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Study of the photochemical and *in vitro* phototoxicity of chlortalidone [2-chloro-5-(1-hydroxy-3-oxo-1-isoindolinyl)benzene sulfonamide]

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The photodegradation process of the phototoxic diuretic drug chlorthalidone was studied. The products of its photolysis under UV-B were isolated and identified. Chlorthalidone was found to be active when examined by photohemolysis on human erythrocytes, but not in the presence of the isolated photoproducts. Inhibition of the photohemolysis process induced by chlorthalidone on addition of reduced glutathione (GSH) or ascorbic acid suggests the involvement of radicals species. The inhibition with 1,4-diazabycyclo [2.2.2] octane (DABCO), sodium azide (NaN_3) sowie 2,5-dimethylfuran proof the participation of singlet oxygen ($^1O_2$) in this process.

1. Introduction

Phototoxixity and photosensitization reactions are well-known side effects of diuretic drugs, especially sulfonamide derived substances. Several clinical cases of photosensitivity caused by sulfonamides can be found in the literature [1–8]. Other studies using NHIK 3025 cells derived from human carcinoma have demonstrated that antidiabetics and diuretics induce a phototoxic effect [9, 10]. We determined the photochemical and phototoxic properties of the diuretic and antihypertensive drug chlorthalidone. Despite its extensive use and the knowledge about possible photosensitizing side effects, neither photochemistry nor the molecular mechanism of *in vitro* phototoxic effects of this drug have not been investigated. The aim of this study was to eastablish the photolability of chlorthalidone in UV-A (320–380 nm) and UV-B (290–320 nm), the nature of the different photoproducts and to give evidence of the mechanism of *in vitro* phototoxicity, the role of the free radical intermediate and the role of the oxygen and its excited products ($^1O_2$, $O_2^{\cdot -}$ and $^{\cdot}OH$).

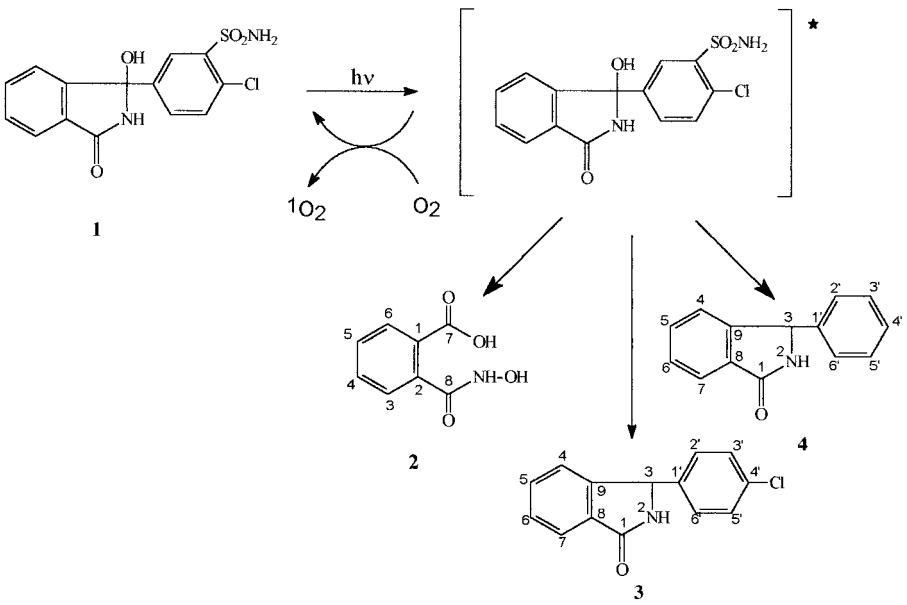
2. Investigations, results and discussion

The phototoxic diuretic drug chlorthalidone (Hygroton®, **1**), a benzenesulfonamide derivative, is photolabile under aerobic conditions and UV-B light. Irradiation of a PBS solution of **1** under oxygen produces the photoproducts **2** to **4** and singlet oxygen (Scheme). Its photolability under UV-B irradiation was followed by the progressive decrease in the main absorption bands at 220 and 280 nm. The isolation of the photoproducts suggests that the photodegradation occurs under radical and oxygen participation.

The formation of singlet oxygen by photolysis of **1** was evidenced by trapping with 2,5-dimethylfuran, which is routinely used as $^1O_2$ scavenger [11–13]. This trapping induced the formation of hexene-2,5-dione (12%) *cis*- and *trans*-3-oxo-1-butenyl acetate (4 and 13%, respectively), and 2-methoxy-5-hydroperoxide-2,5-dimethyl-1,5-dihydrofuran (10%), as determined by GC-MS. In a control experiment performed in the presence of 2,5-dimethylfuran under argon atmosphere no formation of the corresponding products was detected.

Chlorthalidone was screened *in vitro* at different concentrations for UV-Vis-induced phototoxic effects in a photohemolysis test, in the presence and absence of different radical scavengers (reduced glutathione (GSH), vitamin C), singlet oxygen traps (DABCO, NaN_3) and hydroxyl radical quenchers (Fig.). Chlorthalidone shows a photohemolytic effect, not shown by a preirradiated solution of this compound. No photohemolytic effect of the photopro-

Scheme



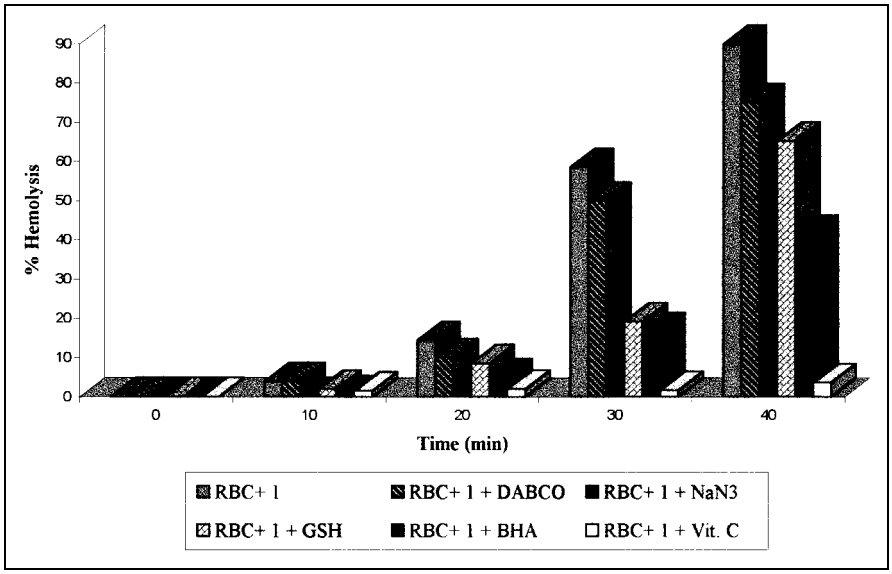


Fig.:
Photohemolysis of RBC (red blood cells) sensitized with 1 (2.5×10^{-4} M) in the presence of oxygen

ducts **2**–**4** was detected. This could be proven when each isolated photoproduct was tested in a photochemolysis experiment. The photohemolysis for **1** was always 28 to 35% higher under aerobic conditions than under argon atmosphere. After addition of GSH, vitamin C, NaN_3, inhibition was highly effective. This suggests the involvement of radicals and a participation of singlet oxygen in the photohemolysis process.

The oxidizing capacity of the radical intermediates is evidently shown by the model studies with GSH, which was converted into the corresponding disulfide. It is well established that the toxicity of many xenobiotics is preceded by depletion of intra and extracellular GSH and vitamin C. The *in vitro* experimental model in the presence of chlorthalidone studied here showed an intrinsisic relation between the phototoxicity of this drug and the antioxidative role of these compounds in the cell, which act as protective agents against drug-induced oxidative stress and free radical-induced damage [14].

On the other hand, chlorthalidone photosensitized the oxidation of histidine (a singlet oxygen scavenger). The photoinduced *in vitro* degradation of this amino acids may be an indicator of the photosensitizing potential of chlorthalidone and of drugs in general [15]. Studies on peripheral blood mononuclear and polymorphonuclear cells (lymphocytes and neutrophils) are in progress.

In a last experiment, the capability of chlorthalidone to act as electron-transferring photosensitizer was tested using NBT as an electron aceptor. Concerning the photochemical reactions under aerobic conditions, the hypothesis is widely accepted that reduction of nitro blue tetrazolium (NBT) to diformazan occurs by electron transfer from superoxide anion that acts as an intermediate. Chlorthalidone has not been shown to photosensitize the reduction of NBT [16]. This suggests that a single electron transfer mechanism is not probably implicated in these processes as well as the possibility of formation of oxygen superoxide [17].

With the intention to reduce the phototoxicity of this drug, a reformulation of chlorthalidone could be recommendable.

3. Experimental

3.1. Chemicals

Chlorthalidone (**1**) (Cas 77-36-1) was extracted from Higroton® (Geigy) in a soxhlet extractor with ethanol and recrystallized from the same solvent. The purity was 99% as determined by elemental analysis (Fisons instruments EA 1108 CHNS-O) and in comparison with a pure commercial sample provided by sigma (St. Louis, MO, USA). Superoxide dismutase (SOD) was purchased from Sigma (St. Louis, MO, USA), while sodium azide (NaN_3), vitamin C, butylated hydroxyanisole (BHA), 1,4-diazabicyclo[2.2.2]octane (DABCO), reduced glutathione, histidine, 2,5-dimethylfuran (DMF) and rose bengal were purchased from Aldrich (Steinheim, Germany). All analytical or HPlC grade solvents were obtained from Merck (Darmstadt, Germany).

3.2. Photolysis

The solution of **1** was irradiated at room temperature for 72 h in methanol (0.120 g, 0.335 mmol in 100 ml) with an Rayonet photochemical reactor equipped with 16 phosphor lamps with a emission between 290–320 nm (23 mW/cm^2 of irradiance) as measured with a model of UVX Digital Radiometer after 1 h of continued illumination, under oxygen atmosphere for 48 h and under argon as well. The course of the reaction was followed by UV-Vis spectrophotometry using a Milton-Roy 3000 instrument, and also by GC and HPLC until the chlorthalidone was completely consumed. After irradiation the solvent was evaporated at reduced pressure (14 Torr) at room temperature and the residue was purified by preparation chromatography (neutral alumina) using methylene chloride/methanol (4 : 2), and the photoproducts were isolated by preparative HPLC. The HPLC system used in all experiments described herein consumed of a Waters Delta Preep 4000 equiped with a 3.9×300 mm, a 10 μm Bondapak C18 column and a CH_3CN/H_2O binary solvent system.

The residue was purified by preparative HPLC. The isolated products **2** to **4** were analyzed by ^{1}H NMR and ^{13}C NMR spectroscopy (Bruker Aspect 3000, 300 MHz), FT IR (Nicolet DX V 5.07) and MS (Carlo Erba/Kratos MS25RFA).

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 incient 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}. For a photolysis of **1** under oxygen atmosphere the quantum yield was $\Phi = 0.12$ with the formation of the photoproduct **2** (yield 12%), the photoproduct 3 (yield 46%) and the photodechlorinated product 4 (yield 42%) (Scheme).

Compound **2** (2-[(hydroxyamino)carbonyl]benzoic acid) was a colorless crystals with a m.p. of 204–206 °C [18], m.p.$_{obs.}$ = 205–207 °C (ethanol). Its showed the following spectroscopic data: IR (KBr): $\nu = 3210$, 3000, 2700, 2500, 1700, 930, 830 cm^{-1}. ^{1}H NMR (CD_3OD, 300 MHz): $\delta = 830$ (m, $J_{6,5} = 8.2$ Hz, $J_{6,4} = 1.3$ Hz, 1 H, CH-6), 8.28 (m, $J_{3,4} = 8.2$ Hz, $J_{3,5} = 1.3$ Hz, 1 H, CH-3), 7.50 (m, $J_{5,6} = 8.2$ Hz, $J_{5,4} = 6.9$ Hz, 1 H, CH-5), 7.48 (m, $J_{4,3} = 8.2$ Hz, $J_{4,5} = 6.9$ Hz, 1 H, CH-4), 3.54 (s, 1 H, −OH), 2.40 (br.s, 1 H, NH). ^{13}C NMR (CD_3OD, 100 MHz): $\delta = 172$ (s, COOH), 169 (s, CONH), 134.1 (d, C-6), 133.6 (d, C-3), 132 (s, C-1), 130.2 (d, C-5), 129.4 (s, C-2), 128.4 (d, C-4). MS: m/z (%) = 181 (15, $M^{+\cdot}$), 164 (10), 163 (100, $M^{+\cdot}$-H_2O), 150 (10, $M^{+\cdot}$-NOH), 149 (80), 135 (8), 104 (12), 92 (14), 77 (20), 41 (12).

Compound **3** (3-(4′-chlorophenyl)-1-isoindolinone) was a white solid with a m.p. = 210 °C [19, 20], m.p.$_{obs.}$ = 210–212 °C (ethanol). IR (KBr): $\nu = 3480$, 3100, 2750, 2400, 1640, 1060, 930, 840, 750 cm^{-1}. ^{1}H NMR (CD_3OD, 300 MHz): $\delta = 7.55$ (m, $J_{7,6} = 7.8$ Hz, $J_{7,5} = 1.0$ Hz, 1 H, CH-7), 7.40 (m, $J_{6,7} = 7.8$ Hz, $J_{6,5} = 5.2$ Hz, 1 H, CH-6), 7.33 (d, 2 H), $J_{3',2'} = J_{5',6'} = 8.1$ Hz, CH-3′ and 5′), 7.28 (d, 2 H, $J_{2',3'} =$, $J_{6',5'} = 8.1$ Hz, CH-2′ and 6′), 7.10 (m, $J_{5,4} = 7.8$ Hz, $J_{5,6} = 5.2$ Hz, 1 H, CH-5), 7.00 (m, $J_{4,5} = 7.8$ Hz, $J_{4,6} = 1.0$ Hz, 1 H, CH-4), 6.40 (s, 1 H, CH-3), 3.20 (br.s,

1 H, NH). ^{13}C NMR (CD_3OD, 100 MHz): $\delta = 168$ (s, CO), 140 (s, C-4′), 135 (s, C-8), 134 (s, C-9), 129 (s, C-1′), 128 (d, CH-7), 126 (d, CH-6), 121 (d, CH-5), 120 (d, CH-4), 119 (d, CH-3′ and 5′), 111 (d, CH-2′ and 6′), 102 (s, CH-3), MS: m/z (%) = 245 (2, M + 2), 244 (16, M + 1), 243 (40, $M^{+\cdot}$), 242 (100, M-1), 208 (5, $M^{+\cdot}$-Cl), 180 (2), 162 (14), 132 (45), 102 (22), 77 (10), 76 (24), 75(18), 50 (5).

Compound **4** (3-phenyl-1-isoindolinone) was a white solid with a m.p. = 218–220°C [21, 22], $m.p._{obs.}$ = 219–220 °C (ethanol). The corresponding data were: IR (KBr): $\nu = 3485$, 3100, 2700, 1580, 1500, 775, 740, 720, 680 cm^{-1}. ^{1}H NMR (CD_3OD, 300 MHz): $\delta = 7.54$ (m, $J_{7,6} = 7.8$ Hz, $J_{7,5} = 1.0$ Hz, 1 H, CH-7), 7.40 (m, $J_{6,7} = 7.8$ Hz, $J_{6,5} = 5.0$ Hz, 1 H, CH-6), 7.32 (m, 2 H, CH-2′ and 6′), 7.23 (m, 2 H, CH-3′ and 5′), 7.11 (m, $J_{5,4} = 7.8$ Hz, $J_{5,6} = 5.0$ Hz, 1 H, CH-5), 7.00 (m, $J_{4,5} = 7.8$ Hz, $J_{4,6} = 1.0$ Hz, 1 H, CH-4), 6.38 (s, 1 H. CH-3), 3.10 (br.s, 1 H, NH). ^{13}C NMR (CD_3OD, 100 MHz): $\delta = 168$ (s, CO), 138 (s, C-8), 135 (s, C-9), 128 (d, CH-7), 127 (d, CH-6), 126.8 (d, CH-5), 127.5 (s, C-1′), 121 (d, CH-2′), 120 (d, CH-3′), 119.6 (d, CH-4), 119 (d, CH-4′), 112 (d, CH-5′), 103 (s, CH-3), 100 (d, CH-6′).

MS: m/z (%) = 209 (30, $M^{+\cdot}$), 208 (100, M-1), 162 (15), 152 (12), 132 (50), 102 (14), 77 (10), 76 (8), 51 (5).

3.3. Singlet oxygen detection

In a separate experiment irradiations were carried out under the same experimental conditions of the photolysis of **1**, in the presence of 2,5-dimethylfuran (2,5-DMF, 5.00 mmol) which is used as a trap for single oxygen ($^1O_2$) [12]. This process was followed by GC and MS.

Rose bengal, a well known $^1O_2$ sensitizer, was used as a standard for comparison with **1** for $^1O_2$ formation, under identical conditions of photolysis.

The irradiation of **1** was also carried out in the presence of 1,4-diazabicyclo[2.2.2]octane (DABCO) and sodium azide (NaN_3) as singlet oxygen quencher, and superoxide dismutase (SOD) as oxygen superoxide scavenger.

Chlorthalidone (**1**) was also irradiated in the presence of rose bengal with an Osram HQL 250 W medium pressure Hg lamp using a potassium chromate solution (100 mg/l) as a filter allowing $\lambda > 400$ nm and maintaining all other conditions. In this way, the reaction of singlet oxygen with **1** can be studied.

3.4. Electron transfer mechanism detection by reduction of NBT

Under the same condition of the photololysis of chlorthalidone ($[\mathbf{1}] = 5.1 \times 10^{-5}$ M), the photoreduction of nitro blue tetrazolium ($[NBT] = 5.1 \times 10^{-5}$ M) was followed in the presence and absence of oxygen, as a function of the irradiation time by determining the increase in absorbance at 560 nm due to the formation of the diformazan product [16, 17].

3.5. Photoinduced hemolysis of red blood cells by chlorthalidone

For the photohemolysis experiments, a red blood cells (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), centrifuging each time the cells at $2500 \times g$ for 15 min and carefully removing the supernatant. For the photohemolysis experiments RBS 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 [23].

The hemolysis rate was determined by measuring the decreasing OD at 650 nm, since the optical density is proportional to the number of intact RBC [23, 24]. Chlorthalidone (**1**) was dissolved in the RBC solution and was irradiated at concentration of 20–80 μg ml^{-1} under aerobic conditions in a Rayonet photochemical reactor equipped with 16 phosphor lamps with an emission between 290–320 nm or alternatively with an Osram HQL 250 Watt medium pressure Hg lamp in a Pyrex inmersion-well photoreactor, for periods ranging between 10–200 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 butylated hydroxyanisole (BHA), reduced gluthatione (GSH) as radical scavenger (10^{-5} M), 1,4-diazabicyclo[2.2.2]octane (DABCO) and sodium azide (NaN_3) as singlet oxygen quencher and vitamin C as antioxydant agent.

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