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The effects of UK 2054 on the multiplication of influenza viruses

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SUMMARY

The isoquinoline compound UK 2054 prevents the uptake of influenza virus by susceptible cells. Pre-incubation of virus particles with 500 μg./ml. UK 2054 at 37° C. for 2 hr. does not reduce virus infectivity. Host cells vary in their responsiveness to the inhibitory effect of UK 2054; virus multiplication is inhibited in chick allantoic cells by lower concentrations than those required to inhibit virus growth in chick embryo fibroblasts. The effectiveness of UK 2054 is reduced by the presence of serum.

It is concluded that inhibition of influenza virus multiplication by UK2054 might result from interaction of the inhibitor with both virus and cells. Any direct combination between inhibitor and virus is completely reversible.

INTRODUCTION

The compound 1-phenoxymethyl-3,4-dihydroisoquinoline (Pfizer compound UK 2054) is a weak, non-competitive inhibitor of bacterial and viral neuraminidases (Brammer, McDonald & Tute, 1968). This feature of the compound led to an investigation of its ability to inhibit virus multiplication in simple cell culture systems. It was found to have a spectrum of antiviral activity which includes those viruses which possess neuraminidase, namely, the influenza and parainfluenza viruses responsible for a large proportion of common respiratory infections. It is also effective however against many other viruses, such as rubella and respiratory syncytial viruses, which do not possess neuraminidase.

Toxicological studies indicate that UK 2054 and related isoquinolines are safe for administration to man (Brammer *et al.* 1968). It has been found that the closely related compound UK 2371 (1-(4-methoxyphenoxymethyl)-3,4-dihydroisoquinoline) is active against influenza type B infection in man (Beare, Bynoe & Tyrrell, 1968).

The mechanism by which isoquinolines inhibit virus multiplication is not understood. In the case of influenza viruses, these compounds are most effective when pre-incubated with virus before infection, and it has been concluded that they act by direct inactivation of the virus particle (Brammer *et al.*, 1968; Hobson, Flockton, & Gregory, 1969). In this paper some experiments are reported which suggest that direct inactivation of influenza virus by UK 2054 is reversible and may not be the only means by which the compound prevents infection.

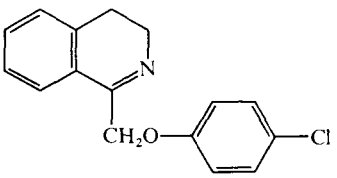
MATERIALS AND METHODS

Virus strains

Three strains of influenza virus were used in this study: influenza A/PR 8/34, A2/Singapore/1/57 and fowl plague virus. These are egg-adapted strains which have been maintained in this laboratory for many years by serial egg passage. Virus-infected allantoic fluid was stored in glass ampoules at $-70°$ C. and thawed rapidly before use.

Anti-viral agents

UK 2054 was prepared by the Chemical Research Division of the Pfizer Group, Sandwich, Kent, and was sent to us through the courtesy of Dr D. Jackson. The chemical structure of UK 2054 is as follows:



Stock solutions were prepared in distilled water at a concentration of 1 mg./ml. and stored at 4° C. Actinomycin D was a gift from Merck, Sharp and Dohme.

Experimental system

Two cell systems were used to test the effect of UK 2054 on influenza virus growth:

(1) 1 sq. cm. pieces of surviving allantois-on-shell, taken from 11-day-old fertile hens' eggs, were incubated in 0·7 ml. volumes of medium 199 dispensed into large Perspex trays (Fulton & Armitage, 1951). Full details of the quantitative aspects of this system have been described (Barry, 1961).

(2) Monolayer cultures of chick embryo fibroblasts were prepared from 11-day-old eggs and grown in 5 cm. plastic Petri dishes. The cultures were grown in medium 199 supplemented with 10 % calf serum and incubated in an atmosphere of 5 % CO_2. Cells were infected by adding 0·02 ml. volumes of the appropriate dilution of virus.

Virus titration

Unless otherwise stated, haemagglutination titrations were carried out 18 hr. after infection, according to the method of Fazekas de St Groth & Graham (1954). In all figures, except Fig. 1, haemagglutination (HA) titres are expressed as the $\log_2$ value per 0·25 ml. Virus infectivity was titrated by the method of Fazekas de St Groth & White (1958).

RESULTS

Degree of inhibition

Various doses of UK 2054 ranging from 0 to 28 μg./ml. were added to egg pieces. Each piece was then infected with one of three strains of influenza virus at

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an estimated multiplicity of 3 ID50/cell. The production of all strains was inhibited to a similar degree by increasing concentrations of UK 2054 (Fig. 1). The production of fowl plague virus and the PR 8 strain were not completely inhibited and in other experiments we frequently found that inhibition of the Asian strain was not complete. This effect is different from that found with inhibitors of DNA function such as actinomycin D, where the degree of inhibition varies directly with the dose of inhibitor and where virus production can be completely inhibited (Barry, Ives & Cruickshank, 1962).

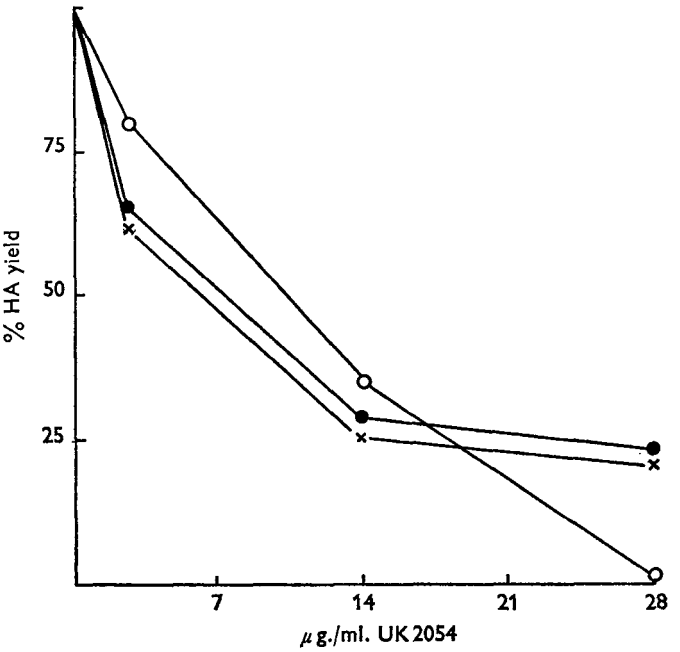

Fig. 1. Inhibition of influenza virus growth by UK 2054. UK 2054 present in culture medium throughout the experiment. ○, A2/Singapore/1/57 influenza virus; ●, fowl plague virus; ×, A/PR 8/34 influenza virus.

Nature of inhibition

To test whether UK 2054 has an all-or-none effect on virus production, the following experiment was carried out. One-step growth experiments, using the A 2/Singapore/1/57 strain at a multiplicity of 3 ID 50/cell, were performed in egg pieces in the presence or absence of 5 and 20 μg./ml. UK 2054. The results are shown in Fig. 2. In untreated egg pieces newly formed virus appears 5 to 6 hr. after infection and is released rapidly up to about 12 hr. after infection. In egg pieces treated with 5 μg./ml. UK 2054 the eclipse phase of the virus is 2 to 4 hr. longer and the rate of virus release is slower. Although there is no reduction in the 24 hr. yield, at 12 hr. after infection the inhibitor-treated cells have produced less than half the yield from control cells. The effect of 20 μg/ml. UK 2054 is to lengthen the eclipse phase and delay the release of virus still further.

This result is quite different from that obtained with actinomycin D, where the rate of virus release is normal but the final yield of virus reduced (Fig. 3). Thus,

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UK 2054 does not have an all-or-none effect on virus production but delays the appearance of newly formed virus and its rate of release. Since this effect is similar to that seen when the multiplicity of virus infection is reduced, it seems likely that the inhibitor is reducing the effective multiplicity of infection by preventing the uptake of virus by host cells. If this is so, then the inhibitor should be effective only during the early stages of the virus growth cycle.

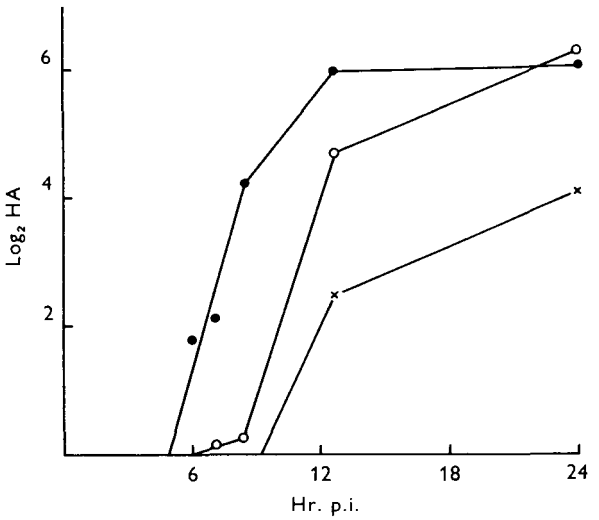

Fig. 2. The effect of UK 2054 on one-step growth curve of A 2/Singapore/1/57 influenza virus. ●, 0 μg./ml. UK 2054; ○, 5 μg./ml. UK 2054; ×, 20 μg./ml. UK 2054.

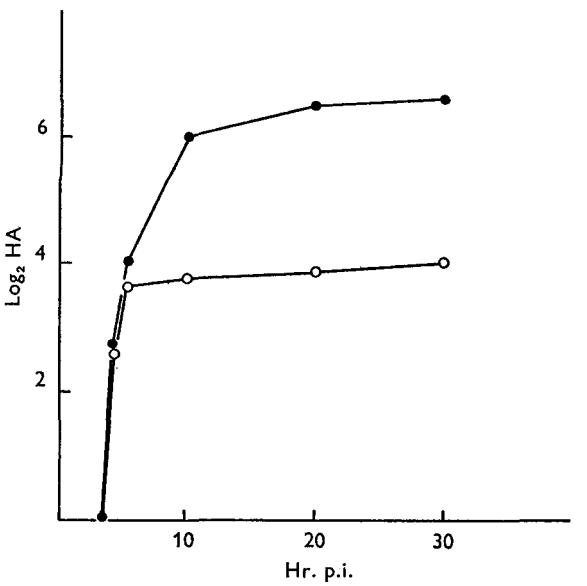

Fig. 3. The effect of actinomycin D on the one-step growth curve of A 2/Singapore/1/57 influenza virus. ●, 0 μg./ml. actinomycin D; ○, 5 μg./ml. actinomycin D.

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Egg pieces were therefore infected with the A 2/Singapore/1/57 strain and treated with 20 μg./ml. UK 2054 either before or shortly after virus infection. The inhibitor was left on the egg pieces for periods of 30 min. and then removed by washing. Fig. 4 shows that the inhibitor is most effective when added before virus infection and that its effectiveness decreases as the eclipse phase proceeds. There is no inhibition of virus yield when UK 2054 is added later than 2 hr. after infection. This result is compatible with the idea that UK 2054 prevents virus uptake.

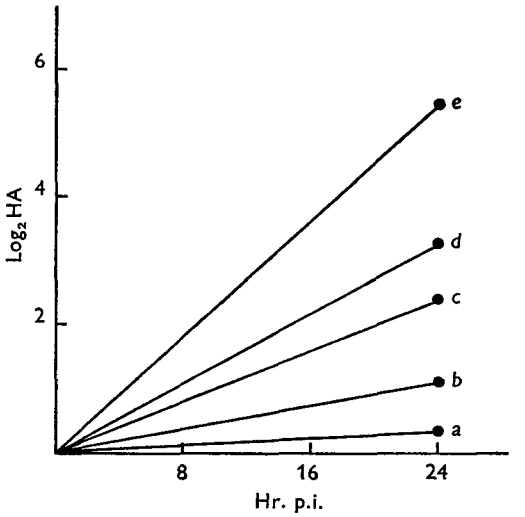

Fig. 4. The effect of UK 2054, at various times before and after virus infection, on the yield of A 2/Singapore/1/57 influenza virus from allantoic pieces. 20 μg./ml. UK 2054 were present from: (*a*) 30 min. before infection to time of infection; (*b*) 0 to 30 min. after infection; (*c*) 30 min. to 1 hr. after infection; (*d*) 1–2 hr. after infection; (*e*) No UK 2054.

TABLE 1. *The effect of UK 2054 (500 μg./ml.) on the infectivity of influenza A 2/Singapore/1/57*

Treatment	Titre (log_{10})/ml.
Virus + saline	7·85
*Virus, treated with and diluted in UK 2054	5·00
Virus, treated with UK 2054, diluted in saline	7·70

* Dilutions were prepared in 0·9 % saline containing 100 μg./ml. UK 2054.

Mechanism of inhibition

The mechanism involved in inhibition could be direct inactivation of the virus particle (Brammer *et al.* 1968), competition for cell surface receptors or interference with an early intracellular event.

Interaction of UK 2054 with influenza virus particles

The A 2/Singapore/1/57 strain was incubated for 2 hr. at 37° C. with 500 μg./ml.

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UK 2054 and the resulting virus infectivity was measured in allantoic cells by finding the highest dilution of virus capable of infecting 50 % of the egg pieces. Serial tenfold dilutions of virus were made either in saline or in saline containing 100 μg./ml. UK 2054. The results are presented in Table 1. When dilutions are made in saline containing UK 2054, the infectivity of isoquinoline-treated virus is reduced one thousandfold. However, when dilutions are made in saline alone, the infectivity titres of treated and untreated virus preparations are indistinguishable. The fact that the inhibitory effect of UK 2054 is absent after dilution suggests that any interaction which may occur between virus and inhibitor is unstable.

Table 2. *The effect of UK 2054 on the production of fowl plague virus in chick embryo fibroblasts (C.E.F.) and chick allantoic cells (C.A.).*

	Host cell	
Inhibitor μg/ml.	C.E.F.	C.A.
0	7·2*	5·7
10	7·5	4·0
20	n.t.	3·2
40	7·2	n.t.

* Yields of virus are expressed as the haemagglutination titre ($\log_2$/ml.) determined 24 hr. after infection.

n.t. = not tested.

The inhibitory effect of UK 2054 in different host cells

If isoquinolines neutralize influenza viruses in much the same way as antibody (Hobson *et al.* 1969), then the degree of inhibition should be independent of the host cell. We therefore tested the effect of various concentrations of UK2054 on the production of fowl plague virus from two cell types. The results are presented in Table 2. UK 2054 at 10 μg./ml. inhibits the production of fowl plague in allantoic cells by about 65 %, whereas doses as high as 40 μg./ml. do not affect the yield of fowl plague from chick embryo fibroblasts. This difference in response suggests that inhibition is not simply due to the irreversible inactivation of virus particles by UK 2054. The chick embryo fibroblasts used in this experiment were infected in medium 199 in the absence of serum.

The effect of serum on inhibition

UK 2054 is bound readily by serum proteins (J. D. Coombes, personal communication). The presence of serum in any culture used to assay the effectiveness of anti-viral activity is therefore likely to complicate the results. An experiment demonstrating the effect of serum on inhibition by UK 2054 of fowl plague multiplication in chick fibroblasts is shown in Fig. 5. It is clear that doses of UK 2054 up to 40 μg./ml. have little or no effect on the yield of fowl plague virus from chick fibroblasts, whether or not serum is present. At higher doses, inhibition of virus growth is achieved only in the absence of serum.

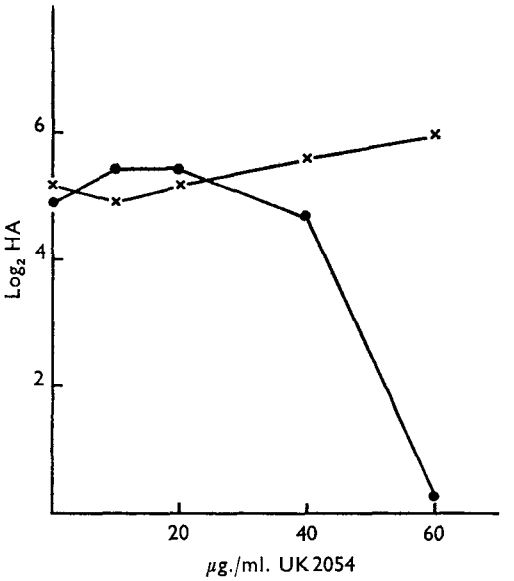

Fig. 5. The effect of serum on inhibition of fowl plague virus by UK 2054 in chick embryo fibroblasts. ×, 10% calf serum; ●, no serum.

DISCUSSION

It is claimed that isoquinoline inhibits the growth of neuraminidase-containing viruses by direct inactivation of the virus particle (Brammer *et al.* 1968; Hobson *et al.* 1969). Prolonged pre-incubation of virus–isoquinoline mixtures produces a greater degree of inhibition than brief pre-incubation, when the mixtures are tested subsequently for their ability to infect cells. The results presented above (Table 1) indicate that virus subjected to prolonged incubation with UK 2054 retains complete infectivity when diluted in saline which does not contain the compound. Consequently, any direct inactivation of virus by the inhibitor (mediated presumably by combination of inhibitor and virus neuraminidase) may be analogous to inactivation of virus by antibody, in that it is reversible when diluted.

The presence of inhibitor in the culture medium prevents virus uptake. However, host cells may vary in their sensitivity to similar mixtures of inhibitor and virus (Table 2). The simplest, but by no means the only, explanation of this phenomenon is that adsorption of inhibitor to the host cell can play a part in preventing virus adsorption. Thus, relatively low doses of UK 2054 may combine with allantoic cells, but not chick embryo fibroblasts, in such a way as to alter the response of the cells to the virus. Combination of inhibitor with host cell rather than with the virus itself could account for the effectiveness of isoquinolines against those viruses which do not contain neuraminidase. In support of the idea that UK 2054 exerts its effect on influenza viruses by combination with cells, as well as with virus, is the fact that concentrations of isoquinoline derivatives greater than 100 μg./ml. agglutinate chick red cells in a manner indistinguishable from that produced by

influenza viruses (unpublished observations). This suggests that isoquinolines may compete for the same cell receptors as influenza viruses.

The effects of UK 2054 on the multiplication of influenza viruses differ from those of amantadine hydrochloride. The effectiveness of amantadine is not dependent on the host cell system used to propagate the virus, and it appears to prevent some step in the replication cycle later than adsorption (Hoffman, Neumayer, Haff & Goldsby, 1965; Neumayer, Haff & Hoffman, 1965). The ability of serum to neutralize the effects of UK 2054 may prove to be a serious drawback to the therapeutic usefulness of isoquinoline derivatives.

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