Antiviral Research, 21 (1993) 119–127

AVR 00629

Antiviral Research

Antiretroviral activities of hypericin and rose bengal: photodynamic effects on Friend leukemia virus infection of mice

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(Received 3 November 1993; accepted 3 February 1993)

Summary

The ability of hypericin to protect mice from splenomegaly resulting from infection with Friend leukemia virus (FLV) was re-examined in light of recent evidence showing that light is absolutely required for this drug's antiviral activity. FLV-induced splenomegaly was not prevented or ameliorated in mice injected with 100 μg hypericin, either mixed with the FLV inoculum or administered 1 day p.i., either under normal laboratory light or in the dark. These results contradict previous findings. Both hypericin and rose bengal, however, inactivated the FLV inoculum at low doses (< 11 μg), provided that the mixture was illuminated for 1 h under a normal fluorescent desk lamp. This procedure protected mice completely from FLV-induced splenomegaly, and provided a possible explanation for the discrepancy between our results and those reported previously. We conclude that for FLV, as for other enveloped viruses studied previously, illumination of hypericin with the virus is absolutely required for hypericin's antiviral (virucidal) effects, thus limiting its potential usefulness as an antiretroviral agent.

Hypercin; Rose bengal; Friend leukemia virus; Mouse

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Introduction

Hypericin is under study and clinical evaluation for possible use in the treatment of AIDS (Gulick et al., 1992). Interest began with the report that hypericin decreased splenomegaly and prevented death in mice infected with Friend leukemia virus (FLV). The drug was coadministered with the infecting virus, or delivered as a single injection around the time of infection. Reported results were dramatic: 50% survival occurred after a 10 μg injection, while complete protection from FLV-induced mortality was conferred by 150 μg (Lavie et al., 1989; Meruelo et al., 1988). Other workers have noted, however, that they were unable to replicate these findings (Tang et al., 1990, note added in proof).

Recent work from this laboratory (Lenard et al., 1993) has characterized the antiviral action of hypericin, based on its well-documented photodynamic action (Blum, 1941a; Knox and Dodge, 1985). It was found that treatment of stock virus with hypericin prevented the infection of cultured cell lines by both HIV and VSV at nanomolar concentrations. Illumination by visible light was absolutely required for any effect, however. It appeared that this antiviral activity resulted from the light-dependent inactivation of viral fusion, since this was induced by similar concentrations of hypericin in 4 different enveloped viruses (Lenard et al., 1993). Rose bengal had very similar effects on both viral infectivity and fusion (Lenard et al., 1993), as was predicted from the fact that its photodynamic action is essentially identical to that of hypericin: both compounds concentrate in membranes, and catalyze the formation of reactive singlet oxygen upon absorption of light. Singlet oxygen causes most or all of the membrane damage induced by either compound (Barratt et al., 1982; Blum et al., 1937; Blum, 1941b; Duran and Song, 1986; Giulive et al., 1990; Valenzeno, 1987; Valenzeno and Pooler, 1982). These results were consistent with earlier reports establishing that both hypericin and rose bengal could inactivate many lipid containing viruses, and that this antiviral activity was enhanced by illumination (Andersen et al., 1991; Carpenter and Kraus, 1991; Hudson et al., 1991; Roat et al., 1987; Schinazi et al., 1990; Tang et al., 1990; Turner and Kaplan, 1968).

Since it was not obvious how a light-dependent mechanism might work in vivo, we have attempted to repeat the previously reported experiments on FLV in mice (Lavie et al., 1989; Meruelo et al., 1988). Contrary to those reports, we could find no protection from the splenomegaly induced by FLV by 100 μg hypericin under several conditions of administration. The inoculating dose of FLV was readily inactivated, however, by prior illumination in the presence of <11 μg of either hypericin or rose bengal.

Materials and Methods

Mice and virus

Adult male BALB/c mice, approximately 20 gm, were obtained from Charles River (Charles River, MA), and were housed under normal conditions with a 12/12 h light/dark cycle. Laboratory chow and water were available ad libitum. The mice were caged by experimental groups. Illumination within the cages ranged from 3 to 5 foot candles in the front of the cage to <1 foot candle in the back of the cage. Illumination levels were measured with a Spectra 'Candela' light meter.

Murine leukemia (Friend) virus, original strain, was obtained from the American Type Culture Collection, VR-245, Lot 9W. The original virus sample was passaged twice in the BALB/c mice prior to use. Virus was harvested by homogenizing infected spleens in Dounce grinders and diluting 1:5 with phosphate-buffered saline, pH 7.2, (PBS). Cell debris was removed by a low speed centrifugation. The supernatant was filtered through 0.45 μ filters and either used immediately or aliquoted and frozen at $-80°$C. for later use. All experiments reported in this paper used virus from the same preparation.

Reagents

Hypericin was obtained from Atomergic (Farmingdale, NY) and rose bengal from Sigma (St. Louis, Mo). Stock solutions of hypericin (6.6 mM) and rose bengal (5.7 mM) were prepared by dissolving the compounds in either 95% or absolute ethanol and diluting with PBS. Solutions were freshly prepared for each experiment under conditions of reduced light, i.e., room lights turned off, but hall and adjacent room lights on. Solutions were held in aluminum foil wrapped tubes. Illumination on the bench top was <1 foot-candle.

Experimental procedures

In all experiments, the inoculum consisted of 280 μl of material, contained in PBS with a final concentration of 1.0–1.2% ethanol; this was injected i.p. into mice. The volume was obtained by mixing 250 μl of infected or uninfected spleen (diluted 1:5 in experiment 1 and 1:10 in experiment 2) with 30 μl of appropriately diluted hypericin or rose bengal, either component being replaced by the PBS-ethanol mixture when appropriate. After the initial injection, mice were visually inspected at least once a day throughout the experiment. The mice were killed by CO_2 asphyxiation on the indicated days p.i., and the spleens were promptly removed and weighed.

The groups used in the first experiment (Fig. 1) were as follows:

Pre-mixed. Hypericin and FLV were mixed in a test tube and then drawn into a syringe, under reduced light. All samples were kept on ice for 60–90 min prior to injection. The syringes for the '+HD' group were immediately wrapped in aluminum foil in order to protect them from light, while those for the '+HL' group were kept unwrapped under normal laboratory illumination (ca. 50 foot-candles).

Sequential. FLV inoculum was injected into mice under identical conditions of volume, buffer and alcohol concentration as used above, but without hypericin. 18 h later, a second injection of 150 μl was administered which contained the indicated amount of hypericin in PBS-ethanol, after storage on ice for 60–90 min in the dark ('+HD') or under normal laboratory illumination ('+HL'), as described above.

The 'FLV' group was injected with appropriately diluted infected spleen, with no hypericin or rose bengal and no subsequent injection.

'C+H' controls were injected with uninfected spleen extracts that had been diluted in the same way as the FLV inoculum, and hypericin, which had been exposed to laboratory light in the same way as the '+HL' samples.

Normal controls were mice of similar age, strain and source as those comprising the above groups, that were kept under identical conditions to the other mice, but were untreated in any way.

For experiment 2 (Figs. 2 and 3), inocula were prepared as described above, placed in plastic petri dishes (35 mm) on ice, and illuminated by placing a fluorescent desk lamp, containing 2 Philips F15 T8/CW 15 watt bulbs, 5 cm above the mixtures for 1 h. Illumination was 8–900 foot-candles. Dark (D) inocula were placed directly into a syringe, wrapped in aluminum foil to protect them from light, and placed on ice along side the illuminated samples.

Statistical analysis

Data is reported as the mean $\pm$ 1 S.D., $n = 3$–5 mice. Statistical differences were determined with a one-way ANOVA, $P < 0.01$.

Results

Several attempts to use hypericin to protect BALB/c mice from FLV-infection are shown in Fig. 1. Splenomegaly was used as a simple and unambiguous assay for FLV infection. FLV induced splenomegaly was evident at both 13 days (580% of (C+H) control) and at 30 days (1660% of control). Spleens from the control mice (C+H), receiving extracts from uninfected spleen mixed with 100 μg hypericin and illuminated were somewhat enlarged (25%) at 13 days but returned to the untreated (normal) size by 30 days. When hypericin was mixed with FLV and exposed to normal levels of laboratory light (premixed, +HL), spleen size increased to 650% of control value by day 13, and 2006% of control value by day 30. When the mixture was held in the dark (premixed, +HD) spleens increased to 1085% of control value by day 13 and 2685% of control value by day 30. When hypericin was exposed to normal laboratory light and administered on the day following infection with FLV (sequential, +HL), spleen size increased to 708% of control value by day 13 and 3188% of control value by day 30. When hypericin was held in the dark and administered on the following day (sequential, +HD), spleen size increased to 1515% of control value by day 13 and 3172% of control value

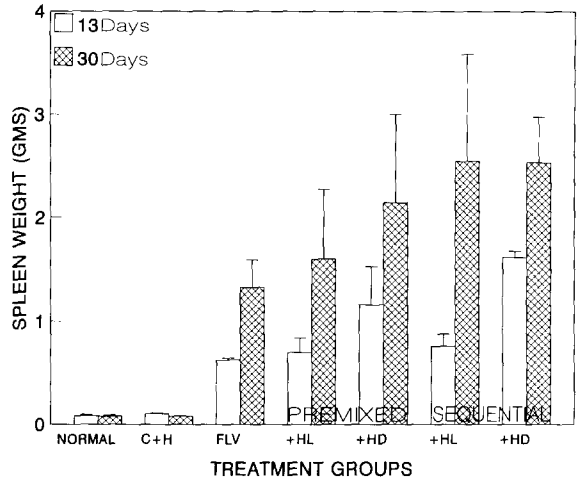

Fig. 1. FLV induced splenomegaly was not inhibited by hypericin (100 μg) under several different conditions of administration. The pre-mixed, sequential, +HD, +HL, C+H and normal groups are described in Materials and Methods. Mean $\pm$ S.D., $n = 3$–4.

by day 30. Thus, in none of these four groups of mice was any protection from FLV-induced splenomegaly observed. On the contrary, the two groups receiving hypericin prepared in the dark (premixed, +HD and sequential, +HD), had a significant enhancement of splenomegaly on day 13 as compared with groups receiving hypericin prepared in the light. This enhancement was no

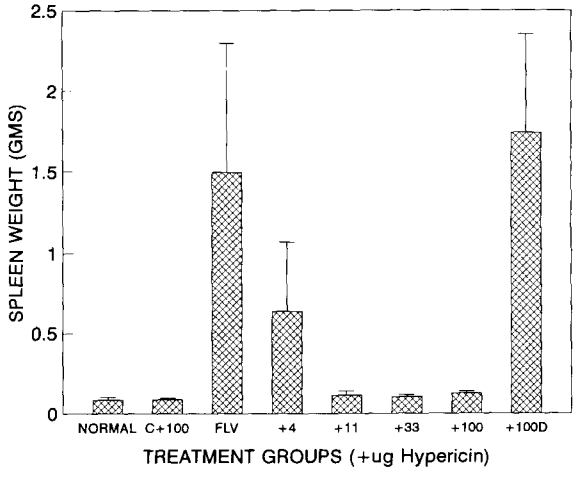

Fig. 2. FLV-induced splenomegaly was inhibited by hypericin premixed with FLV and illuminated with bright light, as described in Materials and Methods. The inocula contained the indicated concentrations (in μg) of hypericin. 'D' refers to mice that received inocula that had been protected from light and not illuminated prior to injection. 'C' refers to mice receiving inocula containing uninfected spleen extract in place of FLV-infected spleen extract, illuminated as described in Materials and Methods. 'Normal' mice were untreated. Mean $\pm$ S.D., $n = 3$–5.

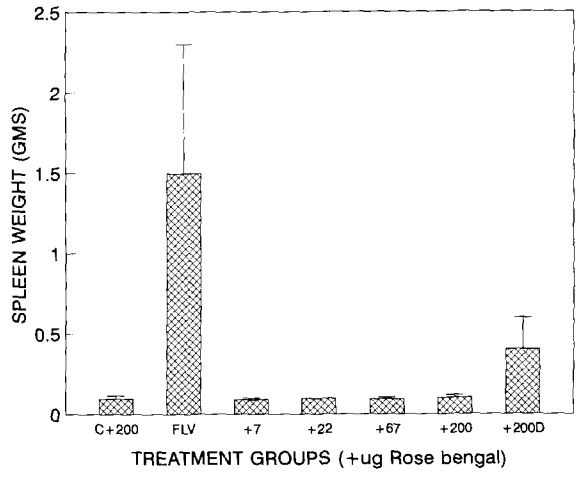

Fig. 3. FLV-induced splenomegaly was inhibited by rose bengal premixed with FLV and illuminated with bright light, as described in Materials and Methods. The inocula contained the indicated concentrations (in μg) of rose bengal. 'D', 'C' and 'normal' groups were as described in Fig. 2.

longer evident on day 30.

In order to test whether hypericin and rose bengal could inactivate FLV upon suitable illumination, different concentrations of these compounds were mixed with FLV inoculum and the mixtures illuminated for 1 h under a fluorescent desk lamp. These conditions were previously shown to inactivate other enveloped viruses (Lenard, et al., 1993). As shown for hypericin in Fig. 2 and for rose bengal in Fig. 3, both compounds inhibited splenomegaly even at the lowest concentrations tested (4 μg hypericin or 7 μg rose bengal). Complete protection was afforded by 11, 33 and 100 μg of hypericin (spleen weights were 117, 106, and 129% of control, respectively). All concentrations of rose bengal, 7, 22, 67 and 200 μg, gave complete protection (spleen weights were 92, 99, 95, 104% of control, respectively). On the other hand, the highest concentration of hypericin tested was completely ineffective in the absence of illumination; spleen weight was similar to that of the FLV group (1777% and 1526% of control value, respectively). Some protective effect of rose bengal was evident even in the absence of illumination, since spleen weight was less than that of the FLV group (411% and 1526% of control value, respectively). Spleen weights of controls receiving drug and uninfected spleen extract (C + 100 for hypericin, C + 200 for rose bengal) were not different from those of the untreated control group. Similar degrees of splenomegaly were observed when the FLV inoculum was premixed with PBS or PBS-ethanol (data not shown).

Discussion

These experiments were initiated to attempt to reconcile the protective effect claimed for hypericin in FLV infection of mice (Lavie et al., 1989; Meruelo et al., 1988), with the more recent finding that light appeared to be absolutely required for all of hypericin's antiviral actions (Lenard et al., 1993). In particular it seemed possible that in the earlier studies the FLV inoculum might have become inactivated during the mixing of drug and virus that was presumed necessary for 'coadministration'. The concentration of hypericin in such a mixture was calculated to be in the millimolar range, whereas only nanomolar levels of this compound were required for photodynamic inactivation of various enveloped viruses (Lenard et al., 1993). It was felt that in the presence such high concentrations of hypericin, the inoculum might be inactivated by brief exposure to normal laboratory light. Although we could not demonstrate such an effect in our experiments, we were likewise unable to demonstrate any protective effect of hypericin administered to the mice in several different ways (Fig. 1).

The finding that administration of unilluminated hypericin to mice caused a significant enhancement of FLV-induced splenomegaly at early times (day 13, Fig. 1) was unexpected. It would appear that hypericin itself is the active principle in this effect, and that illumination converts the compound to an inactive form, even under conditions that do not inactivate the virus and prevent splenomegaly (compare +HD groups with +HL groups in Fig. 1). The mechanism of this effect is unknown. It may be noted, however, that in studies on the effects of hypericin on several other enveloped viruses in vitro, activation was never observed (Lenard et al., 1993). The action is thus likely to be on the mouse rather than on the virus.

In contrast to the results presented in Fig. 1, hypericin did effectively inactivate FLV even at very low doses, provided that it was illuminated for 1 h under a fluorescent desk lamp (Fig. 2). Light was absolutely required for this effect, since splenomegaly was indistinguishable between untreated infected controls and mice treated with the highest concentration of hypericin (100 μg) in the dark (Fig. 2).

Very similar results were obtained (Fig. 3) using rose bengal, another photodynamic agent that partitions into membranes and forms singlet oxygen upon illumination (Barratt et al., 1982; Blum and Pace, 1937; Giulive et al., 1990; Valenzeno and Pooler, 1982; Valenzeno, 1987). Some protection against splenomegaly was observed for unilluminated rose bengal at the highest concentration tested (200 μg). This may indicate an additional antiviral action of this compound. The efficacy of the compound was increased by illumination, however.

The precise basis for the differences between the results found previously (Lavie et al., 1989; Meruelo et al., 1988) and those reported here is not clear. We suggest, however, that light-induced inactivation of the virus inoculum could have been responsible for the effects previously observed, rather than an

anti-viral activity occurring in the animals.

These results, together with those reported previously (Lenard et al., 1993) suggest that any possible therapeutic potential of hypericin is likely to be limited by its need to be activated by light. While positive preliminary observations have been reported using extra-corporeal illumination to activate psoralen in the blood (Bisaccia et al., 1990), the lack of specificity of action of photodynamic agents has been a continuing problem in attempts to use them for disinfecting blood fractions (Corash and Hanson, 1992). In light of the results reported here as well as previously (Lenard et al., 1993), however, hypericin and rose bengal must be considered as members of the photodynamic class of antiviral agents, which include merocyanine-540, certain porphyrin derivatives, and certain phthalocyanine derivatives (O'Brien et al., 1992; North et al., 1992; Horowitz et al., 1991). All these compounds appear to act quite similarly, and should probably be considered as a class and compared with each other in the investigation of any potentially useful antiviral effects.

Acknowledgments

The authors thank Sapan Palepulle and Roger Vanderoef for outstanding assistance and Dr. John Silver for helpful advice. This work was supported in part by NIH grant AI-13002.

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