FUROCOUMARIN DIOXETANES AND HYDROPEROXIDES AS NOVEL PHOTOBIOLOGICAL DNA-DAMAGING AGENTS

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Photoexcitation of psoralen, an excellent DNA intercalator, was achieved in the dark by thermal decomposition of psoralen-functionalized dioxetane 2. The 3,4 monoadduct of psoralen was formed when dioxetane 2 was treated with calf thymus DNA in H_2O at 50 °C. Photobinding of psoralen to DNA was also observed when alkyl-substituted dioxetane 1 was thermally decomposed in the presence of psoralen and DNA. The dioxetanes of trimethyl-substituted furocoumarins 4 showed strong mutagenic activity in the *Salmonella typhimurium* strain TA100 (sensitive to alkylating agents and UV^{260} radiation). The mutagenicity of furocoumarin dioxetanes 4 is comparable to that of benzofuran dioxetanes 5, the latter constitute potent mutagens. Presumably DNA adducts of the intermediary furocoumarin epoxides, generated *in situ* by deoxygenation of the dioxetanes, are responsible for the mutagenicity. The furocoumarin hydroperoxides 8,9, which were synthesized by photooxygenation of alloimperatorin (6) and imperatorin (7), are efficient photoactive DNA-damaging agents. They induced single strand breaks and endonuclease-sensitive modifications in supercoiled bacteriophage PM2 DNA upon irradiation at 360 nm. The DNA damage profiles exhibited by furocoumarin hydroperoxides 8,9 are similar to those of hydroxyl radicals, generated either by γ radiation or by xanthine and xanthine oxidase in the presence of Fe(III)-EDTA. tert-Butanol, an efficient hydroxyl radical scavenger, reduced the formation of DNA modifications significantly. Moreover, in the absence of near-UV radiation, the furocoumarin hydroperoxides did not cause any DNA damage. These results indicate that the hydroxyl radicals produced in the photolysis of the hydroperoxides 8,9 are the ultimate DNA-damaging agents.

Keywords: DNA photobinding; photosensitization; mutagenicity; strand breaks; photo-Fenton reagent.

1,2-Dioxetanes serve as thermal source for the generation of triplet-excited carbonyl species.[1] Such triplet states, generated from dioxetanes, sensitize DNA modifications both by energy and electron transfer directly to DNA and indirectly by energy transfer to molecular oxygen (singlet oxygen).[2] Thus, dioxetanes show genotoxic activity in isolated DNA as well as in cellular and bacterial DNA.[3] Specifically, in supercoiled DNA of the bacteriophage PM2, they produce predominantly oxidative base modifications; pyrimidine dimers, AP sites and strand breaks are minor lesions. In human leukemia cells (HL 60) and in Syrian hamster embryo (SHE) fibroblasts dioxetanes cause single strand breaks, in the latter also micronuclei were observed, which implies genotoxicity at the chromosomal level.[4] While simple dioxetanes are nonmutagenic, derivatives of benzofurans show strong mutagenic activity in the *Salmonella typhynurium* strain TA100.[4]

To enhance the DNA-damaging propensity of dioxetanes, we have pursued the strategy of combining the dioxetane moiety with an intercalative, photoactive chromophore, e.g. psoralen and other furocoumarins. Furocoumarins will facilitate the generation of reactive species in the vicinity of the DNA target through intercalation. Moreover, the furocoumarin chromophore can be sensitized for photobinding with DNA by intramolecular energy transfer from the triplet carbonyl species generated by thermal decomposition of furocoumarin-substituted dioxetane. The synthesis of furocoumarin dioxetanes and their photobiological activity are described herein. Indeed, our preliminary results reveal that this concept of such multifunctional agents is effective for promoting DNA damage.

On the other hand, hydroperoxides can cause DNA cleavage through hydroxyl radicals, when activated by transition metal ions (Fenton reaction).[5] Recently it was reported that phthalimide hydroperoxides generate hydroxyl radicals upon near-UV irradiation (λ>300 nm) and DNA is cleaved efficiently.[6] Consequently, these compounds have been designated as *photo-Fenton* reagents. Our strategy of developing more efficient *photo-Fenton* reagents for genotoxicity studies embraces the same concept as described for dioxetanes, i.e. linkage of the hydroperoxide group to photoactive intercalators, e.g. furocoumarin derivatives. We report herein the DNA-damaging effects of the novel intercalative furocoumarin hydroperoxides. For the identification and quantification of the DNA modifications induced by such *photo-Fenton* reagents specific repair endonucleases and gel electrophoresis were employed.

PREPARATION OF DIOXETANES AND HYDROPEROXIDES

Two different types of furocoumarin dioxetanes **2,4** were employed for the photobiological investigations described here. The psoralen dioxetane **2** was prepared from the chloroformate derivative of 3-hydroxymethyl-3,4,4-trimethyl-1,2-dioxetane (**1**) and *xanthotoxol* as reported previously (Eq. 1).[7]

(1)

1 —$(COCl_2)_3$, pyridine→ chloroformate —xanthotoxol, Et_3N→ 2

Furocoumarin dioxetanes **4** were synthesized by photooxygenation of the corresponding furocoumarins **3** (Eq. 2).[8]

a: R^1, R^4=H; R^2+R^3=A
b: R^1, R^2=H; R^3+R^4=A
c: R^3, R^4=H; R^1+R^2=A
d: R^3, R^4=H; R^1+R^2=B
e: R^1, R^4=H; R^2+R^3=B

(2)

The furocoumarin hydroperoxides **8**,**9**, which were employed in the present photobiological investigations, were prepared by ene reaction of singlet oxygen with *alloimperatorin* (**6**) and *imperatorin* (**7**), cf. Scheme I.[9] Reduction of the hydroperoxides **8** and **9** by triphenylphosphine afforded the corresponding alcohols **10** and **11** in very good yield.

Scheme 1

DNA PHOTOBINDING SENSITIZED BY PSORALEN DIOXETANE 2

For the first time we have shown that psoralen can be photosensitized to form DNA photoadducts in the dark by triplet carbonyl species generated in the thermal decomposition of dioxetanes[10]. Thus, the treatment of calf thymus DNA with psoralen dioxetane **2** in H_2O at 50 °C for 45 min led to the psoralen monoadduct of DNA at the pyrone double bond (Eq. 3),

λ_{ex} = 302 nm; λ_{em} = 410 nm

(3)

as confirmed by fluorescence measurements. On the other hand, irradiation of the cleavage product **12** of dioxetane **2** at 365 nm in the presence of DNA also afforded photoadducts; however, in this case the photobinding occurred at the furan side of psoralen (Eq. 4)[10]. These results are in agreement with earlier observations, in which direct and ketone-sensitized

λ_{ex} = 330 nm; λ_{em} = 397 nm

(4)

irradiation of psoralen gave rise to different regioisomeric photoadducts.[11] For example, triplet-excited psoralen generated by ketone sensitization led to DNA photoadducts at the pyrone ring. Consequently, the thermally generated triplet species derived from the psoralen dioxetane **2** behaved in an analogous way.

Photobinding of psoralen at the 3,4 position was also observed, when this furocoumarin was treated with dioxetane **1** in the presence of DNA (Eq. 5). As expected, in this case the photobinding was less effective than that induced by psoralen

(5)

dioxetane **2**. Clearly, our strategy of attaching the thermal excitation source (dioxetane) to the photoactive chromophore (psoralen) facilitates sensitization through intramolecular energy transfer.[10]

MUTAGENICITY OF FUROCOUMARIN DIOXETANES 4

Furocoumarin dioxetanes **2** and **4** were assayed for mutagenicity by using the *Salmonella typhimurium* (*S.t.*) strain TA100, which mutates mainly by exposure to alkylating agents and UV^{260} radiation.[12] While psoralen dioxetane **2**, like the hydroxymethyl-substituted dioxetane **1** and other alkyl-substituted dioxetanes,[4] did not show any significant mutagenicity, furocoumarin dioxetanes **4a-c** were strongly mutagenic in *S.t.* strain TA100.[8] The mutagenicity of furocoumarin dioxetanes **4** is comparable (Table I) to that of benzofuran dioxetanes **5**,[13] which are structurally related to the furocoumarin dioxetanes **4**. The specific mutagenicity of benzofuran dioxetanes **5** ranges between *ca.* 1000 and *ca.* 370000 revertants/µmol and depends on the substituents in the benzo and furan rings.[13]

The expected UV-type DNA damage, i.e. formation of pyrimidine dimers, was insignificant in the mutagenicity observed for the benzofuran dioxetanes **5**, as confirmed by the lack of repair on attempted photoreactivation.[4] Rather, DNA adducts of the intermediary benzofuran epoxides, generated *in situ* by deoxygenation of the dioxetanes, are presumably responsible for the mutagenicity.[3,14] In fact, control experiments confirmed that authentic benzofuran epoxides, prepared by methionine deoxygenation of benzofuran dioxetane, afforded DNA adducts, as detected by ^{32}P postlabeling.[3] Since the furocoumarin dioxetanes **4** are structurally similar to benzofuran dioxetanes **5** and their mutagenic activities comparable, it is likely that the DNA adducts of furocoumarin epoxides are causing the strong mutagenicity of furocoumarin dioxetanes **4**. Formation of DNA adducts was indeed observed, when the mutagenic furocoumarin dioxetane **4b** was deoxygenated by methionine in the presence of calf thymus DNA, as confirmed by fluorescence measurements (Eq. 6).[15]

Table I. Mutagenicity of furocoumarin **4** and benzofuran dioxetanes **5** in *Salmonella typhimurium* strain TA100

Dioxetanes	Revertants/ μmol[a]	Dioxetanes	Revertants/ μmol[a]
4a	115000	**5a**	170000
4b	50000	**5b**	3700000
4c	10000	**5c**	2000

a) Specific mutagenic activities calculated by linear regression analysis from the dose-response curve[13]; standard deviations are approx. 10% and the spontaneous revertant frequencies per plate 150-200.

4b $\xrightarrow[H_2O]{\text{L-Methionine}}$ $\xrightarrow{\text{DNA}}$ DNA, OH

λ_{ex} = 343 nm; λ_{em} = 425 nm

(6)

DNA MODIFICATIONS INDUCED BY FUROCOUMARIN HYDROPEROXIDES 8 AND 9 UPON IRRADIATION AT 360 NM

In search of efficient DNA photocleaving agents, the novel DNA-intercalating furocoumarin hydroperoxides **8** and **9** were synthesized [9] and their DNA-damaging propensity under photochemical activation was investigated. Supercoiled DNA of bacteriophage PM2 (PM2 DNA) was exposed to near-UV radiation (360 nm) in phosphate buffer in the presence of furocoumarin hydroperoxides **8** and **9**. The exposed DNA was precipitated and analyzed for single and double strand breaks, AP sites (sites of base loss), and base modifications by means of specific repair endonucleases (Table II).[16]

As shown in Table II, the furocoumarin hydroperoxides **8** and **9** (in particular **8a,b**) were very efficient in inducing DNA single strand breaks and FPG-sensitive base modifications; the FPG protein (formamidopyrimidine-DNA glycosylase) detects specifically formamidopyrimidines and 8-hydroxyguanine (7,8-dihydro-8-oxoguanine).[17] Also AP sites and 5,6-dihydropyrimidines were revealed by the exonuclease III and endonuclease III.[18] The formation of 5,6-dihydropyrimidines was inferred by the fact that the number of modifications observed with endonuclease III was significantly higher (Table II) than the AP sites detected by exonuclease III (specific for AP sites), when PM2 DNA was exposed to near-UV radiation in the presence of furocoumarin hydroperoxides **8** and **9**. Control experiments showed that these agents did not induce any significant DNA modifications without near-UV irradiation. Like-

Table II. Endonuclease-sensitive modifications and strand breaks induced in PM2 DNA by furocoumarin hydroperoxides **8** and **9** on irradiation by UV^{360} (4.5 kJ/m^2)[a]

Furo-coumarins	ε[b)]	Modifications/μM[c)]					
		FPG[d)]	EndoIII[e)]	UV-endo[f)]	ExoIII[g)]	SSB[h)]	DSB[i)]
8a	2530	4.90±0.60	3.10±0.60	2.300±0.600	1,80±0.60	4.800±0.300	0.020±0.002
8b	2250	1.30±0.08	0.88±0.05	0.550±0.040	0.58±0.06	1.110±0.020	0.005±0.001
9a	1580	0.38±0.06	0.13±0.04	0.050±0.050	0.09±0.01	0.190±0.010	j)
9b	1350	0.24±0.03	0.06±0.01	0.010±0.004	0.03±0.01	0.060±0.003	j)
10a	2600	(2±8)x10^{-5}	(1.4±1)x10^{-5}	(7±5)x10^{-5}	(3±3)x10^{-5}	(5±4)x10^{-5}	J)
11b	1460	(6.6±0.6)x10^{-4}	(3±1)x10^{-4}	(2.4±0.8)x10^{-4}	(5.6±1.0)x10^{-4}	(0.6±0.6)x10^{-4}	j)

a) Data are taken from Ref. 16; b) Molar absorption coefficient [$M^{-1}cm^{-1}$] at 360 nm; c) Number of modifications (±S.D.) per 10^4 base pairs, calculated by linear regression from the linear portion of the concentration-response relationships; d-g) Modifications sensitive to FPG protein (formamidopyrimidine-DNA glycosylase, provided by Dr. S. Boiteux, Villejuif), endonuclease III (provided by Dr. R.P. Cunningham, Albany), UV endonuclease preparation from M. luteus, endonuclease IV (obtained from Dr. B. Demple, Boston) and exonuclease III (purchased from Boehringer Mannheim, Germany); h) Single strand breaks; i) Double strand breaks; j) Not determined.

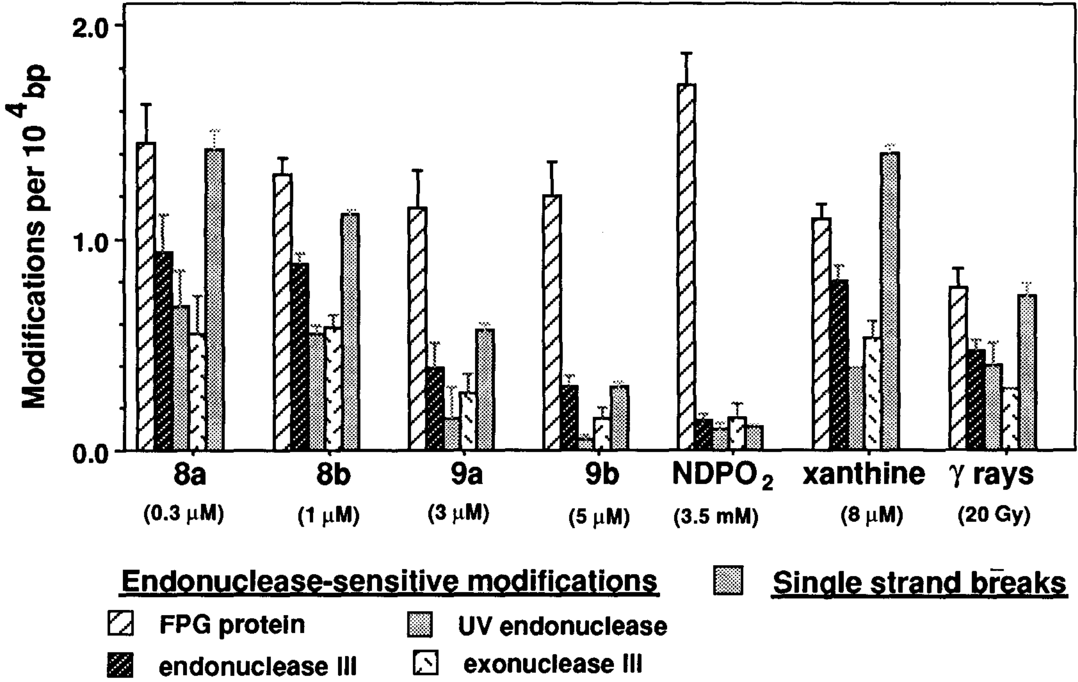

Figure I. DNA damage profiles: Single strand breaks and various endonuclease-sensitive modifications induced in PM2 DNA by treatment with the furocoumarin hydroperoxides 8a,b,[9] and 9a,b under UV^{360} (4.5 kJ/m²); $NDPO_2$ in D_2O buffer (3.5 μM, 2 h, 37 °C); xanthine (8 μM) in the presence of xanthine oxidase and Fe(III)-EDTA (30 min, 20 °C) and γ radiation (20 Gy at 50 μg/ml DNA)[16].

wise, the corresponding alcohols **10** and **11** of the furocoumarin hydroperoxides **8** and **9** did not cause DNA modifications even at high concentrations and upon irradiation at 360 nm.[16]

The DNA damage profiles, i.e. the relative amounts of the various types of modifications induced in PM2 DNA by furocoumarin hydroperoxides **8a,b** and **9a,b**, are exhibited in Figure I. These damage profiles have been compared with those of singlet oxygen, produced by thermal decomposition of the endoperoxide $NDPO_2$, and hydroxyl radicals, produced either by xanthine and xanthine oxidase in the presence of Fe(III)-EDTA or by γ radiation.[19] The damage profiles of the hydroperoxides **8a,b** matched most closely those caused by hydroxyl radicals, while **9a,b** showed more similarities with the damage profile of singlet oxygen.

To assess whether singlet oxygen plays a role in the formation of DNA modifications by furocoumarin hydroperoxides **8** and **9** under irradiation, control experiments were run in D_2O phosphate buffer. No D_2O effect on the formation of FPG-sensitive base modifications and single strand breaks was observed.[16] Therefore, the involvement of singlet oxygen in the generation of DNA modifications can be excluded. Superoxide dismutase and catalase have also no significant effect on the DNA modifications induced by hydroperoxides **8** and **9**;[16] hence, superoxide and hydrogen peroxide are also not involved in this DNA damage. On the other hand, the yield of single strand breaks and FPG-sensitive base modifications were appreciably reduced in the presence of *tert*-butanol, an efficient scavenger of hydroxyl radicals.[16] These results indicate that hydroxyl radicals, efficiently generated by photoexcitation of the furocoumarin hydroperoxides **8** and **9**, are the actual DNA-damaging agents. Therefore, these novel intercalating furocoumarin hydroperoxides serve as effective *photo-Fenton* reagents.

On the basis of the few examples featured herein, it is our contention that multifunctional DNA-damaging agents, which serve on one hand as photoactive intercalators (furocoumarins) and on the other hand either as sources of triplet states (dioxetanes) or hydroxyl radicals (hydroperoxides), show great promise in the study of photochemical genotoxicity. Thus, they constitute novel photobiological tools with potential medical applications as phototherapeutic agents.

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