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Anti-rhinovirus activity of 3-methylthio-5-aryl-4-isothiazolecarbonitrile derivatives

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Abstract

A series of 3-methylthio-5-aryl-4-isothiazolecarbonitriles has been evaluated as anti rhinovirus agents against a panel of 17 representative human rhinovirus (HRV) serotypes, belonging to both A and B groups. No anti rhinovirus activity was detected for 3-methylthio-5-phenyl-4-isothiazolecarbonitrile (**IS-2**). Isothiazole derivatives with bulky substituents (O-Bn or O-But groups) on the *para* position of the phenyl ring were the most effective compounds of this series. In fact, a reduction in virus-induced cytopathogenicity was demonstrated for the O-Bn substituted **IS-50** compound against the majority (88%) of the rhinoviruses tested, whereas the compound with an O-Ts group (**IS-44**) was found to be a specific inhibitor of group B serotypes, exhibiting the lowest IC_{50} against HRVs type 2, 85 and 89. Our studies on the mechanism of action of **IS-44** demonstrated that it prevents the thermal inactivation of HRV 2 infectivity, probably due to a conformational shift in the viral capsid and a decrease in affinity for the cellular receptor, resulting in an inhibition of attachment of the virions.

Keywords: Isothiazole derivatives; Antiviral; Rhinovirus

1. Introduction

The human rhinovirus (HRV) group of the picornavirus family represents the etiologic agent in approximately 50% of all cases of common cold. Although HRV infection of the upper respiratory tract in humans is often a mild and self-limiting affliction, various reports indicate that rhinoviruses are of significance in the occurrence of sinusitis and otitis media and lower respiratory tract diseases (Schmidt and Fink, 1991; Couch, 1996; Nicholson et al., 1996; Gern et al., 1997). In fact, rhinoviruses have been associated with atypical pneumonia and have been reported to occur in up to 40% of exacerbations of chronic bronchitis (Nicholson et al., 1993; Gwaltney and Rueckert, 1997) and to precipitate asthmatic attacks in children (Mertsola et al., 1991; McMillan et al., 1993).

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The production of a vaccine to prevent the common cold has not been possible because there are over 100 immunologically non-cross reactive rhinovirus serotypes. Anti-peptide vaccines, although potentially broad-spectrum in nature, induce only weak neutralizing antibody responses (McCray and Werner, 1989).

Therefore, most effort has been focused on the development of effective antiviral agents for treatment of rhinovirus infections (Al-Nakib and Tyrrell, 1992; McKinlay, 1993; Andries, 1995). A variety of natural or synthetic compounds belonging to different chemical classes have been shown to act as potent and broad-spectrum anti-HRV agents in vitro: e.g. dichloroflavan (BW683C: Bauer et al., 1981; Tisdale and Selway, 1983), enviroxime (Wikel et al., 1980), the pyridazinamine derivatives (e.g. R61837, R77975: Andries et al., 1988, 1992), the chalcones (e.g. Ro 09-0410: Ishitsuka et al., 1982; Ninomiya et al., 1990), the benzamides (Ogata et al., 1986), the piperazine derivatives (e.g. SDZ 35-682, SDZ 880-061: Rosenwirth et al., 1995; Oren et al., 1996), the HRV 3C protease inhibitors (e.g. AG7088: Webber et al., 1996; Patick et al., 1999) and the oxazolinyl isoxazole compounds (e.g. arildone, disoxaril (WIN 51711), WIN 54954, pleconaril (VP63843): Diana et al., 1977, 1985, 1989; Otto et al., 1985; Bailey et al., 1992; Rogers et al., 1999). Some of these compounds have demonstrated anti picornavirus activity both in vitro and in vivo (McKinlay and Steinberg, 1986; Jubelt et al., 1989; Woods et al., 1989; Cox et al., 1996; Pevear et al., 1999).

A number of compounds active against rhinoviruses and enteroviruses have been investigated in clinical studies (Al-Nakib et al., 1989; Turner et al., 1993; Arruda and Hayden, 1995; Rotbart et al., 1998): e.g. intranasal administration of Pirodavir provided protection against experimental rhinovirus colds, but it did not offer any clinical benefit in naturally acquired HRV colds (Hayden et al., 1995). Currently, two promising drugs are close to clinical approval for the treatment of colds: the capsid binding agent pleconaril (VP63843: Hayden et al., 1999; Kaiser et al., 1999) and the 3C protease inhibitor AG7088 (Pithavala et al., 1999).

Mode of action studies have shown that many of these compounds inhibit picornavirus replication by binding to a hydrophobic pocket on the virion surface, thereby preventing viral adsorption or uncoating depending on the viral serotype involved (McSharry et al., 1979; Rossmann et al., 1985; Fox et al., 1986; Smith et al. 1986; Pevear et al., 1989; McKinlay et al., 1992).

Andries et al. (1990) proposed a spectral map analysis technique to rationalize drug screening for capsid binding antirhinovirus agents. A systematic evaluation of 15 rhinovirus capsid binding compounds against all serotype human rhinoviruses revealed the existence of two virus groups, designated group A and B, based on differential susceptibility to antiviral compounds (Andries et al., 1991). Group A consists of rhinoviruses having a more than average susceptibility to elongated molecules (R72440 and WIN compounds), whereas group B consists of rhinovirus serotypes susceptible to short-chain compounds (chalcones, dichloroflavanes, and R61837). The positions of the viruses within the map derived from this multivariate analysis allowed for the selection of a panel of 17 representative human rhinoviruses (six screening serotypes of antiviral group A: HRVs 14, 42, 45, 70, 72, and 86; 11 screening serotypes of antiviral group B: HRVs 2, 9, 15, 29, 39, 41, 51, 59, 63, 85, and 89), for which the antiviral inhibitory values will be highly predictive for all serotypes. In this way, in spite of the heterogeneity of this virus genus, it is possible to obtain a more accurate idea of the real potency and spectrum of activity of a compound thanks to a more rational screening program.

Our previous studies described the synthesis and antiviral activity of some isothiazole derivatives against RNA viruses (Pinizzotto et al., 1992; Garozzo et al., 1994). Recently, we synthesized 3,4,5-trisubstituted isothiazoles with a potent in vitro antiviral activity (Cutrì et al., 1998, 1999). The most active member of the series, 3-methylthio-5-phenyl-4-isothiazolecarbonitrile (coded **IS-2**) was found to be effective against polio 1 and ECHO 9. The anti picornavirus activity led us to investigate the inhibiting effect of some of these compounds against the 17 human rhinovirus serotypes. Among all the new synthe-

sized isothiazole derivatives, we selected six 3-methylthio-5-aryl-4-isothiazolecarbonitriles bearing no substituents or various substituents on the *para* position of the phenyl ring (e.g. O-methyl, O-tosyl, O-benzyl, hydroxy or O-butyl groups) to evaluate their potential anti-HRV activity (Fig. 1).

Our aim was to identify the structural requirements to obtain broad-spectrum anti picornavirus agents.

2. Materials and methods

2.1. Compounds

Fig. 1 shows the structural formulae of the six isothiazole derivatives: 3-methylthio-5-phenyl-4-isothiazolecarbonitrile (coded **IS-2**); 3-methylthio-5 - (4 - methoxyphenyl) - 4 - isothiazolecarbonitrile (coded **IS-21**); 3-methylthio-5-(4-OTs-phenyl)-4-isothiazolecarbonitrile (coded **IS-44**); 3-methylthio-5-(4-OBn-phenyl)-4-isothiazolecarbonitrile (coded **IS-50**); 3-methylthio-5-(4-hydroxyphenyl)-4-isothiazolecarbonitrile (coded **IS-54**); 3-methylthio-5-(4-butoxyphenyl)-4-isothiazolecarbonitrile (coded **IS-79**).

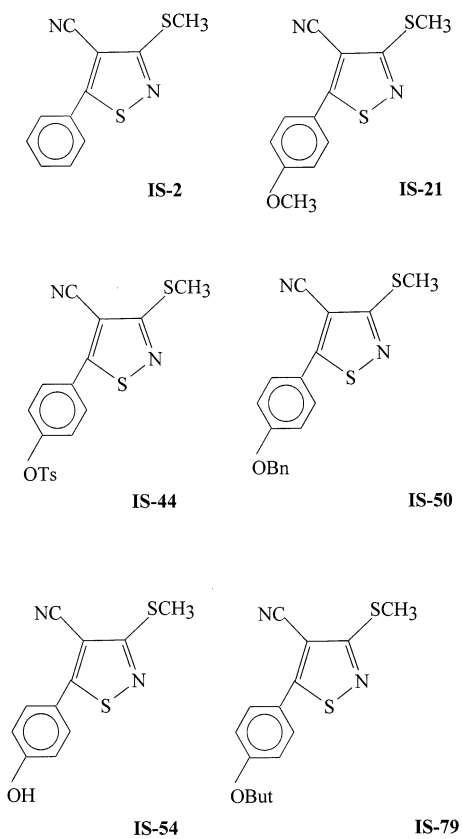

Fig. 1. Structural formulae of the six isothiazole derivatives.

The synthesis and chemical properties of these compounds were previously reported (Cutrì et al., 1998).

All the compounds were initially dissolved in dimethyl sulfoxide (DMSO) and further diluted in maintenance medium before use, to achieve the final concentration needed. Dilution of test compounds contained a maximum concentration of 0.01% DMSO, which was not toxic to the cell line used.

2.2. Viruses and cells

Human rhinoviruses (HRV 2, HRV 9, HRV 14, HRV 15, HRV 29, HRV 39, HRV 41, HRV 42, HRV 45, HRV 51, HRV 59, HRV 63, HRV 70, HRV 72, HRV 85, HRV 86, and HRV 89; originally supplied by Professor Paolo La Colla, Dipartimento di Biologia Sperimentale, Sezione di Microbiologia, Università di Cagliari, Cagliari, Italy) were propagated in human epitheloid carcinoma cervix cells (HeLa-Ohio) at 33°C.

Cells were kept in a humidified 5% carbon dioxide atmosphere at 37°C and grown in Eagle's Minimum Essential medium (MEM) supplemented with 10% heat inactivated fetal calf serum (FCS), 200 μg/ml of streptomycin and 200 U/ml of penicillin G.

For all viruses tested working stock solutions were prepared as cellular lysates using MEM with 2% FCS (maintenance medium).

2.3. Cell viability

The cytotoxicity of the test compounds was evaluated by measuring the effect produced on cell morphology and cell growth. Cell monolayers

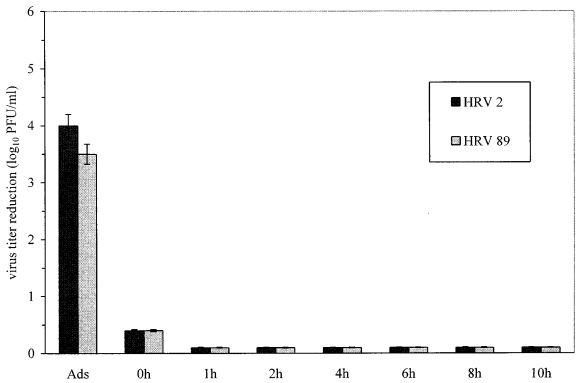

Fig. 2. Effect of time of addition of compound **IS-44** (5 μM) on virus yield from a single-round replication of HRV 2 and HRV 89. Time 0 = post 2 h adsorption period at 4°C. All values are mean ± S.D. for three separate assays.

were prepared in 24-well tissue culture plates and exposed to various concentrations (μM) of the compounds. Plates were checked by light microscopy after 24, 48 and 72 h. Cytotoxicity was scored as morphological alterations (e.g. rounding up, shrinking, detachment). The viability of the cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method (Denizot and Lang, 1986).

The 50% cytotoxic concentration (CC_{50}) was expressed as the concentration of the compound that reduced the absorbance of the control sample by 50%.

2.4. Antiviral activity

Infectivity of virus stock was determined by the MTT method: the reciprocals of viral dilution which resulted in 50% reduction of absorbance of formazan in the infected cells at 48–72 h was determined as infectivity of the virus by MTT ID_{50} (50% infective dose).

The anti-rhinovirus assay was based on the inhibition of virus-induced cytopathogenicity. HeLa-Ohio cells were grown in 96-well tissue culture plates and were allowed to form a subconfluent monolayer by incubation at 37°C in a humidified 5% carbon dioxide atmosphere. Maintenance medium, with or without various concentrations of the test compounds at doses below CC_{50}, was then added followed by a suspension of HRV containing 10 $CCID_{50}$ (50% cell culture infective dose) of the virus stock to produce a complete cytopathic effect within 48–72 h after infection. After incubation at 33°C, the viability of mock-infected and virus-infected cells was quantified by the MTT method. The percentage protection of each compound was calculated by the following formula:

$$\frac{(OD_t)_{HRV} - (OD_c)_{HRV}}{(OD_c)_{mock} - (OD_c)_{HRV}} \times 100$$

whereby $(OD_t)_{HRV}$, $(OD_c)_{HRV}$, and $(OD_c)_{mock}$ indicated the absorbances of the test sample, the virus-infected control (no compound), and the mock-infected control, respectively. The compound concentration required to inhibit virus-induced cytopathogenicity by 50% was expressed as IC_{50} and calculated by dose-response curves using the linear regression technique.

For the experiments on the mechanism of action, the anti rhinovirus activity was assessed using a plaque reduction assay as previously described (Garozzo et al., 1990).

2.5. Addition at different time intervals

Monolayers of HeLa-Ohio cells were grown to confluence in 24-well plates and inoculated with HRV 2 and HRV 89 at a MOI (multiplicity of infection) of 0.1. The plates were incubated for 2 h at 4°C to ensure synchronous replication of the viruses, with or without compound **IS-44** (5 μM), for the adsorption period. Then the inoculum was removed and medium, with or without the compound, was added at various times after the adsorption period, as indicated in Fig. 2. The plates were incubated at 33°C for 12 h, then, cultures were frozen and virus yield was determined by plaque assay.

2.6. Inhibition of virus adsorption

Infective center assays were used to study the effect of the compound on the virus adsorption step. Briefly, a HeLa cell suspension (10^6 cells/ml) was cooled to 4°C for at least 1 h. HRVs 2 and 89 (10^6 PFU/ml), incubated for 60 min at 33°C with

different concentrations of the test compound (1 ×, 10 × and 100 × the IC_{50}), were cooled to 4°C, and subsequently added to the HeLa cell suspension. Cells were incubated with the virus–drug mixtures for 90 min at 4°C to prevent the virus from entering the cells. After the adsorption period, unadsorbed viruses and free compound were removed by washing three times with cold MEM. The cells were then diluted serially and plaque assayed for cell-associated viral cytopathic activity.

2.7. Virucidal activity

To test possible virucidal activity, equal volumes (0.5 ml) of HRV 2 or HRV 89 suspensions (containing 10^6 PFU/ml) and MEM containing compound **IS-44** (10 × and 100 × the IC_{50}) were mixed and incubated for 1 h at 33°C. Infectivity was determined by plaque assay after dilution of the virus below the inhibitory concentration.

2.8. Thermal inactivation

HRV 2 and HRV 89 (10^6 PFU/ml) were incubated for 1 h at 33°C with or without compound **IS-44** (10 × and 100 × the IC_{50}), then shifted to 56°C for 6 min and refrigerated on ice. Aliquots were diluted 10-fold serially in MEM to concentrations of the compound that were not inhibitory and the recovered virus was measured by plaque assay.

Stabilization to heat was assessed by comparing the measured PFU titer with the titer of controls, consisting of virus not pre-incubated with the compound but exposed to heat under the same conditions.

2.9. Extraction with chloroform

For the extraction of the test compound after pre-incubation with rhinoviruses, an equal volume of 100% $CHCl_3$ was added, the sample was vortexed for 1 min at room temperature, and centrifuged at 700 × *g* for 5 min. The aqueous phase, which contained the virus, was collected and the titer was determined by plaque assay.

3. Results

3.1. Effect of isothiazole derivatives on cell proliferation and on virus replication

Table 1 shows the data from previously published papers (Cutrì et al., 1998), which indicate the values of CC_{50} on HEp-2 and L-929 cell monolayers and the values of IC_{50} of the six isothiazole derivatives against picornaviruses polio 1, ECHO 9, Coxsackie B1 and EMC (encephalomyocarditis) virus.

When compared with **IS-2**, bearing an unsubstituted phenyl in the 5-position of the isothiazole nucleus, the aryl derivatives **IS-44**, **IS-50** and **IS-**

Table 1
Anti picornavirus activity of isothiazole derivatives

Compound	CC_{50}[a,c] (μM)	IC_{50}[b,c] (μM)			
	HEp-2\L-929	Polio 1	ECHO 9	Cox B1	EMC
IS-2	20	0.045	0.25	>20	10
IS-21	10	0.6	0.5	>10	>10
IS-44	>25	>25	>25	>25	>25
IS-50	>50	>50	>50	10	20
IS-54	2	0.4	1	>2	>2
IS-79	>50	0.3	1	10	3

[a] CC_{50}, concentration which inhibited HEp-2 and L-929 cell growth by 50% as compared with control cultures. HEp-2 cells were used to propagate polio 1, ECHO 9 and Cox B1 viruses; L-929 cells were used to propagate EMC.

[b] IC_{50}, concentration which inhibited virus plaque formation by 50%.

[c] Values are mean ± 0.5 S.D. (maximal S.D. estimated) for three separate assays.

Table 2
Anti rhinovirus activity of isothiazole derivatives against A group serotypes

Compound	CC_{50}[a,c] (μM)	IC_{50}[b,c] (μM)					
	HeLa-Ohio	HRV14	HRV42	HRV45	HRV70	HRV72	HRV86
IS-2	80	>80	>80	>80	>80	>80	>80
IS-21	12	>12	>12	>12	>12	>12	>12
IS-44	25	>25	>25	>25	>25	>25	>25
IS-50	>50	25	7	>50	25	>50	2
IS-54	10	>10	>10	>10	>10	>10	4.4
IS-79	40	>40	>40	>40	>40	>40	7

[a] CC_{50}, concentration which inhibited HeLa cell growth by 50% compared with control cultures.
[b] IC_{50}, concentration which inhibited virus-induced cytopathogenicity by 50%.
[c] Values are mean ± 0.5 S.D. (maximal S.D. estimated) for three separate assays.

Table 3
Anti rhinovirus activity of isothiazole derivatives against B group serotypes

Compound	IC_{50}[a,b] (μM)										
	HRV2	HRV9	HRV15	HRV29	HRV39	HRV41	HRV51	HRV59	HRV63	HRV85	HRV89
IS-2	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80
IS-21	>12	>12	>12	>12	>12	>12	>12	>12	>12	>12	>12
IS-44	0.3	0.9	1	1	>25	>25	>25	>25	>25	0.3	0.1
IS-50	20	3	3	20	3	32	19	12	10	1	2
IS-54	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
IS-79	5	5	>40	>40	5	>40	>40	>40	>40	1	2.5

[a] IC_{50}, concentration which inhibited virus-induced cytopathogenicity by 50%.
[b] Values are mean ± 0.5 S.D. (maximal S.D. estimated) for three separate assays.

79, with bulky substituents (O-Ts, O-Bn and O-But) on the *para* position of the phenyl ring, showed lower cytotoxicity on HEp-2 cells. However, the presence of these groups caused a diminished (**IS-79**) or a loss of antiviral activity (**IS-44** and **IS-50**) against polio 1 and ECHO 9.

The introduction of an OCH_3 group and an OH group in the *para* position made the respective compounds, **IS-21** and **IS-54**, only slightly active against the above-mentioned viruses. Regarding the anti-EMC activity, **IS-2** and **IS-50** exhibited only a marginal activity, while **IS-79** exhibited the best anti-EMC activity. **IS-50** and **IS-79** were also active against Coxsackie B1 (Table 1).

Tables 2 and 3 show the values of the cytotoxic dose (CC_{50}) on HeLa-Ohio cells and inhibitory concentrations (IC_{50}) of the compounds against both group A (HRV 14, HRV 42, HRV 45, HRV 70, HRV 72, and HRV 86) and B rhinoviruses (HRV 2, HRV 9, HRV 15, HRV 29, HRV 39, HRV 41, HRV 51, HRV 59, HRV 63, HRV 85, and HRV 89).

The unsubstituted compound **IS-2** was ineffective against all the serotypes tested, while the derivatives with various substituents on the *para* position of the phenyl ring showed interesting anti-HRV activity.

In particular, **IS-50** exhibited the broadest anti-rhinovirus spectrum: it was effective against 88% (15 of 17) of the 17 serotypes screened. In fact, this compound was active against all group B serotypes, with a potent inhibitory effect against HRV 85 and HRV 89. The IC_{50} values were found to be 1 and 2 μM, respectively (Table 3). When evaluated against group A serotypes, **IS-50** was found to exhibit the best activity against HRV 42 and HRV 86 and was weakly active against HRV 14 and HRV 70 (Table 2).

The presence of an O-But group (compound **IS-79**) on the phenyl ring resulted in a diminished anti rhinovirus activity with respect to the isosteric analogue **IS-50**. In fact, in spite of showing the broadest antiviral activity against the previous viruses, it was only effective against one of the six group A serotypes (HRV 86) and five of the 11 group B serotypes (Tables 2 and 3).

Interestingly, the introduction of an O-Ts group in the same position (compound **IS-44**) led to the discovery of a compound active against some group B rhinoviruses, with the lowest IC_{50} values for HRVs 2, 85 and 89 (0.3, 0.3 and 0.1 μM, respectively) (Table 3).

Finally, compounds with smaller groups (OCH_3 and OH for compounds **IS-21** and **IS-54**, respectively) were completely ineffective against both group A and B rhinovirus serotypes; the sole exception was compound **IS-54**, which was marginally active against HRV 86 (Tables 2 and 3).

3.2. Effect of time addition of compound IS-44

In order to determine whether compound **IS-44** inhibited the virus yield during a specific period in the virus cycle, the effect of time addition of this compound was studied for HRVs 2 and 89. Results obtained from these experiments clearly demonstrated maximum inhibition of viruses when the compound was added during the adsorption period (4 and 3.5 $\log_{10}$ titer reductions for HRVs 2 and 89, respectively). The inhibitory effect on rhinovirus replication was completely lost when the compound was added after this step (Fig. 2).

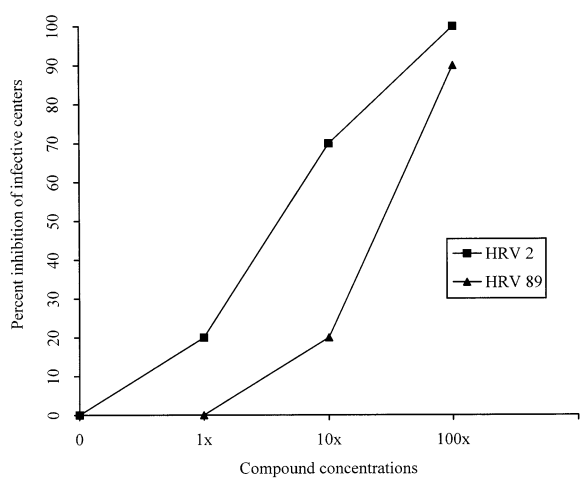

Fig. 3. Effect of compound **IS-44** on the adsorption of HRV 2 (■) and HRV 89 (▲). Infective center assay data were plotted as percentage inhibition relative to the no treatment controls. The concentrations used are ratios with respect to IC_{50} (e.g. 10 IC_{50} is 10 times the IC_{50} of the compound). All values are mean ± S.D. for three separate assays.

3.3. Effect on virus adsorption of HRV 2 and HRV 89

A dose-response curve for a typical infectious center experiment is given in Fig. 3. At concentrations 10 times the IC_{50} of **IS-44**, the adsorption of HRV 2 was inhibited by about 70% and at 100 times the IC_{50}, the inhibition was complete. In contrast, addition of 10 times the IC_{50} of **IS-44** had a weak (20%) effect on the adsorption of HRV 89, while a concentration 100 times the IC_{50} was needed to achieve 90% inhibition of adsorption. At a concentration of 1 × the IC_{50}, adsorption was slightly inhibited (20%) (HRV 2) or not inhibited (HRV 89).

3.4. Effect on virus infectivity and on heat inactivation of HRV 2 and HRV 89

IS-44 stabilized HRV 2 against thermal inactivation. The concentrations that reduced the loss of virus by heat were 10 and 100 times the IC_{50}. In fact, after 6 min of exposure at 56°C, the titer in the untreated controls was reduced by 5 $\log_{10}$, compared with only 1.3 and 0.5 $\log_{10}$ titer reductions in the virus exposed to 10 and 100 times the IC_{50}, respectively. In contrast, the same concentrations of **IS-44** were insufficient to protect HRV 89 from heat inactivation (Table 4). The results obtained after chloroform extraction were similar (data not shown).

Consistent with the ability to recover IS-44-treated HRV 2 after thermal exposure, the compound did not prove virucidal. In fact, **IS-44** did not significantly reduce the titers of HRV 2 after 1 h incubation at 10 and 100 times the IC_{50}.

On the contrary, **IS-44** reduced the viral infectivity of HRV 89 that was completely restored to

Table 4
Effect of **IS-44** on virus infectivity and on heat inactivation of HRV 2 and HRV 89

Virus	**IS-44** concentration[b]	Reduction in titer (log_{10} PFU/ml) compared with that of control[a]		
		Virus inactivation (1 h at 33°C)		Thermal inactivation (6 min at 56°C)
		Before $CHCl_3$	After $CHCl_3$	
HRV 2	0	0.0	0.0	−5.0
	10×	0.0	0.0	−1.3
	100×	0.0	0.0	−0.5
HRV 89	0	0.0	0.0	−5.0
	10×	−3.7	0.0	−5.0
	100×	−4.0	0.0	−5.0

[a] Values are given as differences from the control virus titer and they are mean ± S.D. for three separate assays.
[b] The concentrations used are ratios with respect to IC_{50} (e.g. 10 IC_{50} is 10 times the IC_{50} of the compound).

the original value after extraction of the drug with chloroform at room temperature (Table 4).

4. Discussion

Recently, our contribution to the development of new antiviral agents has led to the discovery of 3-methylthio-5-aryl-4-isothiazolecarbonitriles that were found to be active against RNA viruses. Our compounds exhibited good anti picornavirus activity and, particularly, they were effective against polio 1 and ECHO 9, and weakly active against Coxsackie B1 and EMC.

In order to explore their anti picornavirus spectrum, we tested six compounds with different aryl groups in the 5-position of the isothiazole ring against 17 representative HRV serotypes (Andries et al., 1990, 1991), by trying to establish the necessary structural requirements to obtain a broad-spectrum anti picornavirus activity or a specific inhibitory effect against particular HRV serotypes.

First of all, we chose our lead molecule **IS-2**, with an unsubstituted phenyl (Fig. 1), which showed the highest activity against polio 1 and ECHO 9, although it was only slightly effective against EMC and inactive against Coxsackie B1.

Then, we included in our screening program compounds **IS-21** and **IS-54**, bearing small groups on the *para* position of the phenyl ring, OCH_3 and OH, respectively. These molecules were less active than **IS-2** against the picornaviruses listed in Table 1, but our goal was to evaluate if the presence of OCH_3 or OH resulted in a specific activity against group B rhinoviruses, as has been found for a new series of substituted 5-phenylisoxazoles (Mazzei et al., 1993).

Moreover, in order to study the effect of bulky substituents on the antiviral activity, we included compound **IS-79** (O-But *para*-substituted), endowed with the broadest anti-picornavirus spectrum, its isosteric analogue **IS-50** (O-Bn *para*-substituted) and compound **IS-44**, bearing an O-Ts group, which was completely inactive against the previous viruses tested.

Results obtained from our screening demonstrated that the introduction of bulky groups, such as O-Bn or O-But, in the *para* position broadened the anti-picornavirus spectrum of isothiazole derivatives: compounds **IS-50** and **IS-79** were active against enteroviruses, rhinoviruses belonging to both A and B groups, and EMC. Surprisingly, compound **IS-44** was active against some group B serotypes, exhibiting the best inhibitory effect against HRVs 2, 85 and 89 and showing that an O-Ts group was responsible for an anti-group B specific activity.

On the contrary, the presence of a small group on the *para* position of the phenyl ring generally abolished anti rhinovirus activity, differing from the results obtained for 5-phenylisoxazoles

(Mazzei et al., 1993). In fact, compound **IS-54** was active only against HRV 86 and compound **IS-21** was always inactive against all the rhinoviruses screened.

Unfortunately, our lead compound **IS-2**, which was so effective against polio 1 and ECHO 9, did not exhibit any anti rhinovirus activity, in spite of its lower cytotoxicity for HeLa cells.

Structure–activity relationships suggest that the anti-HRV activity of 5-aryl isothiazole derivatives depends on the presence of particular substituents in the *para* position of the phenyl ring: in fact, the antiviral activity progressively increased when the OH group was replaced by larger groups (O-Ts, O-Bn or O-But).

As peak anti-rhinovirus activity was obtained with the O-Ts derivative **IS-44**, we set up some experiments in order to investigate its mode of action.

Our results with the time of addition experiments indicated that this compound exerted an interference with an early step of the viral replicative cycle of HRV 2 and HRV 89. In fact, compound **IS-44** was active against both viruses tested if added during the adsorption period, while no reduction was observed if the compound was added immediately after this period (time 0) (Fig. 2). Moreover, the influence of the compound on the virus adsorption step, studied by the infective center assays, indicates that it primarily interferes with cellular attachment of HRV (Fig. 3). As the adsorption of HRVs 2 and 89 to HeLa cells is blocked at high concentrations of **IS-44**, it can be deduced that conformational changes of the drug binding site must take place in these serotypes.

Inhibition of attachment is probably only one mechanism by which capsid binding agents act to inhibit the replication of the rhinoviruses. Several hypotheses have been proposed for the mechanism of action of capsid binding compounds. As demonstrated for many of these compounds, binding within the hydrophobic pocket may reduce the capsid flexibility, leading to rigidification and compression of the viral capsid and making the virus more resistant to uncoating (McKinlay et al., 1992; Phelps and Post, 1995; Rotbart et al., 1998). Alternatively, changes in the conformation of the canyon floor as a result of binding within the underlying pocket may affect the attachment of the virus to the host cell receptor (Pevear et al., 1989; Rossmann, 1989; Dewindt et al., 1994). The extent of conformational changes induced in the binding site may be responsible for the observed differences in the mode of action of these capsid-binding compounds. For example, WIN compounds have a dual mechanism of action, inhibiting the adsorption of HRV 14, and the uncoating of HRV 2 and poliovirus type 1 and 2 (Kim et al., 1993). Other capsid binding agents such as SCH 38057 (Rozhon et al., 1993) or 3-methylquercetin (Castrillo et al., 1986) manifest their antiviral effect after the initial stage of picornaviral uncoating, even though they bind to the same site as the WIN compounds and/or pyridazinamines.

Binding of these compounds results in neutralization of viral infectivity that can be reversed by extraction with an organic solvent. Moreover, the increased stability induced by capsid binding is reflected by resistance to thermal inactivation (Rombaut et al., 1985, 1991, 1996).

Our experiments on neutralization of viral infectivity and on thermal stabilization demonstrated that compound **IS-44** has a different effect depending on the serotypes studied. In fact, it was able to directly inactivate HRV 89, and the infectious titer was restored to the original value after extraction of the compound with chloroform. Similar to other capsid binding compounds, such as pirodavir with HRV Hank's (Andries et al., 1992) or chalcone Ro 09-0410 with HRV 2 (Ishitsuka et al., 1982) or isoflavans with HRV 1B (Conti et al., 1988), the binding of **IS-44** results in a neutralization of the viral infectivity, which can be reversed by organic solvent extraction. However, it did not protect the viral infectivity against heat inactivation at the different concentrations used. As many factors (the time and temperature of incubation, the nature of the compound and its concentration, and the virus serotype) can influence the extent of the binding of the drug to the viral particles and its mode of action, it is not surprising that **IS-44** fully protects HRV 2 infectivity from heat inactivation without inactivating virus infectivity. An analogous effect was observed for pirodavir against HRV 1A (Andries et

al., 1992) and for DCF against HRV 1B (Conti et al., 1992), which did not inactivate virus infectivity but stabilized the virion against thermal degradation. These data indicate that these compounds interact directly with the virion and that binding is probably important for the inhibitory action, although it was largely reversible by dilution.

In conclusion, as demonstrated for other capsid binding compounds (Pevear et al., 1989; Dewindt et al., 1994), binding of **IS-44** induces a conformational shift in the viral capsid, resulting in a decrease of affinity for the cellular receptor and, consequently, inhibition of attachment of the virions.

Further studies, using density gradient experiments on radiolabeled compound or X-ray diffraction analysis of crystals of HRV complexed with **IS-44**, are needed to clarify how this drug interacts with the virus to confirm its capsid-binding activity.

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