A COMPARATIVE STUDY OF THE EFFECTS OF CERTAIN HALOGENATED BENZIMIDAZOLE RIBOSIDES ON RNA SYNTHESIS, CELL PROLIFERATION, AND INTERFERON PRODUCTION*

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5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB)[1] has recently been shown to inhibit selectively and reversibly the synthesis of two-thirds of nuclear heterogeneous RNA (hnRNA) in mammalian cells (mouse L-929 and human HeLa S3) (1). The synthesis of messenger precursor RNA is highly sensitive (2), but there is a subfraction of hnRNA which is resistant and which is distributed over the entire broad size range of hnRNA (1, 2). Evidence has been obtained in HeLa cells that DRB inhibits the synthesis of sensitive hnRNA by blocking initiation of new chains (3). DRB (75 μM) prevents the appearance, in the cytoplasm, of almost all poly(A)-containing messenger RNA (2).

In short-term experiments (30 min), DRB has no detectable effect on the rate of protein synthesis, but after treatment for a prolonged period, the rate of protein synthesis declines (1, 4, 5). DRB (75 μM) inhibits the overall rate of cellular DNA synthesis by only 20% in short-term experiments (6). DRB does not block the initiation of new chains of DNA, but reduces the rate of replication fork progression by 20%, possibly through interference with the synthesis of RNA primer sequences.

The biological activity of DRB was first detected by the inhibitory effect of the compound on influenza virus multiplication (7). The early studies with *N*-glycosides of benzimidazoles were undertaken because it appeared likely that benzimidazole ribosides would have a striking effect on virus multiplication (7). Fig. 1 illustrates the structure of DRB as a nucleoside analog. Any departure from the β-D-ribofuranose structure in the benzimidazole glycoside was associated with a reduction in influenza virus inhibitory activity (7–9). However, the inhibitory activity of such derivatives increased with multiple substitution of halogen atoms in the benzenoid ring (7–9), and the bromo-substituted derivatives were, in several instances, considerably more active than the corresponding chloro compounds (9).

We observed early that DRB can reversibly inhibit proliferation of chick cells

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[1] *Abbreviations used in this paper:* DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; FCS, fetal calf serum; MEM, minimal essential medium; PBS, phosphate-buffered saline; poly(I)·poly(C), poly(inosinic acid)·poly(cytidylic acid).

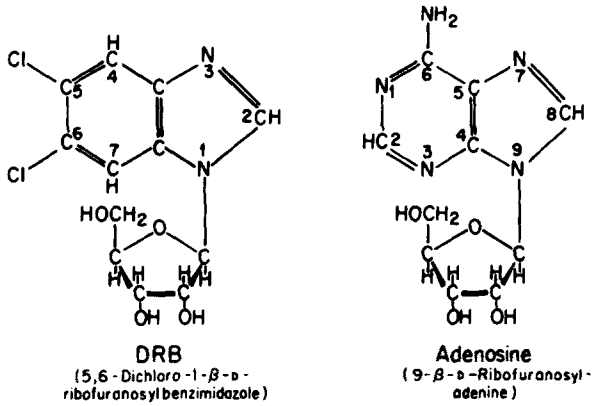

FIG. 1. The structure of DRB as a nucleoside analog.

in culture without affecting cellular oxygen uptake (7, 10, 11). Inhibition of proliferation of L cells has also been reported (12). Recently we have demonstrated that, under certain conditions, DRB can markedly increase interferon production by poly(I)·poly(C)-induced human fibroblasts (4, 5, 13, 14).

The purpose of the present studies was twofold: (*a*) to investigate quantitatively the inhibition of proliferation of human fibroblasts (neonatal foreskin cell strain FS-4) by DRB and two bromo-substituted 1-β-D-ribofuranosylbenzimidazoles, i.e., the 5,6-dibromo and the 5-(or 6-)bromo-4,6-(or 5,7-)dichloro compounds; and (*b*) to determine the effects of these derivatives on RNA synthesis and on interferon production. Our results show that monobromo-dichloro-ribofuranosylbenzimidazole is an extraordinarily active inhibitor of cellular RNA synthesis. The inhibitory effect of halogenated benzimidazole ribosides on cell proliferation and the enhancing (superinducing) effect on interferon production are likely to be mediated through the inhibition of hnRNA and messenger RNA (mRNA) synthesis.

Materials and Methods

Cell Cultures. Cells of a human fibroblast strain (FS-4) capable of producing interferon at a high level (15, 16) were grown in flasks with a 75 cm^2 growth area (3024, Falcon Plastics, Oxnard, Calif.) containing Eagle's minimal essential medium (MEM) (17) with 10% fetal calf serum (FCS). When received from Dr. Jan Vilček (New York University School of Medicine) the FS-4 cells were in their 10th passage. The present experiments were done with cells in their 12th to 17th passage. After reaching confluence these cells can be maintained at 37°C without change of medium for several weeks. They were passaged, using a 4:1 subcultivation ratio, after maintenance for a variable length of time in the confluent state.

Cell growth experiments were done in multiwell tissue culture plates (Falcon 3008). Each plate has four rows of six wells with a 2.1 cm^2 growth area per well. For convenience in microscopy, the first and last wells in each row were not used. FS-4 cells in a flask were dispersed from the monolayer with 0.25% trypsin-0.05% ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS) (18) lacking Ca^{+2} and Mg^{+2}. They were appropriately diluted and introduced in 1-ml vol of Eagle's MEM with 10% FCS into the wells. The plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Microscope Arrangement for Photography of Cells in Multiwell Culture Dishes. An inverted Carl Zeiss, Inc., New York (Plankton) microscope with phase contrast optics was equipped with a photochanger (473051) which was used to split the image between binocular eyepieces and a monocular tube. The monocular tube transmitted the image through a 10× periplan ocular, a

Leitz Microibso camera attachment with deflecting prism, focusing telescope, shutter, and a 1/3× cone to a Nikon 35-mm camera back (M-35 S). The cone contained a photocell that could be placed in the light path. The photocell was connected via a cable to a microammeter (Pace Electrical Instruments Co., Inc., Glendale, N. Y.).

The well bottoms of multiwell tissue culture plates were marked with 1/8 inch circles on the outside of the wells, using a Castell technical drawing pen and a RapiDesign template. Cells within a 0.46 mm^2 portion of a circle in each well were enumerated using the following procedure. To bring a circle into concentricity with the microscopic field, a Plan 2.5× objective was used with a Kpl 8× ocular containing a net micrometer disk (10 × 10 mm; Zeiss 474062). The sides of the micrometer square were superimposed on the circle making rapid centering convenient. Under a 10× phase objective the circle was outside the field of view. After alignment of the phase optics, the cells were viewed and the microscope focused for photography using the focusing telescope of the Leitz Microibso camera attachment. The final magnification on the 35-mm film was 43.3× (as determined by photographing the image of a stage micrometer), and the area of the culture vessel surface photographed was 0.46 mm^2. The light intensity and exposure times were adjusted to give desirable negatives and photographs. Commonly the transformer (Carl Zeiss RIW30) was at 5 V, and the exposure time was 0.5 s. Contact prints were made for survey purposes, but for cell counting the negatives were projected with a slide projector on 8.5 × 13-inch sheets of paper and cells viewed and traced on paper before counting.

Measurement of Cell Multiplication. The technique was based on the procedure of successive enumeration of cells within marked areas of culture dishes (19). It was determined that an inoculum of 1.25×10^4 cells in 1 ml gave a suitable initial number of cells and still readily countable numbers several days later. On the average there were 12 cells per photograph (26 cells/mm^2 of culture vessel surface) on the day after planting. In the great majority of experiments the 24-h cell count was within the range of 20–32 cells/mm^2 of culture vessel surface or $0.42–0.67 \times 10^4$ cells per well after inoculation of 1.25×10^4 cells per well. The variation among the initial (24 h) cell counts can be ascribed largely to two factors: variation in the distribution of cells among different areas of a well and variation in plating efficiency from experiment to experiment. There was a linear relationship between 24- and 48-h cell counts in control cultures. On the 4th day after planting there were on the average 95 cells per photograph (207 cells/mm^2 of vessel surface) in the generally still subconfluent cultures. Thus, on the average the number of cells increased eightfold over a 3-day period of observation.

The mean increase in cell number in control cultures between 24 and 48 h after planting was 1.63, giving an estimated doubling time of 29 h during this period. Cell proliferation proceeded at a constant exponential rate and with a doubling time of 21 h between 48 and 96 h after planting (cf. Figs. 2 and 4). A variable length of time (up to 2 days from planting) was required before the rate of increase in cell number became constant. Therefore the effects of benzimidazole derivatives on the rate of cell proliferation were investigated by measuring the growth rate of cells from 48 to 96 h after planting.

In the Results section the cell counts are expressed in terms of numbers of cells per mm^2 of culture vessel surface. In experiments on the effects of various treatments on the rate of cell proliferation, 4 fields in 4 wells were usually counted per variable, except 8 or 12 fields were counted in controls. Each experiment was carried out at least three times, and mean results were computed.

Measurement of the Rate of RNA Synthesis. Two confluent cultures of FS-4 cells in 35 or 60-mm Falcon Petri dishes were treated with 1 ml each of the test medium for 0.5 or 1 h and were then pulsed for 10.25 min at 37°C with [^{3}H]uridine (New England Nuclear, Bost., Mass., 26.7 Ci/mmol, 50 μCi/ml) in the continued presence of the test medium. At the end of the pulse the cultures were rinsed 10 times with ice-cold PBS and dissolved in 1 ml of 1% sodium dodecyl sulfate. Duplicate aliquots of 10 μl were used to determine total cellular radioactivity, and the remainder was used to measure trichloroacetic acid (10%) precipitable radioactivity (4, 20). The rate of RNA synthesis was calculated as described earlier (4, 20).

Induction of Interferon. Confluent 14-day-old cultures of FS-4 cells in 60-mm Falcon Petri dishes were washed once with warm PBS and then treated with poly(I)·poly(C) (P-L Biochemicals, Inc., Milwaukee, Wis.) at a concentration of 15 μg/ml in 2 ml Eagle's MEM for 1 h as described earlier (20). The cultures were then washed four times with warm PBS, replenished with 2 ml maintenance medium (Eagle's MEM supplemented with 2% heat-inactivated [56°C for 30

min] fetal bovine serum), and incubated at 37°C in a CO_2 incubator. Cycloheximide (Polysciences, Inc., Warrington, Pa.), actinomycin D (Merck Sharp & Dohme Research Laboratories, Rahway, N. J.), and the benzimidazole ribosides were included in the medium at concentrations and for time intervals indicated in the Results section. Interferon was assayed by the semi-micro method of Armstrong (21) as modified by Havell and Vilček (15) using FS-4 cells and vesicular stomatitis virus. Interferon titers are expressed in terms of human reference interferon 69/19 (obtained from Dr. Jan Vilček).

Chemicals. DRB was obtained through the courtesy of Dr. Arthur F. Wagner, Merck Sharp & Dohme Research Laboratories. The dibromo and the monobromodichloro derivatives were obtained from Dr. Karl Folkers, Merck Sharp & Dohme Research Laboratories, 20 yr ago and stored at room temperature before use in the present experiments. All compounds were dissolved in Eagle's MEM by shaking overnight at 37°C. The molecular weights of these compounds are as follows: DRB: 319.15; 5,6-dibromo-1-β-D-ribofuranosylbenzimidazole: 408.07; 5-(or 6-)bromo-4,5-(or 5,7-)dichloro-1-β-D-ribofuranosylbenzimidazole: 398.06.

Results

Inhibition of Cell Proliferation by Benzimidazole Ribosides. The growth rate of FS-4 cells was measured in the presence of benzimidazole ribosides at varying concentrations, chosen on the basis of inhibitory activity on influenza virus multiplication (7–10).

After planting of cells in multiwell dishes, the cultures were incubated for 24 h in control medium before replacement of medium with fresh control medium or with compound-containing medium. The cultures were photographed immediately after medium change and at 24-h intervals thereafter over a 3-day period. In each experiment 12 control wells were examined, and 4 wells were used for each of the three concentrations of three compounds investigated. Results from three experiments were averaged. The mean cell counts per mm^2 of culture dish surface in the controls were as follows: 24 h: 28.7; 48 h: 50.2; 72 h: 119; and 96 h: 239.

Fig. 2 shows the effects of 3-day treatment with benzimidazole ribosides on the rate of proliferation of FS-4 cells. In control cultures the doubling time was 21 h, based on the slope of increase in cell number from 48 to 96 h. The three halobenzimidazole ribosides investigated, i.e. monobromo-dichloro, dibromo, and dichloro, reduced the growth rate of FS-4 cells in a dose-dependent manner. During the first 24-h period after addition of compounds, inconsistent results were obtained in several instances, whereas during the interval from 48 to 96 h the results were consistent and reproducible. Two findings are noteworthy. First, as the drug concentration was increased the growth rate of cells decreased, but remained exponential. Second, the highest concentrations used with two of the compounds (3 μM with the monobromo-dichloro and 60 μM with the dichloro derivative) were sufficient largely to stop cell proliferation. The highest concentrations of the monobromodichloro and dichloro compounds were 50% greater than those (2 and 40 μM, respectively) at which the doubling time of cells was prolonged 2.4-2.9-fold. A different dilution series was used with the dibromo compound, and a concentration sufficient to stop cell proliferation was not employed.

Doubling times, based on the exponential growth rates of cells during the 48–96-h interval, are recorded in Table I. Reciprocals of doubling times were used to construct dose-response curves as illustrated in Fig. 3.[2] On the basis of the

[2] The reciprocal of doubling time is the exponential growth rate constant, expressed as generations per hour (Davis, B. D., R. Dulbecco, H. N. Eisen, H. S. Ginsberg, W. Barry Wood, Jr. Microbiology, second edition. Harper and Row, Hagerstown, Md. See page 97.). It is proportional to the slope of increase in cell number.

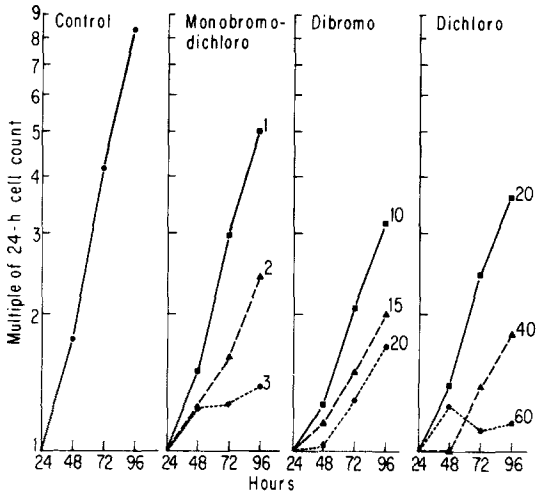

FIG. 2. Growth curves of FS-4 cells in the presence of benzimidazole ribosides. Monobromo-dichloro refers to 5-(or 6-)bromo-4,5-(or 5,7-)dichloro-1-β-D-ribofuranosylbenzimidazole; dibromo: 5,6-dibromo-1-β-D-ribofuranosylbenzimidazole; dichloro: 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole. The numbers refer to the micromolar concentrations of the derivatives.

TABLE I

Doubling Time of Cells in the Presence of Benzimidazole Derivatives at Varying Concentrations

Benzimidazole riboside	Concentration	Doubling time*
	μM	*h*
Control	–	21
Monobromo-dichloro	1	28
"	2	50
"	3	293
Dibromo	10	37
"	15	62
"	20	67
Dichloro	20	35
"	40	56
"	60	∞

* Based on the slope of increase in cell number from 48 to 96 h after planting and 24 to 72 h after beginning of treatment.

interpolated 50% cell growth inhibitory concentrations the relative activities of the monobromo-dichloro, dibromo, and dichloro derivatives are 22, 3.2, and 1, respectively.

Reversibility of the Cell Growth Inhibitory Effects of Halobenzimidazole Ribosides. We have determined the kinetics of cell proliferation after a 24-h period of treatment with halobenzimidazole ribosides at varying concentrations.

24 h after planting of cells in multiwell dishes the medium was replaced with fresh control medium or with drug-containing medium and the cultures photographed. At 48 h from planting

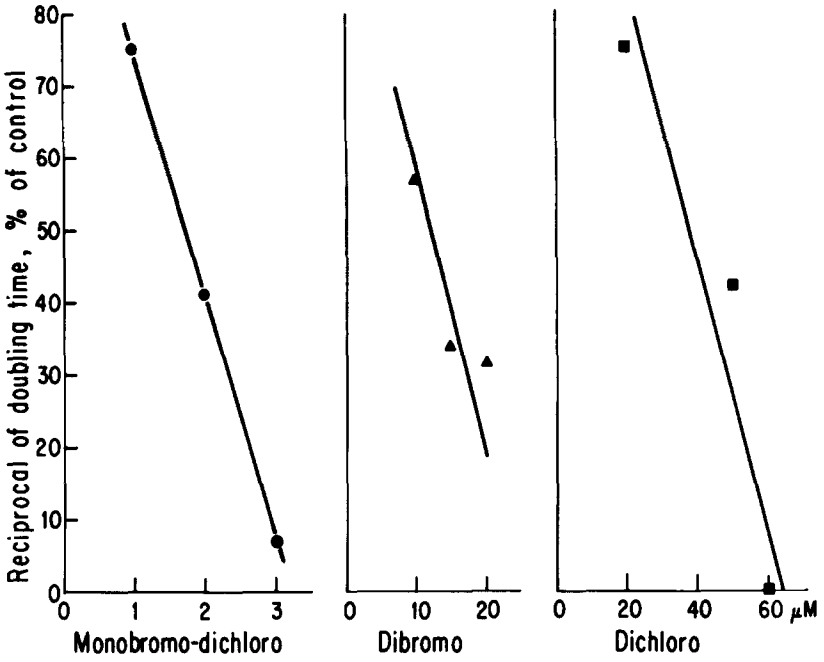

FIG. 3. Relationship between concentration of benzimidazole ribosides and growth rate of FS-4 cells. Doubling times (Table I) are based on the 48–96 h portions of growth curves shown in Fig. 2.

the cultures were photographed for a second time, all media replaced with control medium, and the cultures rephotographed. At 72, 96, and 168 h from planting additional photographs were taken of all cultures. In each experiment 12 wells were used for controls and 4 wells for each experimental variable. Mean results of 4 experiments were calculated. The number of cells per mm^2 in control cultures was as follows: 24 h: 24.9; 48 h (before medium change): 38.6; 48 h (after medium change): 38.7; 72 h: 89.5; and 96 h: 204. At 168 h there were too many cells in a confluent sheet for accurate counting in control cultures as well as in those treated at the lowest drug concentration. The cell loss due to medium change at 48 h was insignificant.

Fig. 4 shows that the doubling time in control cultures (48–96 h from planting) in this set of experiments was 21 h, a value identical to that determined in experiments summarized in Fig. 2. In cultures treated for 24 h with the monobromo-dichloro, dibromo, or dichloro derivative of benzimidazole riboside, the growth of cells was inhibited in a dose-dependent manner, and there was little or no proliferation at the highest compound concentrations used. It should be noted that with the dibromo derivative a concentration series of 7.5, 15, and 22.5 μM was used in these experiments, rather than the series 10, 15, and 20 μM employed in the experiments summarized in Fig. 2.

The kinetics of cell growth after drug removal was characterized by two features: (*a*) the rate of cell proliferation was inversely related to the drug dose which had been used to treat the cells; and (*b*) cell proliferation proceeded at accelerating rates, which was most pronounced after treatment at high dose levels.

The results in Fig.4 clearly show that the inhibition of cell growth by halobenzimidazole ribosides is substantially reversible upon removal of the compounds,

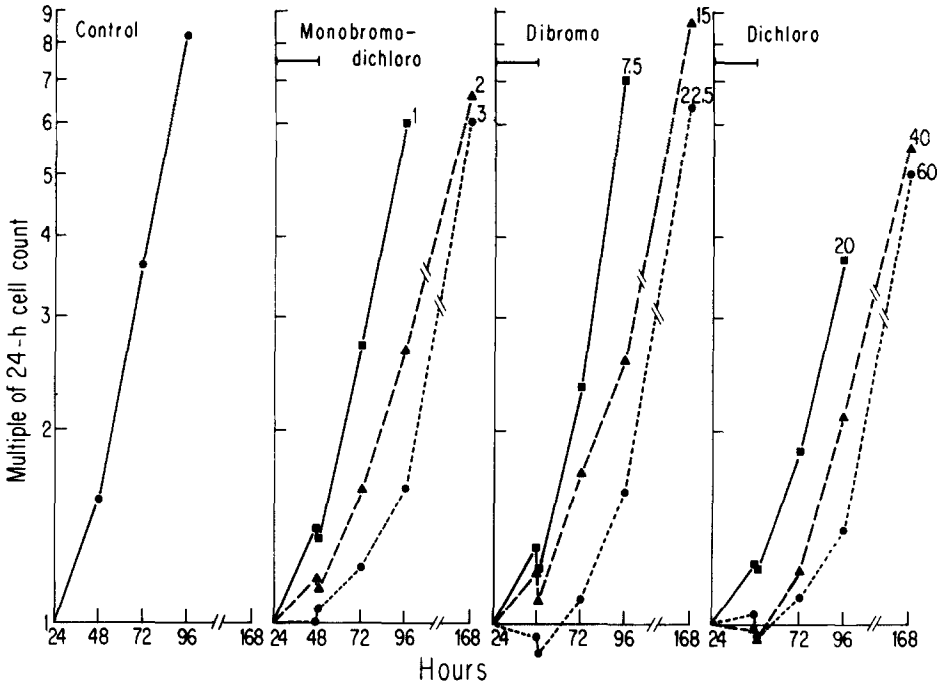

FIG. 4. Effect of 24-h treatment with benzimidazole ribosides on growth kinetics of FS-4 cells. The horizontal bars indicate time of treatment with benzimidazole ribosides. The numbers refer to the micromolar concentrations of the derivatives.

and that the three derivatives used do not differ in the reversibility of their effect.[3]

Inhibitory Effect of Halobenzimidazole Ribosides on RNA Synthesis. Preliminary experiments indicated that monobromo-dichloro, dibromo, and dichloro benzimidazole ribosides at concentrations of 3, 20, and 60 μM, respectively, inhibited the rate of cellular RNA synthesis by approximately 60%. Log dose-response plots of the inhibition of RNA synthesis by the three compounds were constructed in order to determine whether the mode of action of the three derivatives may be similar.

Confluent cultures of FS-4 cells in 35-mm dishes were incubated for 30 min with Eagle's MEM, 1 ml, containing appropriate concentrations of benzimidazole derivatives. The cultures were pulsed with [^{3}H]uridine for 10.25 min, and the rate of RNA synthesis was determined. All such determinations are expressed as fractions of the control rate obtained using inhibitor-free medium.

The log dose-response plots obtained are presented in Fig. 5. It is seen that the three derivatives inhibit the rate of RNA synthesis by 60–70% at the highest concentrations tested. Furthermore, the slopes of the individual dose-response plots are similar. This provides presumptive evidence that the three compounds

[3] The data in the present paper and in previous publications (1, 2, 5) indicate that halobenzimidazole ribosides display the common characteristics of stable reversible inhibitors, in that they cause a definite degree of inhibition, depending on the inhibitor concentration, which is reached rapidly and thereafter is independent of time. (Dixon, M., and E. C. Webb. 1964. Enzymes. Academic Press, Inc., New York. See page 316.).

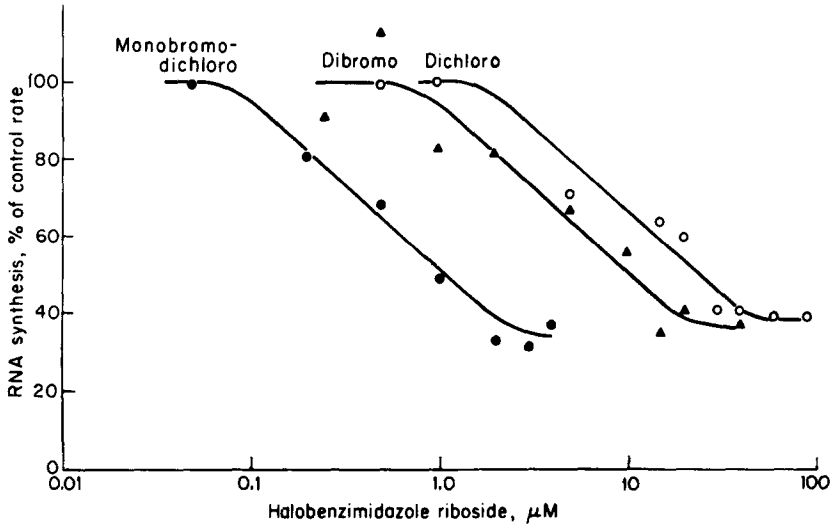

FIG. 5. Dose-response plots of the inhibition of the rate of cellular RNA synthesis by monobromo-dichloro, dibromo, and dichloro benzimidazole ribosides.

have the same mode of action. Finally, the relative potencies of the three derivatives calculated on the basis of the concentrations producing 50% of maximal inhibition are 20:2.3:1 for the monobromo-dichloro (0.45 μM), dibromo (4 μM), and dichloro (9 μM) compounds, respectively. Similar values are obtained when the relative potencies are calculated using concentrations that produce 50% inhibition (Table IV).

In additional experiments we have observed that the three compounds have little or no effect on protein synthesis in cultures exposed to the drugs for 0.5 or 1 h (not shown). Furthermore, monobromo-dichloro ribobenzimidazole appears to inhibit uridine transport to a lesser extent than the dichloro compound at concentrations at which the synthesis of RNA is inhibited to an equivalent degree (Table II).

Stability of Halobenzimidazole Ribosides during Tissue Culture. Solutions of the three benzimidazole compounds in Eagle's MEM with or without added fetal bovine serum are stable for at least 2 wk at −20°C (data not shown). A proper evaluation of the cell proliferation experiments (Fig. 2 and Table I) requires information about the stability of these compounds during the 3-day period of treatment of proliferating cells. Table II provides data that demonstrate that the monobromo-dichloro, dibromo, and dichloro derivatives are stable under the conditions of the cell proliferation assay.

Solutions of the three derivatives at different concentrations were made up in Eagle's MEM containing 10% fetal bovine serum. Half of each was used to carry out one of the cell proliferation experiments included in Fig. 2. The other half was kept frozen at −20°C and is termed 'fresh medium'. At the end of the cell proliferation experiment, the medium in the cell cultures was collected and cellular debris pelleted by centrifugation at 2,000 rpm in a refrigerated centrifuge. The two sets of inhibitor-containing media were then tested for the ability to inhibit cellular RNA synthesis as described in Materials and Methods.

The results presented in Table II clearly show that inhibitor-containing medium obtained after 3 days in tissue culture at 37°C was as potent in

TABLE II

Stability of Halobenzimidazole Ribosides During a 3-day Period of Treatment of Proliferating Cells

Compound	Concentration	[^{3}H]uridine incorporation				
		Total radioactivity		TCA-precipitable radioactivity		
					TCA-prec·cpm/total cpm	
	μM	*cpm/dish*	*% of control*	*cpm/dish*	*%*	*% of control*
A. Fresh medium						
None	–	1,941,000	100	45,018	2.32	100
Monobromo-dichloro	3	1,263,300	65.1	12,696	1.00	43.1
Dibromo	20	1,199,300	61.8	13,547	1.13	48.7
Dichloro	60	941,850	48.5	9,469	1.00	43.1
B. Medium after 3 days growth of cells						
None	–	1,893,700	100	44,446	2.35	100
Monobromo-dichloro	3	1,317,450	69.6	15,437	1.17	49.8
Dibromo	20	1,215,200	64.2	13,986	1.15	48.9
Dichloro	60	1,043,550	55.1	10,382	0.99	42.1

inhibiting RNA synthesis as unused inhibitor-containing medium which had been kept frozen. There was also no difference between the two sets of media in their ability to inhibit [^{3}H]uridine transport. These results emphasize the fact that halobenzimidazole ribosides are highly stable compounds at 37°C. Furthermore, it appears likely that these compounds are neither inactivated nor utilized by cells for metabolic purposes. As the volume of cells in the system is small in relation to the volume of medium, no detectable change in the compound concentration in the medium would be expected through simple dilution, even if the cells were capable of concentrating the compound.

Enhancing Effect of Halobenzimidazole Ribosides on Interferon Production. Earlier data (4, 13, 14) have shown that the dichloro derivative can enhance poly(I)·poly(C)-induced interferon production in FS-4 cells 50-fold. The enhancement of interferon yield was closely correlated with the inhibition of RNA synthesis. We have now measured the ability of the monobromo-dichloro and the dibromo derivatives to enhance interferon yields.

Confluent FS-4 cultures in 60-mm dishes were induced with poly(I)·poly(C) in the presence or absence of cycloheximide at 50 μg/ml for 1 h. The rationale for the induction protocol has been discussed earlier (13). The cultures were washed four times with warm PBS and replenished with 2 ml of maintenance medium with or without cycloheximide (50 μg/ml). At 3 h after the start of the induction the medium in appropriate cultures was changed to one containing both cycloheximide and one of the benzimidazole derivatives at varying concentrations. At 4 h after the start of the induction the medium in two other cultures was changed to one containing cycloheximide and actinomycin D (5 μg/ml). At 6 h after the beginning of induction all cultures were washed four times with warm PBS and replenished with 2 ml of inhibitor-free maintenance medium or with one containing the appropriate concentration of a benzimidazole derivative. The cultures were then incubated for another 18 h at the end of which time the media were harvested and assayed for interferon activity. Two cultures were used for each variable.

Table III shows that the three benzimidazole derivatives enhance interferon yields in a dose-dependent manner. As reported earlier (13), the dichloro com-

TABLE III
Enhancement of Human Interferon Production by Halobenzimidazole Ribosides

Treatment	Drug concentration*	Interferon yield‡
		reference U/ml
A. None	–	16
B. Cycloheximide alone	50	1,448
C. Cycloheximide and actinomycin D	50 5	 23,170
D. Cycloheximide and monobromo-dichloro	50 1 2 3	 2,896 8,192 16,384
dibromo	10 15 20	4,871 9,742 13,777
dichloro	20 40 60	3,444 11,585 11,585

* Concentration of cycloheximide and actinomycin D in micrograms per milliliters and that of the benzimidazole derivatives in micromolar units.
‡ Each titer represents the geometric mean of interferon yields between 6 and 24 h after induction from two cultures, each sample titrated in duplicate.

TABLE IV
Action of Halobenzimidazole Ribosides

Process	Measurement	Concentration, μM			Relative activity		
		Monobromo-dichloro	Dibromo	Dichloro	Monobromo-dichloro	Dibromo	Dichloro
Rate of RNA synthesis	50% inhibition	1.1	10	24	22	2.4	1
Rate of cell proliferation	50% inhibition	1.7	12	38	22	3.2	1
Interferon superinduction	50% of maximum enhancement	1.7	12	30	18	2.5	1
Influenza virus yield*	75% inhibition	1.8	15	38	21	2.5	1

* From reference 9.

pound at a concentration in the range from 40 to 60 μM produces a maximal enhancement. The monobromo-dichloro and the dibromo compounds produce close to a maximal effect at concentrations of 3 and 20 μM, respectively. The maximal interferon yield obtained using any one of the three benzimidazole derivatives differs from that obtained by using the conventional cycloheximide-actinomycin D protocol (13) by at most a factor of two. The relative enhancing activities of the monobromo-dichloro, dibromo, and dichloro derivatives, estimated graphically as concentrations producing half-maximal enhancement, were 18:2.5:1, respectively (Table IV).

Discussion

There are two main questions to be considered in the light of the data presented in the present paper and previously. Is inhibition of cell proliferation by halobenzimidazole ribosides mediated through inhibition of mRNA production? Do different halobenzimidazole ribosides act on cells the same way? There is now strong evidence that the dichloro compound blocks messenger RNA production (2) by inhibiting the synthesis of hnRNA, and that its action on hnRNA synthesis is selective (1, 22–24). The possibility has been considered that DRB may act at the RNA polymerase level and that there may exist a DRB-sensitive and an insensitive component of the RNA polymerase responsible for the synthesis of hnRNA (1–3). It can be seen in Table IV that the monobromo-dichloro, dibromo, and dichloro derivatives of benzimidazole riboside show closely similar structure-activity relationships with respect to inhibition of cellular RNA synthesis and cell proliferation, and that the striking similarity in structure-activity relationships extends also to the enhancing effect of the compounds on interferon production and to inhibition of influenza virus yield (9). The most likely basis for such similarity is that all three compounds act through the same mechanism on the processes examined.

A comparison of the molar concentrations at which 50% inhibition in the rates of RNA synthesis and cell proliferation occurs would be arbitrary, as the conditions for determination of these parameters were necessarily different, and the effect levels for comparisons among the compounds were chosen in accordance with what appeared optimal for the different procedures used. It is noteworthy, however, that the concentrations at which any one of the derivatives produced the defined effects on RNA synthesis, cell proliferation, interferon production, and influenza virus yield are in fact similar.

The hypothesis that halobenzimidazole ribosides inhibit cell proliferation by blocking RNA synthesis is also supported by the following evidence. With the dichloro compound cell proliferation is blocked at 60 μM concentration, while at 40 μM it proceeds at a markedly reduced rate. Similarly, a maximal effect on the synthesis of the drug-sensitive fraction of RNA is obtained at 60–75 μM concentration, while a 40 μM concentration still permits synthesis, albeit at a reduced rate, of such RNA (1, 3). To obtain essentially complete inhibition of mRNA production, a 75 μM concentration of the dichloro compound is required (2). The data on recovery of cell proliferation after a 24-h period of treatment (cf. Fig. 4) agree with the earlier observation that after treatment of FS-4 cells with 40 μM dichlorobenzimidazole riboside for 16 or 24 h there is slow, though complete recovery of protein synthesis (5).

The close correlation between the enhancing effect of the three derivatives on interferon production and their inhibitory effect on RNA synthesis provides further evidence for the involvement of RNA synthesis in the shutoff of poly(I)·poly(C)-induced interferon production in FS-4 cells (4, 5, 13). The data also demonstrate that protocols based on any one of the three benzimidazole ribosides give interferon yields comparable to the conventional cycloheximide-actinomycin D protocol (Table III).

The results summarized in Table IV support the idea of a possible role for host RNA in the multiplication of influenza virus (7, 25), but do not permit a decision as to what role hnRNA may play in virus multiplication.

The very high activity of the monobromo-dichloro derivative is remarkable, as is the reversibility of its action, which it shares with the other derivatives. It appears that halobenzimidazole ribosides provide not only a unique tool for studies of cellular biosynthesis, but an opportunity to explore the possibility that selective chemical inhibition of hnRNA synthesis, either alone or in combination with other approaches, may be a useful avenue to chemotherapy of neoplastic disorders. No other compounds are known which offer such an opportunity.

Summary

5-(or 6-)Bromo-4,5-(or 5,7-)dichloro-1-β-D-ribofuranosylbenzimidazole, 5,6-dibromo-1-β-D-ribofuranosylbenzimidazole, and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole show closely similar structure-activity relationships with respect to inhibition of cellular RNA synthesis, cellular proliferation, and influenza virus multiplication, and also with respect to enhancement of interferon production. The activities of these compounds are ranked 20:2.5:1. The log dose-response curves constructed for inhibition of FS-4 cell RNA synthesis show similar slopes and a leveling off at 60–70% inhibition of RNA synthesis at the highest concentrations of each compound tested. This evidence suggests that these three derivatives act through the same mechanism. It has been shown previously that the dichloro compound selectively inhibits nuclear heterogenous RNA and messenger RNA synthesis.

The concentrations of the benzimidazole ribosides at which the rate of proliferation of human fibroblasts (FS-4) is reduced by 50% are as follows: monobromo-dichloro: 1.7 μM (0.68 μg/ml); dibromo: 12 μM (4.9 μg/ml); dichloro: 38 μM (12 μg/ml). All compounds reduce the exponential rate of cell proliferation in a dose-dependent manner. The inhibition of cell growth is reversible upon removal of the compounds from the medium.

Protocols based on any one of the three halobenzimidazole ribosides give interferon yields from poly(I)·poly(C)-induced FS-4 cells which are comparable to the high yields obtained with the conventional cycloheximide-actinomycin D protocol. The enhancement of interferon yield depends on blocking of the synthesis of RNA which is involved in the shutoff of interferon production.

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