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Antiviral activity of WIN 54954 in coxsackievirus B2 carrier state infected human myocardial fibroblasts

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Abstract

Persistent infections with a cardiotropic enterovirus, e.g. coxsackievirus B2 (CVB2), cause chronic myocarditis and eventually congestive heart failure. Therefore, the antiviral activity of WIN 54954, a capsid binding antiviral agent that inhibits enterovirus uncoating, was studied in persistently CVB2-infected cultures of human myocardial fibroblasts. Cultures displayed a typical carrier state infection with virus titers of $3.9 \pm 1.6 \times 10^5$ plaque forming units (PFU)/ml and 0.99% infected cells. WIN 54954 (0.025–1 μg/ml) application was started 7 days after infection of the cultures. Compared to the WIN 54954 concentration resulting in a 90% plaque number reduction ($EC_{90} = 0.197$ μg/ml) in acutely infected Vero cells, WIN 54954 reduced virus yields of myocardial fibroblast cultures more efficiently, e.g. more than 100 fold (99%) with 0.025 μg/ml after 4 days of application. Antiviral effects of WIN 54954 increased with application time and at 0.025 μg/ml Win 54954 completely inhibited infectious virus progeny after 16 days. Increasing the WIN 54954 concentration up to 1 μg/ml did not cause a greater inhibition of virus replication. In situ hybridization demonstrated that at 0.1 μg/ml WIN 54954 reduced the number of infected cells from 0.99 to 0.18%, although a complete eradication of CVB2-infected cells was not achieved at concentrations as high as 1 μg/ml. In conclusion, the results indicate that low concentrations of WIN 54954 are effective in treating persistent enterovirus infections of myocardial fibroblasts, although a complete eradication of the infection is not achieved with WIN 54954 as a single antiviral agent. © 1998 Elsevier Science B.V.

Keywords: WIN 54954; Myocarditis; Myocardial fibroblasts; In situ hybridization; Coxsackievirus persistence; Polymerase chain reaction

1. Introduction

Many enterovirus infections are probably subclinical, but infections of the heart, especially with the cardiotropic coxsackieviruses of group B

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(CVB, types 1–6) may cause serious diseases, e.g. myocarditis with life-threatening arrhythmias (Abelmann, 1973; Lau, 1983; Kandolf and Hofschneider, 1989; Sato et al., 1989; Muir, 1993; Why et al., 1994). Chronic enterovirus infections of the heart may result in congestive heart failure under the clinical picture of dilated cardiomyopathy and the only remedy then may be heart transplantation (Why et al., 1994; Figulla et al., 1995). Rapid diagnosis of myocardial enterovirus infection was achieved only recently, since routine endomyocardial biopsy with subsequent enterovirus detection by reverse-transcription and polymerase chain reaction (RT-PCR) was introduced as a diagnostic procedure in suspected myocarditis and unclear sudden heart failure (Jin et al., 1990; Schwaiger et al., 1993; Kämmerer et al., 1994; Andreoletti et al., 1996). Therefore, a real chance of early intervention by antiviral therapy has emerged.

WIN 54954 is a capsid binding antipicornavirus agent, which is active against a multitude of rhinovirus and enterovirus serotypes in vitro, obviously by inhibiting virus uncoating (Diana et al., 1989; Woods et al., 1989). As picornavirus infections lead to a rapid lysis of the infected cells, WIN 54954 has so far only been evaluated in in vitro models of an acute infection (Woods et al., 1989). In murine models of coxsackievirus myocarditis and coxsackievirus infections of the central nervous system, WIN 54954 proved to be effective if application was started early, i.e. with infection or as late as 4 days after infection (Woods et al., 1989; See and Tilles, 1992; Pauksen et al., 1993). On the contrary, enteroviruses are capable of establishing chronic infections in vivo (Kandolf and Hofschneider, 1989; Heim et al., 1994; Why et al., 1994; Heim et al., 1997b). So far, it has remained uncertain whether WIN 54954 is active in enterovirus persistence. Therefore, we evaluated the antiviral activity of WIN 54954 in an in vitro model of myocardial enterovirus persistence.

Propagation of human myocardial fibroblasts from small myocardium samples of paediatric-surgical origin was described recently and results in a sufficient number of non-transformed cells for testing antiviral agents (Heim et al., 1995). Infection of human myocardial fibroblast cultures with human cardiotropic enterovirus gives rise to a persistent carrier-state virus replication with a high virus titer in the culture supernatant (Heim et al., 1995). In this type of infection, a small proportion of the cell population is productively infected, thereby resembling enterovirus infection of myocardial interstitial cells in vivo (Kandolf and Hofschneider, 1989; Klingel et al., 1992; Koide et al., 1992). As an effective antiviral therapy of acute and chronic enterovirus myocarditis is urgently needed, we investigated whether at least a 100-fold (99%) reduction of virus replication or a complete suppression of infectious virus progeny may be achieved with WIN 54954 in CVB2 carrier state infected myocardial fibroblasts.

2. Materials and methods

2.1. Experimental outline

Cultures of human myocardial fibroblasts were infected with CVB2 1 week prior to application of WIN 54954 (carrier state type of in vitro virus persistence). Different concentrations of WIN 54954 (0, 0.025, 0.1, 0.5 and 1 μg/ml) were examined over a period of 16 days, each in triplicate cultures. Culture media were changed every other day and WIN 54954 was added to fresh culture media. Culture supernatants obtained at days 4, 8, 12 and 16 were frozen at -20°C and used for virus titration by plaque assays. In addition, RNA was isolated from culture supernatants of day 16 in order to investigate whether CVB2 RNA can be detected by highly sensitive nested RT-PCR in cultures negative for infectious CVB2. On day 16, fibroblasts were harvested by incubation with versene and the effect of WIN 54954 on the proportion of infected cells in the cultures was determined by quantitative in situ hybridization.

2.2. Cells, viruses and antiviral agents

Human myocardial fibroblasts were prepared from paediatric-surgical myocardial tissue without enzymatic tissue disaggregation as described recently (Heim et al., 1995). Fibroblasts were propagated with Dulbecco's modified Eagle's minimal medium (DMEM), supplemented with 15% foetal bovine serum (FBS; Life technologies, Grand Island, NY), non-essential amino-acids, 4.5 g glucose/l and 50 μg kanamycin/ml. Fibroblast cultures were controlled for the absence of mycoplasma contamination with a nucleic acid hybridization kit (Geneprobe, San Diego CA). coxsackievirus B2 (CVB2) Ohio-1 strain (ATCC No. VR-29), transfection generated coxsackievirus B3 (CVB3) Nancy strain (generated from the plasmid CVB3-M1, Kandolf et al., 1985) and CVB3 Woodruff strain, which was a kind gift of Dr Jan Fohlman, University Hospital Uppsala, Sweden, were plaque-purified on Vero cells (ATCC No. CCL 81) as described previously (Kandolf et al., 1985). Confluent monolayers of human myocardial fibroblasts were infected with CVB2 at a multiplicity of infection (moi) of 0.5 plaque forming units (PFU)/cell. WIN 54954 was provided by Sterling Winthrop, (Rensselaer, New York).

2.3. Virus yield assays

Infectious virus titers in culture supernatants of human myocardial fibroblasts were quantitatively determined by plaque assays as described previously (Kandolf et al., 1985). No effort was made to remove WIN 54954 from fibroblast culture supernatants before determining virus yields, since addition of 1 μg WIN 54954/ml to a CVB2 stock solution did not affect the virus titer determined by a plaque assay compared to the virus titer of the WIN 54954 free CVB2 stock solution.

2.4. Sensitivity assays

WIN 54954 sensitivity of different stock virus preparations CVB3 (Nancy strain), CVB3 (Woodruff strain) and CVB2 (Ohio-1 strain) was determined by a plaque reduction method as the 50% and 90% inhibitory effective concentration (EC_{50} and EC_{90}, respectively). In addition, CVB2 isolated from fibroblast cultures after 16 days of WIN 54954 application was tested to search for emergence of WIN 54954 resistance. Briefly, 100 PFU of virus were incubated with various concentrations of WIN 54954 (0.01–2.5 μg/ml) in DMEM for 1 h. Medium was aspirated from confluent monolayers of Vero cells, cells were incubated with the WIN 54954/virus solution for 1 h, washed with PBS^- (phosphate-buffered saline) and overlaid with 1% seaplaque agarose (FMC Corp., Rockland, ME) in DMEM (supplemented with 5% FBS, 20 mM $MgCl_2$ and 50 μg kanamycin/ml) containing various concentrations of WIN 54954. Cells were fixed after 48 h with 5% trichloroacetic acid, stained with 1% crystal violet and examined for plaque count. Median effect plots (regression lines) were calculated from the concentration/plaque number data (Heim et al., 1996). Significant concentration-dependent action of WIN 54954 was confirmed by calculating the correlation coefficient of the regression lines. Median-effect plot parameters were used for computation of effective concentrations resulting in a 50% or 90% reduction of virus yields (EC_{50} and EC_{90}, respectively). In order to control the reliability of EC-values, standard deviations (S.D.) of EC-values were calculated with a computer program (Heim et al., 1996).

2.5. In situ hybridization

After 16 days of application of WIN 54954, fibroblasts were harvested with versene. 1×10^5 cells were applied each to microscopic slides. About 90% of the cloned CVB3 genome (0.06–7.2 kB), which effectively cross-hybridized with CVB2, was labeled with ^{35}S by nick-translation and used as a DNA probe (Kandolf and Hofschneider, 1985; Kandolf et al., 1987. In situ hybridization was performed as described recently (Heim et al., 1992). Slides were examined unstained with an interference-contrast microscope (Zeiss Jenaval, Jena, Germany). A total of 100 cells were counted on each slide at least three times. Counting was performed in a blinded fashion to avoid observer bias. The degree of infection was determined as percent of positive cells accord-

ing to the ratio: (positive cells/(negative cells + positive cells)) × 100%. Intraobserver error and interobserver errors were obtained from repeated measurements and expressed as the percentage of standard deviation. Estimated standard deviations were low in both cases (6.0–9.3%, respectively) and confirmed the reliability of the method employed.

2.6. Reverse transcription and polymerase chain reaction

RNA was extracted from 140 μl cell culture supernatants after 16 days of WIN 54954 application using a silica-membrane technique (Viral RNA kit, Qiagen, Hilden, Germany). The reverse transcription reaction was carried out with 16 μl of the RNA containing eluate, 1 pmol enterovirus specific primer (CX24M, GTTAGGATTAGCCGCATT) and Superscript 2 RNAse-negative Moloney murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD). The buffer delivered with the enzyme was used, containing 0.5 mM (each) deoxynucleotides triphosphates and 1 mM dithiotreitol in a total volume of 30 μl at 48°C for 60 min. A nested PCR was performed under strict conditions to avoid product carry-over and contamination (Kwok and Higuchi, 1989). The reaction buffer contained 10 mM Tris-HCl pH 8.3, 3.5 mM $MgCl_2$, 75 mM KCl (PCR optimization buffer no. 4, Stratagene, La Jolla, CA), 0.25 mM of each deoxynucleotide triphosphate and 0.8 pmol of each primer in a total volume of 80 μl. A hot start PCR was performed with 2.5 U amplitaq gold (Perkin Elmer Cetus, Norwalk, CT). Primers for the PCR were CX1M (ACCTTTGTGCGCCTGTT) and CX24M and for the nested PCR CX3M (CAAGCACTTCTGTTACCCC) and CX4M (TTCAGGGGCCGGAGGA) resulting in a 296-bp product. CX24M, CX3M and CX4M were 100% homologous (or complementary, repectively) to the recently published CVB2 sequence (EBI accession no. Y09512). As the 5′ non-translated region of CVB2 was so far only sequenced in part, CX1M had to be selected for annealing to a highly conserved region of the enteroviral 5′ non-translated region (nucleotides 67–83 of the published CVB3 sequence, EBI accession no. M33854). All oligonucleotide primers were obtained from Eurogentec (Fleurus, Belgium). PCR products were analyzed by electrophoresis on 2% agarose/TAE (0.04 M Tris-acetate, 0.001 M EDTA) gels stained with ethidium bromide. RNA samples extracted from CVB2-infected and mock-infected cell cultures served as internal positive and negative controls, respectively.

2.7. Cell-proliferation and viability assays

Reduction of cell proliferation by WIN 54954 was quantitatively evaluated by use of the cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium-bromide (MTT) to dark blue formazan by viable cells (Denizot and Lang, 1986). Briefly, cells were incubated for a period of 4 days with various concentrations of WIN 54954 (2, 4, 8 and 16 μg/ml with Vero cells and 0.5, 1, 2.5 and 5 μg/ml with human myocardial fibroblasts). Media were removed and cells were incubated with 50 μg/ml MTT in phenol-red and FBS free DMEM for 4 h. Untransformed MTT-DMEM was removed and formazan was extracted from the cell-monolayer by incubation with isopropanol. Absorbance was read at a wavelength of 560 nm in a standard photometer (reference wave length 690 nm). The WIN 54954 concentration resulting in a 50% inhibition of cell proliferation (antiproliferative IC_{50}) was calculated from the concentration/absorbance data as described in detail for the antiviral EC_{50} and EC_{90}. In order to estimate the specifity of the antiviral action the selectivity index (SI) of WIN 54954 was calculated: SI = antiproliferative IC_{50}/antiviral EC_{50}.

3. Results

3.1. WIN 54954 sensitivity of cardiotropic enteroviruses

The WIN 54954 sensitivity of CVB3 Nancy strain, CVB3 Woodruff strain and CVB2 Ohio 1 strain were determined using a plaque reduction assay method with Vero cells. CVB3 Nancy was primarily resistant to WIN 54954 ($EC_{50} > 2.5$ μg/

ml), whereas both CVB3 Woodruff strain and CVB2 were highly sensitive to WIN 54954 (Table 1). Antiviral effects of WIN 54954 on CVB3 Woodruff strain and CVB2 were significantly concentration-dependent. WIN 54954 had only minor effects on the cell proliferation of Vero cells (antiproliferative IC_{50}: 14.9 μg/ml), Selectivity index calculation confirmed the highly specific inhibition of CVB3 Woodruff strain replication (SI: 339) and CVB2 replication (SI: 827).

3.2. Antiviral acitivity of WIN 54954 on CVB2 infectious virus progeny of carrier state infected cell cultures

Antiviral effects of WIN 54954 on virus titers of CVB2 carrier state infected human myocardial fibroblasts are depicted in Fig. 1. For example, WIN 54954 reduced the virus titers of myocardial fibroblast cultures from 3.3×10^5 PFU/ml (SD 1.4×10^5) to 2.96×10^3 PFU/ml (SD $6.0x10^2$) after 4 days of application. The antiproliferative effect of WIN 54954 in myocardial fibroblast cultures was very low ($IC_{50} > 5$ μg/ml) and this confirms the high selectivity of WIN 54954 action. There were no concentration-dependent antiviral effects, but a time-dependent decrease of infectious virus progeny was observed in carrier state infected cell cultures at all concentrations tested Fig. 1). After 16 days of WIN 54954 (0.025–1 μg/ml) application, infectious virus progeny was completely suppressed with the exception of a single culture in the 0.025 μg/ml dose schedule and a single culture in the 0.5 μg/ml dose schedule. A WIN 54954 resistant CVB2 was isolated from the latter culture. The EC_{90} of this isolate as determined in Vero cell had increased significantly to 0.81 μg/ml compared to 0.197 μg/ml of the stock virus and the EC_{50} had increased slightly to 0.026 μg/ml compared to 0.018 μg/ml of the stock virus (standard deviations of EC_{50} and EC $< 5\%$).

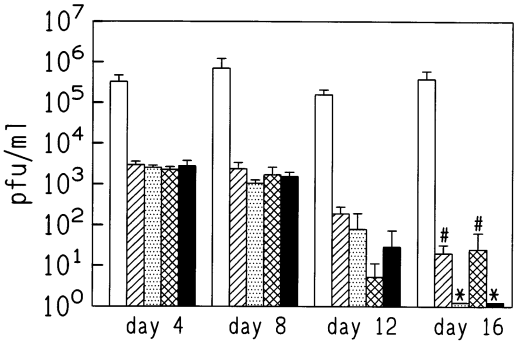

Fig. 1. Reduction of virus titers in CVB2 carrier cultures of human myocardial fibroblasts (mean values of triplicate cultures, error bars indicate standard deviation). Infected control (open bar), 0.025 μg/ml 54954 (hatched bar), 0.1 μg/ml WIN 54954 (dotted bar), 0.5 μg/ml WIN 54954 (criss-cross bar), 1 μg/ml WIN 54954 (solid bar); # complete suppression of infectious virus replication in two of three cultures; * complete suppression of infectious virus replication in all three cultures.

Table 1

Enterovirus (strain)	EC_{50} (μg/ml)	EC_{90} (μg/ml)	SI
CVB3 (Nancy-strain)	>2.5	>2.5	<6
CVB3 (Woodruff-strain)	0.044	0.289	339
CVB2 (Ohio-1 strain)	0.018	0.197	827

EC_{50} and EC_{90} were determined with plaque reduction assays using Vero cells. Standard deviations of EC_{50} and EC_{90} values were $<5\%$, selectivity index (SI = antiproliferative IC_{50}/antiviral EC_{50}). The antiproliferative IC_{50} was 14.9 μg/ml.

3.3. Reduction of CVB-infected cells

In addition to the effects on infectious virus yields, WIN 54954 reduced the number of CVB2 RNA positive cells in carrier state cultures of myocardial fibroblasts. Fig. 2 shows a typical in situ hybridzation of a WIN 54954 treated culture compared to an infected control. Results of a quantitative analysis of in situ hybridization data are depicted in Table 2. A concentration-dependent effect of WIN 54954 was observed on the number of infected cells in contrast to the antiviral effects on infectious virus yields in supernatants (compare Table 2 and Fig. 1).

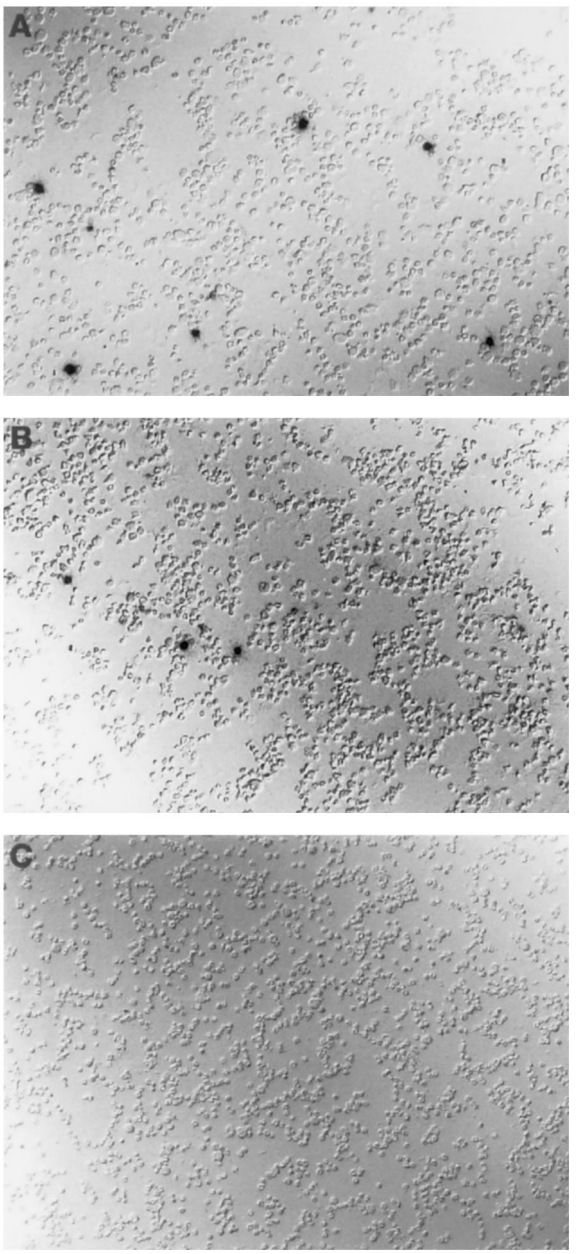

Fig. 2. Autoradiographs of persistently CVB2-infected (A,B) and uninfected (C) cultured human myocardial fibroblasts hybridized in situ with a ^{35}S-labeled, enterovirus specific cDNA probe. (A) Persistently infected CVB2 carrier culture, untreated. (B) Persistently infected CVB2 carrier culture, treated with 0.1 μg/ml WIN 54954 every 24 h over a period of 16 days. (C) Uninfected control cells. Autoradiographic exposure time was 14 days. Interference contrast $\times$ 200.

3.4. Persistence of CVB2 RNA

In spite of the complete suppression of infectious virus progeny by WIN 54954 at 16 days, persistence of CVB2 RNA was demonstrated in myocardial fibroblasts with the help of in situ hybridizations (compare Fig. 1 and Table 2). In addition to the detection of intracellular CVB2 RNA by in situ hybridization, CVB2 RNA persistence was investigated in the cell culture supernatants with a nested RT-PCR, which is highly sensitive, but does not give quantitative results in contrast to the in situ hybridization. Fig. 3 depicts a CVB2 specific amplificate of 296-bp length in all CVB2-infected cultures, including the cultures with a complete suppression of infectious virus progeny after 16 days of WIN 54954 application, but not in the negative control culture. Thus, RT-PCR confirmed the results of in situ hybridizations on CVB2 RNA persistence in WIN 54954 treated cultures.

4. Discussion

Many enterovirus serotypes, most frequently coxsackievirus of subgroup B, have been so far demonstrated as pathogens in myocarditis and associated with consecutive congestive heart failure (Abelmann, 1973, Kandolf and Hofschneider, 1989, Muir, 1993, Why et al., 1994). In the present study, the antiviral activity of WIN 54954 was investigated with an in vitro model system of persistent enterovirus replication to determine whether WIN 54954 is capable to suppress myocardial enterovirus replication if application starts late after target organ infection. WIN 54954

Table 2
Reduction of the proportion of CVB2 RNA positive cells in CVB 2 carrier cultures of human myocardial fibroblasts, as determined by quantitative in situ hybridization

WIN 54954 (μg/ml)	Infected cells (%) $\pm$ S.D.
0	0.99 ± 0.097
0.1	0.18 ± 0.03
0.5	0.15 ± 0.04
1.0	<0.05

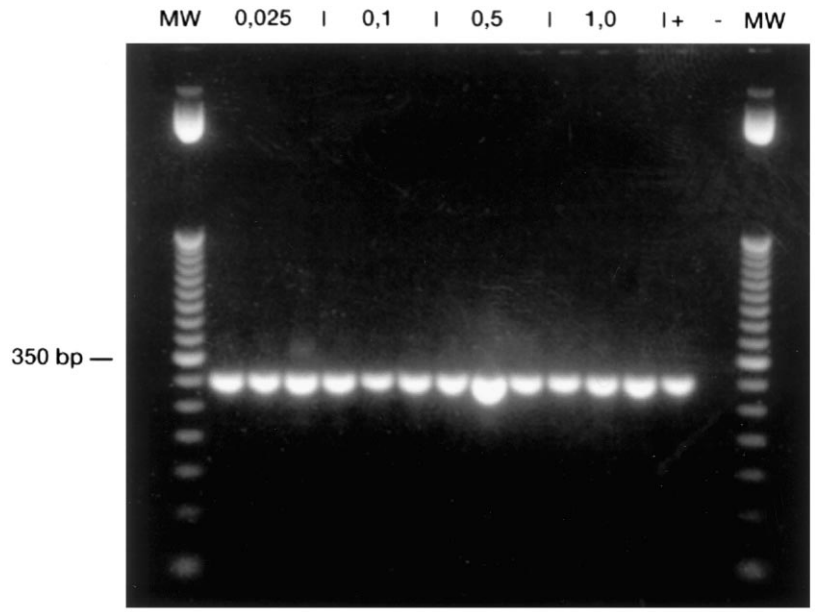

Fig. 3. Gel electrophoresis analysis of RT-PCR products obtained after nested amplification of coxsackievirus RNA. RNA was extracted from supernatants of CVB2 carrier state infected human myocardial fibroblasts after 16 days of WIN 54954 application (0.025, 0.1, 0.5, 1.0 μg/ml), each in triplicates. +: positive control, −: negative control, MW: molecular weight marker, (50-bp ladder), ethidium bromide stain.

was selected as a lead substance of uncoating inhibitors because of its broad spectrum activity against coxsackieviruses in acutely infected cell cultures (Woods et al., 1989). Previously, the antiviral activity of WIN 54954 was demonstrated in murine models of various enterovirus diseases (Woods et al., 1989; See and Tilles, 1992; Pauksen et al., 1993). However, WIN 54954 application was started early after infection of the mice and obviously before the target organ was infected through viremia (See and Tilles, 1992; Pauksen et al., 1993). In contrast to these studies, another uncoating inhibitor, disoxaryl, proved to be effective in a persistent enterovirus infection of the mouse central nervous system (Jubelt et al., 1989).

Our results demonstrate the antiviral activity of WIN 54954 on persistent CVB2 replication using human, organ-specific cells. Surprisingly, WIN 54954 was even more effective in persistent carrier state replication than in acutely infected Vero cells (compare Table 1 and Fig. 1). Recently, a similar result was reported also for another virustatic drug, ribavirin, in carrier state CVB3 replication (Heim et al., 1997a). As carrier state infected cultures need multiple viral replication cycles to maintain their steady state of virus titers, a virustatic inhibition of each single viral replication cycle may add up in carrier state infected fibroblast cultures and eventually results in a time-dependent antiviral effect as demonstrated in the present study (Fig. 1). In contrast to acutely infected Vero cells (Table 1, Woods et al., 1989), antiviral effects of WIN 54954 on carrier state CVB2 replication were not concentration-dependent in fibroblasts cultures. However, the action of WIN 54954 was highly specific and not related to antiproliferative or cytotoxic effects on myocardial fibroblasts (antiproliferative $IC_{50} > 5$ μg/ml). Apparently, a maximum antiviral effect was already achieved in myocardial fibroblasts with the lowest WIN 54954 concentration tested (0.025 μg/ml); which resulted in a complete suppression of infectious virus progeny after 16 days (Fig. 1). In addition to its effects on virus yields, WIN 54954 effectively reduced the number of CVB2 RNA positive cells (Table 2) in carrier state infected myocardial fibroblasts cultures. As WIN 54954 has no direct effect on enterovirus RNA

replication (Diana et al., 1989; Woods et al., 1989), reduction of infected cells is probably due to a protection against CVB2 infection of permissive cells in carrier cultures. In spite of the complete suppression of infectious virus progeny, persistence of CVB2 RNA was detected after 16 days of WIN 54954 application in myocardial fibroblasts by in situ hybridization Fig. 2, Table 2). One possible explanation for this finding is that virus detection by plaque assays is of a limited sensitivity compared to CVB2 RNA detection with in situ hybridization. In general, WIN 54954 carry-over does not interfere with CVB2 detection by plaque assays, probably because WIN 54954 dissociates from the CVB2 virion during the serial dilution with PBS, as already demonstrated with coxsackievirus A 9 and echovirus type 9 (Woods et al., 1989). However, addition of 1 μg WIN 54954/ml to a diluted CVB2 solution (similar to the sensitivity assays, but omitting WIN 54954 from the agarose) reduced the sensitivity of the plaque assay 10-fold (data not shown). Therefore, a very low virus titer (<10 PFU/ml) may be present in cell culture supernatants, which had to be tested undiluted in plaque assays, even if plaque assays give negative results. This view is supported by the detection of CVB2 RNA in the cell culture supernatants by RT-PCR (Fig. 3). Another possible explanation of CVB2 RNA persistence during suppression of infectious virus progeny by WIN 54954 may be a restricted replication of viral RNA that does not result in production of infectious particles (Klingel et al., 1992).

CVB2 was selected as infectious agent for carrier state infection of myocardial fibroblasts because it was demonstrated in human myocarditis, dilated cardiomyopathy and arrhythmogenic right ventricular cardiomyopathy (Lau, 1983; Sato et al., 1989; Heim et al., 1995; Meyer et al., 1997, Heim et al., 1997b). However, CVB2 has only minor myocarditic effects in mice compared to cardiotropic strains of CVB3 (Beck et al., 1990). CVB2 is highly sensitive to WIN 54954 (Table 1) but multiple rounds of CVB2 replication in carrier-cultures with continuous application of WIN 54954 are advantageous prerequisites for in vitro selection of drug resistant strains, which were observed after 16 days. Interestingly, the secondary resistance of CVB2 to WIN 54954 resulted only in a slight increase of EC_{50} values (0.018 to 0.026 μg/ml), whereas, EC50 values increased 4-fold (0.197 to 0.81 μg/ml). In contrast to the secondary resistance of CVB2, one of the cardiotropic CVB3 laboratory strains, CVB3 Nancy, generated from a plasmid containing the complete CVB3 sequence (Kandolf and Hofschneider, 1985), is primarily resistant to WIN 54954 with an $EC_{50} > 2.5$ μg/ml (Table 1). WIN 54954 resistance is not unique for the cloned CVB3 Nancy strain sequence, as a WIN 54954 resistance was also reported for another CVB3 (Nancy strain) stock virus (Fohlman et al., 1996). It may be suspected that CVB3 Nancy strain is also resistant to several other uncoating inhibitors, because a primary resistance of CVB3 Nancy strain was also reported for another capsid binding uncoating inhibitor, SDZ 35682 (Rosenwirth et al., 1995). However, the closely related CVB3 Woodruff strain is highly sensitive to WIN 54954 (Woods et al., 1989). A primary resistance of one CVB3 strain seems to be important concerning the antiviral therapy of enteroviral heart disease, since several studies have emphasized the predominance of CVB3 in the myocardium by sequencing PCR amplicons of the enteroviral 5′ non-translated region (Khan et al., 1994; Nicholson et al., 1995; Heim et al., 1997b). As the conserved 5′ non-translated region sequences of both CVB3 strains are highly homologous to the published CVB3 5′ non-translated region sequences from myocarditis patients (Khan et al., 1994; Nicholson et al., 1995; Heim et al., 1997b), it is not clear so far, whether the primarily resistant CVB3 Nancy strain or the sensitive CVB3 Woodruff strain predominates in enterovirus heart disease. In addition, resistance testing of clinical isolates is not feasible, because enterovirus propagation from the myocardium failed in human myocarditis with a few exceptions (Maller et al., 1967; Sutton et al., 1967; Longson et al., 1969; Abelmann, 1973; Woodruff, 1980). Perhaps, future capsid binding drugs, e.g. derivatives of WIN 54954, can be designed with help of the recently resolved crystal structure of CVB3 Nancy strain (Muckelbauer et al., 1995). Their antiviral spectrum may then include CVB3 Nancy

strain and antiviral therapy of enterovirus myocarditis may be feasible without typing of the infectious agent.

In conclusion, our results indicate that WIN 54954 is capable to suppress persistent enterovirus replication in myocardial fibroblasts. Therefore, WIN 54954 may be active in vivo, even if treatment starts late after the viremic phase, and after the infection of the myocardial interstitium.

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References

Abelmann, W.H., 1973. Viral myocarditis and its sequelae. Annu. Rev. Med. 24, 145–152.

Andreoletti, L., Hober, D., Decoene, C., Copin, M.C., Lobert, P.E., Dewilde, A., Stankowiak, C., Wattre, P., 1996. Detection of enteroviral RNA by polymerase chain reaction in endomyocardial tissue of patients with chronic cardiac diseases. J. Med. Virol. 48, 53–59.

Beck, M.A., Chapman, N.M., McManus, B.M., Mullican, J.C., Tracy, S., 1990. Secondary enterovirus infection in the murine model of myocarditis. Am. J. Pathol. 136, 669–681.

Denizot, F., Lang, R., 1986. Rapid colorimetric assay for cell growth and survival. J. Immunol. Methods 89, 271–277.

Diana, G.D., Pevear, D.C., Otto, M.J., McKinlay, M.A., Rossman, M.G., Smith, T., Badger, J., 1989. Inhibitors of viral uncoating. Pharmacol. Ther. 42, 289–305.

Figulla, H.R., Stille-Siegener, M., Mall, G., Heim, A., Kreuzer, H., 1995. Myocardial enterovirus infection with left ventricular dysfunction: A benign disease compared with idiopathic dilated cardiomyopathy. J. Am. Coll. Cardiol. 25, 1170–1175.

Fohlman, J., Pauksen, K., Hyypiä, T., Eggertsen, G., Ehrnst, A., Ilback, N.G., Frimann, G., 1996. Antiviral treatment with WIN 54954 reduces mortality in murine coxsackievirus B3 myocarditis. Circulation 94, 2254–2259.

Heim, A., Canu, A., Kirschner, P., Simon, T., Mall, G., Hofschneider, P.H., Kandolf, R., 1992. Synergistic interaction of interferon-β and interferon-γ in coxsackievirus B3 infected carrier cultures of human myocardial fibroblasts. J. Infect. Dis. 166, 958–965.

Heim, A., Stille-Siegener, M., Kandolf, R., Figulla, H.R., 1994. Enterovirus-induced myocarditis: Hemodynamical deterioration with immunosuppressive therapy and successful application of interferon-α. Clin. Cardiol. 17, 563–565.

Heim, A., Brehm, C., Stille-Siegener, M., Muller, G., Hake, S., Kandolf, R., Figulla, H.R., 1995. Cultured human myocardial fibroblasts of pediatric origin: natural human interferon-alpha is more effective than recombinant interferon-alpha 2a in carrier-state coxsackievirus B3 replication. J.Mol. Cell. Cardiol. 27, 2199–2208.

Heim, A., Stille-Siegener, M., Pring-Åkerblom, P., Grumbach, I., Brehm, C., Kreuzer, H., Figulla, H.R., 1996. Recombinant Interferons β and γ have a higher antiviral activity than interferon α in coxsackievirus B3-infected carrier state cultures of human myocardial fibroblasts. J. Interferon Cytokine Res. 16, 283–287.

Heim, A., Grumbach, I., Pring-Åkerblom, P., Stille-Siegener, M., Müller, G., Kandolf, R., Figulla, H.R., 1997a. Inhibition of coxsackievirus B3 carrier ctate infection of cultured human myocardial fibroblasts by ribavirin and human natural interferon-α. Antiviral Res. 34, 101–111.

Heim, A., Grumbach, I., Hake, S., Müller, G., Pring-Åkerblom, P., Mall, G., Figulla, H.R., 1997b. Enterovirus heart disease of adults: a persistent limited organ infection in the presence of neutralizing antibodies. J. Med. Virol. 53, 196–204.

Jin, O., Sole, M.J., Butany, J.W., Chia, W.K., McLaughlin, P.R., Liu, P., Liew, C.C., 1990. Detection of enterovirus RNA in myocardial samples from patients with myocarditis and cardiomyopathy using gene amplification by polymerase chain reaction. Circulation 82, 8–16.

Jubelt, B., Wilson, A.K., Ropka, S.L., Guidinger, P.L., McKinlay, M.A., 1989. Clearance of a persistent human enterovirus infection of the mouse central nervous system by the antiviral agent disoxaryl. J. Infect. Dis. 159, 866–871.

Kämmerer, U., Kunkel, B., Korn, K., 1994. Nested PCR for the specific detection and rapid identification of human enteroviruses. J. Clin. Microbiol. 32, 285–291.

Kandolf, R., Hofschneider, P.H., 1985. Molecular cloning of the genome of a cardiotropic coxsackie B3 virus: Full-length reverse-transcribed recombinant cDNA generates infectious virus in mammalian cells. Proc. Nat. Acad. Sci. USA 82, 4818–4822.

Kandolf, R., Canu, A., Hofschneider, P.H., 1985. Coxsackie B3 virus can replicate in cultured human foetal heart cells and is inhibited by interferon. J. Mol. Cell. Cardiol. 17, 167–181.

Kandolf, R., Ameis, D., Kirschner, P., Canu, A., Hofschneider, P.H., 1987. In situ detection of enteroviral genomes in myocardial cells by nucleic acid hybridization: An approach to the diagnosis of viral heart disease. Proc. Natl. Acad. Sci. USA 84, 6272–6276.

Kandolf, R., Hofschneider, P.H., 1989. Viral heart disease. Springer Semin. Immunopathol. 11, 1–13.

Khan, M., Why, H., Richardson, P., Archard, L., 1994. Nucleotide sequencing of PCR products shows the presence of Coxsackie-B3 virus in endomyocardial biopsies from patients with myocarditis or dilated cardiomyopathy. Biochem. Soc. Trans. 22, 176S.

Klingel, K., Hohenadl, C., Canu, A., Albrecht, M., Seemann, M., Mall, G., Kandolf, R., 1992. Ongoing enterovirus-induced myocarditis is associated with persistent heart muscle infection: Quantitative analysis of virus replication, tissue damage, and inflammation. Proc Natl. Acad. Sci. USA 89, 314–318.

Koide, H., Kitaura, Y., Deguchi, H., Ukimura, A., Kawamura, K., Hirai, K., 1992. Genomic detection of enteroviruses in the myocardium-studies on animal hearts with coxsackievirus B3 myocarditis and endomyocardial biopsies from patients with myocarditis and dilated cardiomyopathy. Jpn. Circ. J. 56, 1081–1093.

Kwok, S., Higuchi, R., 1989. Avoiding false positives with PCR. Nature 339, 237–238.

Lau, R.C., 1983. Coxsackie B virus infections in New Zealand patients with cardiac and noncardiac diseases. J. Med. Virol. 11, 131–137.

Longson, M., Cole, F., Davies, D., 1969. Isolation of coxsackievirus group B, type 5, from the heart of a fatal case of myocarditis in an adult. J. Clin. Pathol. 22, 654–658.

Maller, H., Powars, D., Hrowitz, R., Portnoy, B., 1967. Fatal myocarditis associated with echovirus 22 infection in a child with apparent immunological deficiency. J. Pediatr. 71, 204–210.

Meyer, T., Grumbach, I.M., Kreuzer, H, Morguet, A.J., 1997. Giant cell myocarditis due to coxsackie B2 virus infection. Cardiology 88, 296–299.

Muckelbauer, J.K., Kremer, M., Minor, I., Diana, G., Dutko, F.J., Groarke, J., Pevear, D.C., Rossmann, M.G., 1995. The structure of coxsackievirus B3 at 3.5 A resolution. Structure 3, 653–667.

Muir, P., 1993. Enteroviruses and heart disease. Br. J. Biomed. Sci. 50, 258–271.

Nicholson, F., Ajetunmobi, J.F., Li, M., Shackleton, E.A., Starkey, W.G., Illavia, S.J., Muir, P., Banatvala, J.E., 1995. Molecular detection and serotypic analysis of enterovirus RNA in archival specimens from patients with acute myocarditis. Brit. Heart J. 74, 522–527.

Pauksen, K., Ilback, N.G., Friman, G., Fohlman, J., 1993. Therapy of coxsackievirus B3 induced myocarditis with WIN 54954 in different formulations. Scand. J. Infect. Dis. (Suppl) 88, 125–130.

Rosenwirth, B., Oren, D.A., Arnold, E., Kis, Z.L., Eggers, H.J., 1995. SDZ 35–682, a new picornavirus capsid-binding agent with potent antiviral activity. Antiviral Res. 26, 65–82.

Sato, Y., Osaku, A., Koyama, S., Mizakawa, S., Onikura, S., Yagi, H., Suzuki, T., Sawada, T., Uchiyama, T., Kanmatsuse, K., 1989. Complete atrioventricular block associated with regional myocardial scarring in a patient with coxsackie B2 myocarditis. Jpn. Heart J. 30, 935–941.

Schwaiger, A., Umlauft, F., Weyrer, K., Larcher, C., Lyons, J., Muhlberger, V., Dietze, O., Grunewald, K., 1993. Detection of enteroviral ribonucleic acid in myocardial biopsies from patients with idiopathic dilated cardiomyopathy by polymerase chain reaction. Am. Heart J. 126, 406–410.

See, D.M., Tilles, J.G., 1992. Treatment of coxsackievirus A9 myocarditis in mice with WIN 54954. Antimicrob. Agents Chemother. 36, 425–428.

Sutton, G., Harding, H., Trueheart, R., Clark, H., 1967. Coxsackie B4 myocarditis in an adult: successful isolation of virus from the ventricular myocardium. Aerospace Med. 38, 66–69.

Why, H.J.F., Meany, B.T., Richardson, P.J., Olsen, E.G.J., Bowles, N.E., Cunningham, L., Freeke, C.A., Archard, L.C., 1994. Clinical and prognostic significance of detection of enteroviral RNA in the myocardium of patients with myocarditis and dilated cardiomyopathy. Circulation 89, 2583–2589.

Woodruff, J.F., 1980. Viral myocarditis. Am. J. Pathol. 101, 427–479.

Woods, M.G., Diana, G.D., Rogge, M.C., Otto, M.J., Dutko, F.J., McKinlay, M.A., 1989. In vitro and in vivo activities of WIN 54954, a new broad-spectrum antipicornavirus drug. Antimicrob. Agents Chemother. 33, 2069–2074.