色体異常試験やin vitro小核試験での報告がある．遺伝子突然変異検出系としては，CPFX，FLRXならびにLFLXについてTA100，TA102ならびにTA104を用いたAmes試験が実施されており，活性酸素に反応特異性があるTA104で光照射により変異体コロニーの軽微な増加が報告されている（Chételat et al., 1996）．また，他の遺伝子突然変異検出系としては，*Saccharomyces cerevisiae* D7を用いた報告などがある（Chételat et al., 1996；Marrot et al., 2001）．

フルオロキノロンの化学構造と光遺伝毒性の関係を調べていくと興味深い事実につきあたる．それは，キノリン環C8位にハロゲン基がつくことにより，一般的に光毒性および光遺伝毒性が増強することである．光毒性に関しては一般的にクロル基よりもフルオロ基の方が強いとされているが（Domagala, 1994），光遺伝毒性についても，C8位にクロル基を有するCLFXよりもフルオロ基を有するFLRX，LFLXあるいはSPFXの方が光照射による染色体異常誘発増強作用が強いことが報告されている（Snyder and Cooper, 1999；Itoh et al., 2001）．またSnyder and Cooper（1999）は，C8位にフルオロ基をもつLFLXともたないNFLX，C8位にクロル基をもつCLFXともたないCPFXでは，それぞれハロゲン基をもつLFLXおよびCLFXの方が光照射による小核誘発作用が強いことを報告している．

NFLXとCPFXはN1位の置換基がエチル基からシクロプロピル基に変わったものである．NFLXは，光非照射下のV79細胞を用いた染色体異常試験においては陰性であるが，光照射により染色体異常の微増が認められている．一方CPFXは光照射下でも非照射下でも染色体異常の軽微な増加が認められることから，光照射による染色体異常誘発増強作用としてはNFLXの方が大きいと考えられる．（Snyder and Cooper, 1999）．Reavyら（1997）もComet assayにおいては，CPFXよりNFLXの方が光照射によるDNA損傷作用が強いとしており，N1位のシクロプロピル基が光遺伝毒性の増強に関与している可能性が推察される．

また，C8位にメトキシ基を導入したフルオロキノロンは，光安定性が増し，光毒性が減弱することが報告されているが（Matsumoto et al., 1992；Rosen et al., 1997），光遺伝毒性にも同様なことがいえることがItohら（2001）により報告されている．Itohら（2001）は，各種キノロン剤の光遺伝毒性作用をCHL細胞を用いた染色体異常試験で比較し，そのED50値から3つのグループにわけている．すなわち光照射による染色体異常誘発作用が①強いグループ（SPFX，GMFX，CLFX，LFLX，STFX，FLRX，GPFX），②弱いグループ（ENX，LVFX），ならびに③極めて弱いかまたは陰性のグループ（MFLX，TVFX，DK-507k）である．また，メトキシ基を有するMFLXおよびDK-507kとそれぞれ構造が類似しかつメトキシ基を有しないBAYy3118およびSTFXの比較から，C8位のメトキシ基が光照射による染色体異常誘発作用を減弱させることを報告している．

3．光発がん性との関係

フルオロキノロンの光発がん性に関する報告は少ない．Klecakら（1997）は，CPFX，FLRX，LFLX，NAおよびOFLXについて，Skh-1マウスを用いた光発がん性試験を報告している．発生した腫瘍としては，LFLXにsquamous cell carcinomaの増加が認められているが，他のキノロン剤ではpapillomaの増加が主体的であった．また，腫瘍発生時期および腫瘍発生率から，FLRXおよびLFLXは光照射による腫瘍発生のリスクが高いグループ，CPFX，NAおよびOFLXはリスクの低いグループとされ，この傾向は各種報告にみられた光遺伝毒性の強さとほぼ一致していた．

In vivo/in vitro系試験，各種in vitro系試験ならびにその他の試験結果から光発がん性を総合的に推測している研究報告がある．Buleraら（1999）は，CLFX（25 mg/kg），CPFX（200 mg/kg）またはLFLX（20 mg/kg）を投与したSkh-1マウスにUVAを4日間（3時間/日）照射して得られたケラチノサイトを用いてComet assayによりDNA損傷性を検討している．その結果全ての処理で陽性であり，その強さはCLFX＞LFLX＞CPFXであった．また，CHO細胞を用いたin vitro系でのDNA損傷試験（Comet assay）および染色体異常試験でも光照射による遺伝毒性増強作用が認められ，なかでもCLFXが一番強かった．一方，CLFXの光発がん性の可能性については，実験動物を用いた試験では光遺伝毒性作用の強さから考えて光発がん性を有することが予測されるが，ヒトで臨床的に使用した場合には，その血中濃度，皮内濃度等から見積もっても，光発がん性の可能性は極めて小さいと結論している．

光照射による発がんのメカニズムは，遺伝毒性等の直接的な作用に加えて，修復メカニズムの阻害，表皮の保護機能の変化あるいは免疫系の抑制などの間接的な作用も示唆されている．2000年1月に，米国FDAのCDER（Center for Drug Evaluation and Research）より医薬品の光発がん性に関するガイドラインが提示されているが（FDA，2000），そのなかでの光遺伝毒性の位置づけはそれほど高くない．しかし，光発がん性試験はその規模と効果を疑問視する声もあり，事前の光遺伝毒性試験である程度の予測がつくかどうかが問われている．光遺伝毒性試験としては，今までにプラスミドDNAを用いたDNA損傷試験，Comet assay，Ames試験，染色体異常試験，in vitro小核試験ならびに酵母を用いた遺伝子突然変異試験等が開発されている．

フルオロキノロンの光遺伝毒性検出には，その作用メカニズムから考えて，DNA損傷や染色体切断が検出感度において優れていると考えられる．筆者が提案するスクリーニングの手順としては，以下の通りである．

1. UVA/UVBあるいは可視光に吸収性があるか否か
2. 光照射によるin vitro染色体異常（小核）誘発作用があるか否か
3. 光照射によりマウス（SKh1-hr）皮膚に小核誘発作用があるか否か
4. ヒト暴露量（皮内濃度）とin vivo/in vitro系試験成績を総合的に考察

結　　語

光発がんのイニシエーション作用としての光遺伝毒性は，今後その試験方法が確立されていけば，光発がん性検出の有用なツールになると考えられる．すべての遺伝毒性試験に常について回る問題として，in vitro系試験結果の生体，特にヒトへの外挿性がある．光遺伝毒性試験に関しても決して例外ではないが，in vitro系試験における作用発現強度と組織内濃度の解析やin vivo/in vitro系試験結果等からある程度のリスク予測が可能であると考えている．そのためには，特にヒトでの暴露を想定した試験条件の設定が重要なポイントとなる．

医薬品の有用性とその副作用という問題は決して単純に解決できる問題ではなく，医療経済性を含めたリスク／ベネフィットの観点から論じられるべき問題と考えている．副作用の問題は，それを正しく認識し，不幸な事態に陥らないような適切な管理と，もし生じた場合のレスキュー策を考えておく必要がある．フルオロキノロンの光発がん性に関しては，Müllerら（1998）の以下のコメントが参考になる．

フルオロキノロンは光遺伝毒性を有し，そのなかのいくつかは光発がん性のリスクをわずかに増大させる可能性があるが，薬物服用中における太陽光やUV照射を避けることにより，そのリスクを低下できるものと考えられる．

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Original Article

Photogenotoxicity and apoptosis in human HaCaT keratinocytes induced by 8-methoxypsoralen and lomefloxacin

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Summary

The HaCaT cell line is a spontaneously transformed human epithelial cell line from adult skin, which retain some differentiation characteristics of human skin cells. These cells are considered to more closely resemble the in vivo skin condition than other cell lines which are usually employed in genetic toxicity testing and may therefore be particularly appropriate for in vitro photogenotoxicity testing. We investigated chromosomal damage as evidenced by micronucleus (MN) formation in cytokinesis-blocked HaCaT keratinocytes after treatment with 8-methoxypsoralen (8-MOP) or lomefloxacin plus UV irradiation with a UVB/UVA relationship of 1:30. Our results show that 8-MOP induced MN in a dose-dependent manner over a concentration range from 1×10^{-7} to 1×10^{-4} M using low and intermediate UVA/UVB irradiation dose levels. Lomefloxacin (LOM) induced MN in HaCaT cells in the range from 1×10^{-5} to 1×10^{-4} M only in conjunction with high UVA/UVB irradiation doses at which 8-MOP was too toxic to the cells. Both, photoactivated 8-MOP and LOM were also found to be strong inducers of apoptosis in HaCaT cells, probably as a consequence of their DNA damaging activities. Apoptosis was analysed at different days of cell culture, i.e., day 3, 7 or 11, at which HaCaT cells proceed from a proliferation state to differentiation. The highest sensitivity towards apoptosis was found in day-11 HaCaT cells suggesting that keratinocytes become increasingly prone to apoptosis during the differentiation process. In summary, our studies indicate that HaCaT cells are suitable target cells to assess multiple cellular effects, such as clastogenicity, cytotoxicity and apoptosis in response to UV-irradiated photosensitizers.

Keywords: photogenotoxicity, apoptosis, phototoxicity, psoralen, lomefloxacin

Introduction

Conventional genotoxicity testing can be adapted to combination treatment with UV radiation to be used for testing of the photomutagenic potential of pharmaceuticals, cosmetics such as sunscreens and other substances. The use of mammalian cells as targets for measuring photomutagenic potential may be superior to bacteria as target cells due to the differences in processing and repair of oxidative damage (Gocke and Chételat, 2001). However, mammalian cell types usually used in conventional testing, i.e., Chinese hamster fibroblasts, human lymphocytes, or rat hepatocytes are apparently different from the primary target cells for photogenotoxic effects in the skin and may thus show inappropriate responses to photochemical mutagenicity. In vivo testing of photogenotoxicity, on the other hand, is problematic since skin cannot be easily utilized in the standard approaches. In this situation, the use of established keratinocyte cell lines may be a good alternative for in vitro photogenotoxicity testing. Human HaCaT keratinocytes which are used in this study are a spontaneously transformed cell line which behave phenotypically like normal cultured keratinocytes in their pattern of cell proliferation and differentiation (Boukamp et al., 1988). We investigated the induction chromosomal damage evidenced by micronucleus (MN) formation in

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cytokinesis-blocked (CB) human HaCaT proliferating keratinocytes after treatment with 8-methoxypsoralen (8-MOP) and lomefloxacin (LOM) plus UV irradiation. In addition, apoptotic events were characterized in proliferating and confluent HaCaT keratinocyte cultures.

Materials and Methods

Test compounds

8-Methoxypsoralen (8-MOP, $C_{12}H_8O_4$, MW 216.2, CAS No. 298-81-7) and lomefloxacin (LOM, $C_{17}H_{19}F_2N_3O_3$, MW 387.8, CAS No. 98079-51-7) (Sigma, Deisenhofen, Germany) were dissolved in DMSO or PBS, respectively, before use. Final concentrations of DMSO in the cultures did not exceed 1%.

Source of light and irradiation conditions

The Suntest CPS$^+$ accelerated exposure machine was purchased from Atlas (Gelnhausen, Germany). This apparatus is equipped with a Xenon burner as light source which emits a continuous spectrum simulating sunlight. Prior to the experiments the UVA (315-400 nm) and UVB (280-315 nm) doses were measured by using UV detectors RM21 (Gröbel, Ettlingen, Germany) at a distance of 17 cm from the UV source. The Suntest CPS$^+$ machine is equipped with a UVC filter (Atlas, filter number 56009561) and provided a UVB/UVA relationship of 1:13. Due to the presence of a plastic lid during irradiation the UVB/UVA relationship was further altered to about 1:30 when cell cultures were irradiated in Quadriperm culture dishes (quadriPerm^R plus 76077310, Heraeus, Hanau, Germany) and six-well culture plates (Nunc, Roskilde, Denmark).

HaCaT cell culture

Human HaCaT keratinocytes (kindly provided by Dr. N.E. Fusenig, DKFZ, Heidelberg, Germany) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Germany) supplemented with 5% fetal calf serum, 2 mM glutamine and 100 U/ml penicillin/streptomycin (Biochrom, Germany). The cells were seeded on either six-well culture plates (apoptosis assay) or on glass slides in Quadriperm culture dishes (micronucleus assay) at a density of 1×10^4 cells/cm^2 and cultured at 37°C in a gassed (95% air and 5% CO_2) a humidified incubator. The growth medium was changed twice a week.

Micronucleus assay

After 3 days of cultivating HaCaT cells on glass slides in Quadriperm culture dishes cells were treated with serial dilutions of the test chemical at 30 min in the dark and further irradiated with UV light. Following treatment, cells were washed twice with PBS and cultured in DMEM with 5 µg/ml of cytochalasin B (Cyt-B, $C_{29}H_{37}NO_5$, MW 479.6) (Sigma, Germany) for 24 hours. Cells were then rinsed in 1.5% tri-sodium citrate 2-hydrate buffer (Merck, Germany) and fixed in acetic acid:ethanol (1:3) supplemented with 1.25% v/v formaldehyde solution (37%). The air-dried slides were first stained in May-Gruenwald's solution (Merck, Germany) for 3 min and then transferred to a 2.6% Giemsa solution (Merck, Germany) in Weise buffer (3.6 mM KH_2PO_4/7.0 mM $Na_2HPO_4\cdot2H_2O$, pH 7.2) for 20 min. The frequency of binucleated cells was assessed in 1000 cells. Micronuclei were analysed in 1000 binucleated cells per test compound concentration with a light microscopy, in cases of high toxicity only 500-800 binucleated cells were assessed. Micronuclei were identified according to the criteria of Countryman and Heddle (1976; Vian et al., 1993) who describe micronuclei as bodies of a diameter of less than 1/3 of the main nucleus, which do not touch the nucleus, and stain in the same color as the nucleus or lighter.

Apoptosis examination

Apoptosis was measured by both morphological determination using a light microscope and by a photometric enzyme immunoassay for the determination of DNA fragments. Morphological determination of the incidence of apoptosis was done in the slides prepared for the MN assay (see treatment conditions there). Nuclei were screened for normal morphology and apoptotic nuclei comprising those with condensed chromatin and/or chromatin pieces (fragmented nuclei). The percentage of apoptotic cells were scored in 1000 cells per test compound concentration. DNA fragmentation was determined by means of a "cell death detection ELISA" (CDDE, Boehringer-Mannheim, Mannheim, Germany), according to the manufacturers instruction. The assay is based on the measurement of cytoplasmic histon-associated DNA fragments (mono- and oligonucleosomes) contained in the sample, which bind to an monoclonal anti-histon antibody. The DNA-part of the nucleosomes is detected by an anti-DNA-peroxidase. The reaction is visualized with 2,2´-azino-dio-[3-ethylbenzthiazoline sulfonate] and measured photometrically. Briefly, HaCaT cells were seeded on six-well culture plates and cultured 7 or 11 days. After treatment with the chemicals for 30 min in the dark followed by UV irradiation and a further cultivation of 24 hours cells were harvested with 0.1% Trypsin/EDTA (Gibco, Germany). 2×10^5 HaCaT cells were used for each determination in the CDDE test and the samples were measured in duplicate. Nucleosomes enrichment factor were calculated to the following formula.

Nucleosomes enrichment factor =

$$\frac{\text{mU of sample (dying/dead cells)}}{\text{mU of the corresponding control (viable cells)}}$$

mU = absorbance (10^{-3})

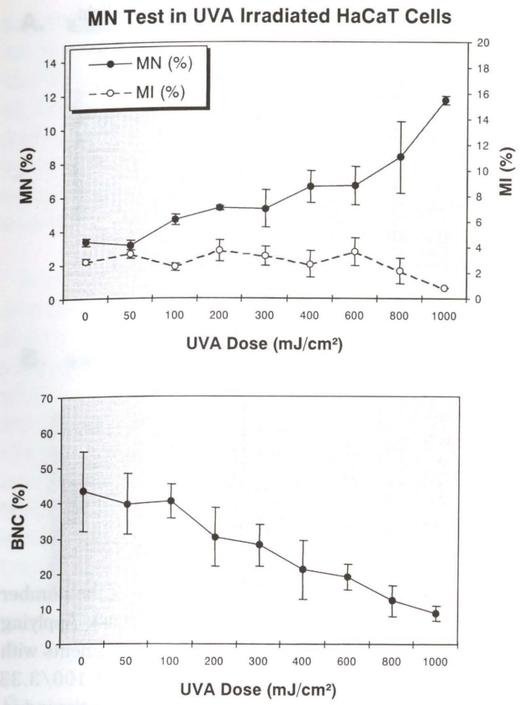

Fig. 1 Micronucleus frequencies (MN %, left ordinate, closed symbols), mitotic indices (MI %, right ordinate, open symbols) and the frequency of bi-nucleated cells (BNC %) in proliferating HaCaT cells after irradiation with UV light using a UVB/UVA relationship of 1:30. Data of two experiments are shown as mean values ± standard deviations.

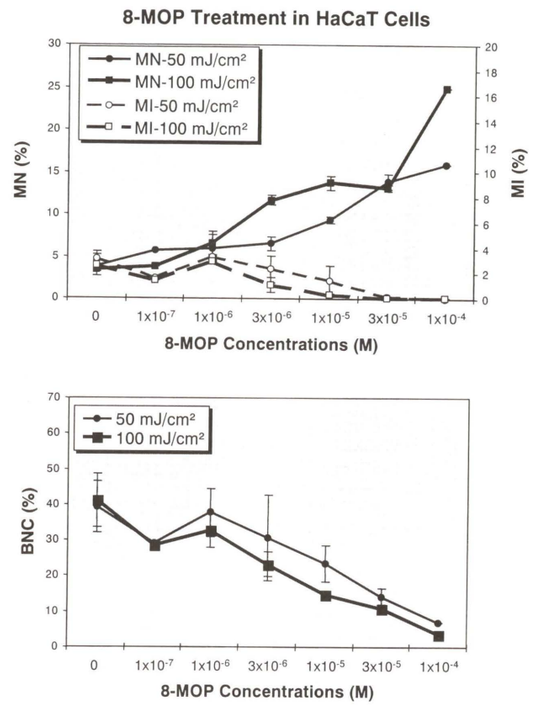

Fig. 2 Micronucleus frequencies (MN %, left ordinate, closed symbols), mitotic indices (MI %, right ordinate, open symbols) and the frequency of bi-nucleated cells (BNC %) in proliferating HaCaT cells treated with 8-MOP and irradiated with UV light. The irradiation condition and data analysis are the same as in Fig. 1.

Results

Induction of MN after UV irradiation

Proliferating HaCaT keratinocytes on day 3 after seeding were irradiated with UV and further incubated with 5 µg/ml of Cyt-B for 24 hours. The frequency of micronuclei in binucleated HaCaT cells steadily increased in the range of 50/1.67 - 1000/33.33 mJ/cm^2 UVA/UVB irradiation (Fig. 1). 400/13.33 mJ/cm^2 UVA/UVB irradiation resulted in 6.6 ± 0.9% MN-containing cells compared with 3.3 ± 0.2% in control cells (Fig. 1). The effect increased further up to 12 ± 0.25% MN containing cells at 1000/33.33 mJ/cm^2 UVA/UVB irradiation (Fig. 1). Irradiation up to 800/26.67 mJ/cm^2 UVA/UVB did not significantly affect the mitotic index (MI) which was in average 2.2 ± 0.98%. Only at 1000/33.33 mJ/cm^2 UVA/UVB the MI was reduced to 0.85% indicating severe phototoxic effects (Fig. 1). The percentage of binucleated cells (BNC) among the HaCaT keratinocytes was in average 43% in untreated cultures. This value was dose-dependently reduced to 8.7% at 1000/33.33 mJ/cm^2 UVA/UVB irradiation (Fig. 1), also indicating treatment-related cytotoxicity.

Induction of MN after treatment with 8-MOP or LOM plus UV irradiation

The frequency of MN containing binucleated HaCaT cells increased in a dose dependent manner with 8-MOP plus using either 50/1.67 or 100/3.33 mJ/cm^2 UVA/UVB irradiation. The maximum percentage of MN cells was 25% induced by 10^{-4} M 8-MOP treatment plus 100/3.33 mJ/cm^2 UVA/UVB (Fig. 2). Without UV irradiation 8-MOP at concentrations up to 10^{-4} M did not induce chromosomal damage in HaCaT cells (data not shown). The level of chromosomal damage by 8-MOP treatment was slightly greater at 100/3.33 than 50/1.67 mJ/cm^2 UVA/UVB irradiation (Fig. 2). A continuous decrease of MI and BNC indicating phototoxic effects were observed at concentrations above 10^{-6} M 8-MOP with both 50/1.67 and 100/3.33 mJ/cm^2 UVA/UVB irradiation (Fig. 2). In 8-MOP treated cultures irradiated with a higher UVA/UVB dose of 400/13.33 mJ/cm^2 very strong phototoxic effects were induced precluding the evaluation of binucleated cells (data not shown). In order to demonstrate the advantage of using the Cyt-B technique, i.e., scoring of only those cells that have completed a nuclear division (binucleated cells) a comparison was done with the convention-

Table 1 Results of micronucleus evaluation with 8-MOP treatment in proliferating HaCaT cells by irradiation with UV light

UVA/UVB Dose (mJ/cm^2)	Concentration (M)	MN (%)	
		In 1000 cells (mono- and bi- nucleated)	In 1000 cells (bi- nucleated)
50/1.67	0	2.4±0.8[a]	3.8±0.6
50/1.67	1×10^{-7}	1.9[b]	5.8
50/1.67	1×10^{-6}	2.5±0.4	6.0±1.6
50/1.67	3×10^{-6}	2.8±0.3	6.6±0.9
50/1.67	1×10^{-5}	3.8±1.0	9.4±0.4
50/1.67	3×10^{-5}	4.3±0.9	13.9±0.9
50/1.67	1×10^{-4}	2.8[b]	16.0
100/3.33	0	2.2±0.4	3.4±0.7
100/3.33	1×10^{-7}	1.4[b]	3.8
100/3.33	1×10^{-6}	2.6±0.8	6.5±1.5
100/3.33	3×10^{-6}	4.5±0.3	11.7±0.6
100/3.33	1×10^{-5}	3.6±0.3	13.8±0.9
100/3.33	3×10^{-5}	2.9±0	13.2±0.4
100/3.33	1×10^{-4}	2.9[b]	25.0

Date of one experiment are shown as mean values of 2 cultures (1,000 cells each).
[a] Mean values ± standard deviations of two independent experiments. [b] Mean values of one experiment.

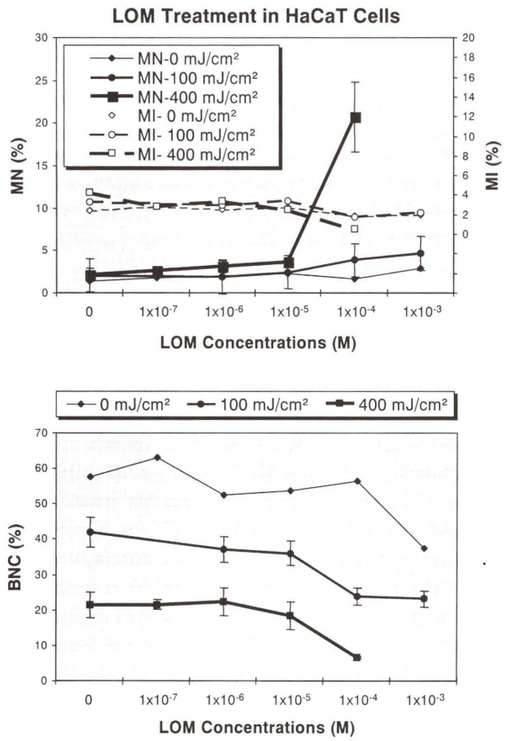

Fig. 3 Micronucleus frequencies (MN %, left ordinate, closed symbols), mitotic indices (MI %, right ordinate, open symbols) and the frequency of bi-nucleated cells (BNC %) in proliferating HaCaT cells treated with LOM and irradiated with UV light. The irradiation condition and data analysis are the same as in Fig. 1.

al technique analyzing all cells irrespective of the number of nuclei (mainly mono- and binucleated cells). Applying this technique to the same slides of the experiments with 8-MOP treatment plus both 50/1.67 and 100/3.33 UVA/UVB irradiation no significant increase in the induction of MN was observed (Table 1) because in this case most of the cells analyzed were mononucleated, i.e., have not undergone a nuclear division and thus cannot express DNA damage as an MN. The effect of the fluoroquinolone LOM on the incidence of micronuclei in HaCaT cells is given in Fig. 3. After irradiation with 400/13.33 mJ/cm^2 UVA/UVB LOM induced a strong increase in the frequency of MN-containing binucleated cells at a concentration of 10^{-4} M. Using 100/3.33 mJ/cm^2 UVA/UVB only a very slight effect was observed at 1×10^{-4} M and 1×10^{-3} M LOM. LOM alone did not induce micronuclei in the concentration range from 1×10^{-7} to 1×10^{-4} M, but at 10^{-3} M of LOM a low effect was detected with 2.9% MN-containing cells in comparison to 1.4% of untreated HaCaT cells (Fig. 3). The percentage of BNC was clearly reduced by LOM at concentrations above 10^{-5} M at both irradiation conditions indicating severe phototoxic activity of LOM at these concentrations (Fig. 3).

Induction of apoptosis after treatment with 8-MOP or LOM plus UV irradiation

Day-3 cultures of HaCaT keratinocytes were treated with different concentrations of 8-MOP or LOM, and further irradiated with UV. Morphological determination of cells undergoing apoptosis revealed strong and concentration-dependent apoptotic effects of 8-MOP after photoactivation with 400/13.33 mJ/cm^2 UVA/UVB (Fig. 4A). When irradiated with 100/3.33 mJ/cm^2 UVA/UVB 8-MOP had no significant influence on the incidence of apoptotic cells

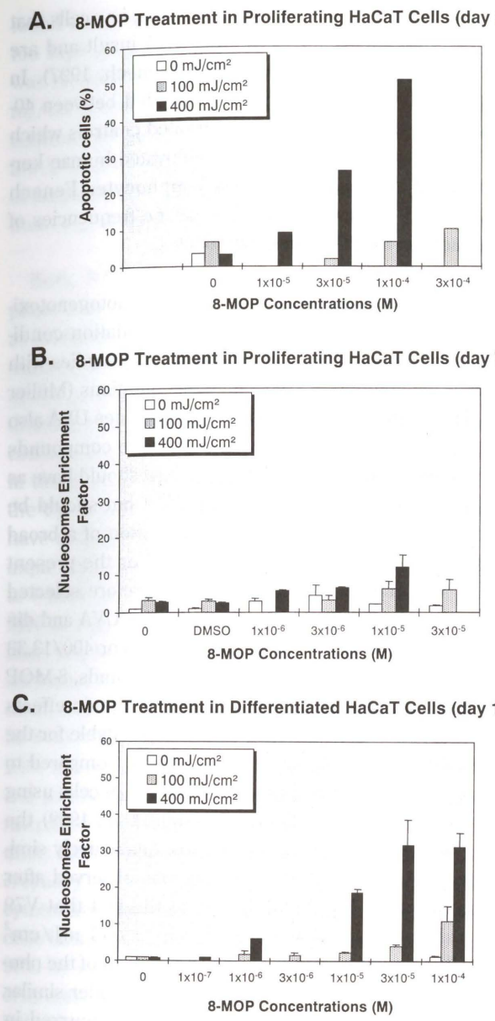

Fig. 4 Induction of apoptosis in UV-irradiated HaCaT cells treated with 8-MOP. The cells were cultured for 3, 7 or 11 days, then treated with different concentrations of 8-MOP plus 100/3.33 or 400/13.33 mJ/cm^2 UVA/UVB irradiation, and harvested 24 h after treatments.
A: Percentage of apoptotic cells were determined by microscope examination of HaCaT cells treated with 8-MOP and UV-irradiation after 3 days of cultivation.
B and **C**: apoptotic cells were determined by the cell death detection ELISA (CDDE) in HaCaT cultures treated with 8-MOP and UV-irradiation after 7 days (**B**) or 11 days (**C**) of cultivation. Results are expressed as mean values ± standard deviations of the 2 independent experiments.

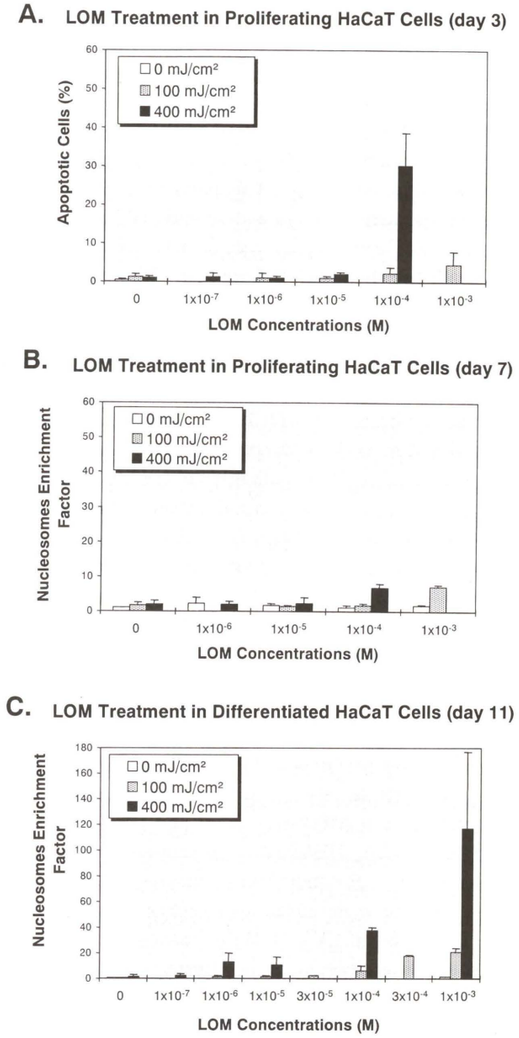

Fig. 5 Induction of apoptosis in UV-irradiated HaCaT cells treated with LOM. The cells were cultured for 3, 7 or 11 days, then treated with different concentrations of LOM plus 100/3.33 or 400/13.33 mJ/cm^2 UVA/UVB irradiation, and harvested 24 h after treatments.
A: Percentage of apoptotic cells were determined by microscope examination of HaCaT cells treated with LOM and UV-irradiation after 3 days of cultivation.
B and **C**: apoptotic cells were determined by the cell death detection ELISA (CDDE) in HaCaT cultures treated with LOM and UV-irradiation after 7 days (**B**) or 11 days (**C**) of cultivation. Results are expressed as mean values ± standard deviations of the 2 independent experiments.

in comparison to cultures treated with UV only. The incidence of apoptotic cells after LOM treatment is shown in Fig. 5A. A large amount of apoptotic cells was found after irradiation with 400/13.33 mJ/cm^2 UVA/UVB at 1×10^{-4} M LOM only. Under the condition of the lower UV dose LOM caused a very slight but concentration-dependent increase in the percentage of apoptotic cells. The apoptotic activity of photoactivated 8-MOP and LOM in day-7 and day-11 HaCaT cell cultures were determined by using the cell death detection ELISA. Treatment with 8-MOP of day-7 HaCaT cultures, i.e., nearly confluent cultures with still proliferating cells resulted in a slight but concentration-related induction of apoptosis starting at a concentration of 1×10^{-6} M using 400/13.33 mJ/cm^2 UVA/UVB and 1×10^{-5} M following irradiation with 100/3.33 mJ/cm^2 UVA/UVB (Fig. 4B). Also LOM treatment induced DNA fragmentation in 100/3.33 or 400/13.33 mJ/cm^2 UVA/UVB irradiated day-7 HaCaT cells, but only at concentrations of 1×10^{-4} M LOM and higher (Fig. 5B). Without UV irradiation, treatment with 8-MOP or LOM even at the highest concentrations did not significantly induce DNA fragmentation (Figs. 4B and 5B) indicating absence of apoptotic cells.

Day-11 HaCaT keratinocytes, i.e., non-proliferating cells which have been reported to exhibit signs of differentiation, responded more sensitive to the apoptotic effects of photoactivated 8-MOP and LOM compared with day-7 cultures (Figs. 4B, 4C, 5B and 5C). Very strong effects were observed after 8-MOP treatment and irradiation with 400/13.33 mJ/cm^2 UVA/UVB. An even more pronounced enhancement in the induction of apoptosis in day-11 cultures was found for LOM. In cultures irradiated with 400/13.33 mJ/cm^2 UVA/UVB the lowest concentration of LOM showing clear apoptotic effects was 10^{-6} M, i.e., a 100-fold lower concentration compared to day-7 cultures. A maximum effect occurred at 10^{-3} M LOM with a more than 100-fold increase in DNA fragmentation level in comparison to controls both with and without irradiation (Fig. 5C) indicating a dramatic increase in cells undergoing apoptosis.

Discussion

In the present study we have employed the in vitro micronucleus test using HaCaT keratinocytes as an easy means to detect clastogenic effects of test compounds which can be activated by UV. Recently the cytochalasin B (Cyt-B) method for MN analysis has increasingly been applied as an in vitro assay for genotoxicity testing (Falck et al., 1997; Fenech, 1997). Following treatment with Cyt-B, cells complete nuclear division and then accumulate as bi-nucleated cells because Cyt-B can inhibit cytokinesis without interfering with nuclear division. The Cyt-B technique enables a good determination of cell division kinetics and a more sensitive and reliable detection of MN since scoring is limited to binucleated cells, i.e., cells that complete one nuclear division after DNA insult and are therefore capable of expressing MN (Fenech, 1997). In this study, Cyt-B treatment for 24 h yielded between 40-60% bi-nucleated HaCaT cells in untreated controls which is in agreement with findings from cultivated human keratinocytes Pelt et al. (1991) and lymphocytes Fenech (1997). Cyt-B did not change the baseline frequencies of micronuclei in the cultured keratinocytes.

A critical issue when performing in vitro photogenotoxicity tests is to determine appropriate irradiation conditions. Based on our earlier photogenotoxicity studies with V79 cells using different UV irradiation conditions (Müller et al., 1998), there is some evidence that besides UVA also UVB is needed for photoactivation of some compounds like 8-MOP. In addition, the UV dose itself should have as little as possible an own genotoxic effect but should be high enough to ensure an efficient activation of a broad spectrum of potential photosensitizers. For the present studies with HaCaT keratinocytes we therefore selected an intermediate relationship of 1:30 of UVB/UVA and different UVA/UVB doses of 50/1.67, 100/3.33 or 400/13.33 mJ/cm^2. Under these conditions both compounds, 8-MOP and LOM were found to exert photoclastogenic effects indicating that HaCaT cells are principally suitable for the in vitro detection of photogenotoxic effects. Compared to our recent findings with Chinese hamster V79 cells using the same treatment conditions (Kersten et al., 1999) the results with HaCaT keratinocytes show qualitatively similar effects. A difference in sensitivity was observed after treatment with 8-MOP mainly due to the fact that V79 cells tolerated irradiation with 400/13.33 mJ/cm^2 UVA/UVB leading to a very sensitive detection of the photoclastogenic effects of 8-MOP at 3×10^{-7} M. Under similar conditions pronounced phototoxic effects occurred in HaCaT cells, resulting in a lack of binucleated cells, i.e., complete inhibition of proliferation, and thus precluding any evaluation of micronuclei. This finding may indicate that HaCaT in comparison to V79 cells are more sensitive to phototoxic effects occuring under extreme treatment conditions. Photoinduction of micronuclei by 4.6×10^{-6} M of 8-MOP in vitro was also demonstrated in primary culture of human fibroblasts following irradiation with 100/200/300 mJ/cm^2 UVA (Stivala et al., 1995). After treatment of HaCaT with LOM a pronounced increase in the frequency of MN containing binucleated cells was found at a concentration of 10^{-4} M only after irradiation with 400/13.33 mJ/cm^2 UVA/UVB. An exactly similar finding was observed with V79 cells (Kersten et al., 1999). The present genotoxicity findings show that it is difficult to recommend one single UV dose as appropriate for an efficient activation of potential photosensitizer. For 8-MOP 50/1.67 mJ/cm^2 and 100/3.33 mJ/cm^2 UVA/UVB were found to be adequate doses, but 400/13.33 mJ/cm^2 was too high giving lethal effects whereas 100/3.33 mJ/cm^2 was ineffective in activating LOM and only at 400/13.33 mJ/cm^2 photogenotoxicity with LOM was observed. We recommend therefore to first perform experiments with a low UVA dose such as 50 and/or 100 mJ/cm^2 and in case of negative results to repeat the test with an increased UV dose, e.g., 400 mJ/cm^2.

Both, 8-MOP and LOM were found to induce strong phototoxic effects in a concentration-dependent manner as evidenced by a decrease in the mitotic index and in the number of binucleated cells indicating delay or inhibition of cell proliferation. Since MN can only be expressed in dividing cells, inhibition of proliferation can be a potential confounding factor in the evaluation of MN. As is shown in the present study this problem can be solved by using the Cyt-B method which allows to score only in cells that have divided. It can be attributed to the use of this technique that it was possible to detect the photogenotoxicity of 8-MOP and LOM in the present study. When scoring MN in a conventional manner in the overall cell population, i.e., irrespective of whether cells are mono- or binucleated no photoclastogenic effects were observed with irradiated 8-MOP. For example, after treatment with 10^{-4} M 8-MOP and irradiation with 100/3.33 UVA/UVB only 2.9% micronucleated cells were found when the overall cell population was analyzed whereas an increase to 25% micronucleated cells was observed when MN scoring was done exclusively in bi-nucleated HaCaT cells. This discrepancy can be explained by the fact that with increasing cytotoxicity the fraction of non-dividing (mono-nucleated) cells incapable of expressing MN is increasing to up to > 90%. By using the conventional method that does not discriminate between dividing and non-dividing cells MN induction at doses that severely inhibit nuclear division would be underestimated or even undetected. At least for primary cells or cell lines like HaCaT which apparently respond more sensitive to cytotoxic effects compared to the widely used and more robust Chinese hamster cell lines (V79, CHO) the use of Cyt-B in the MN assay is highly recommended.

In context of cell survival after genotoxic stress active cell death or apoptosis is another endpoint of importance. HaCaT cells have been reported to be sensitive to the induction of apoptosis in response to UVB irradiation whereas several compounds known as inducers of apoptosis in other cell types did not affect HaCaT cells in this respect (Henseleit et al., 1996). UVB-induced apoptosis may be related to the direct DNA damage in these cell lines (Henseleit et al., 1996). At the very low UVB doses used in the present study no or only very slight increases in apoptosis were induced (see irradiation controls without photosensitizer treatment). However, both, photoactivated 8-MOP and LOM were found to be strong inducers of apoptosis in HaCaT cells. In 3-day HaCaT cultures the morphological examination of apoptotic cells and cells containing MN were done in the same slides thus allowing to compare both endpoints directly. Under conditions of considerable MN-induction (irradiation with 100/3.33 mJ/cm^2 UVA/UVB) 8-MOP did not induce significant apoptosis, only after irradiation with 400/13.33 mJ/cm^2 UVA/UVB which precludes the analysis of MN a massive increase in apoptotic cells was observed. In contrast, LOM induced both MN and apoptosis under exactly the same conditions (10^{-4} M LOM, 400/13.33 mJ/cm^2 UVA/UVB). This different onset of apoptosis in relation to MN formation may reflect the very different DNA lesions induced by both test compounds after UV irradiation. 8-MOP is a DNA intercalator that upon photoactivation can form DNA monoadducts and interstrand crosslinks (Gasparro et al., 1998). Photoactivation of fluorochinolones like LOM result in the formation of reactive oxygen species and may generate 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) which finally can cause DNA strand breaks (Rosen et al., 1997; Umezawa et al., 1997). In the pathway leading from DNA damage to apoptosis the tumor suppressor protein p53 is generally assumed to be a key factor. Also in HaCaT cells p53 has been shown to play a key role in UVB-induced apoptosis (Henseleit et al., 1997). DNA-damaging agents with different modes of action are known to activate p53. Evidence exists that DNA strand breaks either directly induced by ionizing radiation or indirectly in the course of excision repair events are primary signals for activation of p53. More recent evidence suggest that also DNA adducts can trigger p53 induction however compared to strand breaks through a different signal transduction pathway (Mirzayans et al., 1999). Thus, depending on the different DNA lesions induced by photoactivated 8-MOP and LOM different molecular pathways may be involved in the deletion of keratinocytes by apoptosis. It is interesting in this context that etoposide, a very potent inducer of MN in vitro and in vivo (Ashby et al., 1994) was unable to induce apoptosis in HaCaT cells (Henseleit et al., 1996). The genotoxic activity of etoposide is based on its ability to inhibit topoisomerase II activity (Ashby et al., 1994). In other cell types like rat hepatocytes etoposide were found to induce apoptosis (Gill et al., 1998). These findings indicate that different cell types respond quite differently to the apoptotic potential of different genotoxic compounds treatments. In case of HaCaT cells the response to UVB itself and UV-activated phototoxins appear to reflect the physiological role of keratinocytes as the outermost barrier of the body in the deletion of cells specifically damaged by UV.

The induction of apoptosis after treatment with 8-MOP

and LOM was analysed in HaCaT cells at different days of culture, i.e., day 3, 7 or 11. During this time course HaCaT cells were reported to proceed from proliferation to differentiation (Haase et al., 1996). In HaCaT keratinocytes of day 11, which progressively express differentiation-specific keratins and other differentiation proteins such as involucrin, filaggrin and transglutaminese I and II (Haase et al., 1996), considerably higher apoptotic effects were detectable than in those of day 7. A direct comparison to day-3 cultures cannot be made since apoptosis were determined with a different technique in these experiments. The quantitative difference in apoptosis induction between day-7 and day-11 cultures indicates that more differentiated HaCaT cells appear to be more sensitive to those signals triggering active cell death. Terminal differentiation of keratinocytes has been considered to be a form of apoptotic cell death (Haake and Polakowska, 1993; McCall and Cohen, 1991). It may therefore be assumed that keratinocytes during early steps of differentiation become already more prone to apoptosis which might explain the higher sensitivity towards apoptotoc signals derived from DNA damage in day-11 HaCaT cultures compared to undifferentiated proliferating keratinocytes.

In conclusion, the cytokinesis block micronucleus test with HaCaT cells was found to be suitable to assess multiple cellular effects, such as clastogenicity, cytotoxicity and apoptosis in response to the UV-irradiated photosensitizers 8-MOP and LOM.

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Effects of visible light absorbing chemicals in the photo-micronucleus test in Chinese hamster V79 cells

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Summary

The photon energy of visible light is not sufficient to cause direct damage of DNA. However, endogenous or exogenous photosensitizers which absorb in the visible light range may have the potential to damage DNA indirectly. Energy transfer from triplet excited sensitizers to molecular oxygen seems to be the most important mechanism (type II-photoreactions). Excited singlet oxygen may directly damage DNA or other cellular structures and/or give rise to other damaging reactive oxygen species (ROS). The lifetimes of ROS are very short, so that the effects (phototoxic/photogenotoxic) are influenced by the type of molecular targets which are in the immediate vicinity of their site of generation. Photogenotoxic effects induced by visible light absorbing compounds in mammalian cells have been studied only for a few chemicals.

In this study we used the photo-micronucleus assay in V79 cells to investigate photogenotoxic effects induced following treatment of the cells with 5 visible light absorbing chemicals as well as following induction of endogenous porphyrin synthesis by 5-aminolevulinic acid. Irradiation was performed using solar light conditions. Proflavine, neutral red, methylene blue and protoporphyrin IX induced clear photogenotoxic effects in our hands, whereas acridine was negative for this endpoint. All compounds reduced the proliferation index following irradiation, which indicates photocytotoxic effects. Prolonged incubation of V79 cells with 5-aminolevulinic acid (2-6 h) led to clear photogenotoxic effects in the photo-micronucleus assay probably caused by the time-dependent induction of intracellular porphyrin synthesis.

Keywords: photo-micronucleus test, photogenotoxicity, phototoxicity, photosensitizer, 5-aminolevulinic acid

Introduction

Several light-absorbing compounds have long been known to act as photosensitizers and to cause photo(geno)toxic effects. Notably psoralenes (e.g., 8-methoxypsoralene) and chlorpromazine derivatives have been established as photomutagens. When photosensitizers absorb light they may become activated to damaging agents (Evam and Tyrell, 1997) and/or may proceed from the excited singlet state to triplet state and subsequently may transfer energy to other acceptor molecules. In the case of visible light absorbers the energy level of the respective triplet states is not sufficient for a direct energy transfer to DNA as acceptor molecule. Molecular oxygen appears to be the predominant acceptor (type-II photoreaction) because its excited state level is comparatively low (Gocke et al., 1998). Excited singlet oxygen may directly damage DNA or other cellular structures and give rise to other damaging reactive oxygen species (ROS). The lifetimes of ROS are very short, so that the kind of induced effects (phototoxic/photogenotoxic) is influenced by the type of molecular targets which are in the immediate vicinity of their site of generation (Epe, 1993a). Following irradiation with visible light the excitation of endogenous photosensitizers, especially of porphyrines in mammalian cells may lead indirectly to DNA damage as demonstrated

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Table 1 List of compounds tested

Classes of chemicals	Chemicals tested	CAS No.	Molecular weight	Molecular formula	Source	Solvent used[a]
Acridines	acridine	[17784-47-3]	215.7	$C_{13}H_9N \cdot HCl$	Sigma	DMSO
	proflavine	[1811-28-5]	258.3	$C_{13}H_{11}N_3 \cdot 1/2\ H_2SO_4$	Sigma	PBS
Phenazine deriv.	neutral red	[553-24-2]	288.8	$C_{15}H_{16}N_4$	Sigma	DMSO
Phenothiazines	methylene blue	[7220-79-3]	373.9	$C_{16}H_{18}CIN_3S$	Sigma	PBS
Porphyrines	protoporphyrin IX (disodium salt)	[50865-01-5]	606.6	$C_{34}H_{32}N_4Na_2O_4$	Sigma-A.	DMSO
(Inducer)	5-aminolevulinic acid	[5451-09-2]	167.6	$C_5H_9NO_3$	Sigma	PBS

[a], final concentrations of DMSO in the cell cultures did not exceed 1%; deriv., derivative; Sigma, Deisenhofen, Germany; Sigma-A., Sigma-Aldrich, Schnelldorf, Germany.

by means of repair endonucleases (Pflaum et al., 1998). These DNA modifications, predominantly Fpg (formamidopyrimidine-DNA glycosylase)-sensitive modifications, however, gave no rise to mutations in AS52 cells or micronuclei in L1210 cells (Pflaum et al., 1998). Following addition of exogenous visible light absorbers or following induction of endogenous photosensitizers, such as protoporphyrin by treatment of the cells with 5-aminolevulinic acid (5-ALA) DNA damage was increased (Pflaum et al., 1998; Epe et al., 1989; Epe et al., 1993b), which indicates that repair mechanisms may be overloaded. Therefore genotoxic effects may arise; but only a few studies exist for visible light absorbing photosensitizers (Gocke et al., 1998; Noodt et al., 1994; Epe et al., 1989).

For the detection of photochemical genotoxicity in a screening-mode we selected the micronucleus test in vitro as a genetic endpoint known to detect a broad spectrum of genotoxic compounds. A protocol for the in vitro micronucleus test using V79 cells has recently been developed (Kalweit et al., 1999) and further validated in a collaborative study (von der Hude et al., 2000). This protocol has been successfully adapted to photogenotoxicity testing using a Xenon burner providing solar light conditions (Kersten et al., 1999). The data reported for the photo-micronucleus assay proved its suitability to determine the photogenotoxic potential of compounds which are subjected to various photo-activation mechanisms.

In the present study we used the photo-micronucleus assay to investigate photogenotoxic effects induced following treatment of the cells with 5 visible light absorbing chemicals as well as following induction of endogenous porphyrines by 5-aminolevulinic acid (5-ALA). Concomitantly, we investigated the phototoxic potential of the compounds by determining the proliferation index (PI)[1] (Kalweit et al., 1999; Kersten et al., 1999).

Materials and Methods

The V79 cells used and the photo-micronucleus assay were described in detail in Kersten et al. (1999). All experiments described here have been carried out in a similar way.

Table 1 lists the chemicals tested according to their chemical classes and gives the respective sources for purchase. The compounds are known to absorb in the visible light range, however, our conditions for irradiation were not limited to the visible light range but extended into the UVA-range with attenuation in the UVB-area.

Results

The protocol of the photo-micronucleus test described in Kersten et al. (1999) was applied to investigate the photogenotoxic and phototoxic potential of different compounds known as visible light absorbing chemicals (Figs. 1-4). The results from testing of these compounds are summarized in Table 2 in addition to the visible light absorbing compounds hypericin and emodin which were tested already (Kersten et al., 1999).

Both acridines tested, i.e., acridine and proflavine, an acridine amine, induced clearly photocytotoxic effects as indicated by the decrease of the PI as a parameter for cell proliferation (see Fig. 1). Photogenotoxic effects were only detectable for proflavine as evidenced by a steep increase in the frequency of micronucleated cells with the lowest observed effect concentration (LOEC) of 3.16×10^{-9} M following irradiation with 300 mJ/cm^2 UVA.

The phenazine derivative neutral red increased the frequency of micronucleated cells without irradiation at concentrations above 10^{-4} M (see Fig. 2). Following irradiation the LOEC for the induction of micronuclei was reduced to 10^{-7} M indicating photogenotoxic properties.

[1] PI = $(cl_1 \times 1 + cl_2 \times 2 + cl_4 \times 3 + cl_8 \times 4) / (cl_1 + cl_2 + cl_4 + cl_8 + cl_{>8})$; (cl_1), (cl_2), (cl_4), (cl_8), $(cl_{>8})$, number of clones consisting of 1, 2, 3-4, 5-8, or >8 cells respectively.

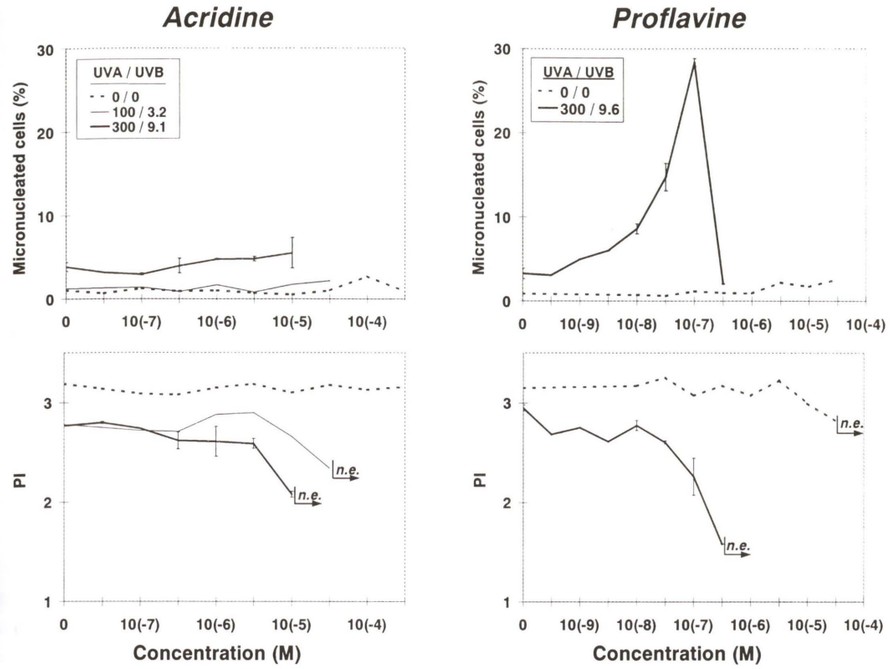

Fig. 1 Effects of 2 acridine derivatives in the photo-micronucleus assay using V79 cells. Data of 1 experiment are shown as mean values of 2 independently treated cultures (1000 cells each). Data of 2 experiments are shown as mean values ± standard deviations. UVA/UVB, UVA or UVB dose in mJ/cm^2; PI, proliferation index; n.e., no evaluation possible due to strong toxic effects of the test compound; 10(-4), 10(-5), etc., 10^{-4}, 10^{-5}, etc.

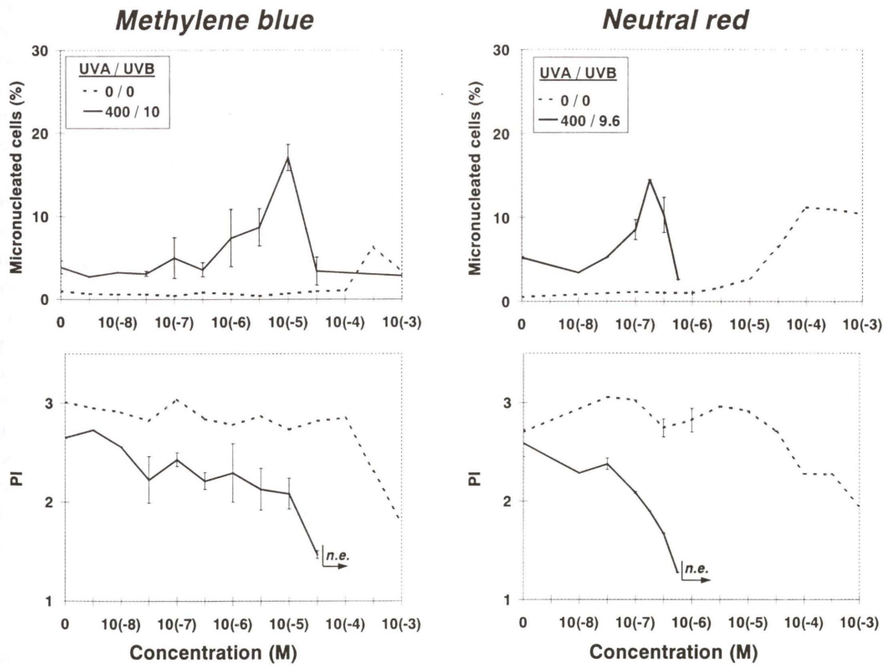

Fig. 2 Effects of neutral red and methylene blue in the photo-micronucleus assay using V79 cells. Further legend: see Fig. 1.

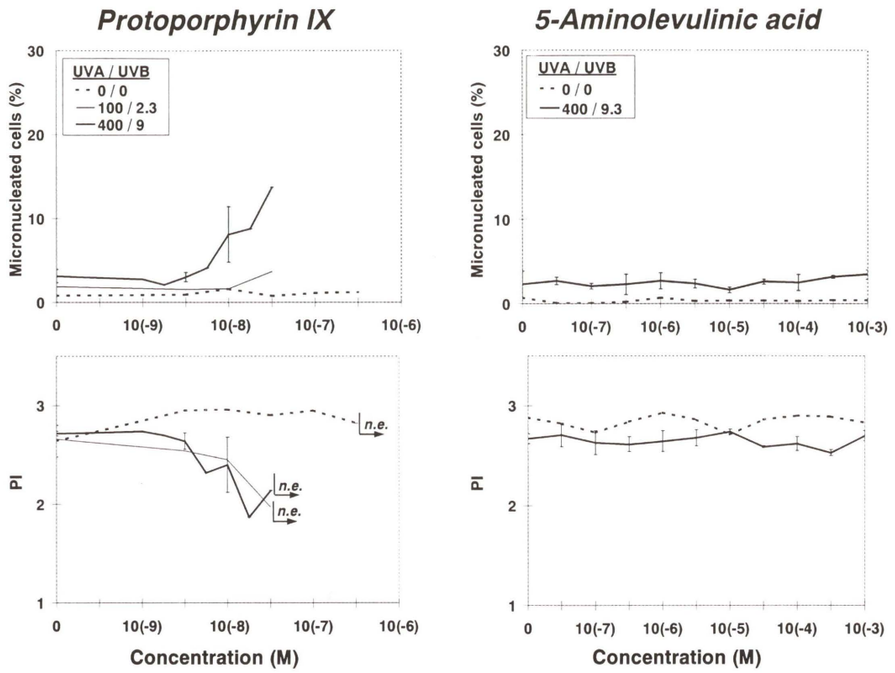

Fig. 3 Effects of protoporphyrin IX and 5-aminolevulinic acid in the photo-micronucleus assay using V79 cells. Further legend: see Fig. 1.

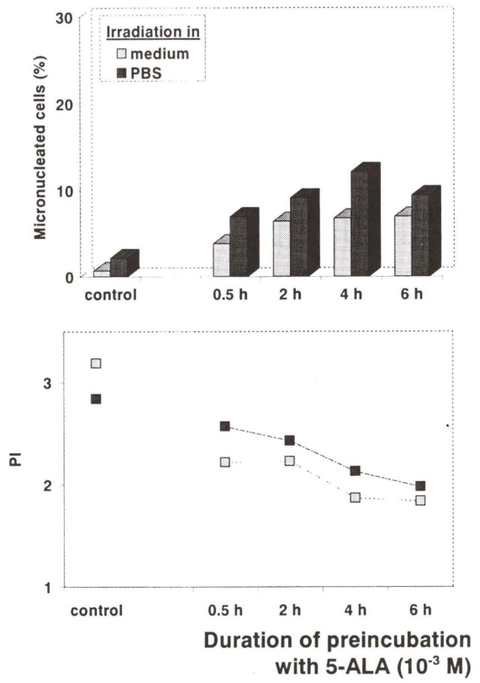

Fig. 4 Photo-micronucleus assay in V79 cells preincubated with 5-aminolevulinic acid (10^{-3} M, in medium or PBS, respectively) for different treatment times and then irradiated with UVA/UVB doses of 400/9.3 mJ/cm^2. PI, proliferation index.

The proliferation of neutral red treated and irradiated cells was inhibited in a concentration dependent manner leading to the classification of the compound as a phototoxin. Methylene blue, a phenothiazine derivative, slightly induced micronuclei and reduced the PI at concentrations above 10^{-4} M in non-irradiated cells (see Fig. 2). Additional irradiation led to a distinct shift of the respective LOECs to lower concentrations and to more pronounced cytotoxic and genotoxic effects.

Without irradiation, protoporphyrin IX showed neither genotoxic nor cytotoxic effects in V79 cells (see Fig. 3). Following irradiation the compound induced micronuclei already at very low concentrations of 10^{-8} M in comparison to the irradiation control. The cell proliferation was inhibited in the same concentration range indicating phototoxic effects. Testing of 5-ALA up to concentrations of 10^{-3} M in the photo-micronucleus assay using the standard protocol (30 min preincubation with the compound) gave clear negative results in regard to (photo)toxicity and (photo)genotoxicity (see Fig. 3). Prolonged incubation of the cells with 10^{-3} M 5-ALA (2-6 hours) before irradiation led to a clear induction of micronuclei in comparison to the irradiation controls (see Fig. 4).

Discussion

For the detection of photochemical genotoxicity of several visible light absorbing compounds we selected a photo-micronucleus test in vitro, which is a reliable test

Table 2 Phototoxicity (Phototox.) and photogenotoxicity (Photogenotox.) of visible light absorbing compounds tested in the photo-micronucleus assay in V79 cells

Chemical class	Compound	Phototox. (PI)	Photogenotox. (MNC)	Photogenotox. LOEC(M)
acridines	acridine			
	proflavine			3.2×10^{-9}
anthraquinones	hypericin*			10^{-7}
	emodin*			
phenazine deriv.	neutral red			10^{-7}
phenothiazine	methylene blue			3.2×10^{-6}
porphyrine	protoporphyrin IX			10^{-8}
(inducer)	5-aminolevulinic acid			
	5-aminolevulinic acid^{pp}			n.d.

shaded boxes, positive results; open boxes, negative results; n.d., not determined; PI, proliferation index; MNC, micronucleated cells; LOEC, lowest observed effect concentration; pp, prolonged preincubation; *, Kersten et al., 1999.

system for this purpose (Kersten et al., 1999). Concomitantly, this assay enables the determination of the photocytotoxic potential of test compounds. Irradiation was performed in the Suntest CPS$^+$ machine equipped with a Xenon burner providing solar light conditions.

Acridine derivatives are dyes which absorb in the UVA to visible light range and are known to intercalate into DNA (Ferguson and Denny, 1991). For different derivatives, e.g., proflavine (2,8-diaminoacridine) and acridine orange (2,8-bis-dimethylaminoacridine) the induction of DNA strand breaks (Uggla and Sundell-Bergman, 1990), chromosomal aberrations (Uggla, 1990), and sister chromatid exchanges in CHO cells (Uggla, 1990; Speit and Vogel, 1979) were described. The photogenotoxic effect of proflavine revealed in the photo-micronucleus assay is in line with these data. Acridine was not photogenotoxic in our test system. No studies on photogenotoxicity of acridine were available from the literature, with the exception of reports on a weak induction of mutations to phage T5 resistance by acridine; but the reported mutation frequency was much lower than that induced by proflavine or other amino derivatives (Webb et al., 1979).

Neutral red is a cationic, vital dye that can be used as a histological stain for proliferating cells. It absorbs in the visible light range and has been used clinically for photodynamic therapy because of its phototoxic properties (VanderWerf et al., 1997). No data on photogenotoxic effects of neutral red came to our attention. In the photo-micronucleus test, the compound was photogenotoxic. Methylene blue is a well-known dye in medicine and has been discussed as an easily applicable drug for the topical treatment in photodynamic therapy (Orth et al., 1998). The compound is a phenothiazine derivative substituted with a dimethylamino group at C3- and C7-position and can probably intercalate into the DNA. Methylene blue absorbs within the visible light area. Production of singlet oxygen has been suggested as the mode of photoactivation leading to photogenotoxic effects (Epe et al., 1989; Gocke, et al., 1998). In the photo-micronucleus test the compound has also been classified as photogenotoxin.

Protoporphyrin IX is the immediate precursor of protoheme in plant and animal cells, which is the prosthetic group of hemoglobin and others. After absorption of visible light the compound excerts photosensitizing properties via formation of ROS (Fuchs et al., 2000). The photoactivation results in strong phototoxic effects, but only moderate photogenotoxic effects were described (Fuchs et al., 2000). The reported predominance of the phototoxic effects has been discussed as a consequence of the prefered location of protoporphyrin in cellular membranes, i.e., far away from the genetic material due to its large size (Gocke et al., 1998). In the photo-micronucleus assay the compound induced photogenotoxic as well as photocytotoxic effects.

5-ALA is a precursor of protoporphyrin in the biosynthetic pathway of heme and induces the intracellular synthesis of especially protoporphyrin IX. The resulting photosensitizing properties are used by clinical photodynamic therapy (Fuchs et al., 2000). 5-ALA itself is not light-absorbing and lacks therefore any photosensitizing properties (Fuchs et al., 2000). In accordance with these data our tests with the standard protocol of the photo-micronucleus assay were clearly negative for photogenotoxicity. However, a prolonged incubation with the compound (2-6 h) led to clear photogenotoxic effects probably caused by the time-dependent intracellular induction of the porphyrin synthesis. Pflaum et al. (1998) could demonstrate that 5-ALA increases the sun light-induced formation of 8-hydroxy-2-deoxyguanosine and induces micronuclei fol-

lowing in vitro incubation and irradiation of L1210-mice cells. Similar studies of the same group using the XPRT-Test in AS52-Zellen did not reveal increasing gene mutation rates induced by 5-ALA.

The generation of ROS has been described for most of the visible light absorbing compounds tested in our system (Table 2). Results with other ROS producing photosensitizers revealed a preference for the induction of clastogenic effects (Chételat et al., 1996; Gocke et al., 1998). Only comparatively weak gene mutation induction has been reported from the same group. Further studies to compare the clastogenic effects indicated by micronucleus induction with mutagenic effects in the same cell type for the compounds described in this study would be useful for risk analysis.

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Original Article

A note on artificial induction of mutation upon testing 7,12-dimethylbenz[*a*]anthracene mutagenicity under fluorescent light in the absence of microsomal enzymes

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Summary

When mutagenicity assay was done under fluorescent lamps commonly used for room lighting, 7,12-dimethylbenz[*a*]anthracene (DMBA) showed mutagenicity without any enzymatic activation. The light between wavelength of 370 and 450 nm is responsible for the photoactivation of DMBA, which is corresponding to the absorption peak of DMBA. The formation of the direct-acting mutagen was inhibited by addition of cofactor for S9mix. These findings have relevance to the routine testing of chemicals for mutagenic activity.

Keywords : photoactivation, photogenotoxicity, DMBA, visible light, mutagenicity

Introduction

Carcinogenic polycyclic aromatic hydrocarbons such as benzo[*a*]pyrene (B[a]P) and DMBA require metabolic conversion to ultimate carcinogens to exert their genetic actions (McCann et al., 1975 ; Pelkonen and Nerbert, 1982). Microsomal cytochrome P450 monooxygenases play a significant role in this biotransformation. DMBA showes therefore mutagenicity in bacterial test systems only in the presence of a metabolic activation enzymes. CYP1B1 is the major isozyme which is involved in metabolic activation of DMBA (Shimada et al., 1996 ; Savas et al., 1997 ; Heidel et al., 2000). DMBA is often used as a positive control in the mutagenicity test of chemicals in the frameshift type of bacterial tester strains in the presence of microsomal metabolizing enzymes.

An appreciable yield of mutants were induced with DMBA in the absence of S9mix. The mutation thus induced was delicately dependent on the experimental procedure in testing mutagenicity. The present study shows the formation of direct-acting mutagens under illumination condition provided by common fluorescent lamps for room lighting.

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Materials and Methods

Materials

DMBA was purchased from Nakalai Tasque Inc. (Kyoto, Japan). DMBA preparations used for experiment were crystallized from acetone-ethanol to give greenish yellow plates ; m.p.123°C.

The liver S9 fraction prepared from rat pretreated with both phenobarbital and β-naphthoflavone were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). The cofactor-I for S9mix was also purchased from Oriental Yeast Co., Ltd. The TA98 tester strain of *Salmonella typhimurium* was kindly provided by Dr. B.N. Ames, University of California, Berkeley, CA.

The illumination meter TM-2D (Topcon Corporation, Tokyo) was used for measurement of the illuminance.

Mutagenicity assay

Mutagenicity of test compounds, at the doses indicated under Results, was evaluated in the *Salmonella typhimurium* TA98, according to the standard plate assay as described by Maron and Ames (1983). All procedures were carried out in subdued light, otherwise mentioned in

Table 1 Production of direct-acting mutagen by exposure to fluorescent light

Irradiation time (min)	Number of His$^+$ revertants/plate
DMBA; 0 μg/plate	
0	42
DMBA; 10 μg/plate	
0	33
20	84
60	249

A mixture of 0.1 ml of DMBA solution (100 μl/ml in dimethyl sulfoxide), 0.5 ml of 0.1 M phosphate buffer (pH 7.4) and 0.1 ml of a fresh overnight culture of *S. typhimurium* TA98 was poured onto a minimal glucose agar plate. After exposure to fluorescent lamps at 430 lx for time indicated in the table, the plates were kept at 37°C in the dark for 2 days, and revertants to histidine-independence were counted.

the text. To 0.1 ml of a fresh overnight culture of the tester strain, 0.1 ml of DMBA solution dissolved in dimethyl sulfoxide and 0.5 ml of 100 mM sodium phosphate buffer (pH 7.4) or S9mix were added and mixed by vortexing. One ml of the S9mix contained $MgCl_2$ (8 μmol), KCl (33 μmol), glucose-6-phosphate (5 μmol), NADPH (4 μmol), NADH (4 μmol), sodium phosphate buffer (100 μmol) and various amounts of S9 fraction. The mixture was then poured onto a minimal glucose agar plate with 2 ml of top agar containing 0.05 mM of L-histidine and 0.05 mM biotin. The plates were placed on a level surface to harden. The plates were inverted and placed in a dark 37°C incubator. After 48 h incubation the number of revertant colonies on the plates were counted. The data represent average values obtained at least in duplicate.

Exposure to light

A 27 W fluorescent-light bulb, FML27 EX-N (Matsushita Electronics Co., Osaka, Japan) was used as a source of fluorescence light. The illuminance on the desk was measured by Illumination Meter IM-2D (Topcon Corporation, Tokyo, Japan). No intensity of UVA light was detected, as measured by an ultraviolet intensity digital radiometer equipped with a 365 sensor (Ultraviolet Products, San Gabriel, CA, USA).

Results

Exposure of the mutation plate after pouring cells with DMBA to fluorescent light

In standard plate incorporation assay described by Maron and Ames (1983), the test compound and the bacterial strain in soft agar were poured onto a minimal glucose agar plate without S9mix, and then placed on a level surface to harden. When the plate was covered with aluminium foil to avoid the exposure to light, no mutagenicity was detected as shown in Table 1. On the other hand, the plate was exposed to fluorescent lamps commonly

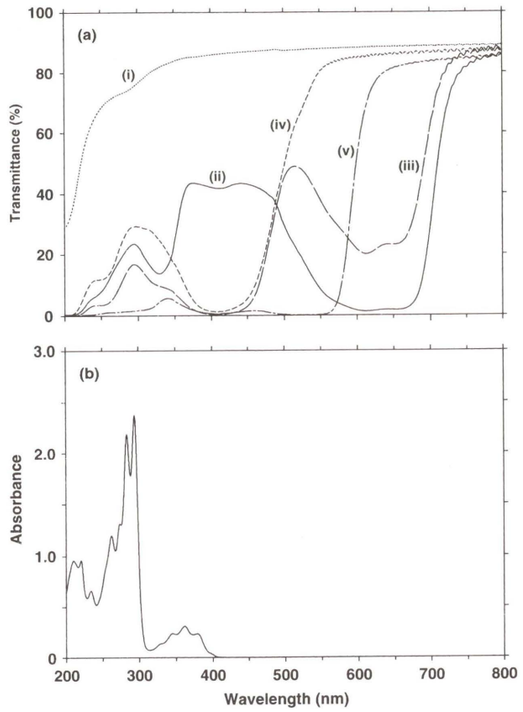

Fig. 1 UV-visible spectra of colored cellophane sheet recorded with a Shimadzu UV-2200 spectrophotometer.
(**a**) transmittance spectra of colored cellophane sheet ; (i) transparent, (ii) blue, (iii) green, (iv) yellow and (v) red, and (**b**) absorption spectrum of DMBA dissolved in methanol at a concentration of 10 μg/ml.

used for room lighting, direct-acting mutagens were formed depending on the length of exposure time.

Dependency of wavelength of light on the production of direct-acting mutagen

Colored cellophane sheets were used in order to determine the range of wavelengths of the light responsible for photoactivation of DMBA. The minimal glucose agar plate poured DMBA with the tester strain was covered with colored cellophane sheet during exposure to the light. Transmittances of cellophan sheet were shown in Fig. 1a. A mutagenic response was observed by interposing blue or transparent filter transmitting wavelength between 370 and 450 nm (Table 2), which is coresponding to minor absorption of DMBA, as shown in Fig. 1b.

Effect of solvent on production of direct-acting mutagen

DMBA is usually dissolved in dimethyl sulfoxide for mutagenicity test. The experiment was carried out by exposure of DMBA solution in dimethyl sulfoxide. Exposure of DMBA solution dissolved in a mixture of

Table 2 Dependency of wavelength on production of direct-acting mutagen from DMBA

DMBA concentration (μg/plate)	Number of His$^+$ revertants/plate Color of cellophane sheet covered				
	transparent	blue	green	yellow	red
0	41				
10	187	211	46	57	52

A mixture of 0.1 ml of DMBA solution (100 μl/ml in dimethyl sulfoxide), 0.5 ml of 0.1 M phosphate buffer (pH 7.4) and 0.1 ml of fresh overnight culture of *S. typhimurium* TA98 was poured onto a minimal glucose agar plate, and then exposed to fluorescent lamps at 2000 lx for 60 min. During exposure to fluorescent lamps, the lidded plates were covered with cellophane sheet. After exposure to the lamps, the plates were kept at 37°C in the dark for 2 days, and revertants to histidine-independence were counted.

Table 3 Effect of solvent on the production of direct-acting mutagen

Irradiation time (min)	Number of His$^+$ revertants/plate	
	DMSO	DMSO + PB
0	30	29
20	33	685

Twenty μg of DMBA were dissolved in 0.6 ml of dimethyl sulfoxide (DMSO) or a mixture of dimethyl sulfoxide and 0.1 M phosphate buffer (pH 7.4) (1 : 5). After exposure to fluorescent lamps at 3250 lx, 0.1 ml of a fresh overnight culture of *S. typhimurium* TA98 were added, and poured onto a minimal glucose agar plate. The plates were then kept at 37°C in the dark for 2 days, and revertants to histidine-independence were counted.

Table 4 Dependency of amount of S9 fraction on DMBA mutagenicity

S9 concentration (μl/ml S9mix)	Number of His$^+$ revertants/plate	
	Fluorescent light	Yellow light
(- S9mix)	875	27
0	44	33
0.5	62	29
1	55	23
5	38	34
10	61	43
20	91	106
50	224	243
100	501	535
solvent control	30	24

A mixture of 0.1 ml of DMBA solution (200 μl/ml in dimethyl sulfoxide), 0.5 ml of S9mix or 0.1 M phosphate buffer (pH 7.4), and 0.1 ml of fresh overnight culture of *S. typhimurium* TA98 was poured onto a minimal glucose agar plate. The plates were then placed under fluorescent light at 390 lx or yellow light at 140 lx for 60 min, kept at 37°C in the dark for 2 days, and revertants to histidine-independence were counted. The experiments indicated by (- S9mix) were carried out by addition of phosphate buffer instead of S9mix.

dimethyl sulfoxide and the phosphate buffer to fluorescent lamps produced direct-acting mutagen (Table 3). On the other hand, no direct-acting mutagen was produced when DMBA was dissolved in dimethyl sulfoxide.

Effect of S9mix

DMBA is often used as a positive control for microsomal preparation in bacterial mutagenicity test system. We therefore examined the effect of exposure to the light on DMBA mutagenicity in the presence of S9mix. In the presence of S9mix, DMBA mutagenicity increased with increasing in the amount of S9 fraction under either fluorescent or yellow lamps, as shown in Table 4. Mutagenic activity of DMBA under fluorescent lamps was not different from that under yellow lamps. It is worth noting that the addition of cofactor that did not contain S9 fraction suppressed DMBA mutagenicity induced by exposure to fluorescent lamps to the background level.

Discussion

We present here the evidence that exposure of carcinogenic polycyclic aromatic hydrocarbon, DMBA, to fluorescent lamps for room lighting results in the formation of direct-acting mutagen which shows mutagenicity without metabolic activation.

Previous studies have shown that certain promutagens or even non-mutagenic compounds can photoactivated to derivatives exhibiting a direct mutagenicity in bacterial cells (McCoy et al., 1979 ; Israel-Kalinsky et al., 1982 ; Strniste et al., 1985 ; De Flora et al., 1989 ; Arimoto-Kobayashi et al., 1997). These compounds include polycyclic aromatic hydrocarbons, heterocyclic amines, aflatoxines and nitrosamines. Some photoactivating products were identified (Warshawsky et al., 1977 ; Tu et al., 1979 ; Okinaka et al., 1984, 1986 ; Strniste et al., 1986 ; Hirose et al., 1990 ; Arimoto and Hayatsu, 1991). Most of the studies were focused on the interactions in extracellular environments between light and chemical carcinogens. Sunlight or artificial sunlight as a source of visible light was therefore used for the photoactivation of environmental mutagens. McCoy et al. (1979) have reported the production of direct-acting mutagen by exposure of DMBA dissolved in dimethyl sulfoxide to fluorescent white light which contains near-UV light. They have also indicated that this photoactivation did not appear to be dependent upon the generation of singlet oxygen, though putative photoproduct, presumably 7,12-epodioxy-DMBA, was produced by illunimation in the presence of singlet oxygen generator. On the other hand, we present here the evidence that direct-acting mutagen did not produced by

exposure to fluorescent lamps when DMBA was dissolved in dimethyl sulfoxide. These results indicate that mechanisms for production of direct-acting mutagen seem to be dependent on the wavelength of the light. In fact, direct acting mutagen was produced by exposure of DMBA solution dissolved in dimethyl sulfoxide to UVA or UVC light (data not shown).

We tested several DMBA samples obtained from different companies. Some of these contained impurity which showed mutagenicity without any enzymatic activation (data not shown). The impurity could be removed by recrystalization. This purified DMBA did not show mutagenic activity after storage using brown screw-capped brown bottle in freezer when dissolved in dimethyl sulfoxide.

The guidelines on testing of chemicals for mutagenicity are published from governments. None of guideline has however described about the room lighting condition where the experiment was carried out. The present results have relevance to the routine testing of chemicals for mutagenicity. In order to avoid artificial mutagenicity, test chemicals must be shielded from light or stored in, manipulation carried out in subdued light or under yellow lamps, and the plate incubated in the dark.

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Original Article

The rapid screening of photogenotoxic compounds using photo plasmid-relaxation assay

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Summary

To develop a simple and rapid screening system for detection of photogenotoxic chemicals, we applied the plasmid-relaxation assay to the in vitro photochemical genotoxicity assay system. Using 54 chemicals as model compounds, including known phototoxic and non-phototoxic chemicals, we evaluated the performance of the plasmid-relaxation assay. The results obtained here were comparable with published data that were assessed from *in vivo* phototoxicity systems. We concluded that the plasmid-relaxation assay is useful as a primary screening method to detect chemicals with photochemical genotoxicity.

Keywords : plasmid-relaxation assay, phototoxicity, photochemical genotoxicity

Introduction

Certain chemicals are known to elicit cytotoxicity and/or genotoxicity in the presence of UVA/visible light irradiation. Many in vitro phototoxicity assays have been proposed to detect such phototoxic chemicals and some have been validated for routine use.

For example, the in vitro BALB 3T3 cell neutral red uptake (3T3 NRU) phototoxicity test has been validated extensively (Spielmann et al., 1994 ; Spielmann et al., 1998) and has been proposed as an OECD test guideline. Photochemical genotoxicity assays are also important for hazard identification of chemicals from the viewpoint of photocarcinogenicity *in vivo*, especially of skin carcinogenesis. For this purpose, ordinary genotoxicity assays are being applied in combination with UVA/visible light irradiation. However, there are still some aspects to be addressed before assays are standardized, e.g., the use of an exogenous metabolic activation system and the limited number of applicable bacterial strains in the Ames test (Gocke et al., 2000).

The induction of photocytotoxicity and photogenotoxicity are closely related to photochemical reactions induced by photosensitization. Thus, biological interaction between cellular substrates such as DNA and protein and reactive oxygen species generated when chemicals absorb a photon of UV/visible radiation, may play an important role in the induction of photochemical DNA damage as an initiating event of phototoxicity. In this study, we aimed to establish a simple and rapid screening system to detect photogenotoxic chemicals in order to validate the application of the plasmid-relaxation assay using 54 model chemicals, some of which are known to be phototoxic.

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Materials and Methods

1) Plasmid DNA

A super coiled plasmid DNA was isolated from *Escherichia coli* XL-I Blue containing the pUCSV-BSD plasmid using QUIAGEN plasmid purification kit (QUIAGEN Inc., Santa Clarita, USA). Immediately before treatment, plasmid DNA was diluted with Dulbecco's phosphate buffered saline without Ca^{++} and Mg^{++} ions (PBS (－) ; Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) to the concentration of 50 ng/μL.

2) Test compounds

Fifty-four chemicals were selected from among environmental pollutants, dyes, drugs, industrial materials, and some miscellaneous chemicals. Some chemicals were known to be phototoxic both in vitro and in vivo. The model chemicals used in this study are listed in Table 1.

3) Positive control

As a positive control, ofloxacin (Sigma, Lot No. : 46H0747) was used. Ofloxacin was dissolved in sterilized

Table 1 The test chemicals used in this experiment

No.	Chemical	CAS No.
1	Naphthalene	91-20-3
2	Phenanthrene	85-01-8
3	Anthracene	120-12-7
4	Pyrene	129-00-0
5	Benz[*a*]anthracene	56-55-3
6	Benz[*b*]anthracene	92-24-0
7	Benz[*a*]pyrene	50-32-8
8	Benz[*c*]pyridazine	253-66-7
9	7,12-Dimethylbenzo[*a*]anthracene(DMBA)	57-97-6
10	3-Methylcholanthrene	56-49-5
11	Erythrosin B	16423-68-0
12	Rose bengal	632-69-9
13	Eosin Y	17372-87-1
14	Promethazine hydrochloride	58-33-3
15	Chlorpromazine	69-09-0
16	Levomepromzine	60-99-1
17	Doxycycline hydrochloride	17086-28-1
18	Tetracycline hydrochloride	64-75-5
19	Nalidixic acid	389-08-2
20	Cinoxacin	28657-80-9
21	Pipemidic acid	51940-44-4
22	Piromidic acid	19562-30-2
23	Norfloxacin	70458-96-7
24	Enoxacin	74011-58-8
25	Acridine orange hydrochloride	494-38-2
26	Neutral red	553-24-2
27	Hexachlorophene	70-30-4
28	Bithionol	97-18-7
29	3,4',5-Tribromosalicylanilide	87-10-5
30	Piroxicam	36322-90-4
31	6-Methylcoumarin	92-48-8
32	8-Methoxypsoralen	298-81-7
33	Pentachlorophenol	87-86-5
34	2,4-Dinitro-1-chlorobenzene	97-00-7
35	Benzidamine hydrochloride	132-69-4
36	Sulfanilamide	63-74-1
37	Pyrithioxin	1098-97-1
38	L-Ascorbic acid	50-81-7
39	Methylene blue	61-73-4
40	Thiourea	62-56-6
41	t-Cinnamaldehyde	14371-10-9
42	Sodium lauryl sulfate(SDS)	151-21-3
43	D-Mannitol	69-65-8
44	L-Histidine	71-00-1
45	p-Dichlorobenzene	106-46-7
46	p-Aminobenzoic acid	150-13-0
47	Butylated hudroxytoluene(BHT)	128-37-0
48	Penicillin G sodium salt	69-57-8
49	2-Hydroxy-4-methoxybenzophenone	131-57-7
50	2-Acetyl aminofluorene(2-AAF)	53-96-3
51	3,4-Dimethyl-3*H*-imidazo[4,5-*f*]quinolin-2-amine(MeIQ)	77094-11-2
52	Bisphenol A	80-05-7
53	Clofentezine	74115-24-5
54	Chlorhexidine dihydrochloride	3697-42-5

distilled water and diluted with PBS(−) (1 : 10). Eighteen μL of diluted positive control solution was then transferred to U-shaped 96 multi-well plates, and 2 μL of plasmid solution (50 ng/μL) was added. The final concentration of ofloxacin was 100 μg/mL.

4) Treatment

Acetone or distilled water was used for solvent according to the solubility of each chemical. In principle, the highest concentration was set at 1000 μg/mL. Test chemical solutions (100 μL) were mixed with 900 μL of PBS (−) (10 v/v %) in 1.5 mL eppendorf tubes. The reaction mixtures were dispensed into U-shaped 96 multi-well plates at 18 μL/well, to which 2 μL of plasmid solution (50 ng/μL) was added. After 1 h of pre-incubation with test chemicals, the plates were exposed to UV/visible light for 50 min at 1.0 mW/cm^2 (3.0 J/cm^2) by using a solar simulator (SOL500, Dr. K. Hönle). The intensity of UVA was measured immediately before irradiation with a UVA meter (Type 37, Dr. K. Hönle). For controls, duplicate samples were incubated at room temperature in the dark. Electrophoresis of the reaction mixtures (10 μL) was performed at constant voltage (100 V) in 0.8% agarose gels with 0.5x Tris-borate EDTA (TBE) buffer. Gels were stained in aqueous solutions of ethidium bromide (1.0 μg/mL) for 15 minutes and photographed with the child CCD camera (C5810, Hamamatsu Photonics, Hamamatsu, Japan).

5) Measurement of DNA damage

The fluorescent intensities of closed circular supercoiled DNA, open circular DNA and linear DNA were measured by using image analysis software (NIH image). The percentage of damaged DNA was calculated from the ratio of the fluorescent intensities of open circular DNA (single strand break) and linear DNA (double strand break).

Results and Discussion

The solvent control (UV−, UV+) data obtained in this study are shown in Fig. 1. Based on the theoretical binomial distribution of the UV− data, the probability of three times higher than average was less than 1% (nominal significance level). Therefore we judged as positive when the percentage of damaged DNA in the treated groups exceeded three times that of solvent control. In order to evaluate UV+ data to the same significance level, we judged as positive in the UV+ group when the percentage of damaged DNA in the treated groups exceeded two times that of solvent control. In cases where DNA damage was not induced in solvent control, we used the historical average value for solvent control instead of the concurrent

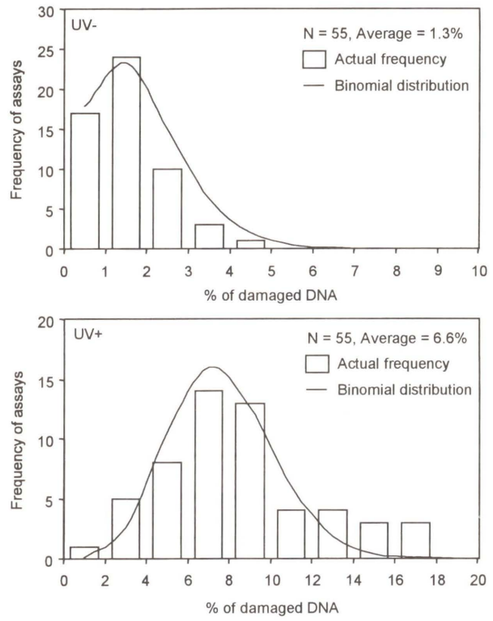


Fig. 1 Frequency distribution of solvent control (UV−, UV+) and theoretical binomial distribution
The theoretical binomial distribution was calculated from the average percentage of damaged DNA. The frequency distribution of actual data was nearly approximated to theoretical value.
From the theoretical distribution of UV−, the probability of three times higher than average (about 4%) was about 1%, we judged positive when the percentage of damaged DNA of treated groups exceed three times higher than solvent control.
Similarly, we judged positive at UV+ group when the percentage of damaged DNA of treated groups exceed two times higher than solvent control (about 13%).

control. The final judgment of the plasmid-relaxation assay was made after assessing the difference of the minimum positive concentration between UV− and UV+ assays, and the intensity of the damaged DNA at the same concentration.

The test results of dose-related DNA damages of chemicals are shown in Fig. 2, and summarized in Table 2. The overall evaluation of in vivo phototoxicity data listed in Table 2 was based on published articles (Spielmann et al., 1994 ; Spielmann et al., 1998 ; Klecak et al., 1997 ; Takayama et al., 1995 ; Cavalieri and Calvin 1971 ; Morton et al., 1951 ; Okamoto et al., 1999). Almost all of the chemicals known to be phototoxic, e.g., PAH (poly aromatic hydrocarbons), Phenothiadine, and Quinlones, clearly induced plasmid DNA breaks under UV/visible light irradiation. Of 17 such chemicals, 16 (including a positive control, ofloxacin) were positive in the present assay. The only exception was neutral red and it was judged as negative. In the electrophoresis, the plasmid DNA treated with a high concentration of neutral red was not detected because the plasmid DNA had migrated to the positive electrode and was degraded.

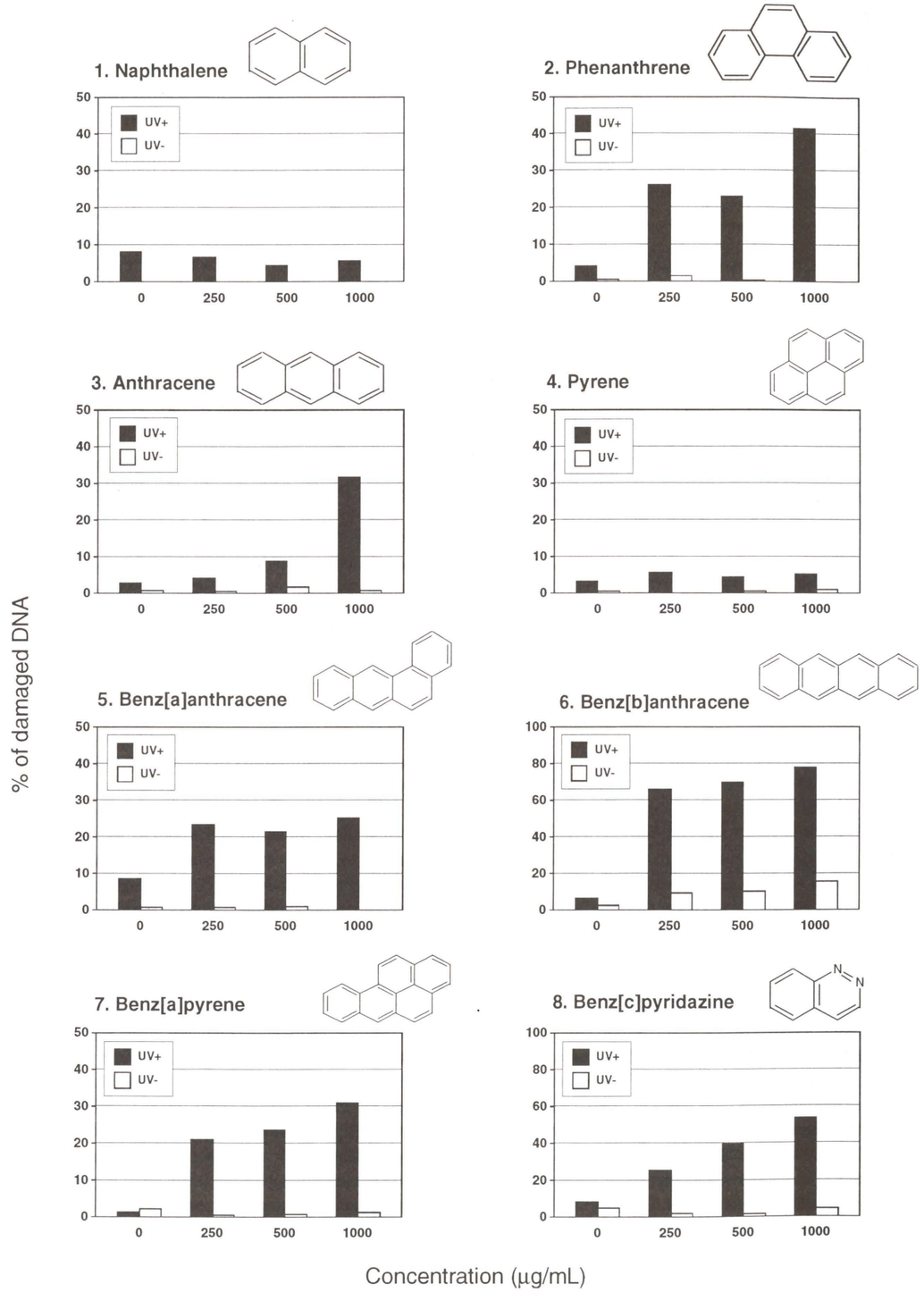
Four chemicals, benz[*a*]anthracene, DMBA, pentachlorophenol, and L-ascorbic acid showed the same levels of minimum positive concentration difference between UV+ and UV− assays. Except for naphthalene and pyrene, PAHs clearly induced DNA damage, and such effects were well correlated with the results of *in vitro* micronucleus and chromosome aberration tests (unpublished data). The reason why naphthalene and pyrene did not induce DNA damage is not well understood but it may be due to physicochemical characteristics, e.g., the absorption pattern of naphthalene shifted to the shorter wavelength side of the UV spectrum.

Among 9 chemicals which are known to be non-phototoxic in animal or human, only 3,4',5-tribromosalicylanilide was positive in the photo plasmid-relaxation assay. This chemical has been reported to be phototoxic (Okamoto et al., 1999) in other *in vitro* phototoxicity tests such as hemoglobin photo-oxidation and 3T3 NRU phototoxicity. Therefore, 3,4',5-tribromosalicylanilide may exhibit phototoxicity under *in vitro*-specific conditions.

The performance of the photo plasmid-relaxation assay in predicting phototoxicity *in vivo* is shown in Table 3. The results are consistent with the *in vivo* data, showing 94% sensitivity, 89% specificity, 94% positive predictivity and 89% negative predictivity.

In general, phototoxic chemicals show their toxicity because of reactive oxygen species generated through photo catalytic action after absorption of a photon of UV/visible light radiation. Okamoto et al. (1999) have shown this to be the case by experimenting with free-radical scavengers. Particularly, Type II photodynamic reactions (e.g., producing singlet oxygen) and the Type I reactions (e.g., reactions of superoxide anion, hydrogen peroxide, and hydroxyradical) are largely involved in phototoxicity. It is suspected that such free radicals and excited singlet oxygen can easily attack DNA and other bio-molecules resulting in cell death.

The test condition of the photo plasmid-relaxation system presented here is quite simple. The test article is simply mixed with plasmid solution, then placed under UV/visible light irradiation. Although it is expected that there are considerable differences between *in vitro* and *in vivo* assays, the present study showed that results in the *in vitro* system reflected those obtained from *in vivo* studies. Another advantages of this method as a screening system is the small scale of experiments, which makes it possible to test many chemicals at one time with less labor. We recommend use of the photo plasmid-relaxation assay as the initial screening method for the photogeno-



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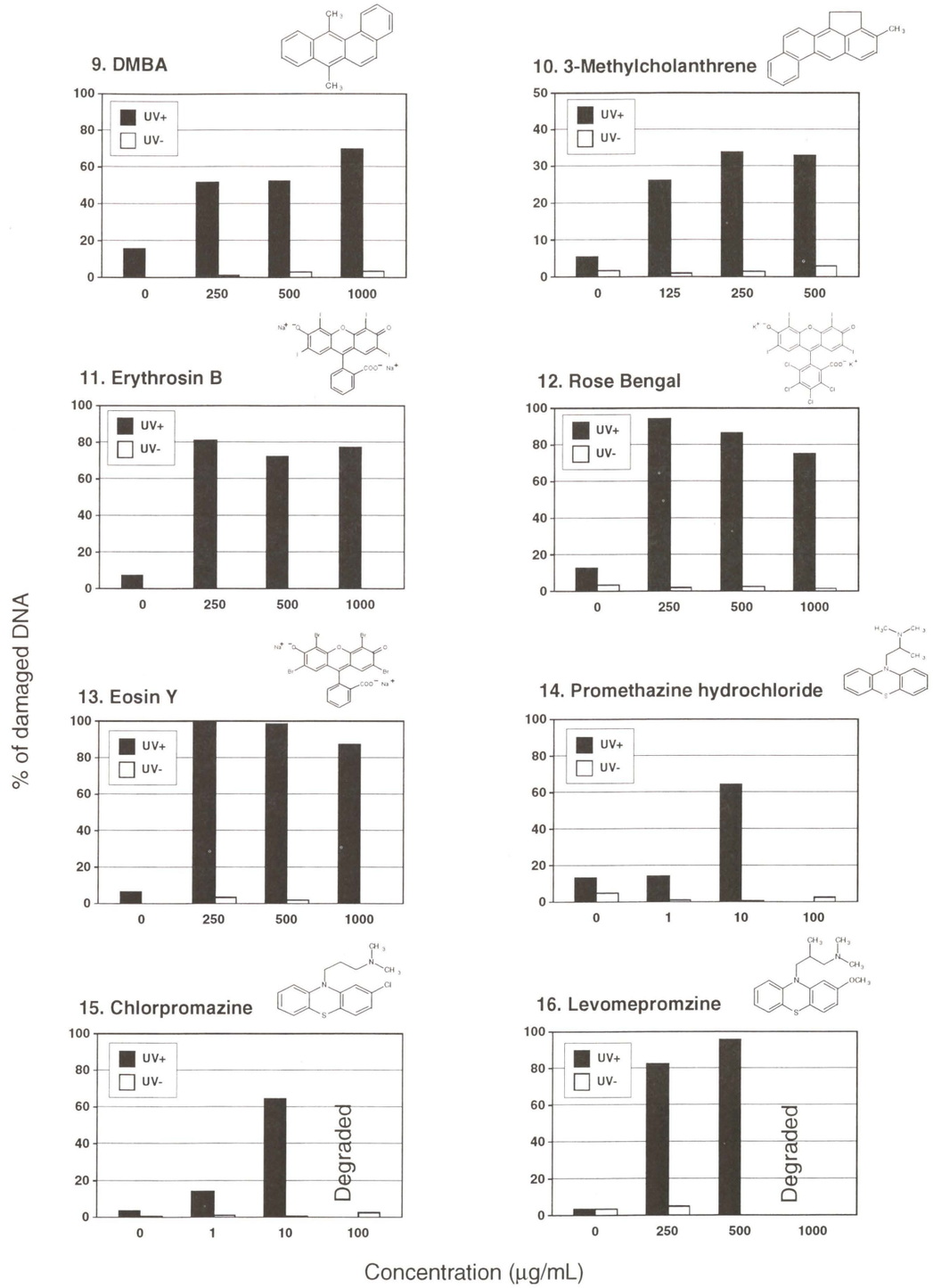

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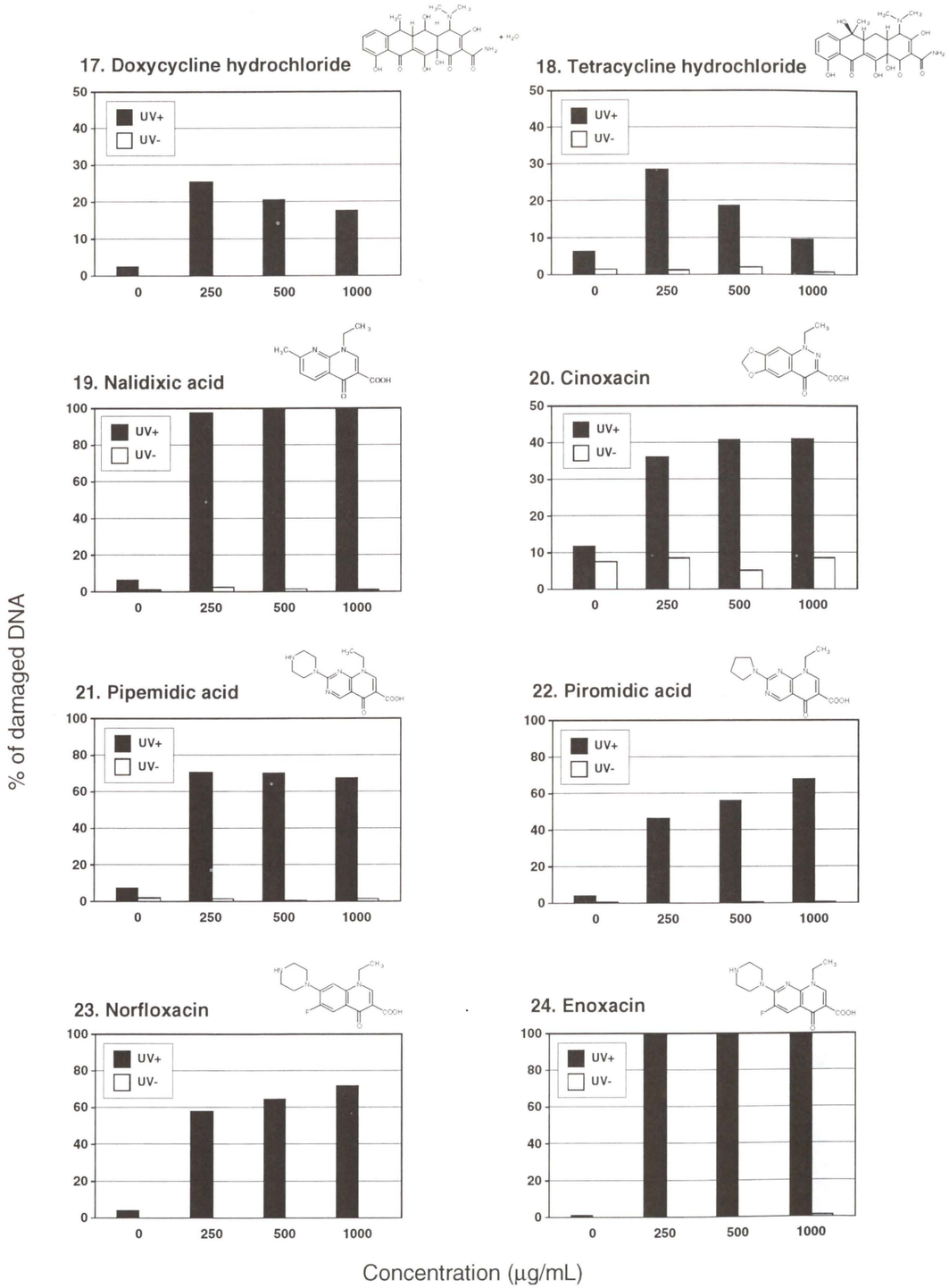

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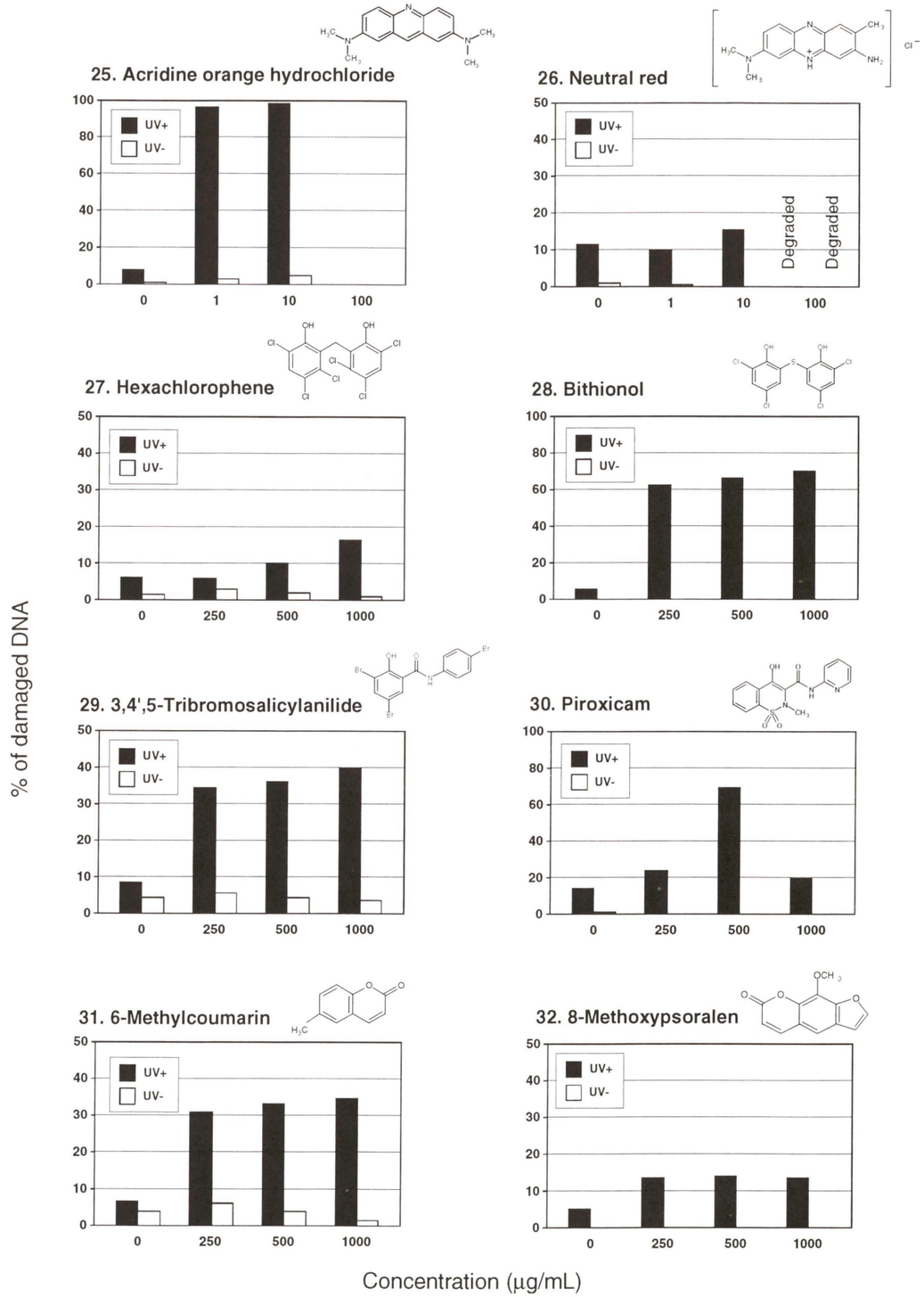

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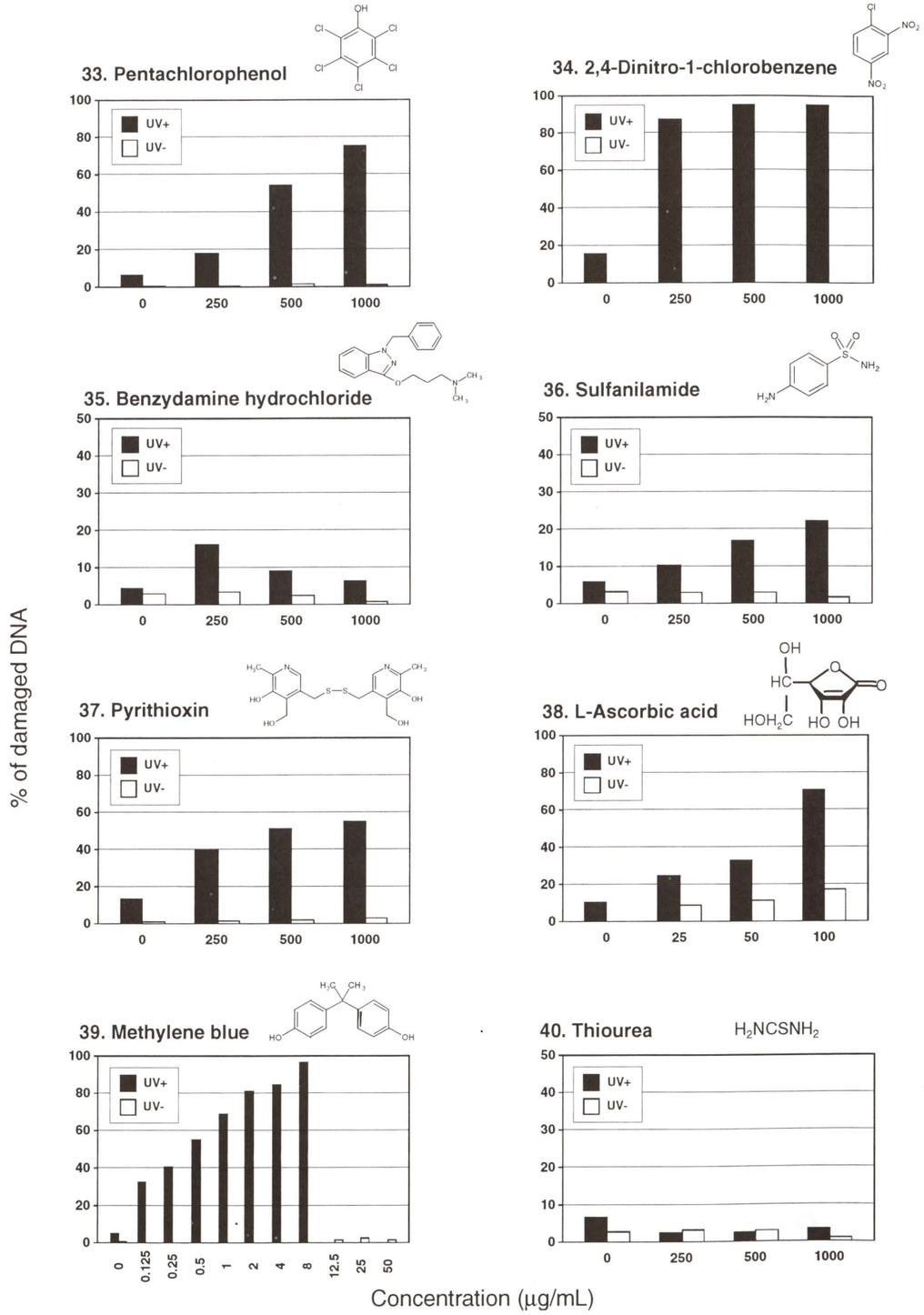

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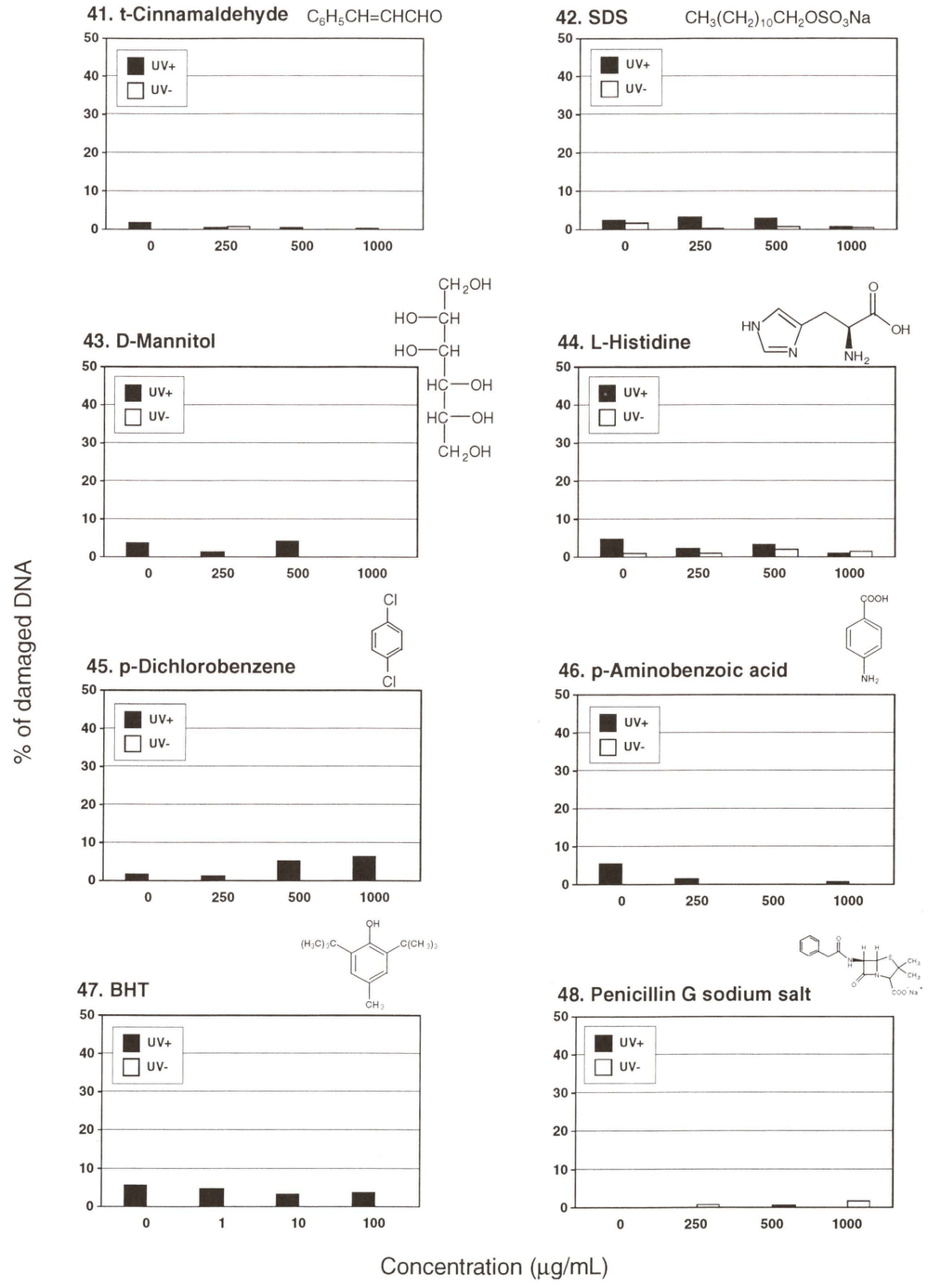

(Fig. 2 continued)

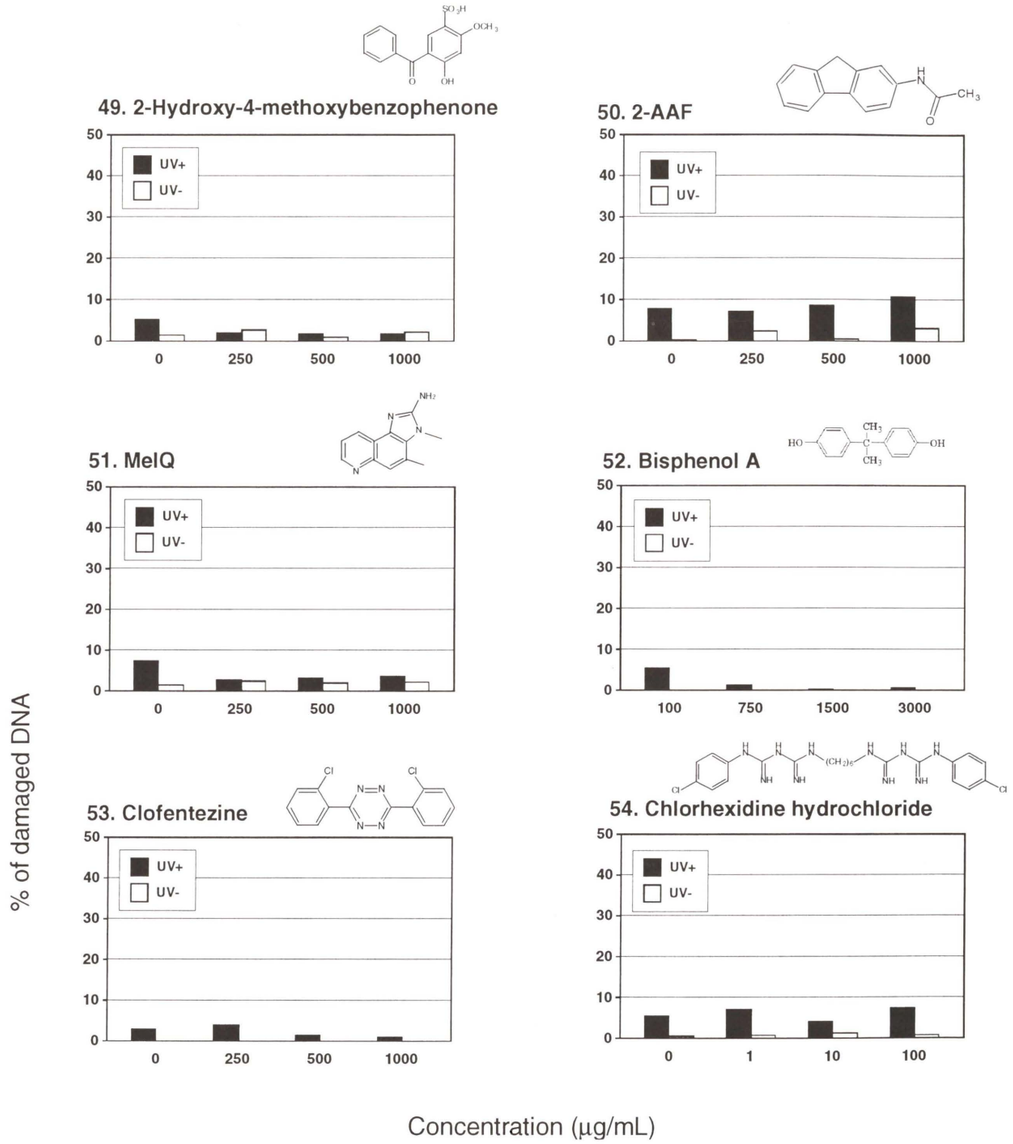

Fig. 2 The results of photo plasmid-relaxation assays of 54 chemicals

toxicity potential of chemicals.

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Table 2 The summary results of photo plasmid-relaxation assays of 54 chemicals

No.	Chemical	Minimum positive concentration (μg/mL) UV+	Minimum positive concentration (μg/mL) UV−	Judgement of Photo relaxation	Phototoxicity in vivo[a]	% of DNA damage of positive control[b] UV+	% of DNA damage of positive control[b] UV−
1	Naphthalene	−	−	−		98.3	0
2	Phenanthrene	250	−	+		89.7	3.2
3	Anthracene	500	−	+	+	90.2	2.8
4	Pyrene	−	−	−		67.3	0
5	Benz[*a*]anthracene	250	−	+		89.7	3.2
6	Benz[*b*]anthracene	250	250	+[c]		98.6	0.3
7	Benz[*a*]pyrene	250	−	+	+	90.2	2.8
8	Benz[*c*]pyridazine	250	−	+		86.3	2.8
9	DMBA	250	250	+[c]		71.7	2.0
10	3-Methylcholanthrene	125	−	+	+	67.3	0
11	Erythrosin B	250	−	+		97.6	0
12	Rose bengal	250	−	+	+	98.6	0.3
13	Eosin Y	250	−	+		88.5	4.9
14	Promethazine hydrochloride	10	250	+	+	93.0	1.0
15	Chlorpromazine	1	100	+	+	90.8	0
16	Levomepromzine	250	−	+		79.8	0
17	Doxycycline hydrochloride	250	−	+		73.7	0
18	Tetracycline hydrochloride	250	−	+	+	92.3	0.7
19	Nalidixic acid	250	−	+	+	84.5	1.7
20	Cinoxacin	250	−	+		86.3	2.8
21	Pipemidic acid	250	−	+		75.8	1.7
22	Piromidic acid	250	−	+		75.8	1.7
23	Norfloxacin	250	−	+	+	94.5	0
24	Enoxacin	250	−	+	+	93.3	0
25	Acridine orange hydrochloride	1	10	+		60.8	1.6
26	Neutral red	−	−	−	+	93.0	1.0
27	Hexachlorophene	1000	−	+	+	84.5	1.7
28	Bithionol	250	−	+	+	73.7	0
29	3,4',5-Tribromosalicylanilide	250	−	+	−	78.3	0
30	Piroxicam	500	−	+	+	75.3	0
31	6-Methylcoumarin	250	−	+	+	81.7	1.2
32	8-Methoxypsoralen	250	−	+	+	98.3	0
33	Pentachlorophenol	500	500	+[c]		79.9	0
34	2,4-Dinitro-1-chlorobenzene	250	−	+		92.0	0
35	Benzidamine hydrochloride	250	−	+		79.8	0
36	Sulfanilamide	500	−	+		78.3	0
37	Pyrithioxin	250	−	+		91.1	1.9
38	L-Ascorbic acid	25	25	+[c]		82.5	0
39	Methylene blue	0.125	25	+		not tested	
40	Thiourea	−	−	−	−	90.5	0
41	t-Cinnamaldehyde	−	−	−	−	94.5	0
42	SDS	−	−	−	−	90.8	0
43	D-Mannitol	−	−	−		78.9	8.2
44	L-Histidine	−	−	−	−	79.9	0
45	p-Dichlorobenzene	500	−	+		78.9	8.2
46	p-Aminobenzoic acid	−	−	−	−	75.3	0
47	BHT	−	−	−		82.5	0
48	Penicillin G sodium salt	−	−	−	−	88.5	4.9
49	2-Hydroxy-4-methoxybenzophenone	−	−	−	−	90.5	0
50	2-AAF	−	−	−		79.3	0
51	MeIQ	−	−	−		79.3	0
52	Bisphenol A	−	−	−		not tested	
53	Clofentezine	−	−	−		93.3	0
54	Chlorhexidine dihydrochloride	−	−	−	−	76.3	0.5
	Ofloxacin	25	−	+	+		

[a] Phototoxic, photoallergic, and photocarinogenic activity to experimental animal or human.
[b] Ofloxacin 100 mg/mL
[c] The minimum positive concentrations of UV+ and UV− were equevalent, however we judged positive because of the clear defference of the % of damaged DNA between UV+ and UV−.

Table 3 Comparison between the results of photo plasmid-relaxation assay and in vivo phototoxicity

	in vivo classification		total
	phototoxic	non-phototoxic	
photo plasmid relaxation assay			
phototoxic	16	1	17
non-phototoxic	1	8	9
total	17	9	26

sensitivity: 94%, specificity: 89%, positive predictivity: 94%, negative predictivity: 89%, prevalence: 1.89, accuracy: 92%

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医薬品における光遺伝毒性試験

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Photogenotoxicity testing for pharmaceuticals

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Summary

Interest in photosafety testing, especially for pharmaceuticals, has grown over recent years in Europe and the US. Therefore, the Genotoxicity Working Group of the Japanese Pharmaceutical Manufactures Association (JPMA) surveyed the area of photogenotoxicity to provide information to members. Current status, literature surveys and questionnaires on photogenotoxicity were conducted. Compounds suspected to be photomutagenic and/or photocarcinogenic (Müller and Kasper, 1998) are shown in Table 1. Questionnaires in JPMA are shown in Table 2. Decision trees in several draft guidance are given in Figs. 1-3. Furthermore, the results of literature surveys are given in Appendix.

Keywords: photogenotoxicity, JPMA, questionnaires

1．はじめに

本稿は，日本製薬工業協会 医薬品評価委員会 基礎研究部会 第三分科会 遺伝毒性ワーキンググループが，2000年度の活動として実施した光遺伝毒性試験に関する現状調査（日本製薬工業協会，2001）をもとに作成した．

2．光遺伝毒性とは

光遺伝毒性とは，遺伝毒性を示さない用量の紫外線あるいは可視光を化学物質に暴露した後に観察される遺伝毒性反応と定義される（Spielmann et al., 2001）．紫外線照射によって誘発される腫瘍原性や遺伝毒性の最も重要な分子学的事象はUVB（290-320 nm）による6-4光産物やピリミジン2量体の形成である．これは，光エネルギーが直接DNA分子に吸収されることによるものと考えられている．また，主にUVA（320-400 nm）および可視光も内因性あるいは外因性の光感受性物質などにより活性酸素種を生成し，酸化的DNA損傷を誘発する可能性がある．

光遺伝毒性ならびに光がん原性が疑われている化合物（群）をTable 1に示す（Müller and Kasper, 1998）．この中には医薬品も多く含まれている．

3．医薬品の光遺伝毒性試験の背景

抗乾癬剤のソラレンや神経弛緩剤のフェノチアジンなど光照射治療で用いられるある種の化学物質は，照射後遺伝毒性作用を誘発する事が1970年代後半より知られていた．ヨーロッパでは，以前より化粧品業界を中心に光毒性に関する関心が高く，光遺伝毒性についても1980年代から，その検出法の検討がなされていた．1990年には，Commission of the European Communities のScientific Committee of Cosmetology（SCC/CEC）がサンスクリーンのための光遺伝毒性試験ガイドラインを発行している（Loprieno, 1991）．1990年代には，フルオロキノロン抗菌剤の光化学的遺伝毒性が報告され，また，オゾン層の破壊による紫外線量の増大に起因する皮膚がんに対する懸念などから，ますます，光がん原性を含む光（遺伝）毒性に関する関心が高まっていったようである．1993年末には，European Centre for the Validation of Alternative Methods（ECVAM）による“In vitro phototoxicity testing”に関するワークショップが開催され，

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Table 1 Compounds and compound groups suspected to be photomutagenic and photocarcinogenic by interaction with DNA upon photoactivation

Compound	Mechanism of photoactivation	Use
Acridines	DNA intercalation	Coloring agents
Amiodarone	Radicals	Antiarrythmic
Anthraquinones	?	Colouring agents, phytotherapy
Benzodiazepines	Activated oxygen species	Hypnotics
Carbamazepine	?	Antiepileptic
Chlordiazepoxide	?	Tranquillizer
Chlorthiazides	?	Diuretic
Fluoroquinolones	Activated oxygen species	Antibiotics
Furocoumarins	DNA intercalation, DNA adducts	Antipsoriatic
Griseofulvin	?	Antifungal
Hydroquinone	Radicals?	Depigmentation
Hypercin	Radicals?	Antidepressant
Metronidazole	Nitro radical?	Antibiotic
Oxicames	?	Antiphlogistic
p-aminobenzoic acid (PABA)	Azodibenzoic acid, intercalation	Sunshield factor
Phenothiazines	Dechlorination, stable radicals	Neuroleptics
Porphyrines	Oxygen radicals	Cytostatics
Sulphonamides	?	Antibiotics
Tetracyclines	?	Antibiotics

Müller and Kasper, 1998

勧告が出された（Spielmann et al., 1994）．1990年代後半には，新規化学物質のための微生物ならびに哺乳類培養細胞を用いた光遺伝毒性試験方法もほぼ確立され，遺伝子突然変異，染色体異常誘発性あるいはDNA鎖切断などの指標による評価が可能となってきた．

しかしながら，これら光遺伝毒性試験においては，処理条件（照射時間，照射量，光源など）が研究者ごとに異なり，統一化された基準は特になかった．このような現状を受け，1999年3月27～28日にワシントンで開催されたInternational Workshop on Genotoxicity Test Procedures（IWGTP）では，光遺伝毒性が検討事項の1つとして取り上げられ，試験法の標準化について国際的な調和がはかられた（Gocke et al., 2000）．加えて，1999年6月22～25日には，ベルリンで第2回目の“In vitro phototoxicity testing”に関するワークショップが開催され，第1回目では主項目として取り上げられなかった光遺伝毒性についても議論がなされた（Spielmann et al., 2001）．

欧米における光（遺伝）毒性に関する産業界および学界における取り組みを受け，欧米の医薬品規制当局も光安全性試験に関するガイダンスを発行するにいたった．すなわち，US FDAによるドラフトガイダンス（FDA, 2000），The European Agency for the Evaluation of Medicinal Products（EMEA）によるコンセプトペーパー（EMEA, 2000），OECDによるドラフト試験法ガイドライン（OECD, 2000）ならびにEMEAによるドラフトNote for Guidance（EMEA, 2001）である．このように，欧米においては光（遺伝）毒性に関する規制当局の動きが活発となっている．

4．海外の医薬品規制当局によるドラフトガイダンス

1）US

FDAのCenter for Drug Evaluation and Research（CDER）が，2000年1月にGuidance for Industryとして光安全性試験のドラフトガイダンスを公表した（FDA, 2000）．これは，第1章：緒言，第2章：背景，第3章：考慮すべき試験および第4章：UV関連皮膚発がんの増大のための試験，から構成され，多数の文献を引用し科学的見地から光安全性を評価することの重要性とその戦略が述べられている．このドラフトガイダンスでは，光遺伝毒性は第4章で扱われており，ヒトで5年以上にわたり使用される可能性のある医薬品で，全身性暴露あるいは直射光に暴露される皮膚に塗布する場合には，当該医薬品が紫外線による発がん性を増大させるかどうかを評価する必要があるとしており，その評価項目の1つとして，光遺伝毒性の有無を挙げている（Fig. 1）．

2）EU

EMEAのCommittee for Proprietary Medicinal Products（CPMP）は，2000年5月25日に光安全性試験に関するガイダンスについてのコンセプトペーパーを発表した（EMEA, 2000）．これは，背景と勧告からなる1ページの簡潔なもので，引用文献も先述のFDAのドラフトガインダンスを含め2つであるが，光安全性を光感受性（光刺激性および光アレルギー）ならびに光遺伝毒性/光がん原性と捉えている．ECVAMやFDAの状況を踏まえ，光安全性試験についてのNote for Guidance

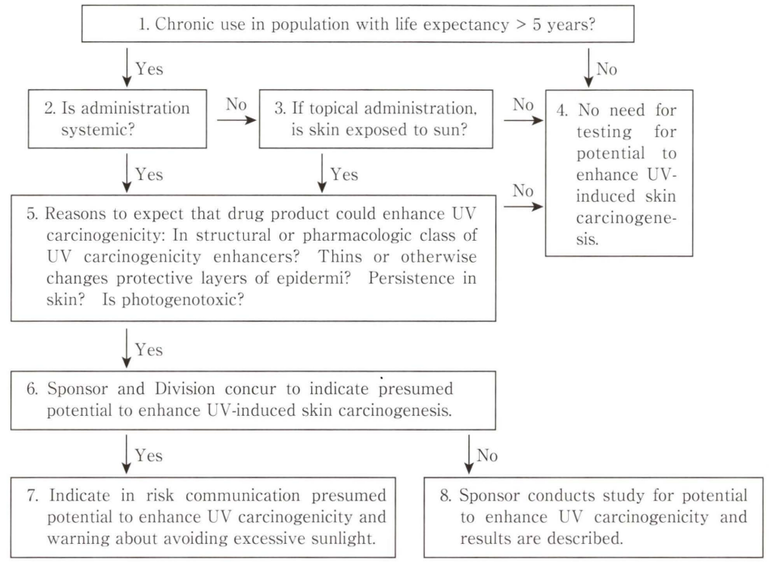

Fig. 1 Testing of nonphotosensitizing drug products for potential * to enhance UV-induced skin carcinogenesis (draft by CDER/FDA)
* Products specifically intended for use in sunlight should be tested for potential to enhance UV carcinogenicity.

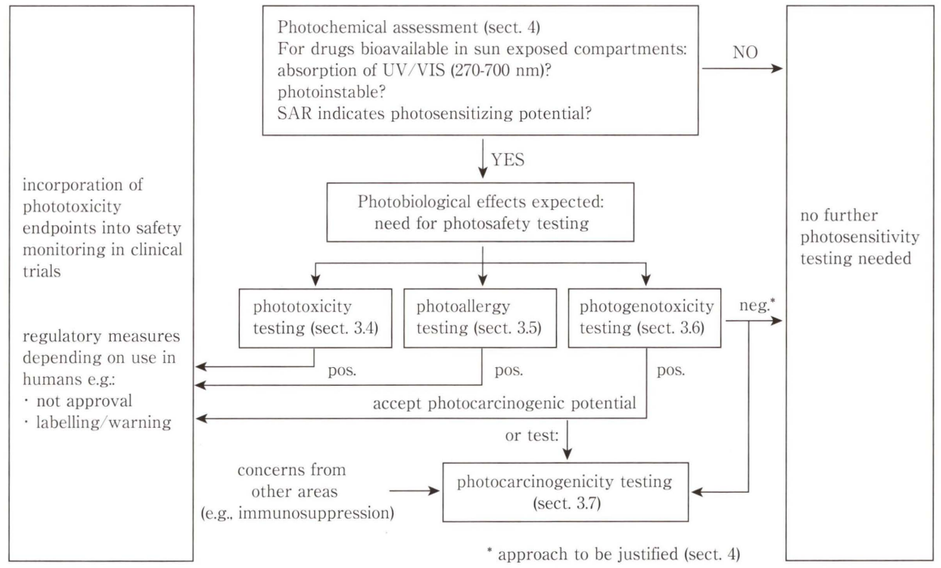

Fig. 2 Flow chart：Assessment of the photosensitizing potential of new active substances（draft by CPMP/EMEA）

（NfG）を作成する予定である事が述べられている．このコンセプトペーパーを受け，2001年3月1日にCPMPはNfGのドラフトを発表した（EMEA, 2001）．このドラフトNfGでは，まず第1章の緒言において，光安全性試験では光毒性（光刺激性），光アレルギー，光遺伝毒性および光がん原性の4つの指標を考慮すべきであることが記され，第2章では光安全性試験が考慮される医薬品の特性，第3章では上記4試験を含めた光安全性試験法，第4章では決定樹を含めた試験戦略（Fig. 2），第5章では当該医薬品の光安全性評価における規制当局の観点，

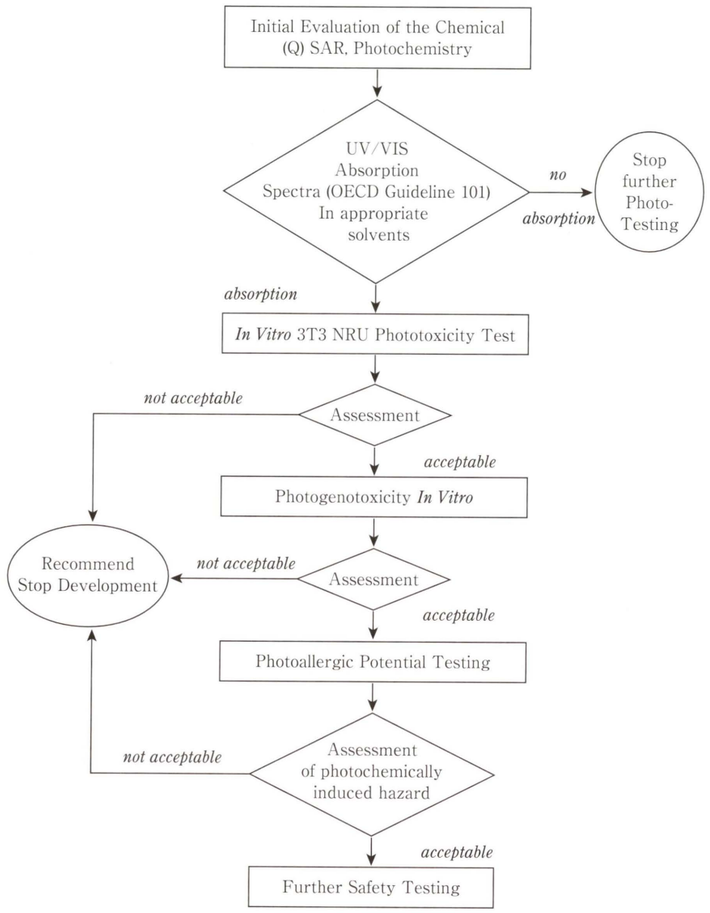

Fig. 3 Annex 2：Role of the 3T3 NRU phototoxicity test in a sequential approach to phototoxicity testing of chemicals

が述べられている．

3）OECD

医薬品ではなく一般化学物質が対象であるが，OECDより2000年2月にDraft Proposal for a New Guidelineとして“In vitro 3T3 NRU phototoxicity test”が発表された（OECD, 2000）．NRUは，Neutral Red Uptakeの意である．これは，当該化学物質が光毒性を有する可能性の有無を検索するために，Balb/c 3T3細胞を用い紫外線照射下および非照射下における細胞毒性をニュートラルレッドの取込みで評価する試験法について記載したものである．その中のAnnex 2として，3T3 NRU phototoxicity testを実施後，必要に応じin vitro光遺伝毒性を評価するという決定樹が記されている（Fig. 3）．

5．光遺伝毒性試験に対する日本の状況

1）アンケート調査

日本製薬工業協会基礎研究部会の遺伝毒性あるいはがん原性ワーキンググループに参加している計32社（国内企業25社，外資系企業7社）を対象に，光遺伝毒性試験に関するアンケート調査（期間：2000年7月～8月）を実施した．そのまとめをTable 2に示す．

アンケート結果によると，光遺伝毒性試験の経験は，国内・外資系企業ともにキノロン系抗菌剤を開発している企業に多く，開発薬剤に依存している傾向が認められた．また，FDAの要求により光遺伝毒性を検討（試験の実施や考察を含む）したケースが多かった．これらの状況から，日本では，規制当局を含め，光遺伝毒性の必要性に関する議論や認識が欧米ほど高くないことが示唆された．

Table 2 Replies to questionnaires in JPMA

Sent to：	32 companies (25 domestic and 7 foreign-affiliated) that participated in the Genotoxicity or the Carcinogenicity Working Group of the JPMA
Period：	July to August 2000
Reply rate：	100% (32/32)

Questions and answers	
Q1：	Have you ever conducted photogenotoxicity test, including those contracted out, for NDA?
A1：	Yes：6 companies (19%, including 3 foreign-affiliated) No：26 companies (81%, including 4 foreign-affiliated)
Q2：	What were the reasons for doing the tests?
A2：	・Quinolones：4 cases ・Phototoxic compound：1 case ・Evidence of photogenotoxicity in related pharmaceuticals：1 case ・Skin application compound with maximum absorption at 400 nm：1 case
Q3：	Have you ever been requested to evaluate photogenotoxicity, including conduct of the tests or discussion, by regulatory authorities in Japan or elsewhere?
A3：	Yes：4 companies (13%), Request from FDA：3 companies including 1 foreign-affiliated Request from MHW：1 company No：28 companies (87%, including 6 foreign-affiliated)

2）今後の展開

IWGTPで光遺伝毒性が取り上げられたこともあり，また，欧米で光安全性試験のガイダンスが制定されつつある現状を踏まえ，光（遺伝）毒性についての規制当局の関心は高まることが予想される．今後は，類薬において光（遺伝）毒性が知られている場合や皮膚外用剤だけでなく，紫外線あるいは可視光吸収の認められる化合物は，光遺伝毒性に関する考察が求められるようになると考えられる．

6．光遺伝毒性関連文献調査

日本製薬工業協会基礎研究部会 遺伝毒性ワーキンググループでは，光遺伝毒性に関連した57文献をリストアップし，その内容を調査した．各文献で検討された化合物リストをAppendix 1に，各文献の概略をAppendix 2に示す．

謝　辞

日本製薬工業協会 医薬品評価委員会 基礎研究部会 遺伝毒性ワーキンググループの下記各委員に感謝いたします：朝波省吾（大塚製薬工場），荒木春美（富山化学工業），伊藤智（グラクソ・スミスクライン），梅原重敬・中野雄司（旭化成工業），大澤浩一（大正製薬），小沢重成（キッセイ薬品工業），笠原義典（帝人），久保欣也（キリンビール），島田弘康（第一製薬），住田浩之（日本化薬），堤俊輔（アベンティスファーマ），西田荘司（東レ），林宏行（明治製菓），松井明子・鷲塚昌隆（ゼリア新薬工業），三宅幸雄（塩野義製薬），森田健（グラクソ・スミスクライン），吉田純一（科研製薬），若田明裕（山之内製薬），松澤利明（部会長，山之内製薬）．

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Appendix 1 Chemicals used in literature on photogenotoxicity

Chemicals	Reference number
A	
Acridine	30, 57
Acridine Orange	57
Acridine Yellow	57
9-Aminoacridine	57
p-Aminobenzoic acid	16, 20, 23, 55
Angelicin	40, 41
Anthracene	30
B	
Bay Y 3118	24, 38
Benzo[*a*]pyrene	3
Bergamot oil	9
Bergapten	47
C	
3-Carbethoxypsoralen	4
2-Chlorophenothiazine	22, 24
Chlorpromazine	5, 16, 22, 24, 26, 27
Ciprofloxacin	10, 12, 20, 29, 32, 38, 52
Clinafloxacin	10, 52
Clonazepam	13
Coal	48, 50
Compazine	22
D	
Dictamine	44, 45
Dihydroxyacetone	55
7,12-Dimethylbenz[*a*]anthracene	55
1,6-Dioxapyrene	3
Doxycycline	24
E	
Emodin	24
Enoxacin	31, 32, 52
Evolitrine	44
F	
α-Fagarine	44, 45
Fleroxacin	12, 29, 31, 32, 38
Flindersiamine	44
Flunitrazepam	13
Fluphenazine	22
G	
Grepafloxacin	24
Griserofulvin	24
H	
Heraclenin	43
Hypericin	24
I	
Imperatorin	43
8-Isoamylenoxypsoralen	1
K	
Khellin	39, 47
Kokusaginine	44
L	
Lomefloxacin	10, 12, 24, 29, 31, 32, 38, 52, 54, 56
M	
Maculine	44
Methoxypromazine	22
5-Methoxypsoralen	4, 9, 17, 35, 46
8-Methoxypsoralen	1, 2, 4, 6, 8, 14, 16, 24, 25, 30, 34, 39, 40, 41, 42, 46, 53, 55
4-Methyl-4',5'-dihydropsoralen	34
5'-Methylangelicin	1
4-Methylbenzylidene camphor	55
Methylene Blue	57
7-Methylprido[3,4-*c*]psoralen	4
4-Methylpsoralen	34
Methyrene blue	20
Metronidazole	24
Mexoryl SL	15
Mexoryl SO	15
Mexoryl SX	15
Musk ambrette	30
N	
Nalidixic acid	29, 31, 38, 52
Nitrazepam	13
Norfloxacin	31, 32, 38, 52
O	
Octyl dimethyl PABA	55
Ofloxacin	29, 52
Oxytetracycline	24
P	
Padimate-O	33
Parasol 1789	11
Parasol HS	11
Parasol MCX	11
Perfumes	17
Perphenazine	22
Petroleum	48
Plant extract	45
Porphyrins	20
Prido[3,4-*c*]psoralen	4
Proflavine	57
Promazine	22
Psoralens	29, 40, 45
Q	
Quinacrine	57
R	
Ro 12-9786	20
Ro 19-8022	20
Ro 47-7737	20
Ro 65-8275	20
Rose bengal	30
S	
Shale oil	48, 49, 50, 51
Skimmianine	44, 45
Spafloxacin	52
Stelazine	22
Sunscreen	17
T	
3,5,3',4'-Tetrachlorosalixylanilide	30
Titanium dioxide	37
3,5,4'-Tribromosalixylanilide	30
Trifluormethylthiazine	22
Trifluorpromazine	22
Triflupromazine	24
4,4',6-Trimethylangelicin	6, 7, 53
4,5',8-Trimethylpsoralen	4, 25, 39
Trovafloxacin	52
TWA	9
TWB	9
V	
Visnagin	47
Others	
Guidance etc.	18, 19, 21, 28, 36

Appendix 2 Outline of each reference

文献	使用化合物	試験方法	結果
1	8-methoxypsoralen (8-MOP), 8-isoamylenoxypsoralen (8-IOP), 5'-methylangelicin (5'-MA)	1) sister-chromatid exchange (SCE) analysis：ヒトリンパ球に各被験物質を暴露し，48時間後に5分間UVAを照射し，SCE頻度を測定. 2) the algal system：緑藻類 (Chlamydomonas reinhardii) に各被験物質を暴露し，その直後から最大120分間UVAを照射し，生存率および変異数を測定.	1) 8-MOPおよび5'-MA：SCEの発生頻度の増加 (陽性)，8-IOP：SCEの発生頻度に変化なし (陰性). 2) 生存率：8-MOP，5'-MA：UV-Aに対して高感受性 (35分照射後で約1%生存)，8-IOP：UVAに対して低感受性 (120分照射後で約1%生存)，変異数：8-MOP＞8-IOP＞5'-MA. 備考：構造；8-MOPおよび8-IOP：linear molecule, 5'-MA：angular molecule
2	8-methoxypsoralen (8-MOP)	UV照射および8-MOP+UVA処理したRAD51-lacZ融合株中のDNA修復遺伝子RAD51の誘導を，DNA二重鎖切断，組替え修復欠損変異rad52，および関連の野生型を用い検討. Saccharomyces cerevisiae; FF181082 (genotype: a RAD$^-$ ura3 + RAD51-51 LACZ LEU2$^-$) およびRAD51-lacZ融合させるrad52 変異株 FF181090 (genotype: a rad52 leu2 trp1 ura3 his7 lys1 + RAD51-51 LACZ LEU2$^-$) を用い，5 μM 8-MOPおよびpeak emission 365 nmのHPW 125 Philips ランプで10^7細胞/mLの試験菌株を処理後，UV-Xデジタルラジオメーターで2 J/m^2/sと測定された，peak emissionが253.7 nmの低圧Philips TUV 30 W 水銀殺菌灯を照射した. 修復遺伝子RAD51遺伝子の誘発は，基質としてo-nitrophenyl-β-D-galactopyranoside (ONPG) を用い，RAD51-lacZ融合株における β-galactosidase活性を調べることにより検討. DNA二重鎖切断は，パルスフィールドゲル電気泳動で調べた.	UVおよび8-MOP/UVA処理後，修復過程に生ずるDNA二重鎖切断生成量が減少．同処理によるrad52変異株中のRAD51遺伝子の誘導は，野生株よりも減少. RAD52修復経路はDNA二重鎖切断だけでなく遺伝子誘導においても重要な役割を果たしているようだ. DNA損傷によりイニシエートされるシグナリング経路とそのプロセシングは，光毒性反応とおそらくリンクしている.
3	1,6-dioxapyrene (1,6-DP), benzo(a)pyrene (B(a)P, 陽性対照)	①細胞毒性試験 (酵母) ②復帰変異試験 (S. thyphimurium；1,6-DP+UVA) ③復帰変異試験 (酵母；1,6-DP+UVA) ④アルカリ溶出法 (酵母；1,6-DP+UVA) ①，③：D7株を1,6-DP存在下 (50 μM) で蒸留水あるいは75% D_2O で15分間インキュベート→O_2あるいはN_2をバブリング→種々の線量の365 nm UVAを照射→コロニー形成能／復帰変異コロニー数／変異コロニー数を測定. ②TA100菌液をソフトアガー中で1,6-DPとS9に混入→最少グルコース培地に播種. ④放射ラベルしたD7株と1,6-DP処理／非処理のプロトプラストについて，アルカリ溶出法により平均DNA分子量を算出し，そこから一本鎖切断を定量 (30℃，30分インキュベーション後). 照射実験：トップアガー固化後，種々の365 nm UVA (250, 500, 1000 J/m^2) を照射. 非照射実験：実験はすべて赤色光下で実施.	①oxygenの存在に強く依存する細胞毒性が認められた. ②1,6-DPは暗所でわずかな変異原性を示したが，S9存在下ではその変異原性が大きく低減した．UVAを照射するとその線量に依存して復帰変異コロニー数が増加したが，高線量では光誘発性と考えられる致死作用により低減した．B(a)Pでは線量に依存した変異原性の上昇は認められなかった．S9を添加すると不活化されるらしくS9非存在下に比べて変異原性能は低減した. ③1,6-DPはB(a)Pと同程度の毒性を示す用量では同程度のphotomutagenicity (ILV$^+$；復帰変異) を示したが，通常の変異 (TRP$^+$) は誘発しなかった. ④1,6-DP + UVA処理によってsingle strand break (SSB) が誘発された．またこの反応はD_2O存在下で$^1O_2$のlife timeが伸びることにより，さらに誘発されるようになる．さらに処理後30分後にアルカリ溶出を行うと，処理によって形成された損傷が酵素的に取り除かれてできたAP site等で鎖切断が起こるためSSBがさらに誘発される結果となる. 1,6-DPはB(a)Pと同程度の毒性用量で同程度のphotomutagenicityを示すことから，同じメカニズムによるものであることが示唆された.

文献	使用化合物	試験方法	結果
4	8-methoxypsoralen (8-MOP), 5-methoxypsoralen (5-MOP), 4,5',8-trimetylpsoralen (TMP), 7-methylprido (3,4-c) psoralen (MPP), 3-carbethoxypsoralen (3-CPs), prido (3,4-c) psoralen (PP)	・突然変異試験：yeast, Chinese hamster V79 cell, dipoloid human fibroblasts. ・マウス光がん原性試験：eukaryotic cells に UVA (365 nm あるいは 405 nm) を照射.	・ヒト皮膚突然変異自然誘発率 (1.2×10^{-4}) に比べ30年間8-MOPで治療したときの突然変異誘発率は約100倍高い (1.3×10^{-2}). ・Monofunctional なソラレンのほうが，Bifunctional なものよりも光突然変異誘発活性は低く，ほとんど活性を示さないこともある. ・Bifunctional なソラレンは，架橋を形成して突然変異を誘発し，さらに光がん原性を示す. ・ソラレンが低用量のとき，UVAによる光突然変異誘発率は増加するが，UVAが低用量のときはソラレンによる光突然変異誘発率は低下する. ・UVAが380 nm以上ならばモノアダクトから架橋への変換ができないので，Bifunctional なソラレンによる光突然変異誘発率は低下する. ・UVAの2回照射は，架橋が増加するので細胞毒性も光突然変異誘発率も増加する. ・活性酸素がソラレンの光突然変異誘発に関与している可能性はあるが，部分的である. 備考：Monofunctial：3-CPs, MPP, PP Bifunctial：8-MOP, 5-MOP, TMP
5	Chlorpromazine (CPZ)	Ames Test：S. typhimurium TA100 を CPZ 10 μg/mL PBS で10分間暗所で処理後，ブラックライト (360 nm 波長極大，エネルギー 10 J/m^2/s) を照射. 照射時間は不明.	陽性．pH と変異原性 (細胞毒性) に相関があり，低pHほど作用が強い.
6	4,4',6-trimethylangelicin (TMA), 8-methoxypsoralen (8-MOP)	S. typhimurium TA102 を用いた復帰突然変異試験. S. cerevisiae D7 を用いた光遺伝毒性検出系. 両試験系に対して両化合物ともに5 μM の濃度で別々に添加し，365 nm の紫外線を照射している．紫外線の照射は，TA102では5, 10, 15分間されており，この場合の照射量はそれぞれ，6, 12, 18 kJ/m^{2}とされている．D7での照射量は，2.4, 4.8, 7.2, 9.6, 12 kJ/m^{2}とされている.	8-MOP, TMA ともに陽性. 備考：TA102の系では8-MOPのほうがTMAより強い光遺伝毒性を示したのに対し，D7の系ではTMAのほうが8-MOPよりも強い光遺伝毒性を示した.
7	4,4',6-trimethylangelicin (TMA)	酵母の lethality, nuclear reversion, gene conversion および petite mutation. 光源：high-pressure mercury vapour lamp HP 125 W, 300-400 nm (最大出力波長365 nm)，1.2 kJ/m^2. 空気のありなし (窒素置換) の条件で光を照射. 観察方法：Isoleucine-free medium ： revertant, Tryptophan-free medium ： convertant, Complete medium ： survival, Tetrazolium overlay technique ： petite	窒素置換するとすべての作用が弱くなる.
8	8-methoxypsoralen (8-MOP)	TA102, D7 を用い，8-MOP を添加し UVA を10分照射.	本論文では8-MOPは光遺伝毒性を惹起する薬物として用いられており，β-カロチン，αトコフェロールの抑制作用および機序を検討しているため，8-MOPの検討結果は不明.
9	5-methoxypsoralen (5-MOP), ベルガモット香油, TWA (化粧水A), TWB (化粧水B)	突然変異試験 (Ames 試験；S. typhimurium TA102, HPRT/Chinese hamster V79 cell)：TA102 もしくは V79 細胞を用い，5-MOP を含むベルガモット香油と2種の化粧水，また5-MOPそのものについて，UVA (355 nm) を照射したときの光遺伝毒性を比較検討した．また，4-isopropyldibenzoylmethane (IDM) と Parsol 1789 (P) を用いてUVAの吸収を阻害した系でも検討した.	・ベルガモット香油および2種の化粧水の光遺伝毒性の原因は5-MOPによる. ・IDMとPの添加により，活性がAmes試験では完全に，V79細胞を用いた試験では50％阻害した．したがって，ベルガモット香油の基剤には，IDMもしくはPなどのUVA阻害剤の添加が必要である.
10	Clinafloxacin, Ciprofloxacin, Lomefloxacin	1) Skh-1 マウスに被験物質を投与して UVA を3時間照射する操作を4日間行った後，ケラチノサイトを単離して DNA 鎖の損傷を測定. 2) CHO 細胞を用い，UVA を60分間照射して DNA 鎖の損傷を測定. 3) CHO 細胞を用い，UVA を60分間照射して染色体異常を観察. 4) CHO 細胞を用い，UVA を60分間照射して水酸化ラジカルの産生を測定.	1) Clinafloxacin, Lomefloxacin, Ciprofloxacin は陽性. 2) Clinafloxacin, Lomefloxacin, Ciprofloxacin は陽性. 3) Clinafloxacin, Lomefloxacin は陽性，Ciprofloxacin は陰性. 4) Clinafloxacin は水酸化ラジカルを産生. ラジカルスカベンジャーで水酸化ラジカルの産生を抑制しても Clinafloxacin の DNA 鎖の損傷および染色体異常は完全には抑制されず，Clinafloxacin の光遺伝毒性にラジカル以外の要因の関与が示唆された.

11	Parasol HS, Parasol 1789, Parasol MCX	Ames test (S.typhimurium TA102, TA100, E.coli WP2)：Plate または suspension に UV 照射. Gene convertion test (S. cervisiae D7)：24 well plate 上で UV 照射.	Photomutagenic 作用なし. UV照射による突然変異誘発作用を抑制. 備考：はじめに8-methoxypsoralen と chlorpromazine を使い条件設定.
12	fleroxacin, ciprofloxacin, lomefloxacin	照射には290～800 nmの発光スペクトルを持つSUNTEST CPSを用いた．UVBをカットするためにAmes testでは3 mmのガラスを，酵母，CAおよびComet testではプラスチックのカバーを通して照射した．曝露量はAmesおよびComet testで0.5 mW/cm^2，CA testで0.4 mW/cm^2，酵母での試験で0.65 mW/cm^{2}だった. Ames testでは，TA100，TA102およびTA104を被験物質と共に照射し，60分間培養後，プレートに播種した. Saccharomyces cerevisiae D7 testでは，D7株を被験物質と共に照射し，寒天培地に播種した. 染色体異常試験 (CA test) では，チャイニーズハムスターV79細胞を被験物質と共に照射し，照射後PBSで2回洗浄し，18時間培養した. Comet assayでは，マウスリンパ腫L5178Ytk$^{+/-}$細胞を被験物質と共に20分間プレインキュベーション後，照射した．照射後，細胞懸濁液をアガロースに滴下し，電気泳動した．ethidium bromideで染色後，4つに分類した. 前照射によるAmesおよびCA testでは，被験物質溶液をUVAで照射した後，TA104またはV79に加えた.	Ames test：TA100；3化合物とも2倍未満の不確定な変化．TA102；照射による増強はなし (ciprofloxacinのみ検討)．TA104；3化合物とも照射により増強. Saccharomyces cerevisiae D7 test：3化合物とも照射による増強はなかった. CA test：3化合物とも照射により増強. Comet assay：3化合物とも照射により陽性. 前照射によるAmesおよびCA test：3化合物とも前照射による増強はなかった.
13	nitrazepam, flunitrazepam, clonazepam, その他の関連化合物	①phototoxicity試験：S. typhimurium TA100の菌懸濁液に，種々用量の被験物質を添加→暗所で37℃，30分間インキュベート→O_2あるいはN_2を1分間バブリング→350 nm光を10分間照射. コロニー形成率測定：菌液をPBSで希釈 (～10^4)→その0.1 mLをプレーティング→暗所で37℃，24時間培養→コロニーをカウントし，生存率曲線を作成. phototoxicity試験：照射後2時間，菌液をインキュベート→以降上記同様に操作. ②toxicity試験：上記①の処理をアルミホイルで遮光した状態で同様に実施. ③Ames試験：上記①と同様に処理→照射後，菌液を最少グルコース培地にプレーティング→暗所で37℃，2日間培養. 各実験は最低3回反復，triplicate.	生存率曲線から，nitrazepamだけでなく，clonazepam, flunitrazepamも細菌に対しphototoxic effectを示すことが明らかとなった．その最大の作用は暗所での放置の有無にかかわらずoxygen-rich培地でみられたものの，oxygen-poor培地ではほとんどみられなかった．つまり化合物の光分解産物がphototoxicityの原因ではないとわかった．このことはさらに追加の光分解産物 (アゾキシ化合物) を用いて調べられた．nitrazepam, clonazepam, flunitrazepamいずれもTA100株に対し変異原性能を示さなかった. 十分な酸素存在下では光誘発致死性が最大値を示すことから，7-nitro化合物は酸素存在下で光照射されることによりphototoxicic effectを示すことがわかった．しかし光照射後2時間放置したほうが非放置のものに比べて生存率曲線が下がっていた．このことからこのphototoxicityは一重項酸素の影響だけによるものではないことが判明した．このことから，短時間で消去されないROSが細胞内の標的と反応することで致死効果をもたらすことが考えられた.
14	8-methoxypsoralen (8-MOP, xanthotoxin)	E. coli K-12/343/113/uvrBを用いた復帰突然変異試験. 1) wild株およびuvrB株を用いて，arginine欠損 (arg+復帰突然変異体を検出) あるいはgalactose添加 (galR-変異体を検出) した培養液下で，照射時間を0～120秒間変化させたときの変異の頻度. 2) D_2OあるいはH_2Oで調製した培養液を用いて，照射時間を0～75秒間変化させたときの酸素存在／非存在下での変異数の頻度. 3) DABCO ($^1O_2$を除去させる) の添加の有無に対して，照射時間を0～75秒間変化させたときの変異数の頻度.	1) uvrB株が感受性が高い. 2) 酸素存在および非存在下とも，D_2O培養液のほうがH_2O培養液に比べ，変異数は多く出現したが，酸素存在下のほうが顕著な差がみられた. 3) DABCOを添加したほうが，変異数は少なかった.

文献	使用化合物	試験方法	結果
15	Mexoryl SL, Mexoryl SO, Mexoryl SX	①大腸菌WP2株を用いた復帰変異試験：菌培養液，被験物質溶液（～5000 μg），S9mixをトップアガーに混入→プレーティング→トップアガーが固化してから種々の線量でUVAあるいはUVA/UVBを照射→37℃，3日間培養→復帰変異コロニー数をカウント．試験は独立して2回実施． ②CHO細胞を用いた染色体異常試験：CHO細胞を暗所で被験物質あるいは陰性/陽性対照とともに37℃，15分間処理→UVAあるいはUVA/UVBを照射→新鮮な培養液に置換→18時間培養→triplicateのうち，1系列をMI測定用，残りを標本作製→1系列あたり陽性対照は25個，陰性対照と被験物質処理群は100個の細胞を観察．	①Mexoryl compoundsはUV下でも復帰変異数を増加させなかった． ②Mexoryl SOおよびSXは5000 μg/mlまで試験したが，Mexoryl SLは4000 μg/ml以上で致死作用を示したため，3654 μg/mlを最高用量とした．これら3種のmexoryl compoundsはUV下でも陰性対照群に比べ，染色体異常出現率を上昇させなかった． 以上のことから，これら3被験物質はいずれも光化学的変化が最大となる条件下でphotomutagenicityを示さなかった． 3被験物質とも，光平衡状態となると最大限の太陽光下でも非常に低速に分解（～1.5%/hr）するようになる．これは1日10時間照射でも5～10%程度の分解に匹敵する． 染色体異常試験では，UVA/UVB照射の組み合わせで%MI値が減少し，致死効果が増大する．
16	8-methoxypsoralen（8-MOP）， chlorpromazine（CPZ）， para-aminobenzoic acid（PABA）	① Ames試験（プレート法）：ソフトアガーで播種後，UV照射（TA100：UVA/UVB（mJ/cm^2）= 0/0, 2.7/0.9, 5.5/1.8, 10.9/3.7, 16.4/5.5，UVA（mJ/cm^2）= 0, 14.3, 28.6, 71.5, 143, 286，WP2：UVA/UVB（mJ/cm^2）= 0/0, 5.5/1.8, 8.2/2.8, 10.9/3.7，UVA（mJ/cm^2）= 0, 229, 458, 687）した．37℃で3日間インキュベート後復帰変異コロニーの計数を行った． ② In vitro染色体異常試験：CHO細胞を被験物質処理し，15分後UV照射（8-MOP：UVA/UVB（mJ/cm^2）= 0/0, 100/20, 200/40, 400/80, 800/160，UVA（mJ/cm^2）= 352, 704，PABAおよびCPZ：UVA/UVB（mJ/cm^2）= 0/0, 200/37.5, 700/0）した．約2時間後，PBSでリンスし培養液を交換した．さらに16時間培養し，コルヒチン添加（1 μg/mL）し2時間培養した．染色体異常観察用と分裂指数観察用の2種類のスライドを作成し観察した．	① Ames試験：8-MOP；陽性（+），PABA；陰性（−），CPZ；陰性（−） ② In vitro染色体異常試験：8-MOP；陽性（+），PABA；陽性（+），CPZ；陽性（+） In vitro染色体異常試験においては，8-MOPおよびCPZはUV照射下でのみ陽性であり，PABAは単独でも陽性であるが，UV照射で陽性反応が増強される．
17	Perfumes：ベルガモット・オイル（bergamot oil）を使って調製されたhydroalcoholic（ethanol：water, 80：20, w/w）model perfumes, 5-methoxypsoralen（5-MOP）， UVA sunscreen：Parsol 1789（4-*tert*-butyl-4'-methoxydibenzoylmethane）， UVB sunscreen：Parsol MCX（2-ethylhexyl-4'-methoxycinnamate）	18人のボランティアの背中の皮膚を用いる光毒性試験（ErythemaとPigmentationに分けて評価し，各々0～4スコアに分類）． ボランティア：20～25歳の健康な男10人女8人の背中に各々25区画を設けた（1区画は2 cm四方で，各区画の間は1 cm空けた）． パヒューム：終濃度5, 15, 50 ppmの5-MOPを含む上記hydroalcoholic model perfumes（controlでは5-MOPなし）． サンスクリーン：上記2種のサンスクリーンを各々終濃度0.5または1%でmodel perfumesに添加． 光照射：2500 Wのxenon arcを通気設備のあるランプハウスにセットし，フィルターでIRとUVCをほぼ除去してsolar-simulated radiation（SSR）とした．UVBの60～300 mJ/cm^{2}およびUVAの5～25 J/cm^{2}に相当するSSRを，光源から90 cmの距離で背中の充分な領域に均一に照射されるようにした（UVAおよびUVBドシメーターで測定）．	1）ヒト皮膚のphotosensitivityはperfume塗布後2時間で光を照射したときに最大である． 2）UVBよりもUVAのサンスクリーンを添加したほうがbergamot oilの光毒性を軽減できる． 3）50 ppmの5-MOPを含むmodel perfumeにサンスクリーンを添加した場合，15 ppmの5-MOPを含むmodel perfumeにサンスクリーンを添加しない場合と同等な光毒性にまで軽減できる． 4）In vitroでの有効性にもかかわらず，低濃度（0.5および1%）のサンスクリーン添加ではbergamot oilの光毒性を完全に抑制することはできない． 備考：初めの検討成績から，光照射後のErythemaおよびPigmentationの評価は各々3日目および14日目が最良と判断され，評価されている．
20	PABA（Para-aminobenzoic acid, monoaromatic compound）， Fluoroquinolones；Ciprofloxacin, Ro47-7737（bis-quinoline compound），Ro19-8022 and derivatives（antipsychotic compound）；Ro19-8022, Ro12-9786, Ro	種々のin vitro試験系（詳細は不明）．	PABA：Photo-ames assay（−）． Ciprofloxacin：S. typhimurium TA104，染色体異常試験，V79/HPRT assay，Comet assayで（+）． Ro47-7737：S. typhimurium TA104（−），Photoclastogenicityとphoto-comet assay（+）． Ro19-8022 and derivatives：Ro19-8022；S. typhimurium TA102（弱い+：通常の室内灯で），S. cerevisiae D7，染色体異常試験，ほ乳類細胞のgene mutation assayおよびComet assayで（+），Vitrotox assay（弱い+），Ro12-9786；Ames testで（+），Vitrotox assay（+），染色体異常試験（+），Ro65-8275：Ames testで（−），Vitrotox assay（−），染色体異常試験（−）．

	65-8275, Methyrene blue, Porphyrins		Methylene blue：Photo-Ames assayで（+）． Porphyrins：Mammalian cellsでのgenotoxicity（+）．
22	Chlorpromazine, Chlorophenothiazine, Trifluormethylthiazine, Stelazine, Promazine, Compazine, Perphenazine, Trifluorpromazine, Fluphenazine, Methoxypromazine	・突然変異試験（S. typhimurium，E. coli, V79/HGPRT，ΦX 174 amber mut. reversion）． ・染色体異常試験（V79，CHO）． ・E. coli K12 differential repair，E. coli K12 derivatives，S. cerev. D7 gene conversion，SCE in V79 hamster cells，UDS in lens epithelial cells，Alk. elution in human P3 cells，Comet assay in L5178Y cells，Biochem. analysis of breaks，Complex formation with naked DNA, double and single strand，Inactiv. of adenovirus in human WT and Xeroderma pig. cells. UV（320～400 nm）照射．	Chlorpromazine（20報文陽性/23報文），Compazine（2報文陽性/2報文）， Chlorphenothiazine（1報文陽性/1報文）， Perphenazine（1報文陽性/1報文）， Trifluormethlthiazine（0報文陽性/1報文），Stelazine（0報文陽性/1報文），Trifluorpromazine（1報文陽性/2報文），Fluphenazine（0報文陽性/1報文）， Promazine（0報文陽性/4報文），Methoxypromazine（0報文陽性/1報文）． 備考：通常の変異原性試験（UV照射なし）； Chlorpromazine（5報文陽性/23報文），Compazine（0報文）， Chlorphenothiazine（0報文），Perphenazine（0報文）， Trifluormethlthiazine（0報文），Stelazine（0報文）， Trifluorpromazine（2報文陽性/9報文），Fluphenazine（1報文陽性/1報文），Promazine（1報文陽性/5報文）， Methoxypromazine（0報文陽性/1報文）．
23	para-aminobenzoic acid（PABA）	Reverse mutation test：S. typhimurium TA98, TA100, TA1535, TA1537, E. coli WP2, WP2（pKM101）． UV source：Oriel xenon arc lamp 250 J/s/m^2 for suspension, 50 J/s/m^2 for plate，Honle arc lamp 20 J/s/m^2 Method：Suspension exposure：mutation plate, survival plate，Direct exposure：plate後に照射	PABAは6菌株に対して，突然変異誘発作用なし．TA100のsuspension exposureではUV照射によりrevertantsが増加するが，PABA添加により抑制． 備考：初めに8-methoxypsoralenとChlorpromazineを使い条件設定．その後PABAを評価．
24	Furocoumarine；8-methoxypsoralen, Fluoroquinolone；lomefloxacin, grepafloxacin, Bay Y 3118, Anthraquinone；hypericin, emodin, Coumaran derivative；griserofulvin, Phenothiazine；chlorpromazine, 2-chlorophenothiazine, triflupromazine, Tetracycline；doxycycline, oxytetracycline, Nitroimidazole；metronidazole	In vitro小核試験：V79細胞を6時間培養後，UVB/UVAを30分照射した．2回洗浄後さらに24時間培養した．1.5%tri-sodium citrateで洗浄後1.24%ホルマリンを含むacetic acid：ethanol（1：3）で固定した．空気乾燥法にて標本作製後染色し1000個の細胞を観察した（UV照射条件は省略）． In vitro染色体異常試験：V79細胞を24時間培養後，UVB/UVAを30分照射した．2回洗浄後さらに16時間培養した．常法に従い標本を作製し100個のmetaphaseを観察した（griserofulvinのみ実施）．	8-methoxypsoralen：陽性（+），chlorpromazine：陽性（+），2-chlorophenothiazine：陽性（+），triflupromazine：弱い陽性（+），lomefloxacin：陽性（+），grepafloxacin：陽性（+），Bay Y 3118：陽性（+），doxycycline：陰性（−），oxytetracycline：陰性（−），hypericin：陽性（+），emodin：陰性（−）， metronidazole：陰性（−）， griserofulvin：陽性（+）&（染色体異常：陰性）．
25	8-methoxypsoralen（8-MOP）， 4,5',8-trimethylpsoralen（TMP）	Ames試験：TA1975，TA1535，TA100，TA1978，TA1538，TA98，TA1977，TA1537，TA2637を用い，UVA（320～400 nm）を10～20分照射した．	TA1977，TA1537，TA2637で陽性．9菌株中，hisC3076変異株に感受性あり．
26	Chlorpromazine（CPZ）	DNA（光）結合：0.075 mM［^{3}H］CPZと1 mMDNAを含む溶液を，室温にてスペクトロフルオリメーターを用い323 nmで0～24分照射し，DNAに結合した［^{3}H］CPZをシンチレーションカウンターで測定した．	CPZとdenatureしたDNAとの共有光付加体の形成は，二本鎖DNAとの光付加体の形成より10倍効率的であった． UV照射により光分解したCPZの約10%がdenatureしたDNAに光結合した． CPZと二本鎖DNAとの複合体の形成は，光分解からCPZを保護し，共有光付加体形成を阻害することを示唆している． 備考：CPZは，antipsychotic drugである．
27	Chlorpromazine（CPZ）	CPZとcalf thymus DNAとを用い，CPZ溶液内でDNAを透析し吸収スペクトル変化，蛍光スペクトル変化から相互作用の種類を検討．	CPZとDNAとの相互作用として，インターカレーション，DNA二重鎖との直接結合，フリー体として存在していることが確認された．光反応生成物はインターカレーションしないことも確認された．

文献	使用化合物	試験方法	結果
29	psoralens；8-MOP, 5-MOP, TMP, 3-CP, ANG, 5-ANG, 4,5'-ANG, DMA, TMA, PP, MPP fleroxacin（FLER）, ofloxacin（OFLOX）, nalidixic acid（NAL）, lomefloxacin（LOM）, ciprofloxacin（CIPRO）	光がん原性，光変異原性および光毒性の関係を検討する． 文献調査による総説のため詳細は不明．	光がん原性：5-ANG＞4,5'-ANG＞8-MOP, 5-MOP＞TMA＞ANG 変異原性：5-ANG, 4,5'-ANG＞8-MOP, 5-MOP＞ANG 光がん原性：LOM＞FLER＞OFLOX＞CIPRO, NAL 光変異原性：LOM, FLER＞CIPRO In vitro 光変異原性の検討は，光がん原性の機序の理解の一助となる．
30	rose bengal, anthracene, Acridine, 8-methoxypsoralen（8-MOP）, 3,5,3',4'-tetrachlorosalixylanilide（T4CS）, 3,5,4'-tribromosalixylanilide（TBS）, musk ambrette	光化学反応：Histidine, tryptophan あるいは glutathione を含む溶液に被験物質を加え，光（medium-pressure mercury arc + Schott WG 305 3 mm ultraviolet filter）を当て，その後の溶液の色をみる．	His / Trp / GSH / 光分解 rose bengal：+ / + / + / ± anthracene：+ / + / + / ± acridine：+ / + / + / + T4SC：− / + / + / + TBS：ND / ND / + / + musk ambrette：− / − / − / ND 8-MOP：± / ± / ± / ND
31	fleroxacin（FLER）, lomefloxacin（LMFX）, norfloxacin（NOR）, nalidixic acid（NA）, enoxacin（ENX）	pBR322DNA を用いた in vitro 照射実験．pBR322DNA とフルオロキノロン系抗生物質を共存下 UVA 照射後 single strand break の生成強度を反応物のアガロースゲル電気泳動により検討している．	FLER＞LMFX＞NA＞＞NOR＞ENX C6，C8 位の F 原子（特に C8 位）の存在が，C6 位のみの F 原子に比べ 10 倍程の強さとなる．活性酸素種は必ずしも必要でない．C8 カルベンが活性中間体と考えられている．
32	lomefloxacin, fleroxacin, enoxacin, norfloxacin, ciprofloxacin	Fluoroquinolone の光分解により生じる fluoride の測定． Xenon arc lamp を用い 320 nm cutoff filter を通して得た＞320 nm の光照射後に光生成される fluoride を fluoride 電極および NMR スペクトルにより測定．	Lomefloxacin および fleroxacin は照射後に fluoride の増加がみられた． Enoxacin は痕跡程度の fluoride が検出された． Norfloxacin および ciprofloxacin では fluoride は検出されなかった．
33	Padimate-O（ethyl-4-dimethylaminobenzoate を含む 2'-ethylhexyl-4-dimethylaminobenzoate）	① M13 mp18 DNA（7.3 kb）あるいは Supercoild pBluescript-Ⅱ SK^+ DNA（Stratagene）に 50 μM Padimate-O，12 J/m^2/s（300～400 nm）を作用． ② plasmid pBSF（pBluescript-Ⅱ SK^+ に 4 塩基挿入，lacZ frameshift mutant とした）50 ng を上記と同様に作用させ，このプラスミドを XL1-Blue cells に導入し，X-gal を分解する復帰変異株を集めて，DNA の変異部位を調べた．	① Free radical による DNA 傷害で，pyrimidine dimer ではなかった． ② 10 個の復帰変異コロニーのプラスミド中に，AT base pair の deletion 1 個，GC base pair の deletion 9 個が検出された． DMSO，EtOH，Tris buffer，mannitol は scavenger として働く．特に，遺伝毒性試験の溶媒として DMSO が用いられているが，光遺伝毒性が検出し難くなり要注意である．
34	8-methoxypsoralen（8-MOP）, 4-methylpsoralen（4-MP）, 4-methyl-4',5'-dihydropsoralen（4-MDHP）	各種突然変異試験：E. coli（uvrA, B, C）, yeast（rad3）, mammalian（Xeroderma pigmentosum）． Psoralens + UVA 照射．	・除去修復の欠損している系では，ソラレンの光による付加体が多くなり，細胞毒性だけでなく，突然変異についてもその感受性は高くなる． ・除去修復が，光によって形成されたソラレンのモノアダクトおよび架橋の除去に関与している． ・架橋形成には組替えが必要である． ・モノアダクトおよび架橋の形成には除去修復，組替え修復および誤りがち修復が関与している． ・光によって形成されたソラレンのモノアダクトは，通常の DNA 複製を停止させるが，バイパスが形成されるので，それが突然変異誘発の原因となる可能性がある．
35	5-methoxypsoralen（5-MOP），ヒト皮膚に塗布後の SBF（Suction blister fluid）	S. cerevisiae を用いた gene mutation test：Cytoplasmic mutation, Intergenic recombination, TRP^+ gene convertants（tryptophane not requiring gene convertanta）, ILV^+ mutants（isoleucine-valine not requiring revertants）． 菌株と SBF を暗黒下で 15 分間処理した後，multiple well plate 上で UV 照射をし，survival と mutation を計測．	ILV^+ のみ陽性．

文献	使用化合物	試験方法	結果
37	Titanium dioxide（TiO_2）particles：p-25（anatase form, average size 0.021 μm）, WA（anatase form, average size 0.255 μm）, WR（rutile form average size 0.42 μm）, TP-3（rutile form, average size 0.42 μm）	Comet assay（SCG）, 染色体異常試験（CA）, Ames 試験, MLA. SCG：L5178 細胞．細胞は TiO_2 と 1 時間インキュベーション後，0.2 mW/cm^2（0.61 J/cm^2），0.4 mW/cm^2（1.25 J/cm^2），0.8 mW/cm^2（2.5 J/cm^2），1.6 mW/cm^2（5 J/cm^2）で 50 分間 UV/可視光を照射．右記の w/o は UV/可視光なし，w は UV/可視光あり，の意味．	SCG / Ames / CA / MLA（w/o / w / w/o / w / w/o / w / w/o / w） p-25：− / + / − / − / − / + / − / − WA：+ / + / − / − / ND / ND / ND / ND WR：− / − / − / − / ND / ND / ND / ND TP-3：− / − / − / − / ND / ND / ND / ND 備考：TiO_2 particles は，マウス in vivo 小核試験において弱陽性（Shelby et al., Environ. Mol. Mutagen, 21, 160-179, 1993）．Ames 試験では UV/vis 光照射の有無に関らず復帰突然変異誘発性は認められなかったことから，光誘発された TiO_2 particles による DNA 損傷は，点突然変異よりも染色体異常を出現させるのかも知れない．
38	lomefloxacin（LOM）, fleroxacin（FLER）, norfloxacin（NOR）, BAYy3118（BAY）, nalidixic acid（NA）, ciprofloxacin（CIP；positive control）	Comet assay：V79 cells（3 × 10^5），35 mmφ dishes で overnight culture，Fresh EMEM に FQ 10～50 μg/mL 添加し 1 時間 37℃暴露，氷冷 PBS に置き換え，氷冷下 UVA（37.5 kJ/m^2，～2 mW/cm^2）を照射し，細胞を pH 12.5 で comet assay を行い，遺伝毒性の強さを検討．	Comet の誘導の強さ：BAY＞NOR＞CIP＞LOM＞FLER＞NA. UV 照射後の DNA repair：UV 照射後，新鮮培地に換え，37℃，0～1 時間後に comet assay 実施した．ほとんどコントロール値と同レベルに減少し，障害は修復されていた．生体では大部分修復され，大きな遺伝毒性につながらないかもしれない． UV 照射後，2.5 μg/mL aphidicolin（DNA polymelase inhibitor）を新鮮培地に加えることにより，comet は完全には減少しない．このことから，遺伝毒性には DNA single strand brake 等よりも，excision repair が関与している可能性あり．
39	khellin, 8-methoxypsoralen（8-MOP）：TA102 用陽性対照，4,5',8-trimethylpsoralen（TMP）：TA1537 用陽性対照	① khellin の photomutagenic activity 検出条件設定のための S. thyphimurium hisC3076，TA1977（rfa），TA1537（rfa，uvrB）株による細胞毒性試験：菌懸濁液を khellin（111 μM）処理→UVA を 5, 10, 15, 20 kJ/m^2 で照射． ②TA1537，TA102 株を用いた復帰変異試験：菌懸濁液に被験物質あるいは各対照物質を所定濃度となるように添加→暗所で 20～30 分間，37℃で振盪しながらインキュベート→この菌液 10 mL をシャーレに分取→種々の線量で UVA を照射． 復帰変異試験：この菌液 0.1 mL をトップアガーに混入→最少グルコース培地にプレーティング→暗所で 37℃，48 時間培養→His^+ 復帰変異コロニー数を計測． 細胞毒性試験：この菌液を PBS で希釈→BHI プレートに播種→37℃でオーバーナイトインキュベート． 以上はいずれも triplicate で実施した．	①UVA 非照射の場合はいずれの菌株にも細胞毒性はみられなかったが，照射した場合には TA1537 のみで生存率が低下した（20 kJ/m^2 で 10％）．そこで UVA 強度を下げた条件下（0～10 kJ/m^2）で TA1537 の突然変異頻度を計測した． ②TA1537 では UVA 照射の場合，細胞毒性が認められたが，khellin による photomutagenicity はみられなかった． TA102 では UVA 照射した場合，細胞毒性がみられず，わずかな復帰変異コロニー数の増加（2 倍）を示した．より低用量での処理では突然変異誘発が認められなかったことから，より高用量（416 μM）で処理したところ復帰変異コロニー数は減少した． 備考： ・khellin は主に thimidine との DNA-photoadduct を形成するため，TA1537（psoralen の mutation を検出）と TA102（A-T の復帰変異）で試験した． ・khellin の photomutagenicity は高濃度域で，かつ非常に高い UVA 照射時にのみ起きた．したがって，psoralens に比べ，khellin の DNA への光親和性は低いと考えられた． ・khellin の photomutagenic potential は 8-MOP よりも弱かった．この低変異原性は真核細胞系での low genotoxic potential や臨床で光毒性が認められないことに一致しており，臨床使用に関して bifunctional な psoralen よりも安全と考えられた．
40	8-methoxypsoralen（8-MOP）, psoralen, angelicin	S. typhimurium TA102 を用いた復帰突然変異試験． 8-MOP, psoralen, angelicin に BC, CX, vitamin A を別々に添加し，365 nm の紫外線を 5, 10, 15 分間照射されている．	8-MOP, psoralen, angelicin ともに陽性． 備考：本文献は 8-MOP, psoralen, angelicin の光遺伝毒性を評価している内容ではなく，BC, CX, vitamin A が光遺伝毒性を軽減するという内容である．BC, CX ともに 8-MOP の光遺伝毒性を 60％程度軽減し，vitamin A は 20％程度軽減している．また，psoralen についても BC, CX ともに光遺伝毒性を 60％程度軽減し，vitamin A は 20％程度軽減している．angelicin については BC, CX ともに光遺伝毒性を大幅に軽減し，vitamin A は光遺伝毒性を 40％程度軽減している．

文献	使用化合物	試験方法	結果
41	8-methoxypsoralen (8-MOP), angelicin	S. typhimurium TA102を用いた復帰突然変異試験. 8-MOPとangelicinにBCとCXを別々に添加し，365 nmの紫外線を5, 10, 15分間照射されている．試験は通常状態と無酸素状態で行われている．	8-MOP, angelicinともに陽性. 備考：本文献は8-MOPとangelicinの光遺伝毒性を評価している内容ではなく，BCとCXが光遺伝毒性を軽減するという内容である．BC, CXともに8-MOPの光遺伝毒性を60％程度軽減し，無酸素状態では8-MOP自身の光遺伝毒性が軽減されている．また，angelicinについてもBC, CXともに光遺伝毒性を大幅に軽減し，無酸素状態では同様にangelicin自身の光遺伝毒性が軽減されている．
42	8-methoxypsoalen (8-MOP)	Ames test：S. typhimurium TA102 プレインキュベーション法： 1) 有酸素(normal condition). 2) 低酸素(hypoxia)：15分間の窒素置換. 3) 無酸素(anoxia)：15時間の窒素置換. プレインキュベーションおよびUV照射中も窒素置換した.	1) 有酸素(normal condition)：+，BCの添加で最大65％の抑制. 2) 低酸素(hypoxia)：+(有酸素の場合の65％の活性)，BCの添加でわずかに抑制. 3) 無酸素(anoxia)：+(有酸素の場合の65％の活性)，BCの添加でも抑制なし.
43	heraclenin, imperatorin	緑藻類Chlamydomonas reinhardtii (arg-1およびstr^d-3-18)変異細胞．UVA($2\ Wm^{-2}$)を照射した.	ともに陽性．S9 activationではcrude shale oilのみ陽性.
44	Dictamnine, Evolitrine, γ-Fagarine, Skimmianine, Kokusaginine, Maculine, Flindersiamine	緑藻類のアルギニン要求性株(arg-1：Chlamydomonas reinhardtii)によるMutation Assay. Tox-Study：Arg-1($1\sim3\times10^6$/mL)に，UVA(30 min)照射後，PBで希釈して，agar plateにまき，7日間培養後colony count. Mutation Assay：Schimmer and Kuhne(1990)に記載. Statistics：Mutation Frequencyについて，F-testで実験間の差を確認した後，3回の実験結果をpoolしてStudent'sのT-testで検定.	すべてで陽性反応．順番は以下のとおり. Dictamnine＞γ-Fagarine＞Maculine＞Evolitrine＞Kokusaginine＞Skimmianine＝Flindersiamine
45	The ethanolic extract(＝tincture) from the herb Ruta graveolens ssp. hortensis（ミカン科ハーブの一種ヘンルーダの市販エキス)，γ-Fagarine, Skimmianine, Dictamnine, Psoralens(ソラレン類：bergapten, psoralen, imperatorin, isopimpinellin)	緑藻類(Chlamydomonas reinhardtii)のアルギニン要求性変異株を用い，UV-Aを30分($3.6\ kJ/m^2$)照射し，アルギニン非要求性の復帰変異株の数から光変異原性が検討された.	チンキ剤中成分の3種のフロクマリン(bergapten, psoralen, imperatorin)および3種のフロキノリン・アルカロイド(dictamnine, fagarine, skimmianine)はすべて光変異原性を示したが，活性は異なっていた．Bergaptenは最も強い光変異原性を示し，dictamnineはフロキノリンでは最も強かったがbergaptenの約10％の光変異原活性であった．これら化合物のチンキ剤中の含有量はbergaptenが最も多かった．各化合物の含有量およびその光変異原活性から，このチンキ剤の主たる光変異原性物質はbergaptenであると結論された. フロキノリン・アルカロイドはフロクマリンよりも弱い光細胞毒性および光変異原性を示し，UV-A存在下でフロキノリンはDNAとmonoadductsしか形成しないが，フロクマリンはDNAとbiadductsも形成することが一因であろう. 備考：フロキノリンがeukaryotic cellで変異原性を示すことの最初の報告．チンキ剤またはエキスの変異原性は，ケルセチンの場合と同様に，必ずしもこの植物や薬剤の変異原性の強さを反映するものではない．(抗変異原物質，細胞毒性化合物，DNA結合サイトでの競合などの影響があるため.)
46	5-methoxypsoralen (5-MOP, bergapten), 8-methoxypsoralen (8-MOP, xanthotoxin)	1) 緑藻類(Chlamydomonas reinhardtii)に各被験物質を暴露し，UVAを1.2～7.2 kJ/m^2照射したときの生存率 2) 緑藻類に各被験物質を暴露し，UVA照射時間および照射量を変えたときの変異数の変化 3) 緑藻類に各被験物質を暴露し，白色光照射時間および照射量を変えたときの変異数の変化	1) 10％生存：5-MOP；3.3 kJ/m^2，8-MOP：6.9 kJ/m^2. 2) 変異数：5-MOP(15分照射)＞8-MOP(30分照射)＞8-MOP(15分照射). 5-MOP($2\ W/m^2$)＞5-MOP($0.5\ W/m^2$)＞8-MOP($2\ W/m^2$)＞8-MOP($0.5\ W/m^2$). 3) 変異数：5-MOP(4600 lux)＞5-MOP(3000 lux)＞8-MOP(4600 lux). (5-MOPの変異数／8-MOPの変異数の比). 白色光＞UVA.

47	visnagin, khellin, bergapten (positive control)	Reverse mutation assay (アルギニン要求性arg-1の緑藻類，Chlamydomonas reinharadtii) ①green alga($1\sim5\times10^6$/mL)，arg-HCL 50 μg/mLを含む最少培地で4～5日培養．cell suspension 100 mLをペトリ皿に分注し，検体を添加し，UVAを照射．照射後，細胞を洗い，寒天プレートに撒く．一部は生存率を，15日間の培養後Arg^+の復帰変異コロニーを10枚のプレートから計測. ②Pre-incubationの場合は，暗所で30分間検体に暴露させた後，UVAを照射．細胞を洗浄後，新鮮培地に懸濁して，再度UVA照射．前記と同様に生存率と復帰変異コロニーを計数. ③UVAの強さと照射時間を変えて復帰変異コロニーの増加を検討.	遺伝毒性の強さ：bergapten＞＞visnagin＞＞khellin エネルギー量が同じ場合，低線量で長時間照射したほうが遺伝毒性は強く現れる．khellinは白斑症の治療に使われている医薬品であるが，光遺伝毒性はほとんどないであろう.
48	Crude petroleum, several coal-derived oils, Shale oil	Ames Test：TA98を用い，DMSOに溶解した各oilで処理し，その溶液の一部を0，10，30分のインキュベーション後，プレートに撒き，また，残りの溶液の一部は，種々の時間(0～120分)，可視光下におき，プレートに撒いた. 可視光にはG.E. 15 W cool whiteを用い，波長は約360 nm, 13.2 w/m^2であった.	Crude petroleum：陽性，several coal-derived oils：一部陽性，Shale oil：陽性.
49	Eastern shale oil	Ames Test：TA98を用い，DMSOに溶解したshale oilと混合し，イルミネーションタイプの蛍光灯を用い，UV-可視光を照射した．照射は760 J/m^2/minで，約0～150 kJ/m^2の範囲であった.	陽性．照射量の増加およびshale oil濃度の増加とともに，復帰変異コロニー数は増加した.
50	実験的にcoalおよびshale oilから誘導して合成されたfuel samples：Solvent Refined Coal-Ⅰ(SRC-Ⅰ)およびSRC-Ⅱ processes由来材料 1. SRC-Ⅱ Heavy Distillate, 2. 蒸留フラクション5種(°F)：300-700, 700-750, 750-800, 800-850および850+, 3. カラム溶出フラクション：A1(脂肪族炭化水素)，A2(中性多環芳香族化合物, neutral PACs)，A3(含窒素PACs, N-PACs)およびA4(水酸基を含むPACs, HPACs)	S. thyphimurium TA98株／哺乳類ミクロソームを用い，光照射下または非照射下で被験物質を菌に経時的に作用させる復帰突然変異試験. TA98株のリン酸緩衝液(0.1 M, pH 7.4)中菌懸濁液($1\sim2\times10^9$ cells/mL)を，(1)蛍光灯,(2)被験物質(暗闇下)，または(3)蛍光灯と被験物質で同時に処理あるいは無処理した．被験物質処理 and/or 光照射の後(室温−33℃)，plate incorporation methodにより経時的に変異率(ヒスチジン非要求性の復帰変異として)および生存率を測定した(37℃で2～3日培養後に復帰変異コロニーおよび生菌数を測定). 光源：General Electric管およびPhilip 15-W cool white蛍光管各2本，18 W/m^2(ほとんどの実験でpyrex covers［280 nm以下の光を吸収］，一部で0.006-in. thick mylar optical filters［320 nm以下の光を吸収］の透過光).	テストサンプルの光変異原性は，その沸点の上昇とともに増強し，neutral PACsフラクションに集中した．光変異原活性は，同じサンプルでのマウスがん原性とよく相関したが，サルモネラ菌TA98／哺乳類ミクロソームでの変異原活性とは相関性が低かった．試験結果から，比較的高い沸点を示すneutral PACsは，synthetic fuels中の主要な光変異原性物質であると考えられた．また，synthetic fuelsのような複雑な混合物中のがん原性物質を検出するための比較的簡単で安価な短期間のバイオアッセイとして，光変異原性試験は有用であると思われた. 備考：人工燃料関連物質の変異原性とtumor-initiating capacityとの間には正の相関が認められているが，false negativeも報告されている．また，PACsの光毒性とがん原活性には正の相関があり，PACsは光変異原活性(光の変異原性を増強する作用)を示すと報告されている．本研究では，人工燃料サンプルのがん原性を短期間で安価に検出できるバイオアッセイ法を開発する目的で，化学的に特徴のある人工燃料フラクション中の光変異原性を測定し，人工燃料中の光変異原性物質の化学的性質および人工燃料の光変異原性とがん原性との関係が調べられた.
51	crude shale oil, hydrotreated shale oil	Ames試験：TA98株を用い，Selbyら(1983)の方法に従ってfluorescent lightを照射した.	ともに陽性．S9 activationではcrude shale oilのみ陽性.

文献	使用化合物	試験方法	結果
52	Ciprofloxacin (CIPRO), Nalidixic acid (NAL), Ofloxacin (OFL), Norfloxacin (NOR), Enoxacin (ENO), Lomefloxacin (LOME), Trovafloxacin (TROVA), Clinafloxacin (CLINA), Spafloxacin (SPAR)	In vitro小核試験．V79細胞を用いてUVG55 Mineralight (290～400 nm) を1～30分(総照射量1250～30000 J/m^2) 照射した．その後citocharacinBを含む新しい培養液に交換し16時間培養後標本を作製した．	化合物 / UV(－) / UV(＋) Ciprofloxacin (CIPRO)： 弱＋ / 中等度＋ Nalidixic acid (NAL)： － / 中等度＋ Ofloxacin (OFL)： － / － Norfloxacin (NOR)： － / － Enoxacin (ENO)： － / － Lomefloxacin (LOME)： 弱＋ / 中等度＋ Trovafloxacin (TROVA)： 弱＋ / － Clinafloxacin (CLINA)： 強＋ / 強＋ Spafloxacin (SPAR)： － / 中等度＋ 総合評価： 光毒性(50％細胞増殖抑制係数，濃度×UV dose)：CLINA＞＞LOME, SPAR＞TROVA, NAL, OFLO, CIPRO＞ENO, NOR. MN(5％MN係数，濃度×UV dose)：CLINA＞＞LOME, SPAR＞TROVA, NAL, CIPRO, OFLO＞NOR, ENO.
53	4,4',6-trimethylangelicin (TMA), 8-methoxypsoralen (8-MOP)	マウスの末梢正染性赤血球およびヒト皮膚線維芽細胞の初代培養を用いた小核試験．UV-A (300～400 nm) を45分間照射．	マウスの末梢正染性赤血球を用いた小核試験で両化合物とも陰性． ヒト皮膚線維芽細胞の初代培養を用いた小核試験で両化合物とも弱陽性．
54	Lomefloxacin	株化されたヒトのケラチノサイトを用い，UVAを10～37.5 kJ/m^{2}照射した．T4 endonuclease Vを用いてピリミジンダイマーを検出した．	10 μg/mLでピリミジンダイマーが産生された．LomefloxacinとUVAによるピリミジンダイマーの産生についての発見はマウスの皮膚癌形成におけるlomefloxacinのイニシエーション活性と関係する．
55	8-methoxypsoralen (8-MOP), p-aminobenzoic acid (PABA), octyl dimethyl PABA, 4-methylbenzylidene camphor, Dihydroxyacetone (DHA), 7, 12-dimethylbenz[a] anthracene (DMBA)	Ames test：TA1537, TA102, WP2を用い，UVA, UVBを照射．	PABAからcamphorは陰性，DHAは弱陽性，DMBAは陽性．
56	Lomefloxacin	ラット肝の培養細胞を用い，被験物質共存下UVA照射により細胞DNA中に生成する7,8-dihydrio-8-oxo-2'-deoxyguanosine (8-oxo-dG) 量を測定している． 一重項酸素捕捉剤 (NaN_3, TMP)，ヒドロキシラジカル捕捉剤 (α-Tocopherol, TBP) 共存下での変化について検討している．	光反応生成物は，一重項酸素捕捉剤共存下で減少し，ハイドロキシラジカル捕捉剤共存下では変化しなかったことより，quinolone系抗生物質のUVA照射時の光毒性メカニズムとして，一重項酸素が関与している．
57	Acridine (A), 9-Aminoacridine (9-AA), Proflavine (P), Acridine Yellow (AY), Acridine Orange (AO), Methylene Blue (MB), Quinacrine (Q)	E.coli B/r (Tl^R trp) のT5ファージ耐性変異検出：E.coli B/r (Tl^R trp) を恒成分培養槽中で化合物と一緒に培養し，経時的にサンプリングしアガープレート上でT5ファージ耐性コロニーを選別する．	作用強度　AY＞P＞AO＞MB＞9-AA＞A＞Q＝Nega Con 環の両側にアミノ基を有している化合物 (AY, P, AO, MB) は強い作用を有する．

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環境変異原研究に関する未発表の「総説」，「一般論文」，「短報」，および「特別企画（受賞講演）」，「論説」，「資料・情報」などを掲載する．なお，投稿論文の採否は編集委員会の審査により決定する．

「総説」は，一つのテーマに関連する多くの研究論文の総括，評価，解説などである．原則として編集委員会より寄稿を依頼する．

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（Letter to Editorも受け付ける．）

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共著者のうちの1人は日本環境変異原学会会員でなければならない．ただし，招待寄稿の場合にはこの限りではない．

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論文原稿の用語は英語または日本語とし，最新の執筆規定に従い簡潔にわかりやすく書く．総説，一般論文，論説は，写真・図表を含めて刷り上がり8頁以内．短報は4頁以内とする．ただし規定の頁数を超えることが明らかな場合は，事前に編集委員に相談する．

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論文原稿は5部と本誌に綴じ込みの「投稿論文チェックリスト」を，下記に（簡易）書留便または宅配便で送付する．

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5. 著作権

本誌に掲載された記事，論文などの著作権は日本環境変異原学会に帰属するものとする．従って，本会が必要と認めた場合は転載し，また外部から引用の申請があった場合には，編集委員会において検討の上許可することがある．ただし，著作者自身が自分の記事，論文などの一部の複製，翻訳などの形で利用することを妨げるものではない．しかし，著作者自身であっても，全文を複製の形で他の著作物に利用する場合には，事前に文書にて学会長に申し出を行い，許諾を求めなければならない．

6. 校正

著者校正は原則として原稿に対する誤植の訂正に限る．原稿にない加筆・変更はしないこと．

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「環境変異原研究 Environmental Mutagen Research」への投稿論文の，投稿から印刷までの時間の短縮を目指し，投稿論文の査読を評議員にお願いすることになりました．それに伴い，投稿規定の見直しも行い，今後は年大会での発表と同様に，投稿論文の分野を著者に申告していただき，同一分野を登録していただいている評議員に査読していただくことになります．**今回投稿時のチェックリストをお届けしますので，投稿論文と一緒に必ずお送り下さい．**

現在7名の編集委員が分担して学会誌，*Jems News* の編集作業を行っていますが，それぞれ自分の仕事を抱えボランティアとして活動しています．そこで，会員の皆様の積極的な雑誌作りへの協力として，年大会で発表されたものを一報でも多く論文にまとめ，本学会誌に投稿していただきますようお願い致します．また，*Jems News* への投稿，情報，意見，公募等もお願いします．

本誌の将来像に関する議論を進めております．英文誌化の話も出ており，編集委員を中心に検討を始めたところですが，皆様のご意見を是非お聞かせいただきたく思います．どんな些細なことでも結構ですから，学会誌に関することでコメント，提案等がありましたら，編集委員までE-mailにてお知らせください．編集委員一同

環境変異原研究 執筆規定

1. 用語は日本語または英語とする．
2. 原稿は原則としてワープロを用いて作成する．
   日本文の原稿：
   原稿はA4判用紙に1行約40字，1頁30～31行で印字する(刷り上がりの約1/2頁に相当する)．ただし，要約は英文(300語以内)とする．また，別に題名，著者名(フルネーム)，所属機関名ならびに所在地を英文で付ける．
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4. 学名，遺伝子記号などはイタリックとし，その他まぎらわしい記号については原稿に適宜指示を与える．
5. 化学物質名は原則として英語とし，一般名を用いる．また，化学物質のCAS登録番号を記載する．文中に用いる英語の単語あるいは句は固有名詞を除いて小文字で書きはじめる(文頭の場合は大文字)．
6. 数字は算用数字を用い，単位は慣用による省略記号を用いる．
7. 略語を使用するときは，論文中にはじめて使用するときに完全な語とその略語を括弧内に示す．
8. 句読点はカンマ(,)およびピリオド(.)とする．
9. 表，図は本文と別にし，それらの挿入箇所を本文の右余白に明示する．グラフ，写真，線画等はすべて図とし，一連の番号Fig. 1, 2, …を付し，説明文(英文)を別紙に添える．
10. 図と写真は原図またはキャビネ大の光沢写真版とし，裏面にFig. 1, 2, …および上下を鉛筆書きする．
11. 表は上部に一連の番号Table 1, 2, …と英文の表題を記入する(文頭のみ大文字)．脚注を要するときに表示の語句の右肩にa，b，c，…を付記し，表の下欄外にそれぞれの説明を記す．
12. 本文中の文献引用は著者名および年号をもってする．
13. 参考文献は筆頭著者名のアルファベット順に配列し，雑誌の省略名はChemical Abstractsの記載方法に従う．記載順序は著者氏名，(年)，題名，雑誌名，巻，頁(単行本の一部引用の場合は著者氏名，(年)，題名，編者名，書名，発行所，発行地，頁)の順とする．単行本そのものを引用する場合は，編者名あるいは著者名，(年)，書名，発行所，発行地の順とする．文献の記載方法は下記の例に従う．

Ames, B.N., J. MaCann and E. Yamasaki (1975) Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test, Mutat. Res., 31, 347-364.

Ashby, J., F.J. de Serres, M. Draper, M. Ishidate Jr., B.H. Margolin, B. Matter and M.D. Shelby (1985) Overview and conclusion of the IPCS collaborative study on in vitro assay systems, In : J. Ashby, F.J. de Serres et al. (Eds), Evaluation of Short-Term Tests for Carcinogens, Elsevier, Amsterdam, pp. 117-174.

Friedberg, E.C., G.C. Walker and W. Siede (1995) DNA Repair and Mutagenesis, ASM Press, Washington, D.C.

藤川和男，梁　治子，近藤宗平(1984)ハエの翅毛スポットテスト―近ごろ注目されている短期試験法，環境変異原研究，6，107-113.

佐々木正夫(1983)環境変異原と染色体異常，外村晶(編)，染色体異常，朝倉書店，東京，pp. 107-113.

松影昭夫(編)(1996)DNA複製，修復と発癌，羊土社，東京．

(改訂　2000年12月)

キリトリ線

投稿論文チェックリスト

論文ご投稿の際，この用紙に必要事項をご記入のうえ，添付してください．

論文名（日本語）：
（英　語）：

窓口著者名：

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分類（該当するものに印をつけてください）

- □ 突然変異のメカニズム
- □ 変異原の検出
- □ 環境汚染物質
- □ 抗変異原
- □ DNA損傷（付加体）
- □ 変異原の代謝
- □ 変異原の修飾，発現
- □ 試験法の開発，改良
- □ その他（　　）

送付前のチェック項目

- □ 投稿規定，執筆規定に沿っていますか（特に引用文献）
- □ 英文校閲はお済みですか
- □ 投稿論文は5部ありますか

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保　　存 S-9は活性の高い酵素系よりなっておりますので、ー80℃で保存して下さい。まれに解凍後分離することがありますが活性には異常がありませんので、よく攪拌して御使用下さい。

●**包装単位**：1.5mℓ×12本詰　●特注品、S-9に関して詰容量は4.5mℓまでお受けいたします。

●**活性データ**

ロット毎に下記の生化学的活性データを添付致します。

分　画	測 定 デ ー タ
S-9 (9,000×g分画)	タンパク質含量 チトクロームP-450含量 DMN脱メチル酵素活性 アニリン水酸化酵素活性 ベンゾ(a)ピレン水酸化酵素活性
ミクロソーム (105,000×g分画)	タンパク質含量 チトクロムP-450含量

ロット毎に下記の変異原活性データ(突然変異株数)を添付致します。

薬　物	菌　　株＊
ベンゾ(a)ピレン	TA-100、TA-98、TA-1537
2-アミノアントラセン	TA-100、TA-98、TA-1537
9,10-ジメチルアントラセン	TA-100、TA-98、TA-1537
自然発生突然変異株数	TA-100、TA-98、TA-1537

※ *Salmonella typhimurium*

エームス試験用凍結S-9MIX

特長 ①エームス試験がより手軽になりました。
②S-9にコファクターミックスを加え無菌的に調整しました。
③解凍後、直ちにエームス試験にご使用いただけます。
④S-9が1mℓとコファクターミックスが9mℓ入っており、20プレート分の試験が可能です。

●**包装単位**：10mℓ×8本、5mℓ×4本

Salmonella typhimurium TA-100,
Benzo(a)pyrene 5μg/plate

ー S-9 Mix

＋S-9 Mix

染色体異常試験用凍結S-9MIX

特長 ①染色体異常試験がより簡単になりました。
②S-9にコファクターミックスを加え無菌的に調整しました。
③解凍後、直ちに染色体異常試験にご使用いただけます。
④S-9が1.05mℓとコファクターミックスが2.45mℓ入っており、7プレート分の試験が可能です。

●**包装単位**：3.5mℓ×3本

カタログNo.	品　　名	包　装	価　格
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S-9 MIXTS	染色体異常試験用凍結S-9 MIX	3.5mℓ× 3 本	**¥12,000**

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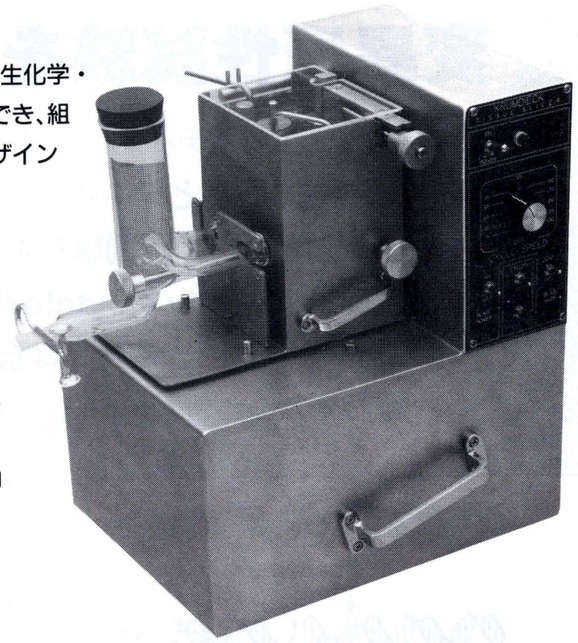

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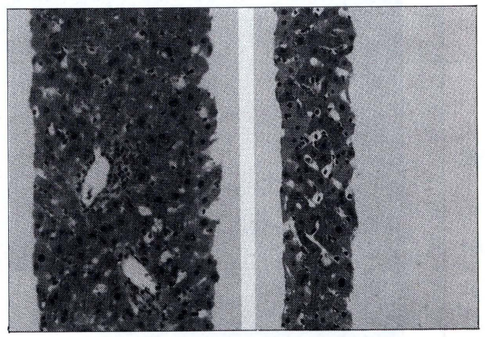
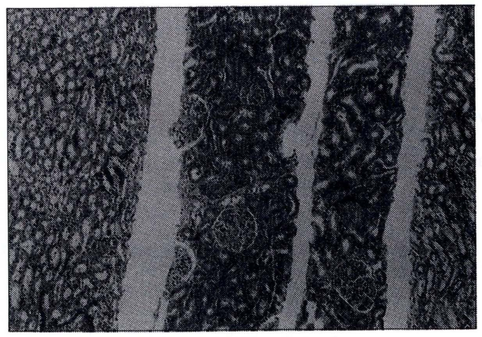
- スライスは再現性良く、バラツキもなく60～1000μmの厚さで作成されます。



ラットの肝臓(倍率430×)



ラットの腎臓(倍率100×)

右の写真はラットの肝臓のスライス(厚さ60μmおよび135μm)で、左の写真はラットの腎臓のスライス(135～200μm)です。どちらも切片面の平行性と美しさ(ダメージがない)に注目して下さい。

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編集後記

1990年代より，ヨーロッパでは光（遺伝）毒性への関心が高かったのですが，2000年にはいり，欧米の医薬品規制当局（EMEA, FDA）ならびにOECDは光安全性試験に関するドラフトガイダンスを相次いで発表しました．日本では，光遺伝毒性への関心はさほど高くありませんが，グローバル化が進む現在，このような欧米の動向を把握することは重要と考え，この分野で活躍しておられる田中憲穂会員および島田弘康会員を特別編集委員としてお迎えし，光遺伝毒性の特集号を組みました．内外の第一人者の先生方のご協力を得ることができ，すばらしい特集号になったと自賛しております．日本における光遺伝毒性に対する関心の契機となれば幸いです．

担当編集委員　森田　健

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環境変異原研究

Environmental Mutagen Research Vol. 23 No. 2 2001

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　　学生会員申込書

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　　執筆規定