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Virucidal activity of hypericin against enveloped and non-enveloped DNA and RNA viruses

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Summary

Hypericin is a polycyclic anthrone first isolated from the plant St. Johnswort and was shown to have dramatic anti-retroviral activity against Friend leukemia virus and radiation leukemia virus in mice. Hypericin displayed marginal activity (IC_{50} = 6 μg/ml) against Moloney murine leukemia virus (Mo-MuLV) in vitro. Hypericin did not display selective antiviral activity against herpes simplex virus, influenza A, adenovirus, or poliovirus. The 50% cytotoxic concentration was approximately 25 μg/ml. When virus was incubated with hypericin before infecting cells, the drug was virucidal to all enveloped viruses tested (herpes simplex, influenza virus A, and Mo-MuLV) at concentrations of 1.56 μg/ml to 25 μg/ml. Hypericin was not virucidal to the non-enveloped viruses tested (adenovirus and poliovirus).

These data indicate that the mechanism of viral inactivation for hypericin is dependent upon the presence of a viral lipid envelope. In vivo, hypericin (50 mg/ml) was effective against FLV or HSV-1 if incubated with the virus for 1 h at 37°C before infecting mice, but was not effective if pre-incubated with virus for 1 h at 4°C or if administered concurrently with virus.

Hypericin, Pseudohypericin, Virucidal agent, Enveloped virus, Non-enveloped virus

Introduction

Hypericin is an aromatic polycyclic anthrone first isolated from the herb *Hypericum triquetrifolium* Turra (St. Johnswort) (Brockman et al., 1974) (Fig. 1). The compound has also been synthesized (Brockman et al., 1957). Hypericin has

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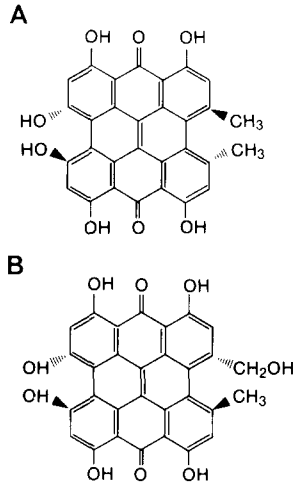

Fig. 1. Chemical structures. A, hypericin; B, pseudohypericin.

been tested and used in patients as an antidepressant with no apparent adverse side effects. The efficacy of this drug as an antidepressant indicates that it can cross the blood-brain barrier (Daniel, 1949). Recently, hypericin and its analog pseudohypericin (Fig 1.) have been shown to have dramatic anti-retroviral activity in mice infected with Friend leukemia virus (FLV) or radiation leukemia virus (RaLV) (Meruelo et al., 1988). Only one treatment of either hypericin or pseudohypericin co-administered with virus infection or given one day post virus infection was required to inhibit completely virus-induced splenomegaly and to prolong survival. In addition, no infectious virus could be recovered from virus-infected mice treated with these drugs. Furthermore, hypericin and pseudohypericin were well tolerated in mice (Meruelo et al., 1988).

In culture, both hypericin and pseudohypericin were effective in inhibiting the release of virus reverse transcriptase into the cell medium (Meruelo et al., 1988). However, no direct effect on reverse transcriptase could be demonstrated in vitro (Meruelo et al., 1988). In addition, these compounds had no measurable effect against transcription, translation, or the transport of viral proteins to the cell membrane (Meruelo et al., 1988). Consequently, it has been suggested that hypericin and pseudohypericin exert their anti-retroviral activity by directly inactivating the virions or by interfering with viral shedding, budding, or assembly at the cell membrane. Recent data suggest that hypericin may interfere with processing of *gag*-encoded precursor polyproteins since hypericin-treated retrovirus-producing cells were found to contain immature or abnormally assembled cores (Lavie et al., 1989). The basis for the mechanism of anti-retroviral activity of these compounds remains unclear, however.

Prompted by observations of the photosensitizing effect of hypericin-like compounds (Duran and Song, 1986) and the possible interaction of hypericin with the cell membrane (Meruelo et al., 1988), the study presented here was undertaken to

assess the in vitro antiviral activity of hypericin using a variety of enveloped and non-enveloped viruses. A differential effect of hypericin against enveloped versus non-enveloped viruses would indicate a potential mechanism of antiviral action.

Materials and Methods

In vitro antiviral activity of hypericin against murine leukemia virus

Hypericin (Aldrich Chemical Co., Milwaukee) was tested in vitro against Moloney murine leukemia virus (Mo-MuLV, clone E7, a generous gift from Marguerite Vogt, The Salk Institute, La Jolla, CA) using an XC assay as described previously (Rowe et al., 1970). Briefly, 3–5 × 10^3 SC-1 cells (CRL-1404; American Type Culture Collection (ATCC)) per well of a 96-well microtiter plate were seeded in minimal essential medium supplemented with 5% fetal bovine serum, penicillin (150 units/ml), streptomycin (150 μg/ml), and polybrene (2 μg/ml) and infected with Mo-MuLV (multiplicity of infection = 10 PFU/cell) the next day. After allowing the virus to adsorb to the cells for 1 h, medium containing serial dilutions of hypericin or medium alone was added to the cells. No semi-solid medium was used. After incubation for 5 days (when cells were confluent), the SC-1 cells were UV irradiated for 10 s and 5–8 × 10^4 XC cells (CCL-165; ATCC) per well were added. Usually, 2 additional days of incubation were required to obtain full virus-induced cytopathic effect.

Virucidal effect of hypericin against murine leukemia virus

The virucidal activity of hypericin was assessed and compared to other antiviral agents. Serial dilutions of hypericin were pre-incubated with serial dilutions of Mo-MuLV for 1 h at 37°C. SC-1 cells were then infected with two-fold dilutions of the various hypericin-virus mixtures. After allowing the virus to adsorb to the cells for 1 h at 37°C, the virus-infected cells were washed three times with Hank's balanced salt solution (HBSS) and the cells were monitored for the appearance of cytopathic effect using the XC cell assay as described above.

Studies with enveloped and non-enveloped DNA and RNA viruses

BSC-1 cells (CCL-26; ATCC) were used in assays to evaluate the activity of hypericin against herpes simplex virus type 1 (HSV-1 strain Mayo 1814, NIAID, V-346-001-015), which is a large enveloped DNA virus, and poliovirus type 1 strain Brunhilde (VR-58; ATCC), which is a small non-enveloped RNA virus. MDCK (CCL-34; ATCC) cells were used in assays to evaluate the activity of hypericin against influenza A/Brazil (Lilly Research Laboratories, Greenfield, IN), an enveloped RNA virus. HeLa cells (CCL-2; ATCC) were used in assays involving adenovirus type 2 (VR-846; ATCC), a non-enveloped DNA virus. Confluent cells in microtiter plates (50000 cells per well) were infected with 150 plaque forming units

of virus and incubated in the presence or absence of various concentrations of hypericin at 37°C in a CO_2-humidified incubator until cytopathic effect (CPE) in hypericin-free control cells was extensive, usually 2–5 days after infection, depending upon the virus. The cells were then fixed, stained, and the antiviral effect against each virus was assessed by comparing the extent of CPE at each drug concentration with the extent of CPE in the absence of drug. The concentration of drug required to inhibit the development of CPE by 50% (IC_{50}) was then determined from the linear portion of each dose-response curve.

Virucidal activity of hypericin

In assays to study the virucidal effect of hypericin against each of the above viruses, each virus inoculum was pre-incubated with or without hypericin before infecting the target cells. The infected cells were then washed extensively 3 times for 5 min with HBSS to remove residual compound and monitored for the appearance of virus induced CPE.

Mo-MuLV reverse transcriptase assay

Recombinant Moloney murine leukemia virus reverse transcriptase was purchased from Pharmacia (Piscataway, NJ). Supernatants were taken from infected SC-1 cells as used in the above XC cell assays and served as a source of reverse transcriptase enzyme. The reverse transcriptase reaction mixture consisted of the following: 50 μl of purified enzyme or cell supernatant; 50 mM Tris-HCl, pH 8.3; 10 mM dithiothreitol; 8 mM $MgCl_2$; 60 mM KCl; 0.05% Nonidet P-40; 2.5 μg/ml oligo$(dG)_{12-18}$:poly(rC); 1 μM dGTP; and 0.01 μM α[^{32}P]dGTP in a total volume of 100 μl. The reaction was incubated at 37°C for 1–2 h and 50 μl of the reaction was spotted directly onto DE-81 chromatography paper (Whatman, Maidenstone, U.K.) using a dot-blotting manifold (Schleicher and Schuell, Keene, NH). The paper was then washed 3 times with 2× saline sodium citrate (SSC; 1× = 0.15 M NaCl, 0.015 M sodium citrate) and twice with 95% ethanol. The paper was then dried under a heat lamp. A Betagen analyzer was used to scan the blots and produce a digital image of the [^{32}P]-dGTP incorporated into homopolymers and bound to the DE-81 paper.

In vivo evaluation of the antiviral activity of hypericin

For the in vivo evaluation of their antiviral activity, hypericin and pseudohypericin were first isolated and purified according to the method S.H. Larsen et al. which will be reported elsewhere. Briefly, St. Johnswort herb powder was obtained from the Chart Corporation, Inc. (Libertyville, IL). Hypericin and pseudohypericin were extracted initially from the powder with ethylene chloride and then with methanol and finally precipitated with ethyl ether. The compounds were then isolated and purified by HPLC, using a μBondapak C_{18} column, and obtained as amorphous powders by lyophilization from tert-butyl alcohol. The lyophilized

material was solubilized in 95% ethanol and diluted to 1% ethanol in phosphate buffered saline. Hypericin and/or pseudohypericin were tested for in vivo activity against Friend leukemia virus (FLV). The FLV used in these experiments is derived from the original isolate which was a gift from Dr C. Friend and has been passaged in our laboratory for over 20 years. Each compound or a mixture of both was given i.p. in a total volume of 0.5 ml/mouse 18 h after i.p. infection with 1.0×10^3 plaque forming units of Friend leukemia virus (FLV)/mouse. The DBA-2 mice used were 4–6 week old males weighing 14–16 g. In other experiments, compound (100 μg/ml) and virus were mixed and either administered i.p. immediately or pre-incubated at 4°C or 37°C for 1 h before infection of mice. Three weeks after infection, a small amount of blood was collected from the retro-orbital plexus and hematocrit readings were taken after centrifugation of the blood sample. Mice were then autopsied and spleen weights were determined for the evaluation of FLV-induced splenomegaly. Pooled plasma from each group of mice was assayed for virus titer using the XC assay described above.

For the HSV-1-induced mouse encephalitis model, CD-1 mice were infected intracerebrally (i.c.) with 70 plaque forming units of virus per mouse immediately after mixing or after pre-incubation with hypericin or pseudohypericin at 37°C or 4°C for 1 h. Mice were observed daily for 15 days and incidences of encephalitis and death were recorded.

Results

In vitro antiviral activity of hypericin and other antiviral compounds

As shown in Table 1, hypericin exhibited only a marginal anti-Mo-MuLV activity with an IC_{50} of approximately 6 μg/ml. Also, pre-treatment of SC-1 cells with hypericin before Mo-MuLV infection did not increase the anti-Mo-MuLV activity (data not shown). In this assay, the 50% cytotoxic concentration of hypericin to dividing SC-1 cells was approximately 25 μg/ml. By comparison, azidothymidine (AZT; BW-A509U; The Burroughs-Wellcome Company) was extremely active and selective against Mo-MuLV with an IC_{50} in the nanogram range (0.006 μg/ml) and no apparent cytotoxicity.

In light of the dramatic in vivo anti-retroviral activity of hypericin/pseudohypericin in mice (Meruelo et al., 1988), the low in vitro anti-retroviral activity of hypericin was not expected. Therefore, the ability of hypericin to inactivate Mo-MuLV directly was evaluated (see below).

In vitro antiviral activity of hypericin against other viruses

Hypericin did not exhibit antiviral activity against herpes simplex virus, influenza, adenovirus, or poliovirus in the CPE inhibition assay (Table 1). Acycloguanosine (acyclovir, ACV), an effective and selective anti-herpes agent (Elion, 1982), was effective against HSV-1 (IC_{50} approximately 0.15 μg/ml), whereas

TABLE 1

Inhibition of virus-induced cytopathic effect by various antiviral compounds

Virus[a]	Compound	Cells	IC_{50}[b]
Mo-MuLV	Hypericin	SC-1/XC	6.00
Mo-MuLV	AZT[c]	SC-1/XC	0.006
HSV-1	Hypericin	BSC-1	20.0
HSV-1	ACV[c]	BSC-1	0.15
A/Brazil	Hypericin	MDCK	>100.0
A/Brazil	Ribavirin	MDCK	6.25
Adeno	Hypericin	HeLa	>100.0
Adeno	Ribavirin	HeLa	12.5
Polio	Hypericin	BSC-1	>100.0
Polio	Enviroxime	BSC-1	0.02

[a]Mo-MuLV, Moloney leukemia virus; HSV-1, herpes simplex virus type 1 strain Mayo 1814; A/Brazil, influenza A/Brazil; adeno, adenovirus type 2; polio, poliovirus type 1 strain Brunhilde.
[b]IC_{50} is the concentration of drug in μg/ml required to inhibit virus induced cytopathic effect by 50%.
[c]AZT, azidothymidine; ACV, acyclovir (acycloguanosine).

hypericin was not (IC_{50} approximately 20 μg/ml). Enviroxime (LY122772; Lilly Research Laboratories), a benzimidazole derivative with potent anti-rhinoviral activity (DeLong, 1984) showed impressive activity against poliovirus (IC_{50} approximately 0.02 μg/ml), whereas hypericin did not (ED_{50} >100 μg/ml). Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide; ICN Pharmaceuticals, Inc., Costa Mesa, CA), a broad spectrum antiviral agent with low toxicity (Witkowski et al., 1972), displayed only modest activity against adenovirus (IC_{50} approximately 12.5 μg/ml) and influenza A/Brazil (IC_{50} approximately 6.25 μg/ml), while hypericin was not effective against either of these viruses (IC_{50} >100 μg/ml). These data reveal that hypericin did not exhibit appreciable in vitro antiviral activity against the enveloped or non-enveloped viruses tested and indicate that hypericin does not interfere selectively with a step in the replication of any of the viruses tested. In these assays, which employ confluent cell monolayers, there was no apparent cytotoxicity associated with hypericin, acyclovir, ribavirin, or enviroxime.

Inactivation of enveloped and non-enveloped viruses by hypericin

The virucidal effect of hypericin against enveloped viruses (Fig. 2) was in sharp contrast to its inability to inhibit virus-induced cytopathic effect (Table 1). At concentrations of 0.78 μg/ml to 1.56 μg/ml, hypericin dramatically inactivated the enveloped viruses tested: HSV-1, influenza A/Brazil, and Mo-MuLV. These viruses were inactivated by more than 95% when pre-incubated with concentrations of hypericin from 3.12 to 50 μg/ml. In contrast, hypericin, in the same concentration range, did not inactivate adenovirus or poliovirus, the non-enveloped viruses tested. None of the other antiviral compounds tested here, (including acyclovir, ribavirin and enviroxime) with the exception of AZT, displayed any virucidal effect against the viruses for which they are known inhibitors of replication (data not shown). AZT, with its potent activity against Mo-MuLV in vitro and in

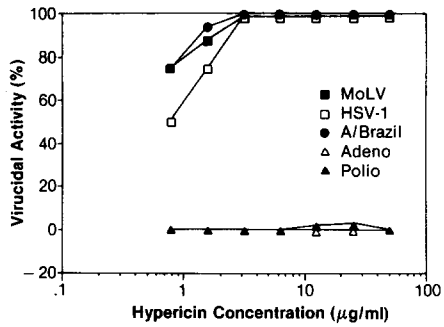

Fig. 2. Virucidal effect of hypericin against Moloney leukemia virus (Mo-LV), herpes simplex virus (HSV-1), influenza A (A/Brazil), adenovirus (Adeno), and poliovirus (Polio). Serial dilutions of hypericin were pre-incubated with serial dilutions of the test virus for 1 h at 37°C after which cells were infected with two-fold dilutions of the various virus-hypericin mixtures. The dilution of virus required to induce 50% CPE in the absence of hypericin was compared to the dilution of virus required to induce 50% CPE in the presence of increasing concentrations of drug. Virucidal activity is defined as per cent reduction in $TCID_{50}$ from the no-drug control.

vivo, displayed a noticeable virucidal effect against this virus at relatively high drug concentrations (0.78–25 μg/ml). The virucidal effect observed with higher concentrations of AZT, may have been due, at least in part, to an incomplete wash-off of drug from the cells and/or extremely rapid entrance of the nucleoside analog into the cells during the 1 h virus adsorption period.

Virucidal effect of hypericin against Mo-MuLV as determined by the release of reverse transcriptase from infected cells

Mo-MuLV was diluted as indicated and pre-incubated with 2-fold dilutions of hypericin for 1 h at 37°C and SC-1 cells were infected as described in Materials and Methods. Five days after infection, cell supernatants were assayed for reverse transcriptase activity. Reverse transcriptase was readily detected in supernatants of cells infected with all dilutions of Mo-MuLV not pre-incubated with hypericin (Fig. 3A). Supernatants of cells infected with virus pre-incubated with hypericin at 2.5 to 20 μg/ml released markedly less reverse transcriptase (Fig. 3A). Interestingly, pseudohypericin did not affect the amount of reverse transcriptase detected in cell supernatants (Fig. 3B). In a separate experiment, SC-1 cells were infected with Mo-MuLV first and then incubated in the presence of hypericin (1.56 μg/ml) for 5 days. The supernatants from these cells were then assayed for reverse transcriptase activity and were found to contain levels of activity equivalent to those in supernatants from cells infected with Mo-MuLV but not treated with hypericin (data not shown). When purified Mo-MuLV reverse transcriptase was assayed in the presence of hypericin or pseudohypericin, no direct inhibition of the enzyme was observed (Fig. 4). These data are consistent with hypericin-induced inactivation of the virion and indicate that hypericin does not inhibit reverse transcriptase directly

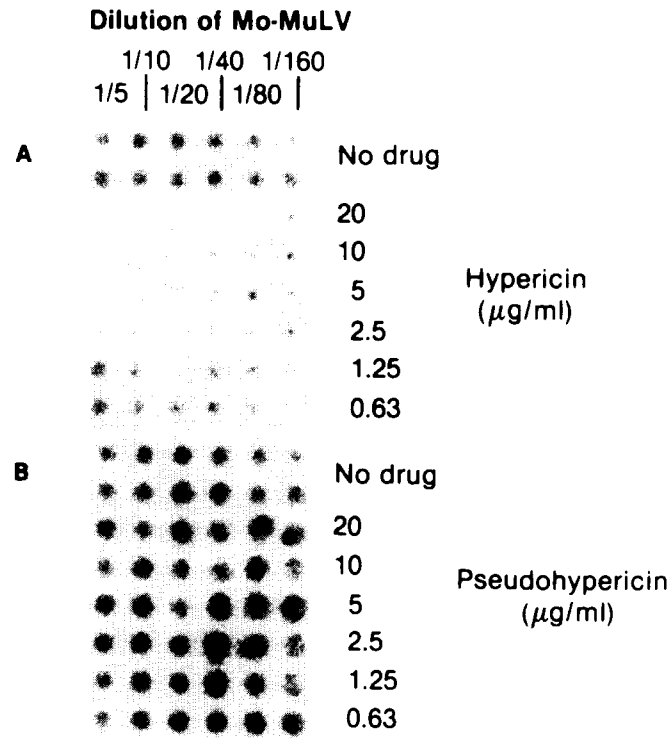

Fig. 3. Virucidal effect of hypericin and pseudohypericin on Mo-MuLV as determined by the release of reverse transcriptase into the cell medium. Beginning with a 1:5 dilution, serial 2-fold dilutions of Mo-MuLV were pre-incubated with 0, 0.63, 1.25, 2.5, 5, 10, or 20 μg/ml of hypericin or pseudohypericin for 1 h at 37°C. SC-1 cells were infected with the virus preparations and five days post infection, supernatant cell medium was harvested and assayed for the presence of reverse transcriptase activity as described in Materials and Methods. A, Mo-MuLV was pre-incubated in the absence or presence of hypericin; B, Mo-MuLV was pre-incubated in absence or presence of pseudohypericin.

or interfere with the replication of the virus once the intracellular replication cycle is underway.

In vivo activity of hypericin and pseudohypericin

The efficacy of hypericin and pseudohypericin against Friend leukemia virus (FLV) in DBA-2 mice was evaluated. When mice were infected with FLV which had been pre-incubated with hypericin or pseudohypericin (50 μg/ml) for 1 h at 4°C or infected with FLV immediately after mixing with either hypericin or pseudohypericin, no inhibition of FLV-induced splenomegaly or viremia was observed (Table 2; treatments 1A, 1B and 1C, and 3A, 3B and 3C) . However, if FLV was pre-incubated with hypericin for 1 h at 37°C before inoculation into mice, a 98% inhibition of splenomegaly and a dramatic reduction of viremia were noted (Table 2; treatments 2A, 2B and 2C). In a separate experiment, mice were infected with

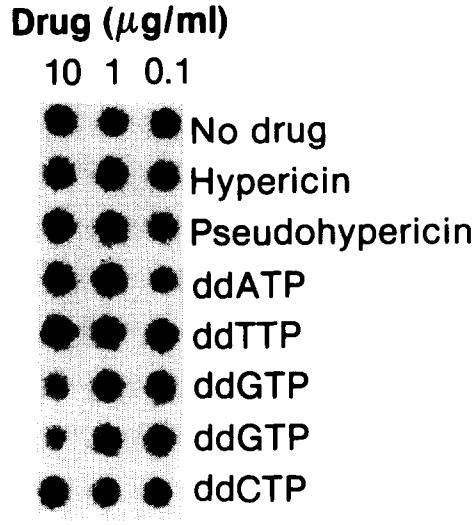

Fig. 4. Effect of hypericin and pseudohypericin on purified Mo-MuLV reverse transcriptase. Reverse transcriptase reactions using purified Mo-MuLV reverse transcriptase were carried out in the presence of 0, 0.1, 1, or 10 μg/ml of hypericin or pseudohypericin as described in Materials and Methods. For comparative purposes, separate reactions were performed with the addition of 10 μg/ml of ddATP, ddTTP, ddGTP, or ddCTP. Since the template used in the reverse transcriptase reaction was poly(rC), only ddGTP showed an inhibitory effect (50% inhibition at 10 μg/ml). Neither hypericin nor pseudohypericin had an inhibitory effect on purified reverse transcriptase activity.

FLV and treated once i.p. with either hypericin (Table 2; treatments 4A, 4B, and 4C), pseudohypericin (Table 2; treatments 5A, 5B, and 5C), or a 1:1 mixture of each (data not shown) 18 h after infection. Mice treated with 25 μg of either compound in such a manner displayed a 35–45% inhibition of splenomegaly. Greater doses of either compound were not more effective. In similar experiments, when given as a single i.p. dose (up to 800 μg/mouse), AZT was ineffective in preventing FLV-induced splenomegaly (data not shown).

The in vivo efficacy of hypericin and pseudohypericin against herpes simplex virus type 1 infection was also studied. When mice were inoculated i.c. with virus immediately after mixing with either hypericin or pseudohypericin, no protective effect was demonstrated in that mortality of these mice was equivalent to that seen in mice infected with virus immediately after mixing with phosphate buffered saline (Fig. 5A). When infection was carried out with virus pre-incubated with hypericin for 1 h at 4°C, death was delayed by 2 days (Fig. 5B). Surprisingly, pseudohypericin, when pre-incubated with HSV-1 for 1 h at 4°C was markedly better in delaying death; mice displayed 50% survival at 16 days post infection (Fig. 5B). Mice inoculated with HSV-1 which had been pre-incubated with hypericin or pseudohypericin for 1 h at 37°C displayed 100% survival whereas control, infected mice displayed 100% mortality by 6 days after infection (Fig. 5C).

TABLE 2

Effect of hypericin and pseudohypericin on Friend leukemia virus infection of DBA-2 mice

Treatment[a]	Average weight gain in mg	Average spleen weight in mg (% inhibition of splenomegaly)	Reciprocal of the plasma dilution containing 1 $TCID_{50}$ of virus	Hematocrit[b] (%)	Average spleen score ± SD (N = number of mice used)[c]
1A	5,900	966	>10,240	56	4.5 ± 1.43 (10)
1B	5,400	1,262	>10,240	59	5.2 ± 1.23 (10)
1C	6,000	1,479	>10,240	62	5.5 ± 0.71 (10)
2A	5,900	1,655	>20,480	66	5.9 ± 0.32 (10)
2B	6,400	100 (98)	<10	48	1.2 ± 0.63 (10)
2C	6,400	1,396 (16)	10,240	60	5.2 ± 1.55 (10)
3A	5,900	970	20,480	56	4.4 ± 1.58 (10)
3B	5,400	1,611	20,480	65	5.9 ± 0.32 (10)
3C	5,500	1,713	20,480	71	5.6 ± 0.52 (8)
Mock-infected	7,700	75	<10	50	1.0 ± 00 (7)
4A	5,600	1,248	Not Determined (ND)	63	5.0 ± 0.47 (10)
4B	4,900	1,050 (16)	ND	65	5.1 ± 0.74 (10)
4C	5,000	702 (46)	ND	57	3.6 ± 1.78 (10)
4D	6,300	1,240	ND	61	4.8 ± 1.12 (20)
5A	5,800	1,018 (19)	ND	61	4.6 ± 0.84 (10)
5B	5,800	890 (30)	ND	63	4.1 ± 1.66 (10)
5C	6,500	824 (35)	ND	66	4.4 ± 1.33 (10)
5D	6,000	1,240	ND	61	4.8 ± 1.12 (20)
Mock-infected	6,300	81	ND	54	0.8 ± 0.46 (8)

[a]*Treatment*:

1. Pre-incubation of virus with the following for 1 h at 4°C before inoculation into mice: A, with phosphate buffered saline; B, with hypericin (50 μg/ml); C, with pseudohypericin (50 μg/ml).
2. Pre-incubation of virus for 1 h at 37°C with the following: A, with phosphate buffered saline; B, with hypericin (50 μg/ml); C, with pseudohypericin (50 μg/ml).
3. Virus inoculated into mice immediately after mixing with the following: A, with phosphate buffered saline; B, with hypericin (50 μg/ml); C, with pseudohypericin (50 μg/ml).
4. Mice treated with the following by i.p. injection 18 h after infection: A, hypericin (100 μg/mouse); B, hypericin (50 μg/mouse); C, hypericin (25 μg/mouse); D, phosphate buffered saline.
5. Mice treated with the following by i.p. injection 18 h after infection: A, pseudohypericin (100 μg/mouse); B, pseudohypericin (50 μg/mouse); C, pseudohypericin (25 μg/mouse); D, phosphate buffered saline.

[b]Hematocrit is expressed as the percentage of packed red blood cells (v/v) after centrifugation of blood collected from the retro-orbital plexus.

[c]Spleen size score: 0: ≤ 0.5 cm; 1: 0.5 to 1.0 cm; 2: 1.0 to 1.5 cm; 3: 1.5 to 2.0 cm; 4: 2.0 to 2.5 cm; 5: 2.5 to 3.0 cm; 6: >3.0 cm. Spleens were measured along the longitudinal axis.

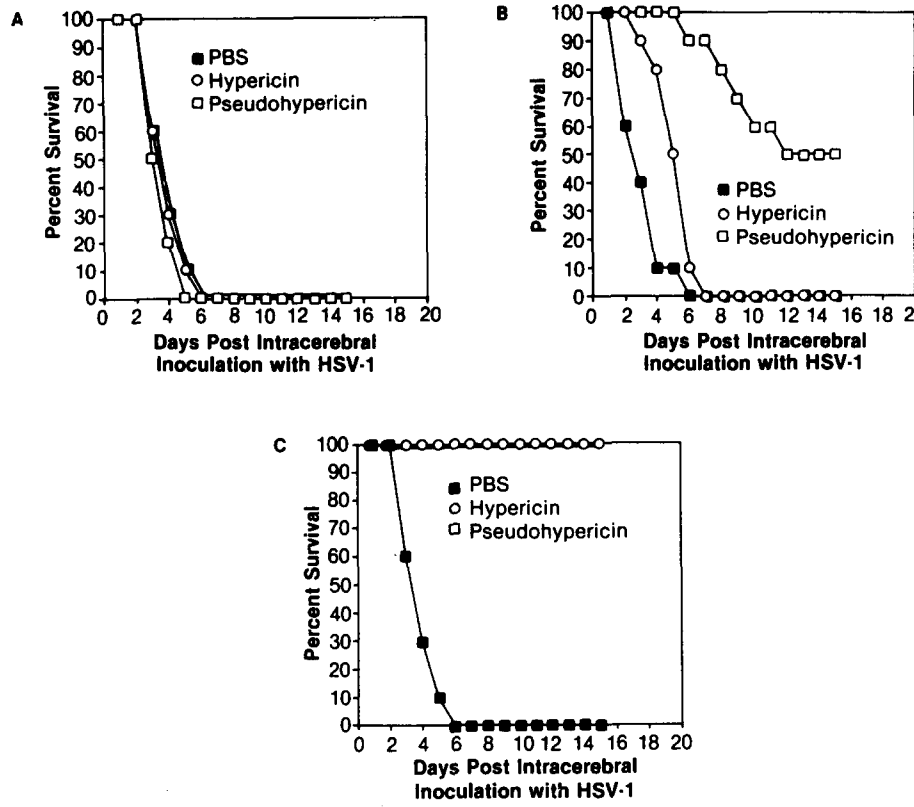

Fig. 5. In vivo activity of hypericin and pseudohypericin, in mice infected with herpes simplex virus type 1 (HSV-1). A, HSV-1 was mixed with phosphate buffered saline (PBS), hypericin, or pseudohypericin and inoculated immediately into CD-1 mice; B, HSV-1 was pre-incubated with PBS, hypericin, or pseudohypericin for 1 h at 4°C before being inoculated into CD-1 mice; C, HSV-1 was pre-incubated with PBS, hypericin, or pseudohypericin for 1 h at 37°C before being inoculated into CD-1 mice. All virus inoculations were by i.c. injection. Mice were monitored for the appearance of HSV-1 induced encephalitis and hind leg paralysis. Dead mice were recorded daily.

Discussion

The data presented in this report support the following conclusions: (1) hypericin and pseudohypericin are marginally active as in vitro anti-retroviral agents and are not active as antiviral agents against other viruses tested (herpes simplex virus 1 (Mayo 1814); influenza A/Brazil; poliovirus type 1 (Brunhilde); adenovirus type 2; (2) when pre-incubated for 1 h at 37°C, hypericin was able to inactivate all enveloped viruses tested (Moloney murine leukemia virus, herpes simplex virus, and influenza virus) but not the non-enveloped viruses tested (adenovirus type 2 and poliovirus type 1) as determined by tissue culture-CPE assay; (3) when pre-incubated with Mo-MuLV for 1 h at 37°C, hypericin was effective in inactivating the virus as determined by the release of reverse transcriptase from infected cells into

the medium; (4) similar to the findings of Meruelo et al. (1988), neither hypericin nor pseudohypericin was directly inhibitory to purified Mo-MuLV reverse transcriptase; (5) in vivo, hypericin, if pre-incubated with virus for 1 h at 37°C, was effective in preventing FLV-induced splenomegaly and viremia in DBA-2 mice. It was not effective if pre-incubated with virus for 1 h at 4°C or if given concurrently with virus; (6) when given i.p. 18 h after infection, hypericin and pseudohypericin were moderately effective in preventing FLV-induced splenomegaly at 25 μg/mouse but were not more effective at higher doses; (7) both hypericin and pseudohypericin, if pre-incubated with virus for 1 h at 37°C, were highly effective in preventing HSV-1 induced encephalitis and death in mice whereas they were only moderately effective if pre-incubated with virus for 1 h at 4°C, and not effective at all if given concurrently with virus.

Our data are consistent with the idea that hypericin and pseudohypericin are effective in inactivating enveloped viruses and that the mechanism of viral inactivation is dependent upon the presence of a viral lipid envelope. The observation that hypericin is able to inactivate both herpes simplex virus and influenza virus is inconsistent with the notion that the mechanism of retrovirus inactivation is mediated through the interference of a unique retroviral function, i.e., the processing of the *gag*-encoded precursor polyproteins (Lavie et al., 1989). A more likely explanation is that hypericin is able to intercalate in the viral envelope thereby resulting in the lysis of the infectious virion. The difference in the activity of hypericin relative to that of pseudohypericin is noteworthy. Pseudohypericin, in contrast to hypericin, was not able to inactivate Moloney murine leukemia virus as determined by the release of reverse transcriptase into the cell medium (Fig. 3B). However, pseudohypericin was equally effective as hypericin in its ability to inactivate herpes simplex virus since mice inoculated with virus that had been pre-incubated with hypericin or pseudohypericin at 37°C for 1 h did not undergo any adverse effects from the herpes virus infection (Fig. 5C). These data indicate that the notion that hypericin and/or pseudohypericin have a 'detergent-like' effect in lysing viral envelopes may be too simplistic.

In contrast to the studies of Meruelo et al. (1988), we could not demonstrate in vivo therapeutic efficacy for hypericin, when given as a single dose, against Friend leukemia virus. In addition, hypericin, when given as a single dose, was not found to have therapeutic efficacy against herpes simplex virus type 1 in vivo. The reason(s) for this discrepancy is not clear but may include the following: (1) differences in the hypericin isolation methods used (For in vivo studies, we used chemically pure hypericin and/or pseudohypericin which were extracted and purified by reversed-phase HPLC); and(2) differences in the strain of mouse employed (BALB/c versus DBA/2).

Since hypericin and pseudohypericin are relatively ineffective antiviral agents therapeutically, intracellular virus may be protected, in some way, from the deleterious effects of these compounds. Consequently, the utility of hypericin and/or pseudohypericin may depend on pre-treating cells with these compounds to create an antiviral state and/or maintaining high blood levels of compound by multiple dosing. The data presented in this report indicate that these compounds, when

given in a single dosing regimen, do not demonstrate the potential for efficacy in the therapeutic treatment of viral infections.

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Note added in proof: We have recently completed studies in which hypericin or pseudohypericin (up to 100 μg/mouse/day) was administered i.p. to Friend leukemia virus (FLV) infected mice once a day for 18 days beginning 2 h prior to virus infection. No effect on hematocrit or virus induced splenomegaly were observed. By comparison, using the same dosing regimen at 100 μg/mouse/day, AZT was found to be active.

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