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Cell culture replication of herpes simplex virus and, or human cytomegalovirus is inhibited by 3,7-dialkoxylated, 1-hydroxyacridone derivatives

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Abstract

The synthetic acridone compound, 5-chloro-1,3-dihydroxyacridone inhibits herpes simplex virus (HSV) replication by inducing the formation of defective viral (B-type) capsids [Antiviral Res. 53 (2002) 113]. In this report, synthetic elaboration of the 1-hydroxyacridone scaffold coupled with antiviral testing led to the identification of 3,7-dimethoxy-1-hydroxy-acridone (**2**) as an inhibitor of low multiplicity human cytomegalovirus (HCMV) infection (ED_{50} value of 1.4 μM (0.5 μg/ml); greater than 35-fold selectivity). Compound **2** was inactive against HSV replication and the efficacy as an anti-HCMV agent at higher viral loads was only apparent if host cells were replicated in the presence of the compound prior to infection. Interestingly, the 3,5-dimethoxy regioisomer inhibited cell replication (mean CC_{50} 33 μM) and was inactive as a selective anti-herpes agent. A limited parallel synthesis and testing of ten 3,7-dialkoxylated compounds closely related to compound **2** led to the discovery of the 3-ethoxy-, 3-propoxy-, 3-isopropoxy- and 3-allyloxy-derivatives as dual inhibitors of both HSV and HCMV (selectivity of the 3-allyloxy analog was greater than 10- and 36-fold, respectively). The 3-benzyloxy-derivative was active (ED_{50} value of 6.9 μM) against HCMV only. Moreover, the corresponding C-7 variable alkoxylated parallel series were either weakly active or inactive antiviral agents suggesting an apparent requirement for a C-7 methoxy substituent in the active structure. Exploratory mode of action studies showed that dual inhibitors were most active against a low multiplicity HSV infection and potent inhibition of viral release likely contributed to this. Furthermore, suppression of late viral protein synthesis by dual inhibitors did not correlate with anti-HSV activity. On the basis of the present findings, the 1-hydroxyacridone scaffold is further expanded as a useful template for the discovery of investigational anti-herpes agents. As a group, the active 3,7-dialkoxylated compounds likely have diverse mechanisms of action, consequently they are of potential medicinal interest.

Keywords: Acridone; Antiviral; HSV; HCMV; *Citrus* alkaloids; Parallel synthesis

1. Introduction

Several types of naturally occurring and synthetic derivatives of 10*H*-acridin-9-one (acridone) are known as investigational antiviral agents and they are of medicinal interest as a group due to their diverse and atypical mechanisms of action (structures are shown in Fig. 1). 10-Carboxymethyl-acridone (10-CMA) is a potent low molecular weight inducer of interferon but may also have other mechanisms of action. For example, adenovirus type 6 is inhibited directly in vitro by 10-CMA (Zarubaev et al., 2003). The replication of human immunodeficiency virus (HIV) in human peripheral blood mononuclear cells is also inhibited by 10-CMA but with only marginal selectivity (Taraporewala et al., 1992). In the same study, derivatives of dercetin, a sponge metabolite, inhibited HIV-1 replication in MT-4 lympocytes with greater than 16-fold selectivity. The dercetin-type of antiviral compound was proposed to inhibit HIV-1 binding to cells as well as exert other actions possibly linked to an interaction with the HIV-1 DNA replication intermediate. Of the 1-hydroxy acridone sub-class, Citrusinine-I, Citpressine-I and related phytochemicals are inhibitors of herpes simplex virus (HSV) and human cytomegalovirus (HCMV) replication in cell culture with apparent selectivity ranging from two- to ten-fold. These agents likely

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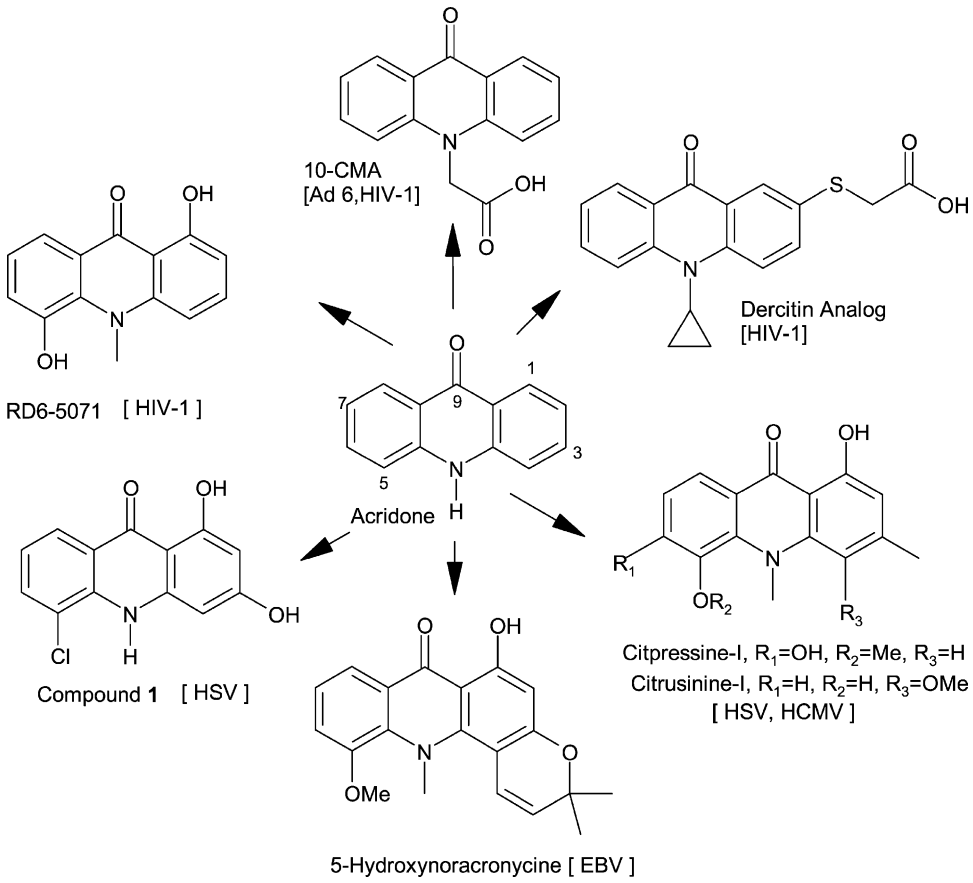


Fig. 1. Acridone derivatives with pre-clinical antiviral activity.

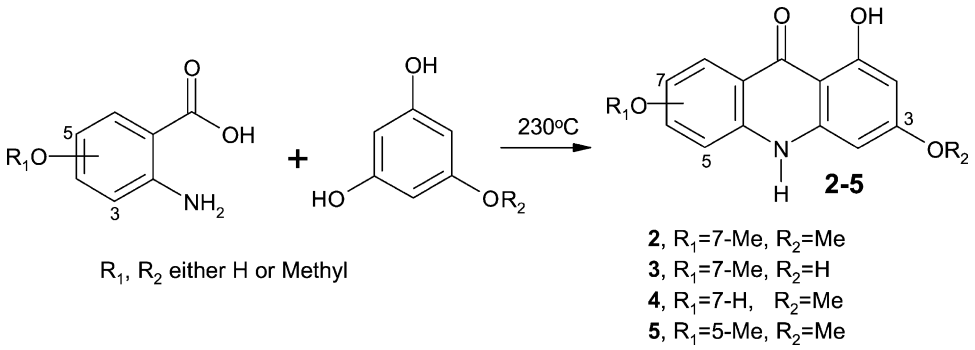
target the viral-encoded enzyme ribonucleoside diphosphate reductase and thereby deplete the host of deoxyribonucleotides used to sustain efficient viral DNA replication (Yamamoto et al., 1989). Another constituent of *Citrus* plants, 5-hydroxynoracronicine, blocks the activation of Epstein-Barr Virus (EBV) early antigen at sub-micromolar concentration but neither the selectivity nor a potential mechanism was elaborated (Takemura et al., 1995). The synthetic 1-hydroxy acridones with antiviral activity include several 1,3-dihydroxyacridone derivatives, which inhibit HSV replication in Vero cells with modest (two- to five-fold) selectivity (Bastow et al., 1994). Two cellular enzymes, protein kinase C (PKC) sub-type δ and DNA topoisomerase II, were proposed as potential drug targets of those analogs but the latter was excluded later primarily on the basis of structure activity information (Akanitapichat et al., 2000). In the same study, 5-chloro-1,3-dihydroxyacridone (**1**) was discovered and designated as the lead compound because of higher selectivity (26-fold) of action. Subsequent definition of the antiviral blockade induced by the lead suggested that an undefined defect in viral (B-type) capsid competency precluded normal HSV DNA packaging in **1**-treated cells (Akanitapichat and Bastow, 2002). Another synthetic series exemplified by RD6-5071 was recently reported to inhibit chronic HIV-1 infection of various myeloid cell lines. The selectivity of RD6-5071 is about 10-fold and the antiviral mechanism occurs in part at the viral transcription level; interestingly, inhibition of cellular PCK was also considered as a possible drug target (Fujiwara et al., 1999).

The principal finding of this report is to extend the antiviral activity spectrum of synthetic 1-hydroxyacridones to include the significant pathogen HCMV. Of this sub-class, C-3 variable bis-alkoxylated derivatives (compounds **6**–**8** and **10**), are the most intriguing because like the *Citrus* alkaloids discovered by Yamamoto et al., they inhibit a productive HSV infection as well. However, on the basis of structure–activity relationships and preliminary information about mode of action, these novel dual inhibitors appear to be unique amongst antiviral acridones and therefore, they are useful templates for anti-herpes drug research and development.

2. Material and experimental procedures

2.1. General chemistry

1H NMR spectra were recorded on a Varian 300 MHz spectrometer with Me4Si as the internal reference. Mass spectra of compounds **2**–**5** were measured using an Hitachi



Scheme 1.

M-80 mass spectrometer and for **6**–**15**, by ESI-MS analysis using a PE-Sciex API-3000 LC/MS/MS with turbo spray ion source operating at −4.2 kV. Elemental analysis of compounds **2**–**5** was performed by Atlantic Microlabs (Norcross, GA). Purity of the parallel series and selected other compounds was monitored using HPLC. This analysis with UV (250 nm) detection in MeOH:water (80:20) used an Agilent 1100 system equipped with an Agilent 4.6 mm i.d. × 15 cm ZORBAX Eclipse XDB -C8 column. The flow rate and run time were 1.0 ml/min and 10 min, respectively.

2.2. Preparation of 1-hydroxyacridone analogs *2*–*5*

The synthetic approach used for single analog synthesis is illustrated in Scheme 1. This thermal coupling reaction is an expedient alternative to more commonly used synthetic routes that involve refluxing in *n*-butanol and zinc chloride (Hughes and Richie, 1951) or *n*-heptanol and *p*-toluene sulfonic acid (Smolders et al., 1984) for the coupling of anthranilic acids with phloroglucinol or resorcinol derivatives. The methodology was optimized to define the structure activity relationship around compound **1** (Lowden, 2002) and was subsequently adapted to a more diverse set of acridone targets including compounds **2**–**5**.

2.2.1. 3,7-Dimethoxy-1-hydroxy-acridone (*2*)

Into a 20 ml vial was added 2-amino-5-methoxybenzoic acid (100 g, 6.00 mmol) and 5-methoxyresorcinol (947 mg, 6.75 mmol). The vial was sealed and heated in an oil bath at 225 °C for 35 min before allowing it to cool to room temperature. The resulting solids were triturated in ethyl acetate and filtered to yield 780 mg yellow powder, 48%. NMR (D_6 DMSO) δ 3.97 (6H, s), 6.25 (1H, s), 6.47 (1H, s), 7.55–7.67 (3H, m), 11.97 (1H, s), 14.41 (1H, s); elemental analysis calculated for $C_{15}H_{13}NO_4$: C 66.41, H 4.83, N 5.16, Found C 66.15, H 4.92, N 5.23. HRMS *m/z* (relative intensity, %) 271 (100) $(M)^+$; calculated for $C_{15}H_{13}NO_4$: 271.0845, Found 271.0840.

2.2.2. 1,3-Dihydroxy-7-methoxyacridone (*3*)

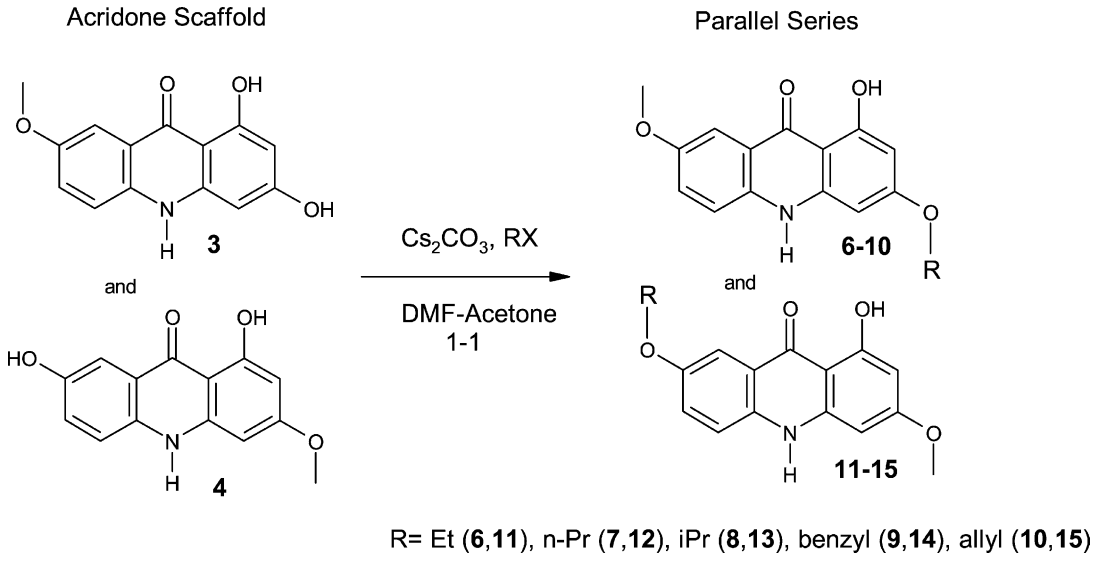
Into a 20 ml vial was added 2-amino-5-methoxybenzoic acid (2.17 g, 6.00 mmol) and anhydrous phloroglucinol (1.64 g, 6.75 mmol). The vial was sealed and heated in an oil bath at 230 °C for 35 min. Upon cooling, the resulting solids were triturated in ethyl acetate and methyl alcohol before filtration. The combined filtrates were evaporated, dissolved in DMF (15 ml) and ethyl acetate (150 ml) and washed with 4:1 H_2O-saturated aqueous sodium bicarbonate (2 × 150 ml) followed by H_2O (2 × 150 ml). The organic phase was dried over sodium sulfate, filtered and the crude product was then flash chromatographed to yield 600 mg yellow powder, 18%. NMR (D_6 DMSO) δ 3.97 (3H, s), 6.10 (1H, d, $J = 2.4$), 6.39 (1H, d, $J = 2.4$), 7.61 (3H, m) 10.55 (1H, s), 11.84 (1H, s), 14.43 (1H, s); elemental analysis calculated for $C_{14}H_{11}NO_4 \cdot 1.5\ H_2O$: C 59.15, H 4.96, N 4.93, Found C 58.76, H 4.58, N 5.01. HRMS *m/z* (relative intensity, %) 257 (7.5) $(M)^+$; calculated for $C_{14}H_{11}NO_4$: 257.0691, Found 257.0688; HPLC purity (retention time) 100% (2.1 min).

2.2.3. 1,7-Dihydroxy-3-methoxyacridone (*4*)

Into a 20 ml vial was added 2-amino-5-hydroxybenzoic acid (281 mg, 1.83 mmol) and 5-methoxyresorcinol (284 mg, 2.02 mmol). The vial was sealed and heated in an oil bath at 230 °C for 35 min. After cooling to room temperature, the resulting solid was then triturated in hot ethyl acetate and filtered to yield the product as a yellow solid, 335 mg, 71%. NMR (D_6 DMSO) δ 3.96 (3H, s), 6.21 (1H, d, $J = 2.4$), 6.45 (1H, d, $J = 2.4$), 7.41 (1H, dd, $J = 2.8, 7.2$), 7.54 (1H, d, $J = 9.1$), 7.61 (1H, d, $J = 2.8$) 9.78 (1H, s), 11.97 (1H, s), 14.48 (1H, s); elemental analysis calculated for $C_{14}H_{11}NO_4 \cdot 0.5\ H_2O$: C 63.16, H 4.54, N 5.26, Found C 63.40, H 4.63, N 5.10. HRMS *m/z* (relative intensity, %) 257 (7.5) $(M)^+$; calculated for $C_{14}H_{11}NO_4$: 257.0688, Found 257.0693; HPLC purity (retention time) 95% (2.1 min).

2.2.4. 3,5-Dimethoxy-1-hydroxyyacridone (*5*)

Into a 4-ml vial was added 3-methoxy-2-aminobenzoic acid (334 mg, 2.00 mmol) and 5-methoxyresorcinol (308 mg, 2.20 mmol). The vial was sealed and heated in an oil bath at 230 °C for 35 min. The resulting solids were dissolved in ethyl acetate (100 ml) and washed with 2 × 100 ml 0.10 M KOH in H_2O. The organic phase was isolated, dried over



Scheme 2.

sodium sulfate and absorbed onto silica gel. The material was then flash chromatographed to yield 70 mg yellow solid, 13%. NMR (D_6 DMSO) δ 3.96 (3H, s), 4.17 (3H, s), 6.28 (1H, d, $J = 2.0$), 7.03 (1H, d, $J = 2.4$), 7.34 (1H, t, $J = 8.0$), 7.47 (1H, d, $J = 7.9$), 7.87 (1H, d, $J = 8.3$), 11.48 (1H, s), 14.35 (1H, s); elemental analysis calculated for $C_{15}H_{13}NO_4 \cdot 0.75H_2O$: C 63.26, H 5.13, N 4.92, Found C 63.07, H 4.71, N 4.96. HRMS *m/z* (relative intensity, %) 271 (8.7) $(M)^+$; calculated for $C_{15}H_{13}NO_4$: 271.0845, Found 271.0851.

2.3. Parallel preparation of compounds *6–15*

The synthetic approach used for solution phase parallel synthesis is illustrated in Scheme 2. Into each of six 20 ml vials with stir bars was added sequentially 1,3 dihydroxy-7-methoxyacridone (**3**, 86 mg, 0.33 mmol), cesium carbonate (98 mg, 0.30 mmol) and 1-1 DMF-acetone (4 ml). Six alkyl halides (ethyl iodide, propyl iodide, isopropyl iodide, benzyl bromide, cyclohexyl bromide, allyl bromide, 0.30 mmol) were then added separately to vials. Reactions were at room temperature for 24 h with stirring. Acetone and some DMF were evaporated with a nitrogen stream from a manifold apparatus while the vials rested in a warm water bath. After adding ethyl acetate (8 ml) and water (8 ml) into reaction vials, they were capped and shaken vigorously. The water was removed and re-extracted in the same manner with ethyl acetate (4 ml). Organic phases combined from both extractions were washed with water (5 ml), then isolated and filtered over a pad of sodium sulfate and silica gel. The solid phase was washed with ethyl acetate (20 ml) and the filtrates were concentrated. Resultant solids were triturated in dichloromethane and filtered to yield the products (**6**–**10**) as yellow solids in 81–99% purity as estimated by peak area using HPLC analysis. A cyclohexyl bromide reaction did not yield any product. The same procedure was carried out using 1,7-dihydroxy-3-methoxyacridone (**4**) as the starting acridone in parallel to yield compounds **11**–**15** in 69–100% purity. Again, the cyclohexyl bromide reaction in parallel was not successful.

2.3.1. 3-Ethoxy-1-hydroxy-7-methoxyacridone (*6*)

NMR ($CDCl_3$) δ 1.57 (3H, t, $J = 7.1$), 4.02 (3H, s), 4.18–4.25 (2H, m), 6.26 (1H, d, $J = 2.4$), 6.36 (1H, d, $J = 2.4$), 7.32 (1H, d, $J = 9.1$), 7.40 (1H, d, $J = 9.1$), 7.84 (1H, d, $J = 2.6$), 8.47 (1H, s), 14.21 (1H, s). LRMS *m/z* (relative intensity, %) $(M)^- = 283.9$ (100); HPLC purity (retention time) 94% (2.3 min).

2.3.2. 1-Hydroxy-7-methoxy-3-propoxyacridone (*7*)

NMR ($CDCl_3$) δ 1.81 (3H, t, $J = 7.1$), 1.93–2.00 (2H, m), 4.02 (3H, s), 4.10 (2H, t, $J = 6.8$), 6.27 (1H, s), 6.38 (1H, s), 7.32 (1H, d, $J = 8.7$), 7.41 (1H, d, $J = 6.5$), 7.84 (1H, s), 8.43 (1H, s), 14.21 (1H, s). LRMS *m/z* (relative intensity, %) $(M)^- = 298.0$ (100); HPLC purity (retention time) 93% (2.4 min).

2.3.3. 1-Hydroxy-3-isopropoxy-7-methoxyacridone (*8*)

NMR ($CDCl_3$) δ 1.50 (3H, s), 1.53 (3H, s), 4.03 (3H, s), 4.73–4.78 (1H, m), 6.27 (1H, s), 6.37 (1H, s), 7.32 (1H, d, $J = 8.7$), 7.43 (1H, d, $J = 7.9$), 7.85 (1H, s), 8.35 (1H, s), 14.20 (1H, s). LRMS *m/z* (relative intensity, %) $(M)^- = 297.9$ (100); HPLC purity (retention time) 81% (2.4 min).

2.3.4. 3-Benzyloxy-1-hydroxy-7-methoxyacridone (*9*)

NMR ($CDCl_3$) δ 4.02 (3H, s), 5.35 (2H, s), 6.62 (1H, s), 7.02–7.19 (2H, m), 7.25–7.59 (6H, m), 7.80–7.88 (1H, m), 8.01 (1H, s), 14.42 (1H, s). LRMS *m/z* (relative intensity, %) $(M)^- = 345.9$ (100); HPLC purity (retention time) 99% (2.3 min).

*2.3.5. 3-Allyloxy-1-hydroxy-7-methoxyacridone (**10**)*

NMR ($CDCl_3$) δ 4.02 (3H, s), 4.71–4.73 (2H, m), 5.44–5.60 (2H, m), 6.12–6.25 (1H, m), 6.30 (1H, d, J = 1.9), 6.39 (1H, d, J = 2.2), 7.29–7.46 (2H, m), 7.85 (1H, d, J = 2.8), 8.45 (1H, s), 14.40 (1H, s). LRMS *m/z* (relative intensity, %) $(M)^-$ = 295.9 (100); HPLC purity (retention time) 91% (2.3 min).

*2.3.6. 7-Ethoxy-1-hydroxy-3-methoxyacridone (**11**)*

NMR ($CDCl_3$) δ 1.59 (3H, t, J = 7.1), 4.00 (3H, s), 4.23–4.31 (2H, m), 6.28 (1H, s), 6.38 (1H, d, J = 2.0), 7.29–7.44 (2H, m) 7.84 (1H, s), 8.36 (1H, s), 14.24 (1H, s). LRMS *m/z* (relative intensity, %) $(M)^-$ = 283.9 (100); HPLC purity (retention time) 92% (2.3 min).

*2.3.7. 1-Hydroxy-3-methoxy-7-propoxyacridone (**12**)*

NMR ($CDCl_3$) δ 1.19 (3H, t, J = 7.1), 1.95–2.02 (2H, m), 3.99 (3H, s), 4.15 (2H, t, J = 7.1), 6.28 (1H, s), 6.38 (1H, d, J = 2.0), 7.29–7.43 (2H, m), 7.84 (1H, d, J = 2.4), 8.39 (1H, s), 14.25 (1H, s). LRMS *m/z* (relative intensity, %) $(M)^-$ = 298.1 (100); HPLC purity (retention time) 77% (2.3 min).

*2.3.8. 1-Hydroxy-7-isopropoxy-3-methoxyacridone (**13**)*

NMR ($CDCl_3$) δ 1.50 (3H, s), 1.52 (3H, s), 3.99 (3H, s), 4.78–4.82 (1H, m), 6.27 (1H, s), 6.38 (1H, s), 7.28–7.41 (2H, m), 7.87 (1H, s), 8.46 (1H, s), 14.26 (1H, s). LRMS *m/z* (relative intensity, %) $(M)^-$ = 298.1 (100); HPLC purity (retention time) 69% (2.3 min).

*2.3.9. 7-Benzyloxy-1-hydroxy-3-methoxyacridone (**14**)*

NMR ($CDCl_3$) δ 4.01 (3H, s), 5.32 (2H, s), 6.28 (1H, s), 6.40 (1H, d, J = 2.4), 7.32–7.63 (6H, m), 7.98 (1H, s), 8.15 (1H, s), 8.24 (1H, s), 14.20 (1H, s). LRMS *m/z* (relative intensity, %) $(M)^-$ = 345.9 (100); HPLC purity (retention time) 99% (2.3 min).

*2.3.10. 7-Allyloxy-1-hydroxy-3-methoxyacridone (**15**)*

NMR ($CDCl_3$) δ 4.02 (3H, s), 4.77–4.81 (2H, m), 5.40–5.63 (2H, m), 6.18–6.22 (1H, m), 6.28 (1H, s), 6.37 (1H, d, J = 10.0), 7.28–7.47 (2H, m), 8.01 (1H, s), 8.32 (1H, s), 14.21 (1H, s). LRMS *m/z* (relative intensity, %) $(M)^-$ = 295.8 (100); HPLC purity (retention time) 100% (2.3 min).

2.4. Reagents and drugs

Acyclovir (ACV) and Foscarnet (PFA) were obtained from Sigma Chemical Co. (St. Louis, MO). 5-Chloro-1,3,-dihydroxyacridone (**1**) and 1,3,7-trihydroxyacridone were prepared as described (Akanitapichat et al., 2000). The original source of the *Citrus* alkaloids, Citrusinine-I and Citpressine-I was Dr. Hiroshi Furukawa (Bastow et al., 1994). For biological testing, all compounds were dissolved in DMSO as 20 mM stock solutions except PFA, which was prepared at similar concentration but in sterile phosphate buffered saline. TRAN^{35}S-LABEL^{TM} (*E. coli* hydrolysate labeling reagent containing 70% L-Methionine, [^{35}S]; >10,000 Ci/mmol) was purchased from ICN Radiochemicals (Irvine, CA). The source and use of the polyclonal rabbit antibody against HSV infected cells was described previously (Akanitapichat and Bastow, 2002). All other chemicals were reagent grade.

2.5. Cells and virus

The African green monkey kidney (Vero 76: ATCC No.: CRL 1587) and human embryonic lung fibroblasts (HEL, ATCC No.: CCL 137) cells were purchased from the UNC Lineberger Comprehensive Cancer Center (Chapel Hill, NC). Cells were routinely cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum and 100 μg/ml of kanamycin (designated "standard" medium) in a humidified 5% (v/v) CO_2 incubator at 37 °C. Throughout the course of experiments, HEL cells were sub-cultured at 1:3 dilution and were not used beyond seven passages from receipt. A high titre stock of HSV-2 (strain 186), a generous gift of Dr. S. Bachenheimer (Microbiology, UNC-CH), was stored frozen as aliquots and used directly for the present work. The same HSV-2 strain was used for the original work on *Citrus* alkaloids (Yamamoto et al., 1985). HCMV (Towne, VR977, Lot 6 W) was purchased from the American Type Culture Collection (Rockville, MD) and a working stock was prepared (2×10^5 PFU per ml) by low multiplicity infection of HEL cells. The source and maintenance of the HSV-1 (KOS) strain was as described (Akanitapichat and Bastow, 2002).

2.6. Virus and cell growth inhibition assays

Established virus culture techniques were used (Huang and Kowalik, 1993; Akanitapichat et al., 1999) but with the following modifications. For the HCMV plaque-elimination assay, HEL cells were plated in standard medium at 60,000 cm^{-2} and infected the following day with 50–100 PFU HCMV for 90 min with occasional agitation. The inoculate was replaced with maintenance medium containing 5% (v/v) fetal calf serum and test agents as indicated (Sections 3.2 and 3.3). After an additional 6–10 days of culture, cells were fixed with formal saline (10% formalin in phosphate-buffered saline; PBS), stained with 0.1% (w/v) toluidine blue in PBS and plaques were scored using an inverted light microscope at 40× magnification (Figs. 2A and 3). For ED_{50} determination, the value was interpolated from dose-response data and is the concentration of compound that reduced plaque formation by 50% relative to control under the specified condition.

To examine whether pre-treatment of cells influenced anti-HCMV activity of 1-hydroxy-3,7-dimethoxyacridone (**2**), a cytopathogenicity (CPE) reduction assay was used. The protocol was like the plaque-elimination assay except HEL cells were replicated in test compound (3–12 days or from 1 to 4 passages) prior to infection, cultures were

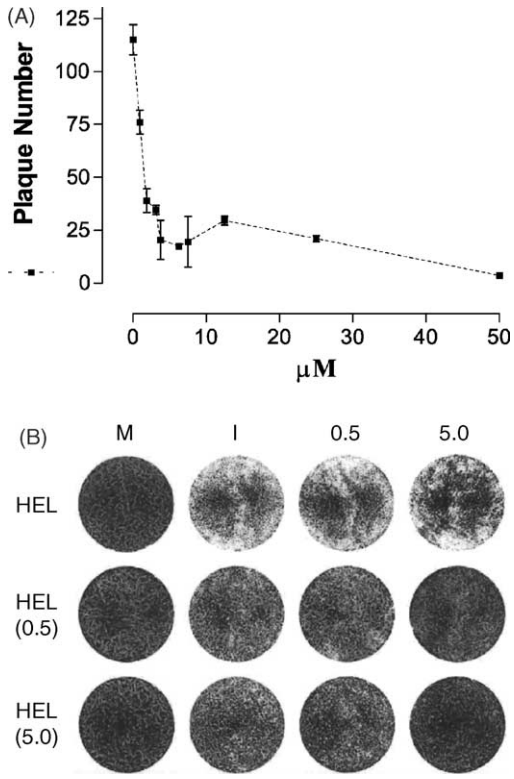


Fig. 2. Inhibition of HCMV plaque formation and cytopathogenicity by 3,7-dihydroxy-1-hydroxyacridone (**2**): HEL cells infected with a low multiplicity of HCMV were treated with various concentrations of compound **2** and plaques were counted after 10 days of continuous treatment. Details of the HCMV plaque-elimination assay are given in Section 2.6. The graphed data shows the dose-dependent antiviral activity of **2**, with values representing the mean and standard deviation of triplicate treatments from two independent experiments (panel A).HEL cells were replicated in the presence of either 0.5 or 5 μM compound **2** for 12 days (four passages) then they were compared to untreated HEL cells as a host for HCMV replication in the presence or absence of the drug. Treatment had no effect on cell morphology or cell viability as reflected by microscopic appearance and by replication rate, respectively. Cultures were infected with 10 times the viral load used for plaque assay and were fixed, stained and photographed after 7 days of infection (panel B). A detailed method is described in Section 2.6. The horizontal rows labeled as HEL, HEL (0.5) and HEL (5.0) represent cells cultured in the absence or presence of 0.5 and 5 μM (**2**), respectively. Labeled columns indicate mock-infected (M), infected (I) and treated after viral infection with the same two concentrations of the compound.

infected with 500–1000 PFU HCMV and after five days, cells were stained with 0.8% (w/v) crystal violet in 50% ethanol in order to achieve contrast for photography. A representative result is shown in Fig. 2B.

Three types of antiviral assay were used for HSV studies. For plaque-elimination, Vero cells (70,000 cm^{-2}) were infected with 50–100 PFU for 30 min with occasional agitation. The inoculate was replaced with medium containing 1% (v/v) fetal calf serum and supplemented with test agents as indicated (Section 3.2). After 2 days of culture, cells were processed using crystal violet staining and plaques were scored by visual examination (Fig. 3). For examination of anti-HSV activity in parallel cultures during biochemical experiments (Section 2.7), the progeny virus obtained from cells and from the culture medium at 23 h post-infection was determined by limiting dilution on Vero cells (Fig. 5A). Macroscopic viral plaques were scored after 2 days of incubation in medium containing 1% (w/v) carboxymethylcellulose and 0.5% (v/v) fetal calf serum. For ED_{50} measurements, the concentration of compound that reduced a single-cycle of viral yield by 50% relative to the control and under the specified condition was interpolated from dose-response graphs (Fig. 4, Table 1). Over the course of 10 independent experiments, the mean cell-associated viral yield was 180 PFU per cell (S.D. = 80) and 10 PFU per cell (S.D. = 5) for HSV-1 and HSV-2, respectively. The percent viral release (into the culture medium) was 16 (S.D. = 7) and 0.9 (S.D. = 0.6) for HSV-1 and HSV-2, respectively. These virologic parameters were independent of viral load and serum concentration.

The effect of compounds on host cell replication (CC_{50}), was measured using Sulforhodamine B-staining and the spectrophotometric method originally developed for the NCIs in vitro anti-cancer drug screening program (Skehan et al., 1990). The CC_{50} value is the concentration of compound that inhibited actively replicating cells by 50% of control respectively after 2 days of continuous treatment.

2.7. Analysis of HSV proteins

Viral protein synthesis was examined using pulse labeling, SDS–PAGE gel separation and phosphor-imaging. Vero cells were infected and treated under conditions specified in Section 3.5 and Fig. 5. After 17 h of infection, 10 μCi/ml TRAN^{35}S-LABEL^{TM} was added directly to culture medium and incubation continued for 30 min more. Radio-labeled cell cultures were carefully washed with PBS pre-warmed to 37 °C then lysed at 2,000,000 cells per ml in the same buffer supplemented with 2% (w/v) SDS. After denaturing cell lysates in Laemmli loading buffer, total proteins recovered from 30,000 cells were separated using either 8% (Fig. 5B) or 10% (Fig. 5D) SDS–PAGE gels (Laemmli, 1970). Proteins were transferred to nitrocellulose and the synthesized proteins were visualized and quantified using a STORM phosphorimager (Molecular Devices, Sunnyvale, CA) and the supplied ImageQuant software according to the manufacturers instructions (Fig. 5, panels B and C). Protein load between samples was compared qualitatively by visual examination of filters stained with India-Ink. A complementary approach for assessing drug effect on viral proteins involved immune detection on a Western-blot using conditions and reagents described previously (Akanitapichat and Bastow, 2002). A representative result is shown in Fig. 5D.

2.8. Statistical analysis

The program Prism^{TM} version 3 (Graphpad Software, Inc., Sand Diego, CA) was used for graphing and statistical analysis of study results.

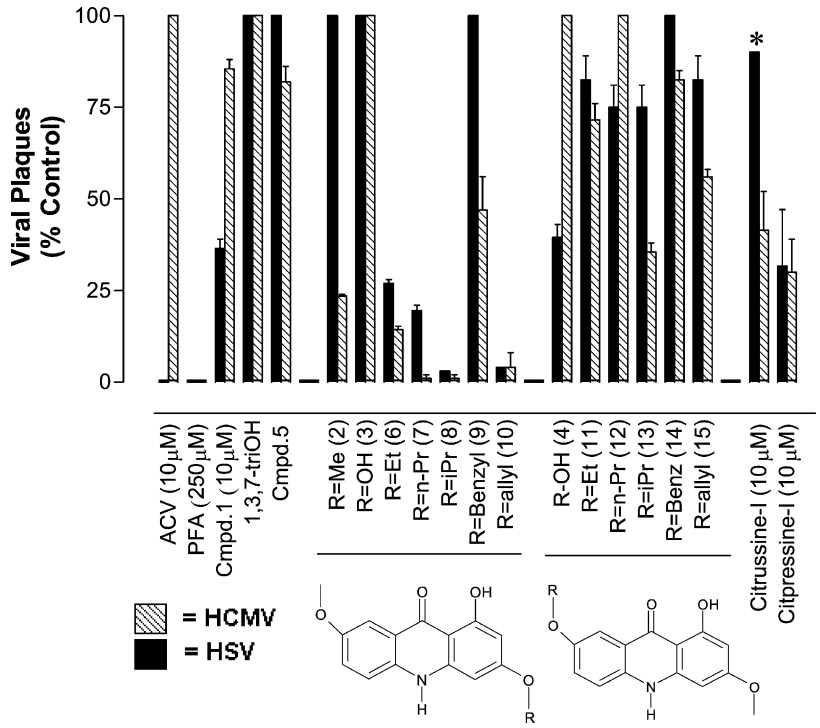


Fig. 3. Comparison between bis-alkoxylated 1-hydroxyacridones as HSV-2 and HCMV inhibitors: A plaque-elimination assay was used to screen compounds as inhibitors of HSV-2 and HCMV in Vero and HEL cells, respectively (Section 2.6). Compounds were tested at five micromolar concentration except for 5-chloro-1,3-dihydroxyacridone (**1**), 1-hydroxy-3,7-dimethoxyacridone (**2**) and acyclovir (ACV) which were tested at 10 μM and for phosphonoformic acid (PFA), which was evaluated at 250 μM. Filled bars and hatched bars are activity against HSV-2 and HCMV, respectively. The values represent mean and standard error of results from two independent experiments conducted several months apart. For control treatments (**1**, **2**, PFA, ACV and 1,3,7-trihydroxyacridone), results were compatible with work reported herein (Fig. 2A, Table 1) and elsewhere (Bastow et al., 1983; Akanitapichat et al., 2000). The asterisk denotes that HSV plaque-size was uniformly smaller in the presence of Citrusinine-I but the number of visible plaques was not reduced.

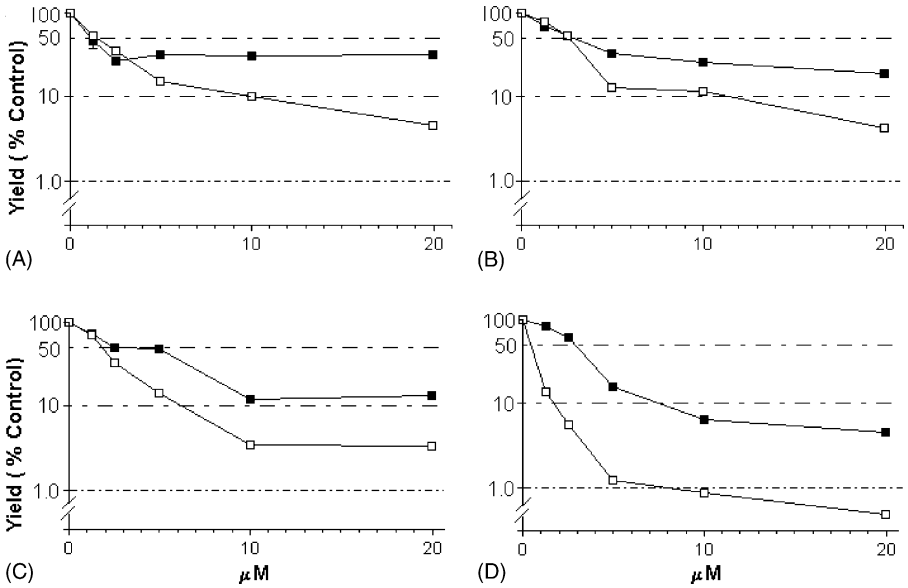


Fig. 4. Effect of viral load and serum concentration on the anti-HSV activity of 3-allyloxy-1-hydroxy-7-methoxyacridone (**10**). Vero cells were infected with HSV-1 at either 1.0 or 0.01 PFU per cell. One hour after infection, either standard growth medium or medium supplemented with 10-fold lower serum (0.5% v/v) and various concentrations of compound **10** was added. The production of cell-associated and released virus at 23 h post-infection was measured by serial dilution and plaque assay as described in Section 2.6. The open squares represent the condition of low multiplicity (0.01 PFU), infection. Panels A and B show the amount of cell-associated virus produced in medium containing 5 and 0.5% (v/v) serum, respectively. Panels C and D show the virus released into medium supplemented with 5 and 0.5% (v/v) serum, respectively. The yield of virus is plotted on a logarithmic scale to more clearly illustrate the differences apparent between treatments.

Table 1
Comparison between inhibitors of HSV and/or HCMV replication in cultured cells

Compound	Parameter (μM)[a]				
	ED_{50} HSV-1	ED_{50} HSV-2	CC_{50} Vero	ED_{50} HCMV	CC_{50} HEL
ACV	0.2	0.8 ± 0.1	>200 (41)	>200 (NA)	>500 (43)
PFA	ND	60 ± 5.0	>500 (11)	70 ± 10	>500 (11)
Citrusinine-I	30 ± 2.0	2.5 ± 0.7	27 ± 4.0	9.0 ± 1.5	41 ± 5.0
5-Chloro-1,3-dihydroxyacridone (**1**)	4.3 ± 0.8	3.8 ± 0.3	>50 (23)	>50(25)	>50 (18)
3,7-Dimethoxy-1-hydroxyacridone (**2**)	>50 (NA)	>50 (NA)	>50 (7)	1.4 ± 0.3	>50 (NA)
3-Alloxy-1-hydroxy-7-methoxyacridone (**10**)	2.3 ± 0.3	3.9 ± 1.0	95 ± 2.0	<2.5 (80)	>50 (33)

[a] Inhibition of HSV replication (1 PFU per cell in 5% (v/v) serum) was measured using a viral yield-reduction assay. Activity against HCMV was the ability to prevent formation of microscopic viral plaques. Cell growth inhibition was evaluated using a protein-dye binding assay. Methods are given in Section 2.6. Results are mean values and standard error obtained from experiments replicated at least once. NA indicates that no activity was observed. The numbers in parenthesis are the percent inhibition observed at the highest concentration tested. ND indicates that a value was not determined.

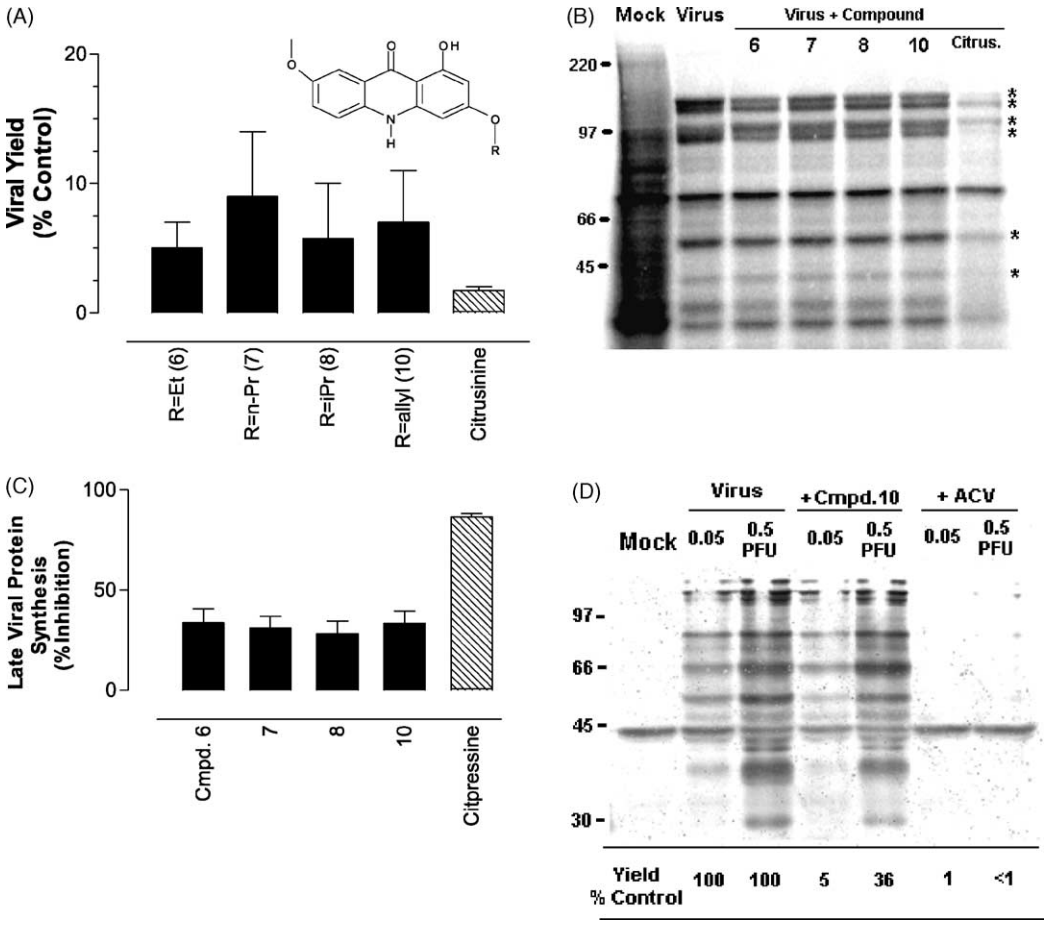


Fig. 5. Effects on HSV protein synthesis and accumulation: Vero cells were infected with HSV-2 at a multiplicity of 0.1 PFU and treated for 22 h with compounds at 20 μM in medium containing 2% (v/v) serum. Cell-associated and released virus was harvested and virus production was quantified by dilution using a plaque-assay. Parallel cultures were pulse-labeled at 17 h post-infection and total cell extracts were subsequently analyzed for late viral protein synthesis. Detailed methods are covered in Section 2.7. The relative amount of progeny virus recovered from drug-treated cultures is shown in panel A (the apparent variation in the activity of **6**–**8** and **10** reflects the significant differential between inhibition of cell-associated vs. released virus under the treatment condition used). The phosphorimage in panel B shows the radio-labeled proteins detected, with treatment condition indicated above each lane. Molecular mass of marker proteins in kDa is indicated in the left margin. Viral proteins denoted with an asterisk in the right margin were used for quantitative analysis of viral protein synthesis and the results obtained are represented graphically in panel C. The image in panel D is a Western immunoblot of infected cell extracts stained with a polyvalent HSV antibody (Section 2.7). Cells were infected with HSV-1 and treated for 16 h either with compound **10** or ACV at 10 μM in medium containing 2% (v/v) serum. Additional details of specific treatments are given above each lane. The relative amount of progeny virus recovered from the medium of parallel cultures is shown under the lanes. Molecular mass of proteins is indicated in the left margin. A cross-reacting cellular protein detected in samples is possibly actin on the basis of high abundance and apparent molecular mass.

3. Results

3.1. Chemistry

In previous work, a variety of 1-hydroxyacridone analogues including **1**, **4** and 1,3,7-trihydroxy acridone were prepared by condensation of anthranilic acid and resorcinol (each appropriately substituted) in *n*-butyl alcohol at reflux in the presence of zinc chloride (Hughes and Richie, 1951; Bastow et al., 1994; Akanitapichat et al., 2000). Compound **2** was then synthesized by selective alkylation of 1,3,7-trihydroxyacridone in 18% yield (Lowden, 1995). Although the Hughes and Richie reaction is somewhat versatile, its efficiency and the ease of product purification was found to vary considerably, therefore, an alternate one-step synthetic route to targets **2**–**5** was investigated (Scheme 1). The reaction, a simple thermal condensation, was originally developed to define the structure activity relationship around compound **1**, the HSV lead (Lowden, 2002). Thermal coupling proved to be a superior route to compounds **2** and **4** and also afforded **3**, the acridone skeleton used to prepare the variable alkoxylated series at C-3 (compounds **10**–**15**), in parallel and compound **5**, the 3,5-regioisomer of the HCMV lead (**2**). The goal of the parallel synthesis was to develop a viable method for the rapid production of compounds closely related to **2** as an exploratory series for preliminary biological evaluation. Pilot studies were undertaken to examine the feasibility of selective *O*-alkylation of compounds **3** and **4**. Use of the Mitsunobu reaction proved to be selective for phenolic alkylation, but removal of the byproducts in a parallel fashion was problematic. Therefore, reaction with alkyl halides was explored as a potential route (Scheme 2). In order to facilitate the selective alkylation of the hydroxyl at C-3 or C-7 over the secondary amine, less than one equivalent of alkyl halide was used in a room temperature reaction. Non-reacted acridone (**3** or **4**) was readily removed with aqueous potassium hydroxide, probably due to the presence of an acidic phenol (the phenol in the 1-position is much less acidic due to hydrogen bonding with the carbonyl). Removal of alkyl halides was accomplished through filtration, subsequent to trituration in dichloromethane. The quantity of bis-alkylated impurities varied depending on the alkylating agent but the trituration step proved to be largely selective for the desired product. On the basis of TLC analysis, the alkylations were usually complete in the first hour; however, alkylating agents with branching on the alpha carbon reacted much slower. Alkylation with cyclohexyl bromide occurred only minimally at the 3-hydroxyl positions of **3** and **4** was not alkylated using cyclohexyl bromide even after heating. Presumably, this result can be attributed to steric hindrance around the bromide leaving group. Yields were approximately 50% for the reaction, and purity ranged from 69 to 100% (Sections 2.3.1–2.3.10). Mass spectral and HPLC analysis indicated that either starting material or bis-alkylated products were the main impurities. The structures of the 10 products that were isolated from the parallel synthesis (**6**–**15**), are depicted in Scheme 2 and Fig. 3.

3.2. Anti-HCMV activity of 3,7-dimethoxy-1-hydroxyacridone (2)

3,7-Dimethoxy-1-hydroxyacridone (**2**), was originally defined as an inactive analog of 1,3,7-trihydroxyacridone, a novel DNA topoisomerase II inhibitor with modest anti-HSV activity (ED_{50} of 40 μM and three-fold selectivity; Lowden, 1995; Vance and Bastow, 1999; Akanitapichat et al., 2000). Subsequent work involving random screening for anti-HCMV agents identified **2** as a candidate inhibitor. The results in Fig. 2A show the activity of **2** measured using a plaque-elimination assay. Compound **2** effectively blocked HMCV plaque formation with an ED_{50} value of 1.4 μM (0.5 μg/ml). Compound **2** was also examined as an inhibitor of cell replication and was inactive at 50 μM against HEL, Vero and an assortment of human tumor cell lines (Table 1 and data not shown). Therefore, compound **2** is a selective (>35-fold) anti-HCMV agent with activity comparable to recently reported values of clinical agents ganciclovir and cidofovir (Zhou et al., 1997; Martinez et al., 2000). Subsequent work using higher viral loads showed that compound **2** did not protect against HCMV cytopathogenicity unless HEL cells were pretreated prior to viral infection. A representative result obtained using a prior exposure of 12 days is shown in Fig. 2B. The culture of cells in concentrations of **2** as low as 0.5 μM subsequently afforded some amelioration of HCMV cytopathogenicity. Interestingly, the protective effect at 5 μM was even apparent in the absence of sustained treatment post-infection. The pre-treatment dependence of **2** at higher viral loads is difficult to interpret without understanding mechanism but the significant activity against HCMV replication prompted the exploration of structure–activity through analog synthesis.

3.3. Antiviral activities of the 3,5-regioisomer (5) and the parallel series (6–15)

Preliminary evaluation of the parallel series as anti-herpes agents showed that several inhibited HCMV replication but unlike **2**, four of the active analogs inhibited HSV replication also. Therefore, antiviral testing was expanded to include examples of the *Citrus* alkaloids that exhibit a similar dual anti-herpes activity (Yamamoto et al., 1989), as well as clinically useful viral DNA polymerase inhibitors with activity against HSV (ACV), or against both HSV and HCMV (PFA). The results obtained for a fixed concentration using a plaque-elimination assay are shown in Fig. 3. Control compounds (ACV, PFA, **1**, 1,3,7-trihydroxyacridone, Citrusinine-I and Citpressine-I gave the expected pattern and level of activity based on published work (Elion et al., 1977; Helgestrand et al., 1978; Yamamoto et al., 1989; Akanitapichat and Bastow, 2002). Compound **2** (10 μM) was designated as a specific HCMV-inhibitor because it

was without effect on HSV even at the highest concentration (100 μM) tested (data not shown). Interestingly, the 3,5-dimethoxy regioisomer (**5**), was inactive against herpes replication at concentrations that did not inhibit host cell replication (mean CC_{50} of 33 μM), suggesting that the C-7 methoxy substituent was important for the anti-HCMV activity of **2**. The two acridone scaffolds used for parallel synthesis were either inactive (compound **3**) or only inhibited HSV replication (**4**). However, compound **4** was not acting as a highly selective antiviral agent because it had significant activity against Vero replication (CC_{50} of 25 μM). Four of the C-3 variable-alkoxylated compounds (**6**–**8** and **10**), inhibited the replication of both types of herpes virus equally, with the 3-isopropoxy-analogues (**8**) and the 3-allyloxy-analogues (**10**) almost completely preventing the formation of microscopic viral plaques. The 3-benzyloxy derivative **9**, inhibited HCMV but not HSV-2 replication and an ED_{50} of 6.9 ± 0.6 μM was subsequently determined. Of the five actives bearing a methoxy substituent at C-7, compound **9** was the least efficacious being about five-fold less active than **2**. Significantly, none of the 3-alkoxylated parallel series significantly inhibited host cell replication (CC_{50} values greater than 50 μM) thereby showing they were acting selectively (at least eight-fold), as viral inhibitors. Of the C-7 variable alkoxylated compounds (**11**–**15**), the isopropoxy-derivative (**13**) was active against HCMV in the screen but a CC_{50} of 12 μM against HEL replication was subsequently determined. In general, the C-7 variable parallel series comprised of either inactive or weakly active antiviral agents and were not evaluated further. A quantitative comparison of anti-herpes activity between compounds **1**, **2** and **10**, Citrusinine-I, AVC and PFA was conducted and results are given in Table 1. ACV was four-fold less active against HSV-2 and was inactive against HCMV, consistent with the known activity spectrum of the drug; the activity profile of PFA was also consistent (Elion et al., 1977; Helgestrand et al., 1978). Citrusinine-I was a selective inhibitor of HSV-2 and HCMV replication but was inactive against the HSV-1 (KOS) strain. Yamamoto et al. reported activity against both HSV sub-types but selectivity was only apparent since cell growth inhibition was tested against a cell line other than the host. The activity spectrum of 5-chloro-1,3-dihydroxyacridone (**1**), against HSV-1 and HCMV was similar to previous results (Akanitapichat et al., 2000) and **1** inhibited the yield of HSV-2 with an ED_{50} value of 3.8 ± 0.3 μM in the present study. By way of comparison, 3-allyloxy-1-hydroxy-7-methoxyacridone (**10**) inhibited replication of HSV-1 and HSV-2 with 41- and 24-fold selectivity, respectively and against HCMV, the analog was at least as active as the 3,7-dimethoxy lead (**2**).

3.4. Parameters influencing the anti-HSV activity of C-3 variable alkoxylated analogs

Previous work with 1,3-dihydroxyacridone derivatives showed that activity against HSV is dependent on both multiplicity and serum concentration, the latter variable likely due to serum protein-binding (Akanitapichat et al., 2000). We observed that compounds **6**–**8** and **10** did not protect against HSV cytopathogenicity under the viral yield assay condition and wondered whether serum concentration and/or viral load could influence the activity of the new compounds also. Results obtained for the 3-allyoxy-derivative **10**, are shown in Fig. 4. Compound **10** inhibited the production of cell-associated HSV-1 and this activity was dependent on viral load but only at concentrations of 5 μM and higher (i.e. the ED_{50} value was not changed; Fig. 4, panels A and B). In contrast, the concentration of serum in culture medium did not affect the inhibition of cell-associated virus (Fig. 4, cf. panel A to panel B). However, both viral load and serum concentration were identified as important variables affecting the amount of virus released into medium in the presence of compound **10** (Fig. 4, cf. panel C to panel D). The study results also show that viral release was inhibited more actively than the intracellular viral replication (cf. panels A to C and B to D) and under the low viral load and low serum treatment condition (Fig. 4 panel D), the ED_{50} concentration for inhibition of viral release was actually decreased about six-fold. On the basis of the results, the inhibition of viral release must have contributed to the antiviral efficacy of **10**, particularly under the plaque-reduction assay condition. Consistent with this interpretation, compound **8** (at 10 μM in 2% (v/v) serum), inhibited a low multiplicity (0.001 PFU per cell) HSV-2 infection. Treatment completely prevented viral release up to 48 h after infection while the production of cell-associated virus was inhibited by 80% over the same period (data not shown).

3.5. Effect of the parallel series on HSV late proteins

The production of HSV proteins are temporally regulated, with the class designated as “late” being dependent upon viral DNA synthesis (Honess and Roizman, 1974). Citrusinine-I, PFA and the active metabolite of ACV all interfere with HSV DNA replication (the latter two acting in a direct fashion) and thereby prevent normal production of late viral proteins (Helgestrand et al., 1978; Yamamoto et al., 1989; Furman et al., 1979). By way of comparison and as an approach to delineate a general mode of action, the effects of compounds **6**–**8** and **10** on HSV proteins were investigated. The results in Fig. 5A–C show the inhibition of HSV-2 replication and late protein synthesis with Citrusinine-I as a positive control treatment. Although viral replication was inhibited by about 90% of control by **6**–**8** and **10** (Fig. 5A), viral protein synthesis was inhibited by only 40–45% (Fig. 5B and C). In contrast, Citrusinine-I inhibited replication and late protein synthesis equally, to about 10% of control values. The activity of compound **10** was also compared to ACV against HSV-1 replication and for effects on viral protein accumulation (Citrusinine-I was not a selective inhibitor of the viral strain used in this work; Table 1). As expected, the level of viral proteins produced from the

culture was dependent upon viral load. Compound **10** had no effect on viral protein accumulation under any condition, despite inhibiting viral replication by 64–95% (Fig. 5D). In contrast, when ACV was used as a positive control, the accumulation of HSV-1 proteins was abolished. The results show that compounds **6**–**8** and **10** at high concentration and in the presence of moderate serum did interfere with normal HSV-2 protein synthesis but this action did not correlate with antiviral activity. However, no effect on viral protein levels was apparent when compound **10** was tested against HSV-1 at a two-fold lower concentration. Overall, the findings suggest that the mechanism of compounds **6**–**8** and **10** is fundamentally different from either Citrusinine-I or ACV.

4. Discussion

The antiviral *Citrus* alkaloids that include Citrusinine-I and Citpressine-I are active against HSV and HCMV replication in cultured cells although the latter compound has only marginal selectivity (Yamamoto et al., 1989). The mechanism of action studies reported showed that the alkaloids block herpes DNA replication and against HSV-2, Citrusinine-I was proposed to act by inhibiting the encoded ribonucleoside diphosphate reductase enzyme. The *Citrus* compounds are relevant to current antiviral drug design and development efforts because they are non-nucleoside structures that do not target viral DNA polymerse (Bastow and Akanitapichat, 1977). However, Citrusinine-I is only 10-fold selective against HSV-2 replication in Vero cells and was inactive against a common laboratory strain of HSV-1 (this study). As a natural product of moderate structural complexity, Citrusinine-I is not readily amenable to optimization via synthesis and this is considered a significant drawback to further development. The findings presented herein confirm and refine the antiviral spectrum of Citrusinine-I, they further show that 3-allyloxy-1-hydroxy-7-methoxyacridone (**10**), has a distinctive antiviral activity profile and they suggest the action of compound **10** and closely related analogs (**6**–**8**), against HSV, must be different from the proposed mechanism of the natural product.

As a group, the active antiviral compounds (**2**, **6**–**10**), were expected to act differently than Citrusinine-I at the biochemical level a priori, on the basis of the known structure activity relationship around the natural product. Yamamoto et al. reported that hydroxyl groups at either the C-5 (R2), or C-6 (R1), position of the acridone skeleton (Fig. 1), was important for selective anti-herpes activity but neither specific substituent is present in the six synthetic actives discovered by us. Accordingly, the novelty of the 3,7-di-alkoxylated 1-hydroxyacridone derivatives was confirmed by experiment. At high antiviral concentration, compounds **6**–**8** and **10** inhibited HSV-2 protein synthesis by less than 50% and compound **10** at five times the EC_{50} concentration had no effect on late protein accumulation in HSV-1 infected cells. In contrast, a marked inhibition of late HSV protein synthesis and accumulation occurred in parallel cultures when Citrusinine-I and ACV, respectively were used as positive controls. Results also showed that compounds **2** and **9** up to 100 μM concentration were inactive against HSV replication. Finally, compounds **2**, **6**–**10** could be differentiated from 5-chloro 1,3-dihydroxyacridone (**1**) also on the basis of activity against HCMV (Fig. 3, Table 1), as well as on the structure–activity relationship around **1** that was recently established (Akanitapichat and Bastow, 2002; Lowden, 2002). The comparisons and results just discussed allow for the following conclusions to be drawn: first is that compounds **6**–**8** and **10**, unlike Citrusinine-I, cause a post-DNA synthesis blockade of HSV replication; second is that compounds **6**–**8** and **10** have a broader spectrum of activity than compound **1** and for compound **10** (one chosen for detailed characterization), the activity spectrum was also distinguishable from Citrusinine-I and third, is that compounds **2** and **9** possibly represent a distinct active sub-series as HCMV-selective inhibitors.

For the cultured cell models of herpes virus infection employed in the present work, cells were routinely treated with compounds after viral adsorption and penetration had occurred. The possibility that interference with one or both of those early events could account for the activity first detected during screening was considered to be unlikely in the case of HSV because delayed treatment with compound **10** inhibited virus produced from a single round of replication. Indeed, subsequent direct measurements confirmed that compound **10** did not affect adsorption and penetration of HSV (Bastow, unpublished). Although a post-viral DNA synthesis blockade of intracellular replication appears to be a major determinant of the anti-HSV activity, it is interesting that for cells maintained in low serum and infected at low multiplicity, treatment with compound **10** caused an augmented inhibition of viral release. The magnitude of this effect was dramatic (about six-fold from comparing ED_{50}s) and was specific for the released viral fraction. We reported that inhibition of total viral yield by 5-chloro-1,3-dihydroxyacridone (**1**) was dependent upon serum concentration and that this property was likely due to the protein-binding affinity of the compound (Akanitapichat and Bastow, 2002). As measured, the serum dependence of **1** would have been largely against the cell-associated viral fraction because the current work shows that only 16% of virions produced from HSV-1 (KOS)-infected cells are released (Section 2.6). Therefore, compounds **1** and **10** have different actions in the presence of low serum and the interference with viral release by **10** must contribute to antiviral efficacy, particularly under the plaque-elimination (screening) assay condition. To our knowledge, a serum-sensitive block of viral release is a unique action for an antiviral agent. On the basis of the results and comparisons just discussed, it is proposed that compound **10** can inhibit HSV spread under certain experimental conditions. Further mechanistic studies are necessary to examine this hypothesis as well as to delineate the critical biochemical

block to intracellular HSV replication that also occurs upon treatment.

The mechanism of the six anti-HCMV actives (**2, 6**–**10**), was not addressed in the present study. Others have reported a pre-treatment dependence for investigational CMV inhibitors (Chen et al., 1988), a property also shared by compound **2**. Although the significance of these types of observation are currently unclear, it is interesting that compounds **2** and **9** were classified as HCMV-selective agents. The 3,5-regioisomer (**5**) was an inactive antiviral agent as were the C-7 variable alkoxylated series of compounds (**11**–**15**). On the basis of this limited structure–activity information, it appears that a C-7 methoxy substituent (Fig. 1), is important for anti-HCMV activity. For the HSV/HCMV inhibitors, the difference between the active C-3 variable alkoxylated series (**6**–**8** and **10**), and the inactive C-7 variable compounds (**11**–**15**), could reflect the importance of the hydroxyl group at C-1, possible as a requirement for target interaction. However, it is interesting that the C-1 hydroxyl is critical for the anti-HSV activity of the 1,3-dihydroxyacridone class and possibly it is important for several other synthetic and natural antiviral acridones (Fig. 1; Lowden, 2002), even though the antiviral mechanisms currently defined for these agents are evidently diverse. The questions whether compounds **2** and **9** comprise a distinct mechanistic sub-class and whether HSV and HCMV inhibition occur via a similar or a different mechanism remain to be addressed.

The six novel anti-herpes compounds described herein (**2**, **6**–**10**), are simple synthetic 1-hydroxyacridone derivatives and as such, they are amenable to further synthetic elaboration. The versatile methodology of thermal condensation developed by us coupled with parallel synthetic approaches could allow for the rapid optimization and definition of these novel classe(s) of anti-herpes compound(s).

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