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Photosensitizing activity of thiocolchicoside: photochemical and *in vitro* phototoxicity studies

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The phototoxic drug thiocolchicoside (2-dimethoxy-2-glucosidoxythiocolchicine, **1**), is photolabile under irradiation with UV-A light from TL 100 W-P Philips bulbs (at λ_{max} 355 nm) light and also with a N_2 laser (at 337 nm) in aerobic and anaerobic conditions. Irradiation of a methanol solution of **1** produces two photoproducts without a glucoside group. One of these lost the methylthio-group, while the other is oxidized (only under aerobic conditions) to sulfoxide. The formation of singlet oxygen by photolysis of **1** was evidenced by trapping with 2,5-dimethylfuran (GC-MS), furfuryl alcohol, 1,3-cyclohexadiene-1,4-diethanoate (HPLC) and by the histidine test as $^1O_2$ scavengers. Thiocolchicoside has been shown to photosensitize the reduction of nitro blue tetrazolium by direct electron transfer mechanism, when irradiated under the same conditions as for photolysis. Oxygen may also be involved in this electron transfer reaction to form the superoxide anion radical. Thiocolchicoside was screened *in vitro* in different concentrations for UV-Vis-induced phototoxic effects in a photohemolysis test, in the presence and absence of different radical scavengers, singlet oxygen and superoxide radical quenchers. In addition, **1** photosensitized the peroxidation of linoleic acid, monitored by the UV-detection of dienic hydroperoxides. Studies on peripheral blood mononuclear cells (lymphocytes) demonstrated phototoxic effects on them. Protection by GSH, DABCO, sodium azide and SOD are indicative of both Type I and II photosensitization pathways mediated by free radicals and singlet molecular oxygen.

1. Introduction

Thiocolchicoside *N*-([5,6,7,9-tetrahydro-1,2-dimethoxy-3-gluocoside-10-(methylthio)-9-oxobenzo[*a*]heptalen-7-yl]-acetamide, **1**) (CAS 2730-71-4), is a phototoxic muscle relaxant drug, which is photolabile under both aerobic and anaerobic conditions. The mechanisms of photosensitization by drugs are being investigated in our laboratory by studying the properties of their excited states, free alkyl-radical, oxygen radical species and singlet oxygen generation that result in model biological systems. In addition to the direct responses to UV-A and UV-B exposure, the human system can be subjected to sunlight-caused effects similar to an exaggerated sunburn or allergy but mediated through an exogenous photosensitizer such as thiocolchicoside [1]. The photooxidation of susceptible biological substrates by singlet molecular oxygen ($^1O_2$) or oxygen radicals as superoxide radical anion ($O_2^{\cdot-}$) pathways is generally believed to lead to the initiation of these adverse responses [2–5].

We examined the photolysis of thiocolchicoside under conditions approximately those encountered in biological systems, namely, neutral, oxygenated media and irradiation with visible light, on erythrocytes, lymphocytes and neutrophils, with the main aims to elucidate the role of reactive oxygen species (singlet oxygen and superoxide radical anion) generated during photolysis, the mechanism pathways of these photoprocesses and the *in vitro* phototoxicity. Nearly all the molecular components of cell membranes are susceptible to sensitized photomodification by free radicals, singlet oxygen and superoxide radical anion. By inference from work in homogeneous solution, five photooxidizable amino acids, tyrosine, tryptophan, histidine, methionine and cysteine, are expected to be particularly vulnerable in membrane proteins to the degree that they are exposed to singlet oxygen [6]. Photoreaction with proteins or with lipids (as constituents of membranes) can lead to cell death, and to pathological diseases such as diabetes, atherosclerosis and neurodegenerative damage. They can trigger an immune response that may eventually lead to allergy reactions. In fact this study has clearly shown that **1** has a phototoxic potential.

The ability of **1** to participate as a sensitizer in several types of photochemical reaction is relevant to the observed clinical phototoxicity and photoallergy of the drug.

2. Investigations and results

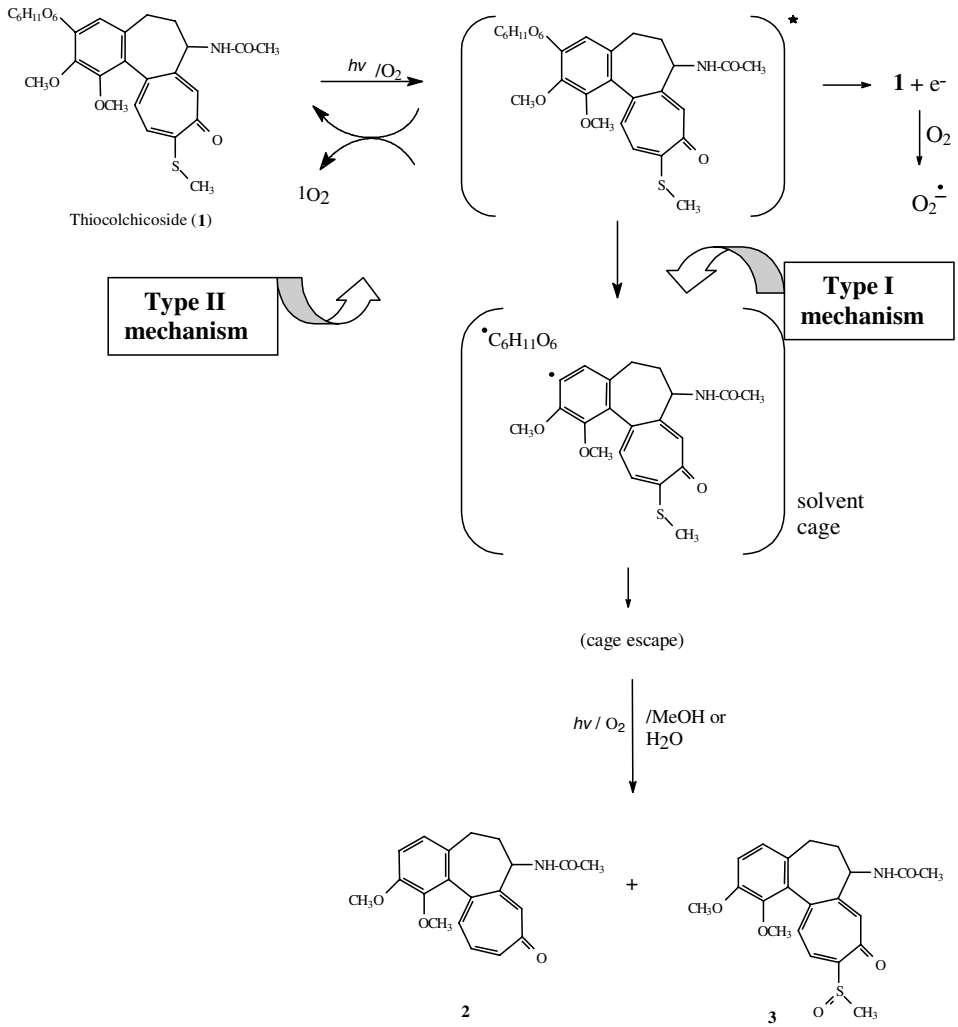
The absorption spectra of thiocolchicoside (**1**) in phosphate buffered saline (PBS) and also in ethanol showed three bands centered at 235, 265 and 368 nm ($\varepsilon = 14000$, 10000 and 8000 $M^{-1}\,cm^{-1}$, respectively) with a tail extending to 450 nm. These spectral characteristics indicate that the drug has the prerequisites to act as an efficient UV-A sensitizer.

As previously stated, the photolysis of **1** was followed by monitoring the disappearance of 235 nm and the decrease at the 368 nm band and the appearance of a increase of the band at 265 nm at 10 min intervals.

Irradiation of a PBS solution of **1** under oxygen atmosphere yields photoproducts **2** (yield 42%) and **3** (yield 58%) and singlet oxygen (Scheme). The quantum yield for thiocolchicoside decomposition was $\Phi = 0.12$. Under argon atmosphere only the photoproduct **2** (yield 60%) was obtained. This result indicate that the loss of the sugar group is attributable to the direct photolysis of the thiocolchicoside. On the other hand, when in a separate experiment, a sample of thiocolchicine was irradiated in the presence of rose bengal and oxygen using a potassium chromate solution (100 mg/l) as a filter allowing $\lambda > 400$ nm and maintaining all other conditions constant, only compound **3** was formed. This reaction indicates that the formation of the sulfoxide **3** in the photolysis of **1** is presumably carried out through an oxidation with singlet oxygen.

The formation of singlet oxygen by the photosensitizing mechanism during the photolysis of **1** was demonstrated

Scheme



by trapping with 2,5-dimethylfuran (DMF) (GC-MS) [7], 1,3-cyclohexadiene-1,4-diethanoate (HPLC) [8] as $^1O_2$ scavengers, furfuryl alcohol (FFA) [9] and by the histidine test [10]. These compounds are substrates for singlet oxygen, and although they are not completely specific, a firm indication of the participation of $^1O_2$ was found. When thiocolchicoside was added to these solutions and kept out of the light, there were no detectable reactions in the dark. Upon irradiation, trapping of singlet oxygen with DMF induced the formation of hexene-2,5-dione (31%), *cis*- and *trans*-3-oxo-1-butenyl acetate (7 and 33%, respectively), and 2-methoxy-5-hydroperoxide-2,5-dimethylfuran (29%), as detected by GC-MS. The measurement with 1,3-cyclohexadiene-1,4-diethanoate (a specific $^1O_2$ trapping chemical) was in water determined with a value of $\Phi^1O_2 = 23\%$ of singlet oxygen formation.

Thiocolchicoside (**1**) was efficient at several concentrations (Fig. 1), for the photooxidation of histidine, which is susceptible to singlet oxygen attack.

The formation of $O_2^{\bullet-}$ was studied by monitoring the photosensitized reduction of nitro blue tetrazolium (NBT). The reduction of nitro blue tetrazolium by $O_2^{\bullet-}$ leads to the formation of a blue-colored nitro blue formazan which can be estimated spectrophotometrically at 560 nm. When NBT in air-satured solution was irradiated in the absence of a photosensitizer, no detectable photoreduction of NBT to diformazan (DF) occurred. With the addition of thiocolchicoside the reduction of NBT could be seen by the appearance of the diformazan at 560 nm. This photosensitized reduction of NBT was more efficient in deoxygenated conditions and quenched in the presence of superoxide dismutase (SOD) (Fig. 2). The quenching of the NBT-reduction was more pronounced as SOD concentrations increased, and its specificity was determined through

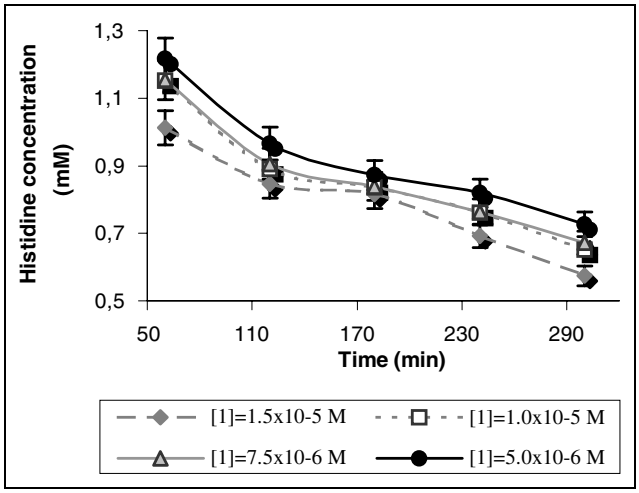


Fig. 1: Histidine test for trapping $^1O_2$ generated by thiocolchicoside. Results are given as mean. Maximum standard deviation was 20%

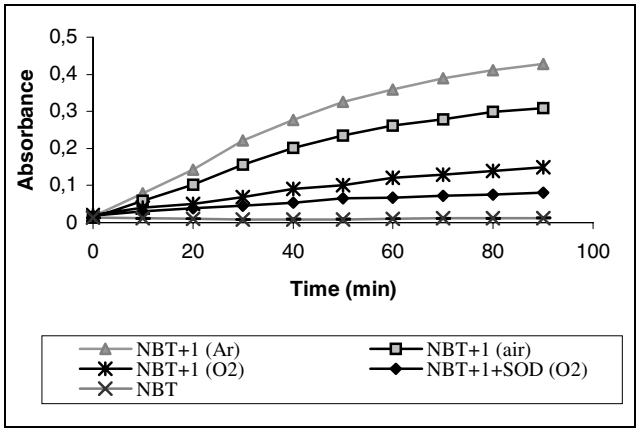


Fig. 2: Photoreduction of NBT (1.0×10^{-5} M) sensitized by thiocolchicoside (**1**) (1.0×10^{-5} M) in PBS solution at 28 °C. [SOD] = 250 units/ml. Values of the standard deviation are omitted for clarity. The standard error of the mean (SEM) was always less than 7%

its inactivation by pre-heating the solution at 42 °C for 30 min (data not shown). The reduction of NBT photosensitized by **1** may possibly be through a direct reaction (direct electron transfer) from the excited state of the sensitizer (**1***) to NBT, but could also involve an initial expulsion of one electron from the photosensitized molecule to the medium and subsequent reaction of the solvated electron with NBT. The partial inhibition of the NBT-photoreduction by SOD suggests that $O_2^{\cdot-}$ could be involved as an intermediate when oxygen is present in the reaction [11, 12].

The reduction of NBT to the formazan by $O_2^{\cdot-}$ through the formation of the cation radical intermediate ($NBT^{\cdot+}$) is a determinant step in the detection of the superoxide anion generated by thiocolchicoside (**1**). This process can be summarised as follows:

$$\mathbf{1} \rightarrow \mathbf{1}^* \rightarrow \mathbf{1} + e^- \xrightarrow{O_2} O_2^{\cdot-} \quad (1)$$

$$NBT + O_2^{\cdot-} \rightarrow NBT^{\cdot+} + O_2 \quad (2)$$

$$2NBT^+ \xrightarrow{H^+} DF \quad (3)$$

All these results implicate electron transfer from the excited states of thiocolchicoside.

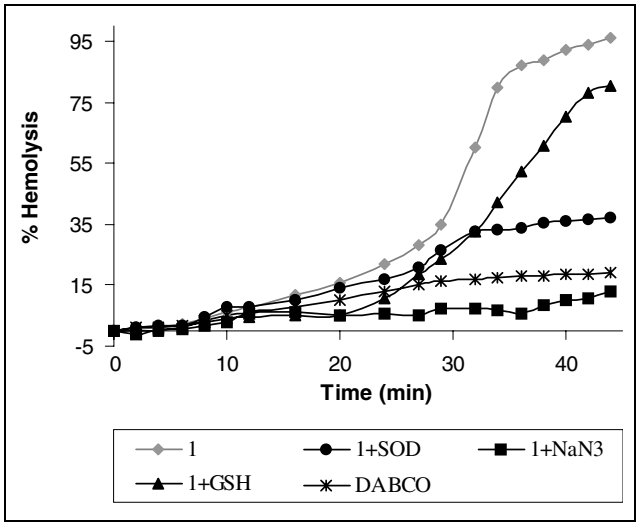


Fig. 3: Photohemolysis of RBC sensitized by **1** (5.3×10^{-5} M) in absence and presence of radical scavengers (GSH, 1.5×10^{-4} M), singlet oxygen quenchers (NAN_3 and DABCO 1.5×10^{-4} M) and superoxide scavenger (SOD, 1.5×10^{-6} M)

Thiocolchicoside (**1**) was able to induce photohemolysis on human erythrocytes (Fig. 3). No lysis was observed when cells were irradiated for 50 min in the absence of **1** or when red blood cells were incubated with **1** but were not irradiated. In the presence of reduced glutathione (GSH), a well established free radical scavengers [13–15], only 50% of the photohemolysis (35 min) was prevented. In the presence of the singlet oxygen ($^1O_2$) quenchers sodium azide (NaN_3) and 1,4-diazabicyclo[2.2.2]octane (DABCO) the photohemolysis process was reduced by 90 and 85% respectively. And in the presence of superoxide dismutase (SOD), an $O_2^{\cdot-}$ scavenger, photohemolysis was inhibited by 65%. The standard error of the mean (SEM) was always less than 8%. The Table 1 shows the protection factor (PF) for the different concentrations of GSH, NaN_3, DABCO and SOD. The values greater than unity indicate protection, whereas values below unity indicate enhancement of the rate of photohemolysis. The results in the Table show that the rate of photohemolysis decreases in the presence of all four additives, and thus type I and II processes appear to be involved.

When thiocolchicoside (**1**) was irradiated in the presence of a PBS solution of linoleic acid significant amounts of dienic hydroperoxides were formed, as shown not only by the detection of conjugated diene through the appearance of a new UV-absorption band that was followed spectrophotometrically at 233 nm [16, 17] but also by the detection of β-aldehydes, which are one of the end products of lipid peroxidation, using the thiobarbituric acid (TBA) assay [18].

Under the same conditions this test was repeated in the presence of the radical scavenger GSH (1.5×10^{-4} M). The process of photoperoxidation was showed down by only about 50%; while in the presence of the same concentration of NaN_3 and DABCO (singlet oxygen quenchers) the photoinduced lipid peroxidation was inhibited by about 60 and 40% respectively (Fig. 4). In the presence of SOD the inhibition of this process was 55%, while that with GSH was only circa 30%. The SEM was always less than 10%. These results show that singlet oxygen has a similar role to that of superoxide radical anion and this centres on the participation of the thiocolchicoside intermediate radicals, generated during the observed photoin-

Table: Effect of additives on photohemolysis induced by thiocolchicoside (1)[a]

Additive	Concentration, M	PF[b]
GSH	0.20×10^{-4}	0.8 ± 0.1
	0.50×10^{-4}	1.2 ± 0.2
	1.20×10^{-4}	1.6 ± 0.1
	1.50×10^{-4}	2.0 ± 0.1
NaN_3	0.20×10^{-4}	0.9 ± 0.1
	0.50×10^{-4}	2.3 ± 0.2
	1.20×10^{-4}	3.5 ± 0.1
	1.50×10^{-4}	6.0 ± 0.1
DABCO	0.20×10^{-4}	0.7 ± 0.1
	0.50×10^{-4}	2.0 ± 0.2
	1.20×10^{-4}	3.8 ± 0.1
	1.50×10^{-4}	4.8 ± 0.1
SOD	0.20×10^{-6}	0.8 ± 0.1
	0.50×10^{-6}	2.3 ± 0.2
	1.20×10^{-6}	3.0 ± 0.1
	1.50×10^{-6}	3.9 ± 0.1

[a] All the data are the mean ± standard of triplicate experiments

[b] PF = Protection factor measured from the ratio between the t_{50} values obtained with and without additive. Irradiation time = 50 min; [1] = 5.3×10^{-5} M; [RBC] = 3.3×10^6 cell ml^{-1}

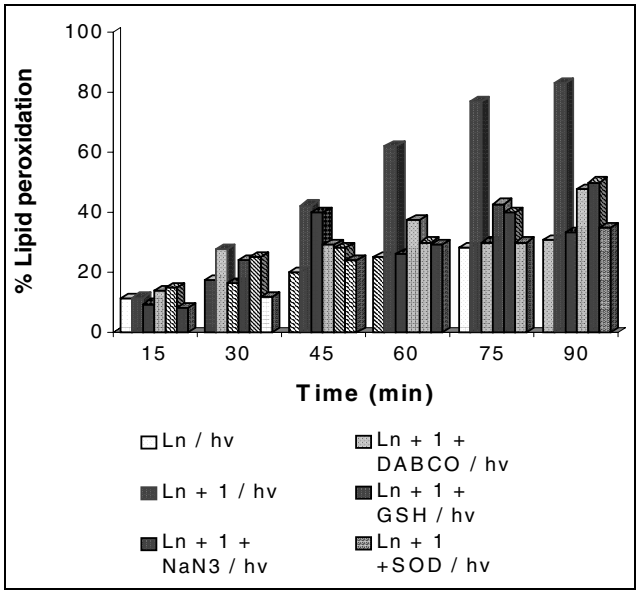


Fig. 4: Photoperoxidation of linoleic acid (1×10^{-3} M) sensitized by thiocolchicoside (1×10^{-5} M) in absence and presence of GSH, NAN_3 and DABCO (1.5×10^{-4} M) and SOD (1.5×10^{-6} M). Results are given as mean of three independent experiments. The SEM was always less than 8%

duced lipid peroxidation. Due to the damaging effects of photoperoxidation on cell membranes, this process is thought to play an important role in skin phototoxicity.

Studies on peripheral blood mononuclear cells (lymphocytes) demonstrated phototoxic effects on them. In addition to the positive phototoxicity demonstrated throughout the test of photohemolysis and lipid peroxidation, the test on mononuclear human cells as lymphocytes showed marked photoxicity. A significant decrease in cell viability was observed up to 240 min of irradiation with UV-A light. Mononuclear cells kept in the dark (with and without thiocolchicoside) were used as controls and no decrease in viability was observed. The number of cells kept in the dark remained constant throughout the experiment (Fig. 5).

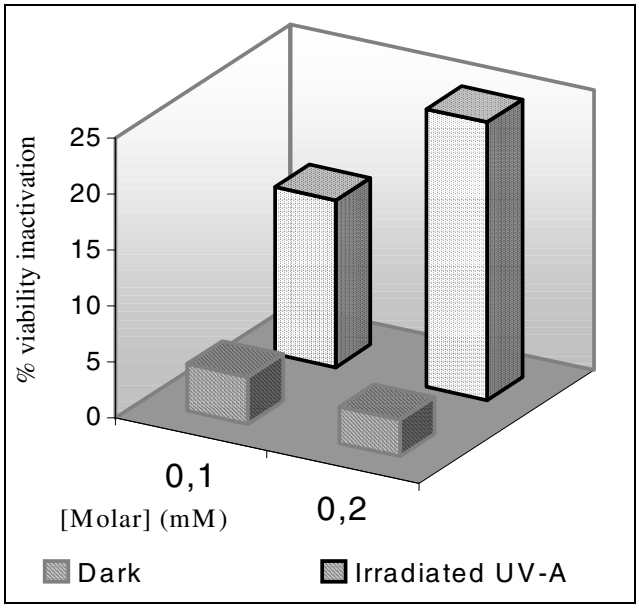


Fig. 5: Photochemical inactivation of lymphocyte viability by thiocolchicoside. Results are given as mean of three independent experiments. The SEM was always less than 8%

3. Discussion

The formation of the photoproducts **2** and **3** is compatible with an initial excitation of thiocolchicoside (**1**) after light absorption, followed in addition to the formation of singlet oxygen, by cleavage of the glucoside – thiocolchicine bond in both cases. Also cleavage of the methylsulfide group, which would give a radical intermediate that could give rise to the compound **2** via hydrogen abstraction, while in the case of the formation of **3** this group is oxidized to sulfoxide.

Our *in vitro* experiments are of great significance as a model for the study of diseases involving photoreactive processes. The photohemolysis induced by thiocolchicoside might reflect extensive photoperoxidation of the membrane lipids. A probable mechanism for these processes, based on a radical chain reaction caused by the oxygen active species photogenerated by **1**, is confirmed by the greater inhibition of the process in the presence of NaN_3 and SOD, (which are well-established singlet oxygen and superoxide radical quenchers) than GSH. These tests certainly correlate with damage produced on cell membrane and therefore with skin phototoxicity.

Two competing pathways of photosensitization namely, singlet oxygen mediated oxidation and electron-transfer mechanisms, are possible when thiocolchicoside is irradiated in aerobic conditions. Triplet thiocolchicoside ^{3}Th* is able to transfer energy to oxygen to form singlet oxygen ($^1O_2$). In some cases, the superoxide anion can be generated by electron transfer from ^{3}Th*. Both oxygen reactive species can be involved in the oxidation of a wide range of important biomolecules. Although the electron-transfer process is probably an important factor in the phototoxic action of **1** in conditions of low oxygen concentration, oxidation by singlet oxygen of biological substrates near the photoexcited molecule (**1***) also has an important role in this process. NBT has been used to detect the formation of superoxide anion radical in this photochemical reaction. The superoxide is formed when molecular oxygen accepts an electron transferred in the reaction. When an adequate electron acceptor is added, the electron transfer is continued, thereby deactivating the superoxide and producing the easily detected reduced product formazan from NBT. SOD will preferentially remove the superoxide and prevent the formation of products from NBT. While the reduction of NBT to formazan generally occurs by electron transfer with oxygen acting as an intermediate, it is apparent that direct electron transfer may occur from the excited state of the thiocolchicoside to NBT in deoxygenated conditions. The relative importance of this pathway will depend on the reactivity of the excited state. The $O_2^{\cdot-}$ generation can be confirmed by using benzoyl peroxide, anthracene or 6-mercaptopurine as reference compounds in the nitro blue tetrazolium reduction reaction [19, 20].

The observed protection by GSH, sodium azide and SOD in the test on RBC, lipid peroxidation and mononuclear cells is indicative of the participation of drug photodegradation and the transient species involved in biosubstrate damage *via* both Type I and Type II mechanisms [15].

The ability of thiocolchicoside to participate as a sensitizer in several types of photochemical reaction is relevant to the observed clinical photosensitivity of the drug. Under relevant biological conditions, thiocolchicoside may generate reactive oxygen species which can probably also arise on DNA. Thus, DNA cleavage by lipid hydroperoxides has been reported on a number of occasions, for ex-

ample in human lymphocytes [21, 22] and the hydroperoxide of linoleic acid has been shown to cause cleavage of pBR322 DNA by an undetermined mechanism [23]. Furthermore, the activity and functionality of neutrophils are severely affected by phototoxicity, for example the inflammatory response. Also, stimulation of neutrophils can induce damage to the bases in DNA [22]. With the determination of $^1O_2$ and $O_2^{\cdot-}$ in the photolysis of **1** and its participation in the observed *in vitro* phototoxicity, we can look forward to a better understanding of the role of singlet oxygen generated in the photolysis of this type of drug in biological systems and their implications for oxidative stress *in vivo*. Oxygen-dependent type II or sensitized reactions, involving the production of reactive oxygens were believed to mediate thiocolchicoside photosensitization reactions.

4. Experimental

4.1. Chemicals

Thiocolchicoside (**1**) was extracted from the commercial drug Coltrax® from Roussel Laboratories, with a soxhlet extractor with aq. methanol and recrystallized from the same solvent. The purity was 99.5% as determined by ^{1}H NMR spectroscopy and UV-vis spectrometry and by comparison with an authentic pure commercial sample. Superoxide dismutase (SOD), reduced glutathione (GSH) and nitro blue tetrazolium chloride monohydrate (NBT) were purchased from Sigma (St. Louis, MI, USA), while sodium azide (NaN_3), 1,4-diazabicyclo[2.2.2]octane (DABCO), histidine, 2,5-dimethylfuran (DMF), and rose bengal were purchased from Aldrich (Steinheim, Germany). A sample of thiocolchicine was generously donated by Hoechst Marion Roussel laboratory (Caracas, Venezuela). All analytical or HPLC grade solvents were obtained from Merck (Darmstadt, Germany).

4.2. Photolysis

Thiocolchicoside (**1**) (Coltrax®, Roussel) was irradiated at room temperature for 72 h in methanol and also in phosphate-buffered saline solution (PBS) pH 7.4 (0.01 M phosphate buffer and 0.135 M NaCl) (0.120 g of **1**, 0.335 mmol in 100 ml) under oxygen and under argon atmosphere, with two TL 100 W-P Philips bulbs, emitting in the range of 305-420 nm, with a emission maximum of 355 nm (25 mW/cm^2 of irradiance) as measured with UVX Digital Radiometer (Melles Griot, USA) after 1 h of continued illumination. Irradiation was also carried out under the same conditions with a Nitrogen Laser with spectral output 337 nm (GL-3300 Photon Technology International, New Jersey, USA) and peak power at 5 Hz of 2.4 MW and 1.45 mJ of energy per pulse. In this case we obtained the same process of photodegradation.

The course of the reaction was followed by UV-vis spectrophotometry using a Milton-Roy 3000 instrument (Milton Roy Company, USA) and by TLC until the thiocolchicoside was completely consumed.

In the determination of quantum yields the photolysis was limited to less than 10% to minimize light absorption and reaction of photoproducts. The photon flux incident on 3 ml of solution in quartz cuvettes of 1 cm optical path was measured by means of a ferric oxalate actinometer and was of the order of 10^{15}–10^{16} quanta s^{-1}.

After irradiation the solvent was evaporated at reduced pressure (14 Torr) at room temperature. Then, the residue was purified by preparative TLC (neutral alumina) using a mixture of methylene chloride/methanol (4 : 2).

4.2.1. Product **2**

M.p. 126–128 °C, yield 0.140 mmol (42%) under oxygen atmosphere and 0.201 mmol (60%) under argon atmosphere, pale yellow crystals (MeOH); was identified as *N*-(5,6,7,9-tetrahydro-1,2-dimethoxy-9-oxobenzo[*a*]heptalen-7-yl)acetamide and its spectroscopic data are as follows: IR (KBr): υ = 3300–3260, 3190, 3110, 3060, 1660, 1540, 840, 770 cm^{-1}. ^{1}H NMR (CD_3OD, 300 MHz): δ = 7.89 (s, 1 H, aromatic-H), 7.86 (d, J = 3.2 Hz, 1 H, aromatic-H), 7.38 (m, 1 H, aromatic-H), 7.35 (d, J = 3.2 Hz, 1 H, aromatic-H), 7.30 (d, J = 4.3 Hz, 1 H, aromatic-H), 7.28 (d, J = 4.3 Hz, 1 H, aromatic-H), 3.89 (s, 3 H. $-OCH_3$), 3.70 (m, 1 H, $-CHN-$), 3.58 (s, 3 H. $-OCH_3$), 2.40 (br s, 1 H, NH), 2.26 (m, 2 H, $-CH_2$), 2.10 (s, 3 H, $-CO-CH_3$), 1.78 (m, 2 H, $-CH_2$). ^{13}C NMR (CD_3OD, 100 MHz): δ = 199 (s, CO), 171 (s, CO), 162 (s, C$-OCH_3$), 160 (s, C$-OCH_3$), 142 (s), 141 (s), 140 (d, CH), 139.5 (s), 139 (s), 138.5 (d, CH), 130 (d, CH), 129.5 (d, aromatic-CH), 125 (d, CH), 114 (d, aromatic-CH), 56.5 (q, OCH_3), 55 (q, OCH_3), 54 (d, CH$-$NH$-$), 29 (t, CH_2), 27 (t, CH_2), 23.5 (q, CO$-$CH_3). MS (70 eV) m/z (%) = 325 (M^+, 10), 324 (M^+-1, 21), 309 (100), 293 (5), 267 (2), 247 (8), 179 (5), 165 (10), 133 (18), 71 (38), 57 (74), 43 (60).

4.2.2. Product 3

M.p. 186-188 °C, yield 0.194 mmol (58%) under oxygen atmosphere, yellow crystals (EtOH); *N*-[5,6,7,9-tetrahydro-1,2-dimethoxy-10-(methylsulfoxy)-9-oxobenzo[*a*]heptalen-7-yl]-acetamide was identified as the main product of the reaction and its spectroscopic data are as follows: IR (KBr): υ = 3310-3280, 3180, 3118, 3060, 1668, 1560, 1060, 860, 785 cm^{-1}. ^{1}H NMR (CD_3OD, 300 MHz): δ = 7.90 (s, 1 H, aromatic-H), 7.88 (d, J = 3 Hz, 1 H, aromatic-H), 7.40 (d, J = 3 Hz, 1 H, aromatic-H), 7.30 (d, J = 4.5 Hz, 1 H, aromatic-H), 7.28 (d, J = 4.5 Hz, 1 H, aromatic-H), 3.91 (s, 3 H. $-OCH_3$), 3.72 (m, 1 H, $-CHN-$), 3.59 (s, 3 H. $-OCH_3$), 2.60 (s, 3 H, SO$-CH_3$), 2.50 (br s, 1 H, NH), 2.26 (m, 2 H, $-CH_2$), 2.18 (s, 3 H, $-CO-CH_3$), 1.76 (m, 2 H, $-CH_2$). ^{13}C NMR (CD_3OD, 100 MHz): δ = 203 (s, CO), 171.2 (s, CO), 162.4 (s, C$-OCH_3$), 159 (s, C$-OCH_3$), 143.5 (s, C$-SOCH_3$), 142.3 (s), 140 (d, CH), 139.7 (s), 139.1 (s), 138.6 (d, CH), 130 (d, CH), 129 (d, aromatic-CH), 125.2 (d, CH), 113.5 (d, aromatic-CH), 56 (q, OCH_3), 55 (q, OCH_3), 53.4 (d, CH$-$NH$-$), 38 (q, $SOCH_3$), 29 (t, CH_2), 27.2 (t, CH_2), 24 (q, CO$-$CH_3). MS (70 eV) m/z (%) = 388 (M^+, 20), 386 (M^+-2, 41), 373 (30), 371 (100), 330 (5), 315 (2), 293 (30), 178 (10), 119 (33), 91 (41), 58 (8), 43 (4), 41 (7).

4.3. Singlet oxygen detection

In a separate experiment irradiation with a Nitrogen Laser with spectral output 337 nm (GL-3300 Photon Technology International, New Jersey, USA) and peak power at 5 Hz of 2.4 MW and 1.45 mJ of energy per pulse, was carried out under the same experimental conditions as the photolysis of **1**, in the presence of 2,5-dimethylfuran (DMF, 5.00 mmol) which is used as a trap for singlet oxygen ($^1O_2$) [7]. This process was followed by GC-MS. The GC-MS analyses were performed using a Carlo Erba/Kratos MS25RFA instrument with a 25-m capillary column of cross-linked 5% phenylmethylsilicone.

Another method for the determination of the quantum yield of singlet oxygen formation developed by Aubry et al. was used with more precision and with smaller uncertainty than other chemical trapping processes. An aqueous solution of sodium 1,3-cyclohexadiene-1,4-diethanoate (10^{-2} M) and thicolchicoside ([**1**] = 0.02 M) was irradiated under oxygen atmosphere in the same conditions as previously described and the determination of the $^1O_2$ quantum yield was carried out by HPLC (Water Delta Prep 4000 equiped with a 3.9 × 300 mm, 10 µm Bondapak C18 column using a CH_3CN/H_2O binary solvent system) by the procedure of Nardello et al. [8].

Singlet oxygen was also detected by trapping with furfuryl alcohol (FFA); this method has been successfully used to detect generated $^1O_2$ in a variety of samples [9]. The comsumption of FFA was followed by HPLC using a 90 : 10 H_2O/CH_3CN mobile phase. The detection wavelength used for monitoring FFA consumption was λ = 222 nm.

Photosensitized degradation of histidine [10] was measured indirectly in the presence of 0.25, 0.50, 1.0 and 1.5 × 10^{-5} M solutions of 1. These solutions were mixed with a equal quantity of L-histidine solution at 0.60 to 0.74 mM in phosphate buffer 0.01, pH 7.4. Samples of this mixture were irradiated with UV-A for periods from 60 to 180 min and the respective controls were maintained protected from light. Histidine was determined by a colorimetric reaction using phosphate buffer, sulfanilic acid, sodium nitrite, sodium carbonate and ethanol as reagents. The absorbance was read on a spectrophotometer at 530 nm against a blank reagent.

4.4. Electron transfer mechanism detection by reduction of NBT

Under the same conditions as for the photolysis of thiocolchicoside ([**1**] = 1.0 × 10^{-5} M), the photoreduction of nitro blue tetrazolium ([NBT] = 1.0 × 10^{-5} M) was followed in argon, air and oxygen atmospheres, and also in the presence of SOD at concentrations of 100, 150, 200, 250 and 300 units/ml, as a function of irradiation time by determining the increase in absorbance at 560 nm due to formation of diformazan product [12, 25].

4.5. Photoinduced hemolysis of RBC by thiocolchicoside

For the photohemolysis experiments, a red blood cell (RBC) suspension from three different samples of freshly obtained human erythrocytes was prepared by washing them four times with a tenfold volume of a phosphate-buffered saline solution (PBS) pH 7.4 (0.01 M phosphate buffer and 0.135 M NaCl), each time centrifuging the cells at 2500 g for 15 min and carefully removing the supernatant. For the photohemolysis experiments the RBC were diluted in PBS containing compound **1** so that the resultant suspension had an optical density (OD) of 0.4–0.8 at 650 nm. An OD value of 0.5 corresponded to 3.3 × 10^6 cell ml^{-1} which was read on a Milton-Roy 3000 spectrophotometer.

The rate of hemolysis and the percentage hemolysis were determined by measuring the decrease in OD at 650 nm, since the optical density is proportional to the number of intact RBC [26]. Thiocolchicoside (**1**) was dissolved in the RBC solution and irradiated in a quartz cuvete (light path of 10 mm) at concentrations of 5.3 × 10^{-5} M under aerobic conditions with a

Nitrogen Laser with spectral output 337 nm (GL-3300 Photon Technology International, New Jersey, USA) and peak power at 5 Hz of 2.4 MW and 1.45 mJ of energy per pulse, for periods ranging between 10–100 min in order to study the photohemolysis effect. Similar experiments were carried out without irradiation and with a preirradiated solution of **1**.

The photohemolysis test was repeated in the presence of reduced gluthatione (GSH) as a radical scavenger, 1,4-diazabicyclo[2.2.2]octane (DABCO) and sodium azide (NaN_3) as singlet oxygen quenchers at concentrations of 0.2, 0.5, 1.20 and 1.5×10^{-4} M and superoxide dismutase (SOD) as an oxygen superoxide scavenger (0.2, 0.5, 1,2 and 1.5×10^{-6} M). The results are expressed as mean ± SEM derived from three observation. The protection factor (PF) was calculated from the ratio between the time needed to produce 50% lysis with and without additive.

4.6. Photosensitized peroxidation of linoleic acid

Linoleic acid 10^{-3} M in PBS was irradiated in the presence of the compound **1** and a pre-irradiated solution of **1** (10^{-5} M), and the formation of dienic hydroperoxides was monitored by UV-spectrophotometry, through the appearance and progressive increase of a new band at $\lambda = 233$ nm [16, 17]. This test was also repeated in the presence of reduced gluthatione (GSH) as a radical scavenger, 1,4-diazabicyclo[2.2.2]octane (DABCO) and sodium azide (NaN_3) as singlet oxygen quenchers and superoxide dismutase (SOD) as an oxygen superoxide scavenger.

The same solution of linoleic acid irradiated with **1** was mixed after the irradiation with 1ml of 0.067% of thiobarbituric acid (TBA) and 0.5 ml 20% trichloroacetic acid and incubated at 100 °C for 20 min. After cooling, the absorbance of the reaction mixture was read at 532 nm. The concentration of TBA-reactive substances was calculated using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} [18].

4.7. Phototoxicity assay on lymphocytes

Peripheral blood mononuclear cells (mostly lymphocytes) have been employed as target cells for *in vitro* phototoxicity testing [27]. The cells were isolated from human blood samples by Fycoll-Hypaque gradient separation. The concentrations of thiocolchicoside were 0.1 and 0.2 mM in PBS, and the irradiation time from 1 to 3 h. Irradiation was carried out with two TL 100 W-P Philips bulbs, emitting in the range of 305–420 nm, with a emission maximum of 355 nm with a radiation dose of 4 J/cm^2 in the presence of lymphocytes (with 9% of monocyte cells) and also of neutrophils. A control experiment was carried out in the dark. Viability was determined by total cell counting and determination of the cell fraction excluding trypan blue. The results were expressed as the mean ± standard error of the mean (SEM) derived from five to six observations. Means were analysed for statistical differences using Student's test.

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