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In vitro virucidal activity of selected anthraquinones and anthraquinone derivatives

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Summary

Anthraquinones and anthraquinone derivatives were characterized for their antiviral and virucidal activities against viruses representing several taxonomic groups. One of these compounds, hypericin, had activity against vesicular stomatitis virus, herpes simplex virus types 1 and 2, parainfluenza virus, and vaccinia virus (from 0.5 to 3.8 $\log_{10}$ reductions in infectivity) at concentrations of less than 1 μg/ml as determined by a direct pre-infection incubation assay. Human rhinovirus was not sensitive to hypericin at concentrations up to 10 μg/ml. Addition of small amounts of Tween-80 to solutions containing hypericin enhanced, by up to 2.6 $\log_{10}$, hypericin's virucidal activity.

Anthraquinones and anthraquinone derivatives with the hydroxyl and alkyl substitution pattern of emodin (i.e. emodin, emodin anthrone, emodin bianthrone and hypericin) were active against the enveloped viruses tested. The following general pattern of activity was found: hypericin > emodin bianthrone > emodin anthrone > emodin. Chrysophanic acid, aloe-emodin, and sennosides A and B did not possess activity against any of the viruses tested.

Anthraquinone; Hypericin; Emodin; Virucidal; Tween-80

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Introduction

The search for compounds useful in combating viral infections has resulted in relatively few successes. Most of the clinically useful compounds discovered so far have been nucleoside analogues, the usefulness of which has often been limited by the development of toxic side effects and the emergence of drug-resistant viruses. Consequently, the discovery of new non-nucleoside compounds, which are less toxic to host cells and have different mechanisms of action than nucleoside analogues, would be of great value.

There are a number of reports pertaining to the antiviral or virucidal activity of hypericin, an anthraquinone dimer first isolated from the plant, St. John's wort (*Hypericum perforatum* and related species) by Brockmann et al. (1951). Halm (1979) has reported that a hypericin-containing topical ointment aided in the healing of herpes simplex infections. Serkedjieva et al. (1990) reported that a mixed plant infusion containing *Hypericum perforatum* inhibited the reproduction of influenza viruses A and B, both in vitro and in vivo, and herpes simplex virus type 1, in vitro. The activity of hypericin and pseudohypericin against several murine retroviruses has been documented (Lavie et al., 1989; Meruelo et al., 1988). It has been recently reported that hypericin also demonstrates activity against herpes simplex virus, influenza virus A and Moloney murine leukemia virus (Mo-MuLV), but not against poliovirus or adenovirus (Tang et al., 1990). A number of other compounds structurally related to hypericin and pseudohypericin have been screened against human immunodeficiency virus (HIV) (Schinazi et al., 1990), but have not been evaluated for activity against other viruses.

Cognizant of these findings, we initiated studies to evaluate the activity of several other anthraquinones and anthraquinone derivatives against viruses representing several taxonomic groups.

Materials and Methods

Cells and viruses

Human epithelioid cervical carcinoma cells (HeLa; Flow Laboratories, Inglewood, CA) and African Green Monkey kidney cells (Vero, CCL 81; American Type Culture Collection, Rockville, MD) were grown in Eagle minimum essential medium (EMEM) with non-essential amino acids (Gibco Laboratories, Grand Island, NY) supplemented with 10% newborn calf serum (NCS) (Intergen Co., Purchase, NY), 20 mM Hepes buffer (pH 7.35) and 50 μg/ml of gentamicin reagent. Parainfluenza virus type 3 (Para-3) strain C-243, human rhinovirus type 2 (HRV-2) strain HGP and vaccinia virus (VV) strain Elstree, each propagated in HeLa cells, and herpes simplex virus type 1 (HSV-1) strain KOS, herpes simplex virus type 2 (HSV-2) strain 333 and vesicular stomatitis virus (VSV) strain Indiana, each propagated in Vero cells, were used in the direct pre-infection incubation assays. Additionally, a VSV pool was grown in HeLa cells for use in the infectious virus

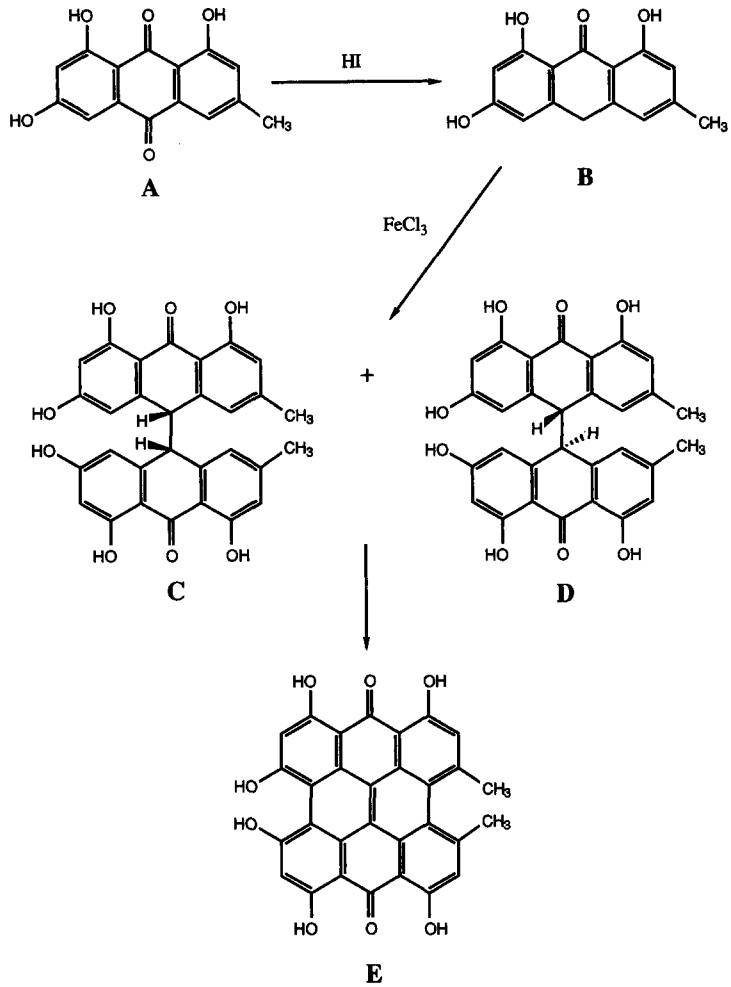
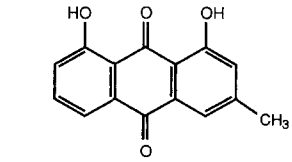


Fig. 1. Chemical synthesis of hypericin and structures of compounds with virucidal activity: A, emodin; B, emodin anthrone; C, *meso*-emodin bianthrone; D, D,L-emodin bianthrone; E, hypericin.

yield reduction assays. Virus-infected cells were cultured in EMEM with 2% NCS. HRV-2 was grown at 33°C in medium supplemented with 30 mM $MgCl_2$. The production of virus pools in this laboratory has been described (Kirsi et al., 1984).

Compounds

Structures of compounds used in this study are found in Figs. 1 and 2. Hypericin, aloe-emodin, and sennosides A and B were purchased from Carl Roth GmbH, Karlsruhe, F.R.G. Emodin and chrysophanic acid were purchased from Aldrich Chemical Co., Milwaukee, WI. Emodin anthrone was synthesized by reducing emodin with hydroiodic acid as described by Jacobson and Adams (1924). Emodin



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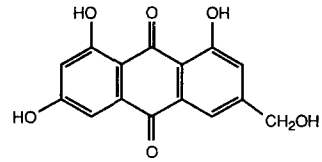

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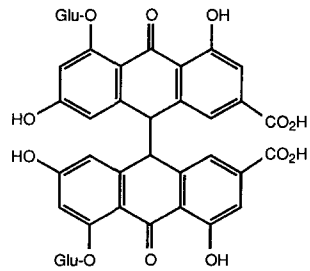

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Fig. 2. Chemical structures of compounds without virucidal activity: F, chrysophanic acid; G, aloe-emodin; H, sennoside A (D,L) and B (*meso*).

bianthrone was synthesized by reacting emodin anthrone with $FeCl_3$ in ethanol as described by Kinget (1967). The resulting diastereomers (*meso*- and D,L-) of emodin bianthrone were separated by reverse phase HPLC on a 5 μ C_{18} column (4.6 mm × 15 cm, Supelco) utilizing a gradient of 60 to 85% solvent B in solvent A. Solvent A was 20% acetonitrile in water containing 0.1% formic acid, while solvent B was 70% acetonitrile in methanol containing 0.1% formic acid. The compounds were detected by UV absorbance at 280 nm, with the D,L pair eluting first at 5.8 min and the meso compound at 6.8 min. The synthesized compounds were characterized by their UV spectra. The structures of the emodin bianthrone diastereomers were assigned based on their relative R_f values on silica gel TLC (Cameron et al., 1976).

Solubilization of the test compounds

Hypericin was initially solubilized in methanol, and the molarity of the resulting solution was calculated by measuring the absorbance at 590 nm and from the

extinction coefficient (ε^{o} = 44000) (Freytag, 1984). The methanol was then removed with the aid of a stream of nitrogen and the hypericin was dissolved in Dulbecco phosphate-buffered saline (PBS, pH 7.4) containing 0.1–0.3% Tween-80 to give a concentration of 200 μg/ml. This stock solution was further diluted to give the desired working concentration and a final Tween-80 concentration of no more than 0.005%. Emodin, emodin anthrone and emodin bianthrone were similarly prepared in PBS, except that the concentrations were calculated using the weights of the compounds rather than extinction coefficients.

In the experiments comparing the activities of emodin bianthrone isomers, samples of *meso*-, DL-, and a mixture of *meso*- and DL-emodin bianthrone were solubilized in ethanol. An aliquot was diluted in methanol and the absorbance was determined at 360 nm. Based on ε^{o} = 27500, the ethanol solutions were diluted in PBS to yield a compound concentration of 50 μg/ml in a 2.5% ethanol solution.

Chrysophanic acid and aloe-emodin were solubilized in ethanol containing 0.01% Tween-80 and subsequently diluted in PBS, with the aid of sonication, to give a compound concentration of 50 μg/ml in a 1% ethanol and 0.001% Tween-80 solution. Sennosides A and B were solubilized in 70% ethanol and diluted in PBS with sonication to give a compound concentration of 50 μg/ml in a 0.7% ethanol solution.

Direct pre-infection incubation (DPI) assay

Aliquots (30-μl) of each virus pool were mixed with 270-μl volumes of the diluted compounds in PBS and incubated at 37°C for 1 h. Typically, titers of virus pools were 5×10^{6} PFU/ml for VV, HRV-2 and HSV-2; 10^{7} PFU/ml for Para-3; 5×10^{7} PFU/ml for HSV-1, and 10^{8} PFU/ml for VSV. PBS containing Tween-80 and/or ethanol at the same concentration as present in the stock solutions of the compounds was used to dilute the solubilized compounds, thus maintaining the concentrations of these solubility-enhancing agents in a given experiment. As a control, each virus was also incubated in PBS containing only Tween-80 and/or ethanol at the appropriate concentrations. Following incubation of the virus-compound mixtures, the samples were serially diluted in PBS and assayed for infectious virus by standard plaque assay techniques using 24-well plates and the same cell lines as those used to propagate the viruses. The minimum amount of virus that could be detected by this assay was 50 PFU/ml. Overlay media (EMEM, 2% NCS) for HRV-2 plaque assays contained 30 mM $MgCl_2$ and 0.9% noble agar, while overlays for the other viruses contained 1% methyl cellulose. Plaques were counted after fixing the cell monolayer with 10% formalin and staining with 1% crystal violet.

Virus yield reduction assay

Virus yield reduction assays were performed using VSV and HSV-1 propagated in HeLa and Vero cells, respectively. Cell cultures were inoculated at an MOI of 0.01 and incubated in medium containing hypericin at 500 ng/ml, a concentration

determined to be nontoxic to both cell lines. Virus titers were determined 24 h post-infection by standard plaque assay techniques after two cycles of freezing and thawing and were compared with nontreated controls.

Results

Inactivation of viruses by pre-incubation with hypericin

The effect of hypericin (Fig. 1, compound E) concentration on the reduction of virus titers as determined by DPI assay is summarized in Fig. 3. With the exception of HRV-2, titers were inversely related to hypericin concentrations in a dose-dependent manner. Of the remaining five viruses, HSV-2 and VSV were the most sensitive to hypericin, with reductions of approximately 3.5 $\log_{10}$ at 500 ng/ml. At the same hypericin concentration, Para-3 and HSV-1 were intermediate in their response, with losses of infectivity approaching 2.0 $\log_{10}$. VV titers were reduced by 0.9 $\log_{10}$ only at a hypericin concentration of 1000 ng/ml. HRV-2 was not sensitive even at concentrations up to 10 μg/ml (data point not shown).

The effect of pre-incubation time on the virucidal activity of hypericin is shown in Fig. 4. The hypericin concentration was adjusted for the sensitivity of the viruses to hypericin (100 ng/ml for HSV-2 and VSV; 1000 ng/ml for Para-3 and VV). At these two hypericin concentrations, the rate of virus inactivation was greater for

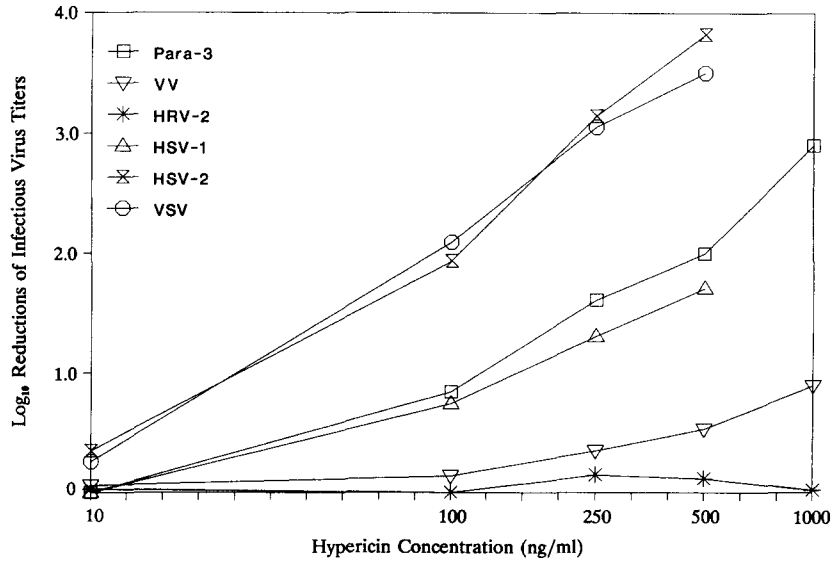

Fig. 3. Dose-dependent reductions of infectious virus titers by hypericin. Reductions of infectious virus titers were determined by DPI assay as described in Materials and Methods. Titers of viruses exposed to hypericin for 1 h were compared with titers of nontreated controls. At a hypericin concentration of 1000 ng/ml for VSV, HSV-1 and HSV-2, no plaques were formed at the lowest virus dilution assayed; therefore, the reductions are greater than can be determined by this assay.

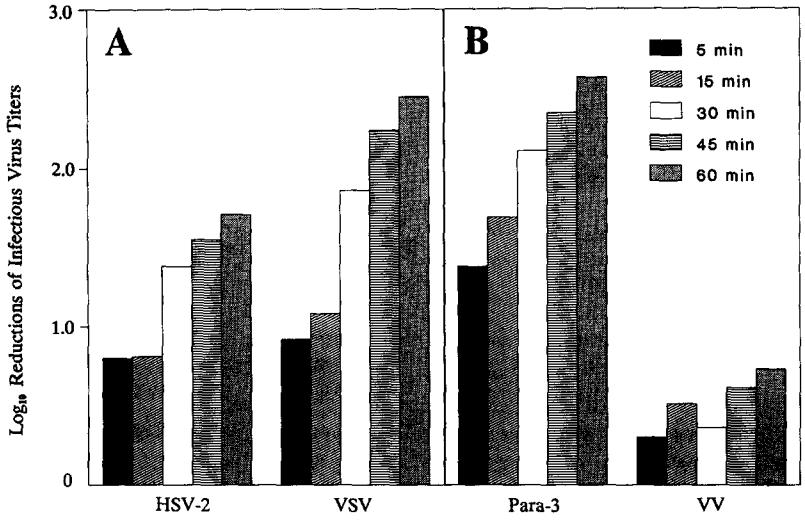

Fig. 4. Time-dependent reductions of infectious virus titers by hypericin. Reductions of infectious virus titers were determined by DPI assay from 5 to 60 min. A: HSV-2 and VSV were incubated with hypericin at 100 ng/ml. B: Para-3 and VV were incubated with hypericin at 1000 ng/ml. Titers of viruses incubated with hypericin were compared with titers of nontreated controls.

VSV than for HSV-2, and greater for Para-3 than for VV. The effectiveness of hypericin in reducing virus infectivity increased (by up to 1.5 $\log_{10}$ as in VSV) with the time of pre-infection incubation during a 60-min period.

Enhancement of hypericin's virucidal activity by Tween-80

Tween-80 enhanced the solubility of hypericin in PBS as determined both visually and spectrophotometrically. The effect of Tween-80 on the virucidal activity of hypericin is summarized in Table 1. Substantial increases (from 0.5 to 2.6 $\log_{10}$) in the ability of hypericin to reduce virus infectivity were obtained when Tween-80 was present in the PBS used to solubilize hypericin. Tween-80 was neither toxic to cell cultures at this concentration, nor did it have any effect on the infectivity of the viruses tested.

TABLE 1

Effect of Tween-80 on the reductions[a] of virus titers by hypericin

Tween-80 concentration[b] (%)	Para-3	VV	HSV-1	HSV-2	VSV
0.001	1.6	0.5	1.7	3.6	3.1
0	0.8	0.0	0.7	1.7	0.5

[a]Log_{10} reductions of virus titers recovered from DPI assays (described in Materials and Methods) were determined by comparing titers of hypericin-treated virus samples with those of virus controls.
[b]The hypericin stock solution in PBS contained 0.3% Tween-80 and was diluted with PBS to a hypericin concentration of 500 ng/ml and 0.001% Tween-80. Virus controls were incubated in PBS with 0.001% Tween-80.

TABLE 2

Log_{10} reductions[a] of virus titers by emodin bianthrone isomers

Concentration (μg/ml)	Isomer	Para-3	VV	HSV-1	HSV-2	VSV
2.5	*Meso*	0.1	ND[b]	0.7	2.5	1.1
	D,L	1.0	ND	2.7	≥3.4[c]	≥5.1
	Mixed[d]	1.0	ND	2.6	≥3.4	4.8
5.0	*Meso*	0.3	0.1	0.7	3.4	2.6
	D,L	1.6	0.5	3.2	≥3.4	≥5.1
	Mixed	1.2	0.4	2.8	≥3.4	4.3
10.0	*Meso*	0.5	0.2	1.0	≥3.4	3.6
	D,L	1.8	0.6	4.0	≥3.4	≥5.1
	Mixed	1.3	0.4	3.0	≥3.4	≥5.1

[a]Log_{10} reductions of virus titers were determined by DPI assay as described in Materials and Methods.
[b]ND designates not done.
[c]A ≥ sign indicates no plaques were formed at the lowest virus dilution assayed; therefore, the reduction is greater than can be determined by this assay.
[d]44% *Meso* and 56% D,L as determined by HPLC.

TABLE 3

Log_{10} reductions[a] of virus titers by hypericin, emodin bianthrone, emodin anthrone and emodin

Compound[b]	Para-3	VV	HRV-2	HSV-1	HSV-2	VSV
Hypericin	≥4.6	1.2	0.0	≥4.0[c]	≥4.3	≥4.6
D,L-Emodin bianthrone	1.8	0.6	0.0	4.0	≥3.4	≥5.1
Meso-emodin bianthrone	0.5	0.2	0.0	1.0	≥3.4	3.6
Emodin anthrone	0.3	0.1	0.0	0.9	≥4.1	1.2
Emodin	0.3	0.3	0.0	0.1	0.5	0.4

[a]Log_{10} reductions of virus titers were determined by DPI assay as described in Materials and Methods.
[b]All compounds were tested at 10 μg/ml.
[c]A ≥ sign indicates no plaques were formed at the lowest virus dilution assayed; therefore, the reduction is greater than can be determined by this assay.

Inactivation of viruses by emodin bianthrone, emodin anthrone and emodin

Initially, the virucidal activity of emodin bianthrone was determined (see Table 2, mixed isomers). Subsequently, the D,L- and *meso*-isomers of emodin bianthrone were separated and their individual activities ascertained. The D,L-isomer was consistently more active than the *meso*-isomer, and a mixture of the isomers (44% *meso* and 56% D,L as determined by HPLC) resulted in an activity intermediate to that of the D,L- and *meso*-isomers (see Table 2).

The virucidal activities of hypericin and those of D,L- and *meso*-emodin bianthrone, emodin anthrone and emodin are compared at 10 μg/ml in Table 3. These compounds are structurally related to hypericin and are intermediates in its synthesis (Fig. 1A–E). D,L- and *meso*-emodin bianthrone, emodin anthrone and emodin were less active than hypericin, with the following general pattern of activity: hypericin > D,L-emodin bianthrone > *meso*-emodin bianthrone > emodin

anthrone > emodin (see Table 3). It is noteworthy that the molar concentrations of emodin and emodin anthrone solutions were approximately twice that of the hypericin or bianthrone solutions. Therefore, comparison of emodin and emodin anthrone to hypericin, and D,L- and *meso*-emodin bianthrone on an equal molar concentration basis should result in even greater disparities in activity than those shown in Table 3.

Inactivation of viruses by other anthraquinone derivatives

Four other compounds, structurally similar to hypericin and emodin, were tested for virucidal activity. Chrysophanic acid, aloe-emodin, and sennosides A and B (see Fig. 2F–H) were not active against any of the viruses tested using the DPI assay at concentrations up to 50 μg/ml. These compounds were not tested at concentrations higher than 50 μg/ml because of solubility limitations.

Antiviral activity of hypericin

The virucidal activity of hypericin was determined by directly mixing virus with hypericin solutions in DPI assays. In other studies, the antiviral activity of hypericin was evaluated by incubating VSV-infected HeLa cells and HSV-1-infected Vero cells in medium containing hypericin. In these yield reduction assays, 500 ng/ml of hypericin resulted in $\log_{10}$ titer reductions of 1.6 and 1.2 for VSV and HSV-1, respectively.

Discussion

The direct inactivation of viruses in vitro by hypericin reported here is consistent with the recently published findings of Tang et al. (1990) and Lavie et al. (1989). Tang et al. (1990) reported the direct inactivation of HSV-1, influenza virus A and Mo-MuLV, but not of adenovirus or poliovirus, while Lavie et al. (1989) reported the direct inactivation of radiation leukemia virus and HIV. Our observation that hypericin was active against Para-3, VV, HSV-1, HSV-2 and VSV, but not against HRV-2, supports the proposal that hypericin is active only against enveloped viruses (Tang et al., 1990). The fact that VV was the least sensitive of the enveloped viruses tested is interesting in that VV possesses an envelope of a more complex nature than these other viruses.

Other investigators have reported that ethanolic or aqueous extracts obtained from *Hypericum perforatum* were effective in inhibiting the replication of tobacco mosaic virus (TMV), both in vitro and in tobacco plants (Schuster and Oschütz, 1979; Wetzler and Schuster, 1981), or of potato viruses X, M and S in potato meristem culture (Borissenko et al., 1985). While these plant viruses are non-enveloped, it should be noted that hypericin-containing crude plant extracts, rather than purified hypericin, were used.

The reductions in infectious virus yields obtained in this study when VSV and

HSV-1 were allowed to replicate in the presence of hypericin do not necessarily indicate an intracellular mode of antiviral action. Rather, reduction may be the result of direct inactivation of virus particles as they mature and are released into the hypericin-containing medium. Lavie et al. (1989) have suggested that hypericin may affect the assembly or processing of intact retrovirions from infected cells. Although hypericin was removed from the medium used to incubate their virus-infected cells, the loss of viral infectivity as measured by reverse transcriptase activity may have been due to disruption of mature viral cores as they bud from cell membranes intercalated with hypericin (Lavie et al., 1989). Tang et al. (1990) reported that hypericin did not show selective antiviral activity against HSV-1, influenza A, adenovirus, or poliovirus as determined by an assay based on the reduction of viral-induced cytopathic effect. Further studies are needed to separate direct inactivation of virus particles from possible specific mechanisms inhibiting steps in the multiplication or maturation of the viruses.

Data in Table 1 dramatically emphasize the significance of Tween-80 in enhancing hypericin's inactivation of viruses. The addition of small, nontoxic amounts of Tween-80 to aqueous hypericin solutions greatly enhanced hypericin's solubility and increased its inactivation of viruses by nearly 2 $\log_{10}$, as with HSV-2. Furthermore, without the addition of Tween-80, we were unable to sufficiently dissolve hypericin in PBS to achieve the stock concentrations used in this study. While we were able to solubilize hypericin in high concentrations of methanol or ethanol, dilution to working concentrations by addition of PBS resulted in the formation of a micro-fine suspension of hypericin which was readily removable by centrifugation. Tang et al. (1990) suggested that their inability to demonstrate the in vivo efficacy of hypericin against Friend leukemia virus as reported by Meruelo et al. (1988) may have been due to differences in hypericin isolation methods and differences in the strain of mouse utilized. However, our experience in solubilizing hypericin in an aqueous diluent leads us to believe that addition of a solubilizing agent such as Tween-80 may facilitate better systemic distribution of the hypericin.

Structure-activity relationships of the anthraquinones and anthraquinone derivatives used in this study have not yet been reported. A recent study by Schinazi et al. (1990) reported that emodin was less active than hypericin in reducing HIV reverse transcriptase activity. This accords with the pattern of activities seen in our study. Lavie et al. (1989) suggested that the hydroxymethyl group on pseudohypericin was the basis of diminished antiretroviral activity of the compound as compared to hypericin. Of the hydroxyanthraquinone and anthrone derivatives we have tested, those possessing the hydroxyl and alkyl substitution pattern of emodin (i.e. emodin, emodin anthrone, emodin bianthrone and hypericin) (see Fig. 1) were able to inactivate the enveloped viruses tested. This activity was enhanced when the structure was in a dimerized form as in emodin bianthrone and hypericin. Furthermore, regardless of whether or not the derivative was in a dimerized form, alteration in the hydroxyl or alkyl substitution pattern resulted in a loss of activity, as seen with chrysophanic acid, aloe-emodin, and sennosides A and B (see Fig. 2).

The D,L pair of emodin bianthrone has a more extended structure similar to hypericin, whereas in the *meso* compound the two rings are folded back on each other

(see Fig. 1C and D). This structural difference is reflected in their relative abilities to inactivate viruses, with the D,L pair showing the greater virucidal activity. Presumably, the relative availability of the ring structures on these compounds for interaction with viral envelopes plays an important part in their ability to inactivate viruses.

Hypericin and related compounds have been shown to possess somewhat selective activity against viruses, both in vitro and in vivo. However, the equivocal results published to date demonstrate that the mechanism for this activity is not yet well defined. Given the need for new compounds which are clinically active against viruses, the data presented here suggest the relevance of further investigation into the mode of action of anthraquinones and their derivatives.

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