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Antiviral Research 49 (2001) 169–178

Antiviral Research

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In vitro antiviral activity of the anthraquinone chrysophanic acid against poliovirus

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Received 13 August 1999; accepted 20 December 2000

Abstract

Chrysophanic acid (1,8-dihydroxy-3-methylanthraquinone), isolated from the Australian Aboriginal medicinal plant *Dianella longifolia*, has been found to inhibit the replication of poliovirus types 2 and 3 (*Picornaviridae*) in vitro. The compound inhibited poliovirus-induced cytopathic effects in BGM (Buffalo green monkey) kidney cells at a 50% effective concentration of 0.21 and 0.02 μg/ml for poliovirus types 2 and 3, respectively. The compound inhibited an early stage in the viral replication cycle, but did not have an irreversible virucidal effect on poliovirus particles. Chrysophanic acid did not have significant antiviral activity against five other viruses tested: Coxsackievirus types A21 and B4, human rhinovirus type 2 (*Picornaviridae*), and the enveloped viruses Ross River virus (*Togaviridae*) and herpes simplex virus type 1 (*Herpesviridae*). Four structurally-related anthraquinones — rhein, 1,8-dihydroxyanthraquinone, emodin and aloe-emodin were also tested for activity against poliovirus type 3. None of the four compounds was as active as chrysophanic acid against the virus. The results suggested that two hydrophobic positions on the chrysophanic acid molecule (C-6 and the methyl group attached to C-3) were important for the compound's activity against poliovirus.

Keywords: Chrysophanic acid; Anthraquinone; Poliovirus; *Picornaviridae*; *Dianella longifolia*

1. Introduction

A number of anthraquinones and anthrones isolated from plants and lichens have been shown to exhibit virucidal activity and non-virucidal antiviral activity against enveloped viruses (Andersen et al., 1991; Sydiskis et al., 1991; Cohen et al., 1996). Sydiskis et al. (1991) showed that aloe emodin inactivated enveloped viruses including herpes simplex virus and influenza virus, but was not virucidal against the non-enveloped viruses adenovirus and rhinovirus. Polycyclic anthrones

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0166-3542/01/$ - see front matter
PII: S0166-3542(01)00125-5

(hypericin, protohypericin and emodin bianthrone), anthraquinones (rhein, emodin, alizarin and quinalizarin) and emodin anthrone, demonstrate non-virucidal antiviral activity against human cytomegalovirus (Barnard et al., 1992). Hypericin exhibits activity against a number of other enveloped viruses including retroviruses, influenza A and herpes simplex virus (Meruelo et al., 1988; Schinazi et al., 1990; Tang et al., 1990; Andersen et al., 1991).

Although these reports describe antiviral activity against enveloped viruses, anthraquinones have not previously been shown to inhibit the replication of non-enveloped viruses such as the picornaviruses. Synthetic derivatives of anthraquinone have been shown to have in vivo antiviral activity against the non-enveloped picornavirus encephalomyocarditis (EMC) virus in mice (Grisar et al., 1974; Sill et al., 1974). These compounds, however, were thought to act as interferon inducers, also exhibiting antiviral activity against a range of other viral types (Grisar et al., 1974).

Previously, in vitro activity against poliovirus (*Picornaviridae*) was reported for a crude extract of the roots of an Australian medicinal plant *D. longifolia* R.Br. (Liliaceae) (Semple et al., 1998). Traditionally, the fleshy roots of this plant have been used by Aboriginal people in Southern Australia as an internal medicine for treatment of colds (Clarke, 1987). Here, the identification of the anthraquinone chrysophanic acid (also known as chrysophanol) as the anti-poliovirus component of the *D. longifolia* extract is reported. An investigation of the antiviral activity of this compound is also described.

2. Materials and methods

2.1. Plant collection and extraction

Plants of *D. longifolia* R. Br. var. *grandis* R. Henderson were collected at two locations near Mount Torrens, South Australia (voucher specimen AD99916108, State Herbarium, Adelaide Botanic Gardens). Within 1 day of plant collection, the roots were removed, frozen at -60°C, then freeze-dried. The extract was prepared by extracting dried powdered root material with ethanol at room temperature.

2.2. Isolation and identification of chrysophanic acid

Activity-guided fractionation of the extract, using an inhibition of poliovirus-induced cytopathic effect assay (Semple et al., 1998), led to the identification of the anthraquinone chrysophanic acid (Fig. 1) as the antiviral component of the extract. The chemical structure was determined by nuclear magnetic resonance (NMR) spectrometry. ^{1}H NMR (Varian Inova, 600 MHz): (CCl_4/acetone-d_6, 9/1). δ (ppm) 2.45 [3H, *s*, C3(C$\underline{H}_3$)], 7.00 [1H, *d*, $J = 1.2$ Hz, C2(H)], 7.18 [1H, dd, $J = 7.2$, 1.2 Hz, C7(H)], 7.52 [1H, *d*, $J = 0.6$ Hz, C4(H)], 7.61 [1H, *t*, $J = 7.2$ Hz, C6(H)], 7.69 [1H, dd, $J =$ 6.0, 1.8 Hz, C5(H)], 11.83 [1H, *s*, C1 (O$\underline{H}$)], 11.93 [1H, *s*, C8(O$\underline{H}$)]. The spectral assignment was confirmed with one bond (gHSQC) and long range (gHMBC) heteronuclear coupling experiments. The ^{1}H-NMR profile and behavior on thin layer chromatography was identical to that of an authentic sample of chrysophanic acid. The commercial material was used for all further antiviral testing described below.

2.3. Cells and viruses

Buffalo green monkey kidney (BGM) and African green monkey kidney (Vero) cells were obtained from the Infectious Diseases Laboratory, Institute of Medical and Veterinary Science, Adelaide, South Australia, Australia. Human embryonic lung (HEL) cells were obtained from

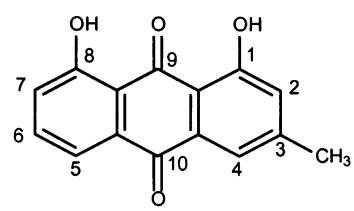


Fig. 1. Chemical structure of chrysophanic acid (1,8-dihydroxy-3-methylanthraquinone).

CSL, Parkville, Victoria, Australia and WISH cells (ATCC CCL-25) from the ATCC, Manassas, VA. Cells were grown in Dulbecco's modified Eagle medium (DMEM) with sodium bicarbonate, 3.7 g/l, glucose, 4.5 g/l, HEPES buffer, 15 mM, or Eagle's minimum essential medium with non-essential amino acids (CSL, Parkville, Victoria, Australia). Media also contained glutamine, 2 mM, gentamicin, 16 μg/ml, penicillin, 12 μg/ml, and heat inactivated foetal calf serum (10% for WISH cells; 5% v/v for BGM and Vero cells; for HEL cells 10% v/v was used to grow the cells, and 2% v/v was used to maintain the cells in antiviral assays). Viruses (with the exception of Coxsackievirus A21) were obtained from the Infectious Diseases Laboratory, Institute of Medical and Veterinary Science, Adelaide, South Australia, Australia. Coxsackievirus A21 (ATCC VR-850) was obtained from the ATCC, Manassas, VA. Poliovirus types 2 and 3 (Sabin), and Coxsackievirus B4 (CVB4, clinical isolate) were propagated in BGM cells; Coxackievirus A21 (CVA21) in WISH cells; Ross River virus (RRV, strain T48) and herpes simplex virus type 1 (HSV-1, clinical isolate) in Vero cells; human rhinovirus type 2 (HRV-2) in low passage number HEL cells. All viruses were grown at 37°C in a humidified atmosphere of 5% CO_2, in air, with the exception of HRV-2 which was grown at 33°C.

2.4. Compounds

Chrysophanic acid and emodin were purchased from Aldrich Chemical Co., Milwaukee, WI. Rhein and aloe-emodin were purchased from Apin Chemicals Ltd, Abingdon, Oxon, UK. 1,8-dihydroxyanthraquinone, and the positive control antiviral compounds guanidine HCl and acycloguanosine (acyclovir) were obtained from Sigma, St. Louis, MO.

2.5. Antiviral testing of chrysophanic acid

Solubilisation of the test compound: Chrysophanic acid was pre-solubilised at 37°C in dimethylsulphoxide (DMSO) to give a stock solution of 2.5 mg chrysophanic acid per ml. This stock solution was further diluted in cell culture media to give the desired working concentration. A final concentration of no more than 1% v/v DMSO was used in the antiviral and cytotoxicity assays.

Cytotoxicity testing and inhibition of poliovirus-induced cytopathic effect: Chrysophanic acid was tested for cytotoxicity to actively growing BGM cells and for inhibition of poliovirus-induced cytopathic effect (CPE) as described previously (Semple et al., 1998). Cells were seeded into 96-well microtitre plates at an initial concentration of 8×10^3 cells per well, and incubated for 4–6 h to allow cell attachment. One series of triplicate wells was then infected with virus at a multiplicity of infection (moi) of approximately 0.02 $CCID_{50}$ units per cell. The second series of wells was mock-infected with media only. Two-fold serial dilutions of chrysophanic acid (with a maximum final concentration of 25 μg/ml) were then added to the two series of triplicate wells to allow simultaneous determination of antiviral and cytotoxic effects. Controls of mock-infected cells without compound treatment, and untreated cells infected with virus were included in triplicate on each plate. Controls for the DMSO concentrations in chrysophanic acid samples were also tested for antiviral and cytotoxic effects.

Cultures were incubated for 42–48 h until the wells containing untreated cells infected with poliovirus showed complete (100%) CPE and the cells in the mock-infected, untreated wells had grown to form a near-confluent monolayer. Mock-infected cells treated with the serial dilutions of chrysophanic acid were examined microscopically for cytotoxic effects. Microtiter plates were then washed with PBS to remove any detached cells and cell debris, fixed and stained with crystal violet 0.5%, in a mixture of water:ethanol:formaldehyde:sodium chloride (400:190:7:1). Cell viability was quantified using the crystal violet cytotoxicity assay method of Matthews and Neale (1987). Absorbance was read at 570 nm using a Titertek® Multiskan photometer.

The percentage inhibition of virus-induced CPE was calculated as:

$$\% \text{ inhibition} = \frac{(A_E)_{virus} - (A_C)_{virus}}{(A_C)_{mock} - (A_C)_{virus}} \times 100,$$

where $(A_E)_{virus}$ is the average absorbance measured with a given chrysophanic acid concentration in virus-infected cells; $(A_C)_{virus}$ is the average absorbance measured for the untreated virus-infected cells; $(A_C)_{mock}$ is the average absorbance measured for untreated, mock-infected cells.

The maximum non-toxic dose (MNTD) for chrysophanic acid was the dilution of the compound at which mock-infected cells showed normal morphology and cell density by microscopic examination when compared to control cells grown without compound, and at least 95% of the absorbance of the mock-infected, untreated control in the crystal violet cytotoxicity assay. The experiment was repeated three times.

The EC_{50} (chrysophanic acid concentration producing 50% inhibition of virus-induced CPE) was determined from the plot of percentage inhibition of CPE versus the logarithm of the compound concentration. The known poliovirus inhibitor guanidine HCl was used as a positive control substance in the antiviral assay (producing greater than 50% inhibition of poliovirus-induced CPE at concentrations above 0.31 mM).

2.6. Testing of chrysophanic acid with other viruses

Chrysophanic acid was tested for its ability to inhibit the induction of CPE by three other viruses of the family *Picornaviridae* — CVB4, CVA21 and HRV-2, and two enveloped viruses — RRV (*Togaviridae*) and HSV-1 (*Herpesviridae*). The inhibition of virus-induced CPE and cytotoxicity assay procedures described above were used to test for inhibition of CVB4, CVA21, HSV-1 and RRV. Complete virus-induced cytopathic effect was obtained for untreated, virus-infected control wells at 42, 50, 65 and 96 h post-infection for CVB4, RRV, CVA21 and HSV-1, respectively. To test for inhibition of HRV-2 induced CPE, near-confluent monolayers of HEL cells were grown in 96-well microtiter plates. One series of triplicate wells was infected with 160 $CCID_{50}$ per well of HRV-2. The second series of wells was mock infected with media only. Chrysophanic acid dilutions were added as described before. The plate was incubated at 33°C until monolayers in the untreated, virus infected wells showed extensive HRV-induced CPE (approximately 72 h post-infection). The plate was scored by microscopic examination for inhibition of CPE and cytotoxicity. Testing with each virus was performed in duplicate. Controls for DMSO concentrations were also performed for each virus.

Guanidine HCl was used as a known inhibitor in the CVB4, CVA21 and HRV-2 assay (antiviral concentration range 0.16–2.5 mM). Acyclovir was used as a known inhibitor in the HSV-1 assay (antiviral at concentrations above 0.02 μM).

2.7. Poliovirus yield reduction assay

To determine the effect of chrysophanic acid on the yield of infectious poliovirus, monolayers of BGM cells were infected with poliovirus type 3 at three multiplicities of infection (0.001, 0.01 or 0.1 $CCID_{50}$ per cell), and treated with chrysophanic acid at final concentrations of 1.0, 0.1 and 0.05 μg/ml. Each chrysophanic acid concentration was set up in triplicate for each moi. Controls of media treatment only, and treatment with DMSO at 0.04% v/v (the concentration of DMSO present in wells treated with 1.0 μg/ml chrysophanic acid) were also set up in triplicate for each moi. Plates were incubated for 42 h then frozen and thawed three times. The contents of each set of three identical wells were harvested, mixed and clarified by low speed centrifugation. To remove any chrysophanic acid that may interfere with the replication of progeny virus, 0.2 ml of harvested virus suspension was shaken for 5 min with an equivalent volume of chloroform (in which the compound is freely soluble, and to which poliovirus is resistant) and then centrifuged at low speed to facilitate separation of the aqueous (virus suspension) and chloroform layers. Controls treated with DMSO or media only were handled in an identical manner as the chrysophanic acid treated samples. The virus suspensions were diluted 10-fold and assayed on BGM cells. The $CCID_{50}$ titer was determined by the method of Reed and Muench (1938). The experiment was repeated three times.

2.8. Determination of the possible mechanism of chrysophanic acid against poliovirus

Virus inactivation: To test whether chrysophanic acid had a direct virucidal effect on poliovirus, equal volumes of poliovirus type 3 stock (approximately 10^7 $CCID_{50}$/ml) were mixed with media containing chrysophanic acid to give final chrysophanic acid concentrations of 1 and 10 μg/ml. Controls of DMSO 0.4% v/v (the concentration of DMSO present in samples treated with 10 μg/ml chrysophanic acid), and media only were also performed. Samples were incubated at 37°C for 1 h. Thereafter, the virus suspensions were diluted ten-fold serially and adsorbed onto BGM cells for 1 h. Any unadsorbed virus was removed by washing monolayers three times with PBS. Cells were overlaid with fresh media, incubated for 48 h and the $CCID_{50}$ titer determined as before. The experiment was performed in triplicate.

Pre-incubation of cells with chrysophanic acid: To determine whether the anti-poliovirus activity of chrysophanic acid resulted from the induction of an antiviral state in cells (prophylactic effect), BGM cells were incubated with serial two-fold dilutions of the compound for 24 h at 37°C. Control wells were treated with media only. A DMSO (1% v/v) control was also performed which corresponded to the concentration of DMSO present at the highest concentration of chrysophanic acid tested (25 μg/ml). After this pre-incubation period, the media was removed and cells washed three times. Cells were infected with poliovirus type 3 (160 $CCID_{50}$ units per well) or mock infected with media only. The remainder of the procedure was identical to that described for the CPE inhibition assay and cytotoxicity assays above. The experiment was carried out in triplicate.

Effect of time of addition of chrysophanic acid on viral inhibition: To determine the point of action of chrysophanic acid in a single cycle of poliovirus replication, BGM cells (2×10^4 cells per well) were incubated overnight at 37°C. Media was then removed from the cells, and they were infected with poliovirus type 3 (2×10^4 $CCID_{50}$ per well) for 1 h at 37°C. Unadsorbed virus was then removed, cells were washed three times and fresh media was added. Chrysophanic acid (at a final concentration of 1 μg/ml) or media only (untreated controls) was added progressively to three replicate wells at hourly intervals during the 8 h viral replication cycle. For one series of wells, chrysophanic acid (or media for controls) was added during the 1 h adsorption period, and replaced immediately following the removal of unadsorbed virus. Eight hours after viral adsorption (1 cycle of poliovirus replication), the plate was frozen. The infectious virus yields for chrysophanic acid-treated wells and untreated controls were determined for each time of addition as described for the virus yield reduction assay.

2.9. Testing of other anthraquinones for antiviral activity against poliovirus

To provide some information about the structure-activity relationship of anti-poliovirus activity of chrysophanic acid, four anthraquinone compounds, differing in structure from chrysophanic acid at only one position on the molecule, were tested for anti-poliovirus activity. Serial two-fold dilutions (at a maximum final concentration of 12.5 μg/ml) of rhein, 1,8-dihydroxyanthraquinone, emodin and aloe-emodin were tested using the inhibition of poliovirus-induced CPE and cytotoxicity assays described previously. Compounds were pre-solubilised in DMSO as described for chrysophanic acid. Each compound was tested in duplicate.

3. Results

3.1. Antiviral testing of chrysophanic acid

Cytotoxicity testing and inhibition of poliovirus-induced cytopathic effect: Chrysophanic acid inhibited the development of poliovirus-induced cytopathic effect at concentrations well below those that inhibited the growth of BGM cells. As shown in Table 1, the EC_{50} for the compound against poliovirus types 2 and 3 was 0.21 and 0.02 μg/ml, respectively. The maximum non-toxic dose

Table 1
Results of virus-induced cytopathic effect and cytotoxicity assays for chrysophanic acid

Virus	Cells	EC_{50}[a]	MNTD[b]
Poliovirus type 2	BGM	0.21	12.5
Poliovirus type 3	BGM	0.02	12.5
Coxsackievirus type A21	WISH	>25	12.5
Coxsackievirus type B4	BGM	>25	12.5
Human rhinovirus type 2	HEL	>25	12.5
Ross River virus	Vero	>25	12.5
Herpes simplex virus type 1	Vero	>25	12.5

[a] Concentration of chrysophanic acid (μg/ml) producing 50% inhibition of virus-induced cytopathic effect.
[b] Maximum non-toxic dose of chrysophanic acid to cells (μg/ml).

Table 2
Log_{10} reductions[a] of poliovirus type 3 titers by chrysophanic acid

Tested concentration (μg/ml)	Multiplicity of infection[b]		
	0.1	0.01	0.001
0.05	ns[c]	1.2 (±0.14)	1.9 (±1.1)
0.1	1.5 (±0.25)	2.0 (±0.25)	3.9 (±0.76)
1.0	3.1 (±0.14)	3.9 (±0.63)	>5

[a] Log_{10} reductions of virus titers compared to untreated controls, expressed as the mean (±SD) from three replicate experiments.
[b] Multiplicity of infection expressed as $CCID_{50}$ units per cell.
[c] ns, not significant (yield reduction less than 1 log_{10}).

(MNTD) for actively growing BGM cells was 12.5 μg/ml. At concentrations above 12.5 μg/ml precipitation of the compound out of the media solution was observed after the 42–48 h incubation period. This made it difficult to quantify cytotoxic effects above this concentration. Additionally, although DMSO at the maximum concentration tested (1% v/v) did not inhibit the development of poliovirus-induced CPE, some slowing of cell growth was observed with this concentration of the solvent.

3.2. Testing of chrysophanic acid against other viruses

Chrysophanic acid did not exhibit significant antiviral activity against HSV-1, RRV, CVB4, CVA21 or HRV-2 in the CPE inhibition assay (Table 1). The cytotoxicity of chrysophanic acid in the other cell lines (Vero, WISH and HEL) agreed with that observed for BGM cells.

3.3. Reduction of poliovirus yield by chrysophanic acid

Chrysophanic acid was also found to reduce the actual yield of infectious poliovirus type 3 produced at each of the three multiplicities of infection in a dose-dependent manner (Table 2). Reductions of virus yield of less than 1 log_{10} were not considered significant. The DMSO control (0.04% v/v DMSO) did not reduce the yield of infectious poliovirus.

3.4. Determination of the possible mechanism of antiviral action against poliovirus by chrysophanic acid

Virus inactivation: Results of the virus inactivation assay (Table 3) showed no difference between the infectious virus titer of the chrysophanic acid treated and non-treated poliovirus stocks. Even when virus was incubated with a relatively high

Table 3
Testing for virucidal effect of chrysophanic acid on poliovirus type 3

Treatment	Virus titer[a] (log_{10} $CCID_{50}$ per ml)
No treatment	6.65 (±0.22)
DMSO control (0.4% v/v)[b]	6.65 (±0.07)
Chrysophanic acid, 10 μg/ml	6.71 (±0.19)
Chrysophanic acid, 1.0 μg/ml	6.70 (±0.09)

[a] Infectious virus titer expressed as mean ±SD for three replicate experiments.
[b] Control for the concentration of DMSO in samples treated with 10 μg/ml chrysophanic acid.

Table 4
Effect of pre-incubation of BGM cells with chrysophanic acid

Treatment	EC_{50}[a]	MNTD[b]
Pre-incubation[c]	0.48	>25
No pre-incubation[d]	0.02	12.5

[a] Concentration of chrysophanic acid (μg/ml) producing 50% inhibition of cytopathic effect induced by poliovirus type 3.

[b] Maximum non-toxic dose of chrysophanic acid to BGM cells (μg/ml).

[c] Cells were pre-incubated with chrysophanic acid for 24 h at 37°C, then washed prior to infection with poliovirus.

[d] Chrysophanic acid present during poliovirus replication with no pre-incubation.

concentration of chrysophanic acid (10 μg/ml), no decrease in infectious virus titer was observed, suggesting that chrysophanic acid does not have a direct, irreversible virucidal effect on poliovirus particles.

Pre-incubation of cells with chrysophanic acid: When chrysophanic acid was pre-incubated with BGM cells for 24 h, then removed prior to infection of cells with poliovirus, some inhibition of poliovirus-induced CPE was still observed (Table 4). The EC_{50} was, however, substantially higher (approximately 24-fold) when the compound was pre-incubated with cells compared to when it was present only during virus replication. These results suggested that the anti-poliovirus activity of the compound was not potentiated by pre-incubating it with the cells. No cell toxicity was observed at the maximum concentration of chrysophanic acid tested.

Effect of time of addition of chrysophanic acid on viral inhibition: The effect of time of addition of chrysophanic acid on the reduction in poliovirus type 3 titer is shown in Fig. 2. The compound was particularly effective when added during the 1 h viral adsorption period, and still produced inhibition when added immediately after the viral adsorption period. One hour after the completion of the viral adsorption period, the addition of the compound did not result in a significant reduction in virus titer. These results suggested that chrysophanic acid acted early in the poliovirus replication cycle.

3.5. Testing of other anthraquinones for antiviral activity against poliovirus

The molecular structure and anti-poliovirus activity of four other anthraquinones, compared with chrysophanic acid are reported in Table 5. None of the other anthraquinones were as active against poliovirus type 3 as chrysophanic acid. Rhein did not inhibit the virus. Aloe-emodin and 1,8-dihydroxyanthraquinone showed some activity, while emodin was the most active of the four anthraquinones, inhibiting poliovirus-induced CPE by 50% at a concentration of 0.25 μg/ml.

4. Discussion

Chrysophanic acid (chrysophanol) is a naturally occurring anthraquinone that has been iso-

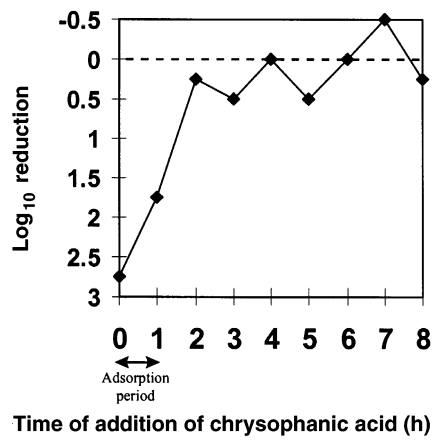
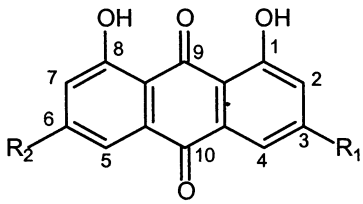


Fig. 2. Monolayers of BGM cells (2×10^4 cells per well) were infected with poliovirus type 3 at an moi of approximately 1 $CCID_{50}$ unit per cell. Virus was adsorbed for 1 h at 37°C. After the 1 h adsorption period, any unadsorbed virus was removed, cells were washed three times with PBS, and fresh culture media was added. Chrysophanic acid at a final concentration of 1 μg/ml or media only (for untreated controls) was added progressively to three replicate wells, at hourly intervals during the 8 h poliovirus replication cycle. For one series of triplicate wells, chrysophanic acid or media (for controls) was added during the adsorption period (time of addition, 0 h), and replaced after the removal of unadsorbed virus. At the completion of the 8 h replication cycle, cells were frozen. Virus yields ($CCID_{50}$ units per ml) were determined for each time of compound addition. Results are expressed as the $\log_{10}$ reduction of virus yield compared to the untreated controls.

Table 5
Structures of anthraquinones, and their activity against poliovirus type 3



Compound	R_1	R_2	EC_{50}[a]	MNTD[b]	SI[c]
Chrysophanic acid	CH_3	H	0.02 (0.08)	12.5 (49.1)	625
1,8-dihydroxyanthraquinone	H	H	0.56 (2.3)	1.6 (6.7)	2.9
Rhein	COOH	H	>12.5 (>44.0)	6.2 (21.8)	na[d]
Emodin	CH_3	OH	0.25 (0.93)	3.1 (11.5)	12.4
Aloe-emodin	CH_2OH	H	0.54 (2.0)	1.6 (5.9)	3.0

[a] Concentration of compound in μg/ml (and μM) producing 50% inhibition of poliovirus-induced cytopathic effect.
[b] Maximum non-toxic dose of compound to BGM cells in μg/ml (and μM).
[c] Selectivity index (MNTD/EC_{50}).
[d] na, not active, no inhibition of poliovirus-induced CPE at non-cytotoxic concentrations.

lated from a number of biological sources including plants, lichens and microbes (Thomson, 1987). In this study chrysophanic acid was identified as the active component of an extract of the medicinal plant *D. longifolia*, which had previously been found to inhibit poliovirus. Although anthraquinones structurally related to chrysophanic acid have been shown to inhibit enveloped viruses by both virucidal and non-virucidal mechanisms (Andersen et al., 1991; Sydiskis et al., 1991; Barnard et al., 1992; Cohen et al., 1996), this is the first report of activity of this type of compound against a non-enveloped virus. Chrysophanic acid was found to inhibit poliovirus in both CPE inhibition and yield reduction assays at concentrations well below those that caused toxicity to actively growing cells. The maximum non-toxic concentration of the compound in the four cell lines tested was found to be 12.5 μg/ml (49.1 μM). This result was not inconsistent with previously reported toxicity data for the compound. Barnard et al. (1992) reported the 50% cytotoxic concentration of chrysophanic acid in MRC-5 cells to be >39.3 μM.

The anti-poliovirus activity of this compound was also of interest because of the use of *D. longifolia* as a traditional medicine for the treatment of the common cold by Aboriginal people in Southern Australia (Clarke, 1987). Some viruses of the family *Picornaviridae*, to which poliovirus belongs, are important causative agents of the common cold. Rhinoviruses are the most frequent cause of the common cold (Rueckert, 1996), and some enteroviruses including Coxsackieviruses and Echoviruses also cause respiratory infections (Chonmaitree and Mann, 1995). In this study, however, chrysophanic acid did not exhibit in vitro antiviral activity against the three other picornaviruses tested: the rhinovirus HRV-2 and the enteroviruses CVB4 and CVA21. This may indicate that the activity of the compound may be limited to poliovirus. Chrysophanic acid did not demonstrate any significant activity against the two enveloped viruses tested (HSV-1 and RRV). This result was consistent with previous findings that the compound lacks significant virucidal or other antiviral activity against enveloped viruses (Andersen et al., 1991; Barnard et al., 1992; Cohen et al., 1996).

Investigation of the mechanism by which chrysophanic produces its anti-poliovirus effect, suggested that the compound did not cause irreversible disruption of viral particles (virucidal effect). When BGM cells were pre-incubated with

chrysophanic acid, inhibition of poliovirus-induced CPE occurred even though the cells were washed to remove the compound prior to the addition of virus. The EC_{50}, however, was higher. This result suggested that chrysophanic acid did not inhibit poliovirus by inducing an antiviral state in cells. The residual activity after washing cells to remove the chrysophanic acid may be explained by the hydrophobic nature of the compound. The poor aqueous solubility of the compound may mean that it has a tendency to accumulate in cells, and is not completely removed by washing of cells with aqueous-based solutions. This has also been observed for other hydrophobic compounds that inhibit picornaviruses (Tisdale and Selway, 1983; Conti et al., 1990).

Testing of four other structurally related anthraquinones was performed in order to obtain some information about the structure-activity relationship of chrysophanic acid when exerting an anti-poliovirus effect. The results suggested that the methyl group attached to C-3 in the molecule was important for activity against poliovirus. Emodin, which retains the C-3 methyl function, but has an extra hydroxyl group attached to C-6, was the most active of the four extra anthraquinones tested. It was, however, approximately 12-fold less active than chrysophanic acid against poliovirus type 3. This suggested that the presence of a free hydroxyl group at the C-6 position hindered the inhibition of poliovirus.

A further experiment was carried out to determine the point at which chrysophanic acted in a single cycle of poliovirus growth. This suggested that the compound acted at an early stage in the poliovirus replication cycle, within the first 1–2 h of viral entry into cells. Such early stages may include viral penetration of the cell, uncoating, early translation of the input viral RNA, or the initial cleavage of the viral polyprotein.

Cleavage of the picornaviral polyprotein to yield the non-structural proteins required for viral RNA replication could be a target for chrysophanic acid. The picornaviral proteases which perform these cleavages (2A and 3C) are cysteine proteases structurally related to the trypsin-like serine proteases (Bazan and Fletterick, 1988; Yu and Lloyd, 1991). The poliovirus and human rhinovirus 14 2A proteases are inhibited by two compounds (elastatinal and methoxy-Ala-Ala-Pro-Val-chloromethylketone) which are specific inhibitors of the mammalian elastases, a group of serine proteases (Molla et al., 1993). As a result, it has been suggested that the poliovirus and rhinovirus 2A proteases have a substrate binding pocket very similar to that of the pancreatic and leukocyte elastases (Molla et al., 1993). While anthraquinones have not previously been reported to inhibit picornaviral proteases, it is interesting that human elastases — human leukocyte elastase (HLE) and cathepsin G, are inhibited by anthraquinone derivatives. Zembower et al. (1992) tested a series of anthraquinone derivatives against these elastase enzymes. Four of the anthraquinones tested were chrysophanic acid, emodin, aloe-emodin, and rhein. The relative potency of these compounds against the HLE enzyme was comparable with the pattern seen for these same compounds against poliovirus in the present study, and may indicate that the 2A protease of poliovirus is a target for these compounds.

Further experiments are required to elucidate the mechanism of action for chrysophanic acid. These could include measurement of poliovirus entry into cells and the cleavage of the polypeptide eukaryotic initiation factor 4G (mediated by the poliovirus 2A protease) in the presence of the compound.

In conclusion, the anthraquinone chrysophanic acid has been found to exhibit in vitro antiviral activity against poliovirus. The compound inhibits an early stage of the poliovirus replication cycle. Further experiments, however, are required to determine the compound's mechanism and target of action.

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