Photodynamic inactivation of infectivity of human immunodeficiency virus and other enveloped viruses using hypericin and rose bengal: Inhibition of fusion and syncytia formation

(vesicular stomatitis virus/influenza virus/Sendai virus/hemolysis)

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ABSTRACT The mechanism of the antiviral activity of hypericin was characterized and compared with that of rose bengal. Both compounds inactivate enveloped (but not unenveloped) viruses upon illumination by visible light. Human immunodeficiency and vesicular stomatitis viruses were photodynamically inactivated by both dyes at nanomolar concentrations. Photodynamic inactivation of fusion (hemolysis) by vesicular stomatitis, influenza, and Sendai viruses was induced by both dyes under similar conditions (e.g., I50 = 20–50 nM for vesicular stomatitis virus), suggesting that loss of infectivity resulted from inactivation of fusion. Syncytium formation, between cells activated to express human immunodeficiency virus gp120 on their surfaces and CD4+ cells, was inhibited by illumination in the presence of 1 μM hypericin. Hypericin and rose bengal thus exert similar virucidal effects. Both presumably act by the same mechanism—namely, the inactivation of the viral fusion function by singlet oxygen produced upon illumination. The implications of this photodynamic antiviral action for the potential therapeutic usefulness of both hypericin and rose bengal are discussed.

As the worldwide epidemic of human immunodeficiency virus (HIV) infection and AIDS grows, the rapid development of effective, affordable agents with anti-HIV activity is an urgent need. To date, the most effective anti-HIV agents have been nucleoside analogs that inhibit the HIV reverse transcriptase (RT). Recently, much attention has been given to classes of potential antiviral agents that target other aspects of the viral life cycle. In this regard, hypericin has been regarded as a promising potential anti-AIDS drug since 1988, when it was reported that a single injection of this compound prevented splenomegaly and death in mice infected with Friend leukemia virus. Hypericin was “coadministered” with the virus; 50% survival was conferred by 10 μg of hypericin, whereas 150 μg was needed for complete protection (1, 2). In addition, biweekly injections of hypericin into mice greatly reduced the viremia resulting from infection with the slower acting LP-BM5 murine leukemia virus (2). Others, however, could not replicate some of these results (ref. 3, note added in proof; N. R. Stevenson and J.L., unpublished results).

Subsequent reports rapidly established two important facts: (i) hypericin inactivated a wide variety of lipid-containing (i.e., enveloped) viruses, but was ineffectiveness against viruses lacking membranes (3–7); and (ii) light increased the virucidal potency of hypericin against several different enveloped viruses by at least 100-fold (6, 7).

Microscopic observations of hypericin’s intrinsic red fluorescence in cultured cells showed it to be localized in plasma membranes (2). This suggests that hypericin acts in a manner quite different from the well-known nucleoside analogs. Observations of hypericin’s activities in vitro have led to suggestions that it may act on viral assembly (1, 2) or by inhibiting RT (4). Since these experiments did not control light as a variable, their interpretation is difficult.

Hypericin has a long and well-documented history as a photodynamic compound. It is the active principle contained in plants of the Hypericum genus; ingestion by grazing animals causes photopoeision (hypericin) leading to skin irritation, fever, and death (8, 9). Hypericin’s photodynamic activity against membranes in vitro was also noted very early, when it was found to be a promoter of light-induced hemolysis (H. F. Blum, quoted in ref. 10). In this regard it resembled rose bengal, another potent inducer of photodynamic hemolysis (11–13).

The similarities between hypericin and rose bengal extend to their mechanism of action. Both compounds catalyze the light-induced formation of singlet oxygen, a highly reactive oxidizing species that is largely or completely responsible for their membrane-disruptive effects (14–16). In a further parallel, rose bengal has been shown to inactivate enveloped viruses upon illumination. The photodynamic inactivation of vaccinia virus by rose bengal was attributed to alterations in viral proteins, as opposed to nucleic acids (17). Rose bengal’s virucidal activity against herpes simplex virus was noted as a complication attending its use in a common diagnostic procedure for ocular disease (18). However, in contrast to hypericin, rose bengal has not previously been evaluated as a potentially useful antiviral agent.

In view of these very similar properties of hypericin and rose bengal, a comparison of their effects on enveloped virus

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Abbreviations: HIV, human immunodeficiency virus; VSV, vesicular stomatitis virus; RT, reverse transcriptase; PMA, phorbol 12-myristate 13-acetate.
infectivity and fusion was carried out. Fusion was chosen for study because it is the membrane-specific process that all enveloped viruses must perform. As reported below, both compounds appear to act by the same mechanism. Both are potent inhibitors of fusion by all the enveloped viruses studied. Loss of fusion activity appears to account for the inactivation of infectivity of both vesicular stomatitis virus (VSV) and HIV, induced in the presence of light, by either of these photodynamic agents.

MATERIALS AND METHODS

Viruses. Sendai (Z strain) and influenza (APR/8/34 strain) viruses were grown in 10- to 11-day-old embryonated eggs and purified on 5–40% potassium tartrate gradients. VSV Indiana (Birmingham strain) was grown in BHK-21F cells and purified as described (19). HIV-1 (LAV strain) was prepared by passage in phytohemagglutinin-stimulated human peripheral blood lymphocytes and was a kind gift of Kathleen Clouse and the Viral Biology Unit (Georgetown University).

Reagents. Rose bengal was obtained from Sigma, and hypericin was from Sigma or Atomergic (Farmingdale, NY). Stock solutions of rose bengal (100 μM) were made up in water. Stock solutions of hypericin (400 μM) were made up by adding 1 vol of methanol or dimethyl sulfoxide to the solid compound, heating gently as necessary (especially with methanol) to dissolve, and then adding 9 vol of phosphate-buffered saline at pH 7.2 (PBS) containing 0.1% Tween 80 (5). Samples were prepared under conditions of reduced light; that is, lights immediately overhead were turned off, but incident light remained sufficient to carry out the required operations (measured illumination, <1 footcandle; 1 footcandle = 10.76 lx).

Illumination. The purified or stock viruses were suspended in translucent plastic tubes or plate wells in 100 μl of PBS containing the desired concentrations of hypericin or rose bengal. Illumination was carried out for 1 h in ice by using a standard fluorescent desk lamp (containing two Philips F15 T8/CW 15-W bulbs) placed 5 cm above the sample. Illumination was 8–900 footcandles, as measured with a Spectra Caneda light meter.

Hemolysis. After illumination of the virus sample, 1.4 ml of buffer and 0.1 ml of a 10% suspension of fresh washed human erythrocytes in PBS were added, and the sample was mixed. PBS was used for Sendai virus-induced hemolysis. Citric acid/phosphate buffer at pH 5.0 (20) was used for influenza virus- and VSV-induced hemolysis; the buffer contained DEAE-dextran (3 mg/ml) for the VSV experiments (21). The suspension was incubated in the dark for 1 h on ice, followed by 1 h at 37°C. The samples were then centrifuged at 1200 × g for 10 min at 4°C, and absorbance of the supernatant was measured at 590 nm. Values corresponding to complete (100%) hemolysis were determined in each experiment from samples lysed in distilled water. The amount of virus used in each experiment (1–5 μg of virus protein) was controlled so as to produce 30–80% hemolysis under these conditions.

Infectivity. Stock VSV was illuminated in the presence of hypericin or rose bengal as described above and then used to infect BHK-21F cells at a multiplicity of infection of 5. The infection was allowed to proceed overnight. The cell medium was then collected, serially diluted, and titrated for plaque-forming units on BHK-21F cell monolayers. Similarly, HIV-1 was treated with hypericin or rose bengal, illuminated, and then added to 4 × 10⁵ MT-4 cells (22) at a multiplicity of infection of 0.01 in 1.5 ml of RPMI 1640 with 10% (vol/vol) fetal calf serum (GIBCO/BRL) in 24-well plates. Culture medium was changed on days 4 and 6, and aliquots of culture supernatant were harvested daily for RT assay. Cell viability was determined by trypan blue dye exclusion.

HIV RT Assay. HIV production in infected cultures was determined by a 32P-based RT assay previously described (23) in which samples were incubated with RT cocktail for 1.5 h and then spotted on DEAE paper and washed as described. RT activity was determined by quantitation of 32P bound to the filter by using a Molecular Dynamics phosphorimager following a 3-h exposure to a phosphorimaging screen.

HIV gp120-Induced Synctium Formation. A culture of ACH-2 cells was induced to active production of HIV by incubation with phorbol 12-myristate 13-acetate (PMA) as described (24, 25). After 24 h of induction, 4 × 10⁴ ACH-2 cells were suspended in 100 μl of buffer in a microtiter dish, treated with hypericin or rose bengal with or without illumination, then mixed with 4 × 10⁵ Sup-T1 cells (a CD4⁺ T-cell line that forms large syncytia upon HIV infection; ref. 26), and incubated overnight at 37°C; “dark samples” were wrapped in foil. Syncytia were identified by microscopic examination, and those exceeding a minimum size of four cell diameters were counted.

SDS/PAGE. SDS/PAGE was carried out by the method of Laemmli (27) using 10% polyacrylamide gels with 6% stacking gels.

RESULTS

Inactivation of Infectivity. Stock preparations of HIV or VSV were mixed with various concentrations of hypericin or rose bengal and illuminated prior to infecting their respective host cells as described. Dark samples were prepared and incubated identically, but were shielded from the light by wrapping in aluminum foil. Results with VSV are shown in Fig. 1, and those with HIV are shown in Fig. 2. Both viruses lost infectivity upon illumination with either photosensitizer, in a concentration-dependent manner.

The concentration of hypericin or rose bengal required to cause 50% inactivation (I₅₀) under standard illumination conditions is shown in Table 1. Values for VSV inactivation were 20 nM hypericin and 50 nM rose bengal. The I₅₀ value for loss of HIV infectivity depended upon the time of assay. Thus, on day 3 postinfection I₅₀ for rose bengal was = 8 nM, while on day 5 postinfection it was 50 nM; a similar increase occurred for hypericin between day 5 and day 7 postinfection. The higher I₅₀ on later days resulted because the lowest concentrations delayed, but did not completely prevent, new virus production (Fig. 2). This delay was due to a 3-log reduction in infective titer in the viral stock, induced by the drug treatment and illumination (data not shown). Complete inhibition of HIV infectivity was achieved by using 30 nM

Fig. 1. Photodynamic inactivation of infectivity of VSV by hypericin (A) or rose bengal (B). pfu, Plaque-forming units.
hypericin or 100 nM rose bengal. No inactivation was seen in the presence of 1 μM hypericin or rose bengal in the absence of light. Further, growth of MT-4 cells was not inhibited by the illuminated HIV stocks containing 1 μM hypericin or rose bengal, showing that inhibition of HIV replication was due to effects on the virus, not on the cells.

Inactivation of VSV, Influenza Virus, and Sendai Virus-Induced Hemolysis. In view of the broad antiviral activity demonstrated by hypericin and by rose bengal against enveloped but not nonenveloped viruses, it was of interest to determine the effects of these agents on viral fusion. This is conveniently measured for many viruses by virus-induced hemolysis (21, 28, 29), since fusion of the viral envelope with the erythrocyte membrane is an essential prerequisite to leakage of hemoglobin. To test for photodynamic inactivation of viral fusion, therefore, hypericin or rose bengal was added to purified virus preparations and illuminated as described; hemolytic activity was then assayed in the dark. As shown in Fig. 3, the fusion activity of VSV and influenza and Sendai viruses was inactivated by nanomolar concentrations of both drugs. Inactivation was absolutely dependent upon light (Fig. 3) and increased with increasing time of illumination (data not shown). I₅₀ values differed for each virus/photosensitizer combination, but most fell in the 20–80 nM range at the virus concentration used (Table 1).

It was possible that the inactivation of fusion shown in Fig. 3 might have resulted from an impaired ability of the virus to bind to the erythrocyte cell surface. To test for this, hemagglutination titers were determined for aliquots of influenza and Sendai viruses that had been photodynamically treated as in Fig. 3. Hemagglutination was unaffected by any concentration of either dye (data not shown). This indicates that fusion rather than binding was inactivated by the photodynamic action of the dyes.

The susceptibility of erythrocytes to direct photodynamic hemolysis (i.e., in the absence of virions) by hypericin and rose bengal were also measured. Both reagents induced direct photodynamic hemolysis (data not shown), confirming previous reports (10–13). I₅₀ values were much higher than those for viral inactivation of hemolysis, however (Table 1). It should be noted that the drug concentrations shown in Fig. 3 were those that were present during illumination of the

Table 1. I₅₀ values for photodynamic effects of rose bengal or hypericin on hemolysis, infectivity, and protein cross-linking

<table>
<thead>
<tr>
<th>Virus</th>
<th>Function</th>
<th>Hypericin I₅₀ (±50%), nM</th>
<th>Rose bengal I₅₀ (±50%), nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Hemolysis at pH 7.2</td>
<td>14,000</td>
<td>350</td>
</tr>
<tr>
<td></td>
<td>Hemolysis at pH 5</td>
<td>6700</td>
<td></td>
</tr>
<tr>
<td>VSV</td>
<td>Inactivation of hemolysis</td>
<td>20</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Inactivation of infectivity</td>
<td>25</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Cross-linking of G</td>
<td>1,000</td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>Cross-linking of M</td>
<td>5,000</td>
<td>1000</td>
</tr>
<tr>
<td>HIV-1</td>
<td>Inactivation of infectivity</td>
<td>&lt;10 (5)*</td>
<td>8 (3)*</td>
</tr>
<tr>
<td>Inf</td>
<td>Inactivation of hemolysis</td>
<td>20 (7)*</td>
<td>50 (5)*</td>
</tr>
<tr>
<td>Sendai</td>
<td>Inactivation of hemolysis</td>
<td>220</td>
<td>35</td>
</tr>
</tbody>
</table>

Inf, influenza.

*The numbers in parentheses are the days at which the assay was performed.
virus; as described in Materials and Methods, they were diluted 15-fold prior to the hemolysis assay.

Inactivation of HIV gp120-Induced Syncytium Formation. Viral fusion activity may also be assayed by the formation of cell syncytia mediated by cells expressing the viral fusion protein on their plasma membranes. For HIV, syncytia formation involves the fusion of uninfected CD4+ cells with cells expressing cell surface HIV gp120. ACH-2 cells are a chronically infected T-cell line in which high levels of HIV protein expression can be induced by treatment with phorbol

Table 2. Effect of hypericin or rose bengal and light on syncytium formation by HIV-producing ACH-2 cells with SUP-T1 cells

<table>
<thead>
<tr>
<th>Treatment of ACH-2 cells*</th>
<th>Syncytia per plate†</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>155 ± 34</td>
</tr>
<tr>
<td>None (no PMA)</td>
<td>3 ± 3</td>
</tr>
<tr>
<td>1 μM hypericin + light</td>
<td>4 ± 4</td>
</tr>
<tr>
<td>1 μM hypericin (dark)</td>
<td>227 ± 64</td>
</tr>
<tr>
<td>1 μM rose bengal + light</td>
<td>38 ± 3</td>
</tr>
<tr>
<td>1 μM rose bengal (dark)</td>
<td>205 ± 17</td>
</tr>
</tbody>
</table>

*Treatment 24 h after induction of HIV with PMA, except as indicated.
†Average ± SD of three plates.

esters (24, 25). Induced ACH-2 cells efficiently form syncytia when incubated with Sup-T1 cells (26). ACH-2 cells were treated with hypericin or rose bengal, with or without light, 24 h after induction of HIV protein expression by PMA, in order to study the effects of these treatments on HIV-induced cell fusion. Syncytium formation between these cells and Sup-T1 cells was completely abolished by 1 μM hypericin, but illumination was required; syncytium formation was unaffected by hypericin in the dark (Table 2). Treatment with 1 μM rose bengal decreased syncytium production by >80% and was similarly light dependent (Table 2).

Cross-Linking of Viral Proteins. Treatment of membranes with rose bengal has been reported to result in the cross-linking of membrane proteins, evidenced by their disappearance from their usual positions in SDS/PAGE and their accumulation at the top of the gel (13, 15). We therefore examined the effects of hypericin and rose bengal on cross-linking of VSV proteins. As shown in Fig. 4, both compounds caused a similar pattern of protein cross-linking. Of the three major VSV proteins, the integral membrane protein G was most readily cross-linked, followed by M, a peripheral membrane protein that partially penetrates the viral bilayer (30). The nucleocapsid protein, N, which binds the genomic RNA but is not membrane-associated, was not significantly cross-linked by either compound. These findings provide another indication that the predominant action of both hypericin and rose bengal is at the viral membrane and that both dyes are exerting similar effects.

**DISCUSSION**

In this report we have provided evidence that the photodynamic inactivation of enveloped viruses by hypericin may
arise chiefly from the destruction of its fusion function. The fusion activity of three different enveloped viruses was photoinactivated by concentrations of hypericin ranging from 20 to 220 nM. HIV gp120-mediated cell fusion, as measured by syncytium formation, was photoinactivated completely by 1 μM hypericin and >80% by 1 μM rose bengal. The higher concentrations used for inhibition of syncytium formation were likely necessitated by the larger amounts of membrane present in the cell samples as compared to viral stocks.

Similar concentrations of hypericin have previously been reported to inactivate Sindbis virus and murine cytomegalovirus in a light-dependent fashion (7). Higher concentrations were required for photodynamic inactivation of equine infectious anemia virus (6), and of other lipid-containing viruses in experiments in which light was not controlled (1–5).

Comparison of the effects of hypericin and rose bengal permitted the following observations. (i) Both dyes act similarly, inactivating infectivity and/or fusion in all four enveloped viruses tested and promoting similar cross-linking of viral membrane proteins (Table 1). Both dyes are efficient producers of singlet oxygen upon illumination (14–16), and both are known to associate with biological membranes. Reactions of singlet oxygen induced by either compound thus presumably cause most or all of the observed effects. (ii) Loss of fusion function may be the general reason enveloped, but not nonenveloped, viruses are inactivated by hypericin and light. In support of this suggestion, the I50 for VSV inactivation of infectivity and that for inactivation of hemolysis were very similar, using either hypericin or rose bengal as photosensitizer (Table 1).

As clinical trials of hypericin are currently under development, it is critical to understand the mechanism by which this drug exerts its antiretroviral effects. Most importantly, we have demonstrated that light is essential for all the antiviral activities of hypericin and rose bengal that were studied. In this connection, it should be noted that the action of hypericin and rose bengal resembles quite closely that of several other photodynamic antiviral compounds: merocyanine-540, certain porphyrin derivatives, and certain phthalocyanine derivatives (31–33). All of these associate with membranes and are thought to work at least in part by generation of singlet oxygen (34). In common with these agents, hypericin and rose bengal might prove to be suitable agents for photodynamic inactivation of enveloped viruses present in blood or blood products, although lack of specificity might be a problem with any of these agents. The possibility of illuminating the blood of HIV patients after administration of a photodynamic dye might also be investigated, as has been reported for psoralen (35).

The close similarity of effects seen with hypericin and rose bengal and their similarity to effects seen with other photodynamic compounds suggests that several of these should be considered together and compared as potential anti-HIV agents, both in vivo and in vitro.

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