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Evaluation of new antiviral agents: I. In vitro perspectives

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Summary

A variety of different methods for the evaluation of antiviral agents in cell culture systems are briefly reviewed. It has been repeatedly noted that many test conditions such as the cell culture system, virus strain, virus challenge dose, virus input multiplicity of infection, and time of harvesting, etc., can substantially affect or even alter the test results, thus making comparative studies and unambiguous evaluations very difficult. Attempts are made to discuss previous test methods together with our recent studies with the aim to simplify test procedures and assay methods. Suggestions are proposed for in vitro evaluation of new antiviral agents. It is hoped that this review will alarm investigators to the problems of assaying new antiviral agents. If the suggestions made in this review can be followed, the screening of the enormous number of promising antiviral compounds may be made more efficiently in the near future.

Chemotherapy; Antiviral agent; Test method

Introduction

Today chemotherapy of viral diseases has become a reality in clinical medicine. Remarkable progress has been made during the past decade in the development of new antiviral agents for the treatment of some common viral diseases. These

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include treatments for herpes keratitis, herpes encephalitis and genital herpes (Allison, 1986; Gold and Corey, 1987; Kaufman, 1962; Whitley et al., 1981). More recently, much effort has been made to develop effective antiviral agents for the treatment of AIDS patients (Hamamoto et al., 1987; Mitsuya and Broder, 1986; Mitsuya et al., 1985; Nakashima et al., 1986b). As more new antiviral agents are synthesized, the pressure for developing suitable and practicable methods to evaluate these new agents will undoubtedly increase. Thus, simple procedures and guidelines for evaluating antiviral agents are urgently needed.

An antiviral agent can be screened first for its effectiveness and toxicity in cell culture systems. In reviewing the literature, it was noted that studies on the assessment of antiviral agents varied greatly from one laboratory to another (Choi et al., 1978; Collins and Bauer, 1977; De Clercq et al., 1980; Sauer et al., 1984; Sidwell, 1986; WHO, 1987). Because there are no standard testing procedures, it is often very difficult to compare the results reported by different laboratories. In this review, attempts are made to discuss the various methods that have been used for in vitro tests and three assay steps are suggested for the evaluation of new antiviral agents in cultured cells.

Methods commonly used for evaluation of antiviral activities in vitro

In order to test the inhibitory activity of a new antiviral agent, it is first necessary to select the host-cell system(s) in which the virus replication can be measured. Viruses vary considerably in their ability to replicate in cultured cells. Many viruses can cause CPE while some of them can form plaques. Others are capable of producing specialized function, i.e. hemagglutination, hemadsorption, or cell transformation. Virus replications in cell cultures may also be monitored by the detection of viral products, i.e. viral DNA, RNA or polypeptides. Thus the antiviral test selected may be based on inhibition of CPE; reduction of plaque formation, reducing virus yield, or other viral functions.

Inhibition of virus-induced cytopathic effect

For viruses that induce CPE but do not readily form plaques in cell cultures, a quantal assay can be used to determine the effectiveness of an antiviral agent (De Clercq et al., 1980; Field et al., 1986). The assay is basically done as follows: a series of quadruplicate cell cultures prepared in culture panels or tubes are infected with a constant dose of about 100 $TCID_{50}$ of virus. After 1–2 h of virus adsorption at 37°C, maintenance media containing various concentrations of an antiviral agent are added to the cultures. If possible, the concentration range should include a low dose in which no apparent antiviral effect is observed, and a high concentration in which the virus replication is maximally inhibited. Virus-induced CPEs are recorded each day until all of the quadruplicate cultures in the virus control (i.e. those cultures without the antiviral agent) show CPE. The 50% effective dose (ED_{50}) of the antiviral agent is expressed as the concentration that inhibits

CPE in half of the quadruplicate cultures. Alternatively, the degree of the virus-induced CPE in certain concentrations of the antiviral agent can be compared and the ED_{50} is the drug concentration which inhibits the CPE by 50%.

A viable cell count assay has been developed for the detection of anti-HIV activity in cultured cells. Because HIV infection results in the decrease in viable cells in infected cultures, protection of viable cells indicates the antiviral activity of an antiviral agent against HIV infection (Hamamoto et al., 1987; Mitsuya et al., 1985; Nakashima et al., 1986b).

Plaque reduction assay

For viruses which form plaques in suitable cell systems, inhibition of plaque formation is a simple and effective means for detecting antiviral activity. This is usually performed in appropriate cell monolayers infected with a constant dose of the virus, for example, 50–100 PFU (plaque forming units), depending upon the size of the cell monolayer. After 1–2 h of virus adsorption at 37°C, an overlay medium with nutrients and 1–2% methylcellulous containing a range of concentrations of the test agent is added to the infected cell monolayers. The infected cultures are then returned to incubation for an appropriate period of time varying with the individual virus. For plaque number enumeration, the infected cultures are fixed and stained for examination. Examples are shown in Fig. 1 which illustrates the inhibitions of plaque formation of herpes simplex virus type 1 (HSV-1) in Vero cell and in guinea pig embryo (GPE) cell monolayers by acyclovir (ACV). By com-

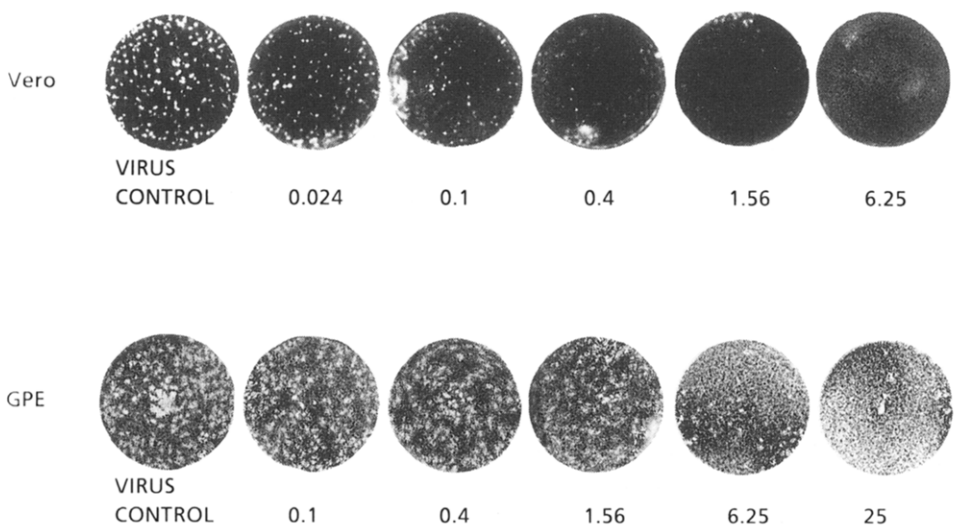

Fig. 1. Plaque reduction assay for evaluation of antiviral agent (ACV) against herpes simplex virus infections in Vero cell and in GPE cell monolayers. A 50% reduction of plaque formation of HSV-1 by ACV at 0.4 μM in Vero cell monolayers (top row) whereas 6.25 μM of ACV was necessary in GPE cell monolayers (bottom row). Unpublished data.

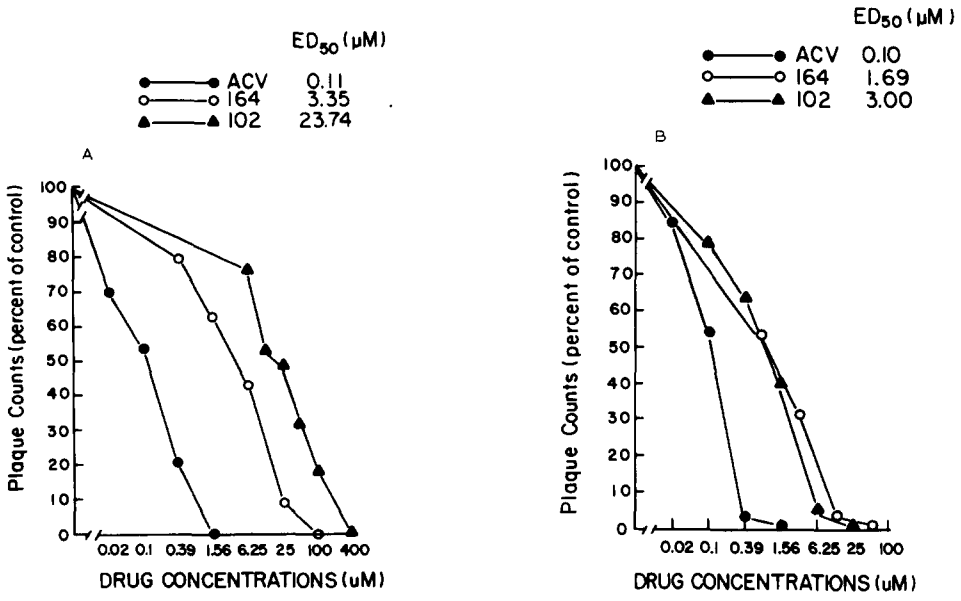

Fig. 2. Dose response curves for three antiviral agents, ACV, and compounds 164 and 102 against HSV-1 replication in (A) Vero cell cultures and (B) in HEF cell cultures by plaque reduction assay. Unpublished data.

paring the plaque number obtained in the virus infected cultures without drug (controls) with the plaque number in cultures containing varying concentrations of the drug a dose response curve is obtained and the dose required to reduce the plaque number by 50%, i.e. ED_{50} is calculated. Representative dose-response curves are shown in Fig. 2, where the effects of ACV, and two new compounds, compound 102 (4-amino-5-bromo-7-(2-hydroxyethoxymethyl)pyrrolo[2,3-d]pyrimidine) (Hu and Hsiung, 1988), and compound 164, 9-[(2-hydroxy-1,3,2-dioxaphosphorinan-5-yl)oxymethyl]-guanine P-oxide (Field et al., 1986) on plaque formation of HSV type 1 (HSV-1) are determined in Vero cell culture system (Fig. 2A) and HEF cell culture system (Fig. 2B). The plaque reduction assay has been shown to be an accurate and reliable technique for the quantitative assay of antiviral activity and the calculated ED_{50} provides a reasonably accurate basis for the comparison of relative drug potency (Boyd et al., 1987; Collins and Bauer, 1977).

A modified plaque forming assay has recently been developed for the titration of HIV (Harada et al., 1985a,b; Nakashima et al., 1986a). Since most of HIV permissive cells do not attach to the surface of culture vessels, poly-l-lysine (Pll) is used to coat the culture dishes and then the HIV permissive cells, such as MT-4, are attached. Plaque reduction assay can then be performed as usual with various concentrations of test agents and 0.6% agarose in the overlay medium, followed by neutral red staining for plaque enumeration.

Virus yield reduction assay

Virus yield reduction assay has been used for a long time for determining the antiviral effect of antiviral agents (Amtman et al., 1987; Boyd et al., 1987; Cheng et al., 1980; Collins and Bauer, 1977). The assay is basically performed as follows: (1) The appropriate cell cultures are infected with a given amount of virus; (2) After virus adsorption for 2 h at 37°C, the unadsorbed virus is removed by washing 3 times with HBSS and a range of concentrations of the test agent are added; (3) After a period of incubation to allow virus replication, total virus yields are assayed; (4) The drug concentrations required to reduce 90% (1 $\log_{10}$ reduction), or 99% (2 $\log_{10}$ reduction) of the virus yield as compared to the virus control (i.e. infected cultures without drug) are determined from the dose-response curves and are expressed as ED_{90} or ED_{99} of the antiviral agent in virus yield reduction assay. Alternatively, the inhibiting effect of the antiviral agent on the virus yield can also be expressed as $\log_{10}$ reductions at certain concentrations of the agent.

Previous reports have demonstrated that the dose-response curves obtained by the virus yield reduction assay were equally satisfactory as the plaque reduction assay for comparing the relative potencies of various antiviral agents (Collins and Bauer, 1977). This is also confirmed by our recent investigation, where the antiviral activities of compound 164, compound 102 and ACV against a guinea pig herpesvirus (GPHLV) infection were evaluated by the two assay methods in parallel (Hu and Hsiung, 1988). Our results showed that the relative potencies of the 3 compounds were essentially the same when assayed by the two methods. It has been shown previously that the plaque reduction test and the virus yield reduction assay revealed contradicting results; thus, these two tests might detect different aspects of viral infection in cultured cells (Boyd et al., 1987). As will be discussed later, certain test conditions, i.e. virus challenge dose (input multiplicity of infection and virus yield harvesting time) must be optimized for each virus-cell system in order to better correlate the evaluation results of the different methods of testing.

Assay systems based on measurement of specialized functions and viral products

A number of viruses do not produce plaques nor cause CPE readily, but may be quantified by certain specialized function based on their unique properties. For example, both hemagglutination and hemadsorption tests have been used to study the antiviral activity against myxoviruses (Bauer, 1972). Various immunological tests detecting viral antigens in cell cultures such as immunofluorescence assay or enzyme-linked immunosorbent assay (ELISA) can be used to determine the extent of virus replication and, thereby, obtain a measure of the inhibitory effect of various antiviral agents on virus replication. These have been used in the anti-EBV, anti-HSV and anti-HIV studies (Farber et al., 1987; Margalith et al., 1980a; Mitsuya et al., 1985; Nakashima et al., 1986b, Rabalais et al., 1987). At the molecular level, the reduction or inhibition in the synthesis of virus-specific polypeptides in infected cell cultures is also an indication of antiviral activity (Hutt-Fletcher et al.,

1986; Lin et al., 1985). The extent of the synthesis of viral nucleic acids in infected cultures can be measured by determining the uptake of radioactive isotope labelled precursors (^{32}P, etc.) or viral genome copy numbers. This method is widely used in anti-EBV studies (Hutt-Fletcher et al., 1986; Lin et al., 1983, 1985, 1987). For viruses such as EBV, which can transform appropriate cultured cells in vitro, the inhibition of cell transformation can also be used to indicate antiviral activity (Margalith et al., 1980a,b). And for HIV, which exhibits the unique reverse transcriptase (RT) activity, the inhibition of RT activity is an indication of the anti-HIV effect (Mitsuya et al., 1985; Nakashima et al., 1986b). As long as a satisfactory dose-response curve can be obtained, all of the above methods may be used to determine the ED_{50} value and, thereby, evaluate the relative potency of new antiviral agents.

Factors influencing evaluation of antiviral agents in cultured cells

Host cell system

Differences in cell culture sensitivity to a given virus infection has been noted by many investigators (Chang et al., 1986; Fayram et al., 1986; Landry et al., 1982; Schwartz et al., 1987) and the response of different cell cultures to a given antiviral agent, including drug metabolism, toxicity, etc., may vary greatly. To be able to perform antiviral testing against a particular virus infection, one must obtain suitable host cell system(s) for that virus infection. The same antiviral agent may behave very differently in different cell culture systems even though the same virus strain is used (Collins and Oliver, 1985; De Clercq, 1982; Harmenberg et al., 1980; Hsiung and Landry, 1986; Person et al., 1970; Schinazi et al., 1987). These dif-

TABLE 1

Comparison of antiviral activities of three nucleoside analogs against herpes simplex virus types 1 and 2 infection in four different cell cultures[a]

Virus	Compound	ED_{50} (μM) in[b]			
		Vero	HEF	GPE	ML
HSV-1	ACV	0.11	0.10	1.80	0.22
	102	23.74	3.00	>400[c]	>25[c]
	164	3.35	1.69	3.54	5.33
HSV-2	ACV	1.82	0.53	3.01	ND[d]
	102	100	10	>400	ND
	164	50	20	50	ND

[a]Unpublished data.
[b]Vero, African green monkey kidney cell line; HEF, human embryonic lung fibroblast cell; GPE, guinea pig embryo cell; ML, mink lung cell line.
[c]Highest drug concentrations tested and were not toxic to the cell cultures.
[d]Not determined.

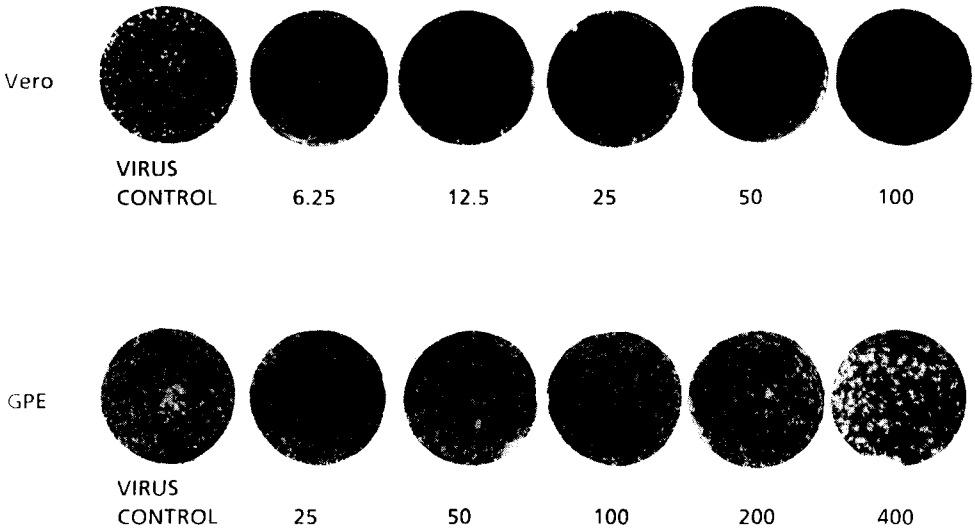

Fig. 3. Effect of compound 102 on HSV-1 plaque formation in two different cell systems: a 50% reduction of plaque formation in Vero cell monolayers was obtained at 23.74 μM (top row) whereas in GPE cells there was little inhibition of the virus plaque formation even at 400 μM (highest concentration which could be given without apparent cytotoxicity). Unpublished data.

ferences are clearly shown in Table 1, where four cell culture systems were used for assaying the antiviral activity of three nucleoside analogs against HSV-1 and -2 infections. Considerable differences are noted among the four different cell cultures in their susceptibility to the anti-HSV effects of the three nucleoside analogs. The largest variability in inhibitory potency was observed with compound 102, which showed an ED_{50} of 3 μM against HSV-1 in HEF cells, 23.74 μM in Vero cells as compared with an ED_{50} of more than 400 μM in GPE cells. Examples are further illustrated in Figs. 1 and 3, where the anti-HSV-1 activity of ACV (Fig. 1) and compound 102 (Fig. 3) in Vero and GPE cells are contrasted. Thus, the importance of the cell culture selected for evaluating the antiviral activity of a new antiviral agent is apparent. Furthermore, such variabilities in the antiviral potencies tested in different cell systems are dependent on the chemical structures of antiviral agents and the virus type used. As shown in Table 1, the highest variability was noted with compound 102, but a moderate degree of differences was noted with ACV; while with compound 164, only a minimal variability of its anti-HSV potency was observed when assayed in four different cell systems. Furthermore, a greater variability was noted in the potencies of ACV and compound 102 against HSV-1 than those against HSV-2 infection when they were tested in the same set of four cell systems.

Virus strain and passage history

The variability of sensitivity to a given antiviral agent has been noted among different strains of HSV and CMV (Harmenberg et al., 1980; Schwartz et al., 1987). Drug-resistant strains have emerged to some antiviral agents, especially in the herpes virus group. The passage history of a virus strain may also affect its sensitivity to some antiviral agents (Person et al., 1970). Preferably a standard strain should be included.

Virus input multiplicity of infection

It was noted that varying virus input multiplicities of infection (MOI) have been used in different reports or even in the same report (Collins and Bauer, 1977; Sauer et al., 1984). However, it has been shown that MOI can influence substantially the evaluation of antiviral activity by the plaque reduction method or the virus yield reduction assay. High MOI will decrease the sensitivity of the virus to an antiviral agent. For example, using the CPE inhibition method in GPE cell culture, compound 164 was shown to have an ED_{50} of 3.2 μM against guinea pig cytomegalovirus (GPCMV) infection if an MOI of 0.001 was used but the ED_{50} increased to 31.8 μM when MOI used was increased to 0.1 (Yang et al., 1988). In another study on the effect of compound 102 against GPHLV replication, we found that a 400 μM concentration of the compound could reduce GPHLV yield by more than 3 $\log_{10}$ $TCID_{50}$ when an MOI of 1.0 was used as the virus challenge dose; whereas the same concentration of this compound could reduce GPHLV yield by only 1 $\log_{10}$ $TCID_{50}$ when the MOI was increased to 10 (Hu and Hsiung, 1988).

Virus yield harvesting time

In the virus yield reduction assay, we also noted that the virus yield harvesting time varied greatly in different reports, ranging from 18 h post-infection to several days or until advanced CPE appeared (Boyd et al., 1987; Collins and Bauer, 1977; Fong et al., 1987). This may also contribute to the disparity among antiviral evaluation results reported by different laboratories. In a recent study, we investigated the effect of compounds 164, 102 and ACV on GPHLV yield, wherein the virus MOI was kept constant (1 PFU/cell) and the virus yield was harvested either at 24 or 48 h post-infection. The results demonstrated that the virus yield harvesting time could influence the results of virus yield reduction assay. When the virus yields were harvested 48 h post-infection, marked inhibition of GPHLV replication was observed with all the three compounds tested, and compound 164 was shown to be much more effective than compound 102 or ACV. However, only limited reduction of the virus yields was obtained and little differences among the potencies of the 3 compounds were observed when the virus yields were harvested at 24 h post-infection (Hu and Hsiung, 1988).

Drug toxicity in cultured cells (cytotoxicity assay) and determination of therapeutic indexes

Thus far, we only discussed methods for measuring antiviral activities of new antiviral agents in cell culture systems. It should be emphasized that the toxic effect of an antiviral agent on the host cell must be considered since an agent may show an apparent antiviral activity by virtue of its toxic effects on the cells. The cytotoxicity assay in cell cultures is basically done by the following two procedures:

Cell viability test

Confluent monolayers of a cell culture are treated with an antiviral agent in various concentrations for about 3 to 4 days. A range of drug concentrations used in the potency assay should be included. The viable cell count is determined daily during the treatment using trypan blue exclusion method. After 3 days of treatment the percentage reduction of the viable cell count is calculated and the drug concentration required to reduce the viable cell count by 50% (ID_{50}, henceforth expressed as CyD_{50} as discussed later) is determined by comparison with the cell count in drug-free control cultures (Chen et al., 1988; Field et al., 1986). Alternatively, the cell viability can also be determined by counting both the dead and viable cells and determining the ratio of viable cells to total cells (dead cell + viable cell count) in each individual culture being treated with the drug (Dargan and Subak-Sharpe, 1985).

Cell growth rate

It has been suggested that the ability of the drug-treated cells to undergo mitotic division may be a more relevant test of the cytotoxic effect of an agent than the cell viability test. Determination of cell growth has been claimed to be the most stringent method for measuring cytotoxicity (Amtman et al., 1987; Cheng et al., 1980; Sauer et al., 1984). This test is performed by adding a range of concentrations of the antiviral agent to rapidly replicating cells (usually 24 h after cell seeding) and continuing the drug treatment for 3 to 4 days. Each day during the log phase of cell growth in either the presence or absence of the antiviral agent, the viable cell count is determined by trypan blue exclusion method. After 3 days' treatment, the drug concentration required to inhibit the cell growth rate by 50% (CyD_{50}) is calculated by reference to the drug-free control cultures.

In addition to the viable cell count, other parameters, such as destruction of cell morphology under microscopic examination, have been used as indicators of drug cytotoxicity (De Clercq et al., 1980). The effect of an agent on the cell growth can also be determined by measuring the DNA synthesis in the treated cells if the antiviral agent is assumed to affect DNA synthesis (Amtman et al., 1987; Harmenberg et al., 1980).

Determination of therapeutic indexes

One of the essential requirements for a prospective antiviral agent is its high therapeutic index. For each virus-host system, this index denotes the ratio:

$$\text{Therapeutic index} = \frac{CyD_{50}}{ED_{50}}$$

where ED_{50} is the minimum drug concentration which is effective to inhibit virus induced plaque formation or cytopathic changes by 50% as discussed above, and CyD_{50} is usually defined as the maximum drug concentration which causes cytotoxic effects in 50% of the cultured cells. Therefore, both the cytotoxicity and the antiviral activity of a test agent must be defined for each virus-cell system. Without the cytotoxicity data, reports of antiviral activity of a given compound even at very low concentrations, are of limited value.

Additional studies on the mode of action of antiviral activity

Virucidal effect

If an in vitro study of an antiviral agent shows antiviral activity, it is necessary to establish whether the virus is inactivated in an extracellular condition (Bauer, 1972). This is done by incubating virus suspensions with various concentrations of the antiviral agent at 25°C and/or 37°C for 1 h to as long as 24 h and determining the rate of loss of infectivity by assaying samples removed at different time intervals (Bauer, 1972; Chen et al., 1988). If the rate of loss of the virus infectivity exceeds that in a control preparation incubating in the absence of the antiviral agent, it is evident that the compound is inactivating the virus before the latter has entered the cell. Antiviral agents in this category would be less applicable to clinical situation for animal studies or patients.

Effect on virus adsorption/penetration, or replication

Some antiviral agents may exert their activity by preventing the adsorption/penetration of the virus to the cell. This can be determined by treating cell cultures before or during virus adsorption. The rate of uptake of the virus by the cell in the presence or absence of the antiviral agent(s) can be measured by assaying supernatant samples removed at various time intervals during the one to two-hour adsorption period.

If an antiviral agent does not inactivate the virus extracellularly and does not block the virus uptake by the cell, it may be assumed that the antiviral action takes place intracellularly. In this case, it is necessary to determine the stage in the virus replication cycle in which the antiviral agent exerts its action. Thus, a single growth cycle of virus replication should be examined. If the antiviral agent is active only

when added in the early stages of the virus replication cycle, it may be inferred that it is inhibiting the synthesis of viral RNA or DNA or of virus-coded RNA or DNA polymerases. If the antiviral agent is still active even when added at a late stage in the growth cycle, it may be inferred that it is inhibiting the synthesis or processing of virus-specific proteins (structural) and is not affecting nucleic acid synthesis. Further information may be obtained by adding the antiviral agent to the culture immediately after virus adsorption, then removing it at increasing time intervals and determining the final virus yield. If the antiviral agent is acting at an early stage in the virus growth cycle, or if it can incorporate into the internucleotide regions of viral DNA, or if the infected cells can conserve the active antiviral agent, inhibition of virus replication can exist even though the antiviral agent is removed soon after virus adsorption. In other cases, the persistent presence of the antiviral agent may be necessary in order to reduce the final virus yield (Boyd et al., 1987).

As mentioned earlier, various immunological assays employing either polyclonal or monoclonal antibodies and electrophoresis assays have been used to determine the synthesis of virus-specific antigens or viral polypeptides in the presence of an antiviral agent. The synthesis and processing (such as glycosylation) of certain viral antigens or polypeptides may be reduced or completely inhibited as a result of the antiviral activity. The synthesis of viral DNA or RNA in the infected cells can be determined by measuring the uptake of radioactive isotope-labelled precursors or viral genome copy numbers. If the amount of radioactivity incorporated or the viral genome copy number is either reduced or suppressed in the presence of an antiviral agent, it is clearly exerting its effect by inhibiting the synthesis of viral DNA or RNA. For viruses that induce virus-specific enzymes in infected cells, such as DNA polymerase induced by HSV and reverse transcriptase induced by HIV, the enzymes can be extracted from the virus-infected cells and tested for their sensitivity to an antiviral agent and extensive kinetic studies are possible (Furman et al., 1979; Hamamoto et al., 1987; Mitsuya et al., 1985). If it can be shown that the antiviral agent inhibits or reduces the activity of the enzyme(s), it may be assumed that the antiviral agent exerts its antiviral activity by inhibiting virus replication through enzyme inhibition.

Interferon induction

Occasionally, a test agent shows its antiviral activity in a virus-cell system only when it is added to the cell culture before virus infection. As discussed earlier, this may be due to the fact that the antiviral agent exerts its activity by interfering with the virus adsorption/penetration or it may induce interferon production in the cells. Therefore, if it has been determined that the agent does not prevent the virus adsorption/penetration, it may instead exert its activity by inducing interferon activity in the treated cell cultures. This can be measured by detection of interferon activity in the drug-treated cell cultures (Chen et al. 1988; Zheng et al., 1985).

Ultrastructural studies

The morphology and morphogenesis of virus-infected cells and virus particles can be observed directly under an electron microscope. If an agent exerts its antiviral activity by inhibiting virus replication in the cells, the morphogenesis of the virus may appear altered. The formation of viral nucleic acid cores and the production of nucleocapsids may be inhibited or reduced. Absence of complete virions in the drug-treated samples is a good indication of the antiviral activity (Dargan and Subak-Sharpe, 1985; Fong et al., 1984, 1987).

Suggestions for the evaluation of new antiviral agents in cultured cells

Selecting suitable virus-cell systems

Preferably, a combination of several different virus strains and several types of cell cultures should be used. Certain well characterized, readily available standard strains should be included simply to facilitate the comparison. Among the cell systems used, human cells and those animal cells in which the in vivo test is going to be performed should be included as long as this is available since there is conceivably a better correlation between in vitro and in vivo testing results when these tests are performed in a homologous system (the same animal species) than in a heterologous system. In the latter, the cell culture is derived from an animal species other than the animal model for in vivo test. When comparing the activity of different antiviral agents, the same cell system(s) should be used for all the antiviral agents against a given virus strain. Obviously, for those viruses which are very host-cell-specific and can grow only in one particular or very few cell culture systems, the selection of cell culture systems should not present a problem. However, if a virus has a broad host-cell spectrum, for example, HSV, proper cautions need to be taken as discussed above.

Establishing dose response curves

Once a virus-cell system has been selected, the various assay methods described earlier can then be used to obtain a dose-response curve for an antiviral agent in that system and the antiviral potency of the agent, expressed as ED_{50}, ED_{90}, ED_{99}, etc. can be determined. Because many test conditions in the various antiviral assays can substantially influence the test results, precautions should be taken with regard to these variables. As to the virus challenge dose, a too high dose of the virus usually overwhelms the antiviral effect of a test material, whereas viral doses that are too low often yield unsatisfactory, and doubtful end points. For the plaque reduction assay, a virus challenge dose of about 100 PFU is usually the choice. For the virus yield reduction assay it is proposed that an MOI of 1 or greater be used. With respect to the virus yield harvesting time, in our hands 48–72 h post-infection is usually the choice for fast growing viruses, such as HSV infection, at which time

the virus-infected control cells already show advanced CPE. The effect of the virus yield harvesting time on the antiviral test is less well documented, and more studies are needed to propose a generalized guideline. In each case, if there is no generally agreed standard procedure available, the specific test method in each report should be described in full detail. It must be emphasized here that the ED_{50} values of the new antiviral agents must be expressed in molar concentrations (μM) in order to compare their relative potencies since the molecular weights of various agents may vary greatly.

Determining cytotoxicity and therapeutic indexes

Cell viability assay, cell growth rate determination, morphological observations and measurement of cellular DNA synthesis, etc. are currently used for measuring cytotoxicity of antiviral agents. The cell growth rate may be the most stringent criterion. After the cytotoxicity of an agent is determined together with its antiviral potency, the therapeutic index of the antiviral agent in a given virus-cell system can be calculated. At present, different designations are being used for representing values for effective antiviral activity (MIC, ID_{50}) and for cytotoxicity (ID_{50}, MTD) of antiviral agents (Sidwell, 1986). To avoid confusion, we suggest here that ED_{50} be used for the antiviral effective dose and CyD_{50} for cytotoxicity dose of the drug.

In addition, the relative potency of a new antiviral agent should also be compared with existing approved drugs. It is suggested that an established drug against a virus infection be tested under the same conditions with the new agent, for example, ACV for anti-HSV evaluation, DHPG for anti-CMV evaluation and AZT for anti-HIV evaluation.

Additional studies can be performed to elucidate the antiviral mechanism or mode of action of the antiviral agents. Investigators will find more and more applications in this aspect as well as in the improved design of protocols for the study of new antiviral drugs.

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