ANTIBIOTICS THAT SPECIFICALLY BLOCK TRANSLATION IN VIRUS-INFECTED CELLS

JUAN CARLOS LACAL, DAVID VÁZQUEZ, JOSÉ MARIA FERNANDEZ-SOUSA* and LUIS CARRASCO

Departamento de Microbiología, Centro de Biología Molecular
C. S. I. C. and U. A. M., Canto Blanco, Madrid-34, Spain
*Antibioticos S. A., Bravo Murilio 34, Madrid, Spain

(Received for publication January 4, 1980)

Several antibiotics including anthelmycin, blasticidin S, destomycin A, gougerotin, hygromycin B and edeine complex, known to powerfully block translation in cell-free systems, did neither inhibit protein synthesis in intact mouse L and 3T6 cells, nor in hamster BHK 21 cells, due to failure to cross the cell plasma membrane. However, after viral infection, these antibiotics exhibited a marked blockade of translation, that is related to the permeability changes induced by viral infection. The inhibition of protein synthesis by hygromycin B in virus-infected cells was studied over the time course of infection, both in encephalomyocarditis virus-infected mouse L cells and in Semliki forest virus-infected hamster BHK cells. We have observed that the entry of hygromycin B into virus-infected cells parallels the inhibition of cellular protein synthesis, *i.e.*, the cells became permeable to this antibiotic at the time the shut-off of host translation occurred. A marked inhibition of picornavirus RNA synthesis by hygromycin B was also noticed, likely as a consequence of the inhibition of the viral replicase synthesis. Finally, a reduction in the virus yield by treatment of virus-infected cells with several antibiotics is also described. All these observations are considered in the context of the interference of viral infection with cellular functions and the potential use of inhibitors non-permeable to normal cells as antiviral agents.

Viral infection induces modifications in the membranes of infected cells[1,2]. Amongst such modifications we found that picornavirus-infected cells lost the permeability barrier to monovalent ions at the onset of viral protein synthesis.[3] This change takes place in such a way, that the translation of viral mRNA occurs in the cytoplasm of a cell with an altered ionic concentration.[3,4] These findings have recently been extended by several investigators to other virus-cell systems.[5,6,7] As a consequence of the modifications induced in membrane permeability after viral infection, several inhibitors for which normal cells are impermeable, readily cross the membrane of infected cells, thereby causing a specific blockage of protein synthesis in those cells.[8,9,10] The alteration of membrane permeability is brought about by a virus-coded component,[8] most likely a virion protein and this phenomenon has now been observed in several virus-cell systems including picornaviruses,[8,9] togaviruses,[8] papovaviruses,[9,10] herpesviruses, rhabdoviruses and paramyxoviruses.[11]

In this contribution we describe two new specific inhibitors of translation in virus-infected cells: anthelmycin and destomycin A. The action of hygromycin B on viral RNA synthesis and virus production is also presented.

Materials and Methods

Cells and viruses

Mouse fibroblast L929, 3T6 cells and hamster BHK 21 cells were cultured in DULBECCO's modified

EAGLE's medium(E_4) supplemented with 10% newborn calf serum (Difco) (E4D10 medium).

Encephalomyocarditis (EMC) virus was grown on L929 cells and after removal of the cell debris by centrifugation, the supernatant was used as our virus stock. Semliki forest virus (SFV) was grown on BHK 21 cells in E_4 medium containing 2% calf serum (E4D2 medium).

Protein synthesis measurement

Cells were grown in 24-well Linbro dishes in 1 ml E4D10 medium. After viral infection at the indicated m.o.i., the cells were incubated in 1 ml E4D2 medium. At the indicated times the medium was replaced by 0.5 ml of methionine-free E4D1 medium and the indicated inhibitor added. Protein synthesis was measured by addition of 0.11 μCi (^{35}S)methionine (1,100 Ci/mmol, 5.4 mCi/ml), after incubation, the medium was removed and the cell monolayer precipitated with 5% trichloroacetic acid, washed twice with ethanol and dissolved in 0.200 ml 0.1 N NaOH; 0.150 ml were taken to estimate the radioactivity in an Intertechnique scintillation spectrometer.

When indicated, the labelled proteins were analyzed by polyacrylamide gel electrophoresis as already described.[10] Briefly, after the labelling period the cell monolayer was washed with saline phosphate buffer, pH 7.4 and dissolved in 0.15 ml 0.02 N NaOH, 0.1% SDS plus 0.15 ml sample buffer. Ten μl were applied to 15% polyacrylamide gels and runned overnight at 25~30 V. After fluorography, the gel was dried and exposed at −70°C to an X-ray film (Kodak). The densitometric tracings were obtained in a digital microdensitometer (Optronics P1700).

RNA synthesis measurement

RNA synthesis was measured by estimating the incorporation of (5,6-^{3}H)uridine (48 Ci/mmol; 1 mCi/ml) into trichloroacetic precipitable material. After viral infection, the medium was removed and replaced by 0.5 ml E4D2 medium containing 5 μg/ml actinomycin D. Hygromycin B was added at the indicated concentrations and 1 μCi (^{3}H)uridine was added. The radioactivity incorporated into RNA was estimated similarly as described above for protein synthesis.

Virus titration

Determination of the virus titer was carried out by the plaque assay method. Serial dilutions of the virus were made in saline phosphate buffer containing 0.05% calf serum and 0.5 ml of each dilution was added to cells grown in 60-mm dishes. After virus absorption during one hour at 37°C the medium was replaced by 5 ml E4D2 medium with 0.8% agar (Gifco). The cell monolayer was stained with neutral red after 3~4 days incubation at 37°C.

Results and Discussion

Screening of a Number of Translation Inhibitors on Virus-infected Cells

The effect of a number of inhibitors of protein synthesis in cultured cells has been already described.[12] We have also shown the effects on translation of several compounds in EMC-infected and uninfected 3T6 cells.[9,10] These results have now been extended to the compounds and virus-cell systems indicated in Table 1. These compounds were tested for the inhibition of translation on EMC-infected 3T6 cells and SFV-infected BHK cells. The antibiotics were present at the time that viral protein synthesis was maximal.

Hygromycin A did not show any effect on our system, as we have recently observed that it is a specific inhibitor of translation in prokaryotic systems (L. CARRASCO, unpublished results). Hygromycin B, destomycin A, anthelmycin, blasticidin S, gougerotin, edeine complex and showdomycin preferentially inhibited translation on infected cells. The chemical structure of some of these compounds is shown in Fig. 1. The aminoglycoside antibiotics paromomycin, neomycin B and gentamicin A had no effect in our system, most probably indicating that although they penetrated into infected cells, they are not potent inhibitors of translation in eukaryotic systems.

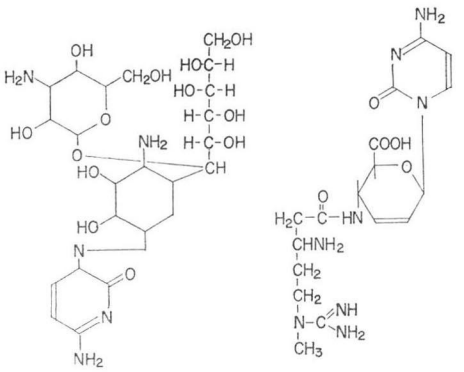
As controls the antibiotics bruceantin, cycloheximide and anisomycin that are permeable to normal

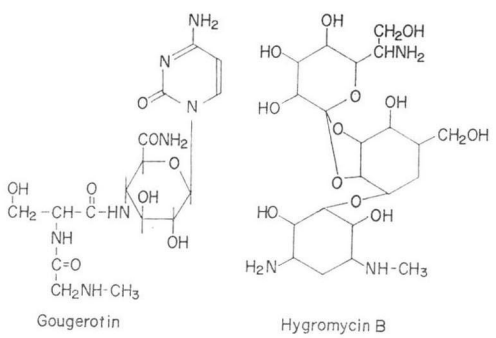
Table 1. Screening of antibiotic action on protein synthesis in virus-infected cells.

EMC-infected 3T6 and SFV-infected BHK-21 cells and 3T6 and BHK-21 mock-infected cells were assayed for protein synthesis in the presence of several antibiotics. After infection or mock-infection, antibiotics were added at 4 1/2 hours post-infection (3T6 cells) or 5 hours p.i. (BHK-21 cells) and incubated for 1 hour until the addition of 0.11 μCi (^{35}S)methionine. One hour later the reaction was stopped and the radioactivity was estimated as described in Materials and Methods: 100% of control represents 96,400 cpm (mock-infected 3T6 cells), 44,700 cpm (infected 3T6 cells), 81,200 cpm (mock-infected BHK-21 cells) and 28,600 cpm (infected BHK-21 cells).

Antibiotic	EMC-3T6			SFV-BHK		
	M	Mock-infected	Infected	M	Mock-infected	Infected
Actinobolin	10^{-4}	102	96	10^{-4}	96	78
Anthelmycin	10^{-4}	100	28	10^{-4}	95	38
Anisomycin	10^{-3}	76	62	5×10^{-3}	69	70
Blasticidin S	10^{-4}	100	11	10^{-4}	68	34
Bruceantin	10^{-7}	21	22	10^{-7}	30	16
Cycloheximide	2×10^{-7}	43	38	2×10^{-7}	39	31
Destomycin A	10^{-4}	96	81	10^{-4}	100	43
Doxycycline	10^{-4}	100	95	10^{-4}	95	96
Edeine complex	10^{-4}	100	100	10^{-4}	102	89
Gentamicin A	10^{-3}	91	89	10^{-3}	94	79
Gougerotin	10^{-4}	105	27	10^{-4}	97	59
Hygromycin A	10^{-3}	93	100	10^{-3}	107	92
Hygromycin B	10^{-4}	104	46	10^{-3}	90	25
Neomycin B	10^{-3}	100	98	10^{-3}	98	81
Paromomycin	10^{-3}	107	100	10^{-3}	101	100
Sinefungin	10^{-3}	100	100	10^{-3}	97	100
Showdomycin	—	—	—	10^{-7}	93	43
Trichodermin	—	—	—	10^{-5}	92	48
Verrucarin A	—	—	—	10^{-8}	32	34

Fig. 1. Chemical structure of anthelmycin, blasticidin S, edeine complex, gougerotin and hygromycin B.





cells did not exhibit a preferential blockade on translation in virus-infected cells as compared to control mock-infected cells. We then chose hygromycin B to continue our studies on its action on virus-infected cells.

Inhibition of Protein Synthesis by Hygromycin B on Virus-infected Cells

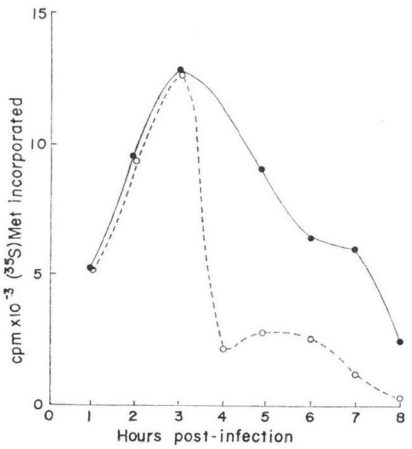
As hygromycin B presented a high specificity in its action against virus-infected cells, it was of interest to determine the time after virus infection in which hygromycin B was able to enter into the cell. EMC-infected L cells were pulse-labelled with (^{35}S) methionine every hour after virus absorption in the presence or absence of 2 mM hygromycin B. Fig. 2 shows that the shut-off of protein synthesis starts at 4 hours post-infection and concomitantly with this inhibition a clear change in membrane permeability, as detected by hygromycin B entry, is observed. Analysis in polyacrylamide gels of the proteins synthesized throughout infection indicates that picornavirus proteins are detectable after the fourth hour post-infection (results not shown). We want to emphasize that in our system, virus absorption takes place before the 0 time. Our unpublished observations indicate that modifications in membrane permeability do also occur during this early period of virus absorption and those changes are dependent on the multiplicity of infection used.

The time course of protein synthesis in SFV-infected BHK cells is shown in Fig. 3. In this system hygromycin B entry occurred early in infection, likely as a consequence of the high m.o.i. used and later on when the shut-off of host protein synthesis and the translation of late viral mRNA was apparent as determined by polyacrylamide gel electrophoresis (Fig. 4).

The conclusion from the above results is that a virus-infected cell becomes permeable to some antibiotics very early during virus absorption and later on at the time when the shut-off of host protein synthesis commences. These results reinforce the idea that the inhibition of cellular functions by viral infection is produced by an unspecific modification of the cell membrane permeability.[13]

Fig. 2. Time course of protein synthesis in EMC-infected L929 cells. Effect of hygromycin B.

EMC-infected L929 cells untreated (●) or treated (○) with 1 mM hygromycin B were assayed for the incorporation of (^{35}S)met into TCA insoluble material. Cells were infected at m.o.i. ~5~10 and incubated 1 hour at 37°C. After incubation, the medium was removed and replaced by 0.5 ml of methionine-free E_4D_1 medium and 1 mM hygromycin B, then 0.11 μCi (^{35}S)met were added. A further 1 hour incubation took place and the reaction was stopped with 1 ml 5% TCA. After washing the cell monolayer twice with 1 ml ethanol and drying under an infrared lamp, the radioactivity was measured as described in Materials and Methods.



Inhibition of RNA Synthesis on Virus-infected Cells

The replication of the picornavirus genome needs at least a virus-coded protein, that is part of

Fig. 3. Time course of protein synthesis in SFV-infected BHK-21 cells. Effect of hygromycin B.

BHK-21 cells were infected with SFV at a m.o.i. of about 3,000 and untreated (●) or treated (○) with 1 mM hygromycin B as described in the legend of Fig. 1.

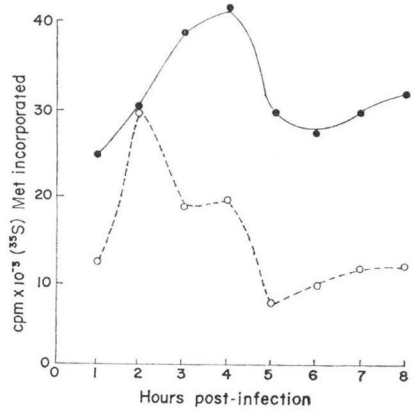


Fig. 4. Densitometric profiles of the proteins synthesized in SFV-infected BHK-21 cells.
BHK-21 cells were infected as described in Fig. 2 legend.
The analysis of the proteins synthesized was carried out as described under Materials and Methods.

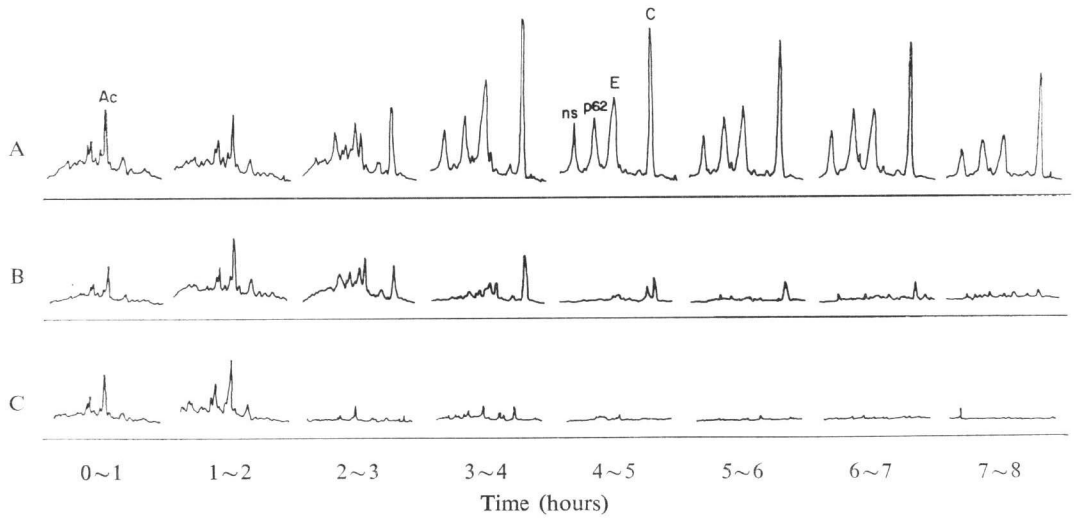


Fig. 5. Inhibition of viral RNA synthesis by hygromycin B in EMC-infected L929 cells.

L929 cells were infected at m.o.i. of 5~10 and treated with actinomycin D (5 μg/ml) after the beginning of infection. Every hour the medium was replaced by E_4D_2 medium and 1 μCi of (5,6-^{3}H)uridine was added and radioactivity measured as described in Materials and Methods.

Untreated cells (●); cells treated with 1 mM (○) or 2 mM (□) hygromycin B.

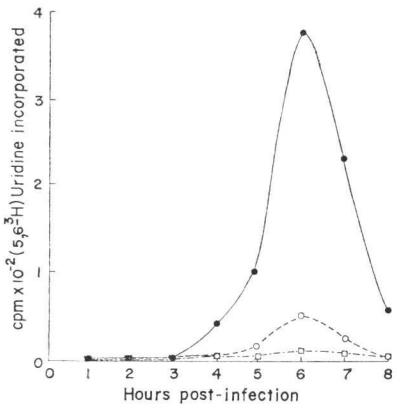


Table 2. Inhibition of virus production in EMC-infected 3T6 cells and SFV-infected BHK-21 cells.

Antibiotics were added at 2 hours p.i. and the virus was collected at 7 hours p.i. The virus titer was determined by plaque forming assay as described in Materials and Methods. 3T6 cells were infected at a m.o.i. of 5 pfu/cell and BHK-21 cells at a m.o.i. of 50 pfu/cell.

Antibiotic	EMC Production		SFV Production	
	M	% Control	M	% Control
Anthelmycin	10^{-4}	27	10^{-4}	24
Blasticidin S	10^{-4}	8	10^{-4}	26
Bruceantin	10^{-6}	14	10^{-6}	17
Edeine complex	10^{-4}	44	10^{-4}	63
Gougerotin	10^{-4}	35	10^{-4}	81
Hygromycin B	10^{-4}	4	10^{-3}	24

the viral replicase.[1,14] We reasoned that if hygromycin B was able to block translation in virus-infected cells at the onset of viral protein synthesis, this would deplete the levels of viral replicase leading to a reduction of viral RNA synthesis. Fig. 5 shows that indeed this was the case. Viral RNA synthesis was measured in the presence of actinomycin D to block cellular RNA synthesis and hygromycin B was present at the beginning of viral infection. Thus, the inhibitor entered into the infected cell once membrane permeability was altered, causing the depletion in əɥɟ viral replicase levels that resulted, in

turn, in a strong inhibition of viral RNA synthesis.

Inhibition of Virus Progeny Production

Since the specific inhibitors selected from Table 1 caused a drastic reduction on viral protein synthesis in infected cells, it was of interest to determine whether such inhibition in the availability of mature virion proteins influenced virus progeny production. The antibiotics shown in Table 2 were added to the culture medium two hours after viral infection and the virus produced was collected seven hours after infection. The virus titration was carried out by the plaque forming assay. The inhibitors tested caused an inhibition in the virus progeny production to varying degrees. Maximal inhibition was observed with hygromycin B, 0.1 mM which caused over 90% inhibition in EMC virus production. These results encourage the search for compounds not permeable to normal cells and with a high inhibitory effect on *in vitro* protein-synthesizing systems. Such inhibitors should act selectively on virus-infected cells and produce a drastic reduction on virus yield.

Acknowledgments

We want to express our acknowledge to Fundación EUGENIO RODRÍGUEZ PASCUAL for financial support, to Ely Lilly for a generous sample of hygromycin B and to Ms. ANGELES RAMOS for expert technical assistance.

References

1) CARRASCO, L. & A. E. SMITH: Molecular Biology of Animal Viris Infection. The Intern. Encyclop. of Pharmacology and Therapeutics. Pergamon Press. (In press) 1980
2) KOHN, A.: Early interactions of viruses with cellular membranes. Adv. Virus Research 24: 223~276, 1979
3) CARRASCO, L. & A. E. SMITH: Sodium ions and the shut-off of host cell protein synthesis. Nature 264: 807~809, 1976
4) EGBERTS, E.; P. B. HACKETT & P. TRAUB: Alteration of the intracellular energetic and ionic conditions by mengovirus infection of EHRLICH ascites tumor cells and its influence on protein synthesis in the mid-phase of infection. J. Virol. 22: 591~597, 1977
5) NAIR, C. N.; J. W. STOWERS & B. SINGFIELD: Guanidine-sensitive Na^+ accumulation by poliovirus-infected HeLa cells. J. Virol. 31: 184~189, 1979
6. GARRY, R. F.; J. M. BISHOP, S. PARKER, K. WESTBROOK, G. LEWIS & M. R. F. WAITE: Na^+ and K^+ concentrations and the regulation of protein synthesis in Sindbis virus-infected chick cells. Virology 96: 108~120, 1979
7) CHERNEY, C. S. & J. M. WILHELM.: Differential translation in normal and adenovirus type 5-infected human cells and cell-free systems. J. Virol. 30: 533~542, 1979
8) CARRASCO, L.: Membrane leakiness after viral infection and a new approach to the development of antiviral agents. Nature 272: 694~699, 1978
9) CARRASCO, L.: The development of new antiviral agents based on virus-mediated cell modifications. *In* "Antiviral Mechanisms for the Control of Neoplasia" pp. 623~631. P. CHANDRA, *ed.* Plenum Press, New York, 1979
10) CONTRERAS, A. & L. CARRASCO: Selective inhibition of protein synthesis in virus-infected mammalian cells. J. Virol. 29: 114~122, 1979
11) BENEDETTO, A.; G. B. ROSSI, A. AMICI, F. BELARDELLI, L. CIOE, G. CARRUBA & L. CARRASCO: Inhibition of animal virus production by means of translation inhibitors impermeable to normal cells. Virology (Submitted for publication).
12) CONTRERAS, A.; D. VÁZQUEZ & L. CARRASCO: Inhibition by selected antibiotics of protein synthesis in cells growing in tissue culture. J. Antibiotics 31: 598~602, 1978
13) CARRASCO, L.: The inhibition of cell functions after viral infection. A proposed general mechanism. FEBS Letters 76: 11~15, 1977
14) SMITH, A. E. & L. CARRASCO: Eukaryotic viral protein synthesis. *In* "Amino acid and Protein Biosynthesis, II" pp. 261~311. H. R. V. Arnstein, *ed.* Intern. Review of Biochem. Vol. 18. University Park Press, Baltimore, 1978