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Research Article

Cytotoxicity Induced by Tetracyclines via Protein Photooxidation

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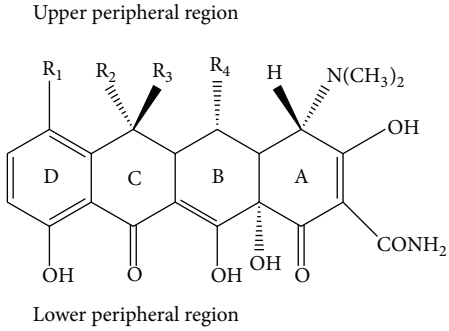
Background. Bacterial ribosomes have been considered the principal targets of tetracyclines. Recently, new clinical data has shown how other biomacromolecules are involved in the cellular damage of bacteria. Researchers are now reconsidering the pharmacological classification of tetracyclines, not only based on their semisynthetic or synthetic generations but also following the new mechanisms of action that are progressively being discovered. *Materials and Methods*. The toxicity properties of seven tetracycline derivatives (tetracycline, oxytetracycline, demeclocycline, chlortetracycline, doxycycline, minocycline, and meclocycline) were investigated *in vitro* using a cell line of human keratinocytes. Cells were irradiated in the presence of tetracyclines for different durations and at three different intensities of light. The investigation of protein oxidation was set up using model proteins to quantify the formation of carbonyl groups. *Results*. After incubation and irradiation with UV light, the viability of keratinocytes was assessed with half the maximal inhibitory concentration for doxycycline, demeclocycline, chlortetracycline, and tetracycline. No phototoxicity was observed for oxytetracycline, meclocycline, and minocycline. *Conclusions*. This study provides evidence that tetracycline's derivatives show different photobehaviour according to their chemical properties due to different reactive groups on the same molecular skeleton.

1. Introduction

Tetracyclines (TCs) are some of the oldest antibiotics still used today. Their safety is well assessed and WHO (World Health Organization) considers them integral members of the essential list of medication for underdeveloped countries [1]. They are relatively safe and are used to treat various infectious diseases [2]. The primary known side effect of TCs is due to their phototoxicity. They increase skin sensitivity to light that can lead to significant discoloration (red and brown spots). Furthermore, the damage can, at times, be permanent and lead to more long-term issues, such as skin cancer [3]. TCs are known to be assimilated in the teeth and bones of young individuals [4]. The aim of this study is to elucidate the mechanisms of toxicity in mammalian cells induced by TCs. It has been postulated that TCs can inhibit cellular growth in bacteria, specifically, by binding to the 30S ribosomal subunit. TCs also change the bacterial membrane integrity and mechanical properties, eventually causing macromolecular dysfunction, cellular lysis, and, inevitably, cellular death [5–9]. TCs have shown profound activity against mammalian mitochondria [10, 11]. The effect of TCs on eukaryotic cell membranes is less famous, and it was thought that the selectivity of TCs as antibiotics was due to their inability to cross mammalian cellular membranes. That notion was dismissed when TCs were found to inhibit the growth of certain mammalian cell lines at concentrations similar to the MIC (Minimum Inhibiting Concentration) values needed to inhibit bacterial growth [12, 13]. It is well-documented that TCs are able to induce photoreactions in human skin and nails [14]. The incidence of skin photosensitivity following treatment with doxycycline and demeclocycline has been reported to be especially high [6]. To our knowledge, there are no clinical reports of light-induced side effects from Minocycline. Phototoxicity *in vivo* is partially oxygen dependent and singlet oxygen is possibly involved [9]. One of the well-studied mechanisms for toxicity in mammalian cells is caused by DNA cleavage due to single and double strand breaks induced by complexes of DNA and photoproducts of TCs [15]. In this study we set

Table 1: Molecular structures of the investigated compounds shown in Scheme 1.

Compound	Prefix	R_1	R_2	R_3	R_4
Oxytetracycline	Oxy	H	OH	OH	OH
Doxycycline	Doxy	H	CH_3	H	OH
Tetracycline	Tetra	H	CH_3	OH	H
Demeclocycline	Demeclo	Cl	H	OH	H
Chlortetracycline	Chloro	Cl	CH_3	OH	H
Meclocycline	Meclo	Cl	$=CH_2$	—	OH
Minocycline	Mino	$N(CH_3)_2$	H	H	H



Scheme 1: Molecular structure of the investigated compounds.

out to determine the role of protein oxidation as another important factor in the phototoxicity mechanism of seven clinically used TCs (Scheme 1 and Table 1). The rates of photochemical degradation and the *in vitro* phototoxicity of the TCs are qualitatively correlated to the clinical phototoxicity [10]. In agreement with other works referenced herein, this paper follows the experimental strategy "from the cell to the biomolecule." The typical TCs, those that act as classic protein-synthesis inhibitors, such as tetracycline, doxycycline, minocycline, and chlortetracycline, exhibit bacteriostatic activity, at least initially in bacteria. Other TCs have been found to be bactericidal, killing bacteria with an atypical mechanism. Atypical TCs are believed to act by disruption of cellular membranes, inhibiting all cellular processes and macromolecular synthesis pathways [16]. Additionally, both typical and atypical TCs have pharmacological effects against eukaryotic cells across multiple cell types; their molecular mechanisms of action are just beginning to be understood. A discussion of the effects of TCs against both bacteria and mammalian cells demonstrate the chemically "promiscuous" nature of the tetracycline molecules, as they can interact with a variety of receptors, both prokaryotic and eukaryotic, to modulate cell processes [16].

2. Materials & Methods

2.1. Chemicals. The investigated compounds belong to the family of TCs (Scheme 1 and Table 1). Tetracycline, oxytetracycline dehydrate, demeclocycline hydrochloride, chlortetracycline hydrochloride, doxycycline hydrochloride, minocycline hydrochloride, and meclocycline sulfosalicylate salt were Sigma-Aldrich products used without further purification. Further purification was not required, as these compounds were purchased with a fluorometric grade [17]. Dimethyl sulfoxide (DMSO) and ethanol (EtOH) were purchased from Fluka and used without further purification. The pH of aqueous solutions was adjusted by Britton buffers in the pH 2–12 range. Bovine serum albumin (BSA) and ribonuclease A (RNase A) were purchased from Sigma-Aldrich (Milano, Italy).

2.2. Cell Strains. Experiments were carried out on an immortalized, nontumorigenic cell line of human keratinocytes (NCTC-2544). The cellular line was grown in Dulbecco's Modified Eagle Medium (DMEM) medium (Sigma-Aldrich), supplemented with 115 units/mL of penicillin G, 115 μg/mL streptomycin, and 10% fetal calf serum (Invitrogen, Milan, Italy). The generation time of NCTC-2544 is approximately 21 h.

2.3. Irradiation Procedure (Light Source). Two HPW 125 Philips lamps, mainly emitting at 365 nm, were used for irradiation experiments. The spectral irradiance of the source was 4.0 mW cm^2 as measured at the sample level by a Cole-Parmer Instrument Company Radiometer (Niles, IL, USA) equipped with a 365-CX sensor.

2.4. Instruments (Spectrophotometer). Absorption spectra were recorded with a Perkin-Elmer Lambda 800 spectrophotometer. Fluorescence emission spectra were measured with a Fluorolog-2 (Spex, F112AI) spectrophotofluorometer.

2.5. Photodynamic Inactivation of Cellular Culture (Cellular Phototoxicity). Phototoxicity experiments were carried out on an immortalized, nontumorigenic cell line of human keratinocytes (NCTC-2544). Cellular line was grown in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich), supplemented with 115 units/mL of penicillin G, 115 ug/mL streptomycin, and 10% fetal calf serum (Invitrogen, Milan, Italy). The generation time of NCTC-2544 is approximately 21 h. Individual wells of a 96-well tissue culture microtiter plate (Falcon; Becton-Dickinson) were inoculated with a complete medium containing NCTC-2544 cells in exponential growth. The plates were incubated at 37°C in a humidified 5% CO_2 incubator for 18 h prior to the experiments. After medium removal, a drug solution, previously prepared in dimethyl sulfoxide and Hank's balanced salt solution (HBSS, pH 7.2), was added to each well and the plates were incubated at 37°C for 30 min and then irradiated. After irradiation, the solution was replaced with the medium, and the plates were incubated for 72 h. After this period, control cells reached a confluence of about 90% and the cell viability was assayed by the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) test [18, 19].

2.6. Protein Oxidation. Solutions of BSA and RNase A (0.5 mg/mL) in phosphate buffer 10 mM were irradiated in the presence of the test compounds for various durations in a quartz cuvette. At different times, an aliquot of

Table 2: log C and log D are data available from the database of http://pubchem.ncbi.nlm.nih.gov according to the Ghose-Crippen method [22, 23]. Serum protein binding and renal clearance data are available from the database of http://www.drugbank.ca/.

Prefix	C log P	K log D	% Serum protein binding	Renal clearance (mL/min)
Oxy	−1.46	−8.00	30	90
Doxy	0.34	−6.84	85	16
Tetra	0.42	−6.37	60	65
Demeclo	0.51	−6.46	75	31
Chloro	0.55	−6.46	55	35
Meclo	0.78	−4.57	—	—
Mino	1.20	−4.57	>90	10

the solution was taken and the degree of protein oxidation was monitored spectrophotometrically, by derivatization with 2,4-dinitrophenylhydrazine (DNPH) [20]. Fluorescence was measured with Fluorolog-2 (Spex, F112AI) spectrophotofluorometer, $\lambda_{\text{excitation}}$ 330 nm and $\lambda_{\text{emission}}$ at 392 nm for BSA and with $\lambda_{\text{excitation}}$ 274 nm and $\lambda_{\text{emission}}$ at 303 nm for RNase A. The rational for protein study via fluorescence quenching is very well-known since the work of Eftink of 1981 [21].

2.7. Statistical Data Analysis. Unless indicated differently, the results are presented as mean ± SEM. The differences between irradiated and nonirradiated sample were analyzed using the two-sided Student's t-test.

2.8. Computational Data Analysis and Method. The partition coefficient (log P) organic phase/aqueous phase was determined using semiempirical software with a computational method according to Ghose-Crippen [22, 23]. The values of log P were then used to calculate the distribution coefficients (log D) using a commercial software (Marvin, Chemaxon Company) for physical chemistry prediction. log D is an important parameter to understand the distribution of drugs in the human body under physiological conditions. The collected data is summarized in Table 2 and is very important to better understand the behaviour of TCs *in vivo* [14, 15].

3. Results

Our approach for elucidating the molecular mechanism of TCs induced phototoxicity was to estimate whether or not a correlation existed between relative clinical phototoxicity of a series of TCs and *in vitro* assay (Scheme 2). The four assays chosen were (a) the relative rates of TCs photodegradation, (b) the relative phototoxicity of TCs to human keratinocytes, (c) the binding to model proteins (BSA and RNase A), and (d) the photoreaction with the proteins and the relative amount of photooxidation.

3.1. Role of Lipophilicity. The aim of this research was to clarify which TCs were phototoxic and which did not show

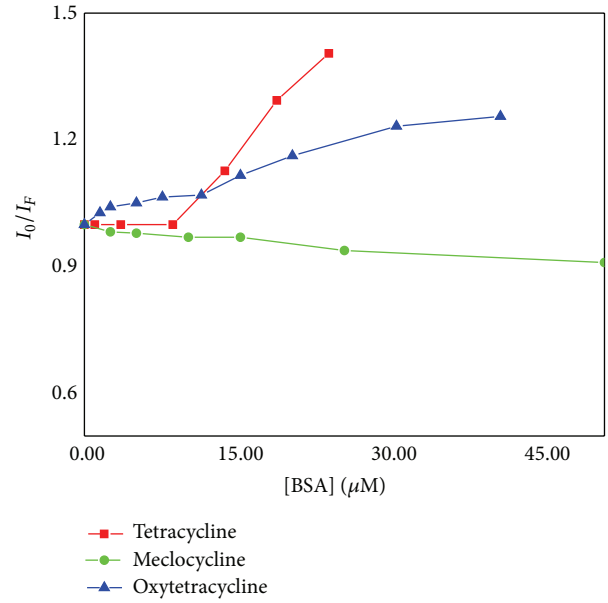
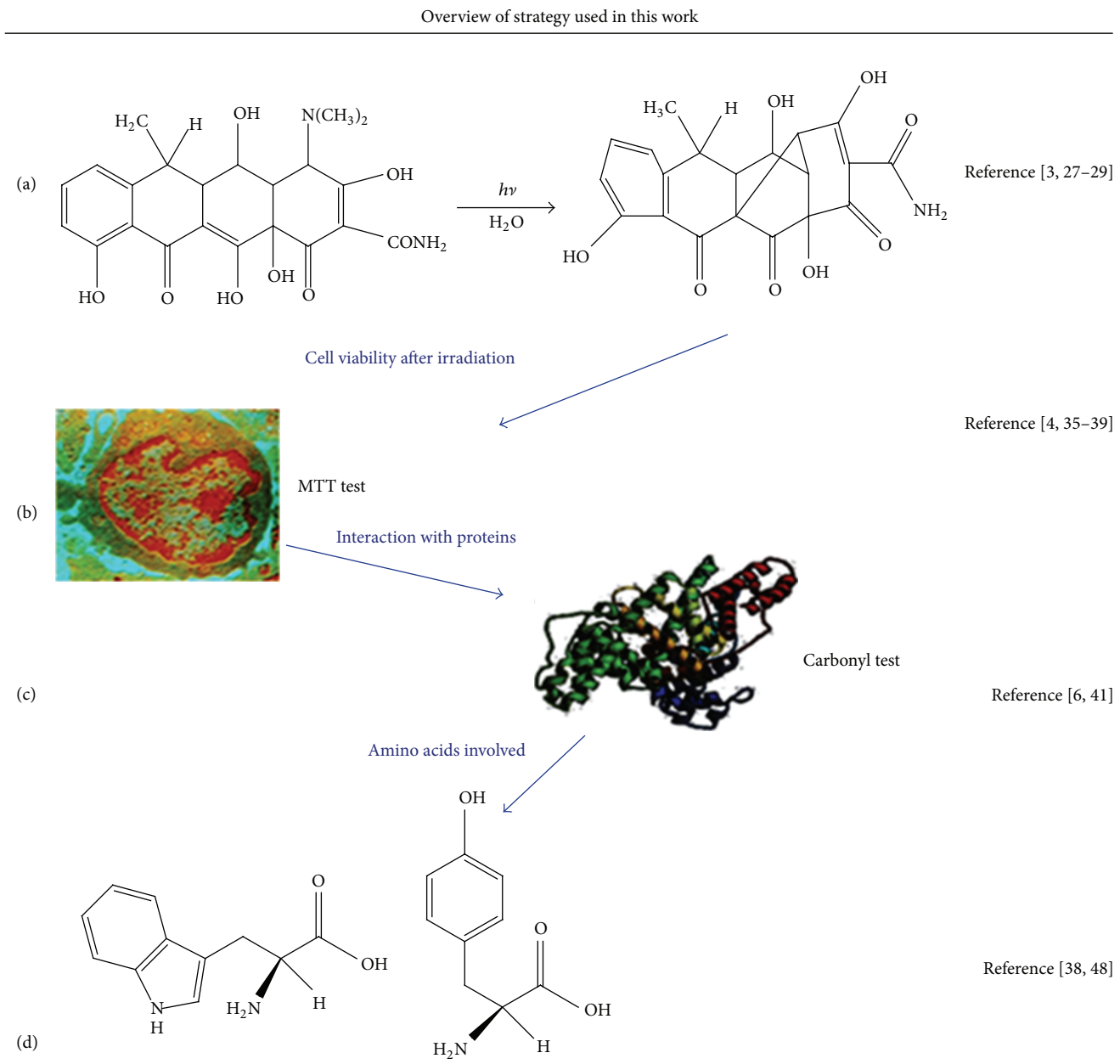


Figure 1: Fluorometric titration of some TCs upon different concentration of BSA. I_F emission with BSA (bound molecule) and I_0 emission without BSA (free molecule). The graph shows that the affinity between the molecules and BSA increases with lipophilicity: tetracycline > meclocycline > oxytetracycline. Data expressed in this graph are in accord with those reported in Table 2.

phototoxicity and to understand the different mechanisms involved in cellular damage. TCs fall within a very similar range of molecular weights, between 444 and 500 Da. However, they differ greatly in their partition coefficients (log P), as well as distribution coefficients (log D), which range from negative to positive values [16, 17, 24]. The degree of lipophilicity was in accordance with the data found in current literature [22]: tetracycline > meclocycline > oxytetracycline (Figure 1). Only data related to photocytotoxicity will be demonstrated and discussed in the following paragraphs. However, in a future paper, currently in preparation, many other physical-chemical measures will be analyzed, namely, in an elaborate table.

3.2. Cytotoxicity and Photocytotoxicity. None of the compounds being investigated have shown a cytotoxic effect in the absence of irradiation. Our results confirm the toxicity of tetracycline, chlortetracycline, doxycycline, and demeclocycline. The relative scale of toxicity magnification ranges from the most to the least toxic (at the maximum irradiation time): doxycycline > chlortetracycline > demeclocycline > tetracycline. Oxytetracycline, meclocycline, and minocycline do not show phototoxicity (Figure 2). The results are in agreement with the clinical data published for these molecules [6] and with other *in vitro* experiments performed on different cellular lines [25, 26]. Specifically, it is possible to observe a trend of toxicity that is similar to the lipophilicity degree, as shown in Table 2. Minocycline is the least phototoxic *in vitro* and clinically. It is not reported to be a photosensitizer



Scheme 2: Strategy of experiment. The four assays chosen were (a) the relative rates of TCs photodegradation, (b) the relative phototoxicity of TCs to human keratinocytes, (c) the binding to model proteins (BSA and RNase A), and (d) the photoreaction with specific amino acids and the relative amount of photo oxidation.

(Figure 2). Doxycycline and chlortetracycline, followed by demeclocycline and tetracycline, are the strongest photosensitizers when tested on normal human keratinocytes.

3.3. Model Protein Binding. Two spectroscopic techniques, fluorescence and fluorescence-quenching, were used to evaluate the binding of TCs with BSA. The fluorescence spectrum of the molecules upon addition of the protein was monitored. In this case, a change in the fluorescence spectrum should be observed for the changed conditions of the bound molecule (Figure 1). The graph in Figure 1 shows that the affinity between the molecules and BSA increases with lipophilicity: tetracycline > meclocycline > oxytetracycline. That rate of affinity shown in Figure 1 is in accordance with the literature data [27–31].

3.4. Photoreaction on Model Proteins (BSA and RNase A). When looking at demeclocycline with BSA, a reduction in the fluorescence peak of 70% at a dose of 15 J/cm^2 of UVA was observed. Contrastingly, with the RNase, a 23% reduction is evident at the same experimental conditions (UVA dose and drug concentration) (Figure 3). For doxycycline and

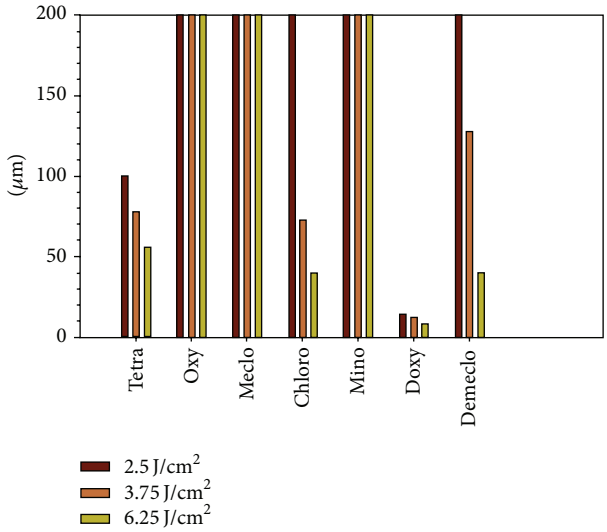


Figure 2: MTT test of photocytotoxicity of seven tetracycline derivatives. Significance: therapeutic agents with an IC_{50} under 10 μM are considered extremely cytotoxic; range between 50 and 100 μM is considered moderately cytotoxic. All TCs studied in this work have noncytotoxic effect on keratinocytes with no light exposure but have toxicity effect in the presence of light. If toxicity is shown upper 200 μM, the compounds are considered safe for their therapeutic purpose.

minocycline with BSA, a reduction in the fluorescence peak of 55% at a dose of 15 J/cm^2 of UVA was observed, whereas, with the RNase, a 19% reduction is evident at the same experimental conditions (UVA dose and drug concentration) (Figure 3). The differences in the photo-behaviour of the proteins can be attributed to the different amino acids on the structure of the proteins (more units of tryptophan in BSA and more units of tyrosine in RNase).

3.5. Determination of the Formation of Carbonyls in Two Model Proteins. The production of carbonyls, and thus the photooxidation induced by demeclocycline, is greater than that caused by doxycycline and minocycline (Figures 4–6). In fact, this difference can be attributed to the activity of photoproducts, especially for doxycycline [32, 33]. Photoreaction in the relationship between the concentration of TCs and induced damage varies from 70% to 20% of maximum fluorescence emission. Literature reports that tryptophan is the most susceptible residue to photooxidation on BSA, while tyrosine is the main photo-damaged residue in RNase A. Results, herein presented, are in perfect accord with the data available in literature [33–35].

3.6. Photodegradation by UVA and UVB Irradiation. It is well known that TCs, when irradiated in solution, photodegrade [36–45]. Particularly in aqueous solution, the most stable of our research compounds is minocycline. However, the medium for cytotoxicity assay is in DMSO and water (50 : 50). Hence, a new trend for degradation was studied in organic medium (Figure 7). To get a preliminary indication of the photolysis, compounds were studied for their absorption spectra after irradiation with increasing doses of UVA (365 nm) and UVB (319 nm). The absorbance after exposure to UV radiation is given by the sum of absorbances of all species present in the solution (Figure 7). Figure 8 shows the kinetics of first-order in which the molecules are photodegraded under UVA condition. The intensity of damage is almost equal for both UVA and UVB (only kinetics plot for UVA are shown in the present paper). Our results suggest that the observed trend of photodegradation is indicative of a scale of toxicity. Under those experimental conditions, we list from the most to the least stable: tetracycline > demeclocycline > chlortetracycline > meclocycline > doxycycline. Except for meclocycline, all the TCs just mentioned are those responsible for the phototoxicity effect on human keratinocytes.

4. Discussion

Despite having the same rigid skeleton, which is founded on four condensate rings, TCs show differences in their physical-chemistry and biological-chemistry [16]. Each TC presents specific chemical groups on the own skeleton, which strongly affect their lipophilicity (Table 2). Those differences in lipophilicity, among the TCs, can explain their reactivity with different biological targets. The lipophilicity also affects oral absorption and the ability to penetrate the blood-brain barrier; only minocycline and doxycycline cross it to a measurable extent [46]. As TCs become more lipophilic, they also become more serum protein bound [28]. This changes their overall bioavailability, maximum detectable concentration, and tetracycline half-life [47]. According to this hypothesis, the role of lipophilicity, pharmacokinetics data was collected to improve our knowledge of the binding characteristics of serum albumin for TCs [28, 48]. These findings, about the biochemistry of TCs, are in agreement with the results of Ljunggren [49]. The correlation of our results, with previous *in vitro* studies using lymphocytes [50] and erythrocytes [51, 52], is fairly good. This experimental data is in agreement with clinical reports and comparative phototoxicity trials in humans [6].

The incidence of photosensitivity reactions to the tetracycline antibiotics varies with the structure of the drug: demeclocycline > tetracycline > minocycline (Figure 2). Chlortetracyclines (demeclocycline and chlortetracycline), the most rapidly photolyzed compounds, are less phototoxic than doxycycline, which is the most potent photosensitizer of the TCs. Because the TCs photodegraded during the phototoxicity assay, it is possible that the photodegradation product contributed to the phototoxicity. Photodegradation is dependent on several factors: concentration of dissolved oxygen, reaction conditions, type of buffer solution, time of irradiation, and presence of antioxidant agents [42]. Meclocycline is a special case since it is the only chlortetracycline derivative without phototoxic effect. In fact, there are no clinical reports available about its phototoxicity. Our approach was to describe the molecular mechanism of TCs induced phototoxicity in order to estimate whether or not a correlation existed between relative clinical phototoxicity of

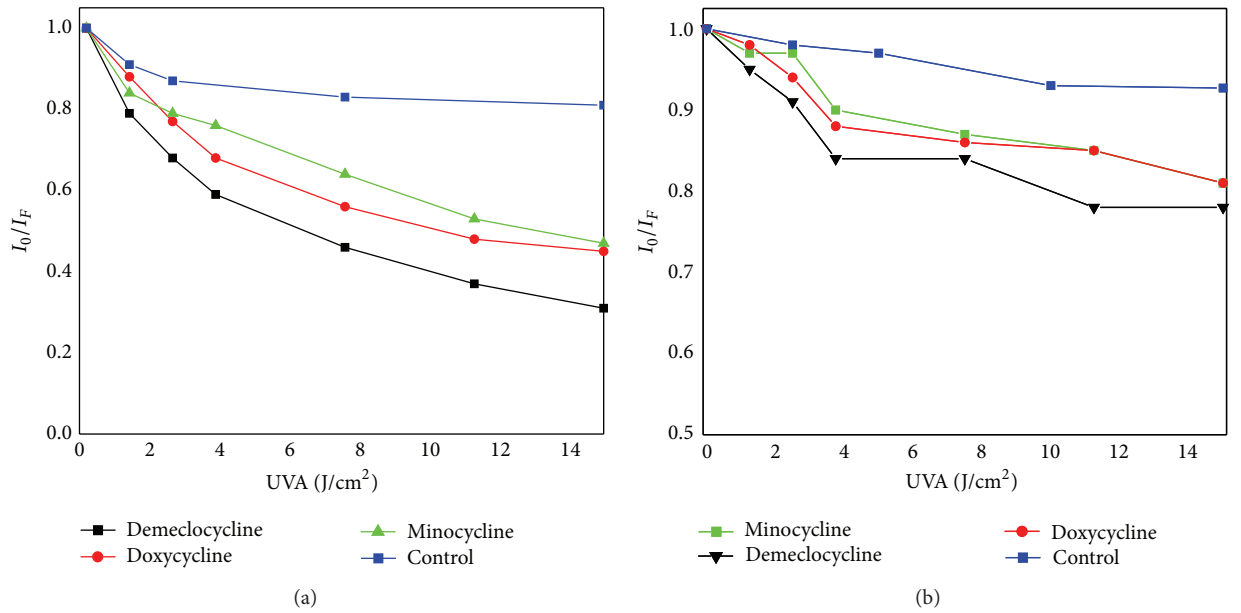


FIGURE 3: Photoreaction of some tetracyclines with same concentration of model proteins at different time of irradiation. On the right, photoreaction upon BSA and on the left upon RNase A. I_F emission with protein (bound molecules) and I_0 emission without protein (free molecule).

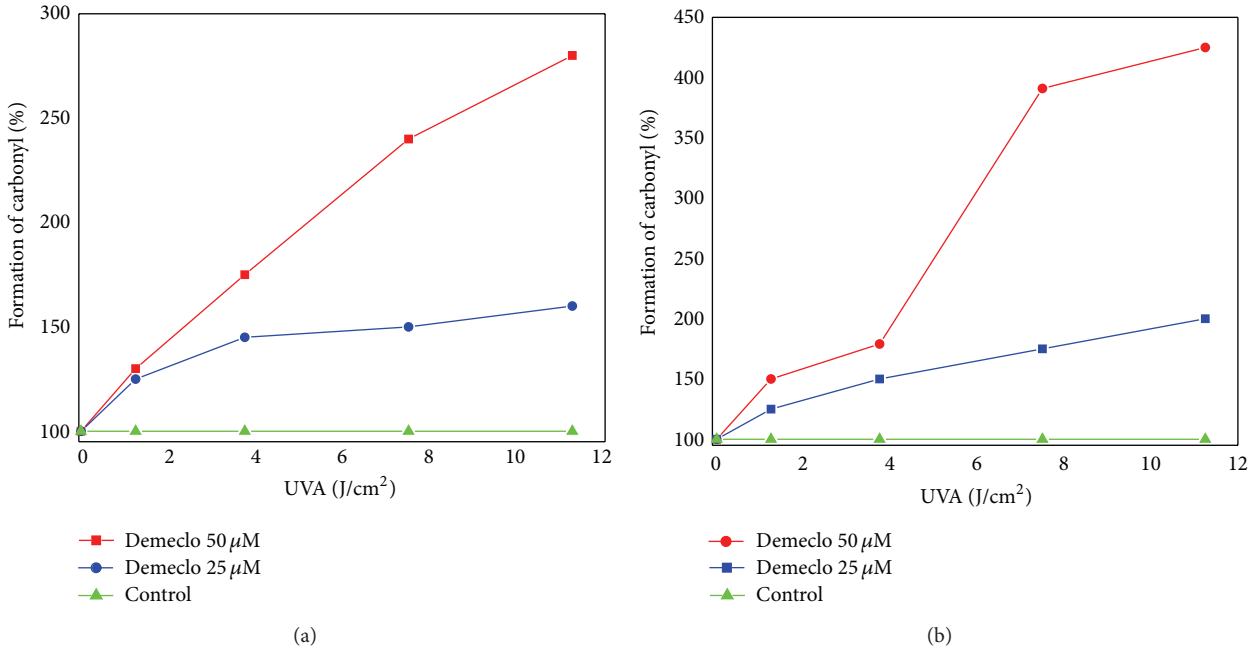


FIGURE 4: Formation of carbonyl of model proteins photo-induced alone (control) and then with different concentrations of demeclocycline. On the right is oxidation effect on BSA and on the left is the same reaction on RNase A.

a series of TCs and an *in vitro* assay. The four assays chosen were (a) the relative rates of TCs photodegradation, (b) the relative phototoxicity of TCs to human keratinocytes, (c) the binding to model proteins (BSA and RNase A), and (d) the photoreaction with the proteins and the relative amount of photooxidation. The clinical photosensitizing ability of all the compounds used in this study was 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 chlortetracyclines (demeclocycline and chlortetracycline). In trial studies, demeclocycline and chlortetracycline-induced photosensitivity were observed in 90%–100% of the subjects and doxycycline-induced photosensitivity in about 20% [6]. The variation of *in vivo* photosensitizing ability

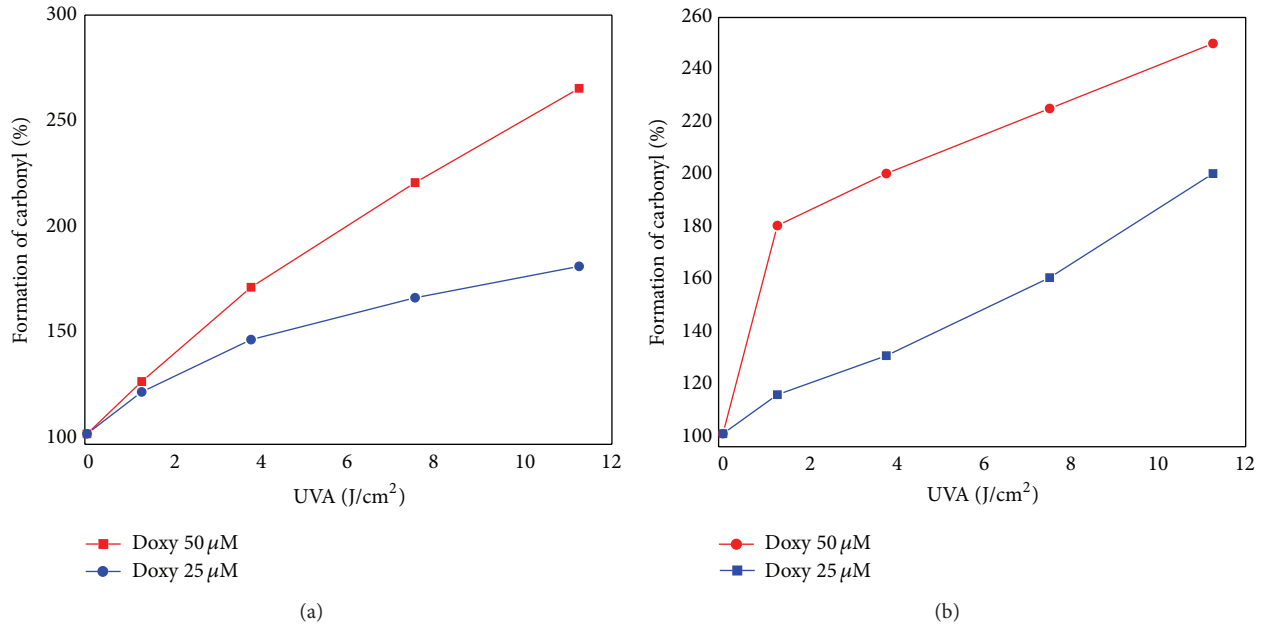


FIGURE 5: Formation of carbonyl of model proteins photo-induced alone (control) and then with different concentrations of doxycycline. On the right is the oxidation effect on BSA and on the left is the same reaction on RNase A.

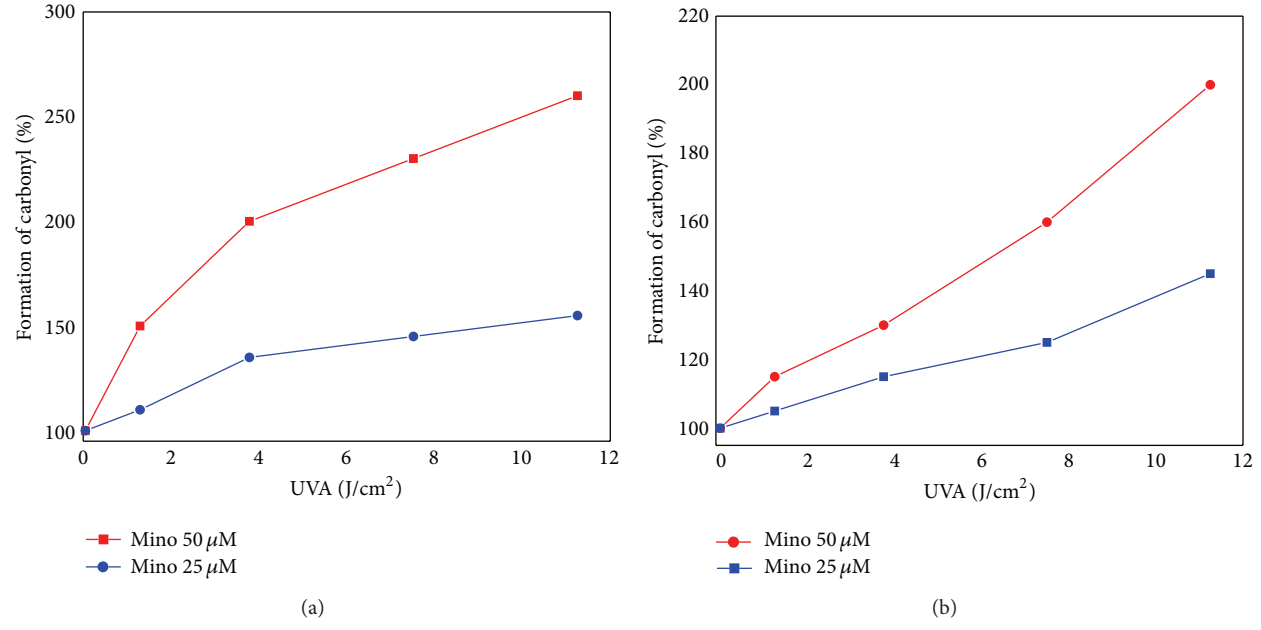


FIGURE 6: Formation of carbonyl of model proteins photo-induced alone (control) and then with different concentration of minocycline. On the right is the oxidation effect on BSA and on the left is the same reaction on RNase A.

agrees with the trend of TCs in relative photodegradation rates that were observed (Figure 8). Indeed, it is possible to deduce that photoproducts contribute significantly to the phototoxic process. In fact, the basis for the reported differences between the *in vivo* action and the *in vitro* behaviour may be in the different structures of relative photoproducts. The contribution of photoproducts of TCs to the mechanism of toxicity has been well known since the 80s [28] and, today, it is even proposed in therapy, as reported by Jiao et al. [53].

To better understand all the factors involved in the mechanism of action of TCs, their interaction with proteins was assessed. Proteins represent 68% of the dry weight of cells and tissues and, therefore, may be accessible targets for different photosensitizers. Photooxidation can induce many changes in a protein: fragmentation, aggregation, oxidation of amino acids, denaturation, changes in its proteolytic susceptibility, alteration of the surface hydrophobicity, and changes in its structure. In this work, the photosensitizing effects of

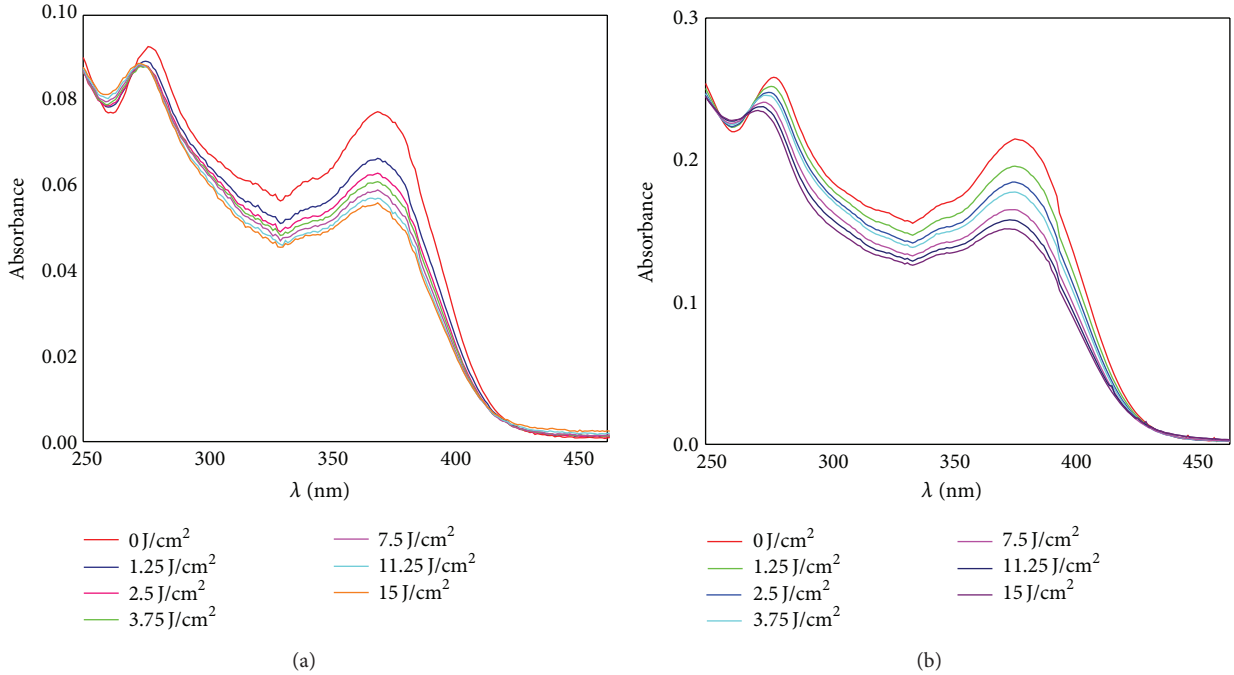


Figure 7: Photodegradation of minocycline dissolved in a mixture of phosphate buffer and DMSO (50 : 50). Graphs show, on the left, exposure at UVA and, on the right, at UVB.

tetracyclines were studied mainly on two models of proteins: bovine serum albumin (BSA, Mw > 60.000 Da, a transport protein, essential to maintaining the osmotic gradient in cell) and bovine ribonuclease A (RNase A, Mw > 14.000 Da, an enzyme capable of hydrolysing the phosphodiester bonds of RNA).

5. Conclusion and Future Perspective

Tetracyclines used at therapeutic concentrations do not have toxicity in mammalian cells, but after UVA/UVB exposure, they show phototoxicity (doxycycline, demeclocycline, chlortetracycline, and tetracycline). This behaviour is caused by their photodegradation products and their reactive nature. The mechanism of cellular damage is associated with an increase of oxidation in biomacromolecules such as albumin (BSA) and RNase A. Not all the tetracyclines, once irradiated, result in phototoxicity. Minocycline is the most photostable compound of this series and does not show phototoxicity. All compounds share the same molecular skeleton composed of four condensed aromatic rings. However, the different reactive groups produce different chemical properties and, in the end, different biological activities. It was determined that tetracyclines have ability to form a new structure with the proteins without irradiation. It was also observed that their affinity for albumin increases with the lipophilicity of tetracyclines. Subsequently, their interaction with proteins following irradiation was studied and then correlated with clinical data. The results of this study will be helpful for all laboratories that are currently developing the next generation of tetracyclines, in order to have the maximum efficacy and fewer side effects. The experiments confirm the literature data and introduce new information about the mechanisms of toxicity in keratinocytes, which proves to be of essential utility to clinical treatments.

Executive Summary

Photobehaviour of Tetracyclines under UVA and UVB Lights. (i) Absorption spectrum of tetracyclines shows different rates of degradation and the same trend under UVA and UVB conditions. These differences are associated to the reactivity of different chemical groups upon the same molecular skeleton.

(ii) Photoaffinity of studied compounds for albumin and others proteins is related to the different lipophilic proprieties shown for the seven tetracyclines derivatives.

Phototoxicity of Seven Tetracyclines Derivatives. (i) Incubation of human keratinocytes for 30 min with decreasing concentration of seven compounds followed by irradiation with blue light (6.25 J/cm^2) was necessary to measure IC_{50}.

(ii) Doxycycline is the most phototoxic compound of the series, followed by demeclocycline, chlortetracycline, and tetracycline. Minocycline is the more photostable compound and has no phototoxicity as well as oxytetracycline and meclocycline.

Protein Oxidation as Primary Target of the Mechanism of Toxicity. (i) Carbonyl assay was performed with two model proteins (BSA and RNase A) to study the entity and quantify the cellular damage via oxidation of specific aromatic amino acids.

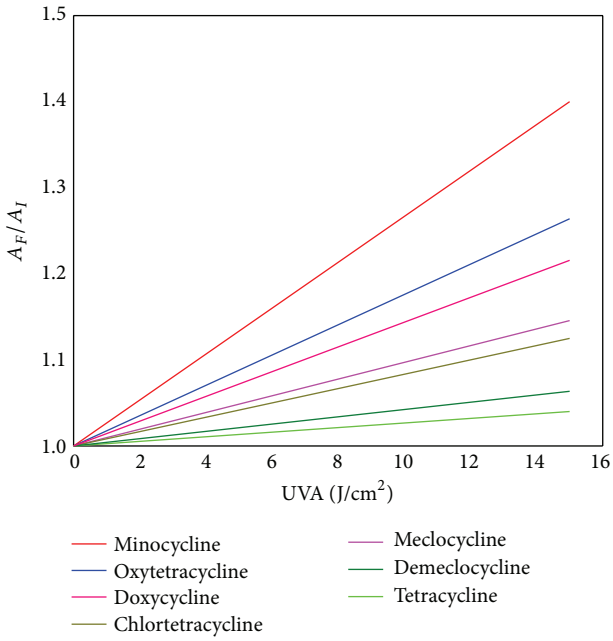


FIGURE 8: Photodegradation of all tetracyclines dissolved in a mixture of phosphate buffer and DMSO (50 : 50). The graph shows the kinetics of first-order in which the molecules are photodegraded (A_F value of absorbance after the solution has absorbed 15 J/cm^2 of irradiation in 30 minutes A_I value of absorbance at 0 J/cm^2).

Conflict of Interests

Author declares no conflict of interests.

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