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COMMENTARY

HALOGENATED BENZIMIDAZOLE RIBOSIDES

NOVEL INHIBITORS OF RNA SYNTHESIS

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Recent studies on the inhibitory effect of halobenzimidazole ribosides on RNA synthesis of viruses and mammalian cells have produced new insights into the regulation of eukaryotic transcription [1–3]. 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB, Fig. 1) appears to block transcription at sites a few to several hundred nucleotides from promoters. In this commentary, we shall focus on the outstanding features of the action of halobenzimidazole ribosides on cells and viruses and indicate directions of future research in this field.

Halobenzimidazole ribosides were synthesized in the early 1950s as part of an intensive investigation into the effects of benzimidazole derivatives on viral replication (see Refs. 4 and 5 for a review of the early work). The starting point of these studies was the realization that the biological specificity of viruses was determined by viral nucleoproteins and that hence it might be possible to inhibit virus multiplication without interfering with host cell function[6]. The benzimidazoles were selected for investigation because this moiety was known to be present in vitamin B_{12} and because by 1950 [7] vitamin B_{12} had been demonstrated to have a close relationship to nucleic acid metabolism. Furthermore, it was noted that both benzimidazole and the purines had the same bicyclic skeleton with respect to size and a common imidazole ring. Hence, analogs of benzimidazole were considered potential selective inhibitors of viral multiplication [6]. The inhibitory effect of 2,5-dimethylbenzimidazole on the multiplication of influenza A virus (PR8 strain) and influenza B virus (Lee strain) was readily demonstrated [6].

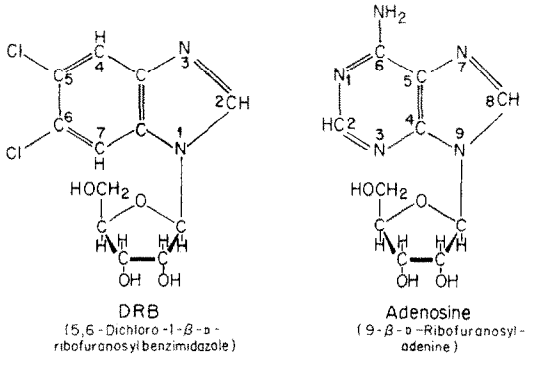


Fig. 1. Structure of a halobenzimidazole riboside—an inhibitor of nuclear heterogeneous RNA and mRNA synthesis. The structure of adenosine is shown for comparison.

This initial result led to a detailed survey of benzimidazole derivatives as selective inhibitors of virus multiplication, particularly that of influenza virus, and was highlighted by the synthesis and examination of halogenated β-D-ribofuranosylbenzimidazoles [4, 5, 8, 9]. The halobenzimidazole ribosides were the most active compounds on a molar basis. The 5-(or 6-)bromo-4,5-(or 5,7-)dichloro derivative inhibited influenza virus multiplication *in vitro* by 75 per cent at a concentration of 1.8 μM (0.72 μg/ml) and was 1950 times more active than unsubstituted benzimidazole [10]. The 5,6-dichloro derivative (DRB), which was used for most of the subsequent studies, inhibited the replication of several RNA and DNA viruses *in vitro* and *in vivo* [1, 4].

While it was clear that the action of DRB and related compounds was biochemically more selective than that of the simpler alkyl derivatives, it was also shown in the early work that DRB inhibited cell proliferation [9]. Therefore, it appeared likely that DRB interfered with the nucleic acid metabolism of both the influenza virus and the host cell [9, 11].

In 1957 it was demonstrated that DRB inhibited the synthesis of cellular RNA [4, 12, 13]. DRB had only a minor effect on protein synthesis after treatment of cells for 3 hr [5, 13, 14]. Thus, it is 20 years since the conclusion was drawn that in the ribofuranoside of dichlorobenzimidazole a new inhibitor of RNA synthesis had been discovered [4]. Because little was known 20 years ago about different species of RNA and their synthesis and function, full exploitation of DRB and related compounds has ensued only after a considerable lag period.

To explain the fact that DRB was able to reduce virus yield not only in cell cultures but also in mice and embryonated eggs without apparent or serious damage to the host, it was suggested that quantitative differences may exist in the metabolic requirements of the virus and of the host [15]. However, it was apparent that DRB and related compounds could not be given to cells or animals in sufficient amounts to cause marked virus inhibition for a prolonged period without toxicity to the host [10, 16]. The search for virus-specific inhibitors of nucleic acid synthesis was directed toward exploration of derivatives containing large substituents at position 2 in the imidazole ring. This led to the discovery of a class of

inhibitors exemplified by 2-(α-hydroxybenzyl)-benzimidazole (HBB), which specifically block the replication of picornavirus RNA (reviewed in Ref. 17).

The demonstration that DRB was a selective and reversible inhibitor of nuclear heterogeneous RNA (hnRNA) synthesis in eukaryotic cells provided a strong stimulus for further work with halogenated ribofuranosylbenzimidazoles [18–21]. It was found that DRB inhibits hnRNA synthesis in mammalian cells by approximately 70 per cent [21, 22], but blocks messenger RNA (mRNA) appearance by > 95 per cent [22, 23]. These observations have led to the hypothesis that approximately one-third of cellular hnRNA may not be precursor to cytoplasmic mRNA [21, 22].

Detailed investigations on the mechanism of action of DRB in chironomid and human cells suggested that DRB inhibits hnRNA synthesis by blocking chain initiation [24–27]. Recently, however, studies on the effect of DRB on adenovirus transcription in human (HeLa) cells have indicated that this compound does not act at sites of RNA chain initiation but acts a short distance downstream from the promoters [3]. Short (400–800 nucleotides), virus-specific RNA continues to be synthesized in drug-treated adenovirus-infected HeLa cells. Since similar DRB-resistant RNA molecules, 330–740 nucleotides in length, have been observed in uninfected HeLa cells [2], it is possible that the DRB-sensitive site may represent a physiological site for transcriptional regulation in mammalian cells.

The main purpose of this Commentary is to draw attention to the unique mode of action of DRB and to those properties of halobenzimidazole ribosides which make them very valuable tools for basic research. The potential usefulness of these compounds in chemotherapy also will be considered.

HALOBENZIMIDAZOLE RIBOSIDES

Synthesis

The synthesis of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole has been described by Folkers and Shunk [8] and Kissman *et al.* [28]. Both prepared the chloromercuri analog of 5,6-dichlorobenzimidazole and condensed it with a 1-chloro-2,3,5-tri-O-acylated-D-ribofuranose. Folkers and Shunk [8] used the tri-O-acetyl analog, whereas Kissman *et al.* [28] employed the corresponding benzoylated derivative. Anhydrous ammonia in methanol was used to unblock the acetylated condensation product; sodium methoxide in methanol was employed to remove the benzoyl groups.

Solubility and stability of derivatives

The solubility of benzimidazole derivatives decreases as the number of halogen substituents in the benzenoid ring is increased [4, 10]. However, this has not interfered with the accurate determination of the activities of 1-β-D-ribofuranosylbenzimidazoles in cell culture systems *in vitro*, because of the high activity of such compounds, and because the activity increases with increased halogenation. There are several convenient ways of solubilizing the compounds in water or cell culture media (before addition of serum), such as by shaking or stirring at 37° for several hours. For example, DRB (mol. wt 319.15) can be dissolved by these means at concentrations up to 500 μM (160 μg/ml). In the authors' laboratory, freshly prepared solutions are commonly used. However, solutions of DRB (e.g. 350 μM) can be stored in aliquots at −20° or, if made up in water, at 4°.

Solutions of three of the 1-β-D-ribofuranosylbenzimidazoles, i.e. the dichloro, dibromo, and monobromo–dichloro derivatives, have been found to be stable for at least 2 weeks at −20° [29]. These halogenated 1-β-D-ribofuranosylbenzimidazoles are also highly stable at 37° under conditions of cell culture experiments. No evidence has been obtained of either inactivation or utilization of such compounds when solutions in cell culture medium were incubated with monolayers of human fibroblasts (FS-4 cells) for 3 days [29]. These compounds have been stored at room temperature for 20 years without loss of activity [29].

Structure–activity relationships

Most of the available information concerns the relationship between structure and inhibitory activity on influenza B virus (Lee strain) multiplication [4, 9, 10, 15, 16]. Table 1 shows that multiple substitution of halogen atoms in the benzenoid ring of 1-β-D-ribofuranosylbenzimidazole increases the virus inhibitory activity of the compounds. In a number of instances, the bromo-substituted derivatives are considerably more active than the corresponding chloro compounds (compare dibromo with dichloro and monobromo–dichloro with trichloro 1-β-D-ribofuranosylbenzimidazole). From the monochloro to the monobromo–dichloro derivative the activity increases 150-fold.

Of the α- and β-forms, the halogenated β-linked ribofuranosides are five to eight times more active than the α-linked compounds. The α-linked ribo- and arabino-pyranosides of 5,6-dichlorobenzimidazole are only $\frac{1}{5}$ and $\frac{1}{30}$ as active, respectively, as 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole.

In conclusion, the highest activity is obtained when the carbohydrate moiety is identical with that present in RNA and when there are multiple bromo and chloro substituents in the benzenoid ring.

A comparison of the relative virus inhibitory activities and morphological cytotoxicities of the derivatives reveals that the values are similar for 5-chloro-benzimidazole; 4,5,6-(or 5,6,7-)trichloro-1-α-D-ribofuranosylbenzimidazole; 5,6-dichloro-1-α-D-ribopyranosylbenzimidazole; and 5,6-dichloro-1-α-D-arabinopyranosylbenzimidazole. On the other hand, the relative virus inhibitory activities are higher than the cytotoxicity values for all β-linked ribofuranosides of halobenzimidazoles examined, i.e. the 5-chloro; 5,6-dichloro; 4,5,6-trichloro; and 5-bromo-4,6-dichloro derivatives of 1-β-D-ribofuranosylbenzimidazole. The conclusion can be drawn that compounds in the first group have broad effects on cells which lead to readily apparent morphological changes, whereas the β-linked halobenzimidazole ribosides have more specific effects

Table 1. Influenza virus inhibitory activity and relative cytotoxicity of benzimidazole derivatives*

Compound	Concentration (μM) at which virus yield is reduced 75 per cent	Relative activity		
		Virus inhibition†	Cytotoxicity CAM‡	MKC§
Benzimidazole	3500	1	1	1
5-Chloro-	750	4.7	6	
5,6-Dichloro-	~250	~14		
1-β-D-Ribofuranosylbenzimidazole				
5-(or 6-)Chloro-	280	13	4.6	
4,6-(or 5,7-)Dichloro-	100	35		
5,6-Dichloro-	38	92	39	47
5,6-Dibromo-	15	230		
4,5,6-(or 5,6,7-)Trichloro-	4.6	760	130	190
4,5,6-(or 5,6,7-)Tribromo-	3.5	1000		
5-(or 6-)Bromo-4,6-(or 5,7-)dichloro-	1.8	1950	~330	~620
1-α-D-Ribofuranosylbenzimidazole				
4,5,6-(or 5,6,7-)Trichloro-	21	165	140	150
5-(or 6-)Bromo-4,6-(or 5,7-)dichloro-	14	250		
1-α-D-Ribopyranosylbenzimidazole				
5,6-Dichloro-	230	15	16	13
1-α-D-Arabinopyranosylbenzimidazole				
5,6-Dichloro-	1100	3.1	2.7	2.6

* Based on data in Refs. 4, 9, 10, 15, 16, 30.

† Unsubstituted benzimidazole as the reference compound was considered to have a virus inhibitory activity equivalent to 1.

‡ CAM = chorioallantoic membrane from embryonated chicken eggs incubated *in vitro*. Benzimidazole causes 2+ macroscopic damage at a concentration of 12,000 μM [30].

§ MKC = primary cultures of rhesus monkey kidney cells. Benzimidazole causes 3+ microscopic damage at a concentration of 5200 μM [30].

which are not associated with morphological changes in cells to the same degree.

5,6-Dimethylbenzimidazole has low influenza virus inhibitory activity (75 per cent inhibitory concentration = 1900 μM) compared with 5,6-dichlorobenzimidazole (75 per cent inhibitory concentration = ~250 μM). Furthermore the 1-D-ribofuranosyl, 1-D-lyxopyranosyl and 1-D-arabinopyranosyl derivatives of the 5,6-dimethyl compound all lack demonstrable inhibitory activity. Also without activity are *N*-glycityl-benzimidazoles such as the 5,6-dimethyl-1-D-ribityl compound and the 1-D-arabityl, 1-D-xylityl and 1-D-sorbityl derivatives of 5,6-dichlorobenzimidazole.

Structure–activity relationships have been determined with three halogenated ribofuranosylbenzimidazoles (dichloro, dibromo and monobromo–dichloro) for their effects on cellular RNA synthesis, influenza virus multiplication, cell proliferation, and superinduction of interferon production [29]. Table 2 shows that there is striking similarity in structure–activity relationships with respect to all of these biological parameters, which suggests that all three compounds act through the same mechanism on the processes examined. It is likely that this mechanism is the selective inhibition of that part of hnRNA synthesis which includes transcription of precursor molecules for mRNA.

EFFECTS OF DRB ON CELLS AND VIRUSES

Inhibition of RNA synthesis

DRB is a selective and reversible inhibitor of hnRNA synthesis in human, murine, avian and insect cells [1]. Treatment of cells with ⩾75 μM DRB blocks the appearance of mRNA in the cyto-

Table 2. Action of halobenzimidazole ribosides*

Process	Measurement	Concentration (μM)			Relative activity		
		Monobromo–dichloro	Di-bromo	Di-chloro	Monobromo–dichloro	Di-bromo	Di-chloro
Rate of RNA synthesis	50% Inhibition	1.1	10	24	22	2.4	1
Influenza virus yield	75% Inhibition	1.8	15	38	21	2.5	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

* From Ref. 29.

plasm by >95 per cent [1]. Detectable inhibition of RNA synthesis is obtained when human (HeLa, FS-4) or murine (L) cells are exposed to DRB at 5 μM. The maximal effect of ~50 per cent reduction in the overall rate of RNA synthesis is obtained in the range of 75 μM DRB [21, 22, 31]. The extent of inhibition at any intermediate concentration remains constant at an intermediate value even after prolonged exposure to DRB [31].

The onset of inhibition of RNA synthesis is very rapid. A detectable effect is seen within 1.5 min of exposure to DRB [21, 27]. The maximum inhibition takes 15–30 min to set in in HeLa cells [22]. Most of this interval probably represents the runoff time of RNA polymerase molecules that have already initiated synthesis of RNA chains. The inhibition of RNA synthesis by DRB is quickly reversed on removal of the compound from a culture that has been exposed to it for 1–2 hr [21, 26, 32, 33]. In HeLa and L cells, complete reversal has been observed within 5 min of removal of DRB by medium change [21]. After longer (~24 hr) exposure to DRB, recovery of RNA synthesis is slower although eventually complete [33].

DRB selectively inhibits the synthesis of hnRNA [18–21]. DRB at 12 μM inhibits hnRNA synthesis in L cells by approximately 40 per cent but has no effect on 45S ribosomal precursor RNA synthesis [21]. At higher concentrations DRB does cause detectable inhibition of pre-ribosomal 45S RNA synthesis, but it has not been established whether this is a primary or a secondary effect [21]. It has also been observed in experiments with α-amanitin in rats and mice that, when hnRNA synthesis catlyzed by RNA polymerase II is inhibited, pre-ribosomal RNA synthesis catalyzed by RNA polymerase I also becomes inhibited even though, *in vitro*, polymerase I is completely insensitive to α-amanitin while polymerase II is highly sensitive [34, 35]. Thus, the possibility exists that synthesis of pre-ribosomal RNA may depend on continuing synthesis of hnRNA.

DRB (65 μM) inhibits hnRNA synthesis almost completely (> 95 per cent) in the salivary gland cells of *Chironomus tentans*, but by only 60–70 per cent in mammalian cells [2, 20–22]. The DRB-resistant one-third of hnRNA that is observed in mammalian cells has the same size distribution, poly(A) content and extent of methylation and capping as hnRNA isolated from drug-free control cultures [22]. In addition to DRB-resistant high molecular weight hnRNA, a class of short RNA molecules is synthesized by RNA polymerase II in HeLa cells which is also resistant to inhibition by DRB [2].

Whereas DRB inhibits only two-thirds of hnRNA synthesis, it inhibits cellular mRNA labeling by > 95 per cent [22, 23]. The almost complete inhibition of mRNA labeling in the presence of continued synthesis of one-third of hnRNA suggests either that the DRB-resistant hnRNA is not a precursor to cytoplasmic mRNA but serves some other function in the mammalian nucleus [21, 22] or that DRB has a post-transcriptional effect on RNA processing. DRB (75 μM) inhibits early and late adenovirus 2 transcription in the cell nucleus by 90–95 per cent, but blocks the appearance of virus-specific RNA in the cytoplasm by > 95 per cent [3, 36]. Thus, it appears that DRB can block transcription from specific message-containing regions of the genome almost completely.

Inhibition of protein synthesis

DRB appears to have only a secondary effect on cellular protein synthesis, caused by inhibition of synthesis of mRNA sequences. Exposure of cells to DRB at high concentration for up to 30 min does not lead to a detectable inhibition in the rate of cellular protein synthesis even though the rate of RNA synthesis is markedly inhibited [21, 31–33, 37]. As would be expected, prolonged exposure of cells to DRB results in the inhibition of the rate of protein synthesis, which reaches a level of 30–40 per cent inhibition after 5 hr of treatment with 60 μM DRB. Removal of DRB from a culture that had been exposed to the compound for 5 hr leads to a fairly rapid and complete recovery of protein synthesis [33]. After long (~ 24 hr) DRB treatment, recovery of protein synthesis is slower though eventually complete [33]. These data support the view that inhibition of protein synthesis by DRB is secondary to the inhibition of mRNA synthesis [20, 31–33].

Inhibition of DNA synthesis

DRB has only a minor immediate effect on cellular DNA replication, and its effect on adenovirus DNA replication appears to be entirely secondary to inhibition of early adenovirus mRNA synthesis.

Inhibition of cellular DNA synthesis. DRB, at concentrations of 60–90 μM, inhibits the overall rate of DNA synthesis in the L-929 line of mouse fibroblasts by only 20 per cent after a 30-min period of treatment [38]. The action of DRB appears to be limited to one aspect of DNA replication, namely DNA chain elongation. A 20 per cent reduction in the rate of DNA replication fork progression in DRB-treated cells has been observed both by density gradient analysis of BUdR- and [^{3}H]thymidine-substituted DNA and by examination of autoradiograms of extended pulse-labeled DNA fibers. DRB does not affect the apparent size of DNA replication units. The mechanism by which DRB retards replication fork progression is not known. DRB may have some effect on the enzyme that is involved in the synthesis of RNA primer sequences in DNA replication. Alternatively, the effect on DNA chain growth may be secondary to inhibition of hnRNA synthesis by DRB [38].

Inhibition of adenovirus DNA synthesis. The synthesis of adenovirus genome sized DNA begins 6–8 hr after infection of the KB line of human oral carcinoma cells with adenovirus type 2 at a multiplicity of 50–150 plaque-forming units/cell and reaches a maximum at approximately 16 hr [36]. DRB, at concentrations of 60–160 μM, has little or no detectable effect on viral DNA replication when added 8 hr after infection or later. However, DRB blocks adenovirus DNA replication almost completely when present from the time of infection. Indeed the synthesis of virus-specific DNA of all four size classes is blocked, i.e. > 100S and 50–90S (viral DNA sequences covalently linked to cellular DNA), 34S (unit genome length viral DNA), and

< 20S (precursor segments of viral DNA). The block in viral DNA replication is reversible upon removal of DRB, but several hours are required before viral DNA synthesis gets under way.

The effects of DRB on viral DNA replication can be explained by the inhibition of transcription of certain "early" genes of adenovirus. DRB, present from the time of infection, reduces the accumulation of early virus-specific RNA in the nucleus by approximately 90 per cent and the appearance of virus-specific RNA sequences in the cytoplasm by > 95 per cent [36].

These results suggest that DRB blocks adenovirus DNA replication by preventing expression of those "early" genes whose products are required for viral DNA replication. That viral gene functions are required for viral DNA replication is well known [39, 40]. There is evidence that one virus-coded single-strand-specific DNA-binding protein, made early in lytic infection, functions in initiation of DNA replication as well as in elongation of nascent viral DNA chains [39].

Inhibition of precursor transport into cells

[^{3}H]*uridine*. DRB inhibits the uptake of [^{3}H]-uridine into avian [37] and mammalian [21, 22, 31, 32, 36] cells in a dose-dependent manner. Uptake of [^{3}H]uridine into HeLa cells is inhibited considerably more than that into L cells [21]. The reduction in the acid-extractable radioactivity of DRB-treated cells is most marked after short periods (< 30 min) of labeling. To estimate the degree of inhibition of RNA synthesis by DRB, determinations of the acid-soluble radioactivity can be used to correct the acid-precipitable radioactivity data [21, 22, 31, 32]. A correction procedure based on incorporation of label into mitochondrial RNA is also satisfactory [22]. Estimates of the rate of RNA synthesis obtained after correction for reduced nucleoside uptake agree closely with those obtained by the use of $^{32}PO_4$ [22] or [^{3}H]methionine, for which no corrections are required.

With labeling times of several hours, equilibrium conditions are reached and no significant differences are observed in acid-soluble counts between control and DRB-treated cells labeled with [^{3}H]uridine [36].

Although direct evidence is not yet available, it is unlikely that DRB interferes with the phosphorylation of [^{3}H]uridine after transport of the precursor into the cell.

[^{3}H]*leucine*. DRB has shown no effect on uptake of [^{3}H]leucine into cells [21, 32].

[^{3}H]*thymidine*. DRB inhibits the uptake of [^{3}H]thymidine into mammalian cells, but does not alter the size of the total dTTP pool [38]. The rate of DNA synthesis in DRB-treated cells can be estimated by correction of acid-precipitable counts for reduced uptake of [^{3}H]thymidine as determined by acid-soluble counts.

Inhibition of cell proliferation

Quantitative studies have shown that DRB, the dibromo and the monobromo–dichloro derivatives of benzimidazole riboside reversibly inhibit the proliferation of the FS-4 strain of human fibroblasts *in vitro* [29]. The dose-dependent effects of these derivatives were determined in exponentially growing cultures. The results summarized in Table 2 show that the relative inhibitory activities of the dichloro, dibromo and monobromo–dichloro derivatives are 1, 3 and 20. The effects of these compounds on cell proliferation can best be explained on the basis of inhibition of mRNA synthesis. For example, 40 μM DRB permits the synthesis of significant quantities of mRNA, while 60 μM DRB permits very little synthesis [22, 31–33]. DRB shows parallel effects on cell proliferation in that 40 μM DRB permits proliferation at a markedly reduced rate, while 60 μM stops cell proliferation.

FS-4 cells resume proliferation with minimal cell loss after treatment with halobenzimidazole ribosides for 24 hr at concentrations sufficient to stop proliferation ([29]; I. Tamm, unpublished observations). These findings are in excellent agreement with data on recovery of protein synthesis in FS-4 cells after prolonged treatment [33].

Recently evidence has been obtained with the HeLa line of human cervical carcinoma cells showing that the bulk of these cells fails to recover after treatment for 24 hr at DRB concentrations sufficient to stop proliferation ($\geqslant$ 60 μM, I. Tamm, unpublished observations). Thus, tumor cells tolerate growth inhibition by DRB less well than normal cells, which raises the possibility that halobenzimidazole ribosides, alone or in combination with other drugs, may be useful in the chemotherapy of some neoplastic diseases. At present there is no evidence that the action of DRB on RNA synthesis is different in cancer cells and normal cells. Rather it appears that the failure of the bulk of HeLa cells to survive a period of treatment with DRB is a reflection of the inability of these cells to maintain themselves under conditions of metabolic restriction.

Inhibition of viral multiplication

A recent survey [1] of the sensitivity of different viruses to inhibition by halobenzimidazole ribosides has shown that viruses can be divided into two classes: (1) those whose multiplication is approximately as sensitive to inhibition as the synthesis of cellular hnRNA; this includes orthomyxo-, oncorna-, adeno- and papovaviruses; and (2) those whose multiplication is less sensitive or completely insensitive to inhibition; this includes paramyxo-, toga-, reo- and picornaviruses.

Available evidence indicates that host RNA polymerase II transcribes adeno- and papovavirus genomes and also the integrated oncornavirus genome. Therefore, it is likely that the sensitivity of these viruses to inhibition by DRB has the same basis as the sensitivity of two-thirds of host cell hnRNA. Orthomyxoviruses possess a viral RNA polymerase, which at least *in vitro* is not sensitive to DRB (L. A. Caliguiri, unpublished observations). It appears that a transcription product of the host cell, whose synthesis is blocked by DRB, is required for an early step in orthomyxovirus multiplication [11].

Since adeno- and papovaviruses contain DNA genomes, whereas orthomyxo- and oncornaviruses are RNA-containing agents, it is clear that sensitivity to DRB does not relate to the type of nucleic acid

present in the virus [14], but probably has to do with the enzymatic process of transcription.

Those viruses not sensitive or only slightly to moderately sensitive to halobenzimidazole ribosides utilize distinctive virus-specific mechanisms for RNA transcription and replication. Somewhat reduced yields of these viruses in the presence of DRB or related compounds may reflect fastidiousness (i.e. variable dependence on the physiological state of the cell) or partial sensitivity of the virus-specific enzyme mechanisms to inhibitions by halobenzimidazole ribosides.

Poxviruses, as exemplified by the vaccinia virus, occupy a unique position in that unglycosylated chlorobenzimidazoles are themselves very highly active inhibitors and their conversion to corresponding ribofuranosides does not further increase their activity [41]. Indeed, vaccinia virus multiplication is inhibited at concentrations of 5-(or 6-)chlorobenzimidazole and the 5,6-dichloro compound which do not cause cytotoxic changes in the appearance of host tissue (chorioallantoic membrane) in culture [16, 30]. Investigations have begun to explore the basis of these remarkable findings (E. Paoletti and I. Tamm, unpublished observations).

Paradoxical enhancement of human fibroblast interferon production

Human diploid fibroblast (FS-4) cultures induced with the double-stranded RNA poly(I) . poly(C) synthesize and secrete interferon into the culture medium (reviewed in Refs. 1 and 42). Interferon production is detectable approximately 1 hr after the beginning of induction. The rate of interferon production reaches a peak by 2.5 to 3.5 hr and is rapidly shut off by 6–8 hr (Fig. 2). Poly(I).poly(C)-induced interferon production involves the transcription of interferon mRNA for approximately the first 3 hr of induction, the translation of this mRNA in the cytoplasm, and the subsequent glycosylation and secretion of interferon along the classical endoplasmic reticulum–Golgi apparatus pathway. The rapid shutoff of interferon synthesis is mediated through a post-transcriptional mechanism which inactivates or degrades interferon mRNA [33, 43,*]. The regulatory mechanism (Fig. 2) is sensitive to inhibitors of RNA and of protein synthesis. Appropriate treatment of induced cultures with inhibitors of RNA or protein synthesis leads to a large paradoxical enhancement of interferon yields, a phenomenon known as "superinduction" [1, 42, 44, 45]. Superinduction of interferon production is thought to result from an inhibition of the synthesis of a labile repressor by inhibitors of macromolecular synthesis (Fig. 2).

DRB under appropriate conditions causes a marked enhancement of human fibroblast interferon yields [29, 31, 32]. The selectivity of DRB in inhibiting RNA synthesis and the reversibility of this inhibition have formed the basis for its use as a valuable tool in the investigation of the regulation of human interferon synthesis [1, 29, 31–33, 43,*] and

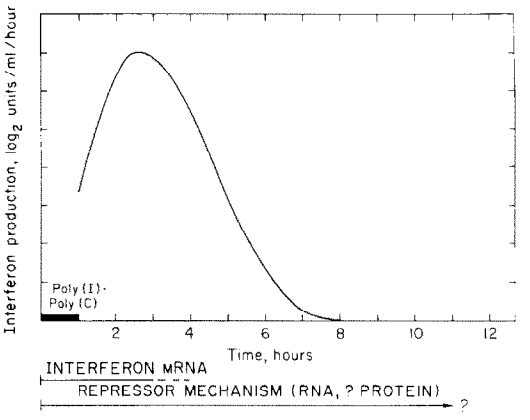


Fig. 2. Scheme of transcription of interferon mRNA and repressor RNA: determinants of interferon synthesis in a culture of FS-4 cells induced by poly(I).poly(C). Taken from Ref. 43.

in the design of a drug regimen that leads to the production of the highest total interferon yield from a culture of diploid human fibroblasts [46].

Inhibition of interferon mRNA synthesis. Treatment of poly(I).poly(C)-induced FS-4 cultures with DRB during the first 2.5 hr of induction under conditions which eliminate superinduction as an experimental variable by the inclusion of cycloheximide and actinomycin D in the protocol leads to an inhibition of interferon yield [23]. The extent of inhibition varies with the concentration of DRB. Detectable inhibition is seen at 5 μM DRB and the yield is inhibited approximately 95 per cent by 60 μM DRB. These results suggested that, although DRB at a high concentration inhibited hnRNA synthesis only partially, it did inhibit the appearance of interferon mRNA and of other cellular mRNA species nearly completely [21–23]. It is important to note that interferon mRNA synthesis is inhibited only partially at 30–40 μM DRB [23], as this is essential for the understanding of superinduction by DRB when treatment of cells with this compound is begun at the time induction with poly(I).poly(C) is commenced.

Superinduction of human interferon production. Induction of FS-4 cultures with poly(I).poly(C) in the presence of DRB with continued maintenance of induced cells in DRB at a moderate concentration (30–40 μM) leads to an increase in total interferon yield compared to DRB-free induced cultures [32]. Under these conditions the rate of interferon production in DRB-treated cultures shows an initial lag compared to control cultures (Fig. 3; [32]). This lag is consistent with the finding that 30–40 μM DRB partially inhibits the synthesis of interferon mRNA. However, the residual amount of interferon mRNA synthesized in the first 3 hr of induction in DRB-treated cultures continues to be translated for up to 4 days (Fig. 3) [31, 32]. Removal of DRB at any time during this period leads to a rapid shutoff of interferon synthesis (Fig. 3). The continued synthesis of interferon in the presence of DRB results in a marked net increase in interferon yields from DRB-treated cultures compared to drug-free controls. The dose–response relationship of this

* P. B. Sehgal, D. S. Lyles and I. Tamm, *Virology*, in press.

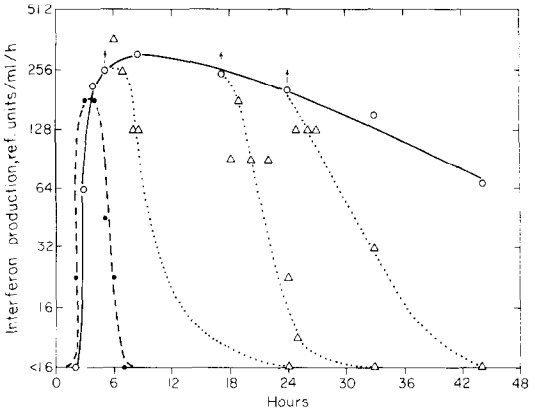


Fig. 3. Rate of interferon production (reference units/ml/hr) during prolonged treatment with 30 μM DRB and effect of termination of treatment. Four 11-day-old cultures (60 mm dishes) were induced with poly(I).poly(C) in the presence (○), and one in the absence (●), of DRB and the rate of interferon production was followed by repeated medium replacement (2 ml, with or without DRB) and interferon titration. At 5, 17, and 24 hr, one dish, which had contained DRB, was washed four times with warm phosphate buffered saline and the rate of interferon production was from then on monitored in the absence of DRB (Δ). Taken from Ref. 32.

paradoxical enhancement of interferon yields by DRB and two other halobenzimidazole ribosides is virtually identical to that of inhibition of RNA synthesis by these compounds (Table 2; [29, 32]). These data add weight to the conclusion that the shutoff of interferon synthesis is mediated by an RNA molecule (Fig. 2). More recently, DRB has been used in experiments that directly establish that the shutoff mechanism involves the irreversible inactivation or degradation of interferon mRNA molecules [33, 43,*]. It is likely that DRB will continue to play a key role in further experiments concerned with the biochemical elucidation of this important translational regulatory mechanism in human cells.

Procedure for large scale production of human fibroblast interferon. A drug regimen based on cycloheximide and DRB has been designed which provides a 50-fold higher yield of human fibroblast interferon than that obtained from drug-free induced cultures [31]. This high yield is similar to that obtainable with the conventional cycloheximide–actinomycin D procedure [31, 45, 46]. However, since the actions of both cycloheximide and DRB are reversible, the same culture of diploid human cells can be superinduced repeatedly using these compounds. FS-4 cultures have been recycled at least four times using the cycloheximide–DRB protocol with the same high yields from each cycle [46]. Actinomycin D-treated cultures cannot, of course, be recycled. At the present time the cycloheximide–DRB procedure used repeatedly provides the highest total yields of interferon from the limited resource represented by a diploid human cell line.

Since DRB alone given at the same time as poly(I).poly(C) will markedly enhance interferon yields, and furthermore, since DRB is a reversible inhibitor of RNA synthesis, it would be of interest to test the therapeutic value of DRB or a related more soluble derivative as a superinducing adjuvant with poly(I).poly(C) or the poly(I).poly(C)–poly-L-lysine complex [47] in animal models of virus infection.

MECHANISM OF INHIBITION OF RNA SYNTHESIS BY DRB

The available evidence supports the view that DRB selectively blocks a step in the synthesis of RNA catalyzed by RNA polymerase II [2, 21, 22].

The mode of action of DRB has been investigated in the salivary gland cells of *C. tentans*, in uninfected HeLa cells, and in HeLa cells infected with adenovirus type 2 (Ad 2). The results obtained in studies of cellular RNA transcription have indicated that DRB inhibits hnRNA synthesis by blocking transcription at or close to the site of RNA chain initiation. A finer analysis of this question using late Ad 2 transcription in HeLa cells as a model system has revealed that DRB does not act at sites of RNA chain initiation but rather acts a short distance downstream from such sites.

Experiments in C. tentans

Salivary gland cells of larvae of the insect *C. tentans* possess giant polytene chromosomes. Specific regions of these chromosomes are transcribed at particular times in development, and this is associated with the appearance of localized puffs on chromosomes. The large amount of RNA synthesized in a single puff is all derived from a single transcription unit.

Elongation of RNA chains in salivary gland explants incubated at 18° is sufficiently slow (approximately 30 nucleotides/sec) so that pulse labeling of cells with tritiated nucleosides for 15–20 min leads to the incorporation of significant amounts of the isotope into nascent unfinished RNA molecules [24, 25]. These nascent labeled molecules are detected on polyacrylamide gel electrophoresis as molecules shorter than full length transcripts. Microdissection of salivary gland chromosomes permits the analysis of nascent molecules derived from one or a few transcription units.

Exposure of salivary gland explants to DRB (65 μM) for brief periods of time (0–15 min) causes preferential inhibition of ^{3}H-incorporation into the shorter nascent RNA during a subsequent pulse (15 min) with [^{3}H]uridine and [^{3}H]cytidine. In contrast, incubation in DRB for 30 min prior to the pulse inhibits ^{3}H-incorporation into both short and long chains of hnRNA [24–26]. As expected, actinomycin D and α-amanitin (known inhibitors of chain elongation) inhibit ^{3}H-incorporation into RNA chains of all sizes, regardless of the length of the treatment period [24].

After removal of DRB, incorporation of [^{3}H]uridine and [^{3}H]cytidine is first largely confined to the shorter RNA chains during a 15-min pulse [26]. Incubation for 30 min in DRB-free medium prior to pulse-labeling restores ^{3}H-incorporation into RNA chains to almost control levels in the entire size range.

* P. B. Sehgal, D. S. Lyles and I. Tamm, *Virology*, in press.

These experiments provided the first evidence that DRB blocks transcription at or close to the site of RNA chain initiation.

Experiments in uninfected HeLa cells

Experiments similar to those described above for *Chironomus* have also been carried out in HeLa cells [27]. The success of these experiments depended on the ability to label nascent hnRNA molecules in mammalian cells. That this could be done was shown by exposing HeLa cells to [^{3}H]uridine at 37° for periods as short as 10–45 sec [48]. In subsequent experiments, HeLa cells were treated with DRB (75 μM) for 45, 90 or 180 sec and pulse-labeled with [^{3}H]uridine for 45 sec [27]. The sedimentation profile of labeled nuclear RNA in sucrose gradients showed that brief treatment with DRB causes a preferential inhibition of incorporation into shorter RNA chains. The longer the DRB treatment, the larger is the size of the diminishing amount of hnRNA which becomes labeled. A brief pretreatment of cells with actinomycin D or 3′-deoxyadenosine does not cause this shift in the sedimentation profile of labeled hnRNA [22, 27]. These results indicate that DRB also inhibits mammalian transcription at or close to the site of RNA chain initiation.

It appears that the synthesis of DRB-resistant high molecular weight hnRNA (> 10S; > 800 nucleotides) and DRB-resistant 7.5 to 9.5S RNA (330–740 nucleotides) in HeLa cells is catalysed by RNA polymerase II, based on the sensitivity of synthesis of these RNA molecules to α-amanitin at low concentrations (1 μg/ml). For these experiments RNA synthesis was monitored *in vitro* in nuclei prepared from DRB-treated HeLa cells [2]. These two RNA classes are, therefore, the synthetic products of the same enzyme which synthesizes the DRB-sensitive hnRNA [2]. The exact biochemical basis for DRB sensitivity or resistance of mammalian hnRNA synthesis is at present not known.

Experiments in Ad 2-infected HeLa cells

Adenovirus type 2 transcription is an excellent model for the study of mammalian RNA synthesis because host cell RNA polymerases are involved in transcribing the Ad 2 genome and because the synthesis and subsequent processing of virus-specific high molecular weight RNA and of hnRNA in uninfected host cells are remarkably analogous (reviewed in Ref. 49). Furthermore, the ability to investigate transcription of specific defined regions of the Ad 2 genome using restriction fragments of the viral DNA permits a detailed analysis of the effect of DRB on RNA synthesis. Since most of Ad 2 transcription late in infection (15–16 hr after infection) is confined to the synthesis of a large (> 25 kilobases) RNA molecule which represents close to 85 per cent of the genome, it is possible to determine the exact site of action of DRB in RNA transcription in a relatively simple manner.

DRB (75 μM) inhibits late Ad 2-specific transcription in HeLa cells by 90–95 per cent and mRNA labeling by > 95 per cent [3]. If DRB inhibits viral RNA transcription at the chain initiation site, then a brief treatment of Ad 2-infected cells with DRB should lead to a greater inhibition of incorporation of [^{3}H]uridine during a 2-min pulse into RNA hybridizing close to the origin of the large transcript as compared to incorporation into RNA corresponding to the terminal portion of this transcript. The results obtained indicate that exposure of virus-infected cells to DRB for 7 or 40 min does cause a graded inhibition of incorporation across the large transcription unit [3]. This is consistent with the hypothesis that DRB acts at or close to the site of RNA chain initiation.

A detailed examination of the effect of DRB on late Ad 2 transcription reveals that ^{3}H-incorporation into RNA complementary to the restriction fragment containing the origin of the large transcript (SmaI f, 11.6–18.2) is not inhibited as strongly as that into RNA transcribed from the adjacent rightward fragment (SmaI b, 18.8–36.7). This apparent anomaly is due to the continued synthesis in Ad 2-infected cells of a short piece of RNA derived from SmaI f even after prolonged treatment with DRB [3]. This short (400–800 nucleotides) RNA was transcribed from the correct, rightward-reading strand of viral DNA. A similar DRB-resistant short RNA fragment has also been observed in the other, smaller late transcription unit located in SmaI e (3.0–11.1) [3]. Furthermore, short, DRB-resistant RNA pieces, transcribed from promoter-containing regions, have also been detected in all of the transcription units that operate early (up to 6 hr after infection) in Ad 2 infection (P. B. Sehgal, N. W. Fraser and J. E. Darnell, unpublished observations). These observations indicate that DRB does not act at the site of chain initiation but blocks transcription at a site a short distance (100–800 nucleotides) distal to the promoter [3]. It is unclear at the present time whether DRB acts at a physiological termination site on the virus genome or whether it causes abnormal termination of RNA chains.

The observation that uninfected HeLa cells synthesize RNA in the size range of 330–740 nucleotides which is DRB resistant and which is made by RNA polymerase II [2] suggests that this class of RNA may be analogous to the DRB-resistant Ad 2-specific short RNA transcribed from regions of DNA containing transcript origins. It is possible that the DRB site represents a physiological regulatory step in eukaryotic transcription. Precedents for such regulatory sites in prokaryotic transcription are found in the tryptophan operon of *Escherichia coli* [50, 51] and in lambda phage transcription [52].

Comment

Inhibition of RNA synthesis by DRB represents a novel biochemical mechanism. The hypothesis that the site at which DRB acts represents an important control step in the synthesis of that portion of mammalian hnRNA which is precursor to mRNA is an attractive one. An intensive investigation into the biochemical basis of the inhibitory effect of DRB on RNA synthesis is clearly indicated. It will also be of great interest to define the molecular structure of the DRB-resistant hnRNA and the 330–740 nucleotide RNA and to relate these findings to the structural features of DRB-sensitive hnRNA. Some of the

outstanding questions are the following. Does DRB-resistant hnRNA contain mRNA sequences? Does the 330–740 nucleotide RNA contain a cap structure identical to that in hnRNA? Can the 330–740 nucleotide RNA be resolved into several specific species, and if so, are these present at 5′-segments in hnRNA? Is DRB itself the active compound or is it metabolized by the cell to an active derivative such as a triphosphate? And finally, is DRB incorporated into RNA?

BENZIMIDAZOLE DERIVATIVES WITH OTHER BIOLOGICAL ACTIVITIES

It is of considerable interest that, in addition to halobenzimidazole ribosides, several other classes of benzimidazoles have distinctive structural features and specific biological activities.

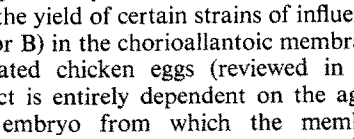
Inhibitors of picornavirus multiplication

2-(α-Hydroxybenzyl)-benzimidazole (HBB, Fig. 4) and certain related derivatives selectively inhibit the multiplication of many, but not all picornaviruses (reviewed in Refs. 1 and 17). HBB does not inhibit the multiplication of viruses belonging to other major groups or the metabolism or proliferation of cells. HBB inhibits the replication of picornavirus RNA without any direct effect on the translation of viral RNA. HBB-dependent virus mutants require the presence of HBB for the replication of their RNA.

The D-(−)-HBB.HCl isomer is two and one-half to three times more active and selective than the L-(+)-HBB.HCl isomer in inhibiting picornavirus multiplication. The overall configuration of HBB is of critical importance for selective virus inhibitory activity. The electron-withdrawing capacity of the entire molecule is also important. Certain 1-substituted derivatives, such as 1-propyl HBB, and 2-(α-methyl-α-hydroxybenzyl)-benzimidazole are more active and to a varying extent more selective than HBB.

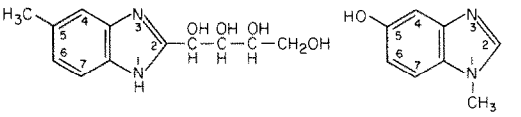
Enhancers of the ability of chick embryo tissue to support influenza virus multiplication

Two kinds of benzimidazole derivatives, exemplified by 5-methyl-2-D-ribobenzimidazole [53, 54] and 5-hydroxy-1-methylbenzimidazole [55] (Fig. 5), increase the yield of certain strains of influenza virus (type A or B) in the chorioallantoic membrane from embryonated chicken eggs (reviewed in Ref. 1). This effect is entirely dependent on the age of the chicken embryo from which the membrane is obtained. The compounds have no enhancing effect



2-(α-Hydroxybenzyl)-benzimidazole (HBB)

Fig. 4. Structure of an inhibitor of picornavirus multiplication.



5-Methyl-2-D-ribo-benzimidazole

5-Hydroxy-1-methyl-benzimidazole

Fig. 5. Structures of two enhancers of influenza virus multiplication.

on the high virus yields produced by membranes from 7-day-old embryonated eggs. Between days 7 and 13 of development, the ability of a unit area of the membrane to support virus multiplication after exposure to a small virus inoculum decreases by 90 per cent. The enhancing compounds partially or completely restore the ability of the membrane from 13-day-old embryos to produce large amounts of virus. As the enhancers are able to affect the state of the membrane prior to infection, it is probable that their action is on host cells rather than directly on the infecting virus. The process affected appears to have a slow turnover rate, in that more than 7 hr is required for the enhancers to show an effect, and, once established, the effect decays slowly.

The important structural feature for the enhancing activity of 5-methyl-2-D-ribobenzimidazole is the presence of the hydroxyl groups in the side chain [55]. The length of the polyhydroxyalkyl side chain is not critical, nor is the presence or absence of methyl groups in the benzenoid ring.

The key structural features of 5-hydroxy-1-methylbenzimidazole, the most active compound, are as follows: the hydroxyl group at position 5 imparts enhancing activity in high degree, whereas the methyl group at position 1 reduces the toxicity of the benzimidazole moiety [55]. Substitution of a hydroxyl group at position 6 instead of position 5 also imparts enhancing activity in high degree; however, there is toxicity associated with substitution at position 6, which severely restricts the maximal enhancing effect obtained.

Inhibitors of the formation or functioning of microtubules

A number of benzimidazole derivatives are effective fungicidal or anthelmintic agents or both (for references see [56]). There is accumulating evidence that compounds such as carbendazim (methyl benzimidazol-2-yl carbamate; Fig. 6) and mebendazole (methyl 5-benzoylbenzimidazol-2-yl carbamate; Fig. 6) react with tubulin and thereby interfere with mitosis [56, 57]. The effect of carbendazim on mycelial growth of *Aspergillus nidulans* is positively correlated with the affinity of the tubulin binding sites for this compound [56]. In drug-resistant mutants the affinity of tubulin for carbendazim is low. The mebendazole-induced changes in the intestinal cells of parasites after treatment of their hosts are probably caused by effects of this drug on cytoplasmic microtubules [58, 59]. It has been pointed out that, although these benzimidazole derivatives appear to interfere with the formation or functioning of microtubules present in all eukaryotic cells, eukaryotes are not equally sensitive to each compound [56]. The molecular basis of the biological

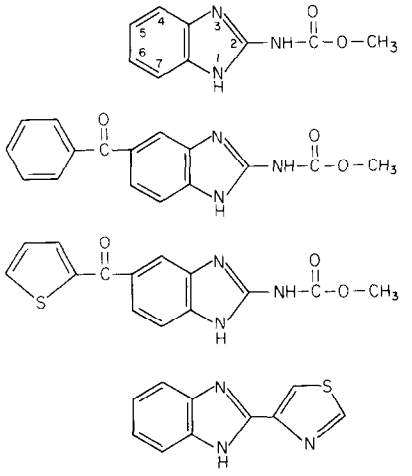


Fig. 6. Structures of benzimidazole derivatives which react with tubulin. From top to bottom:
(1) Carbendazim; benzimidazol-2-yl carbamate.
(2) Mebendazole; methyl 5-benzoylbenzimidazol-2-yl carbamate.
(3) Oncodazole; methyl 5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl carbamate.
(4) Thiabendazole; 2-(4′-thiazolyl)-benzimidazole.

selectivity of these benzimidazole derivatives is not yet known.

Oncodazole [methyl 5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl carbamate; R17934; NSC238159; Fig. 6] has shown significant antitumor activity in several test systems, including L1210 leukemia, lymphocytic P388 leukemia, melanotic melanoma B16 and Lewis lung carcinoma in the mouse [60, 61]. It appeared in the animal experiments that the microtubules of non-dividing normal cells (phagocytes, peritoneal mesothelial cells, liver paryncymal cells, and vascular endothelial cells) were unaffected by drug doses several times higher than those which altered microtubules of neoplastic cells [62]. The animal experiments were undertaken because oncodazole showed an inhibitory effect on the proliferation of mammalian cells in culture, mediated through interference with the structure and function of microtubules [63]. In animal systems, the effects of oncodazole are similar to those of the vinca alkaloid vincristine [61], and the effects on cells in culture are comparable to those of colchicine and the vinca alkaloid [63]. Oncodazole is a potent inhibitor of microtubule assembly *in vitro* [63–65].

Immunopotentiators

Thiabendazole [2-(4′-thiazolyl)-benzimidazole, Fig. 6], an antifungicide and an effective antihelmintic in man, also has shown immunopotentiating properties in a blastogenic system in the mouse using non-specific mitogens, and in the mixed lymphocyte culture [66, 67].

FUTURE PROSPECTS

The value of DRB as an investigative tool in studies of RNA transcription and in the interferon system has been demonstrated. It is likely that the unique properties of this compound will lead to its widespread use in basic biological research. Studies on the biochemical basis of DRB action should continue to provide new insights into the regulation of eukaryotic transcription.

It is also quite apparent that there is little toxicological or pharmacological information on the effects of DRB in the whole animal. This is likely to be the focus of significant future work. Such work would be aided by the synthesis of more soluble effective derivatives of DRB.

The observation that cancer cells (HeLa) largely fail to survive prolonged exposure to DRB whereas a high proportion of normal diploid human cells survive equivalent treatment raises the possibility that DRB, alone or in combination with other drugs, may be of therapeutic value in at least some human neoplasias.

The marked enhancement of interferon yields by DRB provides the basis for producing large quantities of human diploid fibroblast interferon suitable for clinical use. It would also be important to investigate the usefulness of DRB as a superinducing supplement with interferon inducers in animal models of virus infection.

In conclusion, it is likely that the halobenzimidazole ribosides will continue to command keen interest over the next few years.

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Note added in proof—Dreyer and Hausen (*Eur. J. Biochem* **86**, 241 (1978)) have found that transcription by endogenous RNA polymerase II in lysates of Ehrlich ascites cells exhibits two salt optima: one at 0.025 M and another at 0.3 M ammonium sulfate. Preincubation of the cells with DRB results in a selective inactivation of RNA polymerase II active at the lower salt concentration.