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Antiproliferative Actions of 7-Substituted 1,3-Dihydroxyacridones; Possible Involvement of DNA Topoisomerase II and Protein Kinase C as Biochemical Targets†

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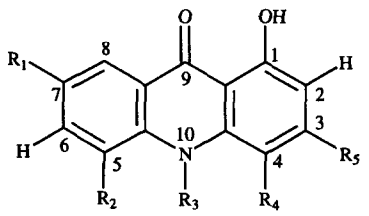
Abstract—7-Chloro-1,3-dihydroxyacridone (**1**) reversibly inhibited growth of KB and vero cell lines with IC_{50}'s of 35 and 40 μM, respectively, and a topoisomerase II-mediated multidrug resistant KB sub-clone was found to be about three-fold more susceptible to **1**. In contrast, two cell lines of lymphoid origin were killed following treatments with 60 μM and at higher concentrations of **1**. KB cell growth inhibition correlated with a rapid, reversible suppression of thymidine incorporation. Uridine but not leucine incorporation was also rapidly suppressed. The *in vitro* activities of DNA topoisomerase II and novel protein kinase C-subtype delta were inhibited at effective concentrations in tissue-culture, but **1** did not stimulate intracellular protein-associated DNA breaks nor interfere initially with topoisomerase II-mediated DNA cleavage in KB cells. In addition to antiproliferative effects against cells, the compound was weakly virustatic for herpes simplex virus type I with an IC_{50} of 8 μM. Limited studies comparing three **1**-congeners and citpressine-I, an acridone alkaloid with reported antiherpes activity, demonstrated that 7-substituted 1,3-dihydroxyacridones are novel antiproliferative agents which share similar biological and biochemical properties.

Introduction

Naturally occurring acridone alkaloids and related synthetic derivatives are known to possess a wide range of biological activities including antitumor, antiparasitic, and antiviral properties.[2] In most instances, the biochemical actions of acridones are not completely understood, but neither DNA topoisomerases nor protein kinase C (PKC) have been implicated as targets in the studies to-date. DNA topoisomerases and PKC iso-enzymes are essential for normal cellular functions involving DNA topological transformations and signal transduction/regulatory mechanisms, respectively, and both types of enzymes are considered to be viable targets for drug development.[3,4] Using a cell-based tissue-culture screen for novel DNA topoisomerase-interactive agents, a series of 7-substituted 1,3-dihydroxyacridones were identified as being candidate inhibitors. One of the compounds chosen for more detailed biological and biochemical evaluations, 7-chloro-1,3-dihydroxyacridone (**1**), was also compared in limited studies to three congeners (**2–4**) and to citpressine-I (**5**), an acridone alkaloid from the plant genus *Citrus* which has reported antiherpes activity[2c] (the structures of all compounds are given in Fig. 1). The present findings demonstrate that 7-substituted 1,3-dihydroxyacridones exhibit interesting growth inhibitory properties, possibly as a result of novel interactions with DNA topoisomerase II and/or inhibition of PKC iso-enzymes.



Compound	R_1	R_2	R_3	R_4	R_5
1	Cl	H	H	H	OH
2	Br	H	H	H	OH
3	CH_3	H	H	H	OH
4	OH	H	H	H	OH
5	H	OH	CH_3	OCH_3	CH_3

Figure 1. Compound structures.

Chemistry

The general procedure used for preparing target compounds **1–4**, as outlined in Scheme I, involved condensation of a 5-substituted 2-amino benzoic acid with phloroglucinol in the presence of $ZnCl_2$.[5] The structures of the final products were secured by spectroscopic and analytical data.

Biological Results

Inhibitory activities of **1** towards KB cell growth and macromolecular synthesis were evaluated using cell

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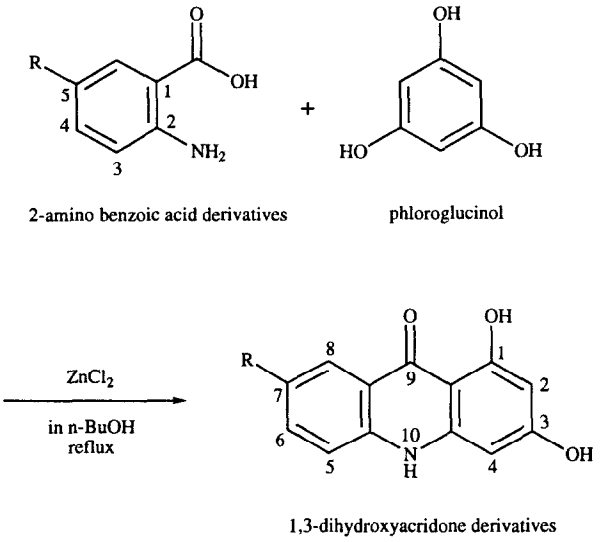

Scheme I. Synthesis of Compounds **1–4.**

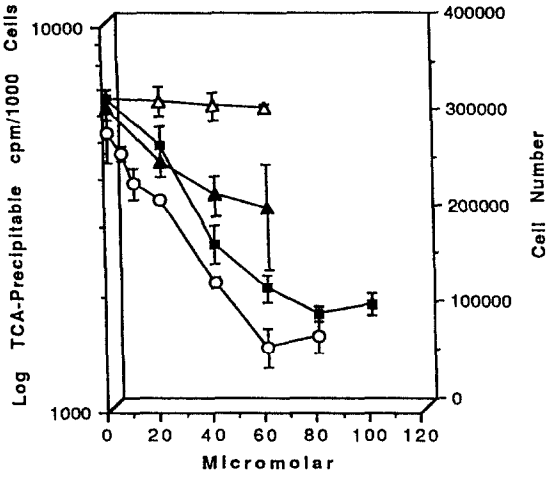

Figure 2. Reversible effects of **1** on KB cell growth and on intracellular thymidine incorporation. Inhibition of cell growth and suppression of thymidine incorporation were assayed as described in the experimental section. All values are mean ± S.D. from tripicate treatments. ■ = Viable cell number in cultures exposed for 2 days to the concentrations of **1** indicated. Cell numbers for one day treatment with 80 μM followed by 2 additional days in the absence of **1** were 3·14±0·02 × 10^5. Pulse-labeling measurements of thymidine incorporation were for 15 min following 1 h exposure to the concentrations indicated (○), and then following reversal for 1 h (▲) and for 2 h (Δ).

growth and radiolabelled precursor incorporation assays respectively. Representative results are given in Figure 2 and Table 1. Growth inhibition by **1** was concentration-dependent with an IC_{50} value of 35 μM. Continuous exposure to 60–100 μM for two days resulted in the complete suppression of cell growth but treatments were not overtly cytotoxic based on cellular exclusion of trypan blue as well as the resumption of normal growth seen when treatment was terminated (Fig. 2 and legend). The inhibition of intracellular thymidine incorporation was rapid, dose-dependent, closely paralleled growth inhibition with an IC_{50} value of 30 μM, and the effect was completely reversed within two hours of terminating treatment (Fig. 2). Residual thymidine incorporation of 30 % control levels observed over the concentration range 60–80 μM was also maintained for treatments of longer duration (60 μM up to two days), and kinetics of recovery after reversal were similar to that seen following the 1 h exposure.[6] Short-term treatment also suppressed intracellular incorporation of uridine but not that of leucine (Table 1). These data show that **1** inhibits KB cell growth reversibly, and a principal biochemical determinant involved was an incomplete, sustainable, apparent block in nucleic acid biosynthesis.

Testing of additional cell lines in two-day growth inhibition assays revealed that different cell types were not uniformly susceptible to **1**, nor was the compounds' antiproliferative effect limited to reversible growth inhibition. Representative results for KB-7d and Molt-3 cells are shown in Figure 3a and b, respectively. The former cell line is an atypical multidrug resistant (at-MDR) sub-clone of KB selected by incremental exposure to the antitumor topoisomerase II poison, VP-16 (etoposide);[3b] these cells are drug-resistant in part because target-enzyme levels are about 60 % lower than normal.[7] The inhibition of KB-7d cell growth by **1** was concentration-dependent (IC_{50} value of 10 μM) and growth was completely suppressed at 20 μM (Fig. 3a). In contrast, Molt 3 cells continuously exposed to **1** in the concentration range of 60–80 μM were killed by the treatment based on altered cell morphology and the inability of treated cells to exclude trypan blue (Fig. 3b). Another human tumor cell line of lymphoid origin, HL-60, was also killed by **1** under similar treatment conditions.[8]

DNA topoisomerases were initially examined *in vitro* as potential biochemical targets for **1**. These studies were

Table 1. Short-term effects of **1** on intracellular uridine (Urd) and leucine (Leu) incorporation

Dose	Urd Incorporation		Leu Incorporation	
(μM)	cpm[a]	% control	cpm[a]	% control
0	8733 ± 128	–	1557 ± 172	–
60	2482 ± 163	28	1300 ± 73	84[b]

[a]Pulse-labelling measurements were for 15 min following 1-h of treatment as indicated. The methodology is described in the experimental section. Values tabulated are the acid-insoluble radioactivity recovered from cultures from 10,000 cells and represent mean ± S.D. from triplicate treatments.
[b]Not significantly different from mock-treatment ($P > 0.1$, Student's *t*-test).

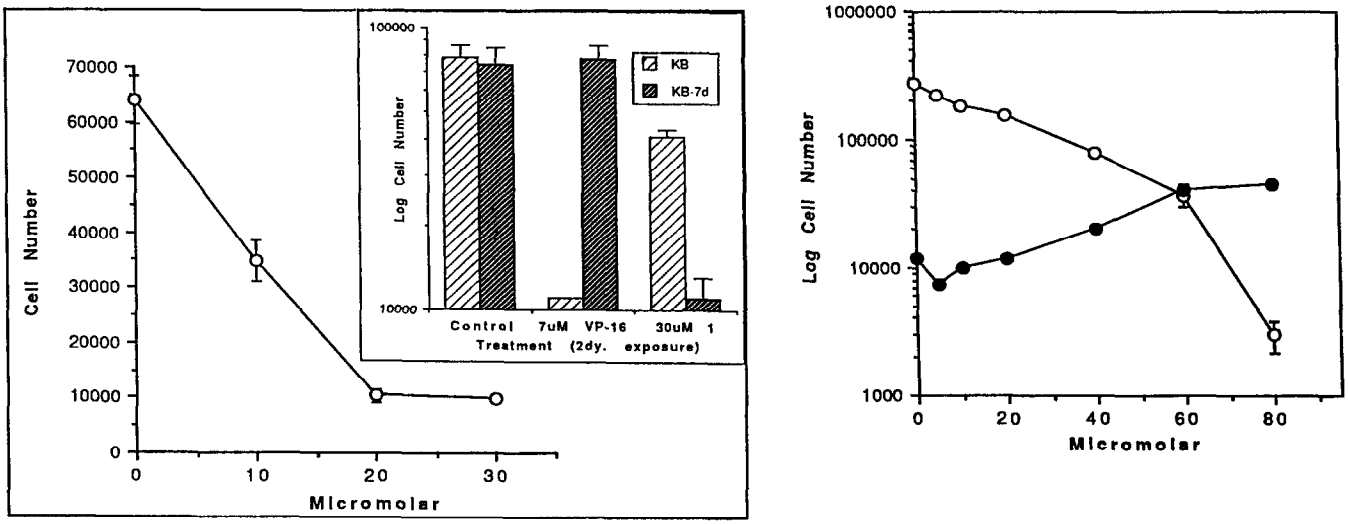

Figure 3. Growth inhibitory effects of **1** on KB-7d and Molt 3 cells. Inhibition of cell growth and assessment of cell viability was determined as outlined in the experimental section. All values are mean ± S.D. from triplicate treatments. (a) Viable cell number in KB-7d cultures exposed for 2 days to the concentrations of **1** indicated. Histogram inset is data from another experiment comparing susceptibilities of KB and KB-7d cells side-by-side to VP-16 (1 μM) and **1** (30 μM). (b) Viable (○) and non-viable (●) cells recovered from Molt-3 cultures treated for two days with various concentrations of **1**.

conducted based on the relative susceptibilities of the two KB cell lines and the observed apparent suppression of nucleic acid synthesis; the latter biochemical effect being shared, in general terms, by topoisomerase inhibitors with diverse chemical structures and different mechanisms of action.[3a,9] DNA topoisomerase II was evaluated first using the DNA unknotting assay and a representative result is shown in Figure 4. Compound **1** at 30 μM inhibited catalytic activity between 30 and 60 % (based on the amount of product formed compared to enzyme controls), a level of inhibition which was similar to a positive control reaction containing VP-16. Neither test compound inhibited catalytic activity significantly at two-fold or lower concentrations (Fig. 4). In contrast, DNA topoisomerase I-dependent DNA relaxation activity was unaffected by concentrations of **1** up to 100 μM, suggesting that the inhibition observed was specific for the type II enzyme.[10]

The majority of DNA topoisomerase inhibitors fall into two general mechanistic classes.[3b] Poisons, like VP-16, stabilize a DNA-enzyme covalent reaction intermediate which can be measured experimentally as protein-associated DNA breaks. Topoisomerase 'antagonists' interfere with the signature reaction of poisons and are less well understood at the mechanistic level.[11] Intracellular topoisomerase inhibition by **1** was evaluated in KB cells using protein-linked DNA cleav-

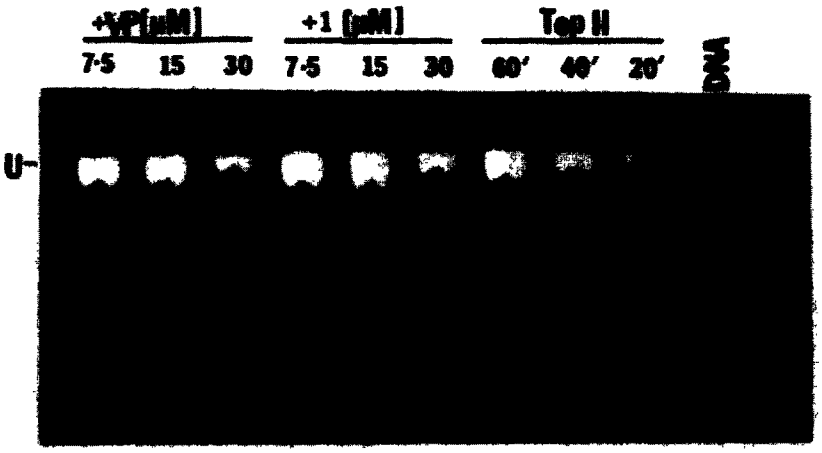

Figure 4. Inhibition of topoisomerase II-dependent DNA unknotting. Inhibition of topoisomerase catalytic activity was assayed as described in the experimental section. A photograph of an ethidium-bromide-stained gel is shown. Enzyme control reactions were terminated for analysis at the times indicated above the tracks. Test reactions treated with either VP-16 (positive control) or **1** at the concentrations indicated were stopped after 1 h incubation and then analyzed. 'U' in the left margin indicates mobility of the unknotted DNA reaction product. The 'DNA' track contains the knotted DNA substrate which migrates as a smear and is barely visible on the photograph.

Table 2. Intracellular effects of **1** on protein-associated DNA cleavage and drug-induced topoisomerase II-mediated DNA cleavage

Treatment[a]	DNA Breaks cpm[b]
MOCK	245 ± 66
1	230 ± 68
1 + VP-16	7663 ± 270
VP-16	8003 ± 891

[a]KB cell cultures pre-labelled with thymidine were either mock-treated or exposed to **1** at 40 μM for 2 h, co-treated with 40 μM VP-16 for the final hour of incubation or were exposed to VP-16 alone for 1 h. Protein-associated cellular DNA breaks were then assayed as potassium/SDS-precipitable radioactivity by the method described under experimental section.
[b]Radioactivity recovered from cultures of 20,000 cells are mean ± S.D. values from triplicate treatments.

age/interference assays and representative results are given in Table 2 and Fig. 5. Topoisomerase II-dependent DNA cleavage was stimulated about 33-fold over background by the VP-16 control treatment but was unaffected following a 2-h exposure to **1**. Since sequential pre- and co-treatment with **1** also did not impact on the formation of VP-16 induced breaks, the data in Table 2 suggest that **1** acts as neither an intracellular topoisomerase poison nor an antagonist of DNA topoisomerase II.[12] However, longer exposures to **1** were found to partially antagonize intracellular VP-16-induced DNA breaks (Fig. 5). Although the mechanism for this delayed interference remains to be resolved, it is possible that **1**-treatment could have downregulated DNA topoisomerase II and/or altered VP-16-enzyme interactions. This possibility prompted the *in vitro* testing of PKC iso-enzymes as potential biochemical targets for **1**, since PKCs and other protein kinases are known to modulate biochemical and pharmacological properties of topoisomerases.[13] Results for PKC activities representative of the three known iso-enzyme classes are shown in Figure 6. The average IC_{50} value was 40 μM for the novel PKC delta sub-type and this value was two-fold less than the extrapolated value for the conventional PKC-alpha-isoform (though in the latter case 'inhibition' was variable and not clearly concentration-dependent).[14] The atypical PKC subtype-zeta also was not inhibited in a concentration-dependent fashion (Fig. 6). Based on results from *in vitro* enzyme inhibition studies, topoisomerase II and/or n-PKC delta-subtype are candidate biochemical targets for **1**.

Acridone alkaloids from plants have been investigated as antiviral agents and one compound, citpressine-I (**5**), was reported to interfere with deoxynucleotide formation in herpes simplex virus (HSV)-infected cells.[2c] In the present work, **1** was also found to suppress nucleotide precursor incorporation, therefore limited studies were carried out to compare bioactivities between **1**, the congeners on hand, and **5**. The susceptibility of HSV type I was examined using plaque-elimination and virus-yield reduction assays in vero cells. In preliminary studies with **1**, host-cell growth inhibition was inhibited in a reversible fashion with an IC_{50} of 40 μM (Fig. 7 and

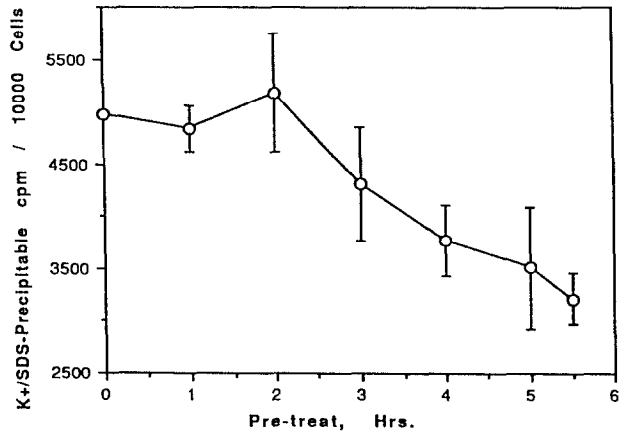

Figure 5. Time-dependent interference by **1** with drug-stimulated intracellular topoisomerase II-mediated DNA cleavage. KB cell cultures pre-labelled with thymidine were exposed to **1** at 50 μM for the times indicated, then were co-treated with 40 μM VP-16 for the final 30 min of incubation. Protein-associated cellular DNA breaks were assayed as potassium/SDS-precipitable radioactivity by the method described in the experimental section. Data are the mean ± S.D. values obtained from triplicate treatments. The control value from mock-treated cultures was 244±44 cpm.

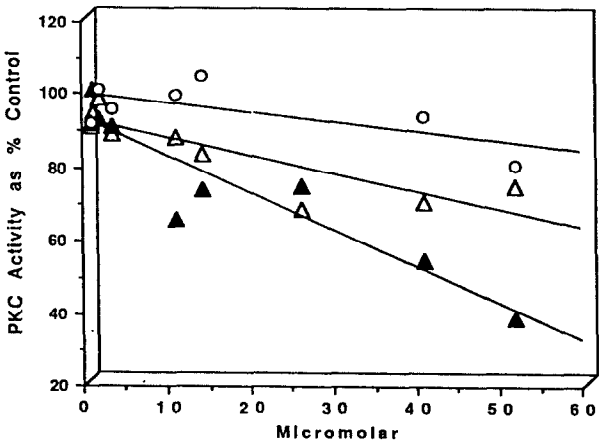

Figure 6. Effects of **1** on recombinant human PKC iso-enzymes *in vitro*. The measurement of PKC activity in a prevesicle enzymes assay is described in the experimental section. Inhibition was evaluated at four different concentrations (up to 166 μM) in each of two or three independent experiments.[14] Data for PKCs representing the three general iso-form classes are presented as scatter plots with dose-responses being computer fitted: Δ, c-PKC-alpha (three experiments); ▲, n-PLC-delta (three experiments); ○, a-PKC-zeta (two experiments).

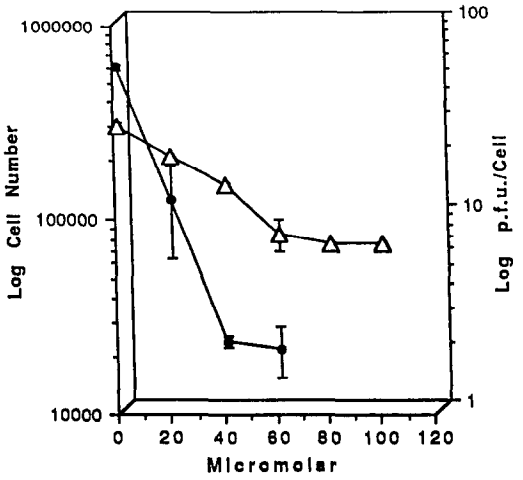

Figure 7. Selective inhibition of herpes simplex virus growth by **1**. The inhibition of host (vero) cell growth and of virus yield were assayed as described in the experimental section. All values are mean ± S.D. from triplicate treatments. Δ = viable cells in vero cultures exposed for 2 days to the concentrations indicated. Cell numbers for a 2-day treatment with 80 μM followed by two additional days in the absence of **1** were $3.1 \pm 0.3 \times 10^5$. Virus growth is denoted by ● and is given as p.f.u. per cell from infected-vero cultures exposed for 1 day to the concentrations shown.

legend). Treatment of infected monolayers over the concentration range 15–30 μM reduced virus plaque size and number (data not shown). From virus-yield measurements made following a single growth cycle, **1** was subsequently found to suppress virus production with an IC_{50} value of 8 μM (Fig. 7). The selective but weak antiviral effect of **1** was dependent upon continual exposure suggesting that the compound acted in a virustatic mode. Preliminary comparative studies using **5** demonstrated non-selective antiviral activity and inhibition of DNA topoisomerase II *in vitro* was not observed[15] (Table 3). Compounds **2–4** shared similar bioactivities with **1** based on limited studies summarized in Table 3.

Discussion

Present findings show that **1** has a major impact on KB cell metabolism, most likely at the level of nucleic acid synthesis but without permanent effects on cell-cycle traverse or other events critical for KB cell survival. The proliferation of cells with a topoisomerase II-mediated mechanism of multidrug resistance was more susceptible to the acridone derivative, which was also found to inhibit the *in vitro* activity of DNA topoisomerase II at growth inhibitory concentrations. Additional evaluation of topoisomerase II-involvement by measuring intracellular protein-associated DNA breaks and interference with VP-16, revealed no enzyme poisoning or antagonistic effects at times when nucleic acid precursor incorporation was markedly suppressed.[12] Overall, these results support the premise that **1** could be a novel topoisomerase inhibitor acting with an unusual mechanism.

The only known DNA topoisomerase II inhibitor with biochemical properties bearing some resemblance to the behavior of **1** is the antibiotic novobiocin, but the action of this drug in mammalian cells is complicated because direct effects on chromatin structure and on α-DNA polymerase have also been reported.[16] DNA topoisomerase II has a unique novobiocin-interaction domain and enzyme inhibition occurs via a unique mechanism.[17] The analogy between novobiocin and **1** is intriguing and is being explored in mechanistic studies currently ongoing.

Biological properties of **1** including possible histeospecific cytotoxicity, anti-HSV action and hyperactivity toward at-MDR cells are unique amongst known topoisomerase II-interactive agents.[18] However, the biochemical mechanism of action for **1** may also be complex. Some, if not all, of the effects attributed to direct interactions between topoisomerase II and **1** may be indirect because the regulatory enzyme n-PKC subtype-delta was also inhibited *in vitro* at concentrations which were effective in tissue-culture. DNA topoisomerases are known to be substrates for protein-kinase action and although the biological function(s) of the n-PKC-isoenzymes are not fully understood, the delta-subtype appears to play a role in nuclear events controlling cell proliferation.[13,19] This information raises the question of whether a regulatory influence of **1** on topoisomerase functions could account for the antiproliferative effects

Table 3. Comparative properties of compounds **2–5**

Compound	Topo II Inhibition[a]	Cleavage/ Interference[b]	PKC Inhibition[c]	Growth Inhibition (μM)	
				Cell (IC_{50})[d]	HSV (IC_{100})[e]
2	+	–	ND	30	20
3	+	–	+	45	30
4	+	–	ND	40	>10
5	–	ND	ND	16	33

[a]Tested at 60 μM against topoisomerase II using the unknotting assay. + = complete inhibition.
[b]Tested in KB cells at 50 μM for a 1 h exposure. For interference measurements, co-treatment with VP-16 (40 μM) was for an additional hour. Protein-associated radioactive cpm recovered from 10,000 cells were 274±150(8) and 4288±835(8) for mock-treatment and VP-16 control, respectively.
[c]IC_{50} values of 48, >166 and >166 μM were determined for PKC subtypes -delta, -alpha, and -zeta, respectively.
[d]Growth inhibitory action against vero cells was determined by direct cell counts following a 2 day exposure. IC_{50} values are the concentrations which inhibit cell growth by 50 % compared to mock-treated controls.
[e]Evaluated using plaque-elimination assays. IC_{100} is the concentration which completely inhibited virus plaques without having cytotoxic effects on preformed cell monolayers. Compound **4** had no apparent impact on plaque size or number at 10 μM and was not examined at higher concentrations.
ND = Not determined, — = no activity

observed. The present studies do not address the possibility of intracellular PKC-antagonism but two of the current findings may be relevant to this proposition. A delayed suppression of VP-16-stimulated DNA cleavage by **1** may indicate topoisomerase II levels were down-regulated and/or VP-16 enzyme interactions were altered in response to treatment. In addition, phosphorylation of topoisomerase I may also have been impacted by treatment because the observed residual level of thymidine incorporation is remarkably similar to an effect seen with the experimental drug, camptothecin; the response to this topoisomerase I poison is quite reproducible but remains to be explained.[20] A resolution of these possibilities awaits direct measurement of topoisomerase phosphorylation and enzyme levels in treated cells.

The hyperactivity of **1** toward KB-7d cells is intriguing and warrants additional investigation. In particular, growth inhibitory effects of co-treatments with **1** and verapamil or with modulators of PKC will help establish contributions of decreased topoisomerase II, atypical **1**-uptake or PKC involvement. The latter two mechanisms are known to modulate the sensitivity of KB-7d cells to VP-16 based on the studies of Ferguson *et al.*[7]

The information obtained from this study concerning the structural requirements of **1** and its derivatives is limited to the observation that the 7-substituents did not impact substantially on the bioactivities examined. More detailed SAR studies are currently in progress.

Experimental

Chemistry

Melting points were determined on a Yanagimoto micro-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.

General procedure for synthesis of 1,3-dihydroxyacridone analogues 1–4

A solution of phloroglucinol dihydrate (15 mmol) in *n*-butanol (100 mL) was heated under reflux for 4 h using a Dean–Stark trap to remove water. 5-Substituted-2-aminobenzoic acid (15 mmol) and freshly prepared zinc chloride (15 mmol) were added and the mixture was further refluxed for 7 h. After cooling, the reaction mixture was filtered and concentrated to give a crude product, which was purified by silica gel column chromatograpy $CHCl_3$:EtOAc, (6:4) yielding 1,3-dihydroxyacridone derivatives (**1–4**).

7-Chloro-1,3-dihydroxyacridone (1)

Yield 70 %; yellow crystals (from EtOAc–Me_2CO); mp > 300 °C; IR (KBr) 3450, 3300, 3200 (br, NH and OH), 1660 (C=O), 1600, 1540, 1470 (aromatic) cm^{-1}; MS *m/z* (rel. int %) 263 (33.4) $[M+2]^+$, 261 (100) $[M]^+$; HR-MS calcd for $C_{13}H_8NO_3Cl$: 261.0193, found m/z 261.0194; ^{1}H NMR (DMSO-d_6) :δ 6.04 (1H, d, J = 2 Hz, H-2), 6.31 (1H, d, J = 2 Hz, H-4), 7.50 (1H, d, J = 9 Hz, H-5), 7.74 (1H, dd, J = 2.5, 9 Hz, H-6), 8.07 (1H, d, J = 2.5 Hz, H-8), 13.94 (1H, s, 1-OH).

7-Bromo-1,3-dihydroxyacridone (2)

Yield 73 %; yellow crystals (from EtOAc–Me_2CO); mp 286–288 °C; IR (KBr) 3450, 3280, 3200 (br, NH and OH), 1660 (C=O), 1600, 1530, 1465 (aromatic) cm^{-1}; MS m/z (rel. int %) 307 (93.8) $[M+2]^+$, 305 (100) $[M]^+$; ^{1}H NMR (DMSO-d_6) :δ 6.03 (1H, d, J = 2 Hz, H-2), 6.30 (1H, d, J = 2 Hz, H-4), 7.43 (1H, d, J = 9 Hz, H-5), 7.84 (1H, dd, J = 2, 9 Hz, H-6), 8.21 (1H, d, J = 2 Hz, H-8), 13.93 (1H, s, 1-OH). Anal calcd for $C_{13}H_8NO_3Br$): C 51.01, H 2.63, N 4.58; Found: C 50.97, H 2.86, N 4.45.

7-Methyl-1,3-dihydroxyacridone (3)

Yield 79 %; yellow crystals (from EtOAc–Me_2CO); mp 272–274 °C; IR (KBr) 3430, 3280, 3200 (br, NH and OH), 1650 (C=O), 1600, 1530, 1470 (aromatic) cm^{-1}; MS m/z (rel. int %) 241 (70.7) $[M]^+$, 126 (100); HR-MS calcd for $C_{14}H_{11}NO_3$: 241.0739, found m/z 241.0769; ^{1}H NMR (DMSO-d_6) :δ 2.41 (3H, s, 7-CH_3), 5.97 (1H, d, J = 2 Hz, H-2), 6.26 (1H, d, J = 2 Hz, H-4), 7.38 (1H, d, J = 9 Hz, H-5), 7.55 (1H, dd, J = 2, 9 Hz, H-6), 7.93 (1H, d, J = 2 Hz, H-8), 14.31 (1H, s, 1-OH).

1,3,7-Trihydroxyacridone (4)

Yield 78 %; yellow crystals (from EtOAc–Me_2CO); mp > 300 °C; IR (KBr) 3450, 3280, 3200 (br, NH and OH), 1650 (C=O), 1605, 1540, 1480 (aromatic) cm^{-1}; MS m/z (rel. int %) 243 (100) $[M]^+$; HR-MS calcd for $C_{13}H_9NO_4$: 243.0532, found m/z 243.0537; ^{1}H NMR (DMSO-d_6) :δ 5.94 (1H, d, J = 2 Hz, H-2), 6.23 (1H, d, J = 2 Hz, H-4), 7.25 (1H, dd, J = 3, 9 Hz, H-6), 7.37 (1H, d, J = 9 Hz, H-5), 7.46 (1H, d, J = 3 Hz, H-8), 14.38 (1H, s, 1-OH).

Biology: enzyme and reagents

Topoisomerase II from HOS cells and the DNA substrate were prepared using standard procedures described previously.[21] Studies with topoisomerase I used the calf thymus enzyme purchased from Gibco-BRL Inc. (Bethesda, MD). Recombinant human PKC isoenzymes were produced using the baculovirus expression system in SF9 cells and were partially purified following established methods.[22] VP-16 was from the Natural Products Laboratory, School of Pharmacy, University of North Carolina at Chapel Hill and citpressine-I (**5**) was prepared and provided for testing by one of us (H.F.). For biological evaluations, all compounds were prepared in DMSO as 20 mM stocks, stored at –20 °C and diluted just before use with either DMSO or buffers as described under specific assay methods. Mock treatments involved a change or supplementation with medium containing DMSO at the highest concentration used in test treatments. Tritium-

labelled radiochemicals for pulse-labelling studies were purchased from ICN Biochemicals Inc. (Irvine, CA) and had specific activities of 37, 60–90 and 60–120 Ci/mmol for uridine, thymidine, and leucine, respectively. [^{32}P]ATP for PKC assays was from Dupont NEN (Boston, MA). Tissue culture reagents were obtained from Sigma Chemical Co. (St. Louis, MO) and Gibco-BRL Inc. All other chemicals were reagent grade.

Cells and virus

The nasopharyngeal carcinoma human KB cell line was provided by M. Fisher (Pharmacology, UNC-CH) and vero (African green monkey kidney cells) and human T-lymphoblast Molt-3 were purchased from the Lineberger Cancer Research Center (UNC-CH). Characteristics of the VP-16 resistant KB sub-clone, KB-7d, obtained from Dr Y.-C. Cheng (Yale University), have been described in detail in an earlier publication.[7] Cultures were kept in a humidified 5 % carbon dioxide atmosphere and were maintained at 37 °C in RPMI-1640 medium supplemented with 5 % or 10 % (v/v) bovine serum (foetal for human lines). Over the course of the experiments, cell lines doubled every 24±4 h. HSV type I (KOS strain) was propagated as previously described.[23]

Cell growth inhibition and reversal assay

Antiproliferative effects were evaluated by direct cell counts following a 2 day treatment. One milliliter of cell suspension (50,000 or 5000 cells) was seeded per 3.8 cm^2 well and incubated overnight. Compounds from DMSO-diluted stocks were added to media at twice the desired final concentration and 1 mL was then added to cultures in triplicate. Cultures were observed microscopically for toxic and/or growth-inhibitory effects at day one and immediately before harvesting. With the exception of Molt 3, cells were trypsinized, and all cells were resuspended in phosphate-buffered saline containing 0.5 % (w/v) Trypan Blue before being enumerated manually using a haemocytometer. Blue-staining cells were scored as being non-viable. For reversal studies, treated cultures were re-fed with fresh replacement medium and incubated for two additional days.

Virus growth inhibition assays

The virus plaque elimination assay used was a standard method except that a carboxymethylcellulose (CMC) overlay was omitted because compounds **1–4** precipitated at 40 μM or greater in CMC-supplemented growth media.[24] For virus-yield reduction, 100,000 vero cells were infected at a multiplicity of 3 plaque-forming units (p.f.u.) per cell and treated in triplicate with **1** from 1 to 24 h post-infection. Microscopical examination showed that 20 μM **1** markedly suppressed the virus cytopathic effect. Virus yield was then titrated using the standard assay method.[23]

Pulse-labelling procedure for macromolecular synthesis

Suppression of precursor incorporation was measured in KB cells cultured in media containing 5 % dialized foetal bovine serum. Media was supplemented with tritiated thymidine, uridine or leucine to 25 μCi/mL for the final 15 min of treatment. Precipitable radioactivity in a portion of the sample applied to GFA-filters (Whatman Inc., Hillsboro, OR) was determined by scintillation spectrometry (counting efficiency 50±2 %) following ice-cold trichloroacetic acid treatment (5 % v/v). For reversal studies, treated cultures were re-fed with fresh media and processed at the indicated times by the same procedure.

Cleavage-interference assay method

Stimulation of intracellular protein-associated DNA breaks and interference with VP-16 induced topoisomerase II-mediated breaks were evaluated using a published method.[25] Briefly, KB cells were labelled overnight with tritiated thymidine (0.5 μCi/mL), chased for 2 h and then treated with test compounds. Protein-associated DNA breaks in samples were measured as potassium/SDS-precipitable radioactivity. To measure interference, VP-16 was added following a pre-treatment period with test compounds alone, then samples were processed at the indicated times by the same procedure.

Topoisomerase assays

Inhibition of DNA strand-passing activity was determined using the standard unknotting and relaxation assay procedures developed for DNA topoisomerases.[26] The specific assay conditions were described previously.[25] With the exception of compound **4**, visible yellow-orange precipitates formed over time in aqueous solution at concentrations greater than 200 μM; therefore, to avoid artifacts arising from solubility problems, careful dilution before addition was required and solubility was monitored by visual inspection throughout incubations. DMSO in the final reactions was maintained at less than 1.5 % (v/v).

Protein kinase C assay

PKC was assayed by quantitating the incorporation of ^{32}P from [γ-^{32}P]ATP into histone type IIIS. The reaction mixture (250 μL) contained 30 μg of phosphatidyl-serine, 20 mM Hepes buffer, 10 mM $MgCl_2$, 47.5 μM EGTA, 100 μM $CaCl_2$, 200 μg/mL histone, 10 μL of DMSO or compound in DMSO, 30 μM [^{32}P] ATP, and the enzyme. The assay was performed for 10 min at 30 °C and terminated with 500 μL of 25 % trichloroacetic acid and 100 μL of bovine serum albumin (1 mg/mL). The reactions were filtered onto glass fiber filters and quantified by counting in a β scintillation counter. Assay controls included a maximal lipid-activated PKC assay and a no-lipid PKC assay. The no-lipid activity was subtracted from the maximal lipid-dependent activity to account for background nonspecific kinase activities. The PKC inhibitor sphingosine, which inhibits all the PKC iso-enzymes, was included as an inhibitor control for all PKC assays.

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4. Cellular responses to various physiological agonists including hormones, growth factors and neurotransmitters occur through elaborate signaling pathways involving vital regulatory enzymes and the PKC family of 10 known enzyme isoforms are widely accepted as being key participants. These enzymes are well characterized in biochemical and biophysical terms but specific biological roles of individual PKC subtypes are largely undefined. Various compounds as well as design strategies for PKC-directed drugs are currently undergoing active investigation. This interdisciplinary field of research is reviewed in the following recent publications. (a) Kittawa, V.; Kishimoto, A.; Nishizuka, Y. *Annu. Rev. Biochem.* **1989**, *58*, 31; (b) Stakel, S.; Parker, P. J. *Pharm. Ther.* **1991**, *51*, 71; (c) Tamaoki, T.; Nakano, H. *Biotechnology* **1990**, *8*, 732; (d) Nishizuka, Y. *Science* **1992**, *258*, 607; (e) Nixon, J. S.; Bishop, J.; Bradshaw, D.; Davis, P. D.; Hill, C. H.; Elliot, L. H.; Kumar, H.; Lawton, G.; Lewis, E. J.; Mulgreen, M.; Westmacott, D.; Wadsworth, J.; Wilkinson, S.E. *Biochem. Soc. Trans.* **1992**, *20*, 419.

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6. A one day treatment with 60 μM **1** reduced thymidine incorporation to 5993±450 cpm from control levels of 18,340 ±1351 cpm. Following 2 h of reversal, thymidine incorporation (17,530±1581 cpm) was not significantly different from control. Values given are from triplicate treatments and are normalized to equivalent cell number.

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8. An IC_{50} value of 25 μM **1** against HL-60 cell growth was determined. The toxicity observed after one day of treatment with 40–80 μM included cell swelling and loss of membrane integrity, morphological characteristics of necrotic cell death.

9. Topoisomerase II inhibitors induce variable effects on macromolecular synthesis. Some, like VP-16, cause a selective, irreversible and incomplete suppression of thymidine incorporation (Kalwinsky, D.K.; Look, A.T.; Ducore, J.; Fridland, A. *Cancer Res.* **1983**, *43*, 1592), whereas others promote a delayed, progressive general inhibition (Fry, D.W.; Boritzki, T.J.; Jackson, R.C. *Cancer. Chemother. Pharmacol.* **1984**, *13*, 171.

10. In these experiments, compounds **1–4** at 200 μM sometimes gave partial 'inhibition' of DNA topoisomerase I. Variable effects possibly were caused by precipitated compounds, since they are poorly soluble in aqueous buffers at higher concentrations. Also see Ref. 14.

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12. Antagonism between topoisomerase II interactive agents in the assay could be mechanism-based or owing to competition for enzyme-binding; the latter would require that the enzyme-binding domains for test compounds are common or overlapping. Novobiocin is the only known inhibitor which has a unique enzyme-interaction domain (see Ref. 17 and Discussion).

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15. The present results differ from an earlier study with citpressine-I which reported a 10-fold selective antiviral effect (Ref. 2c). Since the same host-cell system was used here to measure both virus and cell growth inhibitory activities, this difference between studies could account for the contrasting data.

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