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The importance of light in the anti-HIV effect of hypericin

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Summary

The requirement for light in the anti-HIV-1 activity of hypericin was investigated. The hypericin concentration-dependence and light dosage-dependence of the reaction were measured. Under conditions in which hypericin caused substantial inactivation of HIV-1, there was a strict requirement for visible light. Only when the concentration of hypericin approached the cytotoxic level was there an apparent light-independent antiviral effect. This strict light-requirement for the antiviral effect could explain some of the apparently discrepant results reported by other workers. Furthermore if hypericin is contemplated for use in humans, the importance of light must be considered.

Hypericin; Light; HIV-1

Several groups of investigators have recently reported antiviral activities of the condensed anthraquinone, hypericin (Meruelo et al., 1988; Lavie et al., 1989; Schinazi et al., 1990; Tang et al., 1990; Kraus et al., 1990; Hudson et al., 1991; Anderson et al., 1991). There are however apparent discrepancies between some of these results, and it is conceivable that these discrepancies are due to lack of regard for the light exposure in the various hypericin-virus reactions (Lopez- Bazzochi et al., 1991; Carpenter and Kraus, 1991).

Light is known to be required for the bioactivities of hypericin, since the compound is a photodynamic pigment, which occurs in several species of

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Hypericum (Giese, 1980; Towers, 1984; Knox and Dodge, 1985), and it has been shown that in the presence of visible light, hypericin produces singlet oxygen which can damage membranes (Duran and Song, 1986). Furthermore livestock which consume hypericin-containing foliage in sunny environments can suffer phototoxicity (Ivie, 1982); this is relevant to prospective clinical application.

Most of the fluorescent lamps found in virology laboratories, and in biosafety cabinets, would generate sufficient energy of the appropriate wavelength to photosensitize hypericin and consequently would generate singlet oxygen. If on the other hand hypericin did have significant antiviral activity in the dark, this would suggest alternative pathways and mechanisms not involving singlet oxygen (Hudson, 1989; Lopez-Bazzochi et al., 1991).

In view of these considerations, we decided to analyze the light requirement for the anti-HIV activity of hypericin in more detail.

Hypericin was extracted from the aerial parts of *Hypericum perforatum*, and purified, as described previously (Hudson et al., 1991). It was stored in ethanol at 4°C in dark vials.

The LAV-1 strain of HIV-1, obtained from L. Montagnier (Institut Pasteur, Paris), was propagated and assayed in CEM cells, as described (Schwartz et al., 1988). Stock virus (10^6TCD$_{50}$ per ml) was stored at −70°C as clarified cell-free supernatant, and was thawed and diluted into complete medium (usually with 10% v/v fetal bovine serum) when required.

For photochemical treatments 0.5 ml aliquots of virus, containing 10^3TCD$_{50}$, were added to wells of a 24-well tissue culture tray (Falcon). The hypericin, 1.0 mg/ml in 95% ethanol, was diluted to the desired concentration when required, and 0.5 ml was added to each well. The final concentration of ethanol was 0.1% or less, and this had no effect on HIV-1, ± light. The trays were then covered to exclude light. Following a 30-min preincubation in the dark, virus-hypericin mixtures were exposed to a pair of F12T12CW fluorescent lamps situated at a known distance below the bottom of the tray. The incident radiation, as determined by means of a radiometer, was 5 W/m^2, and the peak emission of the lamps was around 580 nm, coincident with a major absorption peak of hypericin (Giese, 1980; Knox and Dodge, 1985). For the standard exposure time of 30 min (at 20°C) the total incident radiation amounted to 9 Kjoules. No radiation below 400 nm could be detected by means of ultraviolet photometers. After the 30-min exposure to light, 4×10^5 CEM cells, in 1.0 ml of complete medium, were added to each well. Cultures were covered with aluminum foil and maintained at 37°C in a normal cell culture incubator.

Various controls were included. Some culture trays were covered with foil during the exposure to the lamps, to exclude light during the virus-hypericin reaction. Others contained virus only, ± light, or cells only.

All cultures were examined periodically under the microscope to monitor the progress of cpe. The latter was assessed independently by two observers. Every 4 days half of the medium in each well was removed and replaced by complete

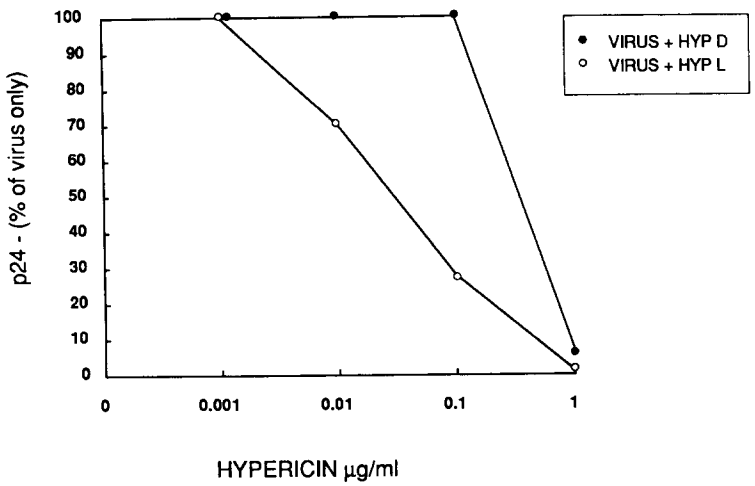

Fig. 1. Anti-HIV effect at different hypericin concentrations, ± light. Aliquots of HIV-1 (10^3TCD_{50}) were incubated with various concentrations of hypericin in light (open circles) or dark (closed circles), under standard conditions, and the mixtures were inoculated into CEM cell cultures. At 6 days p.i. supernatants were removed for p24 assays. 10 μg/ml was cytotoxic in light and dark.

fresh medium (without hypericin). Portions of the supernatants were removed as required for p24 assays.

The relative amounts of HIV-1 p24 protein synthesized in the cultures were determined, on duplicate culture supernatants, by the Abbott HIVAG ELISA system. Absorbance readings were converted to p24 protein concentration by comparison with Abbott standards.

A previous report showed that the antiviral effect of hypericin was considerably enhanced in the presence of light (Hudson et al., 1991). In order to demonstrate specific dependance on light and the compound, it was necessary to show how anti-HIV activity varied with both light dosage and hypericin concentration.

Fig. 1 illustrates the effect of hypericin concentration in the presence and absence of light. Significant antiviral activity was evident at 0.01 μg/ml ($\sim 2 \times 10^{-8}$ M) in light, and more than 75% inactivation was obtained with 0.1 μg/ml + light. In contrast 0.1 μg/ml in the dark showed no antiviral effect whatsoever. These conclusions were substantiated by microscopic assessment of cpe (not shown).

Hypericin concentrations of 0.5–1.0 μg/ml (Fig. 1) in light completely inactivated the virus. In the dark 0.5 μg/ml usually gave little or no effect, whereas 1.0 μg/ml in the dark did invariably cause some inactivation. When the CEM cells themselves were exposed to different concentrations of hypericin + light, but without virus, the cultures looked quite normal with up to 1.0 μg/ml (light or dark) at least for 6 days. At 10 μg/ml, however, the compound was clearly cytotoxic, with and without light.

Table 1 shows the effect of light dosage on the antiviral activities (in the

TABLE 1

Time of exposure to light

Time (min)	% p24[1] synthesized	Viral cpe (% cells)[2]
0	100	75
5	100	70
10	100	70
20	45.3	40
30	48.1	40
60	< 2	0

[1]100% value (untreated virus) = 134 ng/ml measured at 5 days p.i.;
[2]At 5 days p.i. Reactions carried out with hypericin (0.1 μg/ml) in the dark, or without hypericin but + light, were indistinguishable from untreated virus control.

presence of 0.1 μg/ml hypericin). Between 0 and 10 min exposure to the fluorescent lamps had no effect on the virus, which subsequently produced cpe and p24 synthesis at rates that were indistinguishable from untreated virus. 20–30 min exposure, however, caused considerable inactivation of virus, while 60 min exposure (twice the usual standard dose) gave complete inactivation. Hypericin + 60 min in the dark gave no decrease in viral parameters.

Fig. 2 shows the kinetics of virus production, as determined by p24 protein synthesis, following hypericin treatment (0.1 μg/ml) ± light. In the absence of light exposure, normal kinetics of p24 synthesis were seen (open and closed

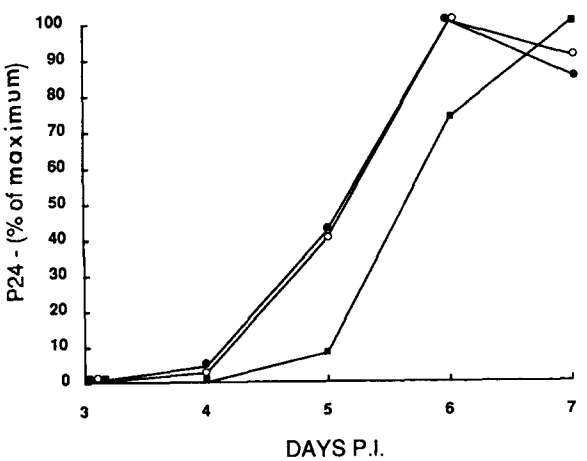

Fig. 2. Kinetics of p24 production. Aliquots of HIV-1 were incubated with or without hypericin, ± light, and the mixtures were inoculated into cell cultures. Starting at 3 days p.i., portions of the culture supernatants were removed, diluted appropriately, and assayed for p24. All cultures were monitored for cpe progress. The p24 values are expressed as percentages of the appropriate maximum. The virus (+ light, − hypericin) cultures showed the same degree of cpe as virus (− light, − hypericin); therefore only the latter were assayed for p24. The baseline values represent <2% of maximum, corresponding to the virus inoculum. Symbols: closed circles, virus (dark) − hypericin; open circles, virus (dark) + hypericin; squares, virus + light + hypericin.

circles), with peak levels at 6 days p.i. The levels decreased after this time. In contrast, virus treated with hypericin + light showed a significant delay in p24 production, indicating substantial inactivation of the original virus, although the maximum level was eventually attained at 7 days p.i. (Fig. 2) followed by a decrease after this (not shown). These conclusions were supported by cpe assessments. These results confirm the importance of light in the antiviral activity of hypericin.

Other workers had previously shown that hypericin could adversely affect certain enzymes, e.g., protein kinase C and reverse transcriptase, although none of these studies examined the role of light (Takahashi et al., 1990; Meruelo et al., 1988; Schinazi et al., 1990). It was for this reason that we decided not to use HIV-1 reverse transcriptase to compare relative virus production. In order to be certain that our p24 measurements were valid, we investigated the effect of hypericin, ± light, on the p24 antigen content of known amounts of HIV-1. However, hypericin showed no effect on the p24 antigen, in light or dark, up to 0.1 μg/ml, although 0.5 μg/ml was slightly inhibitory (20% decrease ± light).

In recent studies on the antiviral effects of photosensitizers, we found that the efficacy of a compound could be significantly decreased by the presence of bovine serum in the reaction mixtures, apparently due to serum components binding the compound (Hudson et al., 1992). Since our HIV-1 stocks are normally stored in serum-containing medium, to conserve viability, we examined the effect of serum removal on antiviral activities in light and dark. This was done by pelleting a stock of HIV-1 and resuspending the virus in medium with or without serum.

The virus preparation without serum did show substantially more anti-HIV effect with hypericin (0.1 μg/ml) + light than did virus to which serum had been added back; but when light exposure was omitted there was still no anti-HIV effect in the absence of serum (data not shown), i.e. the absence of a dark-reaction could not be attributed to the presence of interfering serum components.

Thus the experiments described in this report showed quite clearly that at concentrations of hypericin below 1 μM light was essential for the virucidal activity against HIV-1.

Unfortunately the recent reports on antiviral activities have not taken into account a role for light, and in fact it is not clear from these reports whether virus-hypericin reactions were conducted in the presence of defined doses of light, ambient light, or in the dark (Meruelo et al., 1988; Lavie et al., 1989; Schinazi et al., 1990; Tang et al., 1990; Kraus et al., 1990; Anderson et al., 1991).

Furthermore several of these reports contained apparent discrepancies between the antiviral results and it is possible that some of these could be explained by different lighting conditions. It is also possible that hypericin may have additional light-independent activities that operate by different mechanisms, and this could explain the observed antiviral effects in animals that were given hypericin and virus at different times (Meruelo et al., 1988). Nevertheless,

if hypericin is to be used in humans, the importance of light must be considered.

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