ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 1991, p. 2630–2633
0066-4804/91/122630-04$02.00/0

Vol. 35, No. 12

Hygromycin B Inhibits Synthesis of Murine Coronavirus RNA

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Received 24 May 1991/Accepted 16 September 1991

The aminoglycoside hygromycin B inhibits the infection of mouse hepatitis virus (MHV) A59 both in vitro and in vivo. In probing the mechanism by which hygromycin B exerts its antiviral effect, we describe here studies which point to inhibition of viral RNA synthesis as the key step in virus replication which is affected by the drug. Cells which are infected with MHV do not take up higher levels of hygromycin B than do uninfected ones. Comparative assays of MHV replication and MHV protein synthesis in the presence of hygromycin B and another aminoglycoside, neomycin, indicate that hygromycin B is the more-effective antiviral agent and that its antiviral activity likely does not involve phosphoinositide-mediated processes such as those inhibited by neomycin.

We have previously demonstrated the ability of the aminoglycoside hygromycin B to inhibit infections of mouse hepatitis virus (MHV) A59 (12) both in vitro (11) and in vivo (10). Despite the antiviral potential of hygromycin B against MHV and other virus infections (1), the mechanism(s) of action remains incompletely understood. In this report, we provide some new insights into the mechanism of the antiviral activity of hygromycin B and provide evidence for a previously unsuspected mode of action.

Hygromycin B belongs to a class of semipermeable compounds which have translation-inhibiting properties. Studies with Semliki Forest virus revealed selective inhibition of viral protein synthesis with drugs which do not normally pass through the cell membrane. Easily permeating drugs such as anisomycin, cycloheximide, and trichodermin were found to inhibit both cell and viral-protein synthesis to similar extents (14). Further investigations showing enhanced uptake of alpha-sarcin into virus-infected cells were taken as evidence for increased permeability of virus-infected cells, thereby providing a possible explanation for their selective susceptibility to poorly permeating translation inhibitors such as hygromycin B (15).

Other studies revealed changes in cell membrane permeability to certain substrates (e.g., 2-deoxy-D-glucose) but not others (e.g., amino acids and monovalent cations) as a result of infection with Semliki Forest virus or vesicular stomatitis virus (6). Moreover, selective inhibition of viral-protein synthesis by either guanylyl(β,γ-methylene)diphosphonate (5) or hygromycin B (2) occurred in the absence of differences in the uptake of drug by virus-infected or mock-infected cells.

Few additional insights are currently available to resolve the existing uncertainty surrounding the antiviral effects of hygromycin B. In an effort to partially resolve such uncertainty, particularly with regard to the strong therapeutic potential of this drug against MHV infection and murine hepatitis, we have undertaken a study aimed at identifying the step in MHV infection which is the target of hygromycin B activity.

Cytotoxicity assays of hygromycin B. Viability of L-2 cells exposed to various concentrations of hygromycin B (Sigma) was tested by using both trypan blue exclusion and neutral red uptake assays. Monolayer cultures of L-2 cells were incubated for 12 h at 37°C in medium containing 0, 0.25, 0.5, 1.0, 2.0, 5.0, or 10 mM hygromycin B. The medium was minimal essential medium (prepared from Flow Laboratories' Autopow) supplemented with 5% fetal calf serum (GIBCO), to which were added glutamine (292 μg/ml) and sodium bicarbonate (2 mg/ml). Antibiotics (GIBCO) (penicillin G [100 U/ml], streptomycin [100 μg/ml], and kanamycin [100 μg/ml]) were routinely included (except in the microbiological assays of hygromycin B uptake described below). For the trypan blue exclusion assay, cells were trypsinized, suspended in medium containing 0.1% trypan blue, and examined by light microscopy for enumeration of unstained (viable) and stained cells. For the determination of neutral red uptake (16), monolayers were exposed to neutral red (0.01% in medium) for 30 min at 37°C and washed, and the dye was extracted with a mixture of 1% acetic acid and 50% ethanol. By both trypan blue exclusion and neutral red uptake assays, more than 90% of L-2 cells were viable after exposure to hygromycin B concentrations of up to 5 mM. Cells exposed to 10 mM hygromycin B showed reduced viability (70%), indicating cytotoxicity at this concentration of drug.

Hygromycin B uptake into MHV- or mock-infected cells. L-2 cell monolayers in 75-cm^2 flasks were maintained in antibiotic-free medium for 2 days prior to inoculation with MHV (at a multiplicity of infection [MOI] of 3) and treatment with hygromycin B at various concentrations. Monolayers were washed four times with phosphate-buffered saline and once with Tris-buffered sorbitol (20 mM Tris [pH 7.4], 0.25 M sorbitol) and scraped with 3 ml of Tris-buffered sorbitol. Cells were spun into pellets, suspended in 3 ml of reticulocyte-standard buffer (10 mM Tris [pH 7.4], 10 mM NaCl, 1.5 mM $MgCl_2$), incubated on ice for 10 min, and Dounce homogenized. Homogenates were clarified for 10 min at 2,000 × *g*, and the supernatant cytoplasmic fractions were assayed for hygromycin B content by bioassay with *Bacillus subtilis* as the indicator bacterium (18), with the following modifications. Molten antibiotic agar no. 19 (Difco; 38 g/liter) at 60°C (250 ml) was inoculated with 0.1 ml of *B. subtilis* spores (Difco). The agar (150 ml) was then poured into a bioassay dish (245 by 245 by 20 mm) (Nunc) and allowed to

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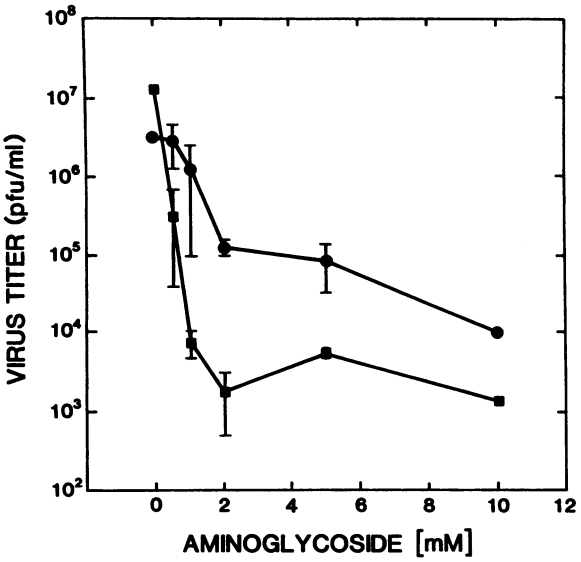

FIG. 1. Comparison of antiviral activities of the aminoglycosides hygromycin B (■) and neomycin (●). MHV-inoculated L-2 cell cultures were incubated in medium containing either hygromycin B or neomycin, and virus in the supernatant medium was quantitated by plaque assay. The result for each concentration is the mean ± standard deviation for three replicate samples.

solidify. Wells were cut in the agar with a sterile 10-mm-diameter cylinder. A standard curve of hygromycin B concentrations was constructed by diluting hygromycin B into minimal essential medium supplemented with 5% fetal calf serum in a range of concentrations from 0 to 50 mM. The bioassay dish was incubated at 32°C for 20 h, and the zones of inhibition were measured with a micrometer. When transformed logarithmically, the values for standard concentrations of hygromycin B plotted against inhibition zone sizes were symmetrically distributed along a straight line. Replicate values of zone sizes obtained for standard hygromycin B values (±2%) and for replicate cell cytoplasmic extracts (±10%) were remarkably consistent even between separate plates, and the lower limit of detection was 1.0 mM hygromycin B. Cytoplasmic hygromycin B levels were assayed in MHV- and mock-infected cells which had been incubated in medium containing 5 or 10 mM hygromycin B for 6 h at 37°C (at which time the MHV-infected cell cultures were completely fused). Cytoplasmic drug concentrations were determined on triplicate samples from each of four separate experiments. No statistically significant differences were found in cytoplasmic concentrations of hygromycin B between mock-infected (mean ± standard deviation, 1.2 ± 0.1 mM) and MHV-infected (1.3 ± 0.1 mM) cells.

Comparison of antiviral activities of the aminoglycosides hygromycin B and neomycin. Certain aminoglycosides, notably neomycin, inhibit phosphoinositide metabolism (3, 8, 17), thereby interfering with some types of transmembrane signal transduction. Since hygromycin B might act by a similar mechanism, we examined the effects of both neomycin and hygromycin B on MHV infection. L-2 cell cultures in 24-well plates were inoculated with MHV (MOI of 3) and incubated postadsorption in medium supplemented with either hygromycin B or neomycin (Sigma) at various concentrations. Virus production was assayed by plaque assay (9) of aliquots removed from supernatant media at 6 h

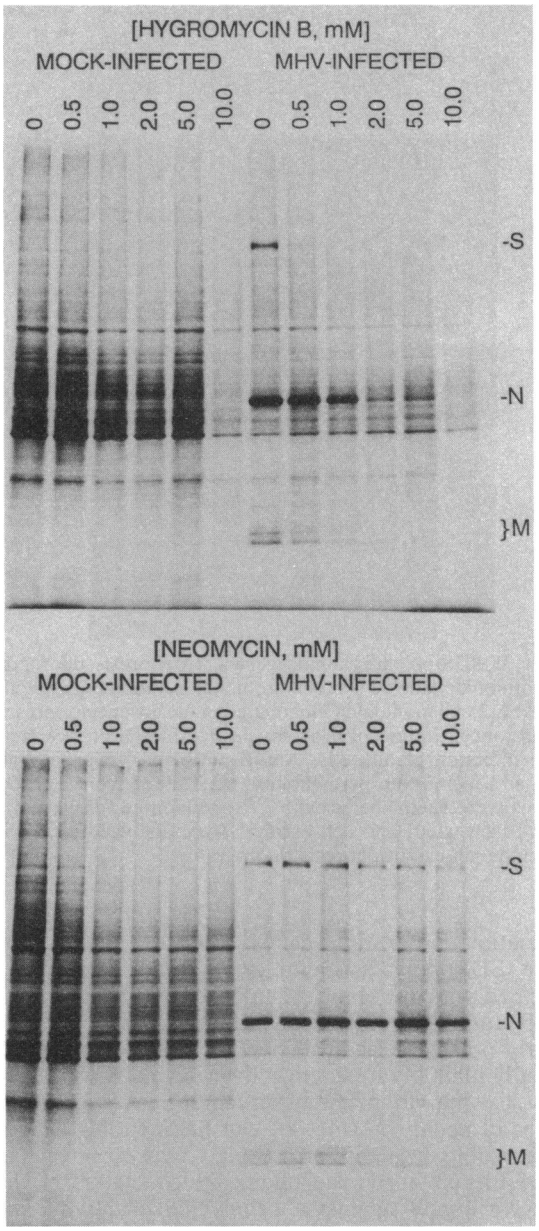

FIG. 2. SDS-polyacrylamide gel electrophoresis fluorograph of [^{35}S]methionine-labeled proteins from L-2 cells either mock or MHV infected (MOI = 3) and incubated in the absence or presence of various concentrations of hygromycin B or neomycin. S, N, and M refer to the three MHV polypeptides.

postinfection (p.i.). As shown in Fig. 1, hygromycin B was the more-effective antiviral aminoglycoside. Concentrations of hygromycin B and neomycin required to effect a 10-fold reduction in virus titer were 0.3 and 1.7 mM, respectively.

Similarly, examination of ^{35}S-labeled-protein synthesis revealed a much greater and more selective inhibition of viral-protein synthesis in response to hygromycin B than in response to neomycin (Fig. 2). Mock- and MHV-infected L-2 cell cultures in 24-well plates were incubated with hygromycin B or neomycin from 0 h p.i., radiolabeled with [^{35}S]methionine (10 μCi/ml) for 30 min at 6 h p.i., and harvested by scraping, and cell pellets were mixed with

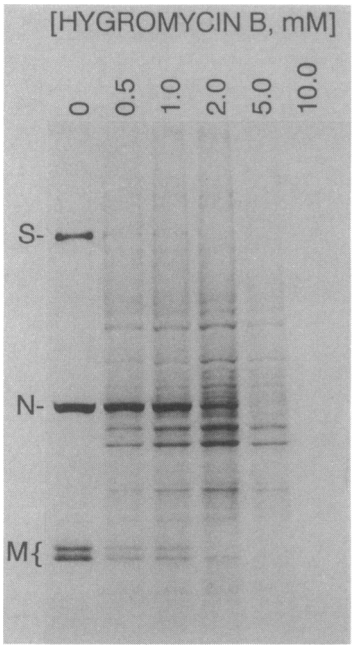

FIG. 3. SDS-polyacrylamide gel electrophoresis fluorograph of [^{35}S]methionine-labeled proteins from anti-S antibody-treated MHV-infected L-2 cell monolayers incubated in the absence or presence of various concentrations of hygromycin B. An MOI of 10 was used to ensure infection of all cells. Anti-S antibody, which inhibits cell fusion and membrane permeability, was added from 3 to 6 h p.i. Cultures were radiolabeled with [^{35}S]methionine for 30 min at 6 h p.i. and harvested, and cell extracts were analyzed by SDS-polyacrylamide gel electrophoresis fluorography.

dissociation buffer for analysis on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels (7). Gels were then fluorographed with En^{3}Hance (Dupont-NEN) according to the manufacturer's directions. Synthesis of viral proteins (particularly noticeable with respect to S and M proteins) was markedly inhibited by 0.5 mM hygromycin B, while considerably less inhibition was observed at much higher concentrations of neomycin (Fig. 2). Our finding that MHV infection is inhibited much more strongly by hygromycin B (not noted for its effects on phosphoinositide metabolism) than by neomycin argues against a neomycinlike antiviral mechanism for hygromycin B. Moreover, a comparison of the effects of neomycin and hygromycin B on protein synthesis suggests that these two aminoglycosides use different mechanisms to inhibit MHV infection. In contrast to hygromycin B, neomycin had little effect on levels of synthesized [^{35}S] methionine-radiolabeled viral protein.

Hygromycin B inhibits viral-protein synthesis and alleviates viral inhibition of host cell protein synthesis. Infection of mouse fibroblast L-2 cells with MHV is accompanied by changes in cell membrane permeability to sodium ions, an effect related to the expression of the MHV S (formerly designated E2) protein (13). To rule out a role for membrane ion permeability changes in the antiviral action of hygromycin B, we examined the effect of hygromycin B on protein synthesis in cells infected with MHV and treated with anti-S antibody from 3 to 6 h p.i. to inhibit cell fusion and cell leakage to sodium ions (13). MHV was used at an MOI of 10 to ensure infection of all cells in culture. As shown in Fig. 3, infection under these conditions, in the absence of drug, resulted in complete inhibition of host cell protein synthesis

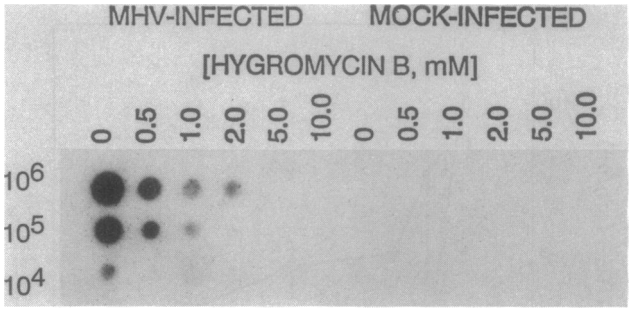

FIG. 4. Autoradiograph of dot blot hybridization of RNA extracted from mock- or MHV-infected cells incubated in the absence or presence of hygromycin B at various concentrations. Aliquots of RNA from 10^6, 10^5, and 10^4 cells were applied to the blot and screened with an MHV-specific [^{32}P]cDNA probe.

(lane 0). With increasing concentrations of hygromycin B, viral-protein synthesis decreased, while host cell protein synthesis recovered (Fig. 3). Thus, the antiviral effect of hygromycin B is not dependent on membrane permeability changes induced by the MHV S protein. Moreover, the hygromycin B-induced recovery of host cell protein synthesis argues against the idea that the drug selectively kills virus-infected cells. Rather, the ability of the host translational apparatus to shift from predominantly viral-protein to predominantly cell-protein synthesis suggests an action of hygromycin B either on the translational apparatus itself or on the relevant mRNAs.

Hygromycin B blocks viral RNA production. In response to the observations given above, we undertook an analysis of levels of viral RNA synthesized in cells treated with hygromycin B. Total RNA was extracted from cultures in 35-mm culture plates at 6 h p.i. and probed for MHV-specific RNA by dot blot hybridization (4) with a cloned, nick-translated MHV nucleocapsid RNA-specific [^{32}P]cDNA. Surprisingly, hygromycin B at a dose of as low as 0.5 mM was found to dramatically inhibit synthesis of MHV-specific RNA (Fig. 4). Higher drug concentrations decreased levels of viral RNA further, such that at 5 mM hygromycin B, no viral RNA could be detected.

The unexpected finding of markedly reduced levels of virus-specific RNA in MHV-infected cells treated with hygromycin B is the effect we consider most likely to account for the antiviral effect of this drug. This finding may be due to a block in translation of the viral RNA polymerase(s), which is likely one of the first products of gene expression in the MHV replication cycle. However, since host cell protein synthesis is not inhibited by the drug, the inhibition of translation of the viral RNA polymerase would have to be selective. Since hygromycin B has binding sites on rRNA, it is possible that it also binds to viral RNA, thereby inhibiting either its transcription or translation. This possibility is heightened by the large size of the MHV genome (currently estimated at >30 kb), which might contain sites homologous to those on rRNA known to bind hygromycin B. Future sequence data for the entire viral genome may permit the identification of such sites.

This work was supported by the Medical Research Council of Canada.

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