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Mechanism of Tetracycline Phototoxicity

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Studies were made to determine factors important in the phototoxicity mechanism of 7 clinically used tetracyclines (TC). The clinical phototoxicity, the rates of photochemical degradation, and the in vitro phototoxicity of the TCs were qualitatively but not quantitatively correlated. Phototoxicity in vitro was partially oxygen-dependent and possibly singlet oxygen is involved. The contribution of photoproducts to the phototoxic process may be the basis for the reported differences between the in vivo action spectrum and the absorption spectrum of demethylchlorotetracycline. A mechanistic model for in vivo phototoxicity is proposed where the absorption of UVA radiation by TC leads to at least two main processes: (i) photosensitization by the drug of biologic molecules to cause phototoxicity; (ii) production of one or more photoproducts which photosensitize by absorption of visible radiation.

The incidence of photosensitivity reactions to the tetracycline (TC) antibiotics varies with the structure of the drug. From clinical reports, demeclocycline (DMCT; 6-demethyl-7-chlorotetracycline) appears to be the most phototoxic. Other TCs are infrequently reported to be phototoxic [1–7]. No reports of phototoxicity from minocycline (MC; 6-demethyl-6-deoxy-7-dimethylaminotetracycline) have appeared. The common manifestations of TC photosensitivity are a tingling, burning sensation [1–7], onycholysis [5,6], papular eruption [3] and, in one case, the presence of multinucleated giant cells [4]. The effective wavelengths for eliciting phototoxicity in vivo are between 320–425 nm [2,7]. However, the most effective wavelength for DMCT photosensitization in a study with mice [8] was at 400 nm, rather than at the absorption maxima of the drug, 278 nm and 375 nm.

The mechanism of TC phototoxicity remains unknown. The available data indicate that the cellular site for phototoxic damage may be ribosomes and cell membranes. Photoactivated TC covalently binds to both eukaryotic and prokaryotic ribosomes [9,10]. TC has also been reported to cause photohemolysis of human erythrocytes by an oxygen-independent mechanism [11]. However, other sites may be involved since, in the dark, TC interacts with other subcellular sites. For example, TC noncovalently binds to both DNA and human serum albumin (HSA) in the presence of divalent cations [12] and appears to affect DNA synthesis in *Escherichia coli* and *Bacillus subtilis* by interacting with cell membrane [13]. Our overall goal is to elucidate the molecular mechanism of TC phototoxicity. In this initial study we have: (1) evaluated the relationship between TC photodegradation rate, in vitro phototoxicity, and clinical photosensitizing potential for 7 clinically used TCs, shown in Table I; (2) evaluated the in vitro TC phototoxicity to lymphocytes and the involvement of oxidative mechanisms and tetracycline photoproduct(s) (PhTC) in this process; and (3) postulated a basis for the action spectrum.

MATERIALS AND METHODS

Tetracyclines

The TC hydrochlorides were either purchased from Sigma or were generous gifts from Charles Pfizer Co., Groton, Connecticut, and American Cyanamid Company, Lederle Laboratories, Pearl River, New York. Anhydrotetracycline was prepared by the acid-catalyzed dehydration of a methanolic solution of TC [14].

Irradiation Sources

UVA irradiations were carried out using a 1.0 kW xenon arc lamp through 10 cm of circulating solution (4% copper sulfate and 4% cobalt sulfate) which limited the irradiance to a 320–400 nm waveband with a peak at 365 nm. The spectral irradiance of this source (average output 75 W/m^2) was measured by a cosine-corrected UV spectroradiometer system (IL 700, International Light, Inc., Danvers, Massachusetts). A SEE 400 detector with a peak sensitivity at 360 nm and half-power points at 330 and 370 nm was used. For visible light irradiations (380–600 nm) a 2500-W xenon arc source (Schoeffel Instruments) with an f/1.5 condensing lens system was used. For reactions with tetracycline (TC) and 5α-6-anhydrotetracycline (AHTC), radiation was filtered through a 10% copper sulfate liquid filter, 3 pieces of 3/8-inch lucite, and a CS1-64 blue glass filter (Corning). The lucite filters sharply cut off wavelengths of less than 380 nm. The CS1-64 transmits maximally between approximately 380 and 420 nm and attenuates radiation of wavelengths above 500 nm. Irradiance was measured using an Eppley thermopile in conjunction with a Keithley 150B microvolt ammeter, and had an average output of 0.3 kW/m^2. For the phototoxicity assay with TC and PhTC, the $CuSO_4$, liquid filter was replaced by water, and the CS1-64 blue glass filter was removed. The average output under these conditions was 0.7 kW/m^2.

Instrumentation

UV-visible absorption spectra were recorded using either a Varian DMS-90 or a Beckman 5270 spectrophotometer. Radioactivity measurements were made on a Beckman LS 150 liquid scintillation counter.

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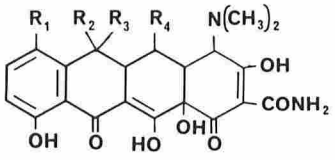
Abbreviations:
AHTC: 5α,6-anhydrotetracycline
BSA: bovine serum albumin
CTC: (chlortetracycline) 7-chlorotetracycline
D_2O: deuterated water
DMCT: (demeclocycline) 6-demethyl-7-chlorotetracycline
DOTC: (doxycycline) 6α-deoxy-5α-hydroxytetracycline
HBSS: Hanks' buffered salt solution
HSA: human serum albumin
MC: (minocycline) 6-demethyl-6-deoxy-7-dimethylaminotetracycline
MTC: (methacycline) 6-demethyl-6-deoxy-5-hydroxy-6-methylenetetracycline
OTC: (oxytetracycline) 5α-hydroxytetracycline
PBMNC: peripheral blood mononuclear cells
PBS: phosphate-buffered saline
PHA: phytohemagglutinin
PhTC: tetracycline photoproducts
PTD_{50}: dose of UVA required to reduce [^{3}H]thymidine incorporation to 50% of that in unirradiated controls
PVD_{50}: dose of UVA required to reduce the number of viable cells to 50% of the number in unirradiated cultures
TC: tetracycline

TABLE I. *Structure of tetracyclines*



Antibiotic	R_1	R_2	R_3	R_4
TC	H	CH_3	OH	H
CTC	Cl	CH_3	OH	H
OTC	H	CH_3	OH	OH
DOTC	H	CH_3	H	OH
DMCT	Cl	H	OH	H
MTC	H	—CH_2—		OH
MC	$N(CH_3)_2$	H	H	H

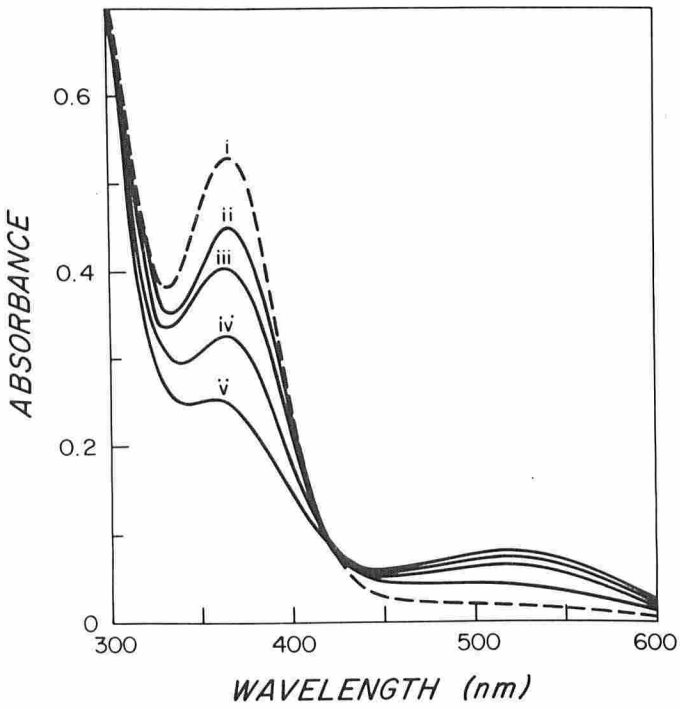


FIG 1. Spectral monitoring of photodegradation of the TCs with increasing UVA radiaton doses (0–40 kJ/m^2). (*i*) initial spectrum; (*ii*), (*iii*), (*iv*), and (*v*) represent spectra after exposure to 5, 10, 20, and 40 kJ/m^2, respectively, of irradiation. Experimental conditions: TCs (40–60μM) in HBSS were irradiated with a 1.0 kW xenon arc lamp set up to emit 320–400 nm waveband irradiation. Spectra were recorded after exposure to the varying doses of radiation.

Photochemical Degradation Rates

The TCs were dissolved in Hanks' buffered salt solution (HBSS) at concentrations of 40–60 μM, at pH 7.0. The solutions were exposed to increasing UVA radiation doses (0–40 kJ/m^2) and their UV-visible spectra recorded after each irradiation (Fig 1). The rate of photodecomposition was evaluated by monitoring the decrease in 365–375 nm absorbance (Fig 2). For determining the effect of protein binding on TC photodegradation, BSA was dissolved in HBSS (2% w/vol) and these solutions mixed with the drug solutions such that the final concentration of the TCs was again 40–60 μM and the bovine serum albumin (BSA) concentration was about 1.2% w/vol. Photolysis rate constants were evaluated as described.

In Vitro Phototoxicity Assay

The in vitro phototoxicity of the TCs to lymphocytes was carried out essentially by the protocol reported by Morison et al [15]. Briefly, human peripheral blood mononuclear cells (PBMNC) were isolated on Ficoll-Hypaque gradients. These cells mixed with drugs (TCs or their PhTCs, typically at concentrations of 20 μg/ml) were incubated at 37°C in an atmosphere of 5% CO_2 for 30 min. Samples were irradiated

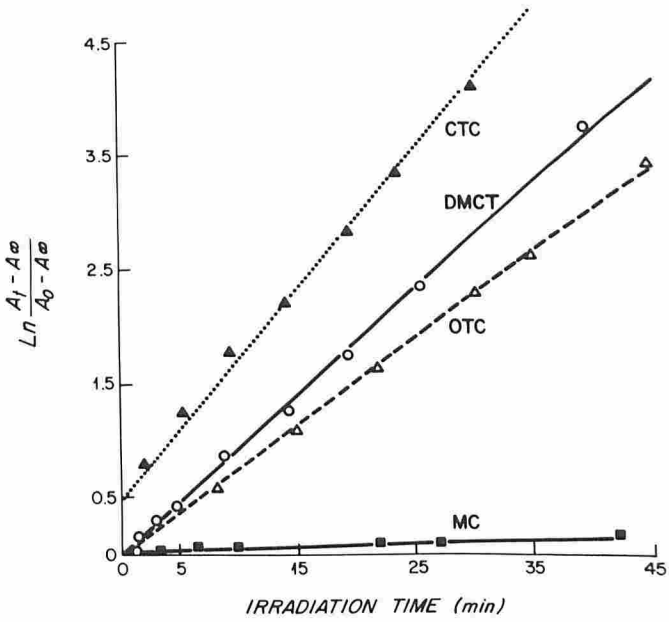


FIG 2. First order plot of the photodegradation of the TCs in HBSS monitored by the decrease in the 365–375 nm absorbance. A_o is the initial absorbance; A_t represents the absorbance at time t, and A_∞ is the limiting value of A_{375} at complete photolysis. The slope of the line gives the first order rate constant. For the sake of clarity, plots for only 4 of 7 compounds are presented.

with 5 doses of UVA, 0.1–8.0 × 10^4 J/m^2, or with the appropriate dose of visible light. (For visible irradiations, samples were cooled in ice.) Cells were then washed with equal volumes of HBSS and centrifuged at 1200 rpm for 10 min. UV-visible spectra were then recorded for the supernatant to determine the amount of TC photodestruction by monitoring the absorbance at 360–375 nm. The pelleted cells were resuspended in complete medium with antibiotics and stimulated by the addition of 0.25 μg/culture of phytohemagglutinin (PHA). After incubation at 37°C in 5% CO_2 for 66 h, 0.5 μCi of [^{3}H]thymidine was added, and the incubation was continued for 6 h. The cells were then harvested using an automated harvester, and the radioactivity measured for each sample. The mean of the radioactive counts per minute of the 3 samples for each condition was calculated. The method of least squares was then used to estimate the radiation dose required to reduce [^{3}H] thymidine incorporation to 50% of that in unirradiated controls. This dose is referred to as the 50% phototoxic dose or PTD_{50}. The viability of the PBMNC was determined by using the trypan blue dye exclusion test. PBMNC cultured with PHA for 72 h after exposure to radiation and drug were harvested and redispersed with a Pasteur pipette. Immediately following redispersion a 50-μl aliquot of cell suspension was mixed with 50 μl of 1% trypan blue dye in saline, and cells that excluded the dye were considered to be viable. The dose of UVA radiation required to reduce the number of viable cells to 50% of the number in unirradiated cultures gave the PVD_{50}.

Oxygen-Dependence Experiments

In order to determine whether or not oxygen is required in the rate-determining step of the phototoxicity response, the lymphocyte phototoxicity assay was performed in an oxygen-depleted atmosphere. Parallel sets of assays were run in which either N_2 or air was bubbled through the samples (cells plus drugs) for 7 min immediately prior to irradiation. Subsequent workup and analyses were identical. The PTD_{50} values under the two experimental conditions were compared.

The involvement of singlet oxygen in the phototoxicity mechanism was evaluated by running parallel sets of lymphocyte phototoxicity assays in deuterated water (D_2O) (Aldrich, 99.8 atom % D) and H_2O. The PTD_{50} in the two series of experiments were compared.

RESULTS

Photochemical Degradation of Tetracyclines

Irradiation of TC solutions in HBSS equilibrated with air changed their electronic absorption spectra, as shown in Fig 3. Typically, the major peak in the 263–268 nm region increased, the major peak in the 365–375 nm region decreased, and a new

TABLE II. *Comparison of the rate constants for the UVA-induced photodegradation of the tetracyclines with their effect on [^{3}H]thymidine incorporation by PBMN cells*

Antibiotic[a]	10^2k_1, min^{-1} ± SD[b]	10^2k^1, min^{-1c} ± SD[b]	PTD_{50}, kJ/m^2 ± SD[b]	PVD_{50}
CTC	11.0 ± 0.7	17.9 ± 1.0	11.6 ± 3.0	25
DMCT	9.6 ± 0.6	22.7 ± 2.0	13.8 ± 6.0	29
DOTC	8.7 ± 0.4	9.5 ± 0.5	5.3 ± 1.2	n.d.
TC	7.0 ± 0.02	2.58 ± 0.02	37 ± 3	n.d.
OTC	6.8 ± 0.7	n.d.	45 ± 12	46
MTC	6.6 ± 0.6	—	19 ± 0.9	25
MC	0.21 ± 0.02	0.21	>80	>80

n.d. = not determined.
[a] [TC] = 5×10^{-5} M; UVA irradiance = 75 W/m^2.
[b] Numbers represent average values from at least 2 determinations.
[c] Photolysis in the presence of 1.2% BSA.

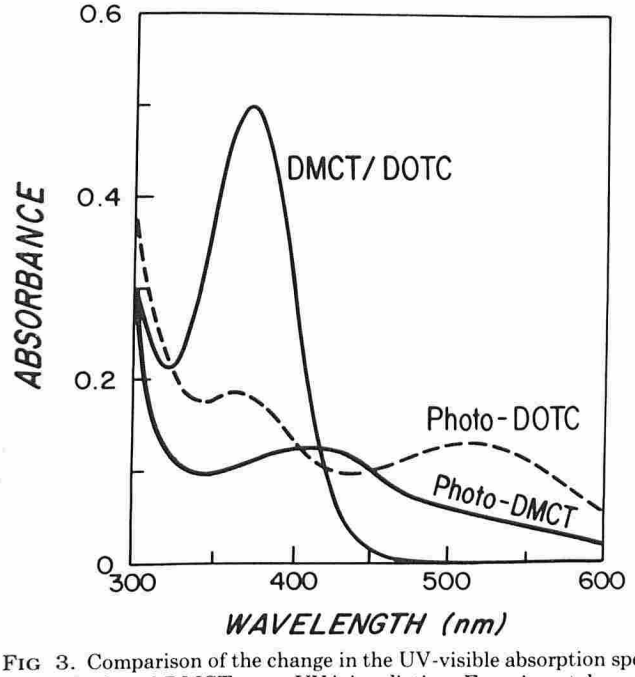


FIG 3. Comparison of the change in the UV-visible absorption spectra of DOTC and DMCT upon UVA irradiation. Experimental conditions identical to those described for Fig 1.

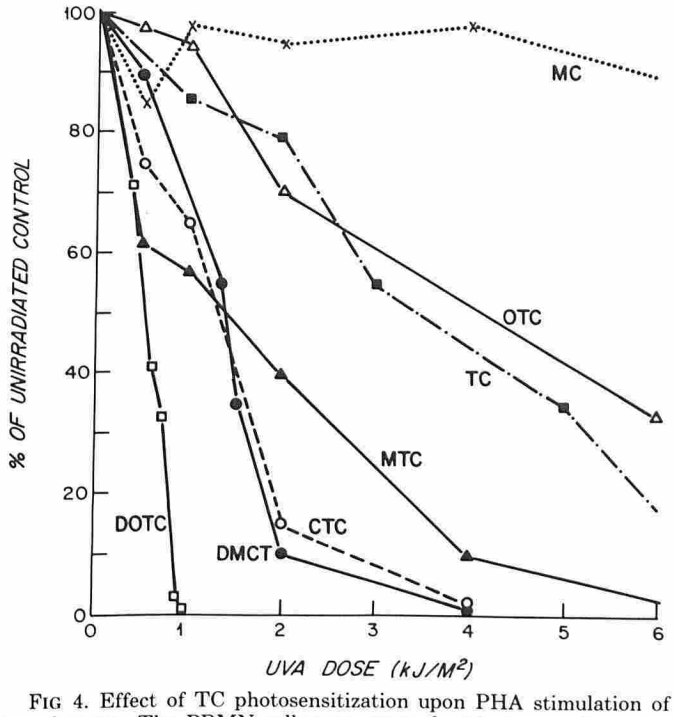


FIG 4. Effect of TC photosensitization upon PHA stimulation of lymphocytes. The PBMN cells were treated with drug and varying doses of UVA and the incorporation of [^{3}H]thymidine measured for each sample. The control was an unirradiated sample of cells treated with drug.

peak at wavelengths >400 nm appeared. The wavelength of maximum absorption for the new peak centered around 530 nm for all the TCs, except the chlorinated compounds (CTC and DMCT) and MC. The major photoproduct under these conditions has been suggested to be a quinone [16]. Photolysis under reducing conditions (in the presence of β-mercaptoethanol), produced a new absorbance peak around 430 nm rather than at 530 nm [10]. The major photoproduct under these conditions is believed to be AHTC.*

For CTC and DMCT the maximum absorption of the new peak is at 410 nm (Fig 3), indicating that different photoproducts are formed from the chlorinated tetracyclines. The MC absorption spectrum is unchanged even after extended photolyses (>200 kJ/m^2), indicating that MC is photostable.

The rate of photodecomposition follows first order kinetics when monitored by the decrease in 365–375 nm absorbance (Fig 2). These rate constants (evaluated from the slope of the straight line in Fig 3) are presented in Table II for the photodecomposition in the presence and absence of added protein (BSA). The salient results are: (i) Photodegradation rate constants vary by a factor of only 1.5 between the 6 TCs, excluding MC. The chloro-substituted TCs photodegraded the fastest; (ii) MC is photostable; (iii) The addition of BSA increases the rate of photolysis of chlorotetracyclines (CTC and DMCT) but not that of the other TCs.

Phototoxicity of Tetracyclines to Lymphocytes

The decrease in mitogen-stimulated incorporation of [^{3}H] thymidine by PBMNC after treatment with drug and varying doses of UVA is presented in Fig 4. The PTD_{50} values for reducing [^{3}H]thymidine incorporation and cell viability are shown in Table II. All of the TCs except MC are photosensitizers. The PTD_{50} values varied by a factor of 8. This contrasts with the factor of 1.5 determined for the relative photolysis ratio and indicates that these two processes may not be related. Chlorotetracyclines (DMCT and CTC), the most rapidly photolyzed compounds, were less phototoxic than doxycycline (DOTC), which was the most potent photosensitizer of the series. In this in vitro assay, MC was the least phototoxic of the TCs.

Because the TCs photodegraded during the phototoxicity assay, it is possible that the photodegradation products contribute to the phototoxicity. Therefore, TC was irradiated with UVA in HBSS to >80% conversion. The photoproduct mixtures were tested for phototoxicity using UVA radiation, and were

* T. Hasan, M. Allen, B.S. Cooperman, manuscript in preparation.

TABLE III. *Comparison of PTD_{50} values for tetracycline and its photoproducts*

Compound	PTD_{50}, kJ/m², ± SD[a]	
	Visible	UVA
TC[b]	122 ± 30	37.0 ± 30
PhTC[b]	55 ± 20	128.0 ± 40
AHTC[c]	2.6 ± 0.5	33.3 ± 4

[a] Average of at least 2 determinations.
[b] [TC material] = 20 μg/ml.
[c] [AHTC] = 4 μg/ml.

found to be less phototoxic than their parent antibiotics by at least a factor of 2 (Table III). However, when the phototoxicity experiments were carried out using visible light (380–600 nm), the photoproducts were about 2 times more potent than the parent drugs (Table III).

We also tested the phototoxicity to lymphocytes of AHTC, a photoproduct of TC under reducing conditions.* AHTC has an absorption maximum at 430 nm and therefore would be expected to be a better sensitizer when excited at wavelengths >400 nm than when UVA excitation is used. When this comparison is made, AHTC is about 12 times as potent with visible light (380–600 nm) as it is when UVA is used (Table III).

Oxygen Dependence of Tetracycline Phototoxicity

The phototoxicity to lymphocytes of TC and DMCT was compared in air-saturated and N_2-enriched samples. The PTD_{50} for the N_2-enriched solutions is 15–17% higher than that for the air-equilibrated solutions, suggesting that oxygen is involved in the photosensitization process. The possibility that singlet oxygen was the reactive species was tested by comparing the phototoxicity to lymphocytes in D_2O and H_2O. The PTD_{50} was determined in D_2O and H_2O with DMCT as the photosensitizer. The PTD_{50} in D_2O was 3 times lower than in H_2O (Fig 5).

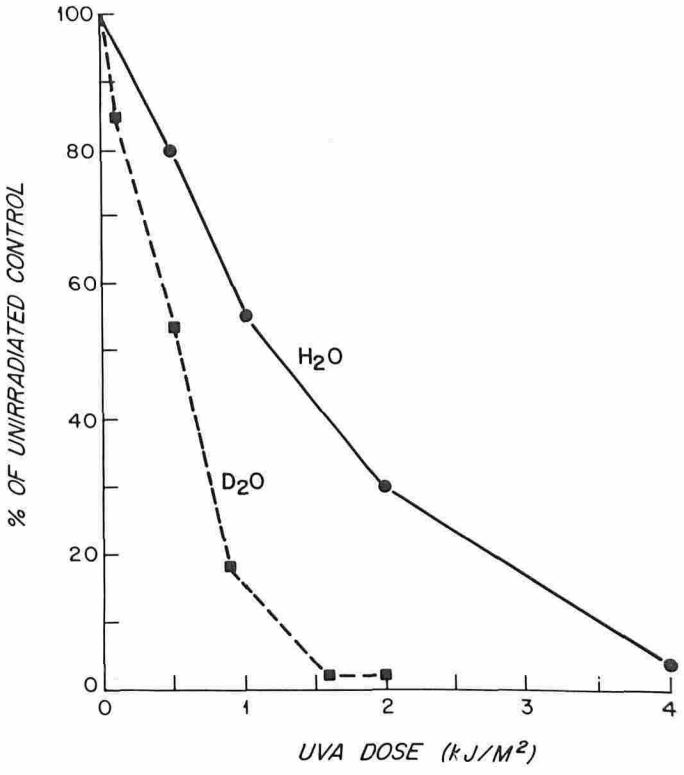


FIG 5. Oxygen dependence of photosensitization by DMCT. The PBMN cells were treated with DMCT and varying doses of UVA in either H_2O or D_2O, and [^{3}H]thymidine incorporation measured for every sample. The control in each case was an unirradiated drug-treated sample of cells.

DISCUSSION

Our first approach to describing the detailed molecular mechanism of TC-induced phototoxicity was to determine whether or not a correlation existed between relative clinical phototoxicity of a series of TCs and in vitro assays. The two assays chosen were: (a) the relative rates of TC photodegradation, and (b) the relative phototoxicity of TC to human lymphocytes. If the relative photodegradation rates reflected the in vivo photosensitizing ability of the series, studies to understand the molecular photochemistry would be pursued. Likewise, if the in vitro assay results paralleled the in vivo response, further studies of the in vitro phototoxicity mechanism would be initiated.

The clinical photosensitizing ability of all the compounds used in this study must be estimated from scattered reports in the literature. It is clear that the members of the TC family most frequently reported to cause photosensitivity are the chloroderivatives, DMCT and CTC [1–7]. In studies of DMCT-, DOTC- and methacycline (MTC)-induced photosensitivity [3,7], DMCT produced photosensitivity in 90–100% of the subjects, DOTC in about 20%, and MTC in about 7%. These variations of in vivo photosensitizing ability with TC structures agree with the trend in relative photodegradation rates we observed (Table II). However, it appears unlikely that the factor of 1.5 difference (excluding MC) between the most rapidly photodegraded compounds (CTC and DMCT) and the least (MTC) can totally explain their significant differences in photosensitizing ability. When the photolysis was performed with BSA present, the rates for CTC and DMCT, but not for the other compounds, were enhanced. This result is consistent with a phototoxicity mechanism involving photoreaction of TCs with ribosomal proteins. Photoaddition of TC to proteins of isolated prokaryotic and eukaryotic ribosomes is known [9,10]. Consequently, photochemical change in the presence of protein may reflect the phototoxicity mechanism.

Comparison of the relative clinical photosensitizing ability with the in vitro PTD_{50} values from our work does not give a strong positive correlation. Consistent with clinical reports, CTC and DMCT appear to be more potent in the in vitro assay than the other derivatives except for DOTC. In this assay, DOTC is the most effective photosensitizer. The high DOTC phototoxicity, although unexpected from clinical reports, is in agreement with the results of Ljunggren and Moller [17]. These workers in their in vivo study of drug phototoxicity by the "mouse tail weight method" found DOTC to be twice as potent as DMCT. Additional factors such as relative lipophilicity of TCs, their absorption in the gut and renal clearance, and their binding to cellular components and serum proteins must be important in determining their photosensitizing ability in vivo. For example, von Wittenau and Yeary [18] determined the binding of a series of TCs to serum proteins in humans and dogs, and determined the relative lipophilicity of the series by measuring their distribution between chloroform and water. Their results show the following order for both lipophilicity and binding to human serum proteins: DOTC > DMCT > TC > OTC. This is the same order as that observed in our phototoxicity assay.

Our results indicate that there is no quantitative correlation between the three aspects of TC phototoxicity discussed above, although there appears to be correlation in the general trend (except for DOTC). CTC and DMCT photodestruct the fastest, are relatively more phototoxic in an in vitro assay, and clinically are most frequently cited as photosensitizers. MC is the most photostable of the series, is the least phototoxic in vitro, and clinically is not reported to be a photosensitizer.

The in vivo action spectrum maximum for DMCT appears to differ from its absorption maximum. Stratigos and Magnus [8] in their study on the action spectrum of DMCT found a

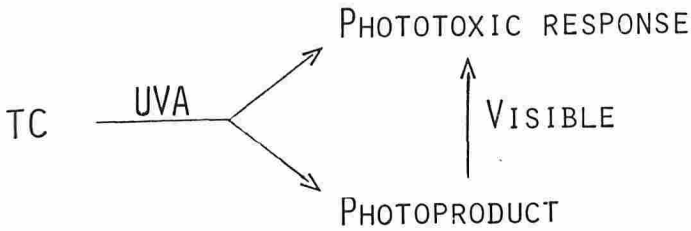


FIG 6. Schematic representation of the possible effect of PhTC on the phototoxic response.

maximal phototoxic effect at 400 nm. They suggested that this maximal phototoxic effect at 400 nm, rather than at the absorption maximum of DMCT at 375 nm, was due to the greater penetration of light through the skin at the longer wavelength. This seems unlikely because the absorptivity of the skin is not significantly different between 375 and 400 nm [19]. Our results, depicted in Fig 6, suggest that the variation between absorption and action spectrum maxima may be due to the intervention of photoproduct(s) that have significant absorptivity at wavelengths ≥ 400 nm (Figs 1, 3). TC photoproducts were more phototoxic than the unphotolyzed drug when visible radiation was employed. This result may indicate that in vivo phototoxicity of TC may be due to at least two main processes: (i) photosensitization by the drug of biologic molecules to cause phototoxicity; (ii) absorption of light by the drug to yield one or more photoproducts. The photoproduct(s) formed then cause photosensitization by absorption of visible radiation. We cannot, however, exclude possibilities such as formation of a skin–drug complex which has spectral characteristics that are different from those of the drug or photosensitization by a TC metabolite which absorbs at wavelengths >400 nm.

Our results indicated that TC photochemistry was different in the presence and absence of oxygen† and that the in vitro phototoxicity was at least partially oxygen-dependent. Singlet oxygen may therefore be involved. All of the 7 clinically used TCs have previously been shown to photosensitize oxidation of the $^1O_2$ acceptors, 2,5-dimethylfuran and limonene [20]. That oxidation by singlet oxygen may be involved in our in vitro lymphocyte phototoxicity assay was indicated by the 3-fold increase in the phototoxic effect observed in D_2O as compared to H_2O (Fig 5). The lifetime of singlet oxygen is 4.2 μsec in H_2O and 55 μsec in D_2O [21]. Consequently, the rates of singlet oxygen-mediated reactions are enhanced in D_2O [11]. For such reactions, up to a 13-fold increase in photooxidation should, in principle, be obtained. However, such large effects will be observed only if the singlet oxygen relaxes mainly by radiationless decay and reacts only with the acceptor molecule. In practice, intermediate values are observed [11]. Singlet oxygen involvement in TC-induced phototoxicity is further supported by recent results of Sandberg et al [22], who demonstrated a 4-fold enhancement of DOTC-sensitized photodamage to neutrophils in D_2O as compared to H_2O.

The subcellular site of TC-photosensitized damage is not known. The initial signs of TC phototoxicity can appear within 2 h of UV exposure if the UV dose rates and drug level are high [1–7]. A short time between exposure and initial phototoxic response is often attributed to membrane damage. Cell membrane damage was also suggested by TC-photosensitized red blood cell lysis [11], membrane alteration in bacteria [13], and photosensitized changes in lecithin films [23]. Oxygen-dependent mechanisms are often associated with membrane damage [24]. However, our results and those in the literature do not exclude other subcellular targets for phototoxicity such as DNA, RNA, ribosomal proteins, and mitochondria. It is conceivable that the photodynamic effect represents the oxidative damage of these targets, in which case direct drug–target interaction is not required.

In summary, in this initial study on the phototoxicity of TC antibiotics we find: (i) there is a general nonquantitative correlation between the clinical phototoxicity, the rates of photochemical degradation, and in vitro phototoxicity; (ii) the photoproduct(s) may contribute to the photosensitizing process and may be responsible for the reported differences between the in vivo action spectrum and the absorption spectrum of DMCT; (iii) the photosensitization is partially oxygen-dependent, and singlet oxygen may be involved.

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