Antiviral Research, 8 (1987) 27–39
Elsevier

AVR 00250

Studies of rhinovirus resistant to an antiviral chalcone

A.L.M. Ahmad[1], A.B. Dowsett[2] and D.A.J. Tyrrell[1]

[1]*MRC Common Cold Unit, Harvard Hospital, Salisbury, Wiltshire, U.K.;* [2]*Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire, U.K.*

(Received 9 March 1987; accepted 4 May 1987)

Summary

During studies of the antiviral activity of chalcone Ro-09-0410 on human rhinovirus type 9 (RV9) chalcone-resistant strains of RV9 were isolated and appeared with a frequency of about 10^{-5} in chalcone sensitive stock. Chalcone-dependent viruses were found after further passage. Some characteristics of the resistant viruses were studied and compared with those of the wild type virus; a number of differences were detected. They produced smaller plaques and grew to lower titre; they were no longer protected by chalcone from inactivation by heat and low pH. The event responsible for drug dependence apparently took place after that responsible for drug resistance. Drug-resistant viruses were still sensitive to dichloroflavan and enviroxime.

Rhinovirus; Drug resistance; Mutant

Introduction

Antiviral chalcones have been synthesised and studied recently (Ishitsuka et al., 1981, 1982; Ninomiya et al., 1984, 1985). The compound Ro-09-0410 (4 ethoxy-2'-hydroxy-4,6 dimethoxy chalcone) is highly active against rhinoviruses of certain serotypes. It is non-toxic to cells at concentrations of 4 μg/ml or less and has been shown to bind specifically to virus particles separated on a sucrose density gradient and to decrease their susceptibility to inactivation by heat or low pH (Ishitsuka et al., 1982; Ninomiya et al., 1984; Ninomiya et al., 1985).

Correspondence to: Dr. D.A.J. Tyrrell, MRC Common Cold Unit, Harvard Hospital, Coombe Road, Salisbury, Wiltshire SP2 8BW, U.K.

We have studied the effect of this chalcone Ro-09-0410 on infection of volunteers with RV9 (Phillpotts et al., 1984) and also its synergistic effect with interferons (Ahmad and Tyrrell, 1986). We now report further in vitro studies in which we have detected populations showing varying degrees of chalcone resistance and, on a number of occasions also found chalcone-dependent virus. We have studied these phenomena since the chalcone is one of a number of compounds which have similar antiviral activities and which are being evaluated for use in man (Phillpotts et al., 1984, 1985; Tisdale and Selway, 1983; Tisdale and Selway, 1984). Clearly the occurrence of drug resistance and the reasons for this, might influence considerably decisions as to whether they were suitable for clinical use.

Materials and Methods

Viruses

Rhinoviruses 2 and 9 were grown in Ohio-HeLa cell monolayers maintained in BME (Gibco) supplemented with 2% foetal calf serum (FCS). Cultures were harvested after incubation for 48 h at 33°C, cells were frozen and thawed three times, clarified by centrifugation and the supernatant was stored at −70°C (Ahmad and Tyrrell, 1986).

Cells

WISH cells were grown at 37°C in MEM (Gibco) supplemented with 10% FCS and antibiotics.

Ohio HeLa cells were grown in BME supplemented with 10% newborn calf serum (NCS) and antibiotics.

Virus titrations

Virus infectivity was assayed by titration in microtitre plates with confluent monolayers of HeLa cells, using half log dilutions and 3 or 4 wells per dilution. Fifty percent end points were calculated by Karber's method. Certain titrations as indicated in the text were carried out after extracting the virus preparation with chloroform (Ninomiya et al., 1984). Antiviral activity was measured as the minimal concentration completely inhibiting cytopathic effect (CPE) induced by 100 $TCID_{50}$ of virus. Drug was present throughout the assay.

Growth curves and time-of-addition experiments

Confluent monolayers in 5 cm petri dishes were inoculated with 0.2 ml of virus to give moi of about 10. No attempt was made to extract drug from the B9 inoculum. Virus was absorbed at room temperature for 90 min with shaking. The monolayers were washed twice with maintenance medium, 5 ml of fresh medium

was added and the plates incubated and this point was taken as time zero. Drug was added or removed by exchanging medium with or without drug. At the indicated time cultures were titrated for infectivity in HeLa cells (see above).

Chemicals

Chalcone Ro-09-0410 was prepared and supplied by Nippon-Roche Research Center, Kamakura, Japan who also supplied tritium labelled compound.

Dichloroflavan 683C77 (DCF) was supplied by Wellcome Research Laboratories, Kent, U.K.

Enviroxime was prepared at Lilly Research Laboratory, Indianapolis, IN, as an equal mixture of syn and anti-isomers.

Compounds were stored as stock solutions in dimethyl sulphoxide and diluted in maintenance medium shortly before use.

Purification and concentration of rhinovirus

Virus-containing tissue fluid was centrifuged for 10 min at 10000 rpm at 4°C and to the supernatant a solution of 30% (w/v) polyethyleneglycol (PEG) was added to give a final concentration of 5% PEG. After 30 min at 4°C the mixture was centrifuged at 10000 rpm for 10 min at 4°C and the pellet was resuspended in 0.05 M Tris, pH 7.2 and an equal volume of a solution containing 0.1 M NaCl, 0.1 M sodium phosphate, 0.2 M sodium pyrophosphate, pH 8.0, and 0.5 mg/ml trypsin (incubated at 37°C for 10 min then cooled to room temperature) and a solution of 10% (w/v) SDS in 0.02 M Tris, pH 7.6, added to give a final concentration of SDS of 1%. The pH was adjusted to between 7 and 8 with 1 M acetic acid and the mixture was centrifuged at 10000 rpm for 10 min. The supernatant was layered over

TABLE 1

Selection of drug resistant and dependent RV9.

RV9 (Code No.)	Chalcone concentration μg/ml in culture on			Log_{10} titre of 3rd passage harvest-titrated in medium containing	
	1st pass.	2nd pass.	3rd pass.	2 μg/ml drug	No drug
RV9 (wild)	0	0	0	1	6.8
B1	0.5	0	0.5	0	3.6
B2	0.5	0	0	0	4.0
B3	0.5	0	1.5	4.2	4.5
B4	0.5	0.5	0	0	3.5
B5	0.5	0.5	0.5	4	4
B6	0.5	0.5	1.5	4	4.5
B7	0.5	1.5	0	3.2	4
B8	0.5	1.5	1.5	3.8	3.2
B9	0.5	1.5	3	5.2	1.3

Virus was passaged when CPE occurred. 0.1 ml of medium was transferred to a fresh roller tube culture.

20% w/v sucrose in 0.02 M Tris, pH 7.6, centrifuged at 105000 *g* for 3.5 h at 4°C and the pellet of virus was then suspended in 0.05 M Tris buffer, pH 7.2.

Electron microscopy

The carbon coated E.M. grid was touched onto a drop of Pansorbium (protein A-bearing *Staphylococcus aureus*, Centre for Applied Microbiological Research, Porton); after incubation for 10 min at room temperature the grid was removed and the excess fluid blotted off. The grid was touched onto a drop of diluted anti-rhinovirus antisera 1/1500 for 1 h at 37°C in a humid chamber. The excess fluid was blotted off and the grid touched onto a drop of virus suspension for 1 h at 37°C as before. The excess fluid was blotted off and the grid was washed with distilled water for a few seconds. Grids were then stained with 1% PTA (pH 7) and examined in the usual way.

Results

Derivation of resistant RV9

Viruses were passaged in roller tube cultures of Ohio-HeLa cells with chalcone containing medium. Various strategies for preparing resistant viruses were tried. It can be seen (Table 1) that after 2 or 3 passages in the presence of the drug highly resistant organisms appeared whereas after single or intermittent exposure of low concentrations they did not (B1, B2 and B4). Strain B9, which was subjected to the strongest selection pressure became drug-dependent since the titre was over 100-fold higher in the presence of 2 μg/ml of chalcone than in its absence. A total of over 25 chalcone-resistant strains of RV9 have been produced. The experiments were repeated using human fibroblast cells and RV2 (data not shown) and again resistant and dependent viruses were found.

Presence of resistant virus in stocks of parental virus

We made approximate estimates of the frequency of drug-resistant virus in our stocks of RV9 by titrating them in normal medium and in the presence of 2 μg/ml of chalcone (data not shown). The difference in titre indicated that the virus was a fraction of $10^{-4.6}$ or $10^{-5.1}$ of the sensitive virus. If this is regarded as a rough index of the mutation rate, it is similar to the high rates which are characteristic of single stranded RNA viruses and probably represents a single point mutation. However, since, as shown above, this mutation reduces the rate of multiplication it is understandable that such mutants do not accumulate in the stock.

Growth characteristics

These viruses were examined in detail and in the first instance attempts were

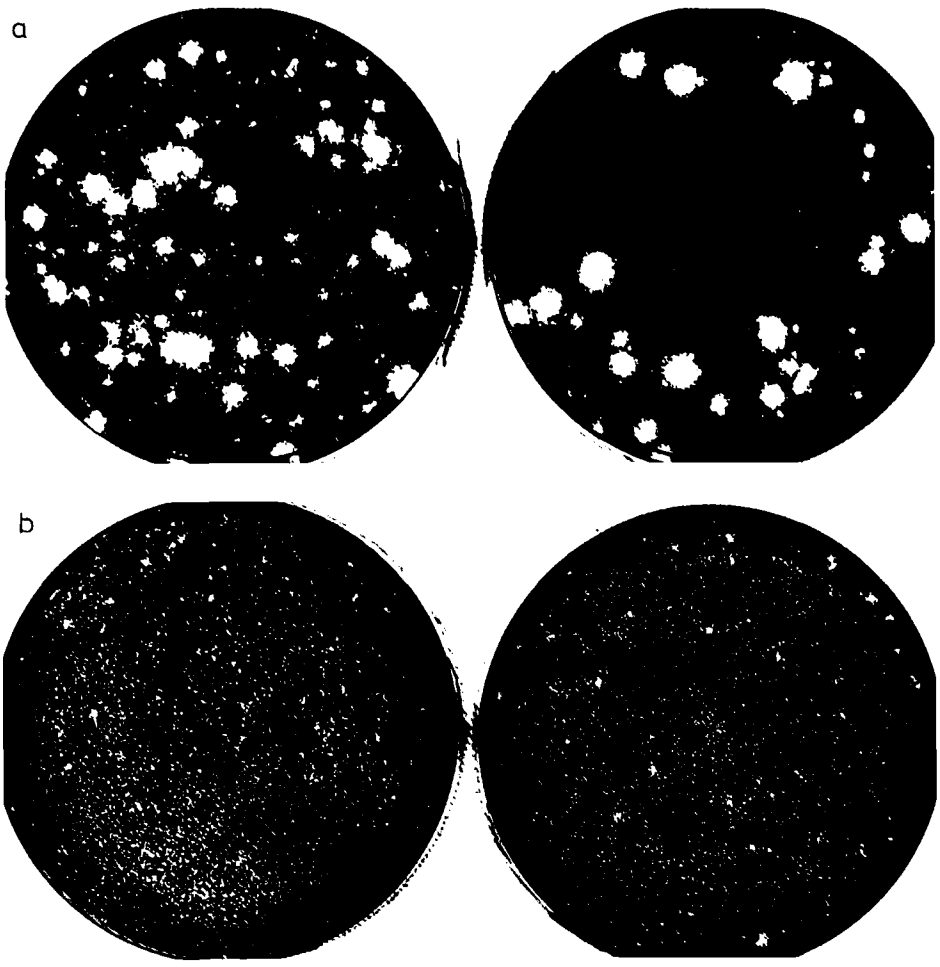

Fig. 1. Plaques produced in Ohio-HeLa cells by wild type RV9 and by drug-resistant mutants B6 and B10. 1a shows typical plaques produced by wild type virus and 1b the restricted plaques produced by B6 and B10 (see text and Table 2).

made to plaque purify them. However, it was found that many either produced no plaques under a standard 0.2% agarose overlay or produced less clear or smaller plaques (Fig. 1). Virus stocks were therefore purified by limit dilution if plaque purification was not practicable.

Since it appeared that these viruses might grow less well than the parents abbreviated growth curves were performed using HeLa cells inoculated at a moi of 10 pfu or 10 $TCID_{50}$/cell. It can be seen from Table 2 that the yield at 33°C was reduced from that of the parent and also that the yield was unaffected by the presence of chalcone, except of course for B9, which required it for growth. The experiment was repeated at 37°C which showed that virus growth was reduced; in

TABLE 2

Growth of wild type and mutants of RV9 and wild type RV2 at 33°C and 37°C with or without chalcone Ro 09-0410.

Virus	1 μg/ml chalcone in culture	Yield of virus (log_{10}) at indicated time (h) after incubation at							
		33°C				37°C			
		6	10	12	24	6	10	12	24
RV9 (wild)	–	3.5	5	NT	6.9	2.5	3	NT	4.25
RV2 (wild)	–	3.5	5	NT	6.5	2.5	3	NT	4
B5	–	1.8	3	NT	4.7	1.5	2	NT	3.1
B5	+	NT	NT	3	5	NT	NT	1.5	1.5
B6	–	2	2.5	NT	4.3	1	1.5	NT	1.85
B6	+	NT	NT	3	5	NT	NT	0	0
B7	–	2	2.9	NT	5	0.5	1	NT	0.7
B7	+	NT	NT	3	5	NT	NT	1.5	2
B8	–	2.2	3	NT	5.6	1	1.5	NT	2
B8	+	NT	NT	3	5	NT	NT	1.5	2.2
B9	+	3	4.2	NT	5.9	3	4	NT	5.7

NT = not titrated.

TABLE 3

Infectivity of virus incubated at pH 4 or at 56°C in presence of 2 μg/ml chalcone.

Virus	Titre of untreated virus in presence of		Titre* (log_{10}) of virus treated			
	Chalcone	None	At pH 4 for 1 h with		At 56°C for 5 min with	
			Chalcone	None	Chalcone	None
RV9	–	6.75	5.5	2.7	5.2	2.5
B3	4.2	4.6	2	1.3	0	1
B5	3.8	4.1	0.5	0.5	0	0.5
B6	4.5	4.8	1.5	1.8	1	0.5
B7	3.5	4.6	1	0.9	0	0
B8	3.8	3.2	1	1.5	1	1
B9	5.2	1.3	2	1.5	1.6	1

* Samples were extracted with chloroform and titrated in the absence of chalcone (RV9-B8) or with chalcone, B9.

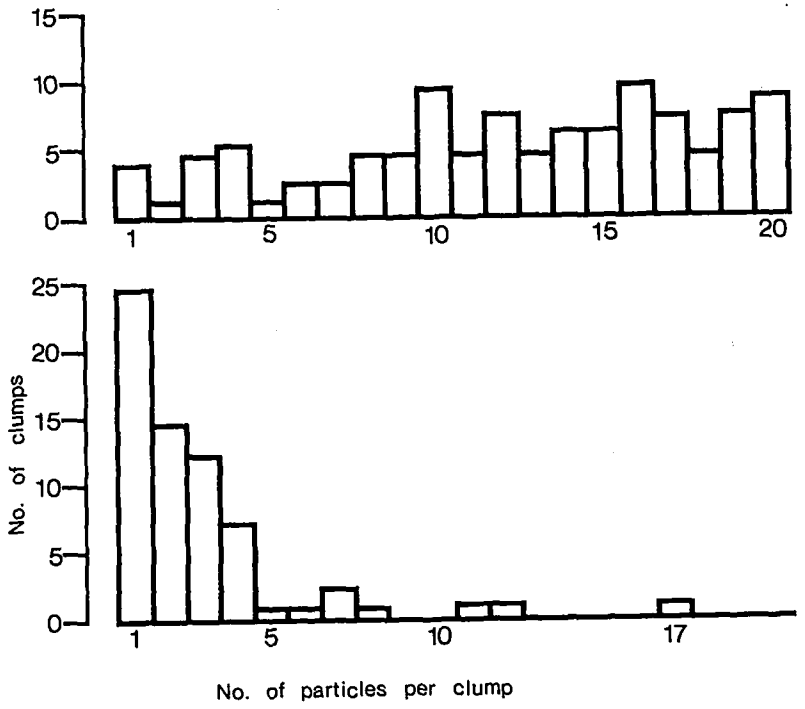

Fig. 2. Aggregation of virus particles. Number of particles per clump detected by immune capture negative contrast electron microscopy when wild type virus was exposed to 2 μg/ml of chalcone (upper section) or untreated (lower section).

other words the viruses were still temperature-sensitive like the parent, except for B9 which was now temperature-resistant. This was not due to the presence of the drug, since in the case of the drug-resistant viruses the presence of drug did not reverse the temperature-sensitive character (see column 2). Two viruses (B5 and B6) became drug-sensitive again at 37°C, but two (B7 and B8) did not.

Stability of virus particles

As the chalcone is believed to act by interacting with a viral particle (Ishitsuka et al., 1981, 1982) it was postulated that a mutation affecting viral protein(s) might occur and confer both drug resistance and a change in the stability of the infectivity of the virus particle.

Virus pools were therefore treated with heat at 56°C for 5 min and with buffer at pH 4 for 1 h both in the presence and absence of drug. Treated and untreated samples were shaken with chloroform to remove the drug and the residual infectivity was titrated. The results are shown in Table 3 which contains several points of interest. In the first place, the top row shows that treating the parent virus in the presence of chalcone protects it from inactivation to a considerable extent (Ninomiya et al., 1984). However, the resistant viruses (B3–B8) were not protected and were inactivated either by heat or by low pH. The dependent virus B9 behaved similarly. It seems plausible that the resistant viruses did not interact with the drug in the same way as the parental virus.

We wished to obtain direct evidence that the drug adsorbs more readily to wild type virus than to drug-resistant virus. Equal amounts of the viruses were mixed with tritium-labelled chalcone, and the mixtures incubated at 33°C for 2 h. After incubation the mixtures was centrifuged and treated as described in Materials and Methods for purification and concentration of viruses. The radioactivity of the pel-

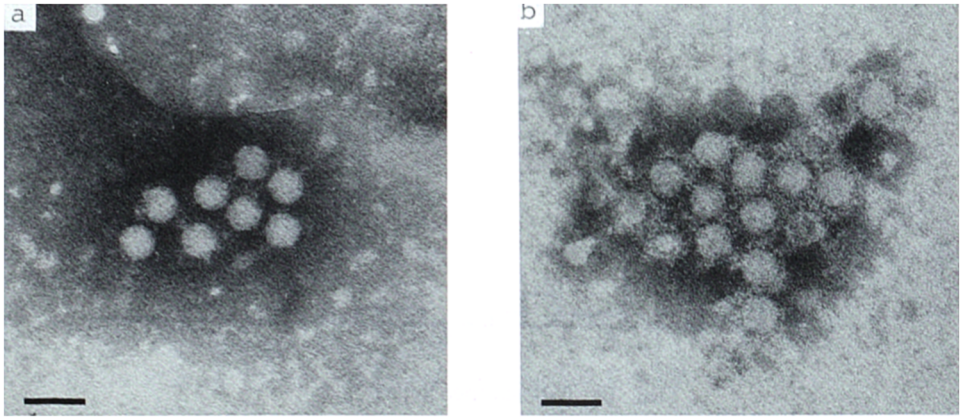

Fig. 3. Electron microscopy of wild type rhinovirus heated at 56°C for 5 min; (a) with 2 μg/ml chalcone and (b) alone. The structure of the particles has been preserved in the presence of the drug. Bar = 50 nm.

leted virus and the supernatant was then estimated with a liquid scintillation counter. Of the drug added 62% was bound to the wild type virus and 17% to the resistant virus.

Electron microscopy

The virus particles from preparations containing 2 μg/ml of chalcone were indistinguishable from those of untreated preparations although they aggregated into larger clumps (Fig. 2). However, when such preparations were heated the chalcone treated particles remained intact while untreated particles showed a blurred surface and sometimes were disintegrated (Fig. 3). Curiously, chalcone-treated heated particles were less aggregated than untreated heated particles (data not shown).

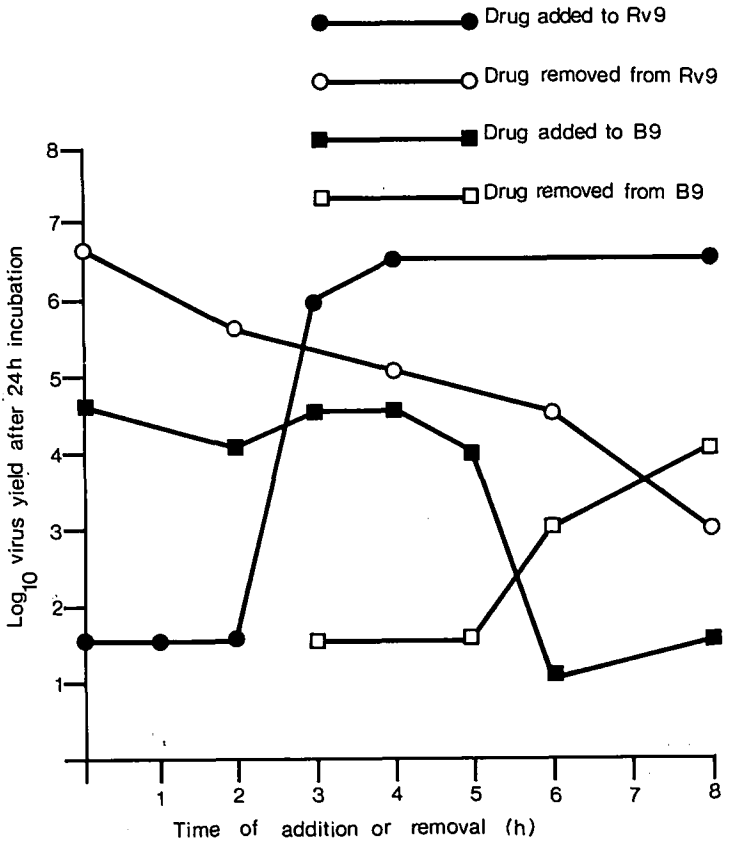

Fig. 4. Yield of infectivity in time-of-addition-or-removal experiments with drug-sensitive and -dependent viruses. The sensitivity event apparently occurs earlier in the growth cycle than the dependent event (see text).

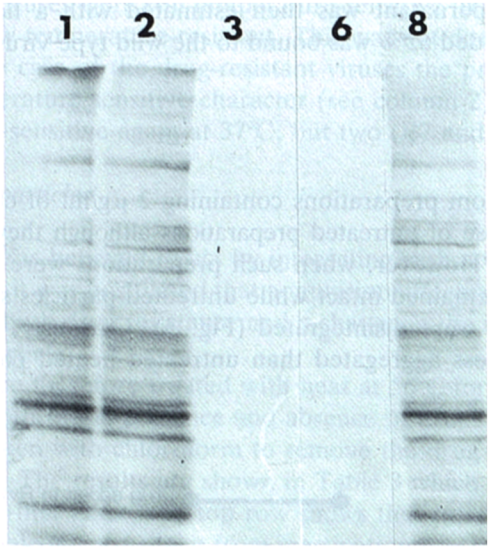

Fig. 5. Autoradiogram of electrophoretically separated ^{35}S methionine labelled immunoprecipitated polypeptides from WISH cells infected with RV9 wild type and RV9 drug-dependent mutant (B9). Rabbit anti RV9 serum was added to cell extracts and precipitated with protein A. The complexes were separated by SDS-PAGE. Polypeptides of B9 (1) with or (2) without 2 μg/ml chalcone; (3) of wild type RV9 treated with 2 μg/ml of chalcone; (6) of RV9 treated with 8 U/ml of HuIFNγ, a positive control; and finally (8) of RV9 without drug.

Resistance to other drugs

It has been suggested that the chalcone interacts with rhinoviruses in the same way as dichloroflavan (DCF) (Bauer et al., 1981; Ninomiya et al., 1985) and that mutations to resistance against one drug confer resistance against the other. We therefore tested some of our resistant viruses against DCF and also against enviroxime, which is believed to have a different mode of action. The results (Table 4) clearly show that the chalcone-resistant and -dependent viruses do not show increased resistance to either DCF or enviroxime (which clearly has a different mode of action), whether or not chalcone is present in the medium.

Relation between resistance and dependence

As a preliminary experiment to determine whether resistance and dependence were due to interactions with the same steps in the virus replication cycle we performed time-of-addition-or-removal experiments on single step growth cycles of the wild type and dependent viruses. The results are summarised in Fig. 4. They show that addition of drug is only effective against wild type virus if added early in the growth cycle. Clearly drug is rapidly lost from cells as full titres are produced if drug is removed early in the cycle. Nevertheless, this type of experiment

gives only an approximate estimate of the time of the step affected. In experiments with B9 there may have been traces of drug derived from the inoculum, but the final concentration would be much lower than that used for propagation. Using arguments similar to those above the time of the drug-*requiring* event in the growth appears to be later and therefore different from the drug-*sensitive* event of wild type virus replication.

The idea that a late event is involved in the effect on drug-dependent virus was also supported by a study of virus polypeptide synthesis. It was found that in the presence of 2 μg/ml chalcone the peptides of a drug-sensitive virus were not formed while the polypeptides of the drug-dependent strain were formed in the presence or absence of 2 μg/ml chalcone (Fig. 5).

Discussion

The results presented here indicate that drug-resistant RV9 can be isolated relatively easily in tissue culture by exposing sensitive wild type RV9 to chalcone. We believe this is due to selection of mutants which occur with a relatively high frequency, i.e. about 10^{-5}. The degree of drug resistance detected was considerable, particularly in virus recovered after three passages in increasing concentrations of chalcone. Some tests showed the development of drug-dependent strains. This implies that if the substance is to be used in man the emergence of drug-resistant virus should be carefully monitored. Such experiments are now in progress, though the fact that the mutants showed impaired growth properties suggests that they may be of reduced virulence and also that in biological terms the advantage of growth in the presence of the drug has been obtained at the cost of a less efficient replication process. However, no definite differences were found by PAGE between viral proteins immunoprecipitated from cells infected with parental and drug-resistant viruses (data not shown).

Chalcone stabilized wild type RV9 to low pH (pH 4) for 1 h and to 56°C for 5 min, and this was presumably due to a change in the properties of the capsid protein. The drug-resistant or drug-dependent strains were not stabilized. The EM changes have not been described before and suggest that the surface properties of the virus are altered by the drug and that the physical structure as well as the infectivity of the virus are preserved by the drug.

Studies with radioactively labelled chalcone suggested that chalcone bound to wild type RV9 but not to drug-resistant or drug-dependent virus. These findings confirm and extend those of Ninomiya et al. (1984), who found that radioactive labelled chalcone bound to wild RV2 but did not stabilize or bind to a drug-resistant derivative.

Time-of-addition-and-removal experiments suggested that chalcone inhibits an early stage of multiplication of wild type virus and may prevent virus penetration or uncoating while enviroxime acts at the latter end of the eclipse phase of virus multiplication (data not shown). This agrees with the results of Ninomiya et al., but unlike them we found no cross-resistance between our mutants and dichloro-

flavan, possibly because they used RV2 and not RV9. However the results suggest to us that only occasional mutations, or perhaps double mutations, affect the site of action of both drugs.

Early studies showed that enterovirus replication could be blocked by molecules such as rhodamine which stabilizes and prevents uncoating of echovirus 12 (Eggers, 1977; Rosenwirth and Eggers, 1979) and arildone which does the same for the poliovirus particle (Caliguiri et al., 1980).

It is interesting that independent lines of research have led to the identification of several different molecules which apparently interact with the rhinovirus capsid and prevent uncoating (Diana et al., 1985; Tisdale and Selway, 1983, 1984). In one instance crystallographic studies have shown how the molecule is inserted into the virus capsid (Smith et al., 1986). Some, such as this chalcone and dichloroflavan can apparently interact with the intact particle and stabilize it to adverse factors such as heat and low pH. There is one (44 081 RP), however, which inhibits uncoating but has no recognized effect on the intact particle (Zerial et al., 1985; Alarcon et al., 1986) – perhaps it only interacts with it after it has been slightly modified as it enters the cell. We suspect that all these, and others with the same mode of action which remain to be discovered, will all be proved to select resistant viruses rather readily. The problem is therefore likely to be of increasing interest and possible practical importance.

The drug-dependent virus is worthy of further study. Timing experiments adding chalcone to, or removing it from, cells infected with a drug-dependent strain suggest that it acts at a late stage after protein synthesis and that it needs chalcone for a step involved in assembly of particles since so many viral polypeptides are formed with or without it.

Our present hypothesis is that drug-resistant viruses emerge as a result of an amino acid substitution in a capsid protein. We therefore plan to test this by studying the nucleotide sequence of parental and resistant viruses.

Acknowledgements

We thank Dr. Peter Shakespeare for help with radioisotope counting and also those who gave us antivirals as mentioned in Materials and Methods.

References

Ahmad, A.L.M. and Tyrrell, D.A.J. (1986) Synergism between antirhinovirus antivirals: various human interferons and a number of synthetic compounds. Antiviral Res. 6, 241–252.

Alarcon, B., Zerial, A., Dupiol, C. and Carrasco, L. (1986) Antirhinovirus compound 44 081 R.P. inhibits virus uncoating. Antimicrob. Agents Chemother. 30, 31–34.

Bauer, D., Selway, J., Batchelor, J.F., Tisdale, M., Caldwell, I.C. and Young, D. (1981) 4',6 Dichloroflavan: a new antirhinovirus compound. Nature 292, 369–370.

Caliguiri, L.A., McSharry, J.J. and Lawrence, G.W. (1980) Effect of arildone on modification of poliovirus in vitro. J. Virol. 105, 86–93.

Diana, G.D., Otto, M.J. and McKinlay, M.A. (1985) Inhibitors of picornavirus uncoating as antiviral agents. Pharmacol. Ther. 29, 287–297.

Eggers, H.J. (1977) Selective inhibition of uncoating of echovirus 12 by rhodanine. Virology 78, 241–252.

Ishitsuka, H., Ninomiya, Y., Ohsawa, C., Ohiwa, T., Fujiu, M., Umeda, I., Shirai, H. and Suhara, Y. (1981) New antirhinovirus agents Ro 09-0410 and Ro 09-0415. In: 12th Int. Congr. Chemother., Florence, Italy. p. 109, Abstr. 318.

Ishitsuka, H., Ninomiya, Y., Ohsawa, C., Fujiu, M. and Suhara, Y. (1982) Direct and specific inactivation of rhinovirus by chalcone Ro 09-0410. Antimicrob. Agents Chemother. 22, 617–621.

Ninomiya, Y., Ohsawa, C., Aoyama, M., Umeda, I., Suhara, Y. and Ishitsuka, H. (1984) Antivirus agent, R0 09-0410, binds to rhinovirus specifically and stabilizes the virus conformation. Virology 134, 269–276.

Ninomiya, Y., Aoyama, M., Umeda, I., Suhara, Y. and Ishitsuka, H. (1985) Comparative studies on the mode of action of the antirhinovirus agents Ro 09-0410, Ro 09-0179, RMI-15, 731; DCF and enviroxime. Antimicrob. Agents Chemother. 27, 595–599.

Phillpotts, R.J., Wallace, J., Tyrrell, D.A.J., Freestone, D. and Shepherd, W. (1983) Failure of oral 4′,6-dichloroflavan to protect against rhinovirus infection in man. Arch. Virol. 75, 115–121.

Phillpotts, R.J., Higgins, P.G., Willman, J.S., Tyrrell, D.A.J. and Lenox-Smith, I. (1984) Evaluation of the antirhinovirus chalcone Ro 09-0415 given orally to volunteers. J. Antimicrob. Chemother. 14, 403–409.

Rosenwirth, B. and Eggers, H.J. (1979) Early processes of echovirus 12 infection: elution, penetration and uncoating under the influence of rhodanine. Virology 97, 241–245.

Smith, T.J., Kremer, M.J., Luo, M., Vriend, G., Arnold, E., Kamer, G., Rossmann, M.G., McKinlay, M.A., Diana, G.D. and Otto, M.J. (1986) The site of attachment in human rhinovirus 14 for antiviral agents that inhibit uncoating. Science 233, 1286–1293.

Tisdale, M. and Selway, J.W.T. (1983) Inhibition of an early stage of rhinovirus replication by dichloroflavan (BW 683C). J. Gen. Virol. 64, 795–803.

Tisdale, M. and Selway, J.W.T. (1984) Effect of dichloroflavan on the stability and uncoating of rhinovirus type 1B. J. Antimicrob. Chemother. 14, 403–409.

Tyrrell, D.A.J., Phillpotts, R.J. and Wallace, J. (1983) Studies on two antirhinovirus substances – dichloroflavan and enviroxime. In: The Beecham Colloquia (Stuart-Harris, Sir C. and Oxford, J. eds.), pp. 265–276. Academic Press, London.

Zerial, A., Werner, G.H., Phillpotts, R.J., Willmann, J.S., Higgins, P.G. and Tyrrell, D.A.J. (1985) Studies on 44 081 R.P., a new antirhinovirus compound, in cell cultures and in volunteers. Antimicrob. Agents Chemother. 27, 846–850.