JOURNAL OF VIROLOGY, Apr. 1980, p. 293-296
0022-538X/80/04-0293/04$02.00/0

Vol. 34, No. 1

# Specificity of Protein Synthesis Inhibitors in the Inhibition of Encephalomyocarditis Virus Replication

TRIPRAYAR V. RAMABHADRAN AND ROBERT E. THACH*
*Department of Biology, Washington University, St. Louis, Missouri 63130*

Effect of protein synthesis inhibitors on encephalomyocarditis virus production in L-cells was studied. Inhibition of initiation by hypertonicity, harringtonine, or pactamycin decreased viral protein synthesis to a lesser extent than that of host. Virus yield was unaffected or actually enhanced by low concentrations of these inhibitors. On the contrary, the elongation inhibitors cycloheximide, anisomycin, and emetine, shown previously to inhibit viral protein synthesis preferentially, had a greater effect on virus yield than on overall protein synthesis. These results support our earlier proposal that the antiviral activity of cycloheximide derives from its specific effect on the rate of elongation of protein synthesis, and that elongation inhibitors in general may show varying degrees of specific antiviral activity.

We have recently reported that cycloheximide, an inhibitor of an elongation step in protein synthesis, specifically suppresses replication of the virulent viruses encephalomyocarditis virus (EMC) and vesicular stomatitis virus when present in low concentrations during the infectious cycle. Cellular protein synthesis, as well as the synthesis of murine leukemia virus proteins, is inhibited to a much less extent at low cycloheximide concentrations. Thus, the effect of low concentrations of this drug was shown to have a specificity similar to that of interferon (20).

The putative basis for the antiviral specificity of cycloheximide has been discussed in detail earlier (6, 20). It was pointed out that the high efficiency of many viral mRNA's in the initiation of protein synthesis (2, 6-8, 11-14) might be used as a signal for the discrimination between viral and host mRNA's. Owing to this rapid initiation capacity, the translation of these viral mRNA's is likely to be limited by elongation, as shown to be the case for EMC messages in vivo (6). In contrast, protein synthesis directed by slowly initiating host mRNA is limited by the frequency of initiation. Given the difference between the two types of mRNA, any decrease in elongation rate should inhibit the translation of elongation-limited viral mRNA's far more than that of the initiation-limited host mRNA's. Consistent with this reasoning, cycloheximide and other elongation inhibitors have been shown to decrease the rate of EMC protein synthesis preferentially (6). The selective inhibition of EMC and vesicular stomatitis virus production by cycloheximide has been ascribed to this phenomenon (20).

According to this interpretation the antiviral specificity of cycloheximide should also be seen with any other agent that causes preferential inhibition of elongation steps. In contrast, agents that inhibit initiation steps selectively should not inhibit virus production more than the overall protein synthesis rate. To test these predictions, we measured the effect of low levels of other elongation and initiation inhibitors on virus production. Data presented in this report establish a correlation between the mode of action of these inhibitors and their differential effects on viral protein synthesis and virion production, confirming and extending previous observations (6, 20).

Anisomycin and emetine are inhibitors of elongation steps in protein synthesis (4, 15, 19). To determine whether, like cycloheximide, they also exhibit specific antiviral activity, their ability to inhibit virus production and overall protein synthesis in EMC-infected cells was determined. Procedures for infection, pulse labeling, and plaque assays have been described earlier (20). Mouse L-cells were infected at a multiplicity of 10 PFU/cell, and different concentrations of the drugs were added immediately after the adsorption period. Incorporation of [$^{35}$S]methionine into acid-precipitable material was determined at 4.5 h postinfection in the presence of the drugs. Virus yield was determined at 12 h postinfection.

It is evident from Fig. 1 that, among the three inhibitors, cycloheximide inhibited the production of virus to the greatest extent while having the least effect on methionine incorporation. With anisomycin and emetine, greater inhibition of total protein synthesis was required to obtain a comparable inhibition of virus yield. The

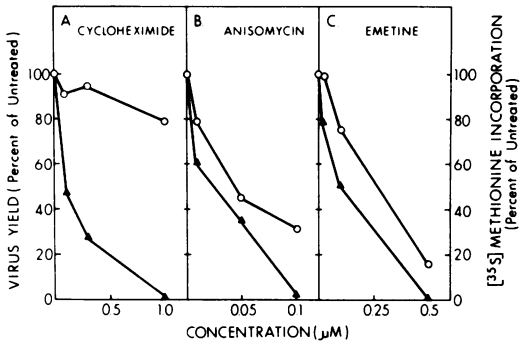


FIG. 1. *Inhibition of virus yield (▲) and [$^{35}$S]methionine incorporation (○) by elongation inhibitors in EMC-infected L-cells. Infected cells were treated with different concentrations of inhibitors starting immediately after infection. Methionine incorporation during a 15-min pulse was determined at 4.5 h after infection, and virus yield was determined at 12 h postinfection.*

[$^{35}$S]methionine incorporation curves included here and in Fig. 3 show that the inhibition of virus production was not simply a trivial result due to nonspecific inhibition of total protein synthesis by prolonged drug treatment. At these low concentrations of inhibitors, very similar inhibition of protein synthesis was observed in uninfected cells treated identically. These determinations were done at 4.5 h postinfection, since in untreated cells at this time a significant amount (20 to 30%) of the total protein synthesis is virus directed. At later times, the protein synthesis in untreated cultures declines, owing to the onset of cell lysis.

It is not obvious why cycloheximide shows greater specificity in inhibiting virus production than anisomycin or emetine. However, the differences seen here are strikingly similar to those reported by Jen et al. (6), where the instantaneous effect of these drugs on viral and host protein synthesis was compared. In those experiments cycloheximide was also found to be most selective, whereas emetine was least. Thus it is clear that the specificities of these elongation inhibitors in reducing virus yield correlate closely with their specificities in the instantaneous reduction of host and viral protein synthesis. This difference is also manifested in Table 1, where the antiviral effects of a variety of inhibitory agents are compared. In addition to the antibiotics shown in Fig. 1, streptovitacin A, a glutarimide antibiotic related to cycloheximide (4, 15), also showed some specificity in inhibiting virus production. However, histidinol and *O*-methyl threonine, competitive inhibitors of charging of histidine and isoleucine tRNA's, respectively (18), showed no specificity. The lack of specificity in these cases is most probably due to the concomitant inhibition of initiation that occurs as a consequence of lower levels of charged tRNA's (18).

The rationale described in earlier paragraphs to explain the specificity of elongation inhibitors in reducing virus protein synthesis and virus yield implies that inhibitors of initiation should not produce specifically antiviral effects. The results of studies using the initiation inhibitors pactamycin, harringtonine (4, 15, 19), and hypertonic initiation block (HIB) with NaCl (16) are shown in Fig. 2 and 3. In the experiments shown in Fig. 2, EMC-infected L-cells were exposed to the inhibitors at 3.25 h after infection and pulse-labeled 15 min later for a 15-min period with radioactive methionine in the presence of the drug. Since the inhibitors were added late in infection, just before pulse-labeling, these data represent an instantaneous effect of the drugs on the rates of protein synthesis. Pulse-labeled proteins were separated on sodium dodecyl sulfate-polyacrylamide gels, and the extent of incorporation into viral and host bands was determined from the scans of autoradiograms as described earlier (6). Equal amounts of radioactivity were analyzed in each gel lane; hence, an increase in relative radioactivity in Fig. 2 represents a greater than average resistance to inhibition by a given agent, and vice versa.

It is clear from Fig. 2 that all three initiation inhibitors caused relative increase in viral bands, indicating that viral translation is more resistant to inhibition by these agents than host translation. The results obtained here are in agreement with earlier reports on the effects of HIB on poliovirus protein synthesis (14). However, as in the case of elongation inhibitors (6), there were

TABLE 1. *Antiviral specificity of protein synthesis inhibitors*[a]

| Inhibitor | Relative virus yield (%) |
|---|---|
| None | 100 |
| Cycloheximide | 27 |
| Anisomycin | 38 |
| Streptovitacin A | 46 |
| Emetine | 63 |
| *O*-Methyl threonine | 80 |
| Histidinol | 86 |
| Blasticydin | 90 |
| HIB | 95 |
| Harringtonine | 96 |
| Pactamycin | 110 |

[a] Experiments were conducted and assayed as in Fig. 1 and 3, except that uninfected L-cells were used to determine the drug concentration that produced a 20% inhibition of protein synthesis. Data are relative virus yield at this drug concentration.

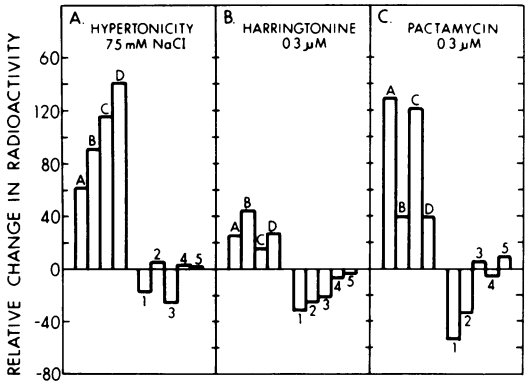


FIG. 2. *Differential effect of initiation inhibitors on EMC protein synthesis. Drugs were added to infected cultures at 3.25 h after infection, and [$^{35}$S]methionine was added at 3.5 h. After a 15-min labeling period, cells were harvested, and labeled proteins were analyzed as described in the text. Overall inhibition of trichloroacetic acid-precipitable incorporation relative to untreated cultures was: HIB, 53%; harringtonine, 50%; pactamycin, 78%. Molecular weights (×10$^3$) were: for EMC proteins, A, 100; B, 90; C, 84; D, 75; for host bands, 1, 47; 2, 53; 3, 60; 4, 80; 5, 93.*

differences among the initiation inhibitors in the specific patterns and the overall extents to which host and viral protein syntheses were affected. It is possible that these differences reflect the varying degrees to which these drugs affect elongation. For example, pactamycin has been reported to have some effects on elongation steps at all concentrations tested in HeLa cells (17). In addition, although harringtonine has been shown to cause polysome runoff in vivo (3, 5, 17), it has been suggested that this may be a consequence of inhibition of early steps of elongation (1).

Figure 3 shows the effect of HIB, harringtonine, and pactamycin on virus production and overall protein synthesis during the replicative cycle of EMC. Experimental details are similar to those described in the legend of Fig. 1. Consistent with the data in Fig. 2, all three initiation inhibitors reduced overall protein synthesis to a greater extent than they inhibited virus production, at least at the lowest doses tested (it is not clear why virus production was severely inhibited above 45 mM additional NaCl; this may be due to a secondary effect of HIB). The same results are seen in Table 1. These results are the converse of those obtained with elongation inhibitors, shown in Fig. 1 and Table 1, and support the previous observation regarding the effect of pactamycin on viral protein synthesis (20). Surprisingly, pactamycin reproducibly stimulated virus production by 30 to 40%. However, we were unable to detect an absolute increase in the rate of viral protein synthesis (data not shown). The reason for this discrepancy is unclear.

It is tempting to explain the specificity of initiation inhibitors in terms of the protein synthesis model of Lodish (10), as has been proposed by Nuss et al. (14) for the effect of HIB on poliovirus translation. In this model, any agent that reduces the concentration of the rate-limiting component for initiation, R*, will inhibit host translation more than EMC viral translation, inasmuch as the viral mRNA is believed to have such a high affinity for R* that its translation is limited by elongation and not by initiation. Since it was previously shown that EMC mRNA does in fact initiate much faster than host mRNA in vivo (6) as well as in vitro (2), this interpretation suggests that pactamycin and harringtonine both cause a reduction in R*, as was proposed for HIB (14). However, there are other possible interpretations which could produce the same result, and the initiation inhibitors frequently produce side effects unrelated to protein synthesis which may complicate the data, especially at the higher concentrations. Thus, until more about the mechanisms by which the initiation inhibitors act is known, it is impossible to draw firm conclusions as to precisely why they appear to be mRNA specific.

In contrast, the results obtained with elongation inhibitors are more readily interpretable, primarily because their mechanism of action is relatively better understood (3, 4, 15, 19). In each case it has been shown that, at low concentrations, polysome size increases as the net translation rate declines (9). Since this can only mean

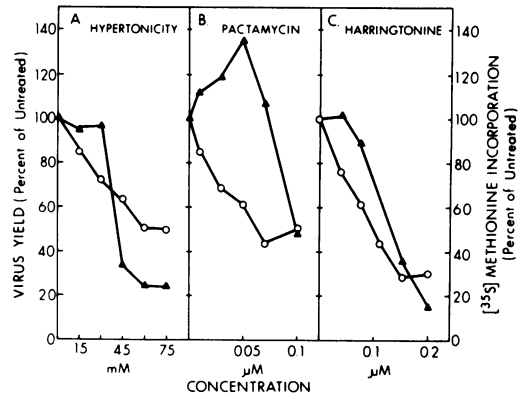


FIG. 3. *Inhibition of virus yield (▲) and [$^{35}$S]methionine incorporation (○) by initiation inhibitors in EMC-infected L-cells. Experimental details as in Fig. 1.*

that the elongation rate decreases relative to initiation rate, and since the drugs appear to act irrespective of amino acid composition of the protein being synthesized, the only explanation for their apparent mRNA specificity must lie in the different rates at which different mRNA's are initiated (i.e., in Lodish's terminology, the value of $K_I$). However, even in the case of elongation inhibitors the reasons for differences in specificity are not clear (see Fig. 1), possibly reflecting differential inhibition of initiation steps, or differences in the reversibility of ribosome binding.

The data presented here and previously (6) demonstrate a clear correlation between the differential effect of the inhibitors on EMC viral protein synthesis on the one hand, and their effects on virion production on the other. These results further support the conclusion that the antiviral activity of cycloheximide derives from its effect on protein synthesis elongation (20), and that elongation inhibitors in general may exhibit varying extents of specific inhibitory activity on virus replication. However, it should be emphasized that these results apply only to viruses which produce mRNA's that initiate unusually rapidly, such as EMC. Recent evidence suggests that reovirus production is not unusually sensitive to elongation inhibitors, and most reo mRNA's initiate very slowly (W. Walden, T. Brendler, T. Godefroy-Colburn, and R. E. Thach, unpublished data).

Cell culture media were provided by the Basic Cancer Research Center of Washington University School of Medicine.

This work was supported by Public Health Service grant CA-13008 from the National Institutes of Health and by National Science Foundation grant PCM 76-03079.

## LITERATURE CITED

1. **Fresno, M., A. Jimenez, and D. Vazquez.** 1977. Inhibition of translation in eukaryotic systems by harringtonine. Eur. J. Biochem. **72**:323–330.
2. **Golini, F., S. S. Thach, C. H. Birge, B. Safer, W. C. Merrick, and R. E. Thach.** 1976. Competition between cellular and viral mRNAs *in vitro* is regulated by a messenger discriminatory initiation factor. Proc. Natl. Acad. Sci. U.S.A. **73**:3040–3044.
3. **Grollman, A. P., and M. T. Huang.** 1973. Inhibitors of protein synthesis in eukaryotes: tools in cell research. Fed. Proc. **32**:1673–1678.
4. **Grollman, A. P., and M. T. Huang.** 1976. Inhibitors of protein synthesis in eukaryotes, p. 125–167. *In* E. H. McConkey (ed.), Protein synthesis. Marcel Dekker Inc., New York.
5. **Huang, M. T.** 1975. Harringtonine, an inhibitor of initiation of protein biosynthesis. Mol. Pharmacol. **11**:511–519.
6. **Jen, G., C. H. Birge, and R. E. Thach.** 1978. Comparison of initiation rates of encephalomyocarditis virus and host protein synthesis in infected cells. J. Virol. **27**:640–647.
7. **Koch, G., H. Oppermann, P. Bilello, F. Koch, and D. Nuss.** 1978. Control of peptide chain initiation in uninfected and virus infected cells by membrane mediated events. Leukemia Res. **2**:541–555.
8. **Lawrence, C., and R. E. Thach.** 1974. Encephalomyocarditis virus infection of mouse plasmocytoma cells. I. inhibition of cellular protein synthesis. J. Virol. **14**:598–610.
9. **Lodish, H. F.** 1971. Alpha and beta globin messenger ribonucleic acid. J. Biol. Chem. **246**:7131–7138.
10. **Lodish, H. F.** 1974. Model for the regulation of mRNA translation applied to haemoglobin synthesis. Nature (London) **251**:385–388.
11. **Metz, D.** 1975. The mechanism of interferon action. Cell **6**:429–439.
12. **Oppermann, H., and G. Koch.** 1976. On the regulation of protein synthesis in vaccinia virus infected cells. J. Gen. Virol. **32**:261–273.
13. **Nuss, D. L., and G. Koch.** 1976. Differential inhibition of vesicular stomatitis virus polypeptide synthesis by hypertonic initiation block. J. Virol. **17**:283–286.
14. **Nuss, D. L., H. Oppermann, and G. Koch.** 1975. Selective blockage of initiation of host protein synthesis in RNA-virus-infected cells. Proc. Natl. Acad. Sci. U.S.A. **72**:1258–1262.
15. **Pestka, S.** 1977. Inhibitors of protein synthesis, p. 467–553 *In* H. Weisbach and S. Pestka (ed.), Molecular mechanisms of protein biosynthesis. Academic Press, New York.
16. **Saborio, J. L., S. S. Pong, and G. Koch.** 1974. Selective reversible inhibition of initiation of protein synthesis in mammalian cells. J. Mol. Biol. **85**:195–211.
17. **Tscherne, J. S., and S. Pestka.** 1975. Inhibition of protein synthesis in intact HeLa cells. Antimicrob. Agents Chemother. **8**:479–487.
18. **Vaughan, M. H., and B. S. Hansen.** 1973. Control of initiation of protein synthesis in human cells. Evidence for a role of uncharged transfer ribonucleic acid. J. Biol. Chem. **248**:7087–7096.
19. **Vazquez, D.** 1978. Translation inhibitors, p. 169–232. *In* H. R. V. Arnstein (ed.), International review of biochemistry; Amino acid and protein biosynthesis II, vol. 18. University Park Press, Baltimore.
20. **Yau, M. P., T. Godefroy-Colburn, C. H. Birge, T. V. Ramabhadran, and R. E. Thach.** 1978. Specificity of interferon action in protein synthesis. J. Virol. **27**:648–658.