The Effects of Substituted Benzimidazoles on the Growth of Viruses and the Nucleic Acid Metabolism of Host Cells

By R. A. BUCKNALL

Imperial Chemical Industries Limited, Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire

(Accepted 17 September 1966)

SUMMARY

The antiviral effects of three substituted benzimidazoles, 4,5,6-trichloro-1-β-D-ribofuranosyl benzimidazole, 2-mercapto-1-(β-4-pyridethyl) benzimidazole and 2-(α-hydroxybenzyl) benzimidazone, have been investigated and compared with their effects on the RNA metabolism of the host cells.

4,5,6-trichloro-1-β-D-ribofuranosyl benzimidazole and 2-mercapto-1-(β-4-pyridethyl) benzimidazole inhibit the growth of influenza A and parainfluenza 1 in calf kidney cells, and vaccinia in L cells, but they also reduce the synthesis of cellular RNA proportionately. They inhibit the growth of EMC in L cells, but only at concentrations at which host cell RNA synthesis is very much reduced. Their action is therefore not specific to a virus induced process.

In contrast, 2-(α-hydroxybenzyl) benzimidazole suppresses the growth of encephalomyocarditis virus in L cells at concentrations which have little effect on host cell RNA synthesis.

INTRODUCTION

The chlorinated ribofuranosyl benzimidazoles are widely quoted as specific antiviral substances (Pienta & Groupé, 1964; Thompson, 1964; Stuart-Harris & Dickinson, 1964; O'Sullivan, 1965). The antiviral effects of these compounds were first described by Tamm et al. (1954) but Tamm now thinks that this class of compound has no specific antiviral action (Tamm & Eggers, 1965). It therefore seemed worth while to examine the metabolic and antiviral effects of some substituted benzimidazoles in order to clarify their status as antiviral agents.

The dichloro- and trichloro-ribofuranosyl benzimidazoles (DRB and TRB) have been reported to be active against influenza A and B and vaccinia viruses in chick chorioallantoic membrane (Tamm et al. 1954; Tamm, 1956a; Tamm & Overman, 1957), and against polio virus and adenovirus 4 in monkey kidney cells (Tamm & Nemes, 1957; Tamm, Nemes & Osterhout, 1960). Following the discovery by Allfrey, Mirsky & Osawa (1957) that DRB strongly inhibited RNA synthesis in isolated calf thymus nuclei, Tamm, Nemes & Osterhout (1960) proposed that the wide spectrum of antiviral activity of DRB was due to its effects on RNA synthesis, and the mode of action of TRB is probably similar.

It seems important here to distinguish between the RNA synthesis of the host cell, and RNA synthesis induced by the invading viral genome. To qualify as true antiviral substances, the ribofuranosyl benzimidazoles must exert a selective effect on virus
induced RNA synthesis so that virus growth is reduced without a corresponding reduction in the parallel metabolic processes of the host cell.

TRB was chosen for this study since it is the most potent of the chlorinated ribosides (Tamm, 1956a; Tamm & Nemes, 1957) and it was compared with two other substituted benzimidazoles: 2-mercapto-1-(β-4-pyridethyl) benzimidazole (MPB), and 2-(α-hydroxybenzyl) benzimidazole (HBB) (Fig. 1). MPB has been shown to be a potent inhibitor of cellular nucleic acid synthesis (Bucknall, 1967), while HBB has little or no such effect (Eggers & Tamm, 1961). To determine whether these compounds have any specific effect on virus induced RNA synthesis, we have treated tissue culture cells with them, and then measured (1) the suppression of cellular RNA synthesis and (2) the capacity of the cells to support the growth of DNA and RNA viruses.

![Chemical structures of TRB, MPB, and HBB](image)

**Fig. 1.** TRB: 4,5,6-trichloro-1-β-D-ribofuranosyl benzimidazole. MPB: 2-Mercapto-1-(β-4-pyridethyl) benzimidazole. HBB: 2-(α-Hydroxybenzyl) benzimidazole.

**METHODS**

**Tissue cultures.** Earle's L cells, and primary calf kidney cells were grown in stoppered 4 x ½ in. tubes with 1 ml. Eagle's (1959) medium containing 8% inactivated calf serum. When confluent monolayers had formed, the cultures were changed to Eagle's medium containing 0.2% bovine plasma albumin.

**Viruses.** Viruses were grown under single cycle conditions to avoid cumulative inhibition of virus growth. The DSP strain of influenza A and the Sendai strain of parainfluenza 1 viruses were adapted to and grown in calf kidney cells. The multiplicity of infection was approximately 100 TCD 50 per cell in both cases. Mouse encephalomyocarditis virus and vaccinia virus (kindly supplied by Dr S. Dales) were grown in L cells. The multiplicities of infection were 10 plaque forming units (p.f.u.)/cell and 100 TCD 50 per cell respectively. Virus infectivity was assayed by TCD 50 in the appropriate cell system except for influenza which was assayed in chorioallantoic membrane pieces (Fazekas de St Groth & White, 1958). The myxoviruses were also assayed by haemagglutination using 0.4% (v/v) guinea pig red blood cells.

**Nucleic acid synthesis.** Benzimidazoles were made up to 500 µg./ml. in Eagle's medium containing 0.2% (w/v) bovine plasma albumin and diluted with the same medium in 2-fold steps to 1.95 µg./ml. One ml. of each dilution was added to six tube cultures which were then rolled for 1, 6 or 24 hr as required. [3H]uridine (50 c./m-mole) or
[\textsuperscript{3}H]thymidine (14.8 c./m-mole) were added to groups of three cultures to give a final concentration of 0-33 \(\mu\)c./ml. After 1 hr at 37\(^\circ\), incorporation was stopped by removing the medium and rapidly washing the cultures with the following ice-cold reagents: 5 ml. saline twice, 5 ml. 10 \%(v/v) perchloric acid, 5 ml. saline. Cells were then treated with 1 ml. 0-25 \%(w/v) trypsin in water for 20 min. at 45\(^\circ\), and then removed from the glass by gentle agitation. Seven ml. dioxane scintillating fluid containing 10 \%(w/v) naphthalene, 1 \%(w/v) PPO and 0-25 \%(w/v) POPOP were then added and the radioactivity measured in a Tri-carb liquid scintillation counter. Results were calculated from the mean 10 min. count of three cultures, and the overall coefficient of variation of the method was 7.9 \%. The specificity of this method for measuring RNA and DNA synthesis was demonstrated by labelling cells grown on coverslips with either [\textsuperscript{3}H] uridine or [\textsuperscript{3}H] thymidine, fixing in absolute alcohol, and treating with ribonuclease at 0-25 mg./ml. in 2-amino-2 hydroxymethyl-propane-1:3-diol (tris) buffer at pH 7-2 for 1 hr at 37\(^\circ\). Cells labelled with [\textsuperscript{3}H]uridine lost 95 \% of their radioactivity while cells labelled with [\textsuperscript{3}H]thymidine lost nothing. The reciprocal experiment with deoxyribonuclease was less convincing, probably because the enzyme was not pure.

**Test system.** Virus inocula were added to cultures and allowed to absorb for 1 hr (vaccinia virus was left for 2 hr). Tubes were then drained, washed three times with warm medium, and benzimidazole solutions added; 1 ml. of each concentration was added to groups of three infected cultures. Compound was also added to a parallel series of uninfected cultures. Calf kidney cultures were rolled, and L cells were kept stationary at 37\(^\circ\). After 6 hr, the rates of RNA and DNA synthesis were measured in the uninfected cultures and, after 12 hr, virus growth in the inoculated cultures was stopped by chilling. Intracellular virus was released by freezing and thawing three times (six times for vaccinia virus) and fluids were clarified by centrifuging before virus assay.

**RESULTS**

The inhibition of nucleic acid synthesis in calf kidney and L cells by TRB and MPB is shown in Fig. 2; there are two points to note. First, TRB exerted a selective effect on RNA synthesis while MPB suppressed both RNA and DNA synthesis simultaneously. Secondly, the concentration required to cause 50 \% suppression of RNA synthesis was approximately the same for each compound. These results were obtained after cells had been treated with compounds for 1 hr. To determine whether the inhibition changed with time, cells were treated for 1, 6 and 24 hr and the rate of RNA synthesis measured. TRB exerted progressively more effect the longer it was left in contact with the cells. In contrast, the inhibition produced by MPB reached a maximum by 1 hr, was unchanged at 6 hr and was waning by 24 hr (Fig. 3). This is an important finding since it showed that the concentration of compound causing 50 \% inhibition of RNA synthesis was changed with time and, if the reduction of virus growth and the reduction of RNA synthesis are compared, this must be taken into account. In practice, since the 4 viruses used in this study all have growth cycles of 8 to 12 hr, the RNA synthesis was measured at 6 hr and taken to be the average value for the duration of each growth cycle.

Fig. 4 shows the effects of TRB and MPB on the growth of influenza A in calf
kidney cells. The concentration of TRB which reduced the yield of infectious virus by 50\% was 3.5 \mu g./ml. and this concentration also inhibited cellular RNA synthesis by 50\%. The yield of haemagglutinin appeared to be slightly less sensitive to TRB since 50\% inhibition occurred at 6 \mu g./ml. Since the accuracy of the haemagglutination titrations was greater than that of the infectivity titrations, haemagglutination was probably the more reliable measure of virus growth. In any event, the inhibition
of haemagglutination and infectivity was not significantly different. Both haemagglutinin and infectious virus yields were reduced to 50% by 11 μg./ml. MPB. This concentration of compound reduced the RNA synthesis of the host cells to 35%. Synthesis of RNA in the calf cell was therefore slightly more sensitive to MPB than was virus growth.

Both compounds reduced the yield of infectious Sendai virus, haemagglutinin and

![Fig. 3. The inhibition of RNA synthesis in calf kidney cells (CK) and mouse L cells (L) by 4,5,6-trichloro-1-β-D-ribofuranosyl benzimidazole (TRB) and 2-mercapto-1-(β-4-pyridethyl) benzimidazole (MPB). The responses after 1 hr (●), 6 hr (○), 24 hr (∆) are shown.](image)

the rate of synthesis of host cell RNA in calf kidney cells, and the 50% inhibitions virtually coincided (Fig. 5). As with influenza, the slight displacement of the haemagglutinin curve was probably not significant.

The result with vaccinia virus in L cells was similar (Fig. 6). As has already been shown (Fig. 2) MPB suppressed synthesis of both RNA and DNA in L cells, while TRB had a selective effect on RNA. Despite the dual action of MPB, the multiplication of vaccinia virus, involving both RNA and DNA synthesis, was no more sensitive
to MPB than to TRB. With both the 50% reduction of virus growth and the 50% reduction in synthesis of cellular RNA virtually coincided.

The results with influenza A, parainfluenza 1 and vaccinia viruses clearly showed that TRB and MPB inhibited both cellular and virus induced RNA synthesis equally. With encephalomyocarditis virus the virus induced RNA synthesis was considerably less sensitive to inhibition than the RNA synthesis of the host cells (Fig. 7).

In contrast to TRB and MPB, 2-(α-hydroxybenzyl) benzimidazole (HBB) was shown to have very little effect on synthesis of cellular RNA, or; indeed, on any other cellular process which has been studied (Eggers & Tamm, 1961). Nevertheless, HBB inhibited the growth of a wide range of enteroviruses, and the specificity of this antiviral activity was therefore investigated for comparison with TRB and MPB. At concentrations between 8 and 62 μg./ml. HBB caused a 20 to 30% stimulation of cellular RNA synthesis. This was followed by a linear fall with a 50% inhibition at 1260 μg./ml. (by extrapolation) (Fig. 8). The growth of encephalomyocarditis virus was affected by concentrations up to 16 μg./ml., but was strongly suppressed at higher concentrations. The 50% end point at 40 μg./ml. clearly indicated a specific effect on virus growth.
DISCUSSION

These results reinforce the opinion (Tamm & Eggers, 1965) that the ribofuranosyl benzimidazoles have no specific action on virus induced RNA synthesis. It now seems that the early indications of specific antiviral effects were due to the methods used for assessing the toxicity of the compounds to the host cells. Most of the developmental work on the benzimidazole glycosides was done by determining the concentration which inhibited the growth of influenza virus in isolated fragments of chick chorioallantoic membrane and comparing this with the concentration which caused toxic effects in these cells. Membrane fragments normally curl and contract when shaken in nutrient medium, and the extent to which this curling was inhibited by added compounds was used as a measure of the compounds' toxicity (Tamm, 1956a). Disturbances in the normal cellular metabolism will inevitably lead to abnormal morphological changes, but the time taken for these to become visible makes this system rather insensitive for measuring toxicity.

Since the primary action of TRB and MPB is on cellular RNA synthesis, it is possible that some other reported biological activities of substituted benzimidazoles may be due to a similar action. For example, Tyrrell & Tamm (1955) showed that the interfering effect of heat inactivated influenza virus in chorioallantoic membrane...
Fig. 6. The inhibition of vaccinia virus growth in L cells by 4,5,6-trichloro-1-β-D-ribofuranosyl benzimidazole (TRB) and 2-mercapto-1-(β-4-pyridethyl) benzimidazole (MPB). Cells were infected, compounds added, and virus harvested after 12 hr. TCD50 = infectivity in L cells (○). Control value was 7.2 log. TCD50/ml. RNA synthesis (△) was measured in uninfected cells after 6 hr. The 50% inhibition points are arrowed.

Fig. 7. The inhibition of encephalomyocarditis virus growth in L cells by 4,5,6-trichloro-1-β-D-ribofuranosyl benzimidazole (TRB) and 2-mercapto-1-(β-4-pyridethyl) benzimidazole (MPB). Cells were infected, compounds added, and virus harvested after 12 hr. TCD50 = infectivity in L cells (○). Control value was 8.9 log. TCD50/ml. RNA synthesis (△) measured in uninfected cells after 6 hr. The 50% inhibition points are arrowed.
Benzimidazoles and virus growth

could be abolished by treating the membrane with dimethylbenzimidazole. Interference was also prevented if membranes were kept at 4°C. It is now thought that many instances of viral interference are mediated through interferon mechanisms, and the production and action of interferon are known to require the continued synthesis of cellular RNA (Taylor, 1964; Wagner, 1964). We find that the RNA synthesis of L cells is inhibited by dimethylbenzimidazole.

Fig. 8. The inhibition of encephalomyocarditis virus growth in L cells by 2-(α-hydroxybenzyl) benzimidazole (HBB). Cells were infected, compounds added, and virus harvested after 12 hr. TCD50 = infectivity in L cells (○). Control value was 8.3 log. TCD50 ml. RNA synthesis (△) was measured in uninfected cells after 6 hr. The 50% inhibition points are arrowed.

Also, Tamm (1956b) reported that 5-methyl-2-ribobenzimidazole at concentrations up to 1000 μg./ml. increased the yield of influenza B virus from chorioallantoic membranes. Tamm suggested that one possible reason for this stimulation of virus growth was that some cellular defence mechanism, which normally operates during virus infection, was damaged by this compound (interferon was then unknown). There have been several recent reports of similar enhancements of virus growth in cells treated with actinomycin (Heller, 1963; Anderson & Atherton, 1964; White & Cheyne, 1965). Actinomycin inhibits synthesis of cellular RNA and so prevents the formation of...
of interferon which normally follows virus infection. In the absence of interferon, virus yields are greater, and it is possible that the increased yields of influenza virus from cells treated with 5-methyl-2-ribobenzimidazole were due to a suppression of interferon production. The stimulation of influenza virus growth by low concentrations of MPB (Fig. 4) may be due to a similar mechanism.

HBB is thought to prevent the growth of enteroviruses, not by direct inhibition of virus RNA synthesis, but by preventing the formation or function of the virus coded polymerase responsible for this synthesis (Baltimore, Eggers, Franklin & Tamm, 1963). Since this enzyme does not occur in uninfected cells, HBB exerts a selective effect on virus growth. In the present study, HBB caused some stimulation of the RNA synthesis of L cells at concentrations which were not antiviral. However, at a concentration of 125 µg./ml., which reduces virus growth by 95%, the synthesis of cellular RNA was normal, which is in sharp contrast to both TRB and MPB. Although it is not possible to calculate a true therapeutic ratio for HBB in this system since the virus inhibition and the RNA inhibition curves are not parallel, the results clearly indicate that HBB, at concentrations between 15 and 162 µg./ml., suppresses encephalomyocarditis virus growth without inhibiting the RNA synthesis of the host cell.

I would like to thank the following people for their valuable contributions to this work: Dr D. L. Swallow, for preparing samples of TRB and HBB, Mr H. Moores, for his excellent technical work, Dr N. B. Finter, for continuous interest and constructive criticism. MPB was prepared and generously supplied by Midland Tar Distillers Ltd.

REFERENCES


Benzimidazoles and virus growth


(Received 15 July 1966)