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The inhibition of bovine herpesvirus-1 by methyl 2-pyridyl ketone thiosemicarbazone and its effects on bovine cells

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Summary

Methyl 2-pyridyl ketone thiosemicarbazone (MPKT) was found to inhibit bovine herpesvirus-1 (BHV-1) at an ED_{50} concentration of approximately 5–10 μM. Several virus strains were shown to have similar sensitivity to the drug and serial passage of virus in the presence of MPKT failed to yield resistant progeny. There was evidence for toxic effects on cells at drug concentrations similar to those required to inhibit virus and passage of cells in low concentrations of MPKT gave results suggesting cumulative toxicity. Pre-incubation of cells in the presence of MPKT produced a residual antiviral effect. Taken together, these observations cast doubt on the selectivity of the drug for BHV-1, at least in the bovine system under test.

Herpesvirus; Bovine herpesvirus-1; Antiviral; Thiosemicarbazone; Cytotoxicity; Resistance

Introduction

Bovine herpesvirus-1 (BHV-1) is a common infection among cattle. It is most frequently associated with the disease infectious bovine rhinotracheitis and (more rarely) the genital manifestation, infectious pustular vulvovaginitis [2]. The virus is typical of the alpha-herpesvirus group in that it establishes latent infections in the peripheral nervous system and recurrences of virus shedding are associated with

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stress and, experimentally, following the administration of corticosteroids [1,6].

Strains of virus with relatively low pathogenicity are widespread among British cattle. However, since the late 1970s new, more pathogenic, strains have been introduced into the UK probably originating from Holstein stock imported into Belgium from Canada [13]. The new strains of virus can readily be distinguished from the earlier British isolates on the basis of restriction endonuclease patterns [4] and these account for much morbidity and significant economic losses.

Vaccines are available but none are completely satisfactory and therefore this disease is a potential target for antiviral therapy. Compounds of the thiosemicarbazone series have long been considered for use as antiviral agents especially in relation to their activity against poxviruses. In fact, isatin-β-thiosemicarbazone and its *N*-methyl derivitive were both shown to be effective inhibitors of BHV-1 [5]. Scientific interest in the thiosemicarbazones was overshadowed by the progress with nucleoside analogues which became the focus of attention for 2 decades, especially in relation to the human herpesviruses. Now there is renewed interest in compounds with alternative modes of action to the nucleosides and also in broadening the spectrum of target viruses to include animal infections. The thiosemicarbazones have been re-appraised and recently the effects of a large series of related compounds were described in terms of their antiviral activity against herpes simplex virus (HSV) relative to several measurements of cellular toxicity [8].

In the present study several thiosemicarbazone derivitives were examined for activity against the bovine herpesvirus, BHV-1. The most active compound was found to be methyl 2-pyridyl ketone thiosemicarbazone (MPKT). MPKT was examined in more detail and while the antiviral effect against several different strains of BHV-1 was confirmed, this was found to be closely associated with effects on the host cells and evidence was obtained suggesting that the compound may not have a specific antiviral activity which can readily be uncoupled from the anticellular activity. These findings may have implications for the use of this and similar compounds against HSV in man.

Materials and Methods

Cell culture

Cells of the Madin-Darby bovine kidney cell line (MDBK) and a bovine tracheal cell line (BT) were maintained in Eagle's minimal essential medium containing 10% new-born calf serum. This serum contained BHV-1 neutralizing antibody. When cultures were infected with virus, serum was either omitted or 5% foetal bovine serum was employed which had been checked and found to be free from neutralizing antibody. Cells were cultured at 37°C in a humidified atmosphere of 5% CO2. After 12–15 passages in culture, fresh cells were resuscitated from frozen stocks.

Virus strains

BHV-1 strains Oxford and 6660 were kindly supplied by Dr E.L. Rollinson of

Cooper's Animal Health, Berkhamsted, U.K. and are laboratory strains representing the typical British endogenous isolate and the new imported strain of the Cooper type respectively. A virus isolated from a case of infectious pustular vulvovaginitis, the Carmarthen strain IPV-C was a gift from Dr Steven Edwards, Central Veterinary Laboratory, Weybridge, U.K. The strain Cl23 was supplied by the Cambridge Veterinary Investigation Centre and was a recent clinical isolate from a local outbreak. All 5 strains were digested with several restriction endonuclease enzymes including *Bam*HI, *Eco*RI, *Hind*III and *Hpa*I. Strains 6660 and Cl23 were found to have restriction patterns which closely resembled the American reference strain, Cooper [3] while Oxford resembled the K-22 reference strain [3]. Strain IPV-C clearly differed from Oxford but in restriction pattern was most closely related to the K-22 type. Virus stocks were stored at −70°C in small aliquots. Virus was thawed immediately before use and diluted in Eagle's medium without serum.

Methyl 2-pyridyl ketone thiosemicabazone

The compound (also known by the trivial name 2-acetylpyridine thiosemicarbazone) was a gift from Dr M.J. Hall of Roche Products, Welwyn Garden City, U.K. It was supplied as a dry powder and was made up as a stock solution in distilled water at 1 mg/ml which was stored frozen and diluted in Eagle's medium for use. The compound has a molecular weight of 194 and the structural formula is shown (Fig. 1).

Determination of antiviral effect by plaque reduction

All virus strains were found to produce satisfactory plaques in 2–3 days. Monolayers of MDBK or BT were established in 24-well plastic multiwell dishes ('Linbro', Flow Laboratories). Approximately 100 pfu virus were added to each well suspended in 0.1 ml Eagle's medium. After 1 h adsorption at 37°C, 1 ml Eagle's medium containing various dilutions of MPKT (thickened with 3% carboxymethyl cellulose to reduce secondary plaque formation) was added to each well. After 2 or 3 days the cells were stained using 0.5% crystal violet for 10–15 min and the plaques enumerated microscopically. The number of plaques expressed as a percentage of the number forming in the absence of drug was plotted versus the log10 MPKT concentration and the ED_{50} determined directly from the curve.

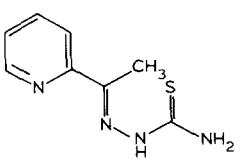

Fig. 1. The structural formula of methyl 2-pyridyl ketone thiosemicarbazone (MPKT). The compound is also known by the trivial name 2-acetylpyridine thiosemicarbazone.

Determination of antiviral effect by reduction in yield

Monolayer cultures of MDBK were prepared in 6 cm plastic tissue culture dishes (Falcon). Before infection the cultures in several dishes were suspended by means of trypsin, ethylenediaminetetra-acetic acid and the cells enumerated by means of a haemacytometer. This then enabled an accurate estimate of the multiplicity of infection. Virus was added in 0.2 ml medium for a period of 1 h adsorption then 5 ml medium containing drug were added to each dish. At daily intervals the cultures were harvested. In some cases the supernatant and cell fraction were separated in order to titrate the cell-associated and cell-free virus independently. The infected cells were harvested and centrifuged at 800 × *g* for 10 min. The cells were resuspended in fresh medium and the virus was released by brief ultrasonic vibration in a sonic waterbath. Samples were stored at −70°C prior to titration by plaque count in further cultures of MDBK cells.

Determination of antiviral effect following preincubation of cells in MPKT

Established monolayers were cultured in the presence of various concentrations of MPKT for 2 days. The cell monolayers were then washed twice with phosphate-buffered saline then virus added in the usual way. After 1 h adsorbtion, fresh medium (free from drug) was added for a further period of 2 or 3 days. At the end of the incubation period either plaques were enumerated or virus yield was determined by titration as above.

Measurement of effect of MPKT on cell growth

The total number of cells seeded on to 6 cm culture dishes was enumerated using a haemacytometer using at least 2 replicate dishes for each count. The cells from further dishes were suspended and counted at intervals after incubation in the presence of various concentrations of MPKT. Two kinds of experiment were carried out. In the first case drug was present at the time of seeding the cells and subsequent counts enabled the cell doubling time to be determined. Alternatively, monolayers were established before adding MPKT-containing medium and the cell counts determined after various times of incubation in the presence of the drug. In some cases 2.5% trypan blue was added to the cell suspension before counting to differentiate between viable (dye-excluding) and non-viable (blue-staining) cells.

Results

The activity of MPKT against several strains of BHV-1

The activity of MPKT was tested by means of plaque reduction assay carried out in preformed monolayer cultures of bovine cells. Four strains of virus were tested comprising the strains Oxford and IPV-C which are, respectively, respiratory and genital isolates of the 'old' type and strains 6660 and Cl23 which represent the new, more pathogenic, strains introduced into the U.K. Cl23 is a recent clinical isolate whereas the others are all fairly well-characterized laboratory strains. The ED_{50} concentration for plaque reduction was in the range 0.9–2 μg/ml (5–10 μM) (Fig.

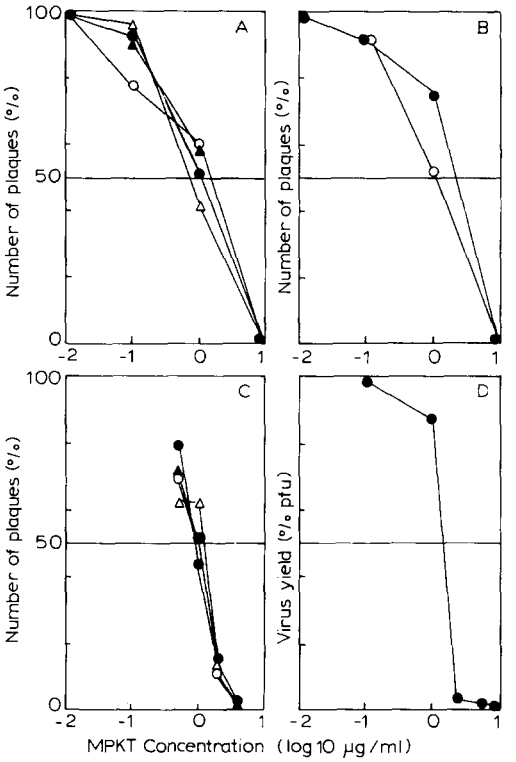

Fig. 2. *Determination of the ED_{50} concentration of MPKT against BHV-1 in bovine cells. (A)* Comparison of four virus strains in MDBK cells by plaque reduction. • = Oxford, ○ = 6660, ▲ = Cl23, △ = IPV-C. (B) The inhibition of two virus strains in BT cells by plaque reduction. • = Oxford, ○ = Cl23. (C) Comparison of parental strain, Cl23 with two viruses which have been passaged in the presence of MPKT. These show no evidence for the acquisition of resistance as judged by plaque reduction assay in MDBK cells. • = Cl23, ▲ = Cl23P4, △ = Cl23P8 following 4 and 8 passages respectively in increasing concentrations of MPKT. (D) The effect of MPKT on the yield of BHV-1 from MDBK cells at low multiplicity of infection. Monolayers of MDBK were infected at 0.1 moi with BHV-1 strain Cl23. The virus yields obtained at 72 h post infection in pfu/culture are plotted as the % yield obtained in the absence of the drug versus Log10 MPKT concentration.

2A). The notable feature of this result was that 4 viruses from different origins had very similar sensitivity to the drug. Repeated tests confirmed that there was no significant difference between sensitivity of the viruses.

The influence of cell type on antiviral activity

BHV-1 has a relatively narrow host-range compared with HSV and cells of other than bovine origin tend to be poorly permissive. The plaque-reduction test was therefore compared in 2 kinds of bovine cell cultures derived from kidney and trachea. The compound was tested against 2 virus strains (Oxford and Cl23) in tracheal cells by plaque reduction (Fig. 2B). Again there was no significant difference in the sensitivity either between the 2 virus types or from the results obtained in MDBK cells.

The antiviral activity of MPKT measured by reduction in virus yield

The measurement of antiviral activity by plaque inhibition has several disadvantages. The method is somewhat subjective, especially when the plaques are reducing in size with increasing concentrations of the inhibitor. Secondly, at higher drug concentrations some cytopathic effects were observed (see below) and there was concern that this might obscure the presence of virus plaques. The measurement of virus yield reduction does not suffer from these possible disadvantages. Accordingly MPKT was tested by this method against a strain of BHV-1 in MDBK cells (Fig. 2D). The experiment was carried out at low moi; this avoided the difficulty of eliminating the inoculum virus. The yields of virus measured 72 h after inoculation showed a reduction with increasing MPKT concentration. Both the shape of the curve and the ED_{50} concentration (2 μg/ml) corresponded closely to the data that had been obtained previously by measuring plaque-reduction.

An attempt to develop MPKT-resistance in BHV-1

One conclusive test for a drug which shows true selective toxicity is to develop mutants which are resistant to the compound in question. This has been done successfully with many different nucleoside analogues and pyrophosphate analogues in relation to HSV and other herpesviruses. For example we have had no difficulty in developing mutants of BHV-1 which are resistant to bromovinyl deoxyuridine or phosphonoformate and such mutants were obtained from strain 6660 and Oxford within 2–5 passages in the presence of a sub-inhibitory concentration of the particular drug (H.J. Field and S. Mittal, unpublished data).

BHV-1 strain Cl23 was passaged 8 times in MDBK cells in concentrations of MPKT of 0.7 and 1.5 μg/ml for the first 2 passages and 3 μg/ml for the third and all subsequent passages (except passage 6 for which the concentration was increased to 4 μg/ml). This is about twice the ED_{50} which had been determined as approximately 2 μg/ml (Fig. 2A,B,D). The virus was cultured once in the absence of drug at each passage level to increase the yield of progeny to a workable concentration. The sensitivity of the virus yield obtained at each passage level was then tested by means of plaque-reduction in comparison with the original virus inoculum but showed that the MPKT sensitivity was unchanged. The results for passage levels 4 and 8 are illustrated by way of example (Fig. 2C) and revealed no decrease in sensitivity compared with the original virus.

Thus no evidence was obtained for the development of resistant progeny in spite of extensive passaging in the presence of an inhibitory concentration of MPKT in a manner which has readily yielded resistance to alternative drugs. One possibility which remained is that a cellular function is sensitive to inhibition which masks the development of virus resistance. For this reason cells were passaged in the presence of MPKT in an attempt to develop an MDBK cell culture adapted to the drug. This approach was abandoned because the cells failed to flourish on passage in the drug and even with low concentrations of MPKT there was evidence of cumulative toxicity (see below).

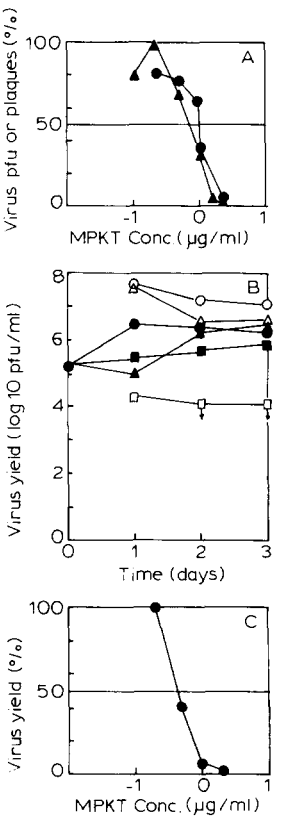

Fig. 3. *The effects of pre-incubation in the presence of MPKT on the growth of BHV-1 in MDBK cells.* (A) ▲ = number of plaques (%) and ● = virus yield (%pfu) from MDBK cells infected with BHV-1 (strain Cl23) at a multiplicity of infection of 0.1 pfu/cell. Preformed monolayer cultures were incubated in the presence of various concentrations of MPKT for 48 h. The drug-containing medium was then removed from the cultures and the cells washed several times in drug-free medium before addition of virus. Plaques were counted at 48 h post infection or cells harvested to determine virus yield. The % plaques or yield is plotted verus log10 MPKT concentration which was present before infection. (B) MDBK cells were pre-incubated in MPKT as above before infection with BHV-1 (strain Cl23) at a multiplicity of infection of 0.1 pfu/cell. At 24 h intervals after infection the cells were harvested and separated from the culture fluid by centrifugation. Virus titres were then measured independently in the culture supernatant and cells. ○ supernatant, ● = cell-associated virus in the absence of drug. △ = supernatant, ▲ = cell-associated virus following pre-incubation with 1 μg/ml MPKT. □ = supernatant, ■ = cell-associated virus following pre-incubation with 2 μg/ml MPKT. log10 virus yields are plotted against time post-infection. (C) Confirmation of the effects of pre-incubation of MDBK cells in the presence of MPKT on the replication of BHV-1 at high multiplicity of infection. Cells were pre-incubated in the various concentrations of MPKT as before. After removal of the drug-containing medium, cultures were inoculated with BHV-1 (strain 6660) at a multiplicity of 5 pfu/cell. Virus yield (cell-associated and supernatant combined) were determined at 48 h post-infection and plotted as percentage yield in the absence of drug versus log10 MPKT concentration.

TABLE 1

The effects of MPKT on the viability of established monolayers of MDBK cells judged by trypan-blue exclusion.

MPKT concentration (μg/ml)	Time (days) after 48 h exposure to drug			
	1	2	3	4
0	5.5*	5.3	5.7	6.5
0.2	3.1	6.6	4.6	7.7
0.5	6.6	4.3	7.2	7.0
0.75	4.3	4.9	4.8	4.3
1.0	5.1	6.7	4.2	4.0
1.5	6.1	7.6	4.5	6.9
2.0	4.2	1.2	0.45	0.33

* Data $\times 10^6$ are counts of cells/culture dish obtained from the mean of 2 samples from 2 dishes for each result. The drug was added for 48 h when confluent monolayers had been established. After removal of the drug by washing twice in PBS the observation period was commenced.

The development of an antiviral state on pre-incubation with MPKT

A number of experiments were carried out to test the effects of removal of the inhibitor at different stages of virus infection. Surprisingly, a period of pre-incubation with MPKT resulted in inhibition of BHV-1 when the drug was absent from the culture medium at the time of infection (Fig. 3A). The ED_{50} concentrations obtained for the inhibition of virus yield and plaque-reduction (1 and 0.9 μg/ml, respectively) in the MPKT pre-treated cells were similar or slightly lower than those obtained in the conventional assay.

In a similar kind of experiment the inhibition of virus yield produced by pre-incubation of cells with MPKT was measured at several times post-infection using 2 different drug concentrations (Fig. 3B). The virus yield from the cells and in the supernatant were measured independently. Virus yields in the presence of MPKT were reduced as expected and a notable observation was that while a reduced yield of virus could be detected in the cellular fraction in cultures pre-incubated with 2 μg/ml MPKT, no virus could be detected in the culture fluid 48 or 72 h after infection following pre-treatment with this concentration of drug.

Finally, using a similar experimental design in which MDBK cell monolayers were pre-incubated in the presence of various concentrations of MPKT the reduction in yield was measured using high multiplicity conditions (5 pfu/cell). The percentage reduction in yield (Fig. 3C) was corrected for the actual number of cells which were counted at the time of virus inoculation. In this case viability was judged by the technique of trypan-blue exclusion. At the highest drug concentration (2 μg/ml) a reduction in cell count was noted in uninfected cultures on the third and fourth day after removal of the drug (Table 1) but this was not sufficient to account for the observed reduction in virus yield. It appears then that there is marked antiviral effect in cells which were morphologically normal at the time of virus inoculation, which could not be accounted for in terms of virus spread from cell to cell.

Evidence for the anticellular activity of MPKT

Careful examination of MDBK cell monolayers showed no obvious morphological changes during 72 h culture in the presence of 1 μg/ml MPKT or less. However, some morphological changes did appear after 72 h in the presence of 2 μg/ml and culture in the presence of 10 μg/ml produced gross morphological changes. It appeared that when the medium was replaced with drug-free medium after 72 h exposure the cells gradually recovered normal morphology. These observations were then pursued by means of quantitative measurements of cell growth.

A sensitive measure of toxicity is to test the ability of cells to form a monolayer

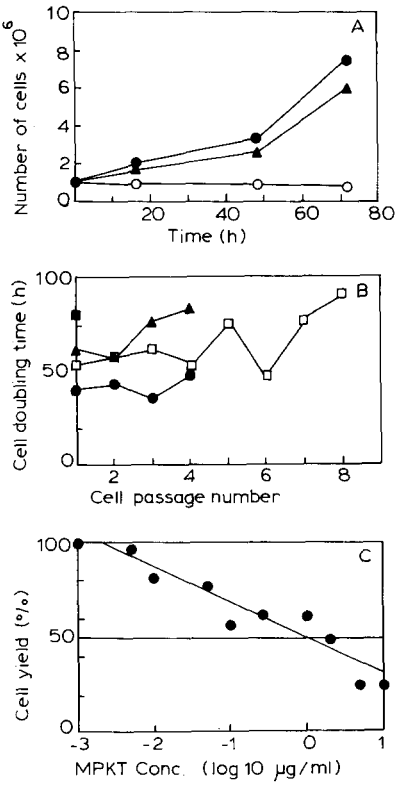

Fig. 4. *The effects of MPKT on the growth of MDBK cells.* (A) The suppression of cell multiplication by MPKT. 1.6×10^6 MDBK cells were plated in replicate cultures in the presence of • = no drug, ▲ = 1 μg/ml and ○ = 10 μg/ml MPKT. At intervals the cultures were suspended by trypsinisation and the cells enumerated. (B) The effects of the presence of MPKT during the repeated passage of MDBK cells. • = No drug present, □ = culture in the presence of 0.2 μg/ml, ▲ = 1 μg/ml, ■ = 2 μg/ml. (C) Determination of the ED_{50} concentration of MPKT required for depression of cell growth of MDBK cell assessed after 72 h incubation. 2.4×10^6 cells were seeded into replicate culture dishes in the presence of various concentrations of MPKT. Cells were enumerated after 72 h incubation and expressed as a percentage (cell number in the absence of drug). This corresponded to 2.2×10^6 cells in the presence of the highest concentration examined (10 μg/ml). The percentage (cell yield) is plotted versus log10 MPKT concentration. The best line was fitted by regression analysis on one predictor using the method of minimum squares.

when sub-cultured in the presence of a potential inhibitor. A suspension containing 1.6×10^6 cells was cultured in medium containing no drug, 1 and 10 μg/ml MPKT. Cultures were harvested at intervals and the cells counted. The results (Fig. 4A) showed a reduction in growth rate at 1 μg/ml and a complete cessation of cell division at 10 μg/ml MPKT.

As mentioned above difficulty was encountered when trying to adapt MDBK cells to grow in the presence of the drug. This was tested more objectively by measuring the doubling rate of cells through several successive passages in the presence of MPKT. It was found (Fig. 4B) that with 2 μg/ml there was insufficient cell growth to enable the doubling rate to be measured after the first passage. With 1 and 0.2 μg/ml the growth rate was significantly decreased and there appeared to be a gradual decrease in growth rate with successive passages. It was notable that in 0.2 μg/ml MPKT the doubling time appeared to increase from 56 h after 1 passage (compared to 40 h in the absence of drug) to 91 h after the 8th. passage. This then suggested that there was a cumulative toxic effect which occurred in a relatively low concentration of the inhibitor.

Finally the ID_{50} concentration for cell growth inhibition was determined by subculturing replicate cultures containing 2.4×10^6 cells and harvesting the cells after 72 h incubation in various concentrations of MPKT. The ID_{50} was determined to be 1 μg/ml (Fig. 4C). At the end of the experiment the untreated cells had completed 2 doublings, those in 1 μg/ml achieved approximately 1 doubling while the cells in the presence of 10 μg/ml MPKT showed no measurable increase in cell number.

Discussion

MPKT, one of a series of thiosemicarbazone derivatives, has previously been shown to be active against HSV in vitro and in a guinea pig skin infection model [7,8]. The primary target for the virus inhibitory effect is thought to be the virus-specified ribonucleotide diphosphate reductase [12] and a selective inhibition of this enzyme compared with the mammalian cellular counterpart has been reported recently [11].

The drugs have also been shown to have toxic effects on mammalian cells and it has been suggested that cellular ribonucleotide diphosphate reductase is the most likely target for these effects [10,11]. In a detailed survey of the antiviral and anticellular effects of a large series of thiosemicarbazone derivatives [8], MPKT was ascribed an in vitro 'therapeutic index' of 144 and 192 against HSV-1 and HSV-2, respectively. However, the [^{3}H]thymidine incorporation assay used by these workers yielded an ED_{50} drug concentration of 2 μg/ml, (approximately twice ED_{50} concentration required for virus inhibition) although this result was not used in the calculation of therapeutic index. The criteria for toxicity used in this paper [8] were essentially 'biochemical' (labelled amino acid incorporation, Lowry, labelled thymidine with diphenylamine DNA assay) after exposure of cultures to the drugs for comparatively short times (18–24 h). Effects on cell numbers or doubling times were not reported by these workers.

In the present study MPKT was found active against several strains of the bovine herpesvirus BHV-1 measured by virus inhibition in bovine cell cultures. The ED_{50} concentration (approx. 1 μg/ml) agrees closely with the published data for HSV [8]. However, a number of other observations arising from this work lead us to doubt the antiviral selectivity of MPKT at least in the bovine systems under consideration. Most important are the following: (1) Several different strains of virus showed identical sensitivity in the same cell cultures. (2) No evidence of resistance development was obtained after repeated passage of virus in the presence of MPKT. (3) Effects on cell growth were obtained at drug concentrations similar to the antiviral ED_{50} and in particular there appeared to be a cumulative toxicity when cells were passaged in low concentrations of the drug. (4) Finally, pre-incubation of cells in the drug produced an antiviral state which could not be reversed by washing the cultures with drug-free medium. It was not clear whether this was due to an irreversible change in the cells or to failure to remove the compound from the cells by simple washing and further work will be required to elucidate this phenomenon. Taken together, the above observations provide strong circumstantial evidence that MPKT does not show true selective toxicity for BHV-1 replicating in bovine tissue cultures.

Ribonucleotide diphosphate reductase has been suggested as a suitable target for the design of antiviral agents [9]. MPKT is an example of a candidate compound for this antiviral strategy. The observations described here clearly sound a note of caution regarding the interpretation of antiviral effects observed in vitro and should be followed up in relation to HSV, the human counterpart.

Acknowledgements

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