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THE ROLE OF OXYGEN IN THE PHOTOINDUCED ANTIVIRAL ACTIVITY OF HYPERICIN

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ABSTRACT

Hypericin displays photoinduced antiviral activity. We examine the photoinduced antiviral activity of hypericin under both oxygenated and hypoxic conditions and observe that hypericin is equally toxic under both conditions. These results indicate that while singlet oxygen may play a role in the antiviral activity of hypericin, it does not play a major role.

INTRODUCTION

The naturally occurring polycyclic quinone, hypericin (Figure 1), possesses important and diverse types of biological activity.[1] It has been shown that hypericin inactivates the human immunodeficiency virus (HIV).[2-5] That antiviral activity requires light was first demonstrated in a lentivirus closely related to HIV, equine infectious anemia virus (EIAV), by Carpenter and Kraus.[6] Hypericin produces singlet oxygen very efficiently (with a quantum yield of 0.73[7]), and many studies have suggested that its antiviral activity is due to the production of singlet oxygen.[2-5]

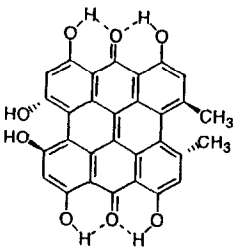

Figure 1. Structure of hypericin.

The excited-state reactivity of hypericin, however, extends well beyond the photosensitization of oxygen to form singlet oxygen.[8-12] Elucidating the reactivity of excited-state hypericin and the subsequent reactivity of molecules that it encounters is essential for understanding the light-induced mechanism of antiviral activity. The mechanism of photosensitization by a given molecule in its excited state can be

classified into two types of processes.[13,14] In Type I processes, the excited-state photosensitizer interacts first with the substrate, which may go on to react with another reagent, which is usually oxygen. In Type II processes, the excited-state photosensitizer interacts first with oxygen, thus producing singlet oxygen, which subsequently goes on to react with the substrate.

We[15,16] have questioned the relative importance of singlet oxygen in the toxicity of hypericin towards HIV and related viruses. We have provided the first detailed investigation that uses both ~1-ps time resolution and a white-light continuum to examine and to unravel the excited-state primary photoprocesses of hypericin and have suggested that the excited-state transients we observe, coupled with data from model compounds, can be interpreted in terms of tautomerization.[15,16] The results and conclusions of these time-resolved studies are of particular interest in the context of earlier observations and suggestions of Song and coworkers[17-19] that the excited states of hypericin-like chromophores produce protons upon photoexcitation. We thus proposed that deprotonation of the tautomer results in the reported pH decrease. Whether such processes are in fact responsible for the antiviral activity of hypericin is as yet uncertain, but it is clear that a detailed investigation of the excited-state chemistry of hypericin is required in order to unravel the mechanism of antiviral activity. In order to determine the relative importance of oxygen for the antiviral activity of hypericin, we have performed experiments where EIAV was challenged with hypericin and light under hypoxic conditions.

EXPERIMENTAL

Titration of infectious virus: All experimental manipulations were done in subdued light. Cell-free stocks of the MA-1 isolate of EIAV[20,21] containing approximately 10^5 focus-forming units/ml (FFU/ml) of EIAV, were diluted 1:10 in Hank's buffered saline solution (HBSS). Hypericin (Carl Roth GmbH & Co.) was added to a final concentration of 10 μg/ml.

Illumination and deoxygenation: Deoxygenation of samples (Table I) by bubbling either N_2 or Ar was done in light-tight containers. Hypericin/EIAV samples were exposed to light from a 300 W projector bulb fitted with a cut-off filter blocking wavelengths shorter than 575 nm. The irradiance at the sample was estimated to be 170 W/m^2 in the spectral range in which hypericin absorbs, 575-600 nm. Alternatively, deoxygenation was effected by using β-carotene (Sigma) as a singlet oxygen scavenger[13,22] (Table II). In neither case was oxygen seen to be required for antiviral activity. Solutions of β-carotene were made up in a Hank's medium/ethanol mixture (95/5 v/v). Samples of EIAV, hypericin, and varying aliquots of the β-carotene mixture were irradiated at 598 nm (24-nm bandpass, ~100 W/m^2) for 20 min. with a Photon Technology International 150-W lamp (LPS-220) coupled to a monochromator.

Oxygen assays: Deoxygenation efficiency was evaluated by two separate techniques. A dissolved oxygen test kit (Hach, OX-2P) showed dissolved oxygen levels after one hour of deoxygenating to have fallen from an initial concentration of 5 mg/L (1.56×10^{-4} M) to below the detection limit of 0.2 mg/L (6.25×10^{-6} M). An alternate method of testing for dissolved oxygen is via the bioluminescence of the firefly luciferase/luciferin reaction. Oxygen is necessary in this system for the production of light.[23] Light output was measured with a liquid-nitrogen cooled charge-coupled device (CCD) (Princeton Instruments LN/CCD-1152UV) mounted on an HR320 (Instruments SA, Inc.) monochromator with a grating (1200g/mm) blazed at 5000 Å. A solution of 1.0×10^{-5} M luciferin and 1.6×10^{-8} M luciferase and a solution of 1.0×10^{-4} M ATP were simultaneously deoxygenated in the same apparatus as the EIAV/hypericin experiments. The reaction was initiated by injecting 0.5 ml of the deoxygenated ATP

solution into the luciferin/luciferase solution. Three successive 30-second integrations yielded spectra that were superimposable on the background spectra of the CCD. Light could be generated from the reaction system by opening it to the atmosphere. Lack of light generation was taken to indicate that oxygen levels were negligible.

A focal immunoassay similar to that previously described was used for quantitation of infectious virus.[6] Results are expressed in terms of FFU/ml supernatant.

RESULTS AND DISCUSSION

TABLE I
Effect of Oxygen on the Antiviral Activity of Hypericin

Illumination time (min) [a]	Infectious virus (FFU/ml)	
	oxygenated [b]	hypoxic [c]
0	10,200	14,500 (N_2)
10	0	25 (N_2)
0	2475	5975 (Ar)
10	0	225 (Ar)

[a] Illumination was effected with a 300 W projector bulb fitted with a cut-off filter blocking wavelengths shorter than 575 nm.

[b] The oxygen content of the sample was determined by letting it equilibrate with the atmosphere.

[c] Hypoxic conditions were obtained by passing argon or nitrogen gas over the samples for 1 hour before and during illumination.

Tables I and II compare the antiviral activity of hypericin under aerobic and hypoxic conditions. Hypericin is toxic in the presence and the absence of oxygen (although the data suggest that hypericin in a hypoxic environment does not inactivate EIAV as effectively as in an oxygenated environment.) While these results do not unambiguously rule out the Type II mechanism, they do demonstrate the importance of the Type I mechanism, particularly that direct interaction of hypericin itself with the virus is important for the remarkable antiviral properties of hypericin. It is of interest that Meruelo and coworkers[5] report that sodium azide (NaN_3), which is believed to scavenge singlet oxygen--as well as to quench other oxidants and sensitizer excited-states,[13,22] inhibits the light-induced activity of hypericin to inhibit reverse transcriptase activity of murine Radiation leukemia virus (RadLV). This argument, however, is based upon the observation that this effect occurs only if NaN_3 is allowed to incubate 10 min. with the sample

before the introduction of hypericin. They argue that since addition of NaN_3 subsequent to a 30-min. incubation with hypericin and exposure to light has little effect on the toxicity that singlet oxygen is responsible for the antiviral activity. An alternative interpretation of the former result is that preincubation of NaN_3 facilitates quenching of the excited state of hypericin and consequently inactivates antiviral activity, which is not necessarily based upon singlet oxygen.

Table II
Effect of a Singlet Oxygen Scavenger on the Antiviral Activity of Hypericin

[β-carotene] (μM)	Infectious virus with hypericin (FFU/ml)	Infectious virus without hypericin (FFU/ml) [a]
80	0	3000
64	0	4800
48	0	6400
32	0	8400
16	0	> 10,000
0	0	10,000

[a] Control experiments.

The antiviral mechanism and the target of hypericin activity are as yet unclear. Meruelo and coworkers[3,4] have observed that in the presence of light hypericin induces significant changes in the HIV capsid protein, p24, and the p24-containing *gag* precursor, p55, as indicated by Western blot analysis. They have also observed that recombinant p24 in the presence of light forms an anti-p24 immunoreactive material of a molecular weight of 48 kilodaltons. They have consequently suggested that cross-linking and other alterations of p24 occur and that such alterations may inhibit the release of reverse transcriptase activity. It is significant that these workers found that under ambient lighting conditions, hypericin did not inhibit the binding of gp120 to CD4 cells and that it did not inhibit the formation of syncytia (large, abnormal multinucleated cells formed by the fusion of infected cells with uninfected CD4 cells).[3,4] On the other hand, inhibition of gp120 binding was observed under conditions of more intense illumination: i.e., when samples were placed ~10 cm away from a fluorescent light source for 30 minutes.[3] The highest concentration of hypericin used by Meruelo and coworkers is 2 μg/ml (~4 μM). Lenard et al.[4], on the

other hand, observed that syncytia formation was inhibited by illumination for 1 hour in the presence of 1 μM hypericin. Because of the similarity in behavior between hypericin and the photosensitizer, rose bengal, Lenard et al. concluded that singlet oxygen was the essential factor in toxicity, even though hypericin proved itself to be much more toxic. These workers quoted an irradiance of 800-900 footcandles, which is 12-14 W/m^2 at 555 nm. This latter figure is an underestimate of the energy that is actually available to be absorbed by hypericin since footcandles are a measure of visible light, whose detectability by eye is a maximum at ~555 nm. In other words, the figure that is quoted[4] gives no information on the irradiance over the spectral range utilizable by hypericin (and transparent to glass) from about 300 to 600 nm. If we assume that the radiant energy provided by the source in this example is constant over this spectral range, then the sample is actually exposed to an irradiance of 3700-4200 W/m^2.

It is useful to consider our experiments in the context of others. The toxicity to *S. coerulus*[17] and to syncytia[3-5] under high, but not ambient or low, light flux and the ability of hypericin to kill HIV and EIAV under conditions of low or ambient light flux suggest that several mechanisms of toxicity may be involved. The relative importance of Type I and II mechanisms in virus inactivation may depend on the intensity of light. Under high irradiance, the formation of singlet oxygen by a Type II mechanism would be predominant, whereas under low irradiance the antiviral activity would be primarily due to a Type I reaction. Such reasoning may explain the differences among investigators in characterizing the antiviral mechanism of hypericin cited above. At high irradiance, there is both inhibition of gp120 binding and inhibition of cell fusion,[3,4] events that depend on interactions between virus and cell membranes. Interestingly, the effects are not reported to occur at low irradiance,[3] where inhibition is associated with alterations in the viral capsid protein. This may indicate that Type II reactions target the viral membrane, whereas Type I reactions may attack other stages in the life cycle of the virus. This may also explain the absolute dependence on oxygen for the anti-tumor effect of hypericin.[14] Thomas and Pardini[14] found that hypericin uptake in EMT6 mouse mammary carcinoma cells was associated with membrane components, both at the cell surface and in intracellular organelles. Although they did not discuss the mechanisms of hypericin-induced cell killing, their observation of an absolute dependence on oxygen for the anti-tumor effect of hypericin in the presence of light (20-30 W/m^2) suggests that toxicity is due to membrane-associated damage produced by singlet oxygen. Much evidence exists that singlet oxygen is the primary mechanism of hypericin-mediated damage to cell membranes.[1]

Our results indicate that under conditions of ambient light (low intensity), oxygen is not required for hypericin to exhibit antiviral activity. While it is certainly likely that singlet oxygen may play a role, especially under conditions of high hypericin (and oxygen) concentrations or intense light fluxes, it is not essential. Much work remains in order to determine whether it is the singlet or triplet state of hypericin that is responsible for its antiviral activity and to determine the mechanism of this activity.

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