Dark and Photohemolysis of Erythrocytes by Furocoumarins

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It has been shown that various furocoumarins are able to cause dark hemolysis in red blood cells (RBC). However, this effect is evident only at relatively high furocoumarin concentrations (4.6×10^{-4} M) – much higher than those used in photosensitization experiments or photochemotherapeutic treatments.

Among the various furocoumarins examined in this study, only psoralen (Ps) and 3-carbethoxypsoralen (3-CPs) showed strong photohemolytic effects, while the other compounds revealed little or no activity. This fact indicates that Ps and 3-CPs are able to induce selective damage to the cell membrane of RBC.

By pre-irradiating furocoumarin in ethanol or isotonic saline solutions and adding the irradiated solutions to a RBC suspension, hemolysis was observed in various compounds. The products of photolysis which form during pre-irradiation may be responsible, in terms of hemolysis, for toxic effects on RBC.

Introduction

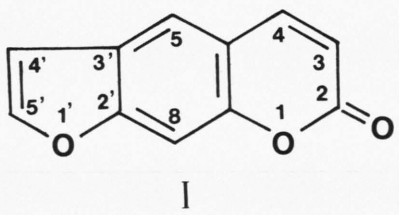
Psoralens (furocoumarins) are a family of naturally occurring or synthetic compounds showing various photobiological effects [1]; many naturally occurring plant psoralens show marked skin photosensitizing activity as a result of erythema followed by dark pigmentation [2] (Fig. 1).

Hindus, Turks, Egyptians and other orientals have exploited this property in the treatment of vitiligo in popular medicine since ancient times [3]. El Mofty [4] rationalized this type of therapy using the active component of *Ammi majus* (8-methoxypsoralen: 8-MOP), isolated in a chemically pure state.

However, scientific interest in photosensitizing psoralens has grown dramatically over the last fifteen years, after the clinical introduction of some of these compounds in the photochemotherapy of psoriasis and other skin diseases, stimulating new studies directed towards better knowledge of their mechanism of action and to more precise evaluation of their toxicity.

In this connection, other than the classic mechanism by which furocoumarins photoinduce selective damage to nucleic acids, other mechanisms have been investigated, such as the ability of furocoumarins to photointeract with proteins, photoinactivate enzymes, generate singlet oxygen and other activated species of oxygen, and cause photodamage to the cell membrane [5].

Among the various mechanisms, the least studied is that involving photodamage to the cell membrane. In this connection, in their study of the photooxida-



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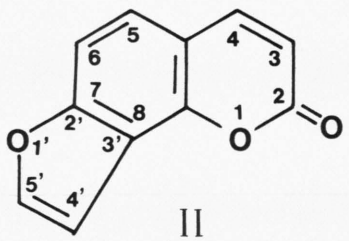

II

Fig. 1. Molecular structure of psoralen I and angelicin II.

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tive properties of furocoumarins on RBC, Musajo *et al.* [6] were unable to show sensitized photohemolysis by furocoumarins. Using 8-methoxypsoralen, Wennersten [7] confirmed these data, even when working in the presence of D_2O. Recently, however, Potapenko *et al.* [8], working in different conditions, have been able to induce sensitized photohemolysis using psoralen. This fact may be connected with the damage that furocoumarins induce in the cell membrane.

Assuming that this type of lesion may be connected with the skin phototoxicity of furocoumarins, we extended our study of photohemolysis to a large series of furocoumarins. The possible correlation between skin phototoxicity and photohemolysis was also considered. Moreover, we studied the ability of furocoumarins to cause hemolysis in the dark, and also post-irradiation dark hemolysis caused by pre-irradiating solutions of furocoumarins and then adding them to suspensions of RBC.

Materials and Methods

Human erythrocytes (RBC) from volunteers were washed in isotonic saline solution and resuspended in the same solution.

For the various experiments, a 10^6 cells/ml suspension was prepared by suitable dilution with isotonic saline solution.

Sample preparation

a) Photohemolysis and dark hemolysis

2 ml of the above suspension of RBC were added to 0.1 ml of ethanol solution of the furocoumarin examined (concentrations ranged between 1×10^{-5} M and 4.6×10^{-4} M). The volume of 0.1 ml of ethanol did not cause hemolysis in the controls.

The samples, in a thermostatically controlled bath at 18 °C, were irradiated for 10 min (photohemolysis) or kept in the dark for the same period of time (dark hemolysis). Irradiation was carried out using two HPW 125 Philips lamps emitting almost exclusively at 365 nm. Irradiation intensity, determined by a chemical actinometer [9], was 59 J sec^{-1} m^{-2}.

At the end of treatment, a dark equilibration was carried out on the two samples, keeping them immersed in a thermostat at 37 °C for 60 min [8]. The cells were then centrifuged at 3000 rpm for 20 min and the absorbance on the supernatant at 415 nm was determined on a Perkin Elmer Lambda 5 spectrophotometer.

A zero level hemolysis value was obtained by using supernatant from an irradiated RBC suspension without added photosensitizer and a 100% hemolysis value from a 1:500 dilution of the stock RBC suspension in distilled water [10].

b) Post-irradiation dark hemolysis (PIDH) in ethanol

An ethanol solution of the examined furocoumarin (9.66×10^{-3} M) was saturated with oxygen and then irradiated for 10 min, following the method described above. 0.1 ml of the irradiated ethanol solution was then added to 2 ml of RBC (final furocoumarin concentration: 4.6×10^{-4} M). The suspension was immersed in a thermostat at 37 °C for 60 min and, after centrifugation, the extent of hemolysis was determined as described above.

Dark hemolysis was carried out using the same ethanol solution as that used for PIDH, without irradiation.

c) Post-irradiation dark hemolysis in isotonic saline solution

The sample furocoumarin was dissolved in isotonic saline solution by the addition of a small volume of concentrated ethanol solution. This solution was then divided into two portions: i) 2 ml were irradiated for 10 min as described above; ii) 2 ml were kept in the dark. The RBC suspension was added to the two samples to reach 10^{-6} cells/ml; the final furocoumarin concentration was 4.6×10^{-4} M. The two samples were then incubated at 37 °C for 60 min, centrifuged, and the extent of hemolysis determined spectrophotometrically.

Results

Dark hemolysis

The effects of concentration were studied in terms of dark and photohemolysis in six compounds – psoralen (Ps), 3-carbethoxypsoralen (3-CPs), 8-methoxypsoralen (8-MOP), 5-methoxypsoralen (5-MOP), 4,5′,8-trimethylpsoralen (TMP), and 4,4′,6-trimethylangelicin (TMA). In particular, Fig. 2 shows their effects on RBC. At a concentration of 10^{-5} M, the various compounds practically do not induce hemolysis. However, by increasing the concentration, an increase in the extent of dark hemolysis is

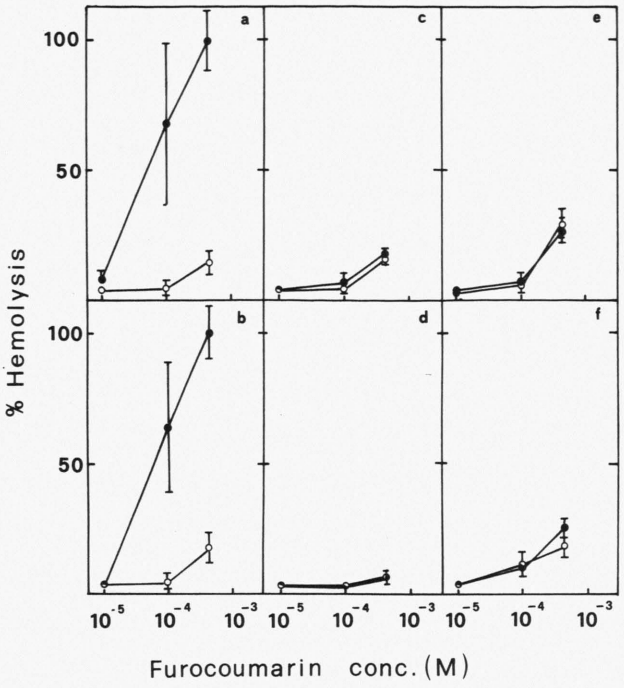

Fig. 2. % hemolysis of RBC caused by various furocoumarins as a function of concentration. ●—● photohemolysis; ○—○ dark hemolysis. a: 3-CPS; b: Ps; c: 5-MOP; d: 8-MOP; e: TMP; f: TMA.

observed. TMP shows the strongest effect, followed by TMA, Ps, 5-MOP and 3-CPs; the effect caused by 8-MOP was much weaker.

In any case, it appears that dark hemolysis is most evident at the highest concentration (4.6×10^{-4} M) which, for furocoumarins, is quite high in biological terms. These compounds are in fact generally used at much lower concentrations in photosensitization experiments and photochemotherapeutic treatments.

Photohemolysis

Fig. 2 also shows that, at a concentration of 1×10^{-5} M furocoumarin, the photohemolytic effect is rather weak, confirming the results obtained by Musajo and Rodighiero [1] who experimented at this concentration; our only exception was 3-CPs which, at this concentration, revealed some useful effects. Photohemolysis gradually increased at higher concentrations (1×10^{-4} M and 4.6×10^{-4} M).

However, taking into account the difference between dark and photohemolysis, 3-CPs and psoralen

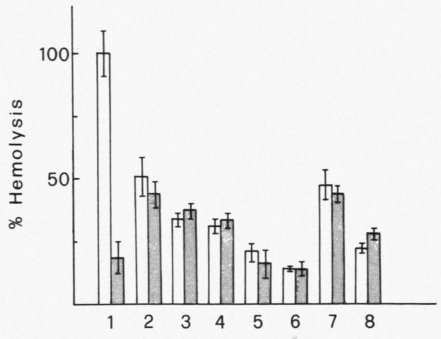

Fig. 3. % hemolysis of RBC caused by various furocoumarins. □ photohemolysis; ▨ dark hemolysis. 1: Ps; 2: BgtOH; 3: 4,8-DMPs; 4: diAcPs; 5: 8-MPs; 6: XtOH; 7: 6,4′-DMA; 8: 4,5′-DMA.

showed strong activity, while TMA showed much weaker effects. The effects of TMP, 5-MOP and 8-MOP under irradiation were practically the same as those in the dark.

The dark and photohemolysis shown by various other furocoumarins used at 4.6×10^{-4} M are shown in Fig. 3. As mentioned above, only Ps and 3-CPs showed strong photohemolysis, while the difference between photo- and dark hemolysis was very small for the other furocoumarins examined (6,4′-dimethylangelicin: 6,4′-DMA; 5-hydroxypsoralen: BgtOH; 8-methylpsoralen: 8-MPs; TMA; 5-MOP). Dark hemolysis was greater than photohemolysis in other compounds such as 4,8-dimethylpsoralen (4,8-DMPs), 5,8-diacetoxypsoralen (diAcPs), TMP, 8-hydroxypsoralen (XtOH) and 8-MOP.

Post-irradiation dark hemolysis

Potapenko *et al.* [11] recently demonstrated the ability of psoralen to cause PIDH by pre-irradiating an ethanol solution of psoralen and then mixing it with a suspension of rat erythrocytes.

We extended our study of PIDH to an ample series of furocoumarins. The results obtained are shown in Fig. 4, in which PIDH is compared with dark hemolysis determined at the same concentration. Ps showed strong PIDH, followed in decreasing order by 6,4′-DMA, 3-CPs, 8-MPs and 4,8-DMPs. The activity shown by 5-MOP, TMA, XtOH, and TMP was much weaker, and that of 8-MOP nil.

The reverse behaviour was shown by diAcPs and 4,5′-dimethylangelicin (4,5′-DMA): dark hemolysis

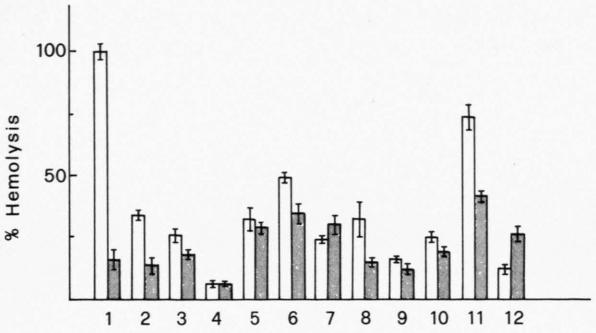

Fig. 4. % of post-irradiation dark hemolysis in ethanol caused by various furocoumarins. □ PIDH; ▨ dark hemolysis. 1: Ps; 2: 3-CPs; 3: 5-MOP; 4: 8-MOP; 5: TMP; 6: 4,8-DMPs; 7: diAcPs; 8: 8-MPs; 9: XtOH; 10: TMA; 11: 6,4′-DMA; 12: 4,5′-DMA.

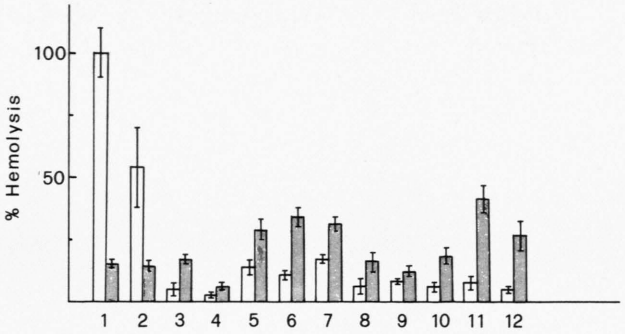

Fig. 5. % of post-irradiation dark hemolysis in isotonic saline solution caused by various furocourmarins. Symbols as in Fig. 4.

was markedly higher than PIDH in these compounds.

Results obtained by studying PIDH in ethanol may be explained by taking into account the fact that, when irradiated in ethanol or aqueous ethanol aerated solutions, furocoumarins undergo photomodification [12].

When PIDH values are higher than those of dark hemolysis, it is suggested that the products of photomodification formed during the photolysis of furocoumarins are more effective in inducing this toxic effect than the same intact furocoumarins (see Fig. 4). Vice versa, when PIDH values are lower than those of dark hemolysis, the products of photolysis of these compounds (diAcPs, 4,5′-DMA) may be less toxic than the intact furocoumarins, so that a decrease in their concentration leads to decreased effects in terms of PIDH.

Post-irradiation dark hemolysis in isotonic saline solution

Parallel experiments were also carried out by preirradiating all the examined furocoumarins in isotonic saline solution.

Results obtained are shown in Fig. 5 which, together with the PIDH data, also gives those on dark hemolysis carried out in the same conditions.

Surprisingly, psoralen and 3-CPs show strong PIDH, while the other furocoumarins give dark hemolysis values which are much higher than those of PIDH.

Conclusions

It has been shown that various furocoumarins are able to induce dark hemolysis in RBC. However, it should be noted that this effect is caused at much higher concentrations of furocoumarins than those used in photosensitizing experiments or photochemotherapeutic treatments. In other words, at high concentrations, even in the dark, furocoumarins may disrupt the bilayer organization of the RBC membrane.

Among the various furocoumarins examined in this study, practically only psoralen and 3-CPs showed strong photohemolytic effects, while the others showed little or no activity.

No correlation may therefore be shown between photohemolytic effects and the skin phototoxicity of the compounds examined. For example, 3-CPs, one of the two most active psoralens in terms of photohemolysis, does not show skin phototoxicity.

Moreover, photohemolysis effects should not be considered as a common property of furocoumarins, but as a peculiar property of some of them, which are able to photoinduce specific damage to the cell membrane of RBC.

Some furocoumarins are able to cause post-irradiation dark hemolysis. This effect too is caused at high furocoumarin concentrations. Bearing in mind that furocoumarins undergo photolysis when irradiated with long ultraviolet light, forming various photolysis products [13], it may be that precisely these photoproducts cause hemolysis.

The differences observed between the various compounds examined here may be ascribed to their different toxicity in terms of hemolysis.

As a general conclusion, it appears that furocoumarins may target the cell membrane in their photosensitizing processes. They may show toxic effects both in the dark and after activation with light. However, the high furocoumarin concentration required to induce hemolysis suggests that the latter toxic effect should not play an important role, in terms of side-effects, in photochemotherapeutic treatments.

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