In vitro activity of R 61837, a new antirhinovirus compound

K. Andries, B. Dewindt, M. De Brabander, R. Stokbroekx, and P. A. J. Janssen

Janssen Research Foundation, Beerse, Belgium

Summary. R 61837 or 3-methoxy-6-[4-(3-methylphenyl)-1-piperazinyl]pyridazine is a new and potent inhibitor of rhinoviruses at concentrations not inhibitory to HeLa cell growth. Different rhinovirus serotypes varied widely in their susceptibility to the antiviral agent. The MICs for 50% CPE reduction ranged from 0.004 to 15 μg/ml. The yields of the most susceptible serotypes were reduced by a factor of 1,000 to 10,000 after single round high multiplicity infections in presence of low concentrations of the compound. The inactivation of some but not all serotypes in a time-, concentration- and temperature-dependent way by R 61837 indicated a direct interaction between the drug and the viral particles. The antiviral activity of the compound was confirmed in the human target cells for rhinoviruses by experiments using nasal polyp explant cultures.

Introduction

Colds are the most common of the acute respiratory infections and probably the most common of all acute infections in man. Rhinoviruses have been shown to be the primary cause [4]. In the current classification system devised for rhinoviruses, 100 serotypes that can each cause colds have been accepted as distinct [8]. The widespread nature of the disease, the economic burden and the impracticality of vaccine development stimulate the search for effective chemotherapeutic agents.

R 61837 or 3-methoxy-6-[4-(3-methylphenyl)-1-piperazinyl]pyridazine (Fig. 1) is a new antirhinoviral compound. The present work describes its marked activity against representative rhinoviruses in HeLa cell culture and human nasal polyp organ cultures.
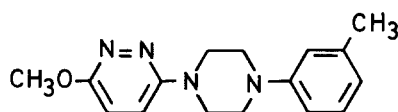

Fig. 1. Chemical structure of R 61837

Materials and methods

Viruses and cells

Laboratory-passaged human coronavirus 229 E and HRV 1 B, 2, 9, 31, and the untyped strains EL, JM, and SM were kindly provided by Dr. D. A. J. Tyrrell from the Common Cold Unit, U.K. The other rhinovirus serotypes used in this study (HRV 1 A, 4, 10, 11, 12, 15, 28, 29, 30, 33, 41, 51, 53, 58, 59, 62, 63, 64, 65, 66, 68, 69, 70, 71, 73, 74, 75, 76, 78, 79, 80, 81, 82, 83, 84, 85, and 88) were obtained from the American Type Culture Collection, Rockville, Maryland. Canine distemper virus, porcine transmissible gastroenteritis virus, pseudorabies virus, and Marek virus were obtained from Dr. M. Pensaert and SV 40 virus from Dr. W. Fiers, both at the University of Ghent. Vaccinia virus, Mengovirus, vesicular stomatitis virus, and polioviruses (type 1 "Saukett", type 2 "Mefie", and type 3 "Mahoney") were obtained from Dr. A. Billiau and herpes simplex viruses (type 1 "KOS" and type 2 "196") from Dr. J. Desmyter at the University of Louvain.

Rhinoviruses and polioviruses were grown in Ohio HeLa cells. Herpes simplex viruses, vaccinia virus, SV 40 virus, and canine distemper virus were cultured in CV-1 cells, Mengovirus in Vero cells, porcine transmissible gastroenteritis virus and pseudorabies virus in SK 6 cells, Marek virus in chicken embryo fibroblast cells, and human coronavirus in MRC 16 cells. All cells were maintained in Eagle's Basal Medium supplemented with 5% foetal calf serum (FCS). Virus stocks were harvested when the cytopathic effect (CPE) involved most of the cell monolayer. Cells were frozen and thawed three times, clarified by centrifugation and the supernatant was titrated to estimate its tissue culture infectivity doses ($TCID_{50}$). Viruses were stored at —70 °C until used.

Chemicals

R 61837 (3-methoxy-6-[4-(3-methylphenyl)-1-piperazinyl]-pyridazine) (M.G. 284.36) was synthesized in the Janssen laboratories by a method described elsewhere (R. Stokbroekx, EP-A 0156433). The maximal solubility in water of this compound is 18.3 μg/ml. The compound was dissolved in dimethylsulfoxide (DMSO) (10 mg/ml) and diluted in growth medium to achieve the final concentration needed. During tolerance and efficacy trials in human volunteers, R 61837 (2.5 mg/ml) was administered as a nasal spray with hydroxy-propyl-β-cyclodextrine (HPβCD) (EP-A 0149197) as the solvent. We therefore conducted some of the experiments reported here with the drug initially dissolved in the same solvent and then further diluted in growth medium till the final concentration needed.

Culture grade Epidermal Growth Factor (EGF) was obtained from Collaborative Research Inc., hydrocortisone, insulin and transferrin from Sigma. Eagle's minimal essential medium, Ham's F 12 medium, and M 199 medium were purchased from Gibco.

Evaluation of cytotoxicity by measurement of cell growth

Ohio HeLa cells were seeded in 25 cm^2 plastic flasks (Falcon) with MEM plus 5% FCS and grown for 24 h at 37 °C to a density of approximately 10^6 cells per flask at the time when R 61837 was added. Both drug dissolved in DMSO and drug dissolved in HPβCD were tested. Stock solutions of the drugs were diluted in medium and a 1 ml sample of each concentration of drug or solvent was applied to each of seven sets of duplicate flasks which were incubated at 37 °C. For sampling at 0, 1 (twice), 2 (twice), and 3 days after the addition of the drug, the growth medium was removed, rinsed once with Ca^{++} and Mg^{++} free phosphate-buffered saline and incubated with 0.25% trypsin. After 15 min at 33 °C, cells were completely detached and separated by gentle agitation and diluted in phosphate-buffered saline. Diluted cell suspensions were counted in duplicate with a Coulter Counter (model ELT 8, Ortho).

Before sampling, each flask was inspected carefully for visible signs of toxicity, such as swelling, granularity, shrinkage, and floating cells.

Minimal Inhibitory Concentration (MIC) assay

60 μl of Ohio HeLa cell maintenance medium (Eagle's Basal medium with 5% FCS) was added to all wells of a microtiter 96-well tissue culture plate. 60 μl of an appropriate starting dilution of drug (initially dissolved in DMSO) was added to duplicate wells and 2-fold dilutions were made in the maintenance medium to cover a wide range of drug concentrations. 120 μl of Eagle's basal Medium with 2% Hepes buffer, 2% FCS, and 30 mM $MgCl_2$ containing approximately 100 $TCID_{50}$ of virus was added to all wells except cell and drug controls. 150 μl of these virus-drug mixtures were then transferred to microtitre plates with subconfluent Ohio HeLa cells, grown in 100 μl of maintenance medium. Appropriate virus controls, cell controls and drug controls were included in each test. Plates were incubated for 4 to 5 days at 33 °C in a 5% CO_2 atmosphere. They were checked by light microscopy without staining and read when the virus controls showed 100% cytopathic effect (CPE) and the virus back titration showed that between 32 and 256 $TCID_{50}$ had been used in the test. The MIC_{50} was taken as the lowest concentration of compound that protected 50% of cells from CPE. The values shown in Fig. 2 represent the median values of at least three separate experiments.

Effect of R 61837 on the yield of virus from a single round of replication

Subconfluent 1-day old monolayers of Ohio HeLa cells in 25 cm^2 flasks (Falcon 3013) were infected with virus at a multiplicity of 1 PFU per cell. R 61837 at the indicated concentrations (Fig. 3) (initially dissolved in DMSO) was added to the viral inoculum 30 min prior to the virus inoculation. After 1 hour adsorption at 33 °C, the inoculum was removed and fresh maintenance medium containing the appropriate concentration of R 61837 was added. The flasks were incubated for an additional 13 h at 33 °C in a 5% CO_2 atmosphere and then frozen and thawed three times. Upon removal of cellular debris, tenfold dilutions of the samples were made and these were quantified by plaque assay on monolayers of HeLa cells. The agar overlay medium consisted of maintenance medium with 0.5% agarose and 30 mM $MgCl_2$. After 5 days incubation at 33 °C, plaques were fixed and stained with a solution containing 0.1% formaldehyde and 0.05% crystal violet in 25% ethanol.

Effect of R 61837 on the yield of virus from multiple rounds of replication

HeLa cells

HeLa cells were infected with 10^3 PFU of HRV 9, previously incubated (30 min, 33 °C) with various concentrations of R 61837, solubilized in HPβCD. Each concentration was tested in triplicate. Fifty percent of the medium (MEM with 5% FCS) was collected at 3 hours (baseline sample), 1 day, 2 days, 3 days, and 4 days after inoculation and replaced by fresh medium with the same concentration of compound. Collected samples were serially diluted and titrated in microtitre plates with subconfluent HeLa cells.

Human nasal epithelial cells

Human nasal polyps were obtained from patients undergoing polypectomy surgery. Polyp tissue fragments (± 2 mm^3) were made, placed in petri dishes (4 to 6 pieces per 35 mm dish), and covered with a few drops of growth factor supplemented (25 ng/ml EGF, 20 ng/ml hydrocortisone, 1 μg/ml insulin, and 10 μg/ml transferrin) F 12 and M 199 (50/50 volume) medium [6]. The use of serum in the medium during the period of outgrowth was omitted to avoid fibroblastic growth [12]. After 1 day of incubation at 37 °C in a 5% CO_2 atmosphere,

more serum-free medium (1 ml) was carefully added to the explants. After 10 days, 24 cultures with comparable areas of growth were selected. Three series of cultures were then infected with 10^3 PFU of HRV 9, previously incubated with various concentrations of the compound (solubilized in HPβCD). Samples were collected and titrated as for the HeLa cell experiment.

Virus inactivation by R 61837

Several rhinovirus serotypes (10^2 or 10^7 PFU/ml) were incubated with or without different concentrations of the test compound in MEM containing 2% FCS and 20 mM HEPES buffer (pH 7.4) at 33 °C. Thereafter, the virus-drug mixtures were diluted serially until non-inhibitory concentrations (as established in the MIC test) of the compound were reached and plaque assayed for remaining infectious virus in HeLa cells.

For chloroform extraction of R 61837 from virus exposed to the compound, the virus suspension was mixed with an equal volume of chloroform, and the mixture was vortexed vigorously for 1 min at room temperature. The mixture was then centrifuged at 1,000 g for 5 min, and the aqueous phase was removed for infectivity titration.

Results

Cell growth in the presence of R 61837

The in vitro cytotoxicity of R 61837 dissolved either in DMSO or in HPβCD was determined by assessing the effect of the drug on cell growth (results not shown). In each case, the cell number for the untreated control increased approximately 10-fold compared to the day 0 level. Inhibition of cell growth, manifested by a 50% decrease in cell number as compared to the controls, occurred at an R 61837 concentration of 15 µg per ml. The results were not significantly different for the two solvents, except for a lower rate of cell death in the samples with the highest drug concentrations (above the maximal solubility in water) dissolved in HPβCD.

Antiviral spectrum

The MIC_{50}s of R 61837 against 40 typed and 3 untyped (EL, JM, and SM) HRVs were assayed in HeLa cell cultures (Fig. 2). The MIC_{50}s ranged from 0.004 to more than 15 µg/ml (maximal concentration tested). R 61837 was highly active against many serotypes, although this activity was very serotype-specific. For example, some serotypes like HRV 9 and HRV 76 were susceptible to concentrations lower than 0.01 µg/ml, whereas other serotypes like HRV 4, 69, and 84 were not inhibited by concentrations as high as 15 µg/ml. The concentrations needed to inhibit 50 or 80% of the rhinoviruses were 2.0 µg/ml and 6.0 µg/ml respectively. Coxsackievirus type A 21 was also susceptible to the compound (MIC 0.5 µg/ml). R 61837 on the other hand was inactive against polioviruses (serotypes 1, 2, and 3) and mengovirus. Representatives from the Coronaviridae (human coronavirus 229 E and porcine transmissible gastroenteritis virus), the Rhabdoviridae (vesicular stomatitis virus), the Paramyxoviridae (canine distemper virus), the Herpesviridae (herpes simplex 1 and 2, Marek virus, pseudorabies virus), the Papovaviridae (SV 40 virus), and the Poxviridae (vaccinia virus) were insensitive to R 61837.
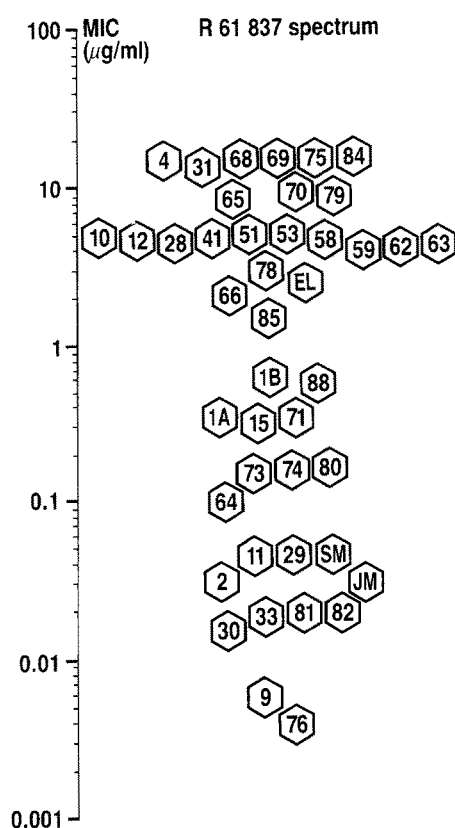
Fig. 2. Antirhinovirus spectrum of R 61837. The sensitivity of 40 typed rhinoviruses (indicated by their serotype number) and 3 untyped strains (EL, JM, SM) was assayed in a MIC test

The MIC_{50} values were not influenced by the nature of the solvent used to dissolve the drug initially. Drug diluted in HPβCD produced the same MICs as drug dissolved in DMSO (results not shown).

Effect of R 61837 on the yield of virus from a single round of replication

The effect of R 61837 on the replication of selected rhinoviruses in a single round of replication was determined (Fig. 3). The viruses selected represent a range of susceptibilities to R 61837 (MIC_{50}s, 0.004 to 8.940 µg/ml). The compound was able to reduce the viral yield by a factor of less than 10^2 (±80%) for the least susceptible serotype (HRV 63) to more than 10^4 for the most susceptible serotype (HRV 76). The 90% effective dose ranged from more than 10 µg/ml for HRV 63 to 0.010 µg/ml for HRV 76 and correlated well with the values determined in the MIC test with multiple replication rounds. Generally, approximately 3 to 5 times the MIC dose was needed to achieve a 90% reduction in a single round of replication. On the other hand, a yield reduction of approximately 10% in a single round of replication was sufficient to achieve a 50% inhibition of CPE after multiple rounds of replication.
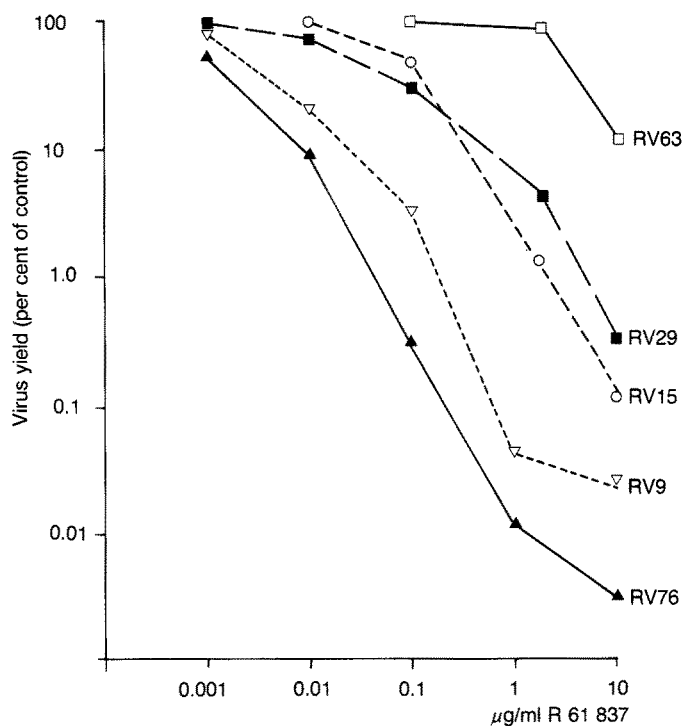
Fig. 3 Effect of R 61837 on the yield of virus from a single round of replication. HeLa cells were infected with the indicated serotypes, previously mixed with the appropriate concentration of R 61837. The cells were incubated for 14 h at 33 °C, frozen and thawed, and the virus yield was plaque assayed

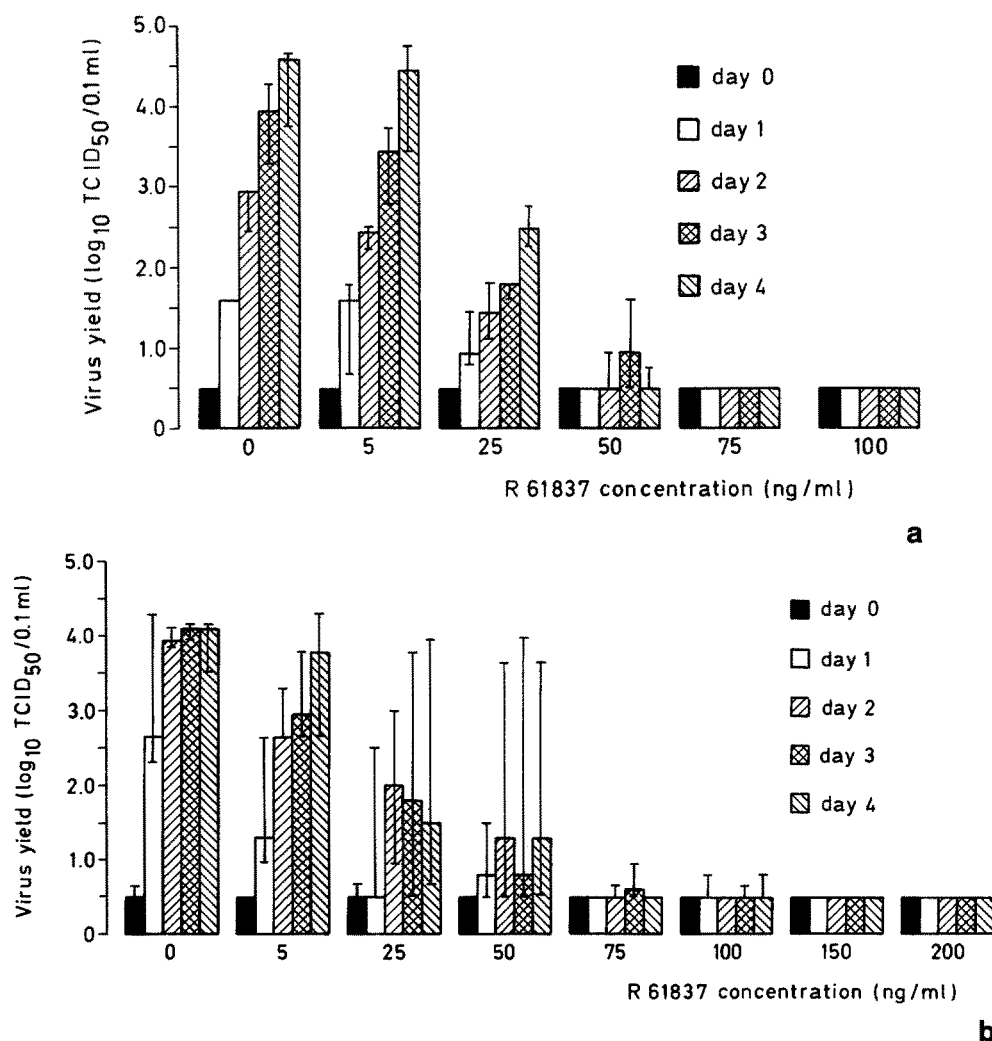## *Effect of R 61837 on the yield of virus from multiple rounds of replication*

HeLa cells

CPE was oberved in the series of cultures treated with 0.005 (100% CPE) and 0.025 ($\pm$ 50% CPE) $\mu g/ml$ R 61837. The results shown in Fig. 4a indicate that a significant reduction (1 $\log_{10}$) of the viral yield correlated well with the concentration needed to reduce the CPE in this experiment (0.025 $\mu g/ml$). At five times this concentration, there was only minimal viral growth in two of the three cultures and yet higher concentrations completely repressed viral multiplication.

Human nasal epithelial cells

After 10 days of incubation, nasal epithelial cells covered large areas of the petri dishes (Fig. 5b). Beating ciliated cells could frequently be seen in the neighbourhood of the original polyp tissue fragment (Fig. 5a).

Upon inoculation of the explant cultures with HRV9, no CPE could be observed. As expected, there was more variation in the viral growth profile in the explant series (Fig. 4b) than in the HeLa cells. The kinetics of the viral growth were different too: maximal titres in the virus controls were reached

Fig. 4. Effect of R 61837 on the yield of virus from multiple rounds of replication in HeLa cells (a) and human nasal polyp explants (b). The cells were infected with HRV 9, previously mixed with various concentrations of R 61837. Each concentration was tested in triplicate. Fifty per cent of the medium was titrated at 3 hours post inoculation (baseline sample) and at days 1, 2, 3, and 4

two days earlier in the human epithelial cells than in the HeLa cells. The reductions of viral yields obtained in human epithelial cells were roughly comparable to those obtained in HeLa cells, although there was a slight tendency towards less inhibition in the former.

Virus inactivation

Effect of incubation time and drug concentration

In an attempt to elucidate the antiviral mechanism of action of R 61837, virus suspensions containing approximately 10^7 PFU of HRV 9 were mixed with
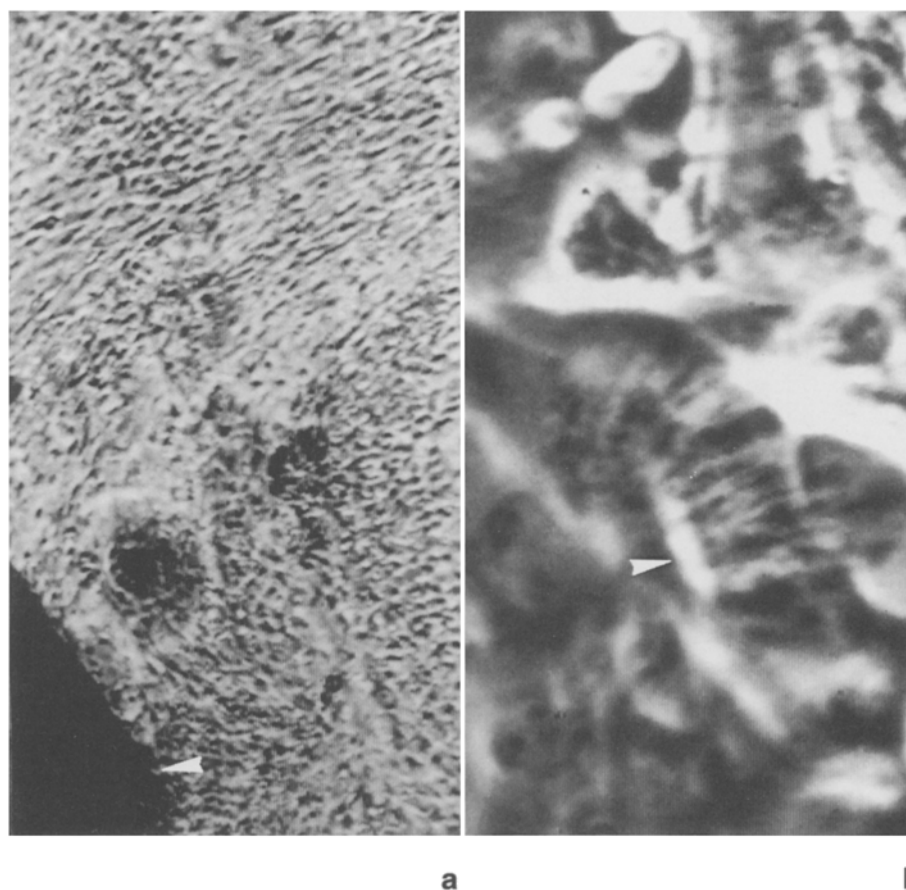
Fig. 5.a Human nasal polyp tissue fragment (arrow) with outgrowing cells (× 100). **b** Human nasal explant tissue with ciliated epithelial cells (arrow) (× 1000)

different concentrations of the compound (solubilized in HPβCD) and incubated for 1, 10, 30, and 60 min at 33 °C. Thereafter, each virus-drug mixture, as well as control virus suspensions with the same solvent concentration but without R 61837, was diluted with MEM until non-inhibitory concentrations of the compound were reached and titrated for residual viral infectivity in HeLa cells. Upon dilution and plaque titration of the virus-drug mixtures, 80% of the infectivity of HRV 9 was lost by interaction with the compound (Fig. 6a). Virus inactivation was time and concentration dependent, rapid at higher drug concentrations (completed within 10 min) and slightly slower (completed within 30 min) with lower drug concentrations. The remaining infectious particles did not appear to be affected by higher drug concentrations or longer incubation times.

Effect of time, temperature, and solvent

To determine whether the reduction in viral infectivity was dependent on the incubation temperature, approximately 10^6 PFU/ml of HRV 9 was allowed to
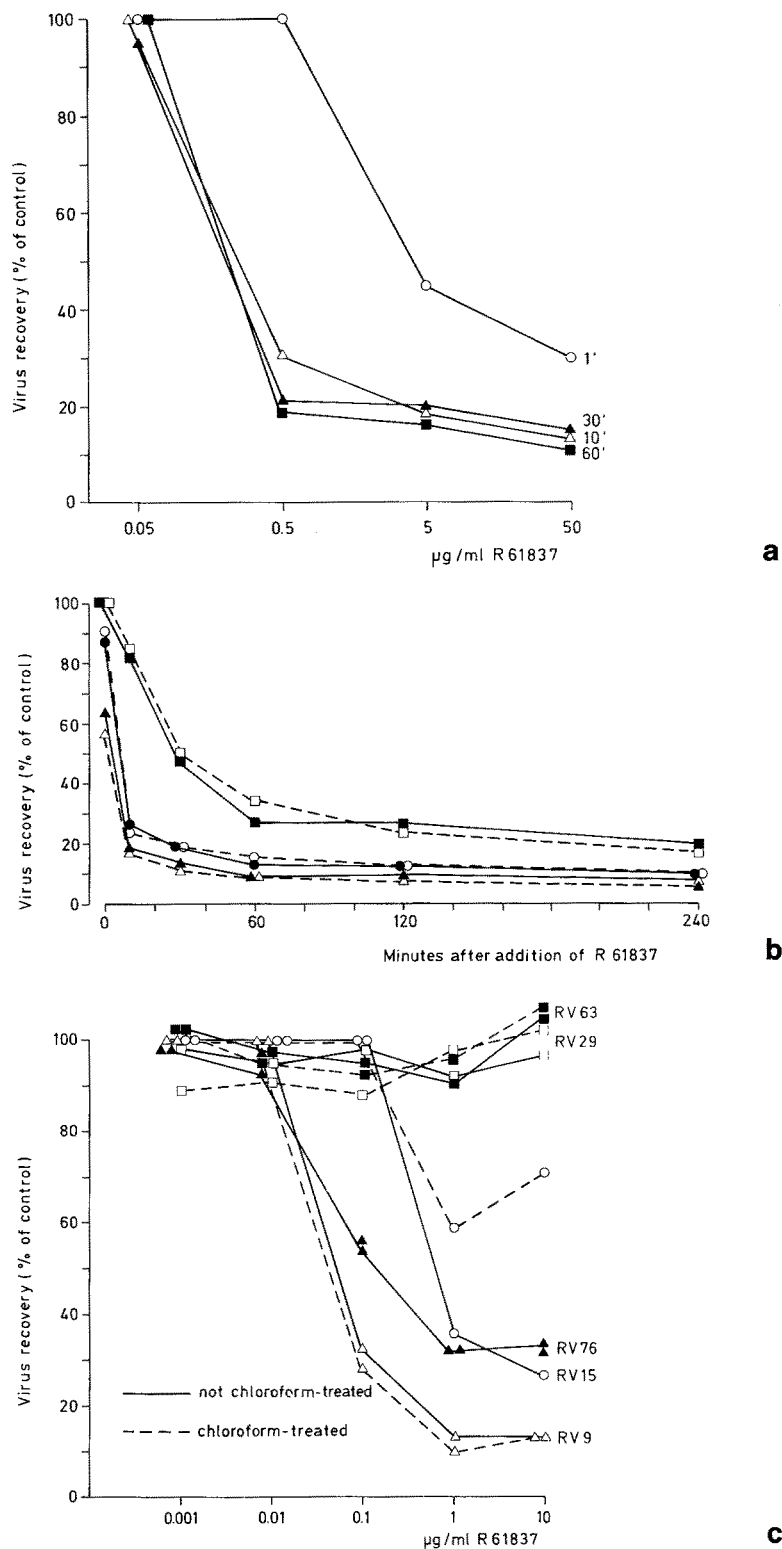
Fig. 6.a Virus inactivation: effect of incubation time and drug concentration. **b** Virus inactivation: effect of time, temperature and solvent. Open symbols: drug dissolved in HPβCD; closed symbols: drug dissolved in DMSO; squares 37 °C, circles 33 °C, triangles 4 °C. **c** Virus inactivation: effect of virus serotype and drug concentration. Approximately 10^6 PFU of the indicated serotypes were incubated for 30 min at 33 °C with various concentrations of R 61837. Part of the samples were chloroform treated in an attempt to extract the drug from the virus. They were then serially diluted until non-inhibitory drug levels were reached and plaque assayed

react with 5 µg/ml of R 61837 (solubilized in DMSO or 10% HPβCD) and incubated at different temperatures (4, 20, and 33 °C for 1, 10, 30, 60, 120, or 240 minutes). Thereafter, each virus-drug mixture, as well as control virus suspensions with the same solvent concentration but without R 61837, was diluted with MEM until non-inhibitory concentrations of the compound were reached and titrated for residual viral infectivity in HeLa cells. As shown in Fig. 6b, the speed of the virus-drug interactions was slower at 4 °C and 20 °C than at 33 °C but eventually, about the same amount of virus was inactivated. The nature of the solvent did not appear to influence the binding between virus and drug.

Effect of virus concentration

To determine whether the reduction in viral infectivity could be confirmed with virus concentrations which could be expected in the nasal washings of experi- mentally infected volunteers, 120 PFU of HRV 9 was allowed to react with different concentrations of R 61837 (solubilized in 10% HPβCD) for 30 min at 33 °C. The virus-drug mixture and the virus controls were first treated with chloroform in an attempt to extract the drug bound to the virus to non-inhibitory levels and then plaque assayed. Preliminary experiments had shown that one chloroform treatment of a R 61837 solution in 10% HPβDC reduced the free drug concentration by a factor of 482. This allowed us to reach non-inhibitory levels of free drug and to determine the remaining infectivity of the viral particles by plaque assay. The results (not shown) indicated that, using this procedure, only 20 to 30 per cent of the original infectivity of low titrated samples of HRV 9 could be regained after interaction with drug levels between 200 ng/ml and 200 µg/ml.

Effect of virus serotype and drug concentration

To determine whether the reduction of viral infectivity and the reversion by chloroform treatment was serotype dependent, 10^7 PFU of the previously se- lected serotypes were incubated with different concentrations of the compound (solubilized in 10% HPβCD) for 30 min at 33 °C. Part of the samples and of the virus controls without the compound was then treated with chloroform in an attempt to extract the drug from the virus. Upon plaque titration of the serially diluted samles, it was clear that the reduction of virus infectivity by the compound was concentration dependent and strongly serotype-specific (Fig. 6c). The infectivity of the less susceptible serotypes (HRV 63 and HRV 29) was not reduced by previous incubation with the compound, while the infectivity of the more susceptible viruses (HRV 9, 15, and 76) was reduced by between 70 and 90%, at least if they were incubated with the highest drug concentrations. Upon exposure of these virus-drug mixtures to chloroform, part of the viral infectivity could be regained in the case of HRV 15. On the other hand, extraction of drug treated HRV 9 or HRV 76 with chloroform had no effect on the viral infectivity.

Discussion

R 61837 represents a new class of antiviral drugs, the pyridazinamines, with significant in vitro efficacy against several members of the picornavirus family. The data presented here show that R 61837 is active against rhinoviruses and coxsackievirus A 21, with MICs as low as 0.005 µg/ml (0.018 µM) for some serotypes. The compound was shown be active in single-round, high multiplicity infections as well as in multi-round, low multiplicity infections.

The antiviral effects of R 61837 were confirmed in two cell types: HeLa cells and human nasal polyp explants. We first used nasal polyp organ cultures to grow rhinoviruses, but parallel cultures from the same polyp did not always produce comparable amounts of progeny virus (results not shown). Human epithelial cells derived from nasal polyps and cultured in a monolayer replicated HRV 9 in a more reproducible way. They represent an easily obtainable alternative to human embryo nasal organ cultures [16] and are very valuable for assessment if a compound exhibits its antiviral activity in the natural host cell of the virus. Compounds which show antiviral activity only if tested in particular cell types have already been described [16, 19].

The effects of R 61837 are clearly specific and can not be ascribed to cytotoxicity. Most of the rhinoviruses were inhibited in subconfluent HeLa cells kept at 33 °C by concentrations of the compound which could not inhibit logarithmic growth of the same cells at 37 °C. Furthermore, the different susceptibilities of the serotypes tested would not be expected if these were due to inhibition of cell metabolism.

The wide range of susceptibility of different HRV serotypes may be surprising at first, but is not very different from the spectrum of antiviral activity of other molecules, known to bind to picornaviruses [3, 9, 10]. Slight variations in the structure of the binding site for these drugs on the viral capsid of different serotypes are likely to affect the strength of the virus-drug bond and therefore the MIC value.

Our study suggests that R 61837 binds to some rhinoviruses in a serotype-, drug concentration-, time- and temperature-dependent way. Comparable properties were described for other rhinovirus binding drugs such as chalcone, RMI 15,731, and dichloroflavan [11]. Chalcone and RMI 15,731 were found to bind to most of the serotypes tested and could be extracted from these viruses by chloroform, while dichloroflavan inactivated RV 16 only, although it was highly active against other serotypes in a CPE reduction test. R 61837 could not be removed by dilution or chloroform extraction from some of the serotypes tested in the present study. As shown for chalcone, the MIC values were lower for serotypes which were bound tighter to R 61837. As for dichloroflavan, not all the serotypes tested bound the drug irreversibly. Susceptible serotypes, inhibited by R 61837 in the CPE reduction test (Fig. 2) and in the virus yield reduction test (Fig. 3) but not inactivated after dilution (Fig. 6c) were apparently bound to the drug in a way which was totally reversible by dilution.

The inability to regain the original infectivity by chloroform extraction after exposure of HRV 9 to the compound, solubilized in the solvent to be used in clinical trials, causes some difficulty in the evaluation of the experimental results. Rhinoviruses, possibly excreted by volunteers and collected in nasal washings, have every opportunity to be bound by the locally applied drug and their infectivity can therefore not be evaluated in a reliable way.

Picornaviruses are associated with a wide spectrum of human and animal diseases. During recent years, several compounds from different chemical classes have been described with very potent in vitro activity against members of this virus family. They include flavans [3], chalcones [9], isoxazoles [13], and pyrano-pyridines [10]. Most of them are suspected to exert their antiviral activity by binding to virions and thereby inhibiting the uncoating event after penetration of the resulting virus-drug complexes into cells [5, 10, 18]. Drug resistant mutants raised against some of these capsid binding compounds sometimes exhibited cross-resistance, indicating a similar mode of action and a sharing of binding sites [17]. The present report and preliminary experiments on the site of action of R 61837 suggest that this compound has similar properties.

Few of the potent compounds mentioned have been advanced to clinical studies and up to now, none of them has shown clinical efficacy in man [1, 14, 15]. The compound described here does have a clinical effect in a double blind, placebo controlled experiment in human volunteers [2]. It can therefore be considered as a new candidate for prophylactic and/or therapeutic control of rhinoviruses in man and will certainly raise a new interest in the compounds mentioned above which have comparable modes of action.

Acknowledgements

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Authors' address: Dr. K. Andries, Janssen Research Foundation, Turnhoutse weg 30, B-2340 Beerse, Belgium.