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Evaluation of the antiviral activity of anthraquinones, anthrones and anthraquinone derivatives against human cytomegalovirus

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Summary

A number of anthraquinones, anthrones and anthraquinone derivatives were evaluated for antiviral activity against human cytomegalovirus (HCMV) as well as for cytotoxicity. Of those compounds evaluated, quinalizarin, emodin, rhein, hypericin, protohypericin, alizarin, emodin bianthrone and emodin anthrone showed antiviral activity against a normal laboratory HCMV strain, AD-169. When tested against a ganciclovir-resistant strain of HCMV, the EC_{50} values for quinalizarin, rhein and alizarin were superior to the values obtained for the AD-169 strain of HCMV. These results suggest that these compounds will be useful as prototypes for synthesizing a class of anti-HCMV drugs that are effective against ganciclovir-sensitive and -resistant strains of HCMV.

Human cytomegalovirus; Anthraquinone; Hypericin; Cytotoxicity; Virucidal

Introduction

Human cytomegalovirus (HCMV) is a major cause of life-threatening infections in immunosuppressed patients such as those receiving organ or tissue transplants, cancer patients, burn patients and those afflicted with AIDS (Colacino and Lopez, 1983; Mills, 1986). In addition, intrauterine HCMV infections (i.e., congenital cytomegalo disease) are second only to Down's

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syndrome as a known cause of mental retardation (Griffiths and Grundy, 1987). Ganciclovir is often the drug of choice for treatment of some of those infections including CMV gastrointestinal infections and CMV retinitis (Mills, 1986; Jabs et al., 1989). Unfortunately, prolonged therapy with ganciclovir causes serious side effects, such as neutropenia, which limits its application (Meyers, 1988). Recently, ganciclovir-resistant strains of HCMV have been isolated from AIDS patients undergoing ganciclovir treatment (Erice et al., 1989). Therefore, this has resulted in an intensive search for compounds that are less toxic and have different modes of action against HCMV.

Recently, naturally occurring anthraquinones and their derivatives have been studied for antiviral activity against human immunodeficiency virus (HIV) and other retroviruses (Nakashima and Yamamoto, 1987; Meruelo et al., 1988; Schinazi et al., 1990; Tang et al., 1990). Some of these compounds have also been reported to possess antiviral activity in vitro against herpes simplex viruses 1 and 2 (HSV-1, HSV-2), influenza virus, vesicular stomatitis virus (Lavie et al., 1987) and Epstein-Barr virus (Konoshima et al., 1989). Murine Friend leukemia virus infections in mice have been reported to be inhibited by a polycyclic anthraquinone, hypericin (Lavie et al., 1989). However, other investigators (Tang et al., 1990) have seen no effect of hypericin on this virus infection in mice. Other anthraquinones have been tested against HIV, but very few have been found to be active against this virus (Schinazi et al., 1990). Tang et al. (1990) have found that some anthraquinones also have virucidal activity against RNA and DNA viruses.

This report describes the in vitro anti-HCMV activity and the cytotoxicity of a number of anthraquinones, anthrones and their derivatives.

Materials and Methods

Compounds

All but three of the compounds evaluated for antiviral activity in this study were obtained from Aldrich Chemical, Inc. (Milwaukee, WI). They were tested without further purification. Four compounds were synthesized at Murdock Healthcare (Springville, UT). Emodin anthrone was synthesized by reducing emodin with hydroiodic acid in glacial acetic acid according to Jacobsen and Adams (1924) and was dimerized to give the 10,10′-coupled emodin bianthrone by reacting emodin anthrone with $FeCl_3$ in ethanol (Kinget, 1967). To assess the purity and confirm the identity of the emodin anthrone and emodin bianthrone, the products of these syntheses were analyzed by reversed phase HPLC on a 5-micron column (4.6 mm × 15 cm; Supelco, Bellefonte, PA) utilizing a gradient of 60% to 85% solvent B in solvent A over a period of 10 min. Solvent A was 20% acetonitrile in water containing 0.1% formic acid, while solvent B consisted of 70% acetonitrile in methanol containing 0.1% formic acid.

Protohypericin was obtained by subsequent oxidation of emodin bianthrone with oxygen in methanol containing triethylamine. To synthesize hypericin, a solution of protohypericin in ethyl acetate was exposed to strong sunlight (Brockmann and Eggers, 1955). Protohypericin and hypericin were also analyzed by reversed phase HPLC (5 micron Microsorb column, 4.6 mm × 25 cm; Rainin, Emeryville, CA) utilizing a gradient of 2% acetic acid in acetonitrile (solvent D) in 2% acetic acid-water (solvent C) for 0–8 min, 20–25% solvent D in solvent C from 8–14 min and 25–90% solvent D in solvent C from 14–20 min. The compounds were detected by UV absorbance at 280 nm. Purity of each compound was determined to be >95%. To characterize the synthesized compounds, the UV and visible spectra of the compounds were compared to the published spectra for these compounds. Protohypericin and hypericin were also characterized by NMR spectra.

Hypericin was also obtained from Roth, GmbH (Karslruhe, F.R.G). Radiolabeled compounds used for biochemical cytotoxicity studies were obtained from ICN Radiochemicals (Irvine, CA).

Cells, virus and medium

Human foreskin fibroblasts (Hs-68) and human lung fibroblasts (MRC-5) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were routinely grown in Dulbecco's modified Eagle medium (DMEM; GIBCO BRL, Grand Island, NY) or basal medium Eagle (BME; GIBCO BRL), respectively. Each type medium was supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT) and $NaHCO_3$ without antibiotics for cell growth. HCMV strain AD-169 from ATCC and a ganciclovir resistant strain, C8805-37 (a gift from Dr. Karen Biron; Burroughs Wellcome, Research Triangle Park, NC), were propagated in MRC-5 cells.

Plaque reduction assay

To test the antiviral effects of each compound, a modified plaque reduction assay was run without the semi-solid overlay. HCMV in DMEM, 2% FBS, 0.1% $NaHCO_3$, 50 μg gentamicin/ml (test medium) was allowed to adsorb (1.0 ml/well with 30–100 PFU/ml) to confluent monolayers of MRC-5 cells in 24-well tissue culture plates (Corning Glass Works, Corning, NY) by centrifugation of the plates at 2200 rpm for 30 min at room temperature. Compounds, except ganciclovir (DHPG), were dissolved in absolute ethanol (EtOH) with either 0.1% or 0.001% Tween 80 (Polysorbate 80, U.S.P.; BBL, Div. Becton, Dickinson and Co., Cockeysville, MD) and diluted in test medium with EtOH and/or Tween 80 added to attain final concentrations of 0.25% EtOH and 0.001% Tween 80 in all test concentrations of compounds other than DHPG. Experiments comparing media with or without the 0.25% EtOH and 0.001% Tween 80 elicited no visible cytotoxicity or antiviral activity due to the EtOH/Tween 80 additives. DHPG was dissolved and diluted in test medium lacking

both EtOH and Tween 80. Each compound was diluted using a one-half $\log_{10}$ dilution series and added to the cells after the virus inoculum was removed. Seven concentrations of compound were used for each test, ranging from 10 μg/ml to 0.01 μg/ml unless cytotoxicity dictated a different range. Each plate included 2 test wells for each concentration of compound used, 2 cell control wells, 4 virus control wells, and 4 wells containing the known positive control compound, DHPG. The plates were incubated at 37°C for 3–7 days, until plaques were well developed in the virus control wells. Before staining, the cells were examined microscopically and graded on a scale of 0% (no evidence of cytotoxicity) to 100% (complete destruction of the cells), by 20% increments, for degree of visible cytotoxicity. The cells were fixed and stained with 0.2% crystal violet in 10% buffered formalin and plaques were counted by use of a dissecting microscope. The percent reduction in plaque number was plotted vs. log concentration. The exponential least squares method was used to fit a curve to those data points bracketing the 50% plaque reduction values, and the EC_{50} value was estimated from the equation of the line. IC_{50} values were determined in a similar manner from visual cytotoxicity data.

Virucidal test

Equal volumes (0.25 ml) of virus (at $10^{6.6}$ PFU/ml) and compound dilutions or diluent without compound were mixed and incubated at 37°C in a water bath for 1 h. These mixtures were then quickly diluted in test medium using a 10-fold serial dilution scheme and 1.0 ml of each dilution (10^{-1} thru 10^{-5}) was added to each of 2 wells of a 24-well plate (Corning) containing confluent monolayers of MRC-5 cells. The plates were centrifuged at 2200 rpm for 30 min at room temperature to allow the virus to adsorb to the cells. The inoculum was aspirated from the cell sheets and fresh test medium (1.0 ml/well) was added. After 6 days of incubation at 37°C in a moist atmosphere of 5% CO_2 and 95% air, the medium was aspirated from each well and the cells were stained with 0.2% crystal violet in 10% buffered formalin. Plaques were counted by use of a dissecting microscope. Virus titers were calculated from the mean plaque count of the duplicate wells which yielded the highest virus titer.

Viable cell count

Compounds were prepared at 4000 μg/ml in absolute ethanol with 0.001% Tween 80. A control tube with absolute ethanol and 0.001% Tween 80 and without test compound was also prepared. These preparations were diluted in DMEM, 2% FBS, 0.1% $NaHCO_3$, 50 μg gentamicin/ml, 0.001% Tween 80 to obtain the concentrations tested. The growth medium in 12-well tissue (Corning) culture plates previously seeded with MRC-5 cells and incubated for 5 h at 37°C was replaced with control medium or test compounds at the desired concentrations (1.0 ml/well). The plates were then incubated for various time periods at 37°C prior to doing viable cell counts. At the appropriate time

periods, medium was aspirated from duplicate (test compound) or triplicate (control) wells to allow counting of the cells as follows. Trypsin (0.25%, 0.5 ml/well) was added and incubated until the cells were rounded. PBS/BSA (0.25 ml) was added without removing the trypsin, and the cells were scraped from the surface with a rubber policeman. The cell suspension was transferred to a snap-cap tube containing 1.0 ml of 0.2% trypan blue in PBS/BSA. An additional 0.25 ml of PBS/BSA was used to rinse the remaining cells from each well into the appropriate tube containing trypan blue stain. Viable cells in samples from each tube were counted using a hemacytometer. IC_{50} values were calculated using the least squares method described above.

Biochemical cytotoxicity assay

The effects of each test compound on the uptake of [^{3}H]thymidine, [^{3}H]uridine, and [^{3}H]leucine into acid soluble and insoluble fractions of cells were determined according to the method of Smee et al. (1983). Approximately 1.5×10^4 cells were seeded per well in 96-well plates (Corning) and incubated at 37°C in a 5% CO_2 humidified atmosphere until cells were confluent. Cells were incubated as before with each compound concentration for 18 h at 37°C. The medium was removed and compound plus the appropriate radiolabeled precursor (10 μCi /ml) in serum-free DMEM (SF-DMEM) were added to cells and incubated as before for 1 h at 37°C. SF-DMEM without leucine was used in the assays involving the uptake of [^{3}H]leucine. Following this incubation, the medium was aspirated from the cells which were rinsed with SF-DMEM and then fixed with 5% trichloroacetic acid for 1 h at 5°C. An aliquot of 50 μl was removed from each well and the incorporation of the radiolabel into the acid soluble fraction was determined using a liquid scintillation counter. The remaining liquid was aspirated from the monolayers and the monolayers were then incubated with 0.5 N KOH at 37°C for a minimum of 2 h. The entire volume for each well was subsequently removed to determine the incorporation of radiolabel into the acid precipitable fraction of cells using a liquid scintillation counter. The concentration of drug that inhibited the uptake of radiolabeled precursors into either TCA-soluble or TCA-insoluble cell fractions by 50% (IC_{50}) was calculated by regression analysis.

Results

Antiviral activity

The in vitro anti-HCMV activity of several anthraquinones and anthraquinone derivatives is shown in Table 1. Quinalizarin, emodin, rhein, hypericin, protohypericin, alizarin, emodin bianthrone and emodin anthrone showed activity against HCMV strain AD-169 distinguishable from cytotoxic effects on cells. Of these, quinalizarin had the highest therapeutic index at 3.4 and emodin

anthrone and emodin bianthrone the lowest therapeutic indices. In 3 separate experiments with emodin anthrone, cytotoxicity varied considerably, making it impossible in some experiments to determine an EC_{50} value. This probably occurred because we were unable to completely solubilize the emodin anthrone, resulting in a colloidal suspension each time with variable amounts of the compound in true solution. The data shown in Table 1 represent one experiment where calculation of an EC_{50} value was possible. Two of the compounds (1,8-dihydroxyanthraquinone, quinizarin) showed slight activity (<50% plaque inhibition) at 41.6 μM and only slight cytotoxicity (graded visually as 20%) at the highest concentration tested. No concentrations were tested which could have provided a calculable therapeutic index for these compounds. Anthraquinone also showed less than 50% inhibitory activity at all concentrations used up to 48 μM, but was not visibly cytotoxic at any concentration. Anthraflavic acid and purpurin showed no antiviral activity, but substantial cytotoxicity at the concentrations tested.

TABLE 1

Evaluation of the antiviral activity of anthraquinones, anthrones and anthraquinone derivatives against human cytomegalovirus strain AD-169 in MRC-5 cells

Compound	EC_{50} (μM)[a]	IC_{50} (μM)[b]	TI[c]
Ganciclovir	10.6 ± 6.3 (14)[d]	>3919.0	>370.0
Quinalizarin	3.7 ± 0.5 (2)	12.5 ± 0.0	3.4
Emodin	4.1	9.6	2.3
Rhein	5.6 ± 2.0 (2)	12.9 ± 1.6	2.3
Hypericin	1.8 ± 0.4 (5)	2.8 ± 1.8	1.5
Protohypericin	5.7	11.1	1.9
Alizarin	24.1	>41.6	>1.7
Emodin bianthrone	1.0 ± 0.4 (3)	1.6 ± 0.6	1.5
Emodin anthrone	2.3	3.5	1.5
Emodin bianthrone (D,L)	3.5	2.4	<1
Emodin bianthrone (*meso*)	3.7	3.5	<1
1,8-Dihydroxyanthraquinone	>41.6	>41.6	?[e]
Quinizarin	>41.6	>41.6	?
Anthraflavic acid	>13.3	14.2	?
Anthraquinone	>15.4	>48.0	?
Aloe-emodin	>37.0	>37.0	?
Anthrarufin	>41.6	>41.6	?
Aloin	>23.9	>23.9	?
Chrysophanol	>39.3	>39.3	?
Physcione	>35.2	>35.2	?
Sennoside A (D,L)	>11.6	>11.6	?
Sennoside B (*meso*)	>11.6	>11.6	?
Purpurin	NA[f]	21.8	NA

[a]The concentration of compound that reduces plaque production by 50%.

[b]The concentration of compound calculated by regression analysis to lie midway between those concentrations showing no evidence of cytotoxicity and those showing complete cytotoxicity, as observed by light microscopy.

[c]TI: therapeutic index (IC_{50}/EC_{50}).

[d]Represents the number of experiments used to derive the mean and S.D. If not noted in parentheses, the compound was tested in one experiment in duplicate.

[e]?: unknown – cannot be calculated.

[f]NA: not active.

TABLE 2

Antiviral activity of ganciclovir and selected anthraquinones, anthrones and anthraquinone derivatives against a ganciclovir-resistant strain of HCMV in MRC-5 cells[a]

Compound	EC_{50} (μM)[b]	IC_{50} (μM)[c]	TI[d]
Ganciclovir	191	>3919	>20
Quinalizarin	1.5	>36.7	>24
Emodin	3.7	12.6	3.4
Rhein	2.0	>40	>20
Hypericin	3.8	57.5	15
Alizarin	3.7	>41.6	>11
Emodin bianthrone	0.8	3.5	4.4
Emodin anthrone	4.7	10.1	2.1

[a]Determined by a plaque reduction assay. Data from a single experiment with each compound tested in duplicate.
[b]The concentration of compound that reduces plaque production by 50%.
[c]The concentration of compound calculated by regression analysis to lay midway between those concentrations showing no evidence of cytotoxicity and those showing complete cytotoxicity, as observed by light microscopy.
[d]TI: therapeutic index (IC_{50}/EC_{50}).

Of the compounds active against the AD-169 strain of HCMV (TI values > 1.0, Table 1), seven were also tested against a ganciclovir-resistant strain of HCMV in parallel with ganciclovir (Table 2). The EC_{50} values of alizarin, quinalizarin and rhein were lower in experiments with the ganciclovir-resistant strain than with the AD-169 virus strain. In addition, the TI values of all the compounds tested were greater in experiments with the ganciclovir-resistant strain than with the AD-169 strain, although the increased IC_{50} values calculated from this set of experiments contributed to the apparent increases in TI values seen in Table 2. This may be so because the C8805-37 strain of HCMV developed plaques more rapidly than did the AD-169 strain. Thus, the cells infected by C8805-37 virus were stained at 3 days instead of the usual 6–7 days post-infection as for strain AD-169. This resulted in reduced visual evidence of chemical cytotoxicity in cells exposed to the compounds for the shorter period of time. Two compounds were tested for virucidal activity by incubation of the virus with compound before virus adsorption (data not shown). Hypericin had an EC_{99} value of 1.2 μM. However, emodin bianthrone was not virucidal at the concentrations showing antiviral activity.

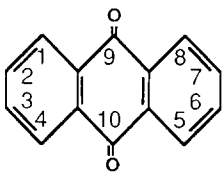
Structure-activity relationships

The structure-activity relationships of the anthraquinones, anthrones and anthraquinone derivatives are shown in Tables 3 and 4. It is noteworthy that all but two (alizarin and emodin anthrone) of the active compounds are similar in structure, with keto groups at C-9 and C-10 and hydroxyl groups at the C-1,8 positions (Table 3) (equivalent positions for dimers: C-10,10′; C-4,5; C-4′,5′ – see Table 4). All but one of these compounds (rhein) have hydroxy substitutions at either the C-2 or C-3 positions of the basic anthraquinone

structure (equivalent positions for dimers: C-2,2′), although rhein does have a carboxyl group at C-3. Dihydroxyanthraquinones with hydroxy substitutions at C-1, and either C-4 or C-8 (quinizarin and 1,8,-dihydroxyanthraquinone) had slight anti-HCMV activity (plaque counts were reduced by no more than 30% relative to untreated control cultures). However, the compound with C-1,4,5,8 hydroxyl group substitution pattern, quinalizarin, showed much greater antiviral activity. Of those compounds found to have no activity against HCMV, none were hydroxy substituted at C-2 or C-3 positions except anthraflavic acid which lacks the C-1,8 hydroxyl groups. Purpurin also has an additional hydroxyl group at C-4, which differentiates its structure from that of

TABLE 3

Structure-activity relationships of anthraquinones and anthrones with antiviral activity against HCMV[a]



Compound	Substituents									
	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10
Active										
Quinalizarin	OH	OH	–	–	OH	–	–	OH	=O	=O
Emodin	OH	–	OH	–	–	CH_3	–	OH	=O	=O
Rhein	OH	–	COOH	–	–	–	–	OH	=O	=O
Alizarin	OH	OH	–	–	–	–	–	–	=O	=O
Emodin anthrone	OH	–	OH	–	–	CH_3	–	OH	=O	2H
Slightly Active[b]										
1,8-Dihydroxy-anthraquinone	OH	–	–	–	–	–	–	OH	=O	=O
Quinizarin	OH	–	–	OH	–	–	–	–	=O	=O
Anthraquinone	–	–	–	–	–	–	–	–	=O	=O
Inactive[c]										
Aloe-emodin	OH	–	CH_2OH	–	–	–	–	OH	=O	=O
Aloin	OH	–	CH_2OH	–	–	–	–	OH	=O	Glucose
Anthraflavic acid	–	OH	–	–	–	OH	–	–	=O	=O
Anthrarufin	OH	–	–	–	OH	–	–	–	=O	=O
Chrysophanol	OH	–	CH_3	–	–	–	–	OH	=O	=O
Physcione	OH	–	O-CH_3	–	–	CH_3	–	OH	=O	=O
Purpurin	OH	OH	–	OH	–	–	–	–	=O	=O

[a]Activity and toxicity were determined as described in Materials and Methods. Compounds were categorized according to the data found in Table 1. The numbering system is used for the sake of comparison and is not necessarily consistent with the nomenclature found in Chemical Abstracts or with IUPAC nomenclature.

[b]Compounds that did not reduce plaque counts by more than 50% relative to untreated controls in a plaque reduction assay.

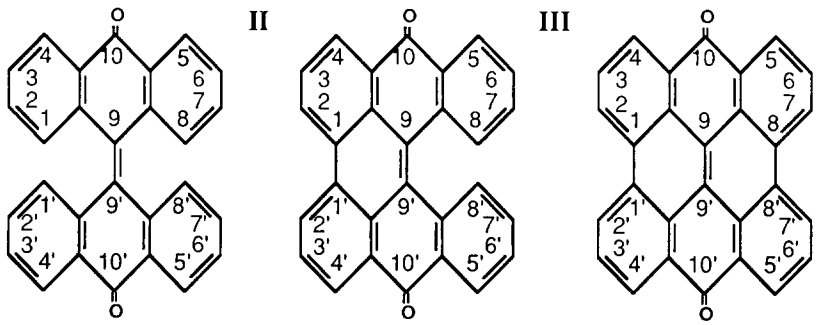
[c]Compounds showed no antiviral activity at the highest concentrations tested in a plaque reduction assay.

alizarin, an active compound. Anthraquinone, without any hydroxy substitutions, and anthrarufin, which also lacks a hydroxyl group at C-8 showed little or no activity at the concentrations tested. Substitutions at the C-3 position of the basic C-1,8 dihydroxyanthraquinone structure appeared to enhance antiviral activity. Substitution with a carboxyl or hydroxyl group resulted in antiviral activity while substitution with a methyl, methyl alcohol or methoxy group led to loss of antiviral activity. No antiviral activity was detected at the concentrations tested when the substitution was a glucose moiety at the C-5 and C-5′ positions of the anthraquinone dimers (Table 4 – structure I) (sennoside A and B).

TABLE 4

Structure-activity relationships of anthraquinone dimers with antiviral activity against HCMV[a]

I II III



Compound	Substituents									
	C-1.1′	C-2.2′	C-3.3′	C-4.4′	C-5.5′	C-6.6′	C-7.7′	C-8.8′	C-9.9′	C-10.10
Active										
Emodin bianthrone (I)	–	OH	–	OH	OH	–	CH_3	–	–	=O
Protohypericin (II)	–	OH	–	OH	OH	–	CH_3	–	–	=O
Hypericin (III)	–	OH	–	OH	OH	–	CH_3	–	–	=O
Slightly active[b]										
Emodin bianthrone (D,L) (I)	–	OH	–	OH	OH	–	CH_3	–	–	=O
Emodin bianthrone (*meso*) (I)	–	OH	–	OH	OH	–	CH_3	–	–	=O
Inactive[c]										
Sennoside A (D,L) (I)	–	COOH	–	OH	Glucose-O	–	–	–	–	=O
Sennoside B (*meso*) (I)	–	COOH	–	OH	Glucose-O	–	–	–	–	=O

[a]Activity and toxicity were determined as described in Materials and Methods. Compounds were categorized according to the data found in Table 1. The numbering system used is for the sake of comparison and is not necessarily consistent with the nomenclature found in Chemical Abstracts or with IUPAC nomenclature.

[b]Therapeutic index < 1.

[c]Compounds showed no antiviral activity at the highest concentrations tested in a plaque reduction assay.

Cytotoxicity

Alizarin appeared to be the least toxic of the compounds tested based on the uptake of radiolabeled macromolecular precursors into cells and subsequent incorporation into cellular DNA, RNA, and protein (Tables 5, 6).

Adding hydroxyl groups at the C-5 and C-8 positions of alizarin to form quinalizarin enhanced the antiviral activity (decreased the EC_{50}, see Tables 1, 2). However, quinalizarin was a more potent inhibitor of cellular DNA repair and/or synthesis (IC_{50} = 18.4 μM) than alizarin (IC_{50} > 41.7 μM). Microscopic examination of quinalizarin-treated cells also revealed marked cytotoxicity.

Emodin, with a hydroxy substitution at C-3 of the basic active

TABLE 5

Effect of selected anthraquinones, anthrones and anthraquinone derivatives on the uptake of radiolabeled macromolecular precursors into TCA-soluble fractions of Hs-68 cells

Compound[b]	IC_{50} (μM)[a]		
	Thymidine	Uridine	Leucine
Quinalizarin	>36.7	>36.7	>36.7
Emodin	35	26.2	5.7
Rhein	>35.2	>35.2	>35.2
Hypericin	9.6	6.8	5
Alizarin	>42	>42	>42
Emodin bianthrone	11.8	13.6	50.5
Emodin anthrone	39	14.8	8.8

[a]IC_{50} is the concentration of drug required to inhibit the uptake of radiolabeled macromolecular precursors into TCA-soluble cell fractions by 50%.
[b]Each dilution of compound was tested four times for inhibition of incorporation of the appropriate [^{3}H]precursor into the soluble fractions of treated cells.

TABLE 6

Effect of selected anthraquinones, anthrones and anthraquinone derivatives on the uptake of radiolabeled macromolecular precursors into DNA, RNA and protein of Hs-68 cells

Compound[b]	IC_{50} (μM)[a]		
	Thymidine	Uridine	Leucine
Quinalizarin	18.4	>36.7	>36.7
Emodin	22	27	30
Rhein	9.3	>35.2	58.2
Hypericin	2.6	7.4	1.3
Alizarin	>42	>42	>42
Emodin bianthrone	3.7	13.3	5.6
Emodin anthrone	8.2	18.5	5.9

[a]IC_{50} is the concentration of drug required to inhibit the uptake of radiolabeled macromolecular precursors into TCA-insoluble cell fractions by 50%.
[b]Each dilution of compound was tested four times for inhibition of incorporation of the appropriate [^{3}H]precursor into the macromolecules of treated cells.

anthraquinone structure and a methyl group at C-6 (Table 3) inhibited [^{3}H]leucine uptake (IC_{50} = 5.7 μM) and consequently protein synthesis (IC_{50} = 30 μM). Elimination of a keto group from the emodin structure to form emodin anthrone resulted in a definite increase in inhibition of macromolecular synthesis when compared to emodin (Table 6). The inhibition of RNA and protein synthesis by emodin anthrone was largely due to the inhibition of the uptake of metabolic precursors into cells (Table 5).

The dimer of emodin, emodin bianthrone, was equally as toxic as emodin anthrone (Tables 5, 6), although they had much different effects on the uptake of thymidine and leucine into soluble cell fractions (Table 5).

Hypericin appeared to be the most potent inhibitor of metabolic precursor uptake and cellular macromolecular synthesis of the compounds tested. The IC_{50} values calculated for hypericin represented the lowest values for each parameter tested for any compound (Tables 5, 6).

Rhein inhibited DNA repair and/or synthesis (IC_{50} = 9.3 μM) at relatively low concentrations, although rhein had relatively little effect on the uptake of metabolic precursors and RNA and protein synthesis when compared with the other compounds tested.

The effects of a number of the anthraquinones on cell viability after 3 or 6 days exposure to the anthraquinone derivatives are shown in Table 7. These results generally agree with those obtained from the biochemical cytotoxicity tests. However, emodin appeared less toxic by viable cell assay. Very little difference was seen between cell counts determined after 3 days of exposure to the anthraquinone derivatives and those determined after 6 days of exposure, except at concentrations of 200 μM for hypericin and 370 μM for emodin. At 200 μM, hypericin exposure reduced the viable cell counts from 20% of control at 3 days to 0% after 6 days of exposure; and exposure to 370 μM emodin reduced the cell counts from 59% of control at 3 days to 18% of control after 6

TABLE 7

Viability of MRC-5 cells after 3 or 6 days of exposure to hypericin, emodin, emodin anthrone or emodin bianthrone

Compound	IC_{50} (μM)		
	Stationary cells[a]		Growing cells[b]
	3 days	6 days	3 days
Hypericin	30.6	18.2	4.3
Emodin	>370	184.6	>117
Emodin anthrone	18.1	13.8	13.1
Emodin bianthrone	<2.0	<2.0	2.1

[a]Cells in untreated control wells reached a maximum cell density at day 2, as estimated by viable cell counts; thereafter, cells remained quiescent.

[b]Cells in untreated control wells did not quite reach maximum cell density at 3 days as estimated by viable cell counts.

days of exposure (data not shown). Growing cells were more sensitive to the effects of the compounds as would be expected. For example, hypericin resulted in a seven-fold lower IC_{50} value when compared to the IC_{50} value for cells in the stationary phase. The IC_{50} values (in growing cells at 3 days) for all the compounds except emodin were close to those values obtained for the effects of the compounds on macromolecular synthesis in stationary cells (see Table 6).

Discussion

A number of anthraquinones and anthraquinone derivatives have now been found to possess antibacterial, antiviral, and antitumor activity (Cudlin et al., 1976; Dhunanjaya and Antony, 1987; Nakashima and Yamamoto, 1987; Meruelo et al., 1988). In addition, Tang et al. (1990) found that certain anthraquinones were virucidal for both DNA and RNA enveloped viruses, but not viruses (polio and adeno viruses) without envelopes. It has been suggested that some of the biological properties of the anthraquinone class of compounds may be related to their ability to form free radicals or to intercalate with nucleic acids (Calendi et al., 1965; Meruelo et al., 1988; Schinazi et al., 1990). Other studies suggest that some anthraquinone derivatives inhibit enzymes such as protein kinase C (Takahashi et al., 1989) and reverse transcriptase (Schinazi et al., 1990). In addition, there are several conflicting reports concerning the ability of some of these compounds to act as mutagens (Betina, 1989) and to initiate (Westendorf et al., 1990) or to promote tumors (Wölfle et al., 1990). It is also possible that the anthraquinones may induce interferon to inhibit HCMV. Jamison et al. (1990) tested a number of anthraquinones for induction of interferon. Emodin, alizarin and rhein did not significantly induce interferon in an human foreskin fibroblast-vesicular stomatitis virus interferon bioassay.

In this study quinalizarin, emodin, rhein, hypericin, protohypericin, alizarin, emodin bianthrone and emodin anthrone were found to possess anti-HCMV activity (non-virucidal activity). Quinizarin and 1,8-dihydroxyanthraquinone possessed marginal activity against HCMV. This antiviral activity was unexpected, since Carpenter and Kraus (1991), Lavie et al. (1987) and Tang et al. (1990) have shown that many anthraquinones inhibit enveloped viruses because of their virucidal properties. Significantly, three of the compounds (quinalizarin, alizarin and rhein) were even more effective against a ganciclovir-resistant strain of HCMV (lower EC_{50} values). This suggests that these compounds or their derivatives may be useful for treating HCMV infections resistant to ganciclovir.

The following generalizations can be made about the structure-activity relationships of these compounds. First, hydroxy substitutions at the C-1 and C-8 positions (equivalent positions for dimers; C-4,5 and C-4′,5′) correlate with anti-HCMV activity since all of the active compounds were hydroxy substituted at the C-1 and C-8 positions except for alizarin. Second, the type of substitution at the C-3 position determines if the compound is active against

HCMV. The active compounds with either a carboxy or hydroxy substitution at C-3 showed antiviral activity against HCMV, while compounds with methoxy, methyl or hydroxymethyl groups at C-3 were not active. Third, an hydroxy substitution at the C-1 position in combination with an hydroxyl group at the C-4 or C-8 position may also contribute to anti-HCMV activity of an anthraquinone derivative. The two compounds that were marginally active, quinizarin and 1,8-dihydroxyanthraquinone, have an hydroxyl group at C-1 in combination with an hydroxy substitution at C-4 or C-8. A similar compound (anthrarufin) with an hydroxyl group at C-1 in combination with an hydroxy substitution at C-5 had no antiviral activity. Fourth, compounds with hydroxy substitutions at C-1 and C-2 are less toxic than compounds with other substitution patterns. The two least cytotoxic compounds (alizarin and quinalizarin), as judged by inhibition of macromolecular synthesis, are hydroxy substituted at C-1 and C-2. One group of investigators (Swanbeck and Zetterberg, 1971) noticed that substitution of anthraquinones with hydroxyl or methoxy groups increases the degree of binding to DNA except when the substitution makes the compound a negative ion at pH 7.2 (at positions 2, 3, 6 or 7), when no binding occurs. For example, emodin has three hydroxyl groups, two of them in positions 1 and 8 promote binding, but the third hydroxyl group at C-3 tends to negate this effect (Anke et al., 1980). A general trend for the compounds found active against HCMV is that most of them have hydroxy substitutions at one of the positions (C-2 or C-3) which could lessen the compounds' affinity for DNA (Toma et al., 1975). This suggests that these compounds may not bind to DNA to inhibit HCMV or to cellular DNA to cause cytotoxicity.

In general, the IC_{50} values for most of the compounds were only 2–3 times greater than the corresponding EC_{50} values. This resulted in a very narrow concentration range between cytotoxicity and antiviral activity. Toxicity was manifested as inhibition of the uptake of radiolabeled precursors into cells and/or actual decrease in cell viability. This suggests that some of these compounds may interact with the membranes of cells resulting in changes of permeability to the labeled precursors, in addition to or in lieu of the apparent affects on macromolecular synthesis.

Wölfle et al. (1990) have shown that anthraquinones with hydroxyl groups at the C-1 and C-8 positions may have tumor initiating or tumor promoting activities, since such compounds stimulated DNA synthesis or induced tumor production. However, they found that emodin and alizarin did not affect DNA synthesis or induce tumors. Similarly, as judged by effects on viable cell counts, we found that emodin was the least toxic of the four compounds tested in this assay, and emodin did not stimulate DNA synthesis at the concentrations tested (data not shown). Alizarin was also relatively non-toxic when assayed for biochemical cytotoxicity and did not stimulate DNA synthesis although uptake of radiolabeled thymidine was greatly enhanced (data not shown).

The results reported here suggest that the anthraquinones, anthrones and anthraquinone derivatives used in this study can be used as prototypes to

synthesize alternative antiviral agents against cytomegalovirus which may be effective against ganciclovir-resistant HCMV.

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