 0014-4754/96/020180-04$1.50 + 0.20

Antiviral activities of anthraquinones, bianthrones and hypericin derivatives from lichens

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Received 23 May 1995; accepted 12 June 1995

Abstract. The antiviral activities of some naturally occurring anthraquinones, bianthrones, and hypericin derivatives were compared by the end-point CPE (viral cytopathic effects) method and plaque assays. Under optimal conditions of exposure to light, hypericin, 7,7′-dichlorohypericin and 5,7-dichloroemodin exhibited strong inhibitory activity against HSV-1 (herpes simplex virus type 1) in both assays. Partial inactivation of the virus was shown by emodin, 7-chloroemodin and 7-chloro-1-O-methylemodin; the bianthrones and other anthraquinones were found to be inactive. Antiviral activity appeared to be positively correlated with increasing substitution of chlorine in the anthraquinone structure. In the absence of light, only hypericin and 7,7′-dichlorohypericin displayed detectable activity.

Key words. Antiviral activity; 7,7′-dichlorohypericin; 5,7-dichloroemodin; hypericin; anthraquinones; bianthrones; lichen; *Nephroma laevigatum*; *Heterodermia obscurata*.

Hypericin, and a number of structurally related anthraquinones and bianthrones, have been shown to possess antiviral activities against several animal viruses with membranes, including HIV-1 (refs 1–7). None of these earlier studies, however, examined the role of light in mediating virucidal activity. Hypericin is a known photosensitizer, and its virucidal activity has been shown to be dependent on light[8–11]. This may also be true for anthraquinones and bianthrones. In addition, the antiviral activity of hypericin has been shown to be affected by a variety of assay parameters[5,9]. Thus, relative activities must be compared under uniform and optimal conditions.

The compounds used in this study (fig.) were either isolated from the lichens *Nephroma laevigatum* and *Heterodermia obscurata*[12,13], or were natural products obtained previously. All of the compounds, with the exception of hypericin, are known constituents of lichens.

Materials and methods

The anti-HSV assays follow the protocols described previously[14]. Briefly, the standard reaction mixtures were prepared by adding a known amount of HSV-1 (100 pfu) to serial two-fold dilutions of the test compound made up in Dulbecco MEM plus 0.1% serum, previously shown to be the optimum conditions for the antiviral activity of hypericin[15]. The range of concentrations of the compounds was 2.0 μg/ml down to 0.06 μg/ml. The reaction mixtures were irradiated at 9 Kjoules, supplied by fluorescent lamps, for 30 min. Controls included reactions kept dark by covering the assay plates with aluminium foil, and virus without compound. All reactions were carried out in duplicate, and every compound was tested at least twice.

The cultures were inspected daily microscopically for residual HSV-1 infectivity (as characteristic CPE), and for comparison with virus only and cells only. In the case of the untreated HSV-1, CPE involved the entire culture by day 3–4. At this time, cultures that still displayed no viral CPE were considered to be free of infectious virus (complete viral inactivation in the reactions).

Compounds that displayed good anti-HSV activity in the end-point tests were evaluated in more detail by use of plaque assays. Reaction mixtures were prepared with several concentrations of the compounds and HSV-1 (10^5 pfu/ml). Following irradiation with light as before, and with appropriate controls, the mixtures were serially diluted to 10^{-1} to 10^{-4}. The 10^{-2} and 10^{-4} dilutions were each inoculated in duplicate onto monolayers of Vero cells in culture dishes (60 mm diameter) to permit adsorption of remaining infectious virus to the cells (60 min at 37 °C). The inocula were then removed by aspiration and replaced by molten agarose overlays (at 42 °C), which consisted of 0.5% final concentration of agarose, Dulbecco MEM and 5% serum. When the overlays had set, the cultures were returned to the incubator until plaques could be visualized (4 days). In order to facilitate the enumeration of plaques, the cell monolayers were fixed in 10% formalin in phosphate-buffered saline, and stained with 1% crystal violet in water. The plaques appeared as discrete round 'holes' in

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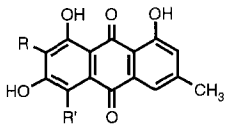
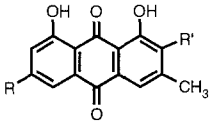
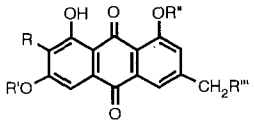


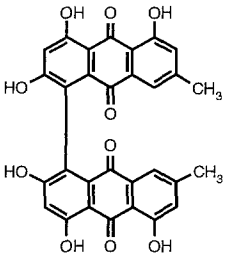
R = R' = H; Emodin (**1**)
R = Cl; R' = H; 7-Chloroemodin (**3**)
R = Cl; R' = Cl; 5,7-Dichloroemodin (**5**)



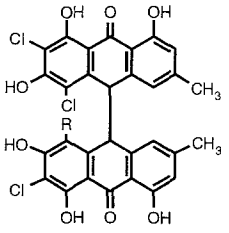
R = OH; R' = COOH; Endocrocin (**7**)
R = R' = H; Chrysophanol (**8**)



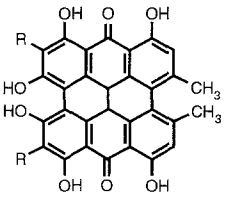
R = R'' = R''' = H; R' = CH_3; 6-O-Methylemodin (**2**)
R = Cl; R' = R''' = H; R'' = CH_3; 7-Chloro-1-O-methylemodin (**4**)
R = Cl; R' = H; R'' = CH_3; R''' = OH; 7-Chloro-1-O-methyl-ω-hydroxyemodin (**13**)



Skyrin (**6**)



R = H; Flavoobscurin A (**9**)
R = Cl; Flavoobscurin B (**10**)



R = H; Hypericin (**11**)
R = Cl; 7,7'-Dichlorohypericin (**12**)

Figure. Structures of anthraquinones, bianthrones and hypericins.

the uniform blue monolayer of intact cells. The number of plaques at each dilution was calculated as pfu/ml, and compared to the corresponding number in the untreated virus control.

Results

Table 1 presents the results of the survey of the lichen compounds for their inhibitory activity against HSV-1 virus. Emodin (**1**), 7-chloroemodin (**3**), and 7-chloro-1-O-methylemodin (**4**) completely inactivated HSV-1 at a concentration of 2 µg/ml. Partial inactivation was also seen for compounds **1** (1 µg/ml), **3** (1 µg/ml) and **4** (0.5 µg/ml). The most active anthraquinone was 5,7-dichloroemodin (**5**). Complete inactivation of the virus was attained at a concentration of 0.25 µg/ml, and 0.125 µg/ml gave partial inactivation. The remaining five anthraquinones (**2, 6–8, 13**) were completely inactive, even at 5 µg/ml. The bianthrones flavoobscurin A (**9**) and flavoobscurin B (**10**) also were inactive at 5 µg/

Table 1. Minimum inhibitory concentrations of lichen compounds against HSV-1 virus.

Compound	Complete inactivation (µg/ml)	Partial inactivation (µg/ml)
Emodin (**1**)	2	1
6-O-Methylemodin (**2**)	inactive	inactive
7-Chloroemodin (**3**)	2	1
7-Chloro-1-O-methylemodin (**4**)	2	0.5
5,7-Dichloroemodin (**5**)	0.25	0.125
Skyrin(**6**)	inactive	inactive
Endocrocin (**7**)	inactive	inactive
Chrysophanol (**8**)	inactive	inactive
Flavoobscurin A (**9**)	inactive	inactive
Flavoobscurin B (**10**)	inactive	inactive
Hypericin (**11**)	<0.06	$\ll 0.06$
7,7'-Dichlorohypericin (**12**)	<0.06	$\ll 0.06$
7-Chloro-1-O-methyl-ω-hydroxyemodin (**13**)	inactive	inactive

ml. Finally, 7,7′-dichlorohypericin (**12**) and hypericin (**11**) showed comparable inhibitory activity against HSV-1; complete inactivation took place at less than 0.06 μg/ml.

Table 2 compares the antiviral activities of the six active compounds in a plaque assay. As with the initial endpoint CPE method, this assay was conducted following irradiation with light. Of the six compounds tested, 5,7-dichloroemodin (**5**), 7,7′-dichlorohypericin (**12**), and hypericin (**11**) completely inhibited HSV-1 at 1.0 μg/ml. Thus, of the 11 anthraquinones and bianthrones tested, only 5,7-dichloroemodin (**5**) exhibited light-mediated anti-HSV activity comparable to that of hypericin (**11**) and 7,7′-dichlorohypericin (**12**).

This is illustrated in table 3, where the effects of light and dark activities of the three compounds are compared. At a higher concentration (1.0 μg/ml), both hypericin (**11**) and 5,7-dichloroemodin (**5**) were appreciably more active than 7,7′-dichlorohypericin (**12**) in the light. In the dark, however, 5,7-dichloroemodin (**5**) was totally inactive; the two hypericins exhibited comparable moderate anti-HSV activity. At an intermediate concentration (0.1 μg/ml), the activities of 5,7-dichloroemodin (**5**) and 7,7′-dichlorohypericin (**12**) in the light started to decrease. At this concentration, all three compounds were inactive in the dark. Finally, at the lowest concentration (0.01 μg/ml), only hypericin (**11**) showed any anti-HSV activity in the light.

Table 2. Inhibition of HSV-1 virus in plaque assay.

Compound[a]	# Plaques[b]	% of Plaques[c]
None (control)	936	100
Emodin (**1**)	784	84
7-Chloroemodin (**3**)	904	97
7-Chloro-1-O-methylemodin (**4**)	1062	113
5,7-Dichloroemodin (**5**)	0	<0.1
Hypericin (**11**)	0	<0.1
7,7′-Dichlorohypericin (**12**)	1	<0.1

[a]1.0 μg/ml.
[b]pfu/ml × 10^{-2} dilution factor.
[c]Relative to control.

Discussion

There are several significant findings in this study. The first observation is the higher inhibitory activity of 5,7-dichloroemodin (**5**), when compared with 7-chloroemodin (**3**) and emodin (**1**), which have comparable activity. Furthermore, there does not appear to be any difference in activity between the monochlorinated anthraquinones and the nonchlorinated analogues. This suggests that, in the emodin series at least, anti-HSV activity is obtained when both the C-5 and C-7 positions are substituted (although 5-chloroemodin has yet to be tested). Substitution of the 1-OH in emodin (**1**) with a 1-OMe (**4**) resulted in an initial partial inactivation; but the plaque assay results suggest that the viral CPE were merely delayed, and that eventually the CPE reached 100%. Thus, for **1**, **3** and **4**, lower concentrations of the compound gave little or no decrease in the progress of CPE, while higher concentrations gave complete alleviation of the CPE.

Modifications of the basic emodin structure (fig) by placing an OMe group at C-6 (**2**), a COOH group at C-2 (**7**), removal of the OH group at C-6 (**8**), and substitution of a CH_2OH for a CH_3 group (**13**) all gave inactive compounds. Of the dimeric structures, only the hypericins (**11**, **12**) exhibited anti-HSV activity; skyrin (**6**) (bianthraquinone) and flavoobscurins A (**9**) and B (**10**) (bianthrones) were completely inactive.

In general, the results suggest that the phenanthroperylenequinone structure of hypericin (**11**) and 7,7′-dichlorohypericin (**12**) is more active than either the less condensed bianthrone structure, or the monomeric/dimeric anthraquinones (table 1). The presence of several chlorine atoms in flavoobscurin A (**9**) and B (**10**) did not influence the antiviral activity of the two compounds in a positive manner, and may, in fact, account for their inactivities. In contrast, substituting chlorines for hydrogens in positions 5 and 7 of emodin (**1**) resulted in greatly enhanced anti-HSV activity for 5,7-dichloroemodin (**5**) (tables 1–3). In the case of 7,7′-dichlorohypericin (**12**), substitution of two chlorine atoms on hypericin (**11**) reduced somewhat the anti-HSV activity of **12** in the light (table 3). Meruelo et al.

Table 3. Effect of light on HSV-1 inhibition at different substrate concentrations.[a]

Compound	Dark/Light	1.0 μg/ml	0.1 μg/ml	0.01 μg/ml
5,7-Dichloroemodin (**5**)	Dark	100	—	—
	Light	<0.01	52	100
Hypericin (**11**)	Dark	34	100	100
	Light	<0.01	0.33	24
7,7′-Dichlorohypericin (**12**)	Dark	43	100	100
	Light	0.17	50	100

[a]Values reported as % pfu remaining.

had shown that pseudohypericin, with CH_2OH groups in place of CH_3, was significantly less active than hypericin[1]. Compound **13**, in which the CH_3 is also substituted by a CH_2OH group, was inactive unlike compound **4**.

In conclusion, this study provides evidence for the anti-HSV activities of several lichen anthraquinones and hypericins. In particular, hypericin (**11**), 7,7′-dichlorohypericin (**12**) and 5,7-dichloroemodin (**5**) exhibited significant anti-HSV activities in light at a concentration of 1.0 μg/ml. Hypericin (**11**) and 7,7′-dichlorohypericin (**12**) also displayed moderate inhibitory activities in the dark at 1.0 μg/ml. Hypericin is known to be a photodynamic compound that mediates biological activities via singlet oxygen[15]. It is not known, however, if the dark reaction follows the same mechanistic course as the light-mediated process; in fact, there may be several different light and dark processes operating concurrently, or under varying reaction conditions. This appears to be supported by the fact that 5,7-dichloroemodin (**5**) showed the same light-mediated anti-HSV activity as hypericin (**11**), but was completely inactive in the dark at a concentration of 1.0 μg/ml.

Acknowledgements. We thank the Natural Sciences and Engineering Research Council of Canada for financial support of this research.

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