The photosensitizing activity of lumazine using 2′-deoxyguanosine 5′-monophosphate and HeLa cells as targets†

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Lumazines are an important family of heterocyclic compounds present in biological systems as biosynthetic precursors and/or products of metabolic degradation. Upon UV irradiation, the specific compound called lumazine (pteridine-2,4(1,3*H*)-dione) is able to generate singlet oxygen ($^1O_2$), which is one of the main chemical species responsible for photodynamic effects. To further assess the photosensitizing capability of lumazine (Lum) experiments were performed using the nucleotide 2′-deoxyguanosine 5′-monophosphate (dGMP) and, independently, cervical cancer cells (HeLa cell line) as targets. In the dGMP experiments, the data revealed that dGMP indeed undergoes oxidation/oxygenation photoinduced by Lum. Moreover, dGMP disappearance proceeds through two competing pathways: (1) electron transfer between dGMP and excited-state Lum (Type I process) and (2) reaction of dGMP with $^1O_2$ produced by Lum (Type II process). The multistep processes involved are convoluted and susceptible to changes in experimental conditions. The independent studies with HeLa cells included fluorescence analysis of cell extracts and phototoxicity experiments performed at the single-cell level. Results showed that, upon Lum uptake and irradiation, photodynamic effects occur. In particular, the mitochondria and cell membrane were perturbed, both of which reflect key stages in cell death. The data reported herein illustrate how the irradiation of an endogenous biological compound can have various effects which, depending on the system, can be manifested in different ways.

Introduction

Pteridines in their multiple forms are widespread in biological systems and play different roles ranging from pigments to cofactors for numerous redox and one-carbon transfer reactions.[1,2] Within the pteridine family,[3] pterins are those compounds derived from 2-aminopteridine-4(3*H*)-one (pterin; denoted Ptr) and lumazines are those derived from pteridine-2,4(1,3*H*)-dione (lumazine; denoted Lum).

Lumazine derivatives are present in cells, since 6,7-dimethyl-8-ribityllumazine is the biosynthetic precursor of riboflavin (vitamin B2). Riboflavin is itself the precursor of flavin mononucleotide and flavin adenine dinucleotide, essential cofactors for a wide variety of redox enzymes.[4] Lumazines are also natural products from the metabolic degradation of pterins.[5]

The participation of pterins in photobiological processes has been suggested or demonstrated in the past decades, and interest in the photochemical properties of these compounds has subsequently increased. Whereas the photophysics and photochemistry of pterins have been studied in detail,[6] little is known about the photochemical behaviour of lumazines.[7,8] In addition, although the capability of pterins to photoinduce oxidation of DNA and its components has been demonstrated,[9–11] to the best of our knowledge, no studies dealing with photosensitization of biomolecules by lumazines have been reported. Given that even subtle molecular alterations can evoke substantive changes in photochemical responses and that lumazine is, in itself, biologically important, we therefore set out to investigate several aspects of lumazine-initiated photochemistry.

Lum presents different acid–base equilibria in aqueous solutions. The only relevant equilibrium at physiological pH involves the neutral form and the monoanion (Fig. 1), with a pK_a value of 7.95.[12] The absorption spectra of these acid–base forms, although quite different, have intense bands in the spectral region of 320–400 nm (Fig. 1).

Singlet oxygen ($O_2(^1\Delta_g)$, denoted throughout as $^1O_2$), the lowest electronic excited state of molecular oxygen, is an important oxidizing intermediate in chemical processes and is one of the main species responsible for the damaging effects of light on biological systems (photodynamic effects).[13] The main source of $^1O_2$ production *in vivo* is photosensitization.[14] In a recent article, the capability of Lum to produce $^1O_2$ was reported.[8] In this work, it was demonstrated that Lum efficiently generates $^1O_2$ upon UV irradiation at physiological pH. Moreover, Lum was shown to be a poor $^1O_2$ quencher and that it does not degrade upon irradiation at wavelengths over the range 320–400 nm. Therefore, taking into account these results and the presence of lumazines in living

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† This paper is dedicated to Professor Esther Oliveros on the occasion of her 60th birthday.

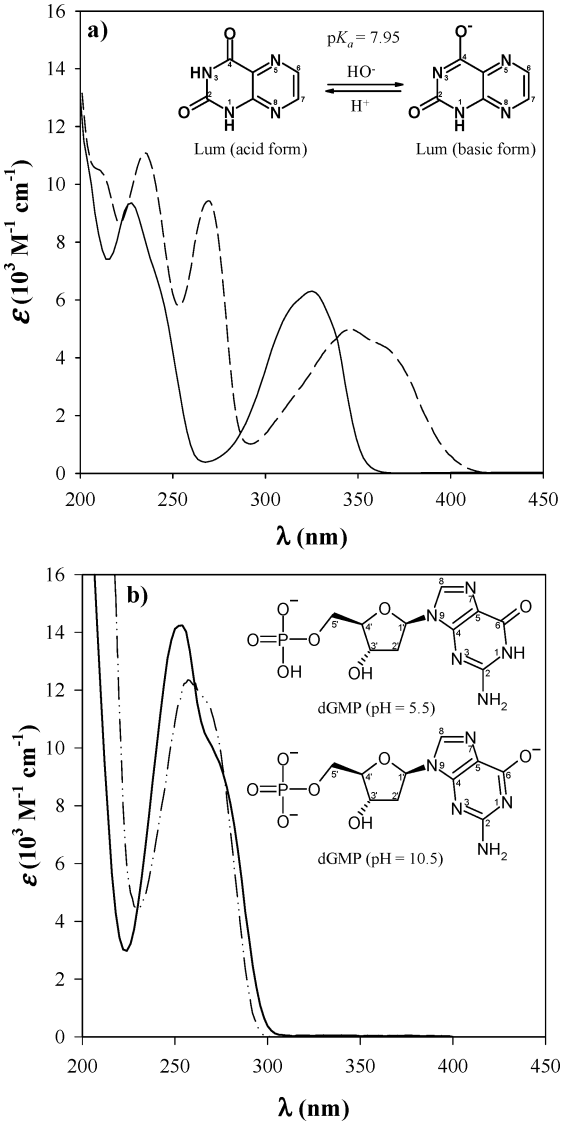

Fig. 1 Molecular structures and absorption spectra in air-equilibrated aqueous solutions of (a) Lum and (b) dGMP. Solid line: pH = 5.5, dashed line: pH = 10.5.

systems, it becomes interesting to study the capability of these compounds to photoinduce damage in biological targets.

To assess the photosensitizing capability of Lum, we first set out to perform experiments using 2′-deoxyguanosine 5′-monophosphate (dGMP) as an oxidizable target. Guanine, the nucleobase of dGMP, is the preferential DNA substrate of photooxidations *via* electron transfer or hydrogen abstraction (type I mechanism)[15] and is the only DNA constituent that significantly reacts with $^1O_2$ (type II mechanism).[16] Therefore, molecules bearing guanine are good substrates to evaluate biologically-relevant photosensitizing properties. In this work, we describe the oxidation of dGMP photosensitized by Lum in aqueous solution under UV radiation and analyze the participation of type I and type II mechanisms in acidic and alkaline media.

Taking into account that $^1O_2$ promotes cell death[17,18] and Lum is a good $^1O_2$ sensitizer,[8] it is logical to expect that this sensitizer has phototoxic properties. However, correlation between the photophysical properties of a given sensitizer and its biological photoactivity is not always simple and depends on many environmental factors.[19] Therefore, irradiation of eukaryotic cell lines in the presence of a given photosensitizer allows one to investigate if photoinduced processes have real effects at a cellular level. As such, in a different series of experiments, we set out to evaluate the cellular uptake and phototoxicity of Lum using cervical cancer cells (HeLa).

Experimental

General

Lum (Shircks Laboratories) and dGMP (Sigma Chemical Co) were of the highest purity available (>98%) and were used without further purification. Superoxide dismutase (SOD) from bovine erythrocytes (lyophilized powder, ≥95% biuret, ≥3000 units per mg protein), rhodamine 123 (Rh123) and trypan blue (TB) were purchased from Sigma Aldrich and used as received.

The pH of aqueous solutions was adjusted by adding drops of 0.1–0.2 M aqueous NaOH or HCl solutions with a micropipette. In all experiments of photosensitization of dGMP (*vide infra*) the ionic strength was *ca.* 10^{-3} M.

Photosensitized oxidation of dGMP

Irradiation. Aqueous solutions containing Lum and dGMP were irradiated in 1 cm path length quartz cells at room temperature with Rayonet RPR lamps (Southern N.E. Ultraviolet Co.) with emission centered at 300 or 350 nm. These differences in irradiation wavelength better facilitate overlap with the respective pH-dependent absorption spectra of Lum (see Fig. 1). In these experiments, spectral discrimination was achieved using filters with bandwidths (fwhm) of ~20 nm. Moreover, a cut-off filter was placed between the lamp and the cell to avoid irradiation below 300 nm, where the nucleotide absorbs (Fig. 1). The experiments were performed in the presence and absence of air. Experiments with air-equilibrated solutions were performed in open quartz cells without bubbling, whereas oxygen-free solutions were obtained by bubbling with Ar gas for 20 min. Oxygen-saturated solutions were obtained by bubbling with oxygen. The acid form of Lum was studied at pH 5.0–5.8, whereas the basic form of Lum was studied at pH 10.2–10.7. The photochemical reactions were followed by UV-vis spectrophotometry and HPLC.

Actinometry. Aberchrome 540 (Aberchromics Ltd.), the anhydride form of the (*E*)-*R*-(2,5-dimethyl-3-furylethylidene)-(isopropylidene)-succinic acid, was used as an actinometer for the measurements of the incident photon flux (P_0) at the excitation wavelength ($P_0^{350} = 6.6 \times 10^{-4}$ einstein L^{-1} min^{-1}, $P_0^{300} = 7.1 \times 10^{-5}$ einstein L^{-1} min^{-1}). The method for the determination of P_0 has been described in detail elsewhere.[20,21] To give a crude estimate of the photon flux absorbed by the sensitizer, P_a, in the respective experiments, we used the following expression:

$$P_a = P_0(1 - 10^{-A}) \quad (1)$$

where A is the absorbance of Lum at 300 or 350 nm.

UV/vis analysis. Electronic absorption spectra were recorded on a Varian Cary-3 spectrophotometer. Measurements were made in quartz cells of 1 cm optical path length. The absorption spectra of the solutions were recorded at regular intervals of

irradiation time. Experimental-difference spectra were obtained by subtracting the spectrum at time $t = 0$ from the subsequent spectra recorded at different times t. Each experimental-difference spectrum was normalized relative to the maximum absorbance value of the absorbance difference, yielding the normalized experimental-difference spectrum.

High-performance liquid chromatography (HPLC). A high-performance liquid chromatograph (Prominence from Shimadzu, solvent delivery module LC-20AT, on-line degasser DGU-20A5, autosampler SIL-20AHT, and UV/vis photodiode array detector SPD-M20A) was used to monitor and quantify the photosensitized reactions and photoproducts. A Pinnacle-II C18 column (250 × 4.6 mm, 5 μm; Restek) was used for product separation, the elution being achieved with a solution containing a mixture of 3% methanol and 97% of a 20 mM potassium phosphate aqueous solution (pH = 5.5). HPLC runs were monitored by UV/vis spectroscopy at different wavelengths (typically at 220, 260, 325 and 340 nm).

Determination of O_2 concentration. The O_2 consumption during irradiation was measured with an O_2-selective electrode (Orion 37-08-99). The solutions and the electrode were placed in a closed glass cell of 130 mL.

Comparison of continuous photolysis in H_2O and D_2O. Solutions of Lum and dGMP were independently prepared in H_2O and D_2O. The solutions containing Lum and dGMP at the same concentration were irradiated under identical experimental conditions. The effect of using D_2O instead of H_2O was evaluated by comparing results of UV/vis spectrophotometric and HPLC analysis.

Detection and quantification of H_2O_2. For the determination of H_2O_2, a Cholesterol Kit (Wiener Laboratorios S.A.I.C.) was used. H_2O_2 was quantified after reaction with 4-aminophenazone and phenol.[22,23] Briefly, 400 μL of irradiated solution were added to 1.8 mL of reagent. The absorbance at 505 nm of the resulting mixture was measured after 30 min at room temperature, using the reagent as a blank. Aqueous H_2O_2 solutions prepared from commercial standards were employed for obtaining the corresponding calibration curves.

Superoxide ($O_2^{\bullet-}$) investigation. Solutions of Lum and dGMP containing SOD (~200 U mL^{-1}) were irradiated. Results of UV/vis spectrophotometric analysis, HPLC, and H_2O_2 determination were compared with those obtained from corresponding solutions lacking SOD.

Experiments with HeLa cells

Cell preparation and culture. The preparation and handling of the HeLa cells used for this study have been described previously.[24] Briefly, cells were grown in a CO_2 incubator at 37 °C in a standard culturing/growth medium (Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and antibiotics) and then plated onto cover slips coated with poly-D-lysine. Experiments on the cells were performed only after a period of 24 h to allow the cells to attach to the cover slips.

For the experiments performed in the presence of sensitizer, 200 μL of Lum solution (either 0.4 or 2.3 mM, depending on the experiment) were added to the cover slips in 2 mL of DMEM. Then the cells were incubated in the CO_2 incubator at 37 °C for 6 h. Finally, the cover slips were washed and kept in a so-called "artificial balanced medium" (ABM) until irradiation. Cell density was typically ~700–1000 cells mm^{-2}.

The ABM is an aqueous solution of 140 mM NaCl, 3.5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgSO_4$, 1.25 mM NaH_2PO_4, 10 mM glucose and HEPES 10 mM (osmolarity = 310 mosmol L^{-1}, pH adjusted to 7.35 at 22 °C using NaOH).

Irradiation. The cover slips with cells in ABM were irradiated at 365 nm (fwhm bandwidth of ~20 nm) for 5 min at room temperature using a Spectroline 4 W lamp (model ENF-240C, Spectronics corporation, NY, USA). The incident flux was enhanced by placing a plano-convex lens ($d = 1''$; $f = 2''$) on the top of the cover slip and a parabolic mirror on the bottom. The resulting incident radiation was about 2 mW cm^{-2}, measured with a digital power meter (fieldMax-TO, Coherent). After irradiation, the medium was changed again to DMEM and the cells were incubated for 2 h at 37 °C. (Cells can not be irradiated when in DMEM due to the presence of a pH indicator that will competitively absorb light.)

Rhodamine 123 assay. The DMEM incubating solution (*vide supra*) was removed, and cells were incubated for 30 min in solutions of ABM (2 mL) containing 20 nM of Rh123, after which the cells were washed with ABM to remove excess Rh123. Fluorescence of Rh123 from the cells was monitored in an imaging experiment (excitation λ of 480 nm, detection λ of 535 nm) (*vide infra*).

Trypan blue exclusion assay. Trypan blue (400 μL of a solution prepared from 0.5 mg Trypan Blue per mL water) was added to the cover slips in 200 μL of ABM. After 2 min, the number of blue stained (dead) and unstained (viable) cells were counted using the light microscope.

Imaging microscopy. An Olympus IX70 inverted microscope was used. Details of the instrumentation used in this study are provided elsewhere.[24–26] Fluorescence imaging experiments were performed by irradiating the cells and their surroundings with a tungsten-halogen lamp (X-Cite series 120, EXFO Photonic Solutions Inc.) using bandpass filters to select the wavelength appropriate for Rh123 excitation (480 nm). Light emitted by the sample was detected through a bandpass filter (535 nm) using a cooled CCD camera (Evolution QEi controlled by ImagePro software, Media Cybernetics) placed at the image plane of the microscope. Bright-field images were recorded using the same CCD camera; back-lighting was achieved with a tungsten lamp.

Preparation of cell extracts. Sets of 12 cover slips in DMEM were incubated in the CO_2 incubator at 37 °C for 6 h in the absence and, independently, in the presence of Lum (40 μM). Cells were washed twice with ABM and, after removing the ABM, the cells were lysed by adding 400 μL of DMSO. After 2 min, the extract in DMSO was placed in a quartz fluorescence cuvette (1 cm path length) containing 2 mL of H_2O.

Fluorescence analysis of cell extracts. Fluorescence analysis of cell extracts was performed using a spectrofluorometer (Fluoromax, Horiba Jobin Yvon). Spectra were recorded over an emission wavelength range of 350 to 600 nm and an excitation wavelength range of 200 to 400 nm.

Results and discussion

Oxidation of dGMP photosensitized by Lum

To ascertain if Lum is able to photoinduce oxidation of dGMP, aqueous solutions containing Lum (50 μM) and the nucleotide (100 μM) were exposed to UV radiation for different periods of time (up to 60 min). To avoid interference between the acid and the basic forms of Lum, the experiments were performed in the pH range 5.0–5.8, where Lum is present at more than 99% in its acid form, and, independently, in the pH range 10.2–10.7, where Lum is present at more than 99% in the basic form. Due to the dependence of the Lum spectrum on the pH (Fig. 1a), acidic solutions were irradiated at 300 nm, whereas alkaline solutions were irradiated at 350 nm. The photochemical reactions were followed by UV/vis spectrophotometry and HPLC.

Significant changes in the absorption spectra of the solutions containing Lum and dGMP were observed upon irradiation in both acidic and alkaline media. The spectral changes registered at pH 5.5 (Fig. 2) revealed that, upon irradiation, the characteristic band assigned to dGMP decreased in intensity, whereas product(s) absorbing at wavelengths longer than 300 nm were formed. Results of experiments performed at pH 10.5 also showed considerable irradiation-induced spectral changes, with a similar strong decrease of the 250–280 nm absorbance assigned to dGMP.

Under the same experimental conditions, the concentration profiles of Lum and dGMP were determined by HPLC (Fig. 3). A decrease of the dGMP concentration was observed as a function of irradiation time, whereas the Lum concentration did not change appreciably in the analyzed time window. The results are consistent with the absorption spectra shown in Fig. 2. These experiments were also repeated under conditions that better approximate those used in experiments on cells (*i.e.* with the addition of 140 mM NaCl and 1.25 mM phosphate buffer), and the results obtained were identical to those shown in Fig. 3.

In the HPLC analysis of the irradiated solutions, compounds other than Lum and dGMP were detected, most of them having shorter retention times than both dGMP and Lum. With the shorter retention times, these compounds should be reasonably polar, most probably because of the incorporation of oxygen into their structures. This would be expected for a photooxygenation reaction of dGMP. Moreover, and as one arguably might expect, there were noticeable differences in the products formed under acidic as opposed to alkaline conditions. To the best of our knowledge, evidence of a lumazine photosensitized oxidation of a nucleotide or other DNA component has not previously been reported.

In control experiments, dGMP (~100 μM) solutions were irradiated in the absence of Lum at 300 and 350 nm. Under both irradiation conditions, no chemical modifications of the nucleotide were detected, thus excluding the possibility that spurious effects of direct light absorption by dGMP could give rise to the observed data. In another set of control experiments, dGMP degradation was not observed in solutions of both Lum and dGMP that were kept in the dark. This excludes the possibility that the data in Fig. 2 and 3 reflect thermal reactions between Lum and dGMP. These experiments were carried out under different experimental conditions (concentration, pH, and time).

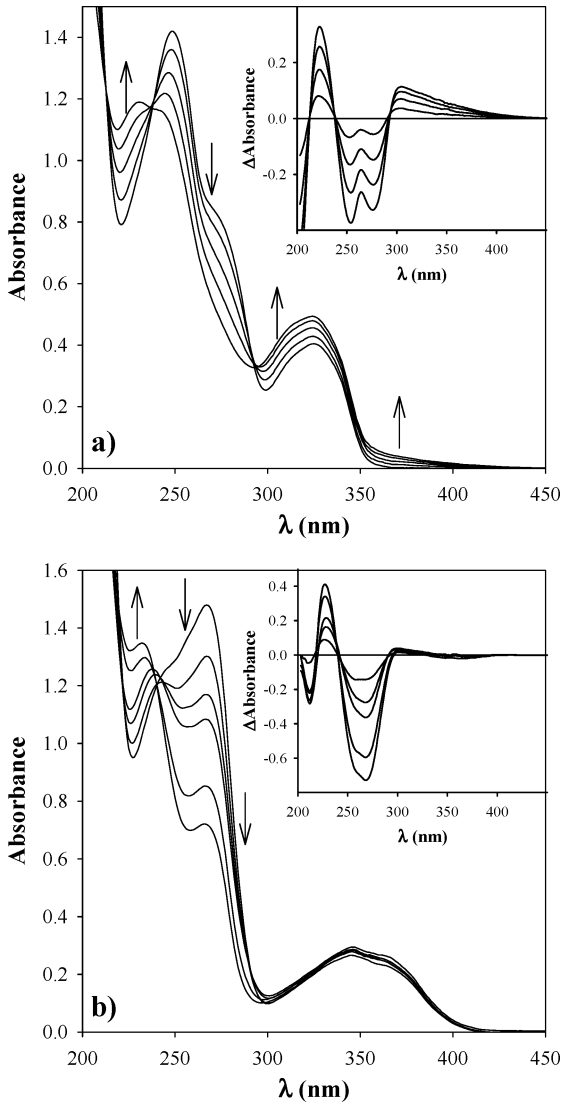

Fig. 2 Time evolution of the absorption spectra of air-equilibrated solutions of dGMP (100 μM) irradiated in the presence of Lum (50 μM). (a) pH = 5.5, spectra were recorded every 15 min; (b) pH = 10.5, spectra were recorded at 0, 5, 10, 15, 30 and 45 min. Arrows indicate the changes observed at different wavelengths, optical path length = 1 cm. Insets: Experimental-difference spectra.

To better elucidate the role of O_2 in these photoinduced processes, Ar-saturated solutions containing Lum (50 μM) and dGMP (100 μM) were irradiated. After 60 min of irradiation, significant changes in the absorption spectra of the solutions were not observed under both acidic (pH 5.5) and alkaline (pH 10.5) conditions. HPLC measurements showed that, in the experiments lacking oxygen, the dGMP concentration did not decrease. Accordingly, no photoproducts were detected.

The evolution of the O_2 concentration during the irradiation of air-equilibrated solutions containing dGMP and Lum was monitored using an oxygen electrode in a closed cell. In both acidic and alkaline media, the O_2 concentration decreased as a function of irradiation time (Fig. 4). Control experiments in the absence of dGMP were performed in order to check the consumption of O_2 resulting from the photolysis of Lum itself.[8] Under both pH

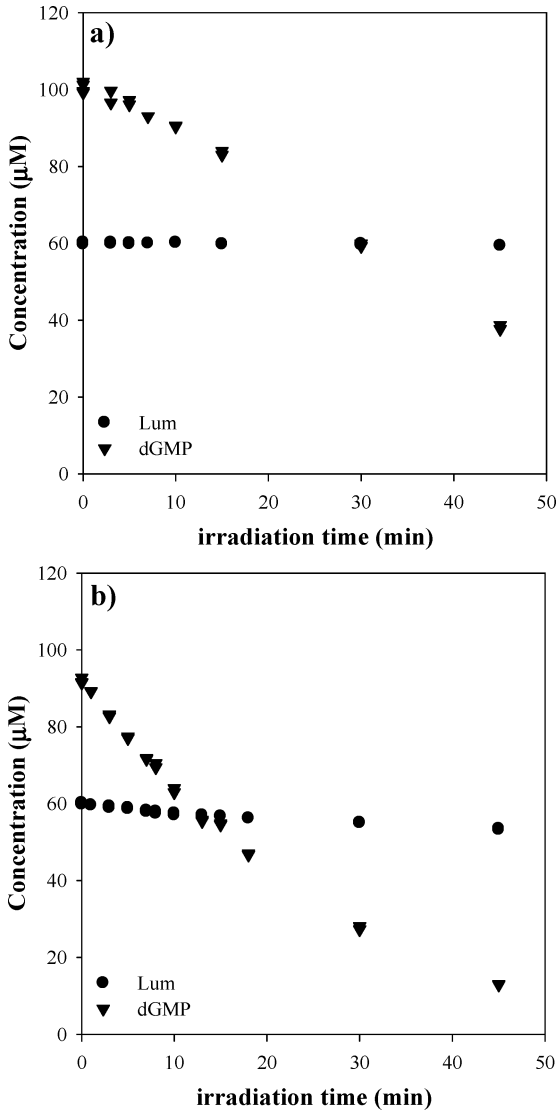

Fig. 3 Evolution of the dGMP and Lum concentrations in air-equilibrated aqueous solutions as a function of elapsed UV irradiation time (concentrations were determined by HPLC analysis): (a) pH = 5.5, λ_{exc} = 300 nm; (b) pH = 10.5, λ_{exc} = 350 nm.

conditions, the decrease of the O_2 concentration was negligible in comparison with that observed in the presence of dGMP (Fig. 4).

The results presented thus far strongly suggest that the photoinitiated Lum-dependent processes that result in dGMP consumption involve oxygenation/oxidation reactions, both at pH 5.5 and 10.5. Although these results are compatible with both type I and type II mechanisms, they do not allow one to assess the relative contribution of each mechanism under acidic or alkaline conditions. We thus set out to address this latter issue by performing a number of exploratory experiments.

Taking into account previously reported values of the rate constant of the chemical reaction (k_r) between $^1O_2$ and dGMP,[11] we can provide a crude estimate for the expected rate of dGMP disappearance if the process depended only on a $^1O_2$ reaction. Eqn (2) expresses the rate of the chemical reaction between $^1O_2$ and dGMP:

$$d[dGMP]/dt = -k_r[^1O_2][dGMP] \quad (2)$$

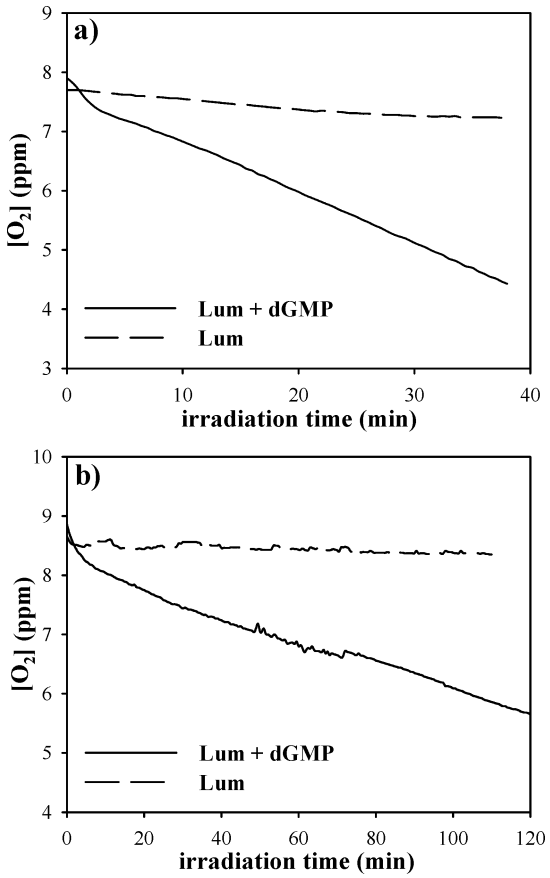

Fig. 4 Evolution of the O_2 concentration in irradiated solutions containing Lum and dGMP as a function of irradiation time. (a) pH = 5.5, [Lum] = 160 μM, [dGMP] = 320 μM; (b) pH = 10.5, [Lum] = 45 μM, [dGMP] = 180 μM. Control experiments: photolysis of Lum at the corresponding concentration in the absence of dGMP.

The steady-state concentration of $^1O_2$ during irradiation of a solution containing Lum and dGMP is given by eqn (3),

$$[^1O_2] = P_a\Phi_\Delta/(k_d + k_t^{Lum}[Lum] + k_t^{dGMP}[dGMP]) \quad (3)$$

where P_a and Φ_Δ are the estimated photon flux absorbed by Lum and the Lum-sensitized quantum yield of $^1O_2$ production, respectively; k_d is the overall rate constant of $^1O_2$ deactivation that reflects the effect of solvent, and k_t^{Lum} and k_t^{dGMP} are the overall rate constants of $^1O_2$ quenching by Lum and dGMP, respectively. Considering the k_t^{Lum} value previously determined,[8] the quenching of $^1O_2$ by Lum is negligible under our conditions in both D_2O and H_2O (*i.e.* $k_t^{Lum}[Lum] << k_d$). Values of k_t^{dGMP} were measured in D_2O at pH 5.5 and 10.5.[11] Therefore, using eqn (3) to obtain $[^1O_2]$, we can provide an estimate for the initial rate of the reaction between $^1O_2$ and dGMP for a given initial [dGMP].[27]

For the respective acidic and alkaline conditions, the calculated initial rate, $(d[dGMP]/dt)_{calc}$, was compared to the corresponding initial rate of dGMP consumption experimentally determined by HPLC analysis, $(d[dGMP]/dt)_{exp}$. The results showed that, in all cases in a given solvent, $(d[dGMP]/dt)_{calc}$ was lower than $(d[dGMP]/dt)_{exp}$ (Table 1). By itself, this suggests that the chemical reaction between dGMP and $^1O_2$, although not negligible, does not appear to be the main pathway responsible for the photosensitized oxidation of dGMP. Comparison of the data recorded in H_2O to

Table 1 Initial experimental rates of dGMP consumption, $(d[dGMP]/dt)_{exp}$, and calculated rates of the chemical reaction between $^1O_2$ and dGMP, $(d[dGMP]/dt)_{calc}$ [a]

Solvent	pH/ pD	[LUM]/ μM	[dGMP]/ μM	$(d[dGMP]/dt)_{calc}$/ μM min^{-1}	$(d[dGMP]/dt)_{exp}$/ μM min^{-1}
H_2O	5.5	83	115	0.05	0.65 (±0.03)
D_2O	5.1	83	115	0.86	2.0 (±0.1)
H_2O	10.5	80	115	0.57	5.0 (±0.1)
D_2O	10.1	80	115	6.68	8.8 (±0.4)

[a] Experiments were performed in air-equilibrated solutions. HPLC was used to determine $(d[dGMP]/dt)_{exp}$, and $(d[dGMP]/dt)_{calc}$ was calculated from eqn (2) and (3).

those recorded in D_2O further support this picture. Specifically, given that the $^1O_2$ lifetime in D_2O is longer than that in H_2O (*i.e.* $k_d(H_2O) > k_d(D_2O)$),[28] the photosensitized oxidation of dGMP should be faster in the deuterated solvent if $^1O_2$ would contribute significantly to the process. Although the $(d[dGMP]/dt)_{exp}$ values increased noticeably as the solvent was changed from H_2O to D_2O, the solvent isotope effects observed are smaller than those expected if the reaction proceeded solely as a type II process.

On the basis of this crude initial assessment, it appears that both type I and type II processes are involved in the Lum-sensitized oxidation of dGMP. Furthermore, on the basis of similar studies on pterins and dGMP,[11] the type I process most probably involves an electron transfer between the dGMP guanine moiety and the excited state of Lum.

With a photoinduced electron transfer mechanism in mind, the formation of H_2O_2 was investigated in aerated solutions containing Lum and dGMP. It is well-established that, in a typical type I process, ground state oxygen will readily quench an organic radical anion to produce the superoxide anion ($O_2^{\bullet-}$).[29,30] H_2O_2 can then be formed upon spontaneous disproportionation of $O_2^{\bullet-}$.[31] Under both pH conditions, H_2O_2 was indeed generated in our Lum/dGMP system and its concentration increased as a function of irradiation time. For the different experiments, the initial rates of H_2O_2 production were of the same order of magnitude as the corresponding rates of dGMP consumption.

Therefore, to investigate the participation of $O_2^{\bullet-}$ in the mechanism, experiments were carried out in the presence of superoxide dismutase (SOD), an enzyme that catalyzes the conversion of $O_2^{\bullet-}$ into H_2O_2 and O_2.[32] The data showed a significant increase in the rate of dGMP consumption when SOD was present in the solution. For example, in an experiment performed irradiating air-equilibrated solutions containing Lum (100 μM) and dGMP (175 μM) at pH 6.0, values of 2.8 (±0.1) μM min^{-1} and 1.91 (±0.05) μM min^{-1} were obtained for the initial rate of dGMP consumption in the presence and in the absence of SOD, respectively. These results indicate that $O_2^{\bullet-}$ is involved in the photosensitized process and provide evidence for the existence of an electron transfer reaction.

Taking into account the results presented thus far, we propose that the Lum-sensitized oxygenation/oxidation of dGMP proceeds by both type I and type II pathways. The electron transfer mediated process can be summarized as shown in eqn (4)–(11), events that have precedence in other dGMP oxidations.[11]

$$\text{Lum} \rightarrow \text{Lum}^* \quad (4)$$

$$\text{Lum}^* + \text{dGMP} \rightarrow \text{Lum}^{\bullet-} + \text{dGMP}^{\bullet+} \quad (5)$$

$$\text{Lum}^{\bullet-} + \text{dGMP}^{\bullet+} \rightarrow \text{Lum} + \text{dGMP} \quad (6)$$

$$\text{Lum}^{\bullet-} + O_2 \rightarrow \text{Lum} + O_2^{\bullet-} \quad (7)$$

$$H^+ + O_2^{\bullet-} \rightarrow HO_2^{\bullet} \quad (8)$$

$$H^+ + O_2^{\bullet-} + HO_2^{\bullet} \rightarrow H_2O_2 + O_2 \quad (9)$$

$$\text{dGMP}^{\bullet+} + O_2^{\bullet-} \rightarrow \text{dGMP} + O_2 \quad (10)$$

$$\text{dGMP}^{\bullet+} + O_2 \rightarrow \text{dGMP(ox)} \quad (11)$$

The excitation of Lum (reaction (4)) is followed by an electron transfer reaction between dGMP and the Lum excited state (reaction (5)), leading to the formation of the corresponding ion radicals ($\text{Lum}^{\bullet-}$ and $\text{dGMP}^{\bullet+}$). In the following step, the electron transfer from $\text{Lum}^{\bullet-}$ to O_2 regenerates the Lum and forms $O_2^{\bullet-}$ (reaction (7)). The latter may disproportionate with its conjugate acid $HO_2^{\bullet}$ to form H_2O_2 (reactions (8) and (9)) or react with the $\text{dGMP}^{\bullet+}$ to regenerate dGMP (reaction (10)). Finally a group of processes, represented schematically by reaction (11) and that include the reactions of $\text{dGMP}^{\bullet+}$ and its deprotonated form ($\text{dGMP}(-\text{H})^{\bullet}$) with O_2 and H_2O, leads to the oxidation of dGMP and consumption of O_2.[15]

To reinforce the complexity of this Lum/dGMP system that exhibits characteristics of both type I and type II behavior, we expanded our exploratory experiments performed as a function of oxygen concentration. Recall that, in the absence of oxygen, we observed no change in the dGMP concentration upon prolonged irradiation of Lum (*vide supra*). Although this result, by itself, is consistent with a process wherein dGMP would be consumed by $^1O_2$ (*i.e.* solely a type II process), it is not immediately apparent that it could also be consistent with our observations of type I behavior. Furthermore, we observe that the rate of Lum-sensitized dGMP disappearance is *greater* in air-saturated solutions than in oxygen-saturated solutions (Fig. 5).

Within the context of the processes shown in eqn (4)–(11), our observation that, in the absence of oxygen, irradiation of Lum does not result in dGMP disappearance suggests, for example, that recombination of the radical ions (eqn (6)) must be a dominant process. The observed low rate of dGMP disappearance at high oxygen concentration could reflect, for example, efficient quenching of the Lum triplet state by oxygen which, in turn, would effectively compete with the electron transfer step shown in eqn (5). It would be only under moderate oxygen concentration (*i.e.* air-saturated solutions) that the type I process could effectively contribute to the overall oxidative removal of dGMP. In any event, the present data clearly indicate that the Lum/dGMP photosystem is characterized by a variety of processes that kinetically compete with each other, and that these respective processes can respond differently to changes in the reaction conditions. More work must be done to fully unravel the mechanistic complexities involved.

Cellular uptake of Lum

The fluorescence of Lum has been studied, and it is known that both acid–base forms have different emission spectra, excitation

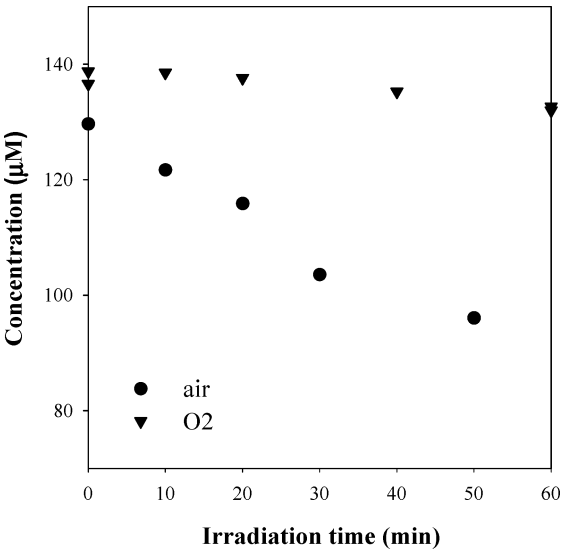

Fig. 5 Plot of the change in dGMP concentration as a function of the elapsed irradiation time of Lum at 300 nm. Data were recorded at pH 5.5 in air-saturated and, independently, oxygen-saturated solutions. Under these same conditions, the concentration of Lum did not change over the irradiation period (data not shown).

spectra and fluorescence quantum yields (Φ_F).[7] We used these fluorescence properties of Lum to investigate its cellular uptake. Extracts of HeLa cells grown in the absence of Lum (control extracts) present weak emission upon excitation in the UV due to fluorophores normally existing in the cells (Fig. 6). We compared emission and excitation spectra of these control extracts to those of HeLa cells grown in the presence of Lum (Lum extracts). In addition, we compared these spectra of cell extracts to those obtained from aqueous solutions of Lum.

Fig. 6 shows that the fluorescence intensity of the Lum extracts is greater than that of the control extract. At the pH of the cell extracts (~7), the acid form of Lum is predominant. For the Lum extracts, the wavelength of the emission maximum of the spectra obtained by excitation into the low-energy Lum band (330 nm), was the same, within experimental error, as that recorded from a standard solution of the acid form of Lum (475 ± 6 nm) (Fig. 6). Likewise, the maximum of the excitation spectra of the Lum extract recorded by monitoring the fluorescence at 470 nm was the same as that obtained from a solution of the acid form of Lum (325 ± 6 nm). For data obtained from the control extract lacking Lum, the emission and excitation maxima did not match with those obtained from the standard Lum solution.

Assuming that the fluorescence detected from the Lum extract is indeed due to the sensitizer, the emission and excitation spectra should change in the same manner as in the standard solution if this Lum extract is made more alkaline. Therefore, in another set of measurements, the fluorescence analysis of a Lum extract was performed after its pH was fixed to 10 by adding drops of NaOH solution. The alkalinization led to a significant increase in the total emission of the cell extract (Fig. 7). This fact can be explained considering that the Φ_F of the basic form of Lum (Φ_F = 0.24) is greater than that of the acid form (Φ_F = 0.08). Moreover, the emission and excitation spectra of the alkalinized Lum extract and those of the standard solution at pH 10 were equivalent, within experimental error.

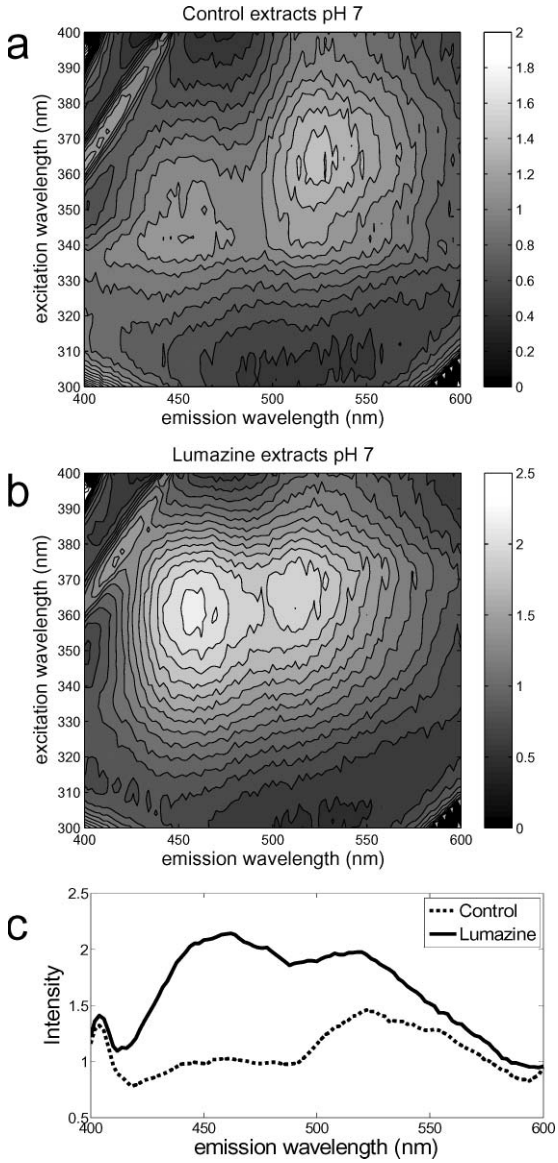

Fig. 6 2D excitation emission matrix of HeLa cell extracts at pH 7. (a) Control and (b) lumazine-incubated (40 μM) cell extracts analyzed directly after preparation. Note different intensity scales. (c) Emission linescans taken from (a) and (b) plotted on the same intensity scale to facilitate comparison of the data (excitation at 366 nm).

These results clearly show that Lum was present in the Lum extracts. Therefore, we assume that, under our experimental conditions, Lum ultimately localizes in intracellular domains when added to the culturing/growth medium. In addition, considering the emission intensities measured, and that the spectra recorded in Lum extracts are equivalent to those of Lum in aqueous solution, the generation of excited states of the sensitizer inside the cells can be assumed.

Photobiological activity of Lum

Cellular phototoxicity of Lum was evaluated using two methods: the rhodamine 123 (Rh123) and the trypan blue assays. The Rh123 method exploits the fact that this fluorescent dye is membrane permeable and that it accumulates in the mitochondria of live cells

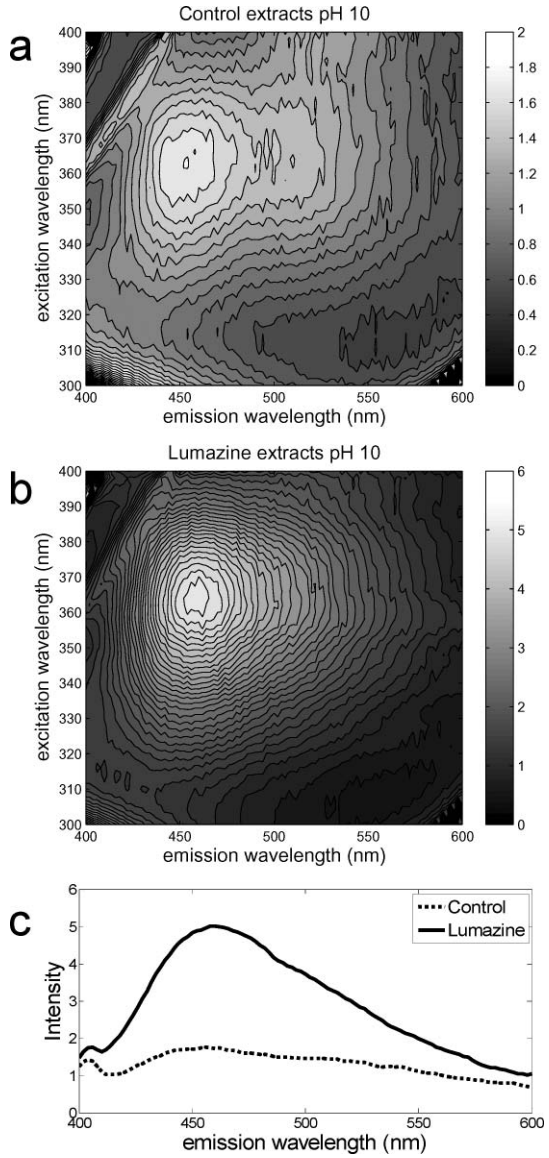

Fig. 7 2D excitation emission matrix of HeLa cell extracts alkalinized to pH = 10 before analysis. (a) Control and (b) lumazine-incubated (40 μM) cell extracts. Note different intensity scales. (c) Emission linescans taken from (a) and (b) plotted on the same intensity scale to facilitate comparison of the data (excitation at 366 nm).

as a result of the mitochondrial trans-membrane potential.[33,34] The manifestation of a "live" cell is a fluorescence image in which the mitochondria are resolved and in which the nucleus does not contain the fluorophore. Moreover, the mitochondria in a viable cell are generally associated with cytoskeletal structures and, as such, appear "strand-like".[24,35] One event in the apoptotic cascade is the loss of the mitochondrial trans-membrane potential which, in turn, is reflected in the delocalization of Rh123. As such, in a "dead" cell, the Rh123 fluorescence is diffuse and can originate, in part, from the nucleus where Rh123 ultimately tends to localize.[24] In cells that have been "compromised", the Rh123 fluorescence is diffuse and it is not possible to resolve the mitochondria even though the nucleus may still appear somewhat dark.

The Rh123 assay was performed for different HeLa cell populations: (i) cell control: cells grown in the absence of Lum and not irradiated; (ii) dark control: cells grown in the presence of Lum and not irradiated; (iii) light control: cells grown in the absence of Lum and irradiated; (iv) light + Lum: cells grown in the presence of Lum and irradiated for different periods of time. For each cell population, at least 500 cells were counted and categorized as "live", "dead", or "compromised". Relevant images are shown in Fig. 8.

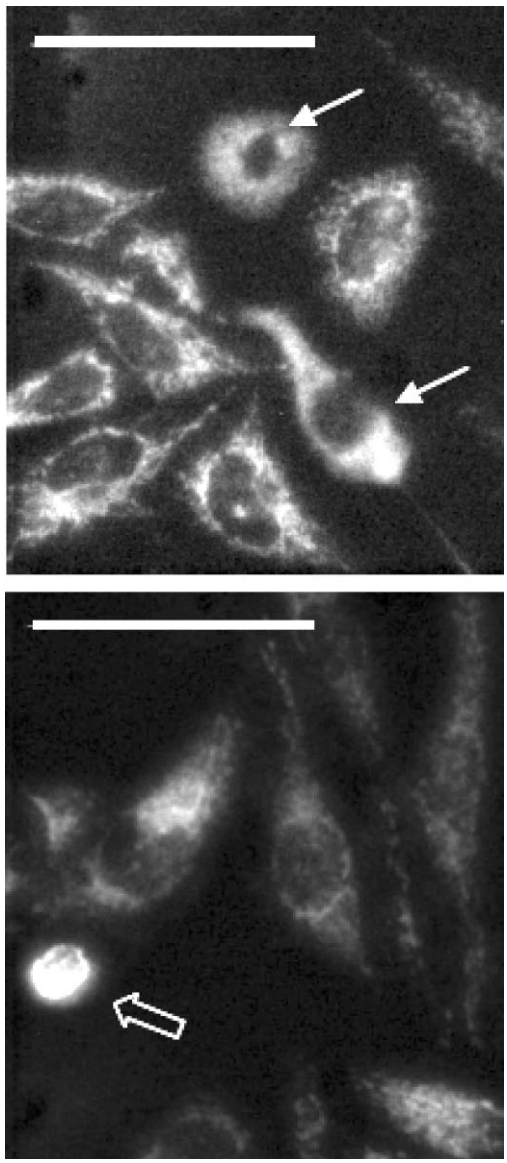

Fig. 8 Examples of fluorescence images obtained from HeLa cells in the Rh123 assay. The full arrows point to cells counted as "compromised", and the empty arrow to a "dead" cell. Scale bar = 100 μm.

The results of the Rh123 assay (Fig. 9) show that, under our conditions in the absence of irradiation (dark control), Lum does not constitute a major perturbation and is not cytotoxic. Results obtained from the "light control" demonstrate that UV radiation in the doses used in our experiments likewise does not increase the amount of altered cells. On the other hand, it is clear that when the cells containing Lum were irradiated, the fraction of "compromised" cells increased with a corresponding decrease in the fraction of "live" cells. In addition, this change in the relative

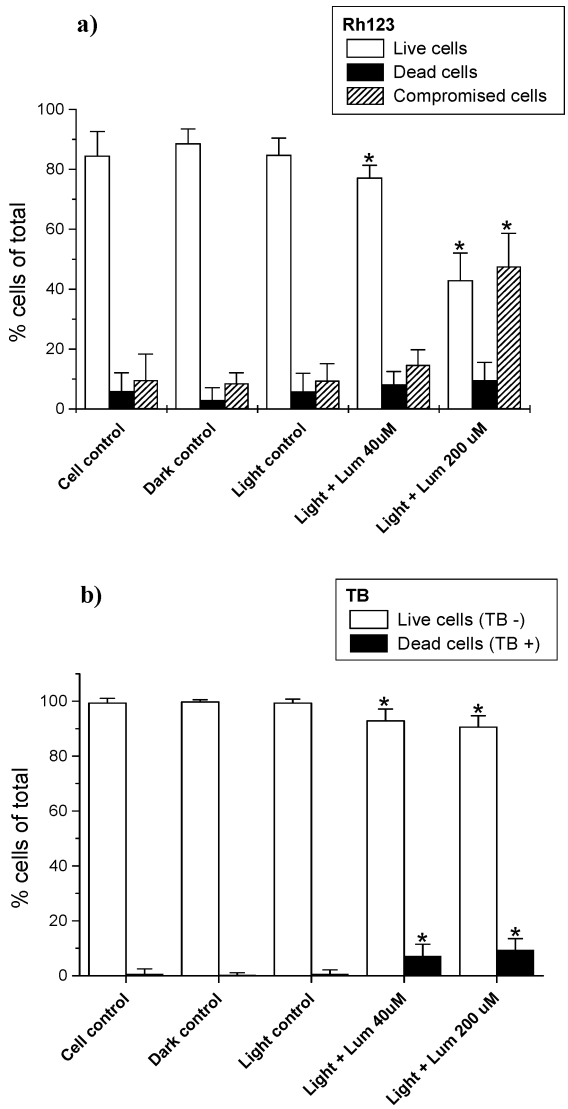

Fig. 9 Histograms showing the results of phototoxicity assays. Four kinds of HeLa cell populations were analyzed: (i) cell control: cells grown in the absence of Lum and not irradiated; (ii) dark control: cells grown in the presence of Lum and not irradiated; (iii) light control: cells grown in the absence of Lum and irradiated for 5 min; (iv) light + Lum: cells grown in the presence of Lum and irradiated for 5 min. For each cell population, at least 500 cells were counted. (a) Rh123 assay, (b) trypan blue exclusion assay. Pylons labeled with an asterisk indicate that there are significant differences between this number and the corresponding numbers obtained from the three control populations of each assay (one-way ANOVA; $p < 0.05$).

amounts of "live" and "compromised" cells was more pronounced for cells containing a higher Lum concentration. These results show that Lum has a photodynamic effect on HeLa cells.

Cellular phototoxicity of Lum was also evaluated by the trypan blue exclusion assay. This method uses the degree of structural integrity of the cell membrane as the parameter to determine cell viability. The dye can not enter cells through an intact membrane and, therefore, stains only cells which have altered membranes. Hence, "dead" cells appear distinctively blue under bright-field microscopic examination. Different types of damage can be evaluated by modifying the time of incubation after treatment.[36] Whereas unspecific cell damage is only detected by this assay after a sufficiently long post-treatment time (more than 24 h), direct membrane damage is observed immediately (0–6 h after the treatment). In our case, we performed the trypan blue assay 2 h after irradiation (see Experimental).

The trypan blue exclusion assay was performed for HeLa cell populations similar to those analyzed using the Rh123 assay (*vide supra*). Specifically, we used three control populations (cell control, dark control, light control) and cells grown in the presence of different Lum concentrations and irradiated (light + Lum). For each cell population, at least 500 cells were counted and categorized, in this case, as "live" or "dead". Pertinent images are shown in Fig. 10.

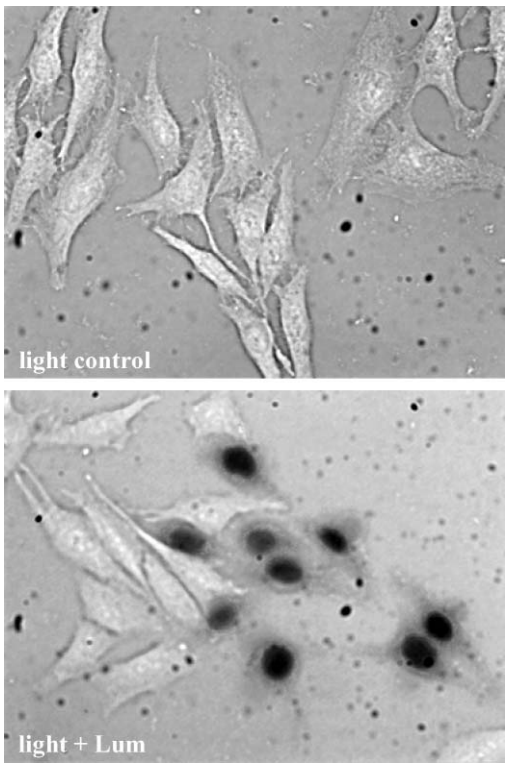

Fig. 10 Examples of images obtained from HeLa cells in the trypan blue exclusion assay. Light control: cells grown in the absence of Lum and irradiated; light + Lum: cells grown in the presence of Lum (200 μM) and irradiated for 5 min.

Data obtained from the trypan blue assay are summarized in Fig. 9. The results show that, in the absence of radiation, Lum cytotoxicity was not significant for sensitizer concentrations up to 200 μM. Likewise, in the absence of Lum, irradiation with the dose used in these experiments did not reduce the viability of the cells. In contrast, the number of dead cells noticeably increased upon irradiation of cells incubated with Lum. These results clearly confirm that Lum has a photodynamic effect on HeLa cells and strongly suggest that direct membrane damage occurred.

Conclusions

The photooxidative sensitizing capability of lumazine (Lum), a compound belonging to a family of heterocycles present in biological systems, was studied. We performed experiments using

a biomolecule (2′-deoxyguanosine 5′-monophosphate (dGMP)) and a cell line derived from cervical cancer cells (HeLa) as targets.

Recorded data unambiguously indicate that Lum can sensitize the photooxidative degradation of dGMP. The photosensitized oxidation occurs through two competing mechanisms: (1) electron transfer between dGMP and excited Lum and (2) reaction of dGMP with $^1O_2$ produced by Lum. This kinetic competition between several convoluted processes defines a complicated system that is susceptible to the effects of many variables. More mechanistic work clearly needs to be done to properly map out the Lum/dGMP response under a given set of conditions. Independently, we have demonstrated that irradiation of intracellular Lum can result in cell death. These results can have important ramifications when studying the photoinitiated effects of endogenous biological compounds.

Acknowledgements

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References

1 W. Pfleiderer, Natural pteridines – A chemical hobby, in *Chemistry and Biology of Pteridines and Folates*, ed. J. E. Ayling, M. G. Nair and C. M. Baugh, Plenum Press, New York, 1993, pp. 1–16.
2 T. J. Kappock and J. P. Caradonna, Pterin-dependent amino acid hydroxylases, *Chem. Rev.*, 1996, **96**, 2659–2756.
3 D. J. Brown, Introduction to the Pteridines, in *The Chemistry of Heterocyclic Compounds, Part 3, Volume 24, Fused Pyrimidines: Pteridines*, ed. D. J. Brown, John Wiley and Sons, New York, 1988, ch. 6, pp. 1–42.
4 K. Kis, K. Kugelbrey and A. Bacher, Biosynthesis of riboflavin the reaction catalyzed by 6,7-dimethyl-8-ribityllumazine synthase can proceed without enzymatic catalysis under physiological conditions, *J. Org. Chem.*, 2001, **66**, 2555–2559.
5 H. Rembold and W. L. Gyure, Biochemistry of the pteridines, *Angew. Chem., Int. Ed. Engl.*, 1972, **11**, 1061–1072.
6 C. Lorente and A. H. Thomas, Photophysics and photochemistry of pterins in aqueous solution, *Acc. Chem. Res.*, 2006, **39**, 395–402.
7 R. Klein and I. Tatischeff, Tautomerism and fluorescence of lumazine, *Photochem. Photobiol.*, 1987, **45**, 55–65.
8 M. P. Denofrio, A. H. Thomas, A. M. Braun, E. Oliveros and C. Lorente, Photochemical and photophysical properties of lumazine in aqueous solutions, *J. Photochem. Photobiol., A*, 2008, **200**, 282–286.
9 K. Ito and S. Kawanishi, Photoinduced hydroxylation of deoxyguanosine in DNA by pterins: sequence specificity and mechanism, *Biochemistry*, 1997, **36**, 1774–1781.
10 G. Petroselli, R. Erra-Balsells, F. M. Cabrerizo, C. Lorente, A. L. Capparelli, A. M. Braun, E. Oliveros and A. H. Thomas, Photosensitization of 2′-deoxyadenosine-5′-monophosphate by pterin, *Org. Biomol. Chem.*, 2007, **5**, 2792–2799.
11 G. Petroselli, M. L. Dántola, F. M. Cabrerizo, A. L. Capparelli, C. Lorente, E. Oliveros and A. H. Thomas, Oxidation of 2′-deoxyguanosine 5′-monophosphate photoinduced by pterin: type I *versus* type II mechanism, *J. Am. Chem. Soc.*, 2008, **130**, 3001–3011.
12 W. Pfleiderer, Pteridine, I. über 2,4-dioxotetrahydropteridine, *Chem. Ber.*, 1957, **90**, 2582–2587.
13 K. Briviba, L.-O. Klotz and H. Sies, Toxic and signaling effects of photochemically or chemically generated singlet oxygen in biological systems, *Biol. Chem.*, 1997, **378**, 1259–1265.
14 E. Cadenas, Biochemistry of oxygen toxicity, *Annu. Rev. Biochem.*, 1989, **58**, 79–110.
15 J. Cadet, M. Berger, T. Douki, B. Morin, S. Raoul, J.-L. Ravanat and S. Spinelli, Effects of UV and visible radiation on DNA – Final base damage, *Biol. Chem.*, 1997, **378**, 1275–1286.
16 J.-L. Ravanat, R. Martinez, M. H. G. Medeiros, P. Di Mascio and J. Cadet, Mechanistic aspects of the oxidation of DNA constituents mediated by singlet molecular oxygen, *Arch. Biochem. Biophys.*, 2004, **423**, 23–30.
17 I. E. Kochevar, M. C. Lynch, S. Zhuang and C. R. Lambert, Singlet oxygen, but not oxidizing radicals, induces apoptosis in HL-60 cells, *Photochem. Photobiol.*, 2000, **72**, 548–553.
18 K. R. Weishaupt, C. J. Gomer and T. J. Dougherty, Identification of singlet oxygen as the cytotoxic agent in photo-inactivation of a murine tumor, *Cancer Res.*, 1976, **36**, 2326–2329.
19 J. C. Stockert, M. Cañete, A. Juarranz, A. Villanueva, R. W. Horobin, J. I. Borrell, J. Teixidó and S. Nonell, Porphycenes: facts and prospects in photodynamic therapy of cancer, *Curr. Med. Chem.*, 2007, **14**, 997–1026.
20 A. M. Braun, M. T. Maurette and E. Oliveros, *Photochemical Technology*, Wiley, Chichester, 1991, ch. 2, pp. 85–88.
21 H. J. Kuhn, S. E. Braslavsky and R. Schmidt, IUPAC technical report on chemical actinometry, *Pure Appl. Chem.*, 2004, **76**, 2105–2146.
22 C. C. Allain, L. S. Poon, C. S. G. Chan, W. Richmond and P. C. Fu, Enzymatic determination of total serum cholesterol, *Clin. Chem.*, 1974, **20**, 470–475.
23 H. M. Flegg, An investigation of the determination of serum cholesterol by an enzymatic method, *Ann. Clin. Biochem.*, 1973, **10**, 79–84.
24 S. Hatz, J. D. C. Lambert and P. R. Ogilby, Measuring the lifetime of singlet oxygen in a single cell: Addressing the issue of cell viability, *Photochem. Photobiol. Sci.*, 2007, **6**, 1106–1116.
25 E. Skovsen, J. W. Snyder, J. D. C. Lambert and P. R. Ogilby, Lifetime and diffusion of singlet oxygen in a cell, *J. Phys. Chem. B*, 2005, **109**, 8570–8573.
26 J. W. Snyder, E. Skovsen, J. D. C. Lambert and P. R. Ogilby, Subcellular, Time-resolved studies of singlet oxygen in single cells, *J. Am. Chem. Soc.*, 2005, **127**, 14558–14559.
27 Notice that the estimation of d[dGMP]/dt must be carried out separately for the two pH conditions because P_a, Φ_Δ and k_r have different values. In particular, the reactivity of dGMP towards $^1O_2$ depends on pH due to charge effects as a consequence of the different acid–base equilibria of the nucleotides in solution. The corresponding pK_a values for dGMP have already been reported (p$K_{a1}(PO_3H_2) \approx 0.65$, p$K_{a2}(PO_3H_2) = 6.29$, p$K_a(N1) = 9.56$).[37,38] At pH 5.5, the dGMP molecule has a charge of −1, with the charge on the phosphate group and the purine base in its neutral form. At pH 10.5, the total charge of the dGMP molecule is −3 and the guanine moiety is deprotonated due to the lactam group (Fig. 1). The k_r values at pH 5.5 and 10.5 have been reported to be $1.7 (\pm 0.3) \times 10^7$ M^{-1} s^{-1} and $9.6 (\pm 0.8) \times 10^7$ M^{-1} s^{-1}, respectively.[11]
28 P. R. Ogilby and C. S. Foote, Chemistry of singlet oxygen. 42. Effect of solvent, solvent isotopic substitution, and temperature on the lifetime of singlet molecular oxygen ($^1\Delta_g$), *J. Am. Chem. Soc.*, 1983, **105**, 3423–3430.
29 E. K. Hodgson and I. Fridovich, The role of $O_2^{\bullet -}$ in the chemiluminescence of luminal, *Photochem. Photobiol.*, 1973, **18**, 451–455.
30 J. Eriksen, C. S. Foote and T. L. Parker, Photosensitized oxygenation of alkenes and sulfides *via* a non-singlet-oxygen mechanism, *J. Am. Chem. Soc.*, 1977, **99**, 6455–6456.
31 B. H. J. Bielski, D. E. Cabelli, R. L. Arudi and A. B. J. Ross, Reactivity of HO_2/O_2^- radicals in aqueous solution, *Phys. Chem. Ref. Data*, 1985, **14**, 1041–1100.
32 I. Fridovich, Superoxide radicals, superoxide dismutases, and the aerobic lifestyle, *Photochem. Photobiol.*, 1978, **28**, 733–741.
33 Z. Darzynkiewicz, G. Juan, X. Li, W. Gorczyca, T. Murakami and F. Traganos, Cytometry in cell necrobiology: Analysis of apoptosis and accidental cell death (necrosis), *Cytometry*, 1997, **27**, 1–20.
34 L. V. Johnson, M. L. Walsh and L. B. Chen, Localization of mitochondria in living cells with rhodamine 123, *Proc. Natl. Acad. Sci. U. S. A.*, 1980, **77**, 990–994.
35 T. Breitenbach, M. K. Kuimova, P. Gbur, S. Hatz, N. B. Schack, B. W. Pedersen, J. D. C. Lambert, L. Poulsen and P. R. Ogilby,

Photosensitized production of singlet oxygen: spatially-resolved optical studies in single cells, *Photochem. Photobiol. Sci.*, 2009, **8**, 442–452.
36 C. Marzano, F. Baccichetti, F. Carlassare, A. Chilin, S. Lora and F. Bordin, DNA damage induced by 4,6,8,9-tetramethyl-2*H*-furo[2,3-*h*]quinolin-2-one, a new furocoumarin analog: biological consequences, *Photochem. Photobiol.*, 2000, **71**, 263–272.
37 B. Song, G. Oswald, M. Bastian, H. Sigel and B. Lippert, Extent of the acidification by N7-coordinated *cis*-diammine-platinum(II) on the acidic sites of guanine derivatives, *Met.-Based Drugs*, 1996, **3**, 131–141.
38 B. Knobloch, H. Sigel, A. Okruszek and R. K. O. Sigel, Acid–base properties of the nucleic-acid model 2′-deoxyguanylyl(5′→3′)-2′-deoxy-5′-guanylate, $d(pGpG)^{3-}$, and of related guanine derivatives, *Org. Biomol. Chem.*, 2006, **4**, 1085–1090.