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Antiviral activity of the prostanoid clavulone II against vesicular stomatitis virus

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Summary

Prostaglandins of the A series exhibit the most pronounced antiviral activity in cells infected with RNA or DNA viruses as compared to other prostaglandins. Clavulone is a prostaglandin A analog found in the soft coral *Clavularia viridis*. Using vesicular stomatitis virus in mouse L929 fibroblasts as a model system, 50% inhibition of viral yield was seen at a concentration of 1–1.5 μM, whereas 50% cytotoxicity required 50–70 times higher inhibitor concentrations. For a further elucidation of the antiviral mechanism a temperature-sensitive mutant, tsG 41, was used, which is replication-negative at the restrictive temperature. Results obtained with this mutant suggest that inhibition of VSV replication occurs at the level of transcription.

Vesicular stomatitis virus; Prostaglandin; Clavulone

Introduction

An antiviral effect of prostaglandins was first reported by Luczak et al. (1975), who observed inhibition of parainfluenza virus by PGE_2 and $PGF\alpha$. Subsequently, prostaglandins of the A series (PGAs) were found to inhibit Sendai (Santoro et al., 1980,1989a), vesicular stomatitis (Santoro et al., 1983a; Bader and Ankel, 1990), vaccinia (Santoro et al., 1983b; Benavente et al., 1984), encephalomyocarditis (EMC) (Ankel et al., 1985), herpes simplex II (Yamamoto et al., 1987) and influenza A virus (Santoro et al., 1988).

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Of particular interest is the fact that both DNA and RNA viruses were inhibitable by PGs which were effective even after infection had occurred. This indicated that PGs do not affect adsorption of the virus to the cell or penetration into the cytoplasm (Santoro et al., 1980,1983a; Ankel et al., 1985). However, different mechanisms of antiviral activity have been discussed for different viruses which include impaired RNA synthesis (EMC), inhibition of immediate early transcription (HSV), and alteration of protein glycosylation (Sendai). In the case of VSV-infected cells treated with PGA_1, inhibition of primary transcription in vivo as well as in vitro has been suggested (Bader and Ankel, 1990).

Recently a great number of pharmacologically active substances that occur in marine invertebrates have been reviewed (Scheur, 1990). Among them are prostaglandins which are produced in a variety of soft corals in amounts as great as 1% of wet weight (Nelson et al., 1982). PGA_2 was isolated from *Plexaura homomalla*, the sea whip found in the Caribbean sea (Weinheimer and Spraggins, 1969). The clavulones, a new type of prostanoids were initially isolated from *Clavularia viridis*, a Japanese stolonifer (Kikuchi et al., 1982). Subsequently, these authors reported the absolute stereochemistry of the clavulones which were structurally distinct in regard to their chiral centers (Kikuchi et al., 1983).

PGAs were first shown to inhibit tumor growth (Stein-Werblowsky, 1974; Eisenbarth et al., 1974) and to induce tumor cell differentiation (Santoro et al., 1979). Fukushima and Kato (1984) suggested a requirement for a reactive α,β unsaturated carbonyl group in PGs that exerted antiproliferative activity, which was subsequently confirmed in more detail (Honn and Marnett, 1985). Consequently, coral-derived clavulone was found to have significant antileukemic activity when cell growth of normal cells was compared to that of human cancer cells (Honda et al., 1985,1987). Therefore, it was interesting to study whether clavulone, similar to PGAs, would also exert antiviral activity.

Materials and Methods

Prostaglandin A_1, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), and methyl cellulose were obtained from Sigma. Clavulone II was chemically synthesized (Nagaoka et al., 1984). Mass spectral analysis indicated that the material was more than 99% pure. Its structure is compared to that of PGA_1 in Fig. 1. Prostaglandins were dissolved in ethanol and kept at $-20°C$. Fetal bovine serum (FBS), minimal essential medium (MEM), and F-12 medium were purchased from Gibco. Antibody against the *Indiana* serotype of VSV (VSV_{Ind}) was purchased from Lee BioMolecular Research Inc., San Diego. [α-^{32}P]dCTP (>3000 Ci/mmol) and [^{3}H]thymidine (100 Ci/mmol) were purchased from Amersham or ICN. [^{35}S]Methionine (>800 Ci/mmol) was from ICN. [^{125}I]Protein A was generously supplied by Dr. A. Haas, Medical College of Wisconsin. Kits for in vitro translation and nick translation as well

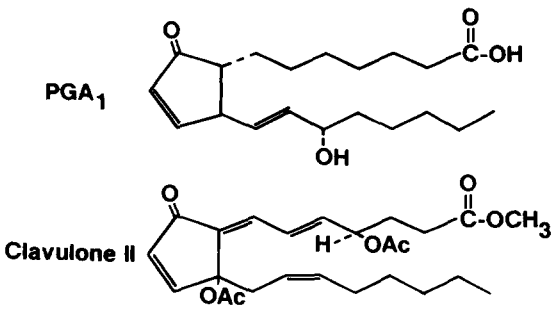


Fig. 1. Structures of PGA_1 (top) and the prostanoid clavulone II (bottom) derived from *Clavularia viridis*.

as RNA (0.24–9.5 kb) and protein (15–200 kDa) molecular weight markers were purchased from BRL. Cloned cDNAs to **N** (pN4), **NS** (pNS319), **M** (pM309) and **G** (pG1) mRNA of VSV were generously provided by J. Rose, Yale University, New Haven (Rose and Gallione, 1981; Gallione et al., 1981; Rose and Bergmann, 1982). cDNA to **L** mRNA (pSV-VSV**L**) was a gift from M. Schubert, NIH, Bethesda (Schubert et al., 1984). Immobilon-P membrane was purchased from Millipore and Nytran membrane from Schleicher and Schuell. The RNA extraction kit with cesium triluoroacetate (CsTFA) was supplied by Pharmacia. *Staphylococcus aureus* suspension was purchased from Calbiochem, San Diego.

Cells and viruses

L929 cells were grown as monolayers in tissue culture flasks (25 cm^2, Costar) in MEM or RPMI supplemented with 10% FBS. MDBK cells were also grown as monolayers in F-12/10% FBS. The *Indiana* serotype of VSV was propagated in L929 cells at a multiplicity of infection (MOI) of 0.1. The temperature-sensitive mutant tsG 41 of VSV was a gift from P. Marcus, University of Connecticut, Storrs, and was also propagated in L929 cells at the permissive temperature of 30°C.

Plaque assays

Standard assays consisted of a one-hour infection period with VSV at an MOI of 10–20. Unadsorbed virus was removed and PGs were added in serum-free medium for the remainder of the incubation period. Viral yields were determined in miniplaque assays as described (Langford et al., 1981).

Cytotoxicity measurements

These employed the conversion of MTT tetrazolium salt to MTT-formazan as a measure of cell integrity essentially as described by Denizot and Lang

(1986). Triplicates of confluent monolayers of uninfected L929 cells in 96-well microtiter plates were incubated with the indicated clavulone concentrations in serum-free medium (0.1 ml/well) for 6 h. Then medium was replaced by 0.12 ml of a 0.13% MTT solution in phosphate-buffered saline and incubation continued for 2 h. Supernatants were removed, colored crystals that had formed were dissolved in 0.04 N HCl in isopropanol, and the absorbance was measured in an ELISA reader at 570 nm. 50% cytotoxicity was determined from graphic extrapolation of the values obtained and is defined as the clavulone concentration that yielded 50% reduction in absorbance. Thymidine incorporation was carried out in routine assays as described above employing 20 μCi/ml of the labeled nucleoside in addition to the clavulone, likewise for an incubation period of 6 h. After collection of the cells with an automatic harvester and repeated washing with water, incorporated radioactivity was determined by scintillation counting.

SDS-PAGE and Western blots

Supernatants of cells were concentrated approximately 100-fold in Amicon A25 concentrators to a final volume of 40 μl and mixed with an equal volume of twice-concentrated SDS sample buffer (0.5 M Tris-HCl, pH 6.8, 10% glycerol, 10% SDS [w/v], 5% β-mercaptoethanol). Dialyzed cell extracts (see below) were mixed with an equal volume of twice-concentrated SDS sample buffer. Polyacrylamide gel electrophoresis in the presence of SDS was performed on 10% gels (acrylamide/bisacrylamide, 30:0.8). The blotting procedure onto Immobilon-P was identical to the one previously described by Haas and Bright (1985), except 10% methanol was present during transfer. The concentration of anti-VSV_{Ind} was optimized and typically used at a 1:10 000 dilution. [^{125}I]Protein A had a specific activity of 2×10^5 cpm/ml. Following autoradiography, films were quantitated by scanning in a densitometer (Ultrascan, LKB).

Preparation of RNA

Supernatants of L929 monolayers (approx. 5×10^6 cells) were separated from cells which were dissolved in 7.6 M guanidinium-HCl (pH 5.0) and 0.1 M potassium acetate in a final volume of 1.8 ml, and passaged ten times through a 1-ml insulin syringe (26 gauge). The homogenate was layered over a mixture of 670 μl Cs-trifluoroacetate (density 1.52 g/ml) and 1330 μl CsCl (7.6 M) in SW 60 rotor tubes, and centrifuged for 24 h at 35 000 rpm. Thereafter, dissolved proteins in the upper third of the gradient were removed and dialyzed against 50 mM Tris-HCl (pH 7.8), containing 150 mM NaCl, 10 mM EDTA and 20 mM β-mercaptoethanol. Then, tubes were inverted, remnants of salt were removed and the visible RNA pellets were redissolved in 30 μl TE (10 mM Tris-HCl, pH 8, 1 mM EDTA). Tubes were rinsed with an additional 30 μl TE. Both RNA-containing solutions were combined and stored at -20°C.

In vitro translation

Aliquots of total RNA extracts were used to program an in vitro reticulocyte translation system in the presence of [^{35}S]methionine according to the BRL protocol. Aliquots of 5 μl undiluted anti-VSV antibody were added and complexes were allowed to form at 4°C overnight. A second incubation with addition of 20 μl of *Staphylococcus aureus* suspension for 2 h at room temperature was followed by centrifugation and three washes of the pellet with PBS. The final pellets were dissolved in SDS sample buffer, denatured at 95°C for 5 min, chilled on ice and loaded onto a 10% gel as described above. After electrophoresis, gels were fixed in 10% TCA, rinsed in H_2O, soaked in Autofluor (NEN, Boston) dried and subjected to fluorography at −80°C.

Northern analysis

Equal amounts (5–10 μg) of RNA in SDS sample buffer were separated on 0.8% TBE-agarose gels, visualized with ethidium bromide and ^{32}P-labeled by nick translation under UV light for 5 min. Subsequently, RNA was blotted onto Nytran following the manufacturer's protocol and baked at 80°C. Filters were pre-hybridized for 2 h at 65°C in 50% formamide, five times concentrated SSC (150 mM NaCl, 15 mM sodium citrate), 1% SDS, 2% Denhardt's solution and 10 μg/ml sonicated salmon sperm DNA. This solution was removed and replaced by a fresh aliquot containing one of the five probes ^{32}P-labeled by nick translation (see below). Hybridization was allowed to occur for >8 h at 35°C. After incubation, the filters were washed twice in twice-concentrated SSC and once in twice-concentrated SSC containing 0.1% SDS, for 30 min each at room temperature. Wet filters were wrapped in plastic foil, and Konica A medical X-ray film was exposed in the presence of DuPont Lightning Plus intensifying screens at −20°C or −80°C. For reprobing, membranes were boiled three times for 10 min in TE, blocked and hybridized.

Nick translation of cDNA probes

Plasmids containing inserts of **N**, **NS**, **M** and **G** were linearized with *Pst*I, pSV-VSVL with *Xho*I. Approximately 10 μg of DNA per reaction was nick-translated according to the manufacturer's protocol using 100 μCi [α-^{32}P]dCTP, typically yielding a specific activity of approx. 10^8 cpm/μg DNA. Unincorporated label was removed in spin columns as described by Maniatis et al. (1982). Labeled probes were boiled and added directly to the hybridization solution.

Results

The antiviral activity of clavulone

In standard assays L929 cells were infected with VSV, then unadsorbed virus was removed and incubation continued for 6 h with various concentrations of clavulone. As determined in plaque assays, a dose-dependent reduction of viral yield could be observed. In comparison to PGA_1 which had already been shown to be antiviral against VSV (Santoro et al., 1983a; Bader and Ankel, 1990), clavulone appeared to be five times more potent (Fig. 2). A clavulone concentration of 4 ± 1 μM was sufficient to reduce viral yield from VSV-infected cells by more than 90% (for PGA_1, it was 22 ± 3 μM). The antiviral effect is clearly not the result of cytotoxicity of clavulone on the host cells as judged from assays employing the conversion of MTT tetrazolium salt to MTT-formazan as a measure of cell integrity. As shown in Fig. 3, whereas 50% reduction in virus yield occurred at a concentration of 1 to 1.5 μM clavulone, a 50 to 70-fold higher concentration was required to result in 50% cytotoxicity under conditions identical to those employed in the antiviral assays. Determination of thymidine incorporation into uninfected cells in the absence and in the presence of up to 50 μM clavulone revealed no inhibition, and trypan blue exclusion tests corroborated these findings (data not shown). In agreement with results of antiviral studies against Sendai virus (Santoro et al., 1980) and EMC virus (Ankel et al., 1985) no antiviral effect was seen when cells were preincubated only with clavulone prior to infection for as long as 6 h. Similarly,

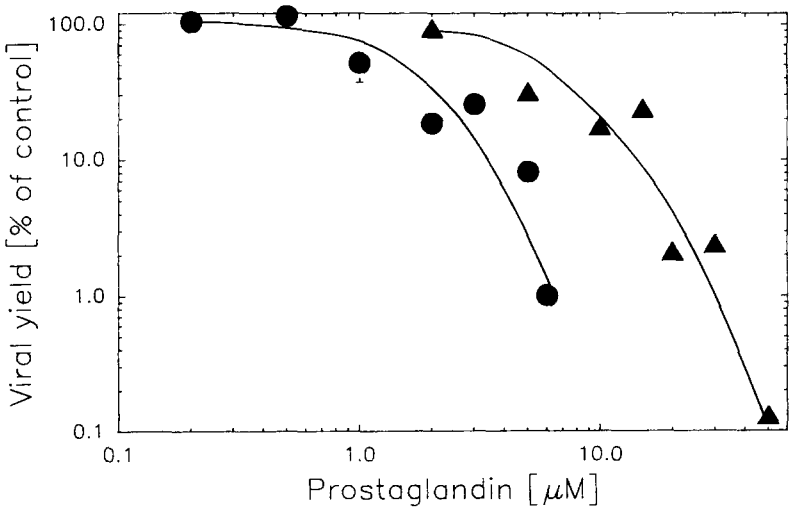


Fig. 2. Effects of clavulone II and PGA_1 on VSV yield in L929 cells. Confluent monolayers of L929 cells were infected at an MOI of 10–20. One hour after infection, unadsorbed virus was removed and cells were further incubated with PGs at the indicated concentrations. After 6 h, cultures were frozen and thawed. VSV yield was determined by plaque assay. Symbols are clavulone (●) and PGA_1 (▲).

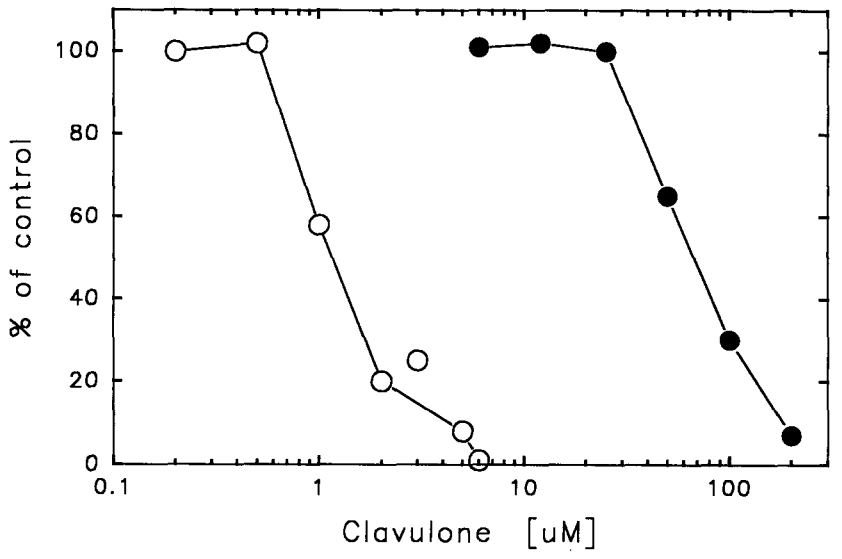


Fig. 3. Comparison of cytotoxic and antiviral effects of clavulone II. Antiviral assays were performed as in Fig. 2. Cytotoxicity was determined in uninfected L929 cells at the indicated concentrations of clavulone under identical conditions. After 6 h of incubation with clavulone, the MTT assay was carried out as described under Materials and Methods. Cell survival: (●); virus yield: (○).

no effect was observed when clavulone was present only during the infection period of 1 h. Fig. 2 compares the antiviral effects of clavulone and PGA_1 on VSV in L929 cells. Percent inhibition was graphed versus the concentration of PGs. Clearly, inhibition of viral yield by clavulone parallels that by PGA_1 at approximately 5-fold lower concentrations.

Distribution of viral proteins

A clavulone dose-dependent reduction of viral yield could be the result of an overall reduced virus production, release of non-infectious virus from cells or inhibition of viral release per se. To distinguish between these possibilities, Western analysis was employed to analyze viral protein patterns both in supernatants and cell extracts. Supernatants were concentrated and resolved by SDS-PAGE, blotted to nitrocellulose and immunostained with anti-VSV followed by [^{125}I]protein A. The same analysis was performed with cell extracts which were processed to yield RNA and protein from the same sample after CsCl/CsTFA density centrifugation. Under these conditions RNA forms a pellet whereas proteins remain dissolved at the top of the gradient. After dialysis of the protein fraction, aliquots could be separated directly on 10% SDS gels.

Fig. 4 illustrates the resulting protein pattern of untreated and clavulone-

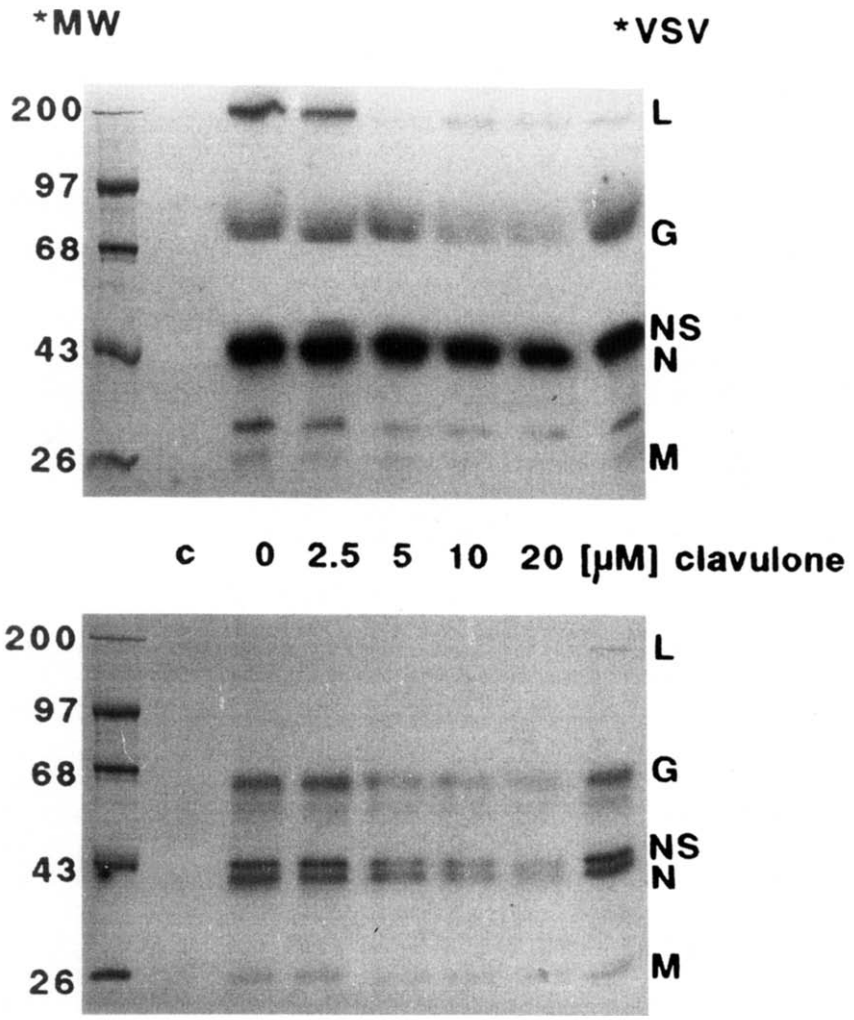


Fig. 4. Western analysis of viral proteins after clavulone treatment. L929 cells were infected for 1 h at an MOI of 10–20, then virus was removed. Clavulone at the indicated concentrations was administered to the cells for 6 h. Then cells and supernatants were processed for Western blots. The upper panel is an autoradiogram of the supernatant, the lower panel is from cell extracts. Relative molecular masses in kDa are on the left, authentic ^{14}C-labeled VSV proteins on the right.

treated cells both in supernatants (top panel) and cell extracts (bottom panel). With increasing concentrations of clavulone, no major change in the ratios of individual viral proteins was visible, rather the amounts of all viral proteins decreased in parallel as compared to the untreated infected controls. Accumulation of viral proteins within the cells or in the supernatant was not observed. This eliminates the possibility of impaired budding of virus or release

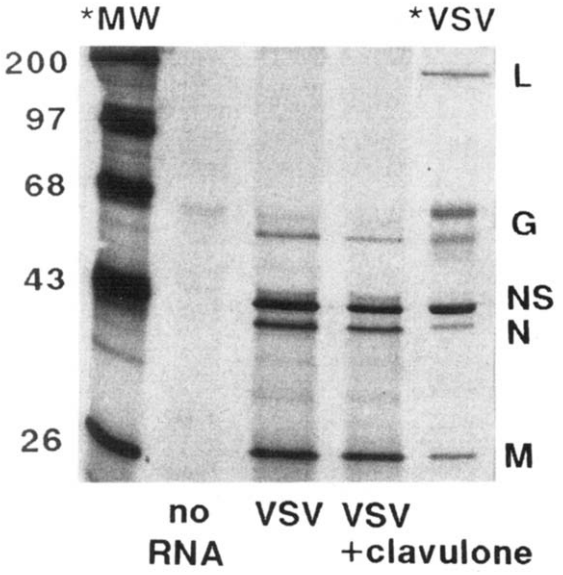
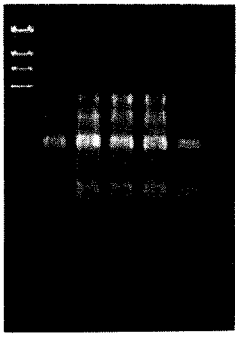
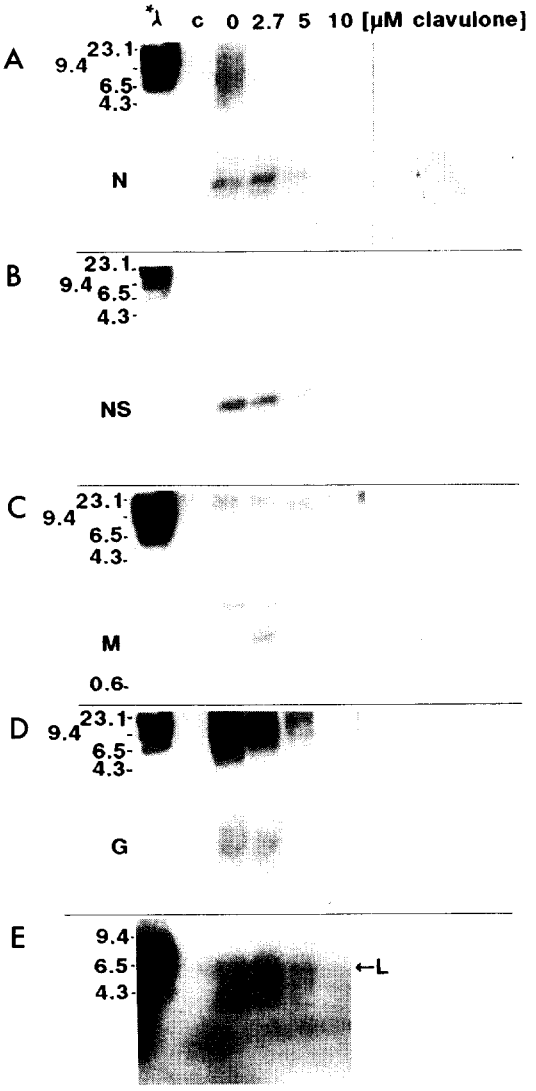


Fig. 5. In vitro translation of VSV RNA in the absence and presence of clavulone. RNA was purified from infected L929 cells. Viral mRNA was used to program a rabbit reticulocyte lysate in the absence and presence of 3 μM clavulone. No exogenous RNA was added to a control reaction. Synthesis products were separated after immunoprecipitation by SDS-PAGE followed by fluorography. On the left side are relative molecular masses in kDa (*MW). On the right side ^{14}C-labeled authentic VSV proteins are separated (*VSV). Note that this system is unable to glycosylate the G protein.

of non-infectious particles, respectively. Rather, the data suggest that clavulone inhibits at a level at or prior to translation of viral message. The fact that increasing concentrations of clavulone caused a uniform decrease of viral proteins without a major change of their ratios supports this conclusion.

In order to explore whether a decrease in viral proteins is a result of inhibition of the translation process itself, in vitro translation reactions were performed. Viral mRNA from infected L929 cells was used to program rabbit reticulocyte lysate in the absence and presence of 3 μM clavulone, a concentration able to inhibit viral yield by approximately 90%. ^{35}S-methionine-labeled synthesis products were precipitated with anti-VSV antibody, resolved on SDS gels and fluorographed. Fig. 5 shows the result of such an experiment. No significant effect on in vitro translation was observable when clavulone was present in the reaction mixture. Therefore, the disappearance of viral proteins cannot be accounted for by a direct interference at the level of translation, but supports the idea that transcription rather than translation is inhibited by clavulone as indicated by the delayed cpe. Note that the in vitro system does not perform glycosylation. As a consequence, the G protein migrates with the unglycosylated VSV G marker on the right side.




*λ c 0 2.7 5 10 [μM clavulone]
A
9.4
23.1
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4.3
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C
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Northern analysis of viral messages

To investigate any antiviral activity prior to translation, Northern analyses were performed with VSV-specific probes to the individual messages. VSV carries out primary transcription as initial step after entry into the cell. Coupled translation regulates full length transcription of (+)RNA which serves as a template for genomic (−)RNA (Banerjee, 1987). Secondary transcription synthesizes more viral message from this genomic template. A temperature-sensitive VSV mutant, tsG 41 (Pringle, 1970), was used to discriminate between these two transcriptional events. At 30°C, this mutant behaves like wild-type VSV (Masters and Samuel, 1983a; Marcus and Sekellick, 1978). At the nonpermissive temperature of 39°C, tsG 41 is only able to carry out primary transcription and synthesizes mRNA from the parent genome, whereas full-length transcripts cannot be made due to a heat labile N protein (Knipe et al., 1977). In plaque assays at the permissive temperature tsG 41 responded to clavulone treatment in the same dose-dependent manner as the wild-type (data not shown).

For the analysis of viral mRNA, L929 cells were infected with tsG 41 at 30°C for 1 h. Since the mutant cannot amplify its genome at the restrictive temperature, the MOI was adjusted to approx. 100 to overcome a lower level of mRNA synthesis. After the infection period, unadsorbed virus was removed and clavulone was added at the indicated concentrations. Then samples were shifted to 39°C so that no secondary transcription could take place. After 6 h of incubation, viral yield was determined in plaque assays at 30°C and 39°C to confirm that no VSV revertants occurred and the RNA was processed as described before. Fig. 6 shows autoradiograms of Northern blots of the five viral messages in the presence of clavulone. A dose-dependent reduction of all viral mRNAs occurs with increasing clavulone concentrations. One exception is seen on panel C for the M-mRNA: although membranes were reprobed and showed a dose dependency, the lane of RNA from untreated infected cells gave only a poor signal for unknown reasons. Despite a high MOI, long exposure times were necessary to visualize the probed messages. Moreover, higher molecular weight RNA hybridized under the experimental conditions.

←

Fig. 6. Northern analysis of viral mRNA from clavulone-treated tsG 41-infected L929 cells. Cells were infected with VSV mutant tsG 41 at 30°C at an MOI of approx. 100 for 1 h. Unadsorbed virus was removed and fresh medium containing clavulone at the indicated concentrations was added. Then, cells were kept at 39°C for 6 h and subsequently processed as described in Materials and Methods. The ethidium-bromide-stained gel is representative for the experiments to show equal loading of lanes. Membranes for the Northern analysis were probed and reprobed in the order NS, M, N and G whereas L was probed against RNA from another experiment. Molecular weight standards are *Hin*dIII digested radioactive fragments of (*λ), the numbers indicate kilobases (kb).

Discussion

Clavulone II, derived from soft coral and previously evaluated for its antiproliferative activity, has now been demonstrated to have antiviral activity. The molecular basis of the antiviral effect against VSV in murine L929 fibroblasts was examined in detail. The results obtained suggest that clavulone inhibits viral transcription which prevents both synthesis of viral RNA and coupled synthesis of viral proteins. This ultimately leads to a dose-dependent reduction of released virus particles from the cell.

As reported previously, clavulone has a higher antiproliferative potency than PGA (Honda et al., 1987). The antiviral activity of this prostanoid as established here is also greater than that of PGA. In comparison with PGA_1, clavulone was approximately five times more potent. Since treatment of VSV-infected cells resulted in significant reduction of infectious viral particles and of viral proteins, it appears that not infectivity per se but the total number of virus particles released per cell was reduced. Clearly the antiviral effect of clavulone is not a consequence of cytotoxicity, since approximately 50 to 70-fold lower concentrations are required to block 50% of the virus than to destroy 50% of the cells. Thus it represents a true antiviral phenomenon which is unrelated to any cellular effects of the compound.

Using Western analysis viral protein content in supernatants and cell extracts of infected control cells was compared to that in clavulone-treated infected cells. Supernatants showed a dose-dependent decrease of viral protein reflecting a reduced release of virus from the cell. The latest point in the VSV multiplication cycle where viral inhibition could conceivably take place would be the virion maturation and release from cells (Masters and Samuel, 1983b). Interference of clavulone at this point would result in an accumulation of viral proteins synthesized during the infectious cycle. This was not found when cellular extracts from clavulone-treated cells were analyzed. Treatment resulted in uniform reduction of all five viral proteins. This indicates that large amounts of viral proteins were never synthesized in the presence of clavulone.

Two possibilities could account for the observed overall reduction of viral proteins: either the translation process itself was impaired in the presence of clavulone leading to inefficient protein synthesis, or the amount of viral mRNA was limiting in translation. To test whether translation was inhibited, in vitro experiments were performed. In the presence of clavulone no change in the amounts of immunoprecipitable proteins was observed. On the other hand, Northern analysis revealed a dose-dependent reduction of viral RNA. With increasing clavulone concentrations the amount of detectable viral RNA diminished, thus explaining reduction of VSV proteins in both supernatant and cells extract. Although higher molecular weight RNA appeared upon hybridization, no signs of increased degradation of viral message was observed. Thus our data suggest molecular inhibition at the level of transcription, similar to the effect of PGA_1 in VSV-infected L-1210 leukemia cells (Bader and Ankel, 1990). Since cytotoxic effects are absent during the

incubation period, clavulone should be considered equivalent with other antivirally active PGs. Prostaglandins of the A series were described as inducing a 70-kDa heatshock protein (Ohno et al., 1988; Santoro et al., 1989b). Interestingly, clavulone also induced this protein (data not shown), underscoring its similarity to PGAs.

The observation that PGAs and clavulone have similar antiviral properties supports the hypothesis that the reactive α,β-unsaturated carbonyl group that is common to both compounds is responsible for these similar effects. The fact that clavulone exceeded antileukemic and antiviral effects of PGA might be due to a higher reactivity or to greater stability of this prostanoid, a question that remains to be resolved.

Little is known about the in vivo effects of clavulone, thus its eventual application as an antiviral drug would depend on appropriate animal models. Studies of its antitumor activity have been carried out in sarcoma-bearing mice (Honda et al., 1988). Daily intraperitoneal treatment of ICR mice bearing sarcoma 180 with lipid microsphere-entrapped clavulone II at 40 μmol/kg/day for 5 consecutive days resulted in increased survival time and decreased tumor growth in these mice. Since in vitro 4-μM concentrations are sufficient to cause 90% inhibition of VSV, it appears feasible that antiviral concentrations of this compound could also be achieved in vivo.

Acknowledgements

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