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Induced photolysis of rabbit red blood cells by several photosensitizers

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The photolysis of rabbit red blood cells induced by several photosensitizers has been studied. Membrane photohemolysis was found to be dependent on light dose (J/cm^2) for hematoporphyrin (HP), methylene blue (MB) and toluidine blue (TB). No significant hemolysis was detected when mesotetra (4*N*-methylpyridyl) porphine (T_4MP_yP) plus light was used. Sodium azide inhibited, whereas deuterium oxide potentiated membrane lysis with HP, MB and TB. In the case of T_4MP_yP no differences were observed when using sodium azide or deuterium oxide.

Key words: Membrane, photohemolysis, photosensitizers, singlet oxygen.

Introduction

During recent years there has been a growing interest in the search for new photosensitizing agents for application in photodynamic therapy of cancer. Although photodamage can occur at different cellular levels, cytotoxic effects of several photosensitizers involve DNA damage.[1] Nevertheless, biological membranes (plasma membrane, mitochondria, lysosomes, etc.) also seem to be important targets for many antineoplastic and most of the photosensitizer agents.[2–6] In this sense, mammalian erythrocytes constitute an attractive and suitable model system to study membrane photomodification. The membrane lysis is easily detected by estimating the free hemoglobin by using spectrophotometry. In the present note, we have studied the photohemolysis of rabbit red cells induced by two thiazines, methylene blue (MB) and toluidine blue (TB), and two porphyrins, hematoporphyrin (HP) and mesotetra (4*N*-methylpyridyl) porphine (T_4MP_yP).

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Materials and methods

The sensitizers used belong to the thiazine group, methylene blue (MB, Fluka, Chemie AG, Buchs, Switzerland) and toluidine blue (TB, Sigma, St Louis, MO, USA) and the porphyrin group, HP (Sigma) and mesotetra (4N–methylpyridyl) porphine tetraiodide (T_4MP_yP, Ventron-Alfa Produkte, Karlsruhe, Germany) (Figure 1a–d). All drugs were prepared in water with the exception of hematoporphyrin, which was dissolved in 0.13 M NaCl with 0.02 M NaOH and brought to pH 7.4.[7]

Rabbit erythrocytes, not older than 3 days, were used for the experiments. After extraction, the whole blood was centrifuged (5 min at 3000 r.p.m.) to remove plasma, washed once with phosphate buffered saline (PBS) solution (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 and 8 mM Na_2HPO_4) and centrifuged again. Erythrocytes were resuspended in saline solution (0.9% NaCl) to get a concentration of 10^9 red blood cells/ml and maintained at 4°C until use.

Reactions were carried out at room temperature, preparing H_2O or deuterium oxide (D_2O) solutions in PBS with the sensitizer at the appropriate concentration and erythrocytes at a final concentration of 10^8 cells/ml. Sodium azide (NaN_3) was added to the suspensions, as a scavenger of singlet oxygen ($^1O_2$) formation, to get a final concentration of 5×10^{-3} M. Mixtures were incubated for 30 min in the dark prior to irradiation. Irradiation was carried out in plastic tissue culture flasks of 2 cm path length using a Kodak slide projector with a 250 W lamp (Osram). The light was filtered through a 3.0 cm water layer (to absorb heat) and a selected wavelength filter. At the treatment site, the light intensity was 100 mW/cm^2 for the red ($\lambda > 600$ nm) and 28 mW/cm^2 for the blue ($360 < \lambda < 460$ nm) filter (M8-Spectrum-Power-Energymeter). At fixed times, aliquots of 3 ml were separated from the irradiated solutions and maintained in the dark at room temperature for 24 h. After centrifugation,

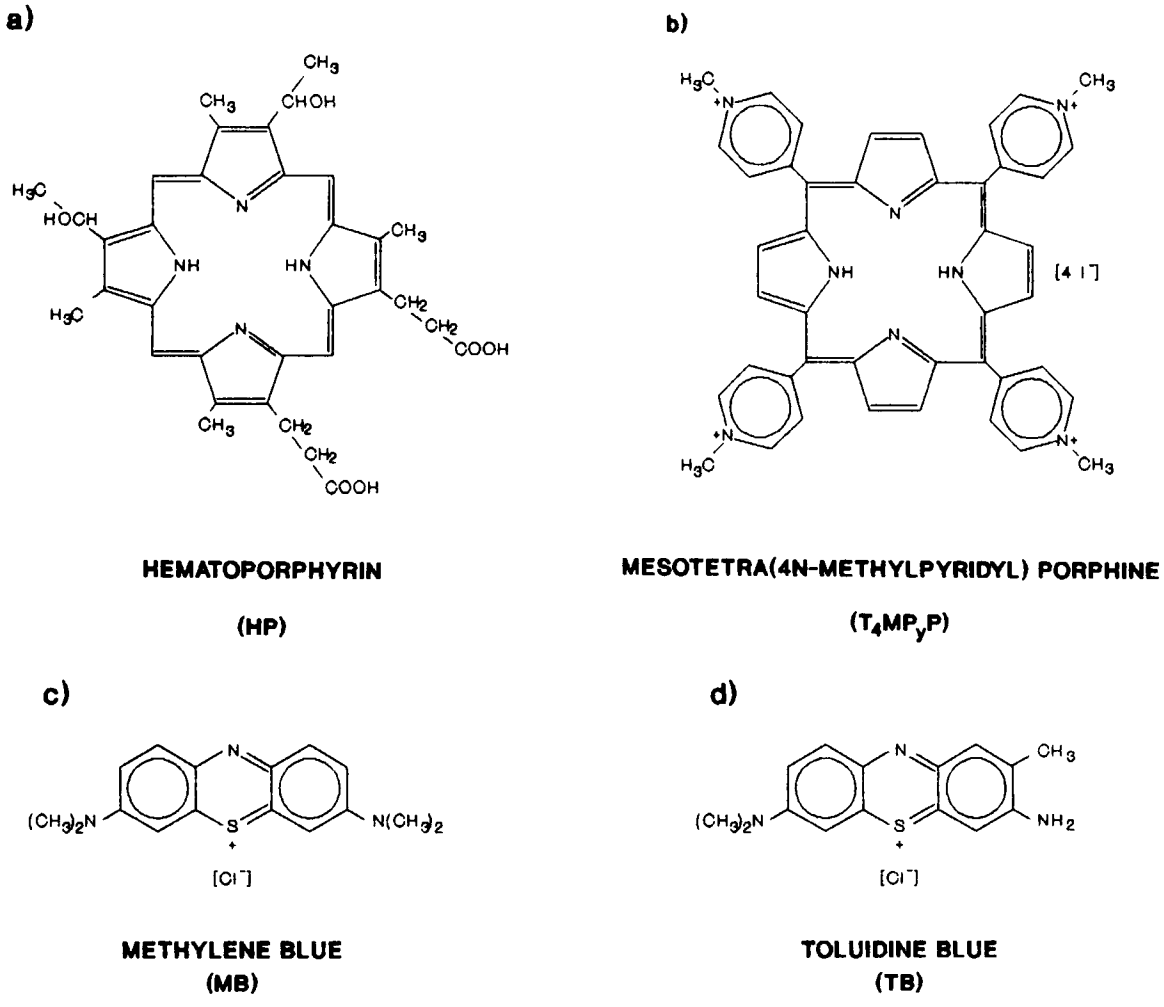

Figure 1. Structural formulae of the compounds used in this work.

the supernatant containing the free hemoglobin was measured at 413 nm in a Perkin-Elmer 551-S UV/VIS spectrophotometer.

Percentage of hemolysis (%H) as a function of the light dose for a given drug concentration [A] or the drug concentration for a given light dose [B] was calculated as

$$\%H = \frac{H_t - H_B}{H_T - H_B} \times 100 \qquad [A]$$

$$\%H = \frac{(H_c - (H_B) - (H_0 - H_B)}{H_T - H_B} \times 100 \qquad [B]$$

where H is the hemoglobin absorption at 413 nm. The subscripts correspond to the solution measured: H_B, hemolysis in PBS; H_T, 100% lysis in distilled or D_2O water; H_c, hemolysis induced by the sensitizers at the indicated concentrations plus light; H_0, hemolysis induced by the sensitizer without irradiation (in the dark); and H_t, after an irradiation time.

Results and discussion

The hemolysis induced by the sensitizers studied was assayed from the amount of hemoglobin released to the medium and measured at 413 nm. Hemolysis due to the effect of the sensitizer alone, without light, was low and independent of drug concentration for MB, TB and HP (<8% in relation with the total hemolysis in distilled water), at the conditions considered. In the case of T_4MP_yP, the hemolysis obtained in dark conditions seemed to be related to the sensitizer concentration, but in any case was higher than 15 ± 0.53%.

Figure 2 shows the cellular photolysis as a function of the sensitizer concentration using a light dose of 9 J/cm^2. With the exception of HP, membrane photolysis seemed not to be dependent on the concentration used. On the contrary, elevated quantities of free hemoglobin were detected when blood cells were treated with high

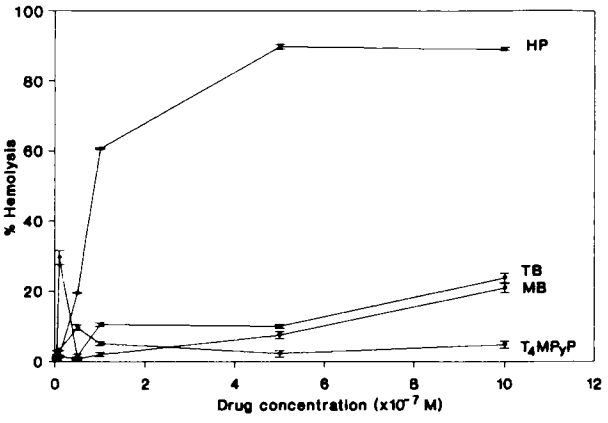

Figure 2. Photolysis of membrane erythrocytes by the sensitizers as a function of concentration (M). Same light dose: 9 J/cm^2. The cellular lysis was estimated by measuring the free hemoglobin at 413 nm.

concentrations of HP and subsequently irradiated. Concentrations of 5×10^{-7} M for porphyrins and 10^{-6} M for thiazines were chosen for the rest of the experiments, because photolysis of membranes was kept relatively low using these concentrations.

The photolysis of erythrocytes induced by HP, MB and TB as a function of light dose in H_2O solutions is shown in Figure 3. HP shows a rate of hemolysis that is initially very rapid, reaching a plateau at doses over 9 J/cm^2. The kinetics of MB are slow at doses under 9 J/cm^2 and the plateau seems to be reached at very high doses (>27 J/cm^2). The curve obtained with TB has a similar shape than that of MB, though the kinetics of TB are faster. The 50% photohemolysis was observed using doses of 4 J/cm^2 for HP, 18 J/cm^2 for MB and 12 J/cm^2 for TB. The membrane hemolysis induced by 5×10^{-7} M T_4MP_yP was independent of light dose, being almost undetectable (data not shown). When sodium azide was added to red blood suspensions in the presence of the photosensitizers, photohemolysis was inhibited, as also shown in Figure 3. The kinetics of the sensitizers with NaN_3 are quite similar but shifted to higher doses than those observed without the inhibitor. It is known that sodium azide is capable of intercepting $^1O_2$ once formed.[8,9]

However, the induction of membrane photohemolysis was activated when suspensions were prepared in D_2O in relation to H_2O (Figure 4). Using doses corresponding to the 50% hemolysis in H_2O for HP, MB and TB, the erythrocytes lysis increased for all the sensitizers, except for T_4MP_yP.

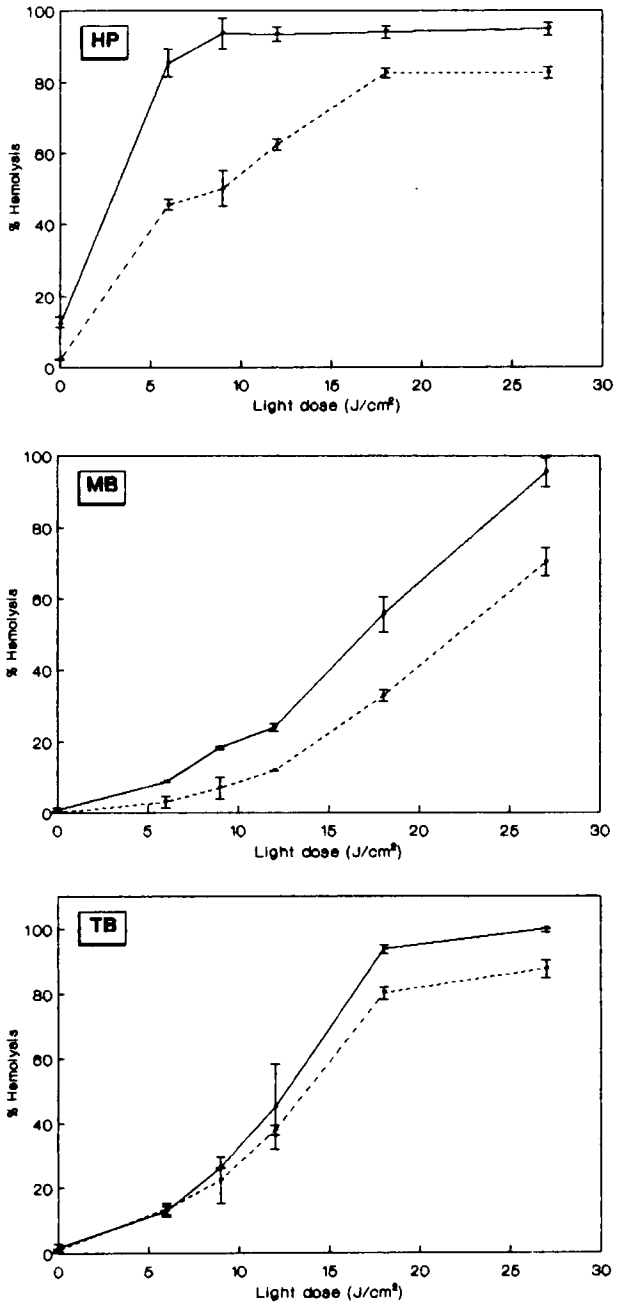

Figure 3. Percentage of photohemolysis induced by HP (5×10^{-7} M), MB and TB (10^{-6} M), and inhibited by sodium azide (5×10^{-3} M) as a function of light dose (J/cm^2). —, sensitizer; ---, NaN_3.

Since the lifetime of $^1O_2$ in D_2O is longer than in H_2O,[10] the photohemolysis is greater when D_2O is used as solvent.

The comparison between the two porphyrins studied (T_4MP_yP and HP) in relation to their ability to induce membrane lysis when exposed to blue or red light is shown in Figure 5. In the case of

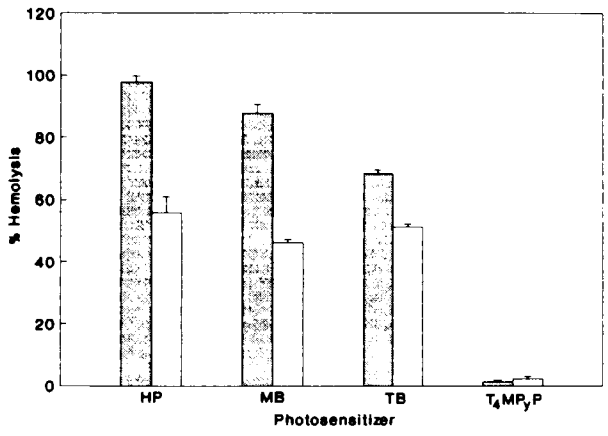

Figure 4. Induction of photohemolysis by the sensitizers in H_2O or D_2O suspensions. Sensitizers: HP and T_4MP_yP, 5×10^{-7} M; MB and TB, 10^{-6} M. Light dose: HP and T_4MP_yP, 4 J/cm^2; MB, 18 J/cm^2; TB, 12 J/cm^2. ▨, D_2O; □, H_2O.

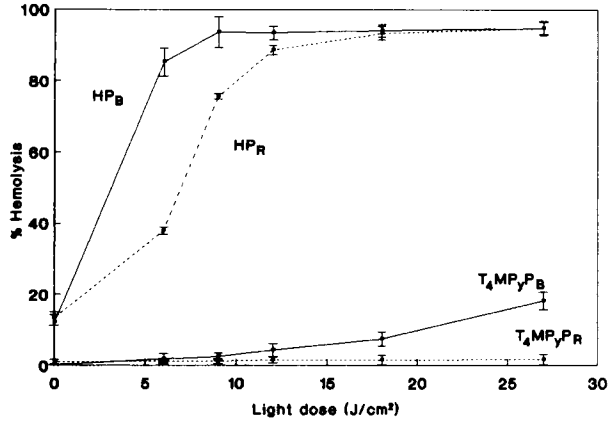

Figure 5. Hemolysis of red blood cells produced by T_4MP_yP and HP (5×10^{-7} M) plus blue (B) or red light (R) as a function of light dose (J/cm^2).

T_4MP_yP, no differences between blue or red light were found; in both cases, the percentage of lysis was virtually zero. However, HP induced a greater hemolysis under blue than under red irradiation light, at the same light doses.

The results described indicate that $^1O_2$ seems to play an important role in the process of membrane photolysis induced by HP, MB and TB, although other factors must be implicated. In this sense, the differences observed relating to the photohemolysis effectiveness could result from the localization of HP, TB and MB, which are probably associated at different levels within the erythrocyte membrane. Under that environment, the irradiated sensitizer transfers energy to molecular oxygen, generating $^1O_2$, which leads to lysis. T_4MP_yP, however, although known to be able to generate $^1O_2$,[11] seems not to be associated with the membrane, thus no photolysis can be detected. However, except for HP, the rest of the photosensitizers, MB, TB and T_4MP_yP, are able to interact with DNA.[12–14] Therefore, in cellular systems, HP acts mainly at the membrane level, MB and TB at both DNA and membrane levels, whereas T_4MP_yP especially produces DNA damage. Thus, photosensitizers that act by damaging membranes as well as DNA or the combination of two photosensitizers with both different targets, could be an interesting strategy for photodynamic treatment of cancer.

References

1. Villanueva A, Juarranz A, Díaz V, *et al.* Photodynamic effects of a cationic mesosubstituted porphyrin in cell cultures. *Anti-Cancer Drug Des* 1992; **7**: 297–303.
2. Deuticke B, Henseleit U, Haest CWM, *et al.* Enhancement of transbilayer mobility of a membrane lipid probe accompanies formation of membrane leaks during photodynamic treatment of erythrocytes. *Biochim Biophys Acta* 1989; **982**: 53–61.
3. Santus R, Reyftmann JF. Photosensitization of membrane components. *Biochimie* 1986; **68**: 843–8.
4. Valenzeno DP, Pooler JP. Cell membrane photomodification: relative effectiveness of halogenated fluoresceins for photohemolysis. *Photochem Photobiol* 1982; **35**: 343–50.
5. Daoud SS. Cell membranes as targets for anti-cancer drug action. *Anti-Cancer Drugs* 1992; **3**: 443–53.
6. Gomer CJ. Preclinical examination of first and second generation photosensitizers used in photodynamic therapy. *Photochem Photobiol* 1991; **54**: 1093–107.
7. Moan J, Waksvik H, Christensen T. DNA single-strand breaks and sister chromatid exchanges induced by treatment with hematoporphyrin and light or by X-rays in human NHIK 3025 cells. *Cancer Res* 1980; **40**: 2915–8.
8. Foote CS, Fujimoto TT, Chang YC. Chemistry of singlet oxygen—XV—irrelevance of azide trapping to mechanism of ene reaction. *Tetrahedron Lett* 1972; **1**: 45–8.
9. Dahl TA, Midden WR, Hartman PE. Some prevalent biomolecules as defenses against singlet oxygen damage. *Photochem Photobiol* 1988; **47**: 357–62.
10. Piette J. Mutageic and genotoxic properties of singlet oxygen. *J Photochem Photobiol B: Biol* 1990; **4**: 335–42.
11. Verlhac JB, Gaudemer A, Kraljic I. Water-soluble porphyrins and metalloporphyrins as photosensitizers in aerated aqueous solutions. I. Detection and determination of quantum yield of formation of singlet oxygen. *Nouv J Chim* 1984; **8**: 401–6.
12. Fiel RJ, Howard JC, Mark EH, *et al.* Interaction of DNA with a porphyrin ligand: evidence for intercalation. *Nucleic Acids Res* 1979; **6**: 3093–118.
13. Mueller W, Crothers, DM. Interactions of heteroatomic compounds with nucleic acids. I. The influence of heteroatoms and polarizability on the base specificity of intercalating ligands. *Eur J Biochem* 1975; **54**: 267–77.
14. Stockert JC, Cytochemistry of nucleic acids: binding mechanisms of dyes and fluorochromes. *Microscopia Electronica Biologia Celular* 1985; **9**: 89–130.

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