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On the complex nature of the antiviral activity of coumermycin A_1: its interference with the replication of herpes simplex virus type 1

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Summary

The mechanism of inhibition of the replication of herpes simplex virus type 1 (HSV-1) by coumermycin A_1 (CA_1), an inhibitor of bacterial DNA gyrase, has been investigated. Concentrations of antibiotic slightly higher than those needed for 50% inhibition of viral growth were able to inhibit viral DNA synthesis in infected cells. This effect was accompanied by a depressed synthesis of viral polypeptides. Protein synthesis was also inhibited in uninfected cells, especially after long exposure to the drug, but not in a cell-free system.

In vitro assays of highly purified HSV-1 DNA polymerase in the presence of the drug, provided evidence that the enzyme was a target of CA_1. The viral polymerase was in fact inhibited by the antibiotic to an extent comparable to that of viral DNA synthesis in intact cells. In contrast, DNA polymerase α, the enzyme involved in chromosomal DNA replication, was relatively insensitive to CA_1. The drug was also shown to bind to protein and to viral and cellular DNA.

coumermycin; DNA polymerases; topoisomerases; HSV

Introduction

The possible intervention of DNA topoisomerase during the replication of some DNA viruses, namely SV_{40} [8], adenoviruses [7,13] and herpes simplex virus type 1, has been recently emphasized [11]. Topoisomerases are enzymes modulating a variety of

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topological transitions in DNA both from prokaryotes and eukaryotes; in prokaryotes they are primarily responsible for the supercoiling of DNA [5]; such a supramolecular organization in eukaryotic cells arises mainly by the wrapping of DNA around histone octamers that results in the nucleosome structure [17]. The idea that a step of viral replication may be dependent on a topoisomerase activity was derived essentially from the sensitivity of the above mentioned DNA viruses to novobiocin and coumermycin A_1 (CA_1), two related coumarin antibiotics [28,29] which are specific inhibitors of bacterial DNA gyrase (a topoisomerase II) [12,31]. Whilst the genomes of SV_{40} and adenovirus are associated with cellular or virus proteins to form nucleosomal structures, the DNA of herpes simplex virus does not seem to be packed into nucleosomes [10,19]. The anti-HSV effect of novobiocin and CA_1, was therefore ascribed to an inhibitory effect on a gyrase-like function involved in maintaining HSV-DNA in a supercoiled state in spite of its lack of association with histones [11]. However, no definite evidence that HSV-DNA has such topology has been presented thus far. Relevant to this point are recent experimental data which have suggested that a topoisomerase activity might indeed be induced by HSV-1 [3,18] but these data do not indicate the sensitivity of this enzyme to the above mentioned drugs. Accordingly, results of drug sensitivity which suggest a role for such enzymes as drug targets during the replicative cycle of HSV should be considered with respect to at least three sets of findings. First, topoisomerases of type II, which have been found so far in T_4 and eukaryotic cells, are relatively insensitive to novobiocin and coumermycin [20,21,29,30]; second, the concentrations required to inhibit HSV-1 replication are more than 50 times higher than those effective against *E. coli* DNA gyrase [14]. Finally, we have recently noted pure non-competitive inhibition of lactate dehydrogenase activity by CA_1 and binding of the drug to serum albumin as well as to chromatin constituents, DNA and RNA by means of different techniques [23].

These observations have prompted us to reinvestigate the anti-HSV effect of the coumarin antibiotics. Our attention has been addressed to the compound CA_1, whose structure (Fig. 1) resembles that of the analogue novobiocin in that it contains two specular moieties of novobiocin. Of these two antibiotics CA_1 is the most potent both as an antigyrase and antibacterial agent [4,31] and as an anti-HSV drug [11].

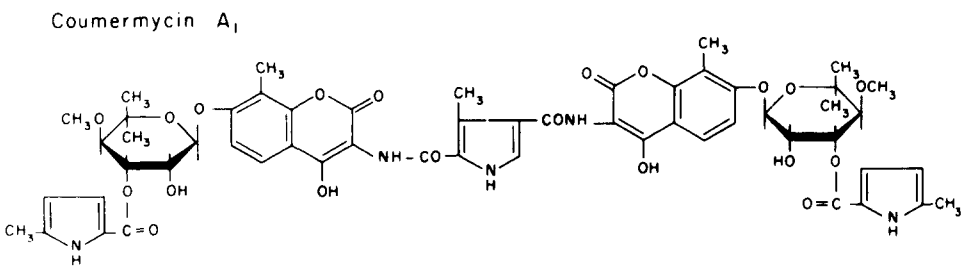

Fig. 1. Molecular structure of CA_1.

Materials and Methods

Cells and viruses

Vero, HeLa, HEp-2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS) (Gibco), antibiotics and L-glutamine. HSV-1 was a clinical isolate identified by its digestion pattern with restriction enzyme Bam HI.

Chemicals and radioisotopes

CA_1, a kind gift from Dr. W.F. Minor (Bristol-Myers, Syracuse), was checked for purity by means of HPLC; it was dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C. [^{3}H]dThd, L-[4,5-^{3}H]leucine and [^{3}H]amino acid mixture were from Amersham, and DTT from Boehringer Mannheim.

Inhibition of HSV-1 plaque formation and assay of virus yield reduction by CA_1

Confluent monolayers of Vero cells in 50-mm diameter Petri dishes were inoculated with 350, 250, 100 plaque-forming units (PFU) of HSV-1 in 0.2 ml of medium (DMEM). After 1 h absorption at 37°C inoculum was removed and replaced with a medium containing 2% (v/v) FCS, 0.1% (v/v) pooled human immunoglobulin and increasing concentrations of CA_1. After 48–72 h cells were fixed with methanol and stained with Giemsa and plaques were enumerated using an inverted microscope.

Virus yield measurements were performed on Vero cells infected with a m.o.i. of 0.5 treated with various amounts of the compound.

From 48 to 72 h following the infection, cells were harvested in the culture medium and disrupted by two cycles of freezing and thawing and mild sonication. Virus yields were determined by plaque assay. The amount of CA_1 required to reduce plaques by 50% (viral ED_{50}) was derived from the best linear plot of a least-square fit relating surviving plaques to CA_1 concentration [6].

Cell cytotoxicity

The effect of CA_1 on the growth of Vero, HeLa and HEp-2 cells was determined by a 3-day culture experiment in the presence of increasing amounts of the compound. Cells were seeded at a concentration of 5×10^4 cells in 20-mm diameter Costar wells and counted at the end of the 72 h by a conventional haemocytometer after trypsinisation of the monolayers. The overall rate of DNA synthesis, as a measure of the cell metabolic activity, was also determined on confluent monolayers that were incubated in the presence of increasing drug concentrations and 1 μCi/ml [^{3}H]dThd for a period of 16 h. After this period, the cells were extensively washed with a cold hypertonic NaCl solution, and three times with 10% (v/v) TCA and methanol; monolayers were then lysed with 0.1 N NaOH and the samples, neutralized with 1 N HCl, were added to Instagel scintillator (NEN) and the radioactivity counted in a liquid scintillator spectrometer. Measurement of cell ED_{50} was obtained from the plot relating surviving cells and [^{3}H]dThd incorporation to the concentration of CA_1 by means of a least-square fit.

Measurement of viral and cellular DNA synthesis

Incorporation of [^{3}H]dThd into cellular and viral DNA was measured in Vero cells infected at a m.o.i. of 20 and incubated from 9 to 11 h p.i. with 5–10 μCi/ml of [^{3}H]dThd and CA_1. After the labelling interval cells were lysed with 0.5% (w/v) SDS and digested with 1.5 mg/ml pronase and DNA fractionated by isopycnic centrifugation on a NaI gradient essentially as described [33]. Fractions were collected from the bottom of the gradient by the aid of a peristaltic pump and aliquots were spotted onto GF/C filters. The filters were washed three times with TCA and ethanol and, once dried, immersed into an Instagel scintillator in order to count TCA-precipitable radioactivity.

Protein synthesis in infected and uninfected cells

HEp-2 cells were infected at a m.o.i. of 20 and immediately after the absorption period exposed to increasing amounts of CA_1 in DMEM with 2% (v/v) FCS. In order to study the synthesis of α, β and γ polypeptides, cultures of infected cells were pulse-labelled from 1 to 3 h p.i., from 5 to 7 p.i. and from 16 to 18 h p.i., respectively, with 10 μCi/ml of tritiated amino acid mixture in DMEM containing 1/10 of the amino acid concentration and 2% (v/v) dialyzed FCS, to which a known amount of CA_1 was added. Samples were prepared and SDS-PAGE carried out as described [16]. The polypeptide nomenclature was that adopted by Honess and Watson [15].

Absorption measurements on autoradiographic images of separated polypeptides were made with a laser densitometer LKB 2202 Ultroscan equipped with a peak integrator (Hewlett Packard). Uninfected HEp-2 cells grown in DMEM with 2% (v/v) FCS were exposed to 5 and 10 μg/ml CA_1 and pulse-labelled either from 1 to 3 h or from 16 to 18 h thereafter. Samples were prepared as for infected cells and aliquots spotted onto GF/C filters to count TCA-precipitable radioactivity.

Effect of CA_1 on protein synthesis in reticulocyte lysate

Rabbit reticulocyte lysate (nuclease treated, mRNA dependent) was purchased from Amersham as was tobacco mosaic virus (TMV) RNA. The reaction mixture contained 35 μl of lysate, 1 μl of TMV RNA, 100 mM potassium acetate and 50 μM of amino acid mixture deficient in L-leucine in 50 μl final volume; it was added to plastic tubes where 50 μCi of L-[4,5-^{3}H]leucine (spec. act. 165 Ci/mmol) had been previously dried. CA_1, in a dose range from 5 to 50 μg/ml, was added to the reaction tube prior to incubation. The assay conditions were as recommended by the manufacturer, with incubation being performed at 30°C for variable intervals, after which TCA-precipitable radioactivity was measured.

Purification and assay of DNA polymerases from HeLa cells and HSV-1

DNA polymerase of HeLa cells and DNA polymerase of HSV-1 were a kind gift from Dr. Spadari and Dr. Pedrali-Noy (CNR, Pavia). The purification method for both enzymes involves a final step of affinity chromatography on single-stranded DNA and has been described in detail elsewhere [25].

The assay conditions were the following: 5–10 μl of enzyme (2600 U/ml of α-DNA polymerase, 3000 U/ml of HSV-1-DNA polymerase) were incubated in 100 μl of

reaction mixture containing either 50 mM Tris, pH 8.1 (for viral polymerase) or 20 mM KH_2PO_4/K_2HPO_4 buffer, pH 7.2 (for α-polymerase), 7 mM $MgCl_2$, 250 mM KCl (for the HSV-1 polymerase), 0.5 mM DTT, 6.85 μg of activated calf thymus DNA, 100 μM dTTP, dATP, dGTP and 10 μM [^{3}H]dCTP (1160 cpm/pmol) and various amounts of CA_1.

In some experiments the same Tris buffer was used for both reactions and the drug was also preincubated with either the enzyme or the template-primer before adding the reaction mixture. Incubation was performed at 37°C and samples were taken after 15 and 30 min in order to measure the enzyme activity in a linear phase. Samples were spotted onto GF/C filters and TCA-precipitable radioactivity measured as described above.

Interaction of CA_1 with viral and cellular DNA and measurement of the thermodynamic parameters of binding

Viral and cellular DNA were separated on a NaI gradient which contained 0.5 μg/ml of ethidium bromide by means of isopycnic centrifugation as described previously. The two bands were visualized with an UV lamp and collected with a 5-ml syringe and a 19-gauge needle. The samples were diluted with distilled water and DNA was precipitated by addition of two volumes of ethanol and centrifugation at 3000 r.p.m. at room temperature. The pellet was dissolved in 10 mM Tris/1 mM EDTA (pH 7.5) and the solution extracted three times with a mixture of phenol/chloroform/isopentanol (25/24/1). DNA was again precipitated, washed with 70% (v/v) ethanol and dissolved in TE buffer and used for spectroscopic studies.

The concentration of CA_1 in DMSO was determined spectrophotometrically using a molar extinction coefficient of 60 000 at 282 nm.

Spectrophotometric measurements were carried out at 25°C with a Perkin-Elmer 576 spectrophotometer, using 1-cm quartz cells. CA_1 aqueous solutions were freshly prepared and 10 μl of a concentrated solution of the drug (2.3 mM) in DMSO were directly diluted to the desired concentration in the cuvette. Measurement of solutions containing nucleic acids was made against a nucleic acid blank.

In order to obtain correlation between the biological activity and some physico-chemical parameters of interaction, also at low DNA/drug (P/D) ratios, the equation of Papaphilis and Shaw [24] was used. The titration method applied was the same as previously reported [23].

All solutions were prepared with double-distilled water.

Results

Antiviral activity

Table 1 reports the effect of CA_1 on the growth of HSV-1. Values of viral ED_{50}, as measured by plaque reduction assay, were between 2.60 and 2.75 μg/ml. A significantly lower ED_{50} (1.03 μg/ml) was obtained when virus yield reduction was measured.

TABLE 1

ED_{50} of CA_1 for virus replication*

Virus yield reduction** (μg/ml ± S.E.M.)	Plaque reduction (μg/ml ± S.E.M.)	(Size of the inoculum) (PFU/well)
	2.75 ± 0.46	(100)
1.03 ± 0.24	2.61 ± 0.90	(150)
	2.60 ± 0.87	(350)

* Vero cells were employed for both virus yield and plaque reduction assays.
** Cells were infected at a m.o.i. of 0.5. Untreated controls gave an average yield of 300 PFU/infected cell.

Cytotoxicity

The cytotoxic effect of CA_1 has been measured in the three cell lines which were used in all our experiments. The effects of the drug on cell growth and the incorporation of labelled thymidine were measured on cells cultured in a medium containing either 2% (v/v) or 10% (v/v) FCS. These different culture conditions were chosen in view of the well known ability of coumarin compounds to interact with serum protein [29] and to make a comparative assessment of the antiviral and cytotoxic action of the drug.

Inspection of Table 2 indeed shows that the serum concentration was a significant variable. ED_{50} values of CA_1 for cell growth and DNA synthesis increased 1.5–3-fold when the culture medium contained 10% as compared to 2% FCS. Furthermore, each cell line had a different level of susceptibility towards CA_1, HeLa 229 being relatively the most sensitive and HEp-2 the most resistant cells.

Effect on the synthesis of viral and cellular DNA

Mock-infected and virus-infected Vero cells were maintained in a medium containing 2% (v/v) FCS and pulse-labelled with [^{3}H]dThd as described above. The effects of the antibiotic on the incorporation of the radiolabelled precursor into viral and cellular DNA isolated by isopycnic centrifugation are illustrated in Fig. 2A and B.

The incorporation of the labelled precursor into viral (Fig. 2A) and cellular (Fig. 2B)

TABLE 2

ED_{50} of CA_1 for cell growth and DNA synthesis

Cell lines	Fetal calf serum (%)			
	Reduction of growth rate		Reduction of [^{3}H]dThd incorporation	
	2	10	2	10
HeLa 229	2.74 ± 0.71	6.20 ± 1.15	4.17 ± 0.70	12.1 ± 2.12
HEp-2	6.25 ± 1.64	15.9 ± 3.72	13.5 ± 1.55	20.5 ± 2.03
Vero	3.29 ± 0.93	8.87 ± 2.04	6.96 ± 0.64	19.6 ± 2.98

Values are expressed as μg/ml ± S.E.M.

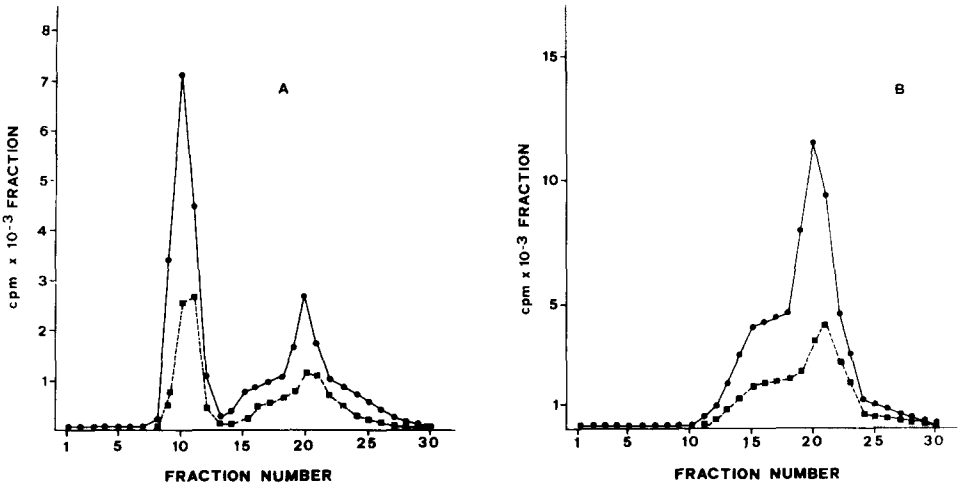

Fig. 2. NaI density gradient profiles of DNA labelled in infected and uninfected Vero cells (B). Cells infected with HSV-1 at a m.o.i. of 20 and mock infected cells were labelled with 5 μCi/ml [^{3}H]dThd from 9 to 11 h following infection in the presence of 10 μg/ml CA_1 (■---■) and in its absence (●---●). DNA was separated as described in Materials and Methods and fractions were collected from the bottom of the centrifuge tubes. The denser peak in A represents virus DNA.

DNA was reduced to a comparable extent (over 50%) by a concentration of 10 μg CA_1/ml. Residual synthesis of cellular DNA after virus infection (Fig. 2A) was equally sensitive to this drug amount which is close to the ED_{50} value obtained by measuring [^{3}H]dThd uptake in intact cells grown in 2% (v/v) FCS (Table 2).

These observations might favor the idea that similar functions of both viral and cellular origin could be affected by CA_1. To explore this possibility we have studied the action of the antibiotic on the enzymes which are primarily involved in the replication of DNA.

Effect on the activity of purified cellular and viral DNA polymerases

Highly purified HSV-1 polymerase was inhibited by increasing concentrations of CA_1 (Fig. 3A) with the concentration of the drug required to inhibit the activity by 50% being in the range from 5 to 10 μg/ml, i.e. concentrations similar to those required to inhibit virus and host DNA synthesis in cell culture.

However, the activity of the DNA polymerase α isolated from HeLa cells, which had a similar specific enzyme activity, was significantly less sensitive to CA_1 (Fig. 3B). The drug doses required for a 50% reduction of this activity (20 and 50 μg/ml) were also significantly higher than the ED_{50} for [^{3}H]dThd incorporation into the same cell line (see Table 2).

Albeit relevant to the mode of action of CA_1, the present results using polymerases do not allow any conclusion concerning selectivity, because the different salt conditions in the two assays might affect the inhibition.

In addition, some indirect evidence for more complex effects of the inhibitor, likely

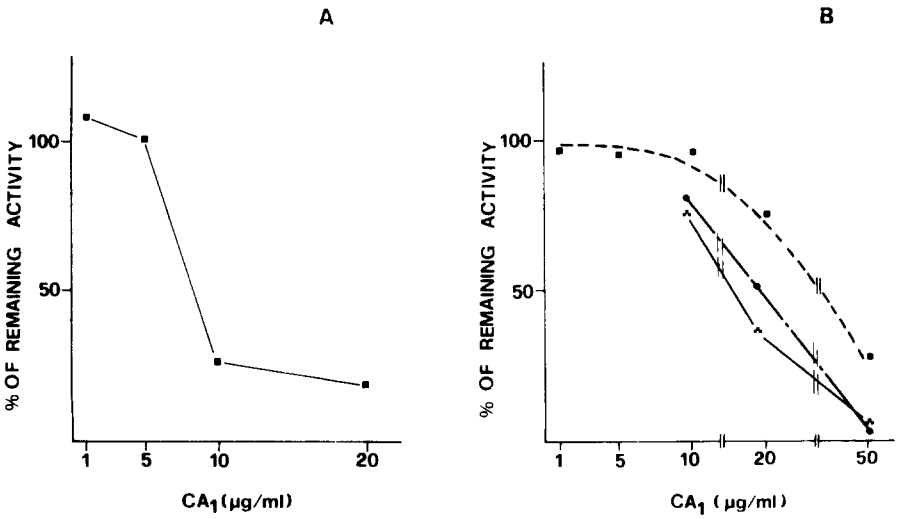

Fig. 3. Effect of CA_1 on the activities of HSV-1 DNA polymerase (A, ■——■) and HeLa cell DNA polymerase α (B, ■---■) in the presence of various concentrations of CA_1 and (B) following preincubation of the drug for 10 min at 4°C with either HeLa cell DNA polymerase α (●)---●) or activated calf thymus DNA (■ ■—■ ■). Activities were measured after incubation of the reaction mixtures for 30 min at 37°C. The incorporation was normalized to the value of the control reaction in the absence of the drug; 100 represents 125644 cpm (HSV-1 DNA polymerase) and 112610 cpm (HeLa cell DNA polymerase α) of [^{3}H]dCMP incorporated per h at 37°C. The assay conditions were as reported in the text. Experimental points represent the mean of triplicate experiments.

to take place also in intact uninfected cells, was obtained by a different assay. This consisted in the measurement of α-polymerase activity after preincubation of CA_1 with either the enzyme itself or activated DNA for a period of 10 min at 4°C.

As shown in Fig. 3B, such a treatment increased the relative potency of the inhibitor which produced the most marked effects after preincubation with the template-primer.

Sensitivity of viral polypeptides synthesis to CA_1 and effect of the antibiotic on protein synthesis in uninfected cells

To characterize further the mechanism of the antiviral activity of CA_1, the effects of the drug on the synthesis of representative virus-specific polypeptides in cells infected with HSV-1 was examined. In the presence of a drug concentration of 5 μg/ml sufficient to prevent virus growth, the synthesis of immediate-early (α) and early (β) polypeptides was not diminished (Fig. 4, lanes 1, 2, 3, 4). However, when cells treated with 5 μg/ml of the drug were pulse-labelled 16–18 h after infection, synthesis of icp 5 and 10 (γ polypeptides), and icp 9 and 12 was selectively reduced (Fig. 4, lanes 5, 6).

When infected cells were exposed to a dose of CA_1 (10 μg/ml) that inhibits viral DNA replication, it appeared that the incorporation into all virus specific proteins was reduced with synthesis of icp 4 (α) and icp 6 (β) being relatively less sensitive than the synthesis of the other virus proteins (Fig. 4, lane 7).

Protein synthesis in uninfected cells is also affected by CA_1, as shown in Table 3. The

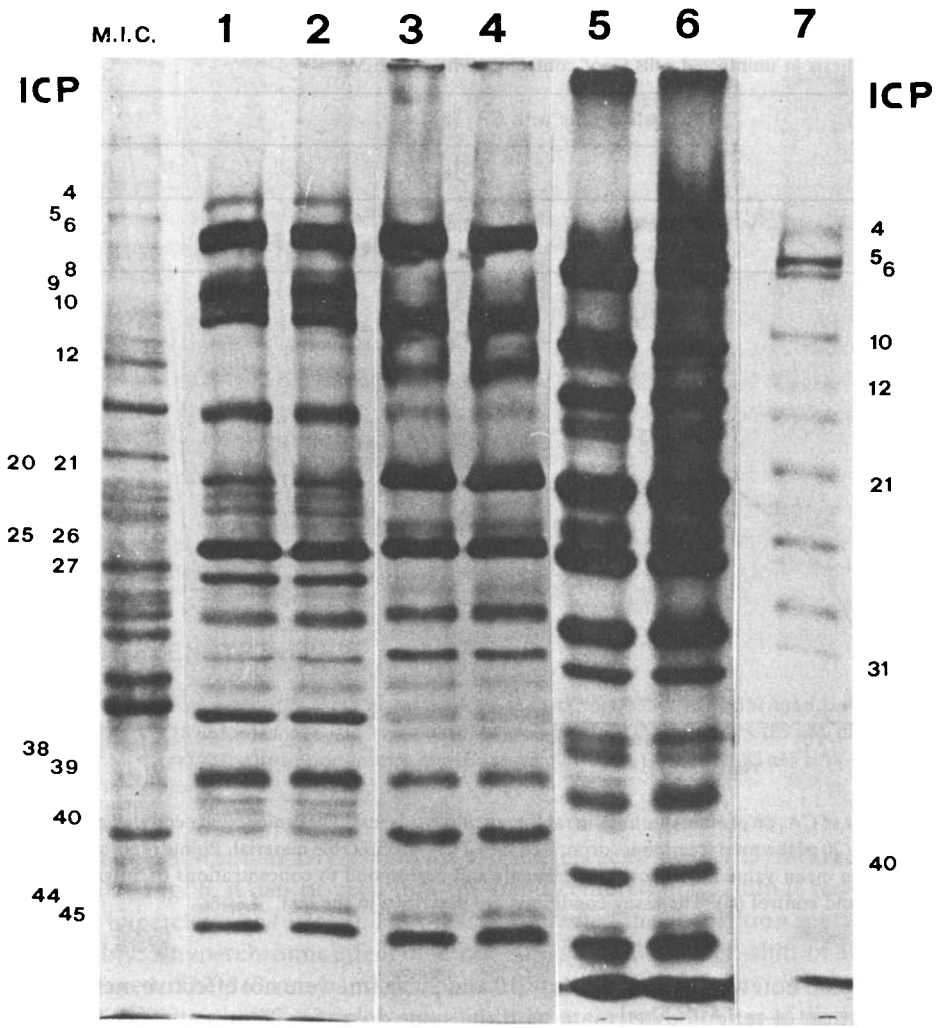

Fig. 4. Autoradiogram of labelled polypeptides separated on a 8.5% polyacrylamide gel slab from lysates of HEp-2 cells labelled with 10 μCi/ml [^{3}H]amino acid mixture from 1 to 3 h (lanes 1,2), 5 to 7 h (lanes 3,4), and 16 to 18 h (lanes 5, 6, 7) after infection with 20 PFU of HSV-1. M.I.C. shows profile of mock infected cells. Cells were either untreated (lanes 1, 3, 5) or maintained with 5 μg/ml (lanes 2, 4, 6) and 10 μg/ml (lane 7) of CA_1 for the whole period following infection. Laser densitometer measurements showed a 41% reduction in the amount of icp 5, a 65.6% reduction of icp 9–10 and a 51.8% reduction of icp 12.

inhibitory activity of the antibiotic is clearly more pronounced with longer periods of exposure to the drug, i.e. after 18 h. At this time, however, there is no significant difference in the effect of drug dose.

Effect on in vitro protein synthesis

As shown in Fig. 5, there is no drug concentration dependence of protein synthesis in

TABLE 3

Protein synthesis in uninfected cells % of control activity ± S.E.M.

CA_1 (μg/ml)	Incubation time with CA_1 (h)	
	0–3	0–18
5	97.2 ± 3.4	55.4 ± 8.6
10	68.7 ± 5.0	51.7 ± 8.5

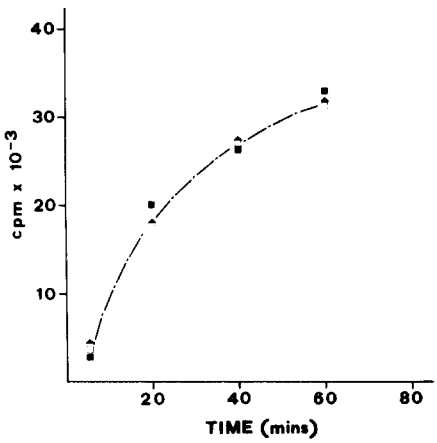

Fig. 5. Effect of CA_1 on protein synthesis in rabbit reticulocyte lysate. The radioactivity counted at each time represents 1/20 of the total fraction incorporated into TCA-precipitable material. Points reported in figure represent the mean value of triplicate experiments and correspond to concentrations of 5 μg/ml (▲), 50 μg/ml (■), and control (□). The assay conditions are described in the text.

vitro. It is also noteworthy that doses of 10 and 20 μg/ml were not effective, neither was preincubation of reticulocyte lysate with the same doses for 20 min at room temperature (data not shown).

Interaction with cellular and viral DNA

The results of the experiments so far described indicate that CA_1 inhibits viral and cellular DNA synthesis but have not clarified if this is the major mechanism responsible for the antiviral activity of the drug. In this regard, it should be considered that viral ED_{50} values were much lower than the concentration of CA_1 giving a 50% inhibition of viral polymerase activity in a cell-free system and viral DNA synthesis in intact cells. A still bigger discrepancy exists between the rate of inhibition of DNA polymerase α in vitro and the cytotoxic potency of the compound. The possibility that CA_1 interacts with DNA has therefore been examined. The absorption spectrum of a dilute aqueous solution of coumermycin A_1 (11.4 μM), which has an UV peak at 282 nm and a shoulder at about 344 nm, was modified upon binding to double-stranded DNAs. The

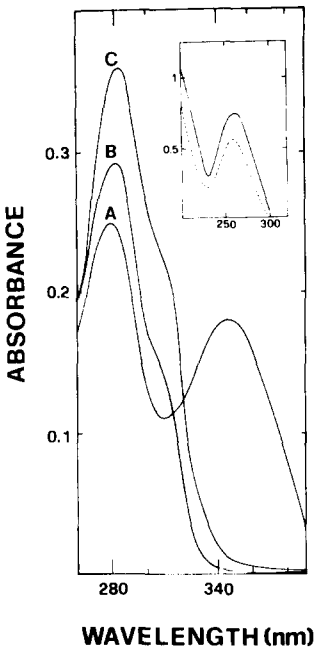

Fig. 6. UV difference spectra of CA_1 (11.4 μM) in 99.5% H_2O/0.5% DMSO, pH 6.55 (A) and in presence (B) of cellular DNA (115 μM, pH 6.62), $K_{app} = 5.1 \times 10^4\ M^{-1}$, $n = 0.93$; (C) HSV-1 DNA (85 μM, pH 6.62), $K_{app} = 8.1 \times 10^4\ M^{-1}$, $n = 0.93$. Absorption spectra of 115 μM cellular DNA (——) and 85 μM HSV-1 DNA (---) are shown in the inset.

spectrum of CA_1–cellular DNA complex at a nucleic acid/dye (P/D) ratio equal to 10, is shown in Fig. 6; it can be seen that the band centered at 282 nm is red-shifted (3 nm) and hyperchromic (+15%). HSV-1 DNA affected the absorption spectrum more appreciably: a hyperchromic effect of about 30% along with a red-shift of about 5 nm was observed. The 347 nm band disappeared in both cases.

Both K_{app} values of the complexes with cellular and viral DNA are of the same order of magnitude, as reported in the legend of Fig. 6, while the number of molecules interacting with DNA remains the same ($n = 0.93$).

Discussion

The results obtained in this study contribute to a better understanding of the anti-HSV-1 activity of CA_1. If one considers virus yield reduction or inhibition of plaque formation, it can be concluded that the therapeutic activity of the drug is rather low. The question should therefore be addressed whether the antiviral properties of CA_1, as it seems from our data, have to be related to some extent to its cytotoxicity. This latter in fact is especially pronounced when cells were cultured in the presence of 2% (v/v) FCS. The differences in the cytotoxic level found when using 2% or 10% FCS

in cultures point to the significance of relative concentration of protein in the environment where CA_1 is added. This could explain why our therapeutic index is markedly lower than the one reported [11].

The data on the incorporation of radiolabelled precursors into DNA clearly show that inhibition of DNA synthesis is a prominent feature of the antibiotic, both in infected and uninfected cells. As for the virus, such an effect could well derive, as shown in this paper, from an interaction of the antibiotic with either the replicase or DNA itself (Figs. 3A and 6C). However, all the attempts to isolate mutants resistant to CA_1, useful to elucidate further the mode of action of the drug at the enzyme level, have not been successful and this in turn argues against selectivity of CA_1. The effect of the compound on protein synthesis is less pronounced and depends on the length of time CA_1 was left to interact with the cells. In particular the inhibition of viral polypeptide synthesis is prominent only at a late stage of viral growth coinciding with a long exposure to CA_1. At 5 μg/ml the 16–18 h pulse appears to show elevated levels of α4 and β38–39 and reduced levels of γ5, 10 and 12. However the synthesis of other proteins (particularly γ21, normally very sensitive to inhibitors of DNA synthesis, but also γ31, 44, 45) appears to be unaffected. The same conclusion applies to the 10 μg/ml dose (slot 7) which, in addition, shows a very marked general inhibition of all viral polypeptides. These effects on protein synthesis are quite unlike the ones observed with phosphonoacetic acid [16], cytosine arabinoside or mutants with *ts*-defects at not permissive temperature [1,27] and seem therefore to suggest a more complicated mechanism of CA_1 action than a simple inhibition of DNA synthesis. On the other hand, the hypothesis that the antibiotic has a direct effect on the translation process itself seems not reasonable in the light of the results of in vitro protein synthesis (Fig. 5). However, it cannot be excluded that transcription might be hampered to some extent by the coumarin binding to DNA, thus leading to a late effect on protein synthesis. In the light of the data presented in this paper it appears that the mechanism of inhibition of HSV-1 replication by CA_1 is rather complex and non-specific since it involves metabolic pathways common to the normal host cell. In this regard it is noteworthy that CA_1 is also active, at a similar range of concentrations (1–5 μg/ml), against influenza virus type A [26] and a number of murine retroviruses [32]. This consideration along with previous studies [23] showing that CA_1 interacts with a wide range of macromolecules, suggest that the anti-HSV-1 activity of the drug cannot be considered as evidence of an inhibitory effect of CA_1 on topoisomerase activity.

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