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1,3-Dihydroxyacridone derivatives as inhibitors of herpes virus replication

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Abstract

The nuclear enzyme DNA topoisomerase II is a candidate pharmacological target for treating herpes virus infections and the novel catalytic inhibitors, 7-chloro-1,3-dihydroxyacridone (compound **1**), and 1,3,7-trihydroxyacridone (**2**) are potential lead compounds [Bastow, K.F., Itoigawa, M., Furukawa, H., Kashiwada, Y., Bori, I.D., Ballas, L.M., Lee, K.-H., 1994. Antiproliferative actions of 7-substituted 1,3-dihydroxyacridones; possible involvement of DNA topoisomerase II and protein kinase C as biochemical targets. Bioorg. Med. Chem. 2, 1403–1411; Vance, J.R., Bastow, K.F., 1999. Inhibition of DNA topoisomerase II catalytic activity by the antiviral agents 7-chloro,1,3-dihydroxyacridone and 1,3,7-trihydroxyacridone. Biochem. Pharmacol. 58, 703–708]. In this report, four new 1,3-dihydroxyacridone analogs with functional groups at either the 5-, 6- or 8-positions (compounds **3**–**6**) were synthesized. Target compounds, three other analogs including compounds **1** and **2** and three anticancer drugs that inhibit DNA topoisomerase II (etoposide, amsacrine and aclarubicin) were then evaluated as selective inhibitors of herpes simplex virus (HSV) replication in cell culture and as enzyme inhibitors in vitro. Etoposide and amsacrine inhibited HSV but acted non-selectively. In general, the activities of 1,3-dihydroxyacridone derivatives as selective anti-HSV agents and as enzyme inhibitors varied inversely suggesting that DNA topoisomerase II probably is not the critical antiviral target. The 5-Cl congener (compound **3**) was the most selective agent (about 26-fold under a stringent assay condition) but was not an enzyme inhibitor. Results of exploratory mechanistic studies with compounds **1** and **3** show that HSV replication was blocked at a stage after DNA and late protein synthesis. The acridone derivatives were also tested against human cytomegalovirus (HCMV) replication but none of them were active.

Keywords: Topoisomerase II; Acridone; Antiviral

1. Introduction

Targeting critical cellular functions of viral replication is an alternative approach to rational antiviral drug design and is becoming more important to consider due to the increased preva-

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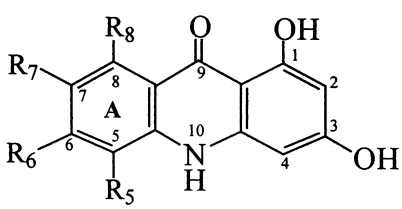
lence of viral drug resistance (Baba, 1997; Erice, 1999). Antiviral agents so developed are generally less safe yet optimizing leads can generate candidate drugs with respectable selectivity of action (reviewed in Akanitapichat and Bastow, 1997).

The nuclear enzyme DNA topoisomerase II (p170 isoform) is a potential cellular target of drugs to treat herpes simplex virus (HSV) infection since biochemical studies show that the enzyme is a functional host factor for HSV replication (Ebert et al., 1990, 1994). Several clinically useful anticancer drugs referred to as enzyme 'poisons' act by stabilizing a ternary complex between DNA topoisomerase II, the drug and the DNA. Once formed, this intermediate called a cleavable complex is recognized as a cytotoxic lesion that ultimately causes cellular death (reviewed in Froelich-Ammon and Osheroff, 1995). Etoposide (VP-16), a prototypical poison was shown to inhibit replication of HSV, and human cytomegalovirus (HCMV; Nishiyama et al., 1987; Huang et al., 1992). Ellipticine, another enzyme poison was shown to inhibit Epstein Barr virus (EBV) replication (Kawanishi, 1993). Although it was not implicitly stated in the cited reports, the effect of enzyme poisons on cell replication precludes their development as useful antiviral agents. A second class of DNA topoisomerase II inhibitor includes compounds that block catalysis without stabilizing cleavable complexes (reviewed in Andoh and Ishida, 1998). These agents are generally considered to be better prospective leads for antiviral drug development. A specific catalytic inhibitor recently reported to be active against HSV replication is ICRF-193, a bisdioxopiperazine derivative. The action of this compound is unusual and can be considered diagnostic for enzyme inhibition in cells (Hammarsten et al., 1996). However, antiviral selectivity of ICRF193 again appears to be marginal based on cytotoxic activity reported in an earlier study (Ishida et al., 1991).



Compound	R_5	R_6	R_7	R_8
1	H	H	Cl	H
2	H	H	OH	H
3	Cl	H	H	H
4	H	Cl	H	H
5	H	H	H	Cl
6	H	H	H	CH_3
7	H	H	CH_3	H

Fig. 1. Structures of compounds **1**–**7**.

The synthetic acridone derivatives, 7-chloro-1,3-dihydroxyacridone and 1,3,7-trihydroxyacridone (compounds **1** and **2**, respectively, Fig. 1), were identified as a novel class of DNA topoisomerase II catalytic inhibitor using both in vitro and cell-based (SV40 -infection) assays (Vance, 1998; Vance and Bastow, 1999). Although compounds **1**, **2** and ICRF-193 are all regarded as catalytic antagonists, the mechanism of enzyme inhibition by each drug class is distinctly different (Roca et al., 1994; Vance and Bastow, 1999). Compounds **1** and **2** were also found to be active against HSV replication but it remains unclear whether the primary antiviral action involves DNA topoisomerase II inhibition (Bastow et al., 1994). This question is difficult to examine directly because intracellular effects of catalytic inhibitors are not simple to measure and both the time and role of DNA topoisomerase II involvement in HSV replication are unclear (Ebert et al., 1990, 1994; Hammarsten et al., 1996). In the present work, a series of 1,3-dihydroxyacridone derivatives were evaluated and compared as enzyme inhibitors and as antiviral agents against HSV and HCMV. The results show that all the antiviral compounds specifically affect HSV replication and that the two of them further investigated do so by inducing a late blockade of the viral life cycle. Despite sharing similar antiviral properties with ICRF-193, DNA topoisomerase II is unlikely to be the critical antiviral target of 1,3-dihydroxyacridone derivatives based on the present findings.

2. Material and experimental procedures

2.1. General chemistry

Melting points were determined on a MEL-TEMP melting point apparatus and are uncorrected. IR spectra were taken on a Perkin Elmer 1320 infrared spectrometer. ^{1}H NMR spectra were recorded on a Bruker AC-300 spectrometer. The chemical shifts are presented in terms of ppm with Me_4Si as the internal reference. Mass spectra were measured on a Hitachi M-80 mass spectrometer.

2.2. Preparation of 1,3-dihydroxyacridone analogs *3*–*6*

2-Aminobenzoic acid derivative (13.56 mmol) anhydrous phloroglucinol (13.56 mmol) and anhydrous zinc chloride (20.0 mmol) were diluted in 40 ml anhydrous *n*-butyl alcohol in a flame dried round bottom flask under nitrogen. The reaction was refluxed for 20 h. After cooling, the reaction mixture was filtered and concentrated to give a crude product, which was purified by silica gel flash chromatography eluting with dichloromethane: methanol. Products were then recrystallized in hexanes: ethyl acetate yielding 1,3-dihydroxyacridone derivatives (**3**–**6**). The identification of the desired product amongst byproducts during the purification was facilitated by the yellow color of the former. Yields were not optimized and only isolated yields are reported in Sections 2.3, 2.4, 2.5 and 2.6. The actual yields may be somewhat higher in the case of compounds **3** and **6**, but problems such as insolubility of the products and a high affinity for silica gel probably contributed to low recovery. An improved synthesis was recently devised and will be reported elsewhere. The spectral and microanalytical data are listed below.

2.3. 5-Chloro-1,3-dihydroxyacridone (*3*)

Yield 70 mg; yellow crystals; mp > 300°C; Rf value = 0.25 in 8:2 hexane:ethyl acetate; NMR (D_6 DMSO) δ 6.08 (1H, s, Ar), 6.82 (1H, d, $J = 1.53$, Ar), 7.26 (1H, t, Ar), 7.90 (1H, d, $J = 7.56$, Ar), 8.16 (1H, d, $J = 7.86$, Ar), 10.67 (1H, s, 3-OH), 11.01 (1H, s, NH), 13.98 (1H, s, 1-OH); elemental analysis calculated for $C_{13}H_8NO_3Cl{\cdot}0.5H_2O$: C 57.70, H 3.33, N 5.18, Found C 57.92, H 3.26, N 5.23.

2.4. 6-Chloro-1,3-dihydroxyacridone (*4*)

Yield 1.42 g; yellow crystal; mp > 300°C; NMR (D_6 DMSO) δ 6.02 (1H, d, $J = 1.98$ Hz, Ar), 6.27 (1H, d, $J = 2.1$ Hz, Ar), 7.25 (1H, d, $J = 8.7$, Ar), 7.46 (1H, d, $J = 1.86$ Hz, Ar), 8.15 (1H, d, $J = 8.7$, Ar), 10.65 (1H, s, 3-OH), 11.82 (1H, s, NH), 13.98 (1H, s, 1-OH); elemental analysis calculated for $C_{13}H_8NO_3Cl$: 261.0193, found m/z 261.0195.

2.5. 8-Chloro-1,3-dihydroxyacridone (*5*)

Yield 1.45 g; yellow crystal; mp > 300°C; Rf = 0.7 in 7:3 hexane:ethyl acetate; NMR (D_6 DMSO) δ 5.99 (1H, d, $J = 1.41$ Hz, Ar), 6.24 (1H, d, $J = 1.86$ Hz, Ar), 7.21 (1H, d, $J = 7.56$, Ar), 7.39 (1H, d, $J = 8.37$ Hz, Ar), 7.59 (1H, t, Ar), 10.58 (1H, s, 3-OH), 11.83 (1H, s, NH), 14.26 (1H, s, 1-OH); elemental analysis calculated for $C_{13}H_8NO_3Cl{\cdot}2H_2O$: C 52.47, H 4.04, N 4.71, Found C 52.56, H 4.27, N 4.53.

2.6. 8-methy-1,3-dihydroxyacridonelacridone (*6*)

Yield 130 mg; yellow crystal; mp > 300°C; Rf = 0.2 in 7:3 hexane:ethyl acetate; NMR (D_6 DMSO) δ 2.83 (3H, s, CH3), 5.94 (1H, d, $J = 1.8$ Hz, Ar), 6.22 (1H, d, $J = 1.8$ Hz, Ar), 6.94 (1H, d, $J = 7.05$, Ar), 7.28 (1H, d, $J = 8.27$, Ar), 7.52 (1H, t, Ar), 10.42 (1H, s, 3-OH), 11.59 (1H, s, NH), 14.60 (1H, s, 1-OH); elemental analysis calculated for $C_{14}H_{11}NO_3{\cdot}2H_2O$: C 60.67, H 5.42, N 5.05, Found C 60.89, H 5.81, N 5.02.

2.7. Reagents and drugs

Etoposide was obtained from the Natural Products Laboratory, University of North Carolina (UNC) at Chapel Hill. Amsacrine and aclarubicin were obtained from the National Cancer Institute (Bethesda, MD). Compounds were prepared in DMSO as 20 mM stocks, except phosphonoacetic

acid (PAA) that was prepared as a sterile 1 M stock in water. Enzymes were from commercial sources as indicated. All other chemicals were reagent grade.

2.8. Cells and virus

The African green monkey kidney cell line (Vero, strain 186) and human embryonic lung fibroblasts (HEL) were obtained from the tissue culture facility at the UNC Lineburger Comprehensive Cancer Center (Chapel Hill, NC). Cells were cultured in RPMI-1640 supplemented with 5% (v/v) calf serum (for Vero) or 5% (v/v) fetal calf serum (for HEL) and 100 μg/ml of kanamycin in a humidified 5% CO_2 incubator at 37°C. Working stocks of HSV-1 (strain KOS) and HCMV (Towne) were propagated at 0.01 plaque-forming units (pfu) per cell using Vero and HEL cells as hosts, respectively, and were prepared using standard procedures (Bastow et al., 1986; Huang et al., 1992). The titers of viral stocks were measured using plaque assays as described below.

2.9. Plaque and viral yield assays

For viral plaque assays, confluent cell cultures (1×10^5 cells/cm^2) were infected with 50–100 pfu of virus. For HSV, the inoculate was removed at 1 h post-infection and replaced with overlay media (RPMI-1640, 0.5% (v/v) calf serum and 1% (w/v) carboxymethylcellulose) in either the absence or presence of test compounds. After incubating cultures for 2–3 days, monolayers were stained with 0.8% (w/v) crystal violet in 50% (v/v) ethanol and de-stained with water. For HCMV, the inoculate was removed at 3 h post-infection and replaced with medium (5% v/v FCS) in either the absence or presence of test compounds. Medium was replaced at day 4 post-infection and on either day 7 or 8, cultures were fixed and stained as described. Plaques were scored by visual examination.

For measuring anti-HSV activity using a viral yield assay, duplicate overnight cultures plated at confluence were infected with HSV at 1.5 pfu per cell. The inoculate was removed at one hour post-infection, replaced with growth medium containing 5% (v/v) calf serum and infected cultures were then incubated for an additional 18 h. Cultures were harvested by freeze thawing three times before titrating virus by limiting (ten-fold)-dilution using the plaque assay. Experimental variation of data given under 'Results' is either between replicates within single experiments or between several independent determinations as indicated. The EC_{50} value is the concentration of compound that inhibits viral replication by 50% following 17 h of continual treatment.

2.10. Cell growth inhibition

Cell growth inhibition by test compounds was determined using a sulforhodamine microtitre plate assay method according to the procedure described by Skehan et al. (1990). Under the seeding conditions used (3×10^3 cells/cm^2) the control untreated cultures replicate continuously during the course of the experiment. Fixed cells were stained with sulforhodamine B, an anionic protein stain and optical density was recorded at 562 nm using a UVmax spectrophotometer (Molecular Devices, Menlo Park, CA). Each determination was for triplicate treatments and experimental variation of data given in Section 3 is either between replicates within single experiments or between several independent determinations as indicated. The EC_{50} value is the concentration of compound that inhibits cell growth by 50% following a 3-day period of continuous treatment.

2.11. Evaluation of drug effects on HSV DNA production

Confluent cells were infected with virus at a multiplicity of 20 pfu per cell. At either 8 or 18 h after infection, cells were scraped and collected using centrifugation ($500 \times g$ for 5 min). Pellets were washed with phosphate buffer saline (PBS) and suspended in a buffer solution containing 10 mM Tris–HCl (pH 8), 10 mM EDTA and 10 mM NaCl. Cells were lysed upon addition of SDS to 0.4% (w/v), and digested with proteinase K (Gibco BRL, Gaithersburg, MD) at a concentration of 100 μg/ml at 37°C overnight. Digested DNA was extracted twice with phenol, and the

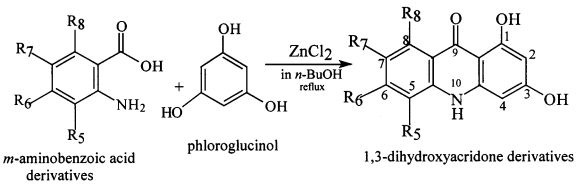
aqueous layer (containing nucleic acids) was dialyzed at 4°C against TEN buffer (50 mM Tris–HCl (pH 8), 10 mM EDTA and 10 mM NaCl) with two buffer changes. Dialyzed samples were treated with 20 μg/ml of RNase A (Boehringer Mannheim, Indianapolis, IN) at 37°C for 1 h, and then were re-digested with proteinase K in the presence of SDS (0.4% w/v). DNA Samples were re-extracted with phenol followed by dialysis against TEN buffer but containing 10 mM Tris–HCl (pH 8). The amount of DNA was measured using a fluorometer (Hoefer Scientific Instruments, San Francisco, CA) with lambda DNA (Gibco BRL, Gaithersburg, MD) as the standard. Ten microgram aliquots of DNA were precipitated with ethanol, dissolved in TE buffer (10 mM Tris–HCl (pH 8), 1 mM EDTA) and subsequently digested overnight with *Hin*dIII according to the manufacturer's recommendation (Promega, Madison, WI). Digested DNA was electrophoresed on 0.7% (w/v) agarose gels in 1 × TBE buffer (89 mM Tris base, 89 mM boric acid and 2 mM EDTA (pH 8)). Separated DNA was denatured by soaking the gel twice for 15 min each in 0.5 M NaOH, 1.5 M NaCl and the gel was then neutralized by soaking twice for 15 min in 3 M NaCl, 0.5 M Tris–HCl (pH 7). Denatured DNA was transferred to a nitrocellulose membrane by blot diffusion (Southern, 1975), and hybridized with a [^{32}P]-labeled *Bam*HI-digested HSV-1 genomic probe. The probe was prepared by rapid small-scale isolation (Kinter and Brandt, 1994), restriction and random primed DNA labeling (using a commercial kit, Boehringer Mannheim, Indianapolis, IN). The amounts of viral DNA were measured by scanning fragments on autoradiographs using a model GS 300 scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA). Data was normalized using a cross-hybridizing cellular DNA (about 2 Kbp) fragment detected in mock-infected and infected samples.

2.12. Evaluation of drug effects on HSV protein synthesis

Confluent cell cultures were infected with virus at a multiplicity of 20 pfu per cell for 1 h and the inoculate was then replaced with high serum media (calf 5%, v/v) either in the presence or in the absence of compounds. At 13 h post-infection, viral proteins were pulsed-labeled by adding growth medium (Basal media eagle (Gibco BRL, Gaithersburg, MD) Containing 10 μCi/ml of [^{3}H] L-amino acid mixture (1 mCi/ml; ICN Radiopharmaceuticals, Irvine, CA)) either in the presence or in the absence of compounds. After pulse labeling for 30 min, cells were scraped and pellets were suspended in PBS. The amount of protein in samples was measured spectrophotometrically using the Bio-Rad protein assay reagent according to the manufacturer's instructions (Bio-Rad, Hercules, CA). Protein samples were denatured upon adding 2 × SDS-PAGE sample buffer (125 mM Tris–HCl pH (6.8), 4% SDS, 10% glycerol, 0.02% bromophenol blue and 4% (v/v) β-mercaptoethanol). After boiling for 5 min, 50 mg amounts of total cell lysates were electrophoresed on SDS-PAGE by the standard method (Laemmli, 1970). Following separation, proteins were fixed in situ using a solution containing 50% methanol, and 10% acetic acid for 1 h. Subsequently, the gel was soaked in a solution of acetic acid containing 20% (w/w) 2,5-diphenyloxazole (Sigma, St. Louis, MO) for 10 h and then washed for an additional 10 h under running tap water. The gel was dried under vacuum on a paper support and exposed to X-ray film at −70°C.

2.13. Inhibition of DNA topoisomerase II catalytic activity

Inhibition of human topoisomerase II activity (p170 isoform; TopoGEN, Columbus, OH) was determined using the standard P4 DNA unknotting assay procedure (Liu and Miller, 1981) under the specific conditions as described previously (Kashiwada et al., 1993). DMSO concentration in the reactions was maintained at less than 1.5% (v/v), a concentration without effect on enzyme activity. Inhibition was determined semi-quantitatively using limiting (two-fold) serial dilutions of drugs and by visual comparison of products formed in the presence of drugs to enzyme control reactions. The IC_{50} value is the estimated concentration of drug that inhibits the production of unknotted-DNA by 50%.



Scheme 1. Synthesis of compounds **3**–**6**.

3. Results

3.1. Chemistry

The A-ring substituted acridones were synthesized as an exploratory series to examine the electronic and, or steric effects of variable substitution on some of the biological activities reported previously for compounds **1**, **2** and **7** (Bastow et al., 1994). The general procedure used for preparing target acridone analogs (**3**–**6**), outlined in Scheme 1, is based on the Hughes and Ritchie reaction (Hlubucek et al., 1970). The synthesis involved condensation of a substituted 2-aminobenzoic acid with phloroglucinol in the presence of $ZnCl_2$. The structures of the final products were secured by spectroscopic and analytical data.

3.2. DNA topoisomerase II inhibitory activities of 1,3-dihydroxyacridone analogs

Compounds **1**–**7** were evaluated as in vitro inhibitors of human DNA topoisomerase II-catalyzed unknotting of DNA using etoposide and compounds **1** and **2** as the inhibitor controls (Table 1). Two of the target compounds (**4** and **6**) were more active enzyme inhibitors than etoposide. Compounds **5** and **7** were relatively weak inhibitors and the 5-Cl congener (**3**) was inactive when tested at 50-micromolar concentration. Results for compounds **1**, **2** and **7** were consistent with previous estimates (Bastow et al., 1994). Although enzyme inhibition was dependent on both the position and the functionality of the A-ring substituent, the limited structural diversity of test compounds prevents a more substantive comparison of structure and activity at this time.

Table 1
Inhibition of viral replication, of cell growth and of DNA topoisomerase II catalytic activity by compound **1**, related 1,3-dihydroxyacridone derivatives (**2**–**7**), antitumor DNA topoisomerase II inhibitors and PAA

Compound	Inhibition of vero cell growth EC_{50} (μM)	Inhibition of HSV replication EC_{50} (μM)	Antiviral activity selectivity index[a]	Inhibition of DNA topoisomerase II IC_{50} (μM)
7-Cl (**1**)	60 ± 3	16 ± 4	4	30
7-OH (**2**)	40 ± 2[b]	15 ± 2[b]	3	20
5-Cl (**3**)	52 ± 6	4 ± 1	13	NA[c]
6-Cl (**4**)	29 ± 4[b]	10 ± 1[b]	3	20
8-Cl (**5**)	70 ± 4[b]	10 ± 2[b]	7	75
8-CH_3 (**6**)	60 ± 4[b]	44 ± 2[b]	1	10
7-CH_3 (**7**)	60 ± 4[b]	13 ± 1[b]	5	50
Etoposide	2.5 ± 0.5[b]	>20[e]	$\ll 1$	35
Amsacrine	3 ± 1[b]	3 ± 1[b,d]	1	ND
Aclarubicin	0.01[c]	0.25–5[b,d,f]	$\ll 1$	ND
PAA	>500	50 ± 3	>10	ND

[a] Selectivity index is the ratio of EC_{50} for inhibition of cell replication/EC_{50} for inhibition of viral replication as determined under conditions specified under experimental procedures.

[b] Mean ± S.E.M.

[c] NA, not active at 50 μM; ND, not determined.

[d] Antiviral activity was measured using a plaque-elimination assay (Bastow et al., 1983).

[e] Treatment with 20 μM etoposide inhibited viral replication by $45 \pm 17\%$ (S.E.M.).

[f] More than 50% of plaques developed in the presence of 0.25 μM aclarubicin. At or above 0.5 μM, aclarubicin treatment completely blocked formation of plaques but treatment also affected the integrity of the cell sheet.

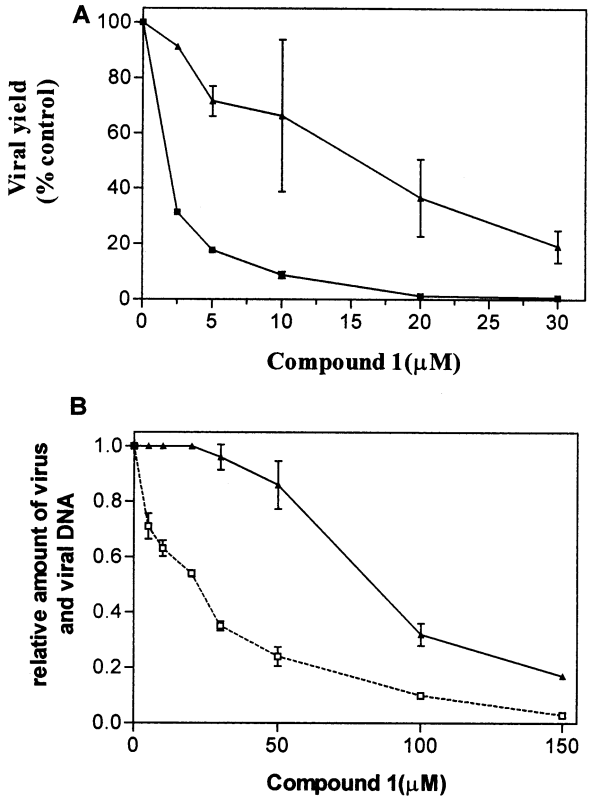


Fig. 2. Serum-dependent antiviral activity of compound **1** and drug effect on HSV DNA production. A: Dose-dependent antiviral activity of compound **1** under different serum conditions. Confluent Vero cell cultures were infected at a multiplicity of 1.5 pfu per cell and treated with compound **1** in media containing calf serum 0.5% (■) and 5%, v/v (▲).Viral production was determined using the standard titration method. Viral yields in pfu per cell are expressed as a percentage of control. Data is the mean and standard deviation from duplicate treatments and two independent experiments. B: Effect of compound **1** on total viral DNA. Cells were infected at a multiplicity of 20 pfu per cell and treated with either compound **1** in high serum media (calf 5%, v/v) for either 8 or 18 h. Isolated DNA was digested with *Hin*dIII, separated using agarose gel electrophoresis and analyzed by Southern blot hybridization. ^{32}P-Labeled genomic HSV DNA digested with *Bam*HI was used as a probe. Antiviral activities were measured at the same time in parallel cultures using the standard titration method. The amounts of viral DNA and viral production are denoted by ▲ and □, respectively. Data are the mean and standard deviation from four independent experiments.

3.3. Antiviral activities of 1,3-dihydroxyacridone analogs

Compounds **1**, **2** and **7** were identified as inhibitors of HSV replication in cell culture but a quantitative assessment of antiviral selectivity was not accurately determined (Bastow et al., 1994). Therefore studies were carried out to measure and compare antiviral activity and selectivity within the series of seven analogs. During the course of the work, the antiviral activity of compound **1** was observed to be about ten-fold higher than reported previously. This apparent discrepancy was traced to the serum concentration of the culture medium. As shown in Fig. 2A, the EC_{50} of compound **1** in medium supplemented with high (5% v/v) and low (0.5% v/v) serum concentration was 16 ± 4 μM and less than 2 μM, respectively. A similar serum effect was observed using compound **3** (the EC_{50}s measured in medium supplemented with high (5% v/v) and low (0.5% v/v) serum was 4 ± 1 μM and less than 1 μM, respectively). Simply using bovine serum albumin to increase protein load of the culture medium (data not shown) could mimic the serum-dependence however. Therefore the serum effect is likely due to drug-protein binding rather than serum responsiveness of a critical pharmacological target, like DNA topoisomerase II for example (Hsiang et al., 1988; Woessner et al., 1991). Based on the initial findings, the activity of test compounds as inhibitors of both cellular and viral replication were studied under the 5% (v/v) serum condition and an index of antiviral selectivity was calculated. PAA a selective herpes viral DNA polymerase inhibitor was used as the positive drug control. Three anticancer drugs that target DNA topoisomerase II (etoposide, amsacrine and aclarubicin) but which inhibit the enzyme by mechanisms distinct from compound **1**, were also included for comparison. Results are shown in Table 1. The anticancer agents were more active as inhibitors of cellular replication than the 1,3-dihydroxyacridone derivatives and none of them were selective anti-HSV agents. The result for etoposide is consistent with an earlier report (Nishiyama et al., 1987). In contrast, the acridone derivatives with the exception of the 8-methyl congener (**6**) blocked productive HSV infection and their ac-

tions were selective (range three- to 13-fold). The 5-C1 analog (**3**) was comparable to PAA as a selective anti-HSV agent although the ten-fold selectivity of the positive drug control was only a minimum estimate. The antiviral activity of compounds **1** and **3** was evaluated further under a stringent assay condition by using rapid-growing cells rather than confluent cell cultures to support infection. The approach allows for a more rigorous test of antiviral selectivity, since the growth of rapid replicating cells is inhibited by the agents (Table 1). Results of two independent experiments showed that compounds **1** and **3** were more active antiviral agents in rapidly dividing host cells then in resting (either serum-deprived or confluent) infected cells by factors of 2.1 ± 0.5-fold and 2.5 ± 0.3-fold, respectively. In contrast, neither the activity of PAA nor the efficiency of productive infection was affected by the growth status of Vero cells (data not shown). Based on these findings, compounds **1** and **3** were about eight- and 26-fold selective as antiviral agents under the stringent assay condition. A comparison amongst 1,3-dihydroxy acridone derivatives as enzyme inhibitors and as inhibitors of either cellular or viral replication showed no obvious trends or relationships (Table 1). However, their activities as enzyme inhibitors (IC_{50}s) and as selective antiviral agents (selectivity indices) were closely correlated (Fig. 3). The significance of this observation will be discussed. Compounds were also evaluated as inhibitors of HCMV replication. Although the rank-order activity of compounds **1**–**7** as inhibitors of HEL and Vero cell replication were similar, 1,3-dihydroxyacridone derivatives were inactive against HCMV replication at a 20-μM test concentration. Five test compounds (**1** and **4**–**7**) were toxic based on visual and microscopic examinations of the stained cultures. In contrast, 50 μM PAA blocked development of macroscopic viral plaques and the agent acted with apparent selectivity (data not shown). Based on the overall results, compounds **1** and **3** were selected as the prototypical and lead anti-HSV agents respectively and exploratory mechanisms of action studies were conducted.

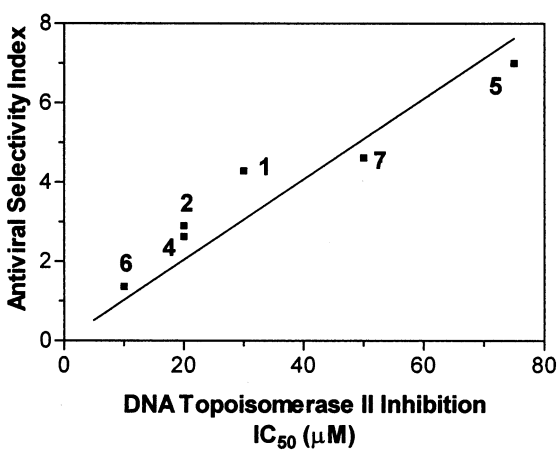


Fig. 3. Antiviral selectivity of acridone-derived DNA topoisomerase II inhibitors. Enzyme inhibition (IC_{50}) as a function of selectivity index (Table 1) was plotted. The computer-fitted line was highly significant ($P = 0.0002$) based on linear regression analysis.

*3.4. Effect of compounds **1** and **3** on HSV DNA production*

Cell-based mechanistic studies of antiviral agents generally use a high multiplicity infection to facilitate the analysis of biochemical effects. Since cells were infected with 1.5 pfu for antiviral measurements, experiments were done to evaluate the multiplicity-dependence of antiviral activity as a prelude to biochemical work. In two independent trials, activity of compounds **1** and **3** were significantly lower by factors of 1.7 ± 0.4 and 2.4 ± 0.5, respectively, for viral loads of 10–20 pfu per cell as compared to the standard condition. Since variability in the efficiency of infection could impact on the analysis of drug action, the effect of drug treatment on virus DNA production was measured as a function of dose and the antiviral activity was measured in parallel. Restriction endonuclease-digested DNA samples prepared from infected cells either at 8 or 18 h were separated by agarose gel electrophoresis and analyzed by Southern blot hybridization. As shown in Fig. 2B, viral DNA was reduced by 70% in cells treated with 100 μM compound **1**, a concentration that inhibited 90% of viral replication. At concentrations of compound **1** that inhibited viral replication by 80% or less (up to 50 μM), drug treatment marginally reduced the amount of viral

Table 2
Comparison of compounds **1**, **3**, PAA and ICRF-193 as inhibitors of HSV DNA production in infected cells[a]

Compound	Inhibition of HSV replication EC_{50}, (μM)	Inhibition of HSV DNA production IC_{50} (μM)
1	22 ± 2	83 ± 7
3	10 ± 3[c]	>30 (20 ± 5)[b,c]
PAA	57 ± 5[d]	50 ± 4[d]
ICRF-193	<2 (90)	>10 (47)[b,e]

[a] Results presented are for comparison and taken from published data (Hammarsten et al., 1996).
[b] The IC_{50} was greater than the highest concentration tested. The bracketed values are the percent inhibition of viral DNA observed. Data from current work are the mean and standard deviation calculated from four independent experiments.
[c] Data are the mean and standard deviation calculated from four independent experiments, respectively.
[d] Data are the mean and standard deviation calculated from three independent experiments, respectively.
[e] Cells were infected at different multiplicity of infection in the presence of 10 μM ICRF-193. The amount of viral DNA at 16 h post-infection was determined by Southern blot and phosphoroimaging analysis.

DNA produced at either early or late times of infection. Results of similar studies using compound **3** and PAA for comparison are summarized in Table 2. As for compound **1**, there was no correlation between the antiviral activity of the 5-Cl analog (**3**) and the intracellular effect on viral DNA production. In contrast, treatment with the viral DNA polymerase inhibitor (PAA) induced a dose-dependent inhibition of viral DNA (data not shown) and this action correlated with inhibition of viral replication (Table 2). Interestingly, substantial HSV DNA replication also occurs in cells treated with effective antiviral concentrations of ICRF-193 (based on our analysis of data from the study of Hammarsten et al., 1996; Table 2). The overall results demonstrate that inhibition of HSV DNA replication is not a primary biochemical action of the two 1,3-dihydroxyacridone derivatives.

*3.5. Effect of compounds **1** and **3** on late viral protein production*

Compounds **1** and **3** induce a post-DNA replication blockade of HSV infection (Fig. 2B, Table 2). In order to define the biochemical action of these agents further, drug effects on both the synthesis and steady-state levels of viral proteins were investigated. The results in Fig. 4 are representative of two independent experiments on late viral protein synthesis. Viral proteins synthesized at 13 h of infection in **1**-treated cultures (lane 3–4), even at 100 μM (lane 4) were indistinguishable from those produced in the control infection

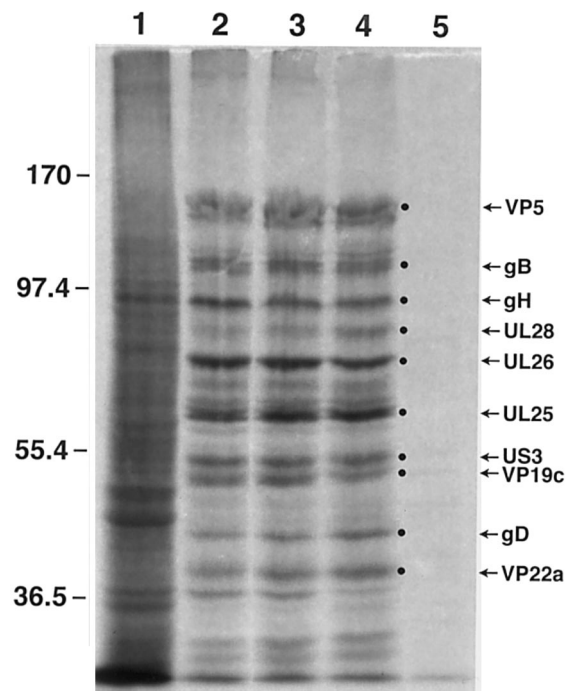


Fig. 4. Effect of compound **1** on the synthesis of viral late proteins. Mock-infected (lane 1) or HSV infected (lanes 2–5) cells were incubated at 37°C in the absence of any compounds (lanes 1 and 2, respectively) and in the presence of 30 μM **1** (lane 3), 100 μM **1** (lane 4) and cyclohexamide 100 μg/ml (lane 5). At 13 h post-infection, cultures were pulse labeled for 30 min with [^{3}H] L-amino acids. Total cell lysates were subjected to 7.5% SDS-PAGE and synthesized proteins detected by fluorography. Numbers on the left indicate the sizes of molecular weight markers in kDa. Viral proteins indicated by ● on the autoradiograrn are tentatively assigned in the right margin based on apparent molecular weights reported in published works (Sherman and Bachenheimer, 1988; Nishiyama, 1996; Homa and Brown, 1997). The viral yield in parallel cultures treated with 30 μM compound **1**, 100 μM compound **1**, and cyclohexamide were 22, 3 and 0.4% of control, respectively.

(lane 2). As expected, no production of viral late proteins was detected in infected cells treated with cyclohexamide (a non-specific inhibitor of viral protein synthesis) at 100 μg/ml (lane 5). These results were in agreement with the lack of apparent effect of either 100 μM compound **1** or 30 μM compound **3** on viral protein accumulation measured using Western blotting and detection with a polyvalent HSV antibody (data not shown). Based on these observations, neither of the compounds have general or specific effects on the production of late viral proteins at effective antiviral concentrations.

4. Discussion

The type II DNA topoisomerases are essential nuclear enzymes that resolve topological problems in cellular DNA as they occur during replication, transcription, chromosome mechanics and recombination (Wang, 1996). One of the enzymes (the p170 isoform) localizes to HSV replication compartments around 4 h post-infection (Ebert et al., 1994). Biochemical analysis also shows that DNA topoisomerase II is preferentially engaged and localized non-randomly on progeny viral DNA during the later stages of infection (Ebert et al., 1990). Although the precise biochemical function(s) of the enzyme in HSV-infected cells is (are) not yet understood, studies using the catalytic inhibitor ICRF-193 suggest that DNA topoisomerase II is required around 4 h post-infection and it is not essential for viral DNA replication (Hammarsten et al., 1996). Aclarubin, a catalytic antagonist that binds to DNA and interferes with enzyme-substrate interaction (Sorensen et al., 1992), was found in the present study to be without effect on HSV replication. Interestingly, the ultimate mechanism of DNA topoisomerase II inhibition by aclarubicin is similar to that of compounds **1** and **2** but the acridone derivatives are more selective since they interact with the enzyme and only weakly with DNA (Vance, 1998; Vance and Bastow, 1999). Whether the added selectivity of 1,3-dihydroxyacridone derivatives as enzyme inhibitors can account for their unique antiviral action is unclear and was not addressed directly in the present work. However, current results do show that DNA topoisomerase II catalytic inhibition by 1,3-dihydroxyacridone derivatives is not required for antiviral activity and apparently it is detrimental for selective antiviral action. These conclusions are based on the following observations. First, the 5-Cl congener (compound **3**) is the most active and selective inhibitor of viral replication but the agent does not inhibit in vitro enzyme activity. Second, enzyme inhibition does not correlate with the inhibition of either cellular or viral replication (Table 1) but it is closely related to selective antiviral activity. As shown in Fig. 3, the correlation is an inverse relationship, which implies the most active enzyme inhibitors of the 1,3-dihydroxyacridone class are the least useful antiviral leads.

Exploratory mechanism of action studies showed that the antiviral properties of the two isomeric compounds, **1** and **3**, were similar. Both were multiplicity-dependent antiviral agents, both had marginal effects on viral DNA and late protein production at effective antiviral concentrations and both were maximally effective when given at 6 h post-infection or earlier (Akanitapichat and Bastow, unpublished observation). These properties are similar to those of ICRF-193 and could indicate that compound **1** does act as a DNA topoisomerase II inhibitor to block HSV replication. The more parsimonious conclusion however is that compound **1** shares an antiviral target in common with six other 1,3-dihydroxyacridone derivatives (excluding compound **6**). Studies are currently ongoing to identify the late blockade of HSV replication that is induced by these agents.

In the present work, 5-chloro,1,3-dihydroxyacridone (compound **3**) was identified as a novel antiviral lead molecule. Antiviral selectivity was about 26-fold under a stringent assay condition and likely would be higher if measured under a low serum condition as is typically used in cell culture screens for HSV agents. Although compound **3** pales by comparison to agents in current clinical use as safe and effective treatments for productive HSV infections (reviewed in Akanitapichat and Bastow, 1997), the compound is a non-nucleoside structure that does not target viral

DNA replication. Therefore, further optimization of 1,3-dihydroxyacridone derivatives as well as identification of their primary antiviral target appears warranted.

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