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Synthesis of new 3-methylthio-5-aryl-4-isothiazolecarbonitriles with broad antiviral spectrum

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Abstract

The isothiazole derivative 3-methylthio-5-(4-OBn-phenyl)-4-isothiazolecarbonitrile, coded **IS-50**, which in previous studies had exhibited a broad antipicornavirus spectrum of action, was selected as the model for the synthesis of a new series of 3-methylthio-5-aryl-4-isothiazolecarbonitriles. These compounds were prepared in good yield (from 66 to 82%) by alkylation of 3-methylthio-5-(4-hydroxyphenyl)-4-isothiazolecarbonitrile with suitable bromides in the presence of acetone; only the 4-cyanophenoxy derivatives were obtained in a yield of less than 30%. All the compounds were screened against a panel of 17 representative human rhinovirus (HRV) serotypes belonging to both A and B groups, enteroviruses polio 1, ECHO 9 and Coxsackie B1, cardiovirus EMC, measles virus, and herpes simplex virus type 1 (HSV-1). Our results demonstrate that HRV 86 (group A) and HRVs 39 and 89 (group B) are the rhinovirus serotypes more susceptible to the action of these compounds. Isothiazole derivatives with a longer intermediate alkyl chain exhibited good activity against polio 1 and ECHO 9. The compound bearing a butyl group between the two phenoxy rings showed the lowest IC_{50} against Coxsackie B1 and measles viruses. No activity against HSV-1 was detected with any of the compounds screened. © 2002 Published by Elsevier Science B.V

Keywords: Isothiazole derivatives; Picornavirus; Measles

1. Introduction

Over the last few years, our interest has been focused on the identification of new synthetic antiviral agents and in our research program we have decided to investigate the potential antiviral properties of the isothiazole nucleus. Many isothiazole derivatives have been reported in literature to have antimicrobial properties (Walsh and Wooldridge, 1972); thus, we have been exploring antiviral activity of new synthetic isothiazoles, discovering new biological properties of the isothiazole ring. Previously, we synthesised the 3-mercapto-5-aryl-isothiazoles, with potent antipoliovirus activity (Condorelli et al., 1967; Pinizzotto et al., 1992). Further studies have demonstrated that the presence of a cyano group in the 4-

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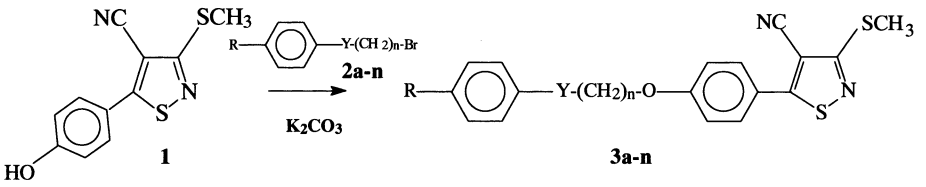


2-3a : Y = CH_2 n = 1 R = H
2-3b : Y = CH_2 n = 2 R = H
2-3c : Y = O n = 2 R = H
2-3d : Y = O n = 3 R = H
2-3e : Y = O n = 4 R = H
2-3f : Y = O n = 2 R = NO_2
2-3g: Y = O n = 3 R = NO_2
2-3h: Y = O n = 4 R = NO_2
2-3i : Y = O n = 2 R = COOEt
2-3j : Y = O n = 3 R = COOEt
2-3k : Y = O n = 4 R = COOEt
2-3l : Y = O n = 2 R = CN
2-3m : Y = O n = 3 R = CN
2-3n : Y = O n = 4 R = CN

Fig. 1. Synthesis of isothiazole derivatives **3a**–**n**.

position of the isothiazole ring improved antiviral activity against polio 1 and broadened the spectrum of action; in fact, some new 4-isothiazolecarbonitriles proved effective against enteroviruses ECHO 9 and Coxsackie B1, cardiovirus Encephalomyocarditis (EMC) and measles virus (Cutrì et al., 1998, 1999). The antipicornavirus activity led us to investigate the inhibitory effect of some of these compounds against rhinoviruses. Recently, we reported that the antirhinovirus activity of the 5-aryl-4-isothiazolecarbonitrile derivatives depends on the presence of bulky substituents at the *para* position of the phenyl ring. In particular, 3-methylthio-5-(4-OBn-phenyl)-4-isothiazolecarbonitrile, coded **IS-50**, showed the broadest antirhinovirus spectrum of action (Garozzo et al., 2000). The primary goal of our studies being the discovery of compounds with a broad spectrum of action, we selected **IS-50** as the prototype of a new generation of 3-methylthio-5-aryl-4-isothiazolecarbonitriles. In the present study we report the synthesis and the antiviral activity of these new compounds. All the newly synthesised isothiazoles were screened against 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) (Andries et al., 1990, 1991), enteroviruses polio 1, ECHO 9 and Coxsackie B1, cardiovirus (EMC), and measles virus. The compounds were also tested against herpes simplex virus type 1 (HSV-1).

2. Materials and methods

2.1. Chemicals

Melting points were determined on a Büchi 510 apparatus and are not corrected. Elemental analyses for all new compounds were performed on a C. Erba Model 1106 elemental analyzer and the data of C, H, N and S are within $\pm 0.3\%$ of calculated values. Thin layer chromatography (TLC) was used to monitor reactions. IR spectra were recorded as KBr pellets using a Perkin–Elmer 281 spectrophotometer. Mass spectra (MS) data were run on a C. Erba/Kratos Ms.

2.2. General procedure for synthesis of isothiazoles **3a–n** (Fig. 1)

A solution of 3-methylthio-5-(4-hydroxyphenyl)-4-isothiazolecarbonitrile (Cutrì et al., 1998) (**1**) (1.5 mmol) in acetone was added with K_2CO_3 (1.5 mmol) and suitable alkylating reagents (Ashley et al., 1942, 1958; Berg and Newbery, 1949; Caldwell and Jackson, 1957; Belleau, 1959; Sharpe et al., 1971; Iizuka et al., 1980; Bang et al., 1994) **2a–n** (1.5 mmol), and was refluxed for 48 h. After cooling, the solution was partitioned between water and diethyl ether and the combined ethereal layers were washed with NaOH 2 M. Then, the organic phase was concentrated to dryness to yield products **3a–n**, which were purified by crystallisation.

The following compounds were obtained:

3-*Methylthio*-5-[4-(2-*phenyl*-1-*ethoxy*)*phenyl*]-4-*isothiazolecarbonitrile* (**3a**): Yield 79%; m.p. 97–99 °C (ethanol); IR (KBr) 2221 (CN) cm^{-1}; MS *m/e* 352, 231, 105.

3-*Methylthio*-5-[4-(3-*phenyl*-1-*propoxy*)*phenyl*]-4-*isothiazolecarbonitrile* (**3b**): Yield 81%; m.p. 109–111 °C (cyclohexane); IR (KBr) 2222 (CN) cm^{-1}; MS *m/e* 366, 233, 91.

3-*Methylthio*-5-[4-(2-*phenoxy*-1-*ethoxy*)*phenyl*]-4-*isothiazolecarbonitrile* (**3c**): Yield 80%; m.p. 148–149 °C (cyclohexane); IR (KBr) 2224 (CN) cm^{-1}; MS *m/e* 368, 121, 107.

3-*Methylthio*-5-[4-(3-*phenoxy*-1-*propoxy*)*phenyl*]-4-*isothiazolecarbonitrile* (**3d**): Yield 78%; m.p. 97–98 °C (cyclohexane); IR (KBr) 2224 cm^{-1}; MS *m/e* 382, 135, 107.

3-*Methylthio*-5-[4-(4-*phenoxy*-1-*butoxy*)*phenyl*]-4-*isothiazolecarbonitrile* (**3e**): Yield 78%; m.p. 122–126 °C (cyclohexane); IR (KBr) 2223 (CN) cm^{-1}; MS *m/e* 396, 149, 107.

3-*Methylthio*-5-[4-[2-(4-*nitrophenoxy*)-1-*ethoxy*]*phenyl*]-4-*isothiazolecarbonitrile* (**3f**): Yield 82%; m.p. 186–190 °C (acetonitrile); IR (KBr) 2217 (CN) cm^{-1}; MS *m/e* 413, 166, 152.

3-*Methylthio*-5-[4-[3-(4-*nitrophenoxy*)-1-*propoxy*]*phenyl*]-4-*isothiazolecarbonitrile* (**3g**): Yield 82%; m.p. 166–168 °C (acetonitrile); IR (KBr) 2217 (CN) cm^{-1}; MS *m/e* 427, 381, 180.

3-*Methylthio*-5-[4-[4-(4-*nitrophenoxy*)-1-*buthoxy*]*phenyl*]-4-*isothiazolecarbonitrile* (**3h**): Yield 80%; m.p. 150–153 °C (acetonitrile); IR (KBr) 2215 (CN) cm^{-1}; MS *m/e* 441, 395, 194.

3-*Methylthio*-5-[4-[2-(4-*ethoxycarbonilephenoxy*)-1-*ethoxy*]*phenyl*]-4-*isothiazolecarbonitrile* (**3i**): Yield 66%; m.p. 155–157 °C (acetonitrile); IR (KBr) 2217 (CN), 1715 (C=O) cm^{-1}; MS *m/e* 440, 395, 193.

3-*Methylthio*-5-[4-[3-(4-*ethoxycarbonilephenoxy*)-1-*propoxy*]*phenyl*]-4-*isothiazolecarbonitrile* (**3k**): Yield 80%; m.p. 138–141.5 °C (acetonitrile); IR (KBr) 2218 (CN), 1717 (C=O) cm^{-1}; MS *m/e* 454, 289, 207.

3-*Methylthio*-5-[4-[4-(4-*ethoxycarbonilephenoxy*)-1-*buthoxy*]*phenyl*]-4-*isothiazolecarbonitrile* (**3j**): Yield 75%; m.p. 126–129 °C (acetonitrile); IR (KBr) 2218 (CN), 1709 (C=O) cm^{-1}; MS *m/e* 468, 423, 221.

3-*Methylthio*-5-[4-[2-(4-*cyanophenoxy*)-1-*ethoxy*]*phenyl*]-4-*isothiazolecarbonitrile* (**3l**): Yield 29%; m.p. 184–187 °C (acetonitrile); IR (KBr) 2218 (CN) cm^{-1}; MS *m/e* 393, 275, 118.

3-*Methylthio*-5-[4-[3-(4-*cyanophenoxy*)-1-*propoxy*]*phenyl*]-4-*isothiazolecarbonitrile* (**3m**): Yield 20%; m.p. 171–174 °C (acetonitrile); IR (KBr) 2218 (CN) cm^{-1}; MS *m/e* 407, 247, 118.

3-*Methylthio*-5-[4-[4-(4-*cyanophenoxy*)-1-*buthoxy*]*phenyl*]-4-*isothiazolecarbonitrile* (**3n**): Yield 28%; m.p. 171–173 °C (acetonitrile); IR (KBr) 2218 (CN) cm^{-1}; MS *m/e* 421, 247, 118.

R 77975 (Pirodavir) (Andries et al., 1992) was kindly provided by Janssen Research Foundation (Beerse, Belgium) and was used as reference compound.

All the compounds were dissolved in DMSO and diluted in maintenance medium 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 lines used.

2.3. Viruses and cells

Poliovirus 1 (Brunhilde strain), echovirus 9 (Hill strain), Coxsackie virus B1 and measles (Edmonston strain) were obtained from the American Type Culture Collection (ATCC) and propagated in human epidermoid carcinoma larynx cells (HEp-2). Encephalomyocarditis (EMC strain)

and herpes simplex type 1 (F strain) were obtained from the ATCC and propagated in mouse connective tissue cells (L-929) and African green monkey kidney cells (Vero), respectively. HRVs (HRVs 2, 9, 14, 15, 29, 39, 41, 42, 45, 51, 59, 63, 70, 72, 85, 86, and 89), kindly 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.

Cell lines were kept in a humidified 5% carbon dioxide atmosphere at 37 °C and grown, except for HeLa cells, in Dulbecco's modified Eagle's Minimum Essential medium (DMEM) supplemented with 6% heat inactivated fetal calf serum (FCS), 200 μg/ml of streptomycin and 200 units/ml of penicillin G. HeLa cells were grown in Eagle's Minimum Essential medium (MEM) supplemented with 10% heat inactivated FCS, 200 μg/ml of streptomycin and 200 units/ml of penicillin G.

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

2.4. Cytotoxicity assay

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

Cell growth was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method (Denizot and Lang, 1986; Garozzo et al., 1994). The cells were seeded at 1×10^4/ml (100 μl/well) in 96-well tissue culture plates such that cell replication remained logarithmic for the 3-day incubation period.

The 50% cytotoxic dose (CC_{50}) was expressed as the highest concentration of the compound that resulted in 50% inhibition of cell growth.

2.5. Antirhinovirus activity: cytopathic effect inhibition assays

Infectivity of HRV 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 MTT ID_{50} (50% infective dose).

The antirhinovirus assay was based on the inhibition of virus-induced cytopathogenicity on HeLa-Ohio cells, as previously described (Garozzo et al., 2000). Briefly, subconfluent monolayers grown in 96-well tissue culture plates were treated with or without various concentrations of the test compounds at doses below $CC_{50,}$ and then infected with 10 $CCID_{50}$ (50% cell culture infective dose) of HRV stock to produce a complete cytopathic effect within 48–72 h after infection. After incubation at 33 °C, the viability of mock- and virus-infected cells was quantified by the MTT method. 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.

2.6. Antiviral activity: plaque assays

The assay of the antiviral activity against the viruses polio 1, ECHO 9, Coxsackie B1, EMC, measles and HSV-1 was carried out by the 50% plaque reduction assay, as previously described (Cutrì et al., 1998).

The compound concentration required to inhibit virus plaque formation by 50% was expressed as IC_{50} and calculated by dose–response curves using the linear regression technique.

2.7. Addition at different time intervals

Monolayers of HeLa-Ohio cells were grown to confluency in 24-well plates and inoculated with HRV 89 at a multiplicity of infection (MOI) of 0.1. The plates were incubated for 2 h at 4 °C to ensure synchronous replication of the viruses, with or without compound **3a** (5 μM) (virus adsorption period). Then, the inoculum was removed, and medium and compound were added at various times after the adsorption period, as indicated in

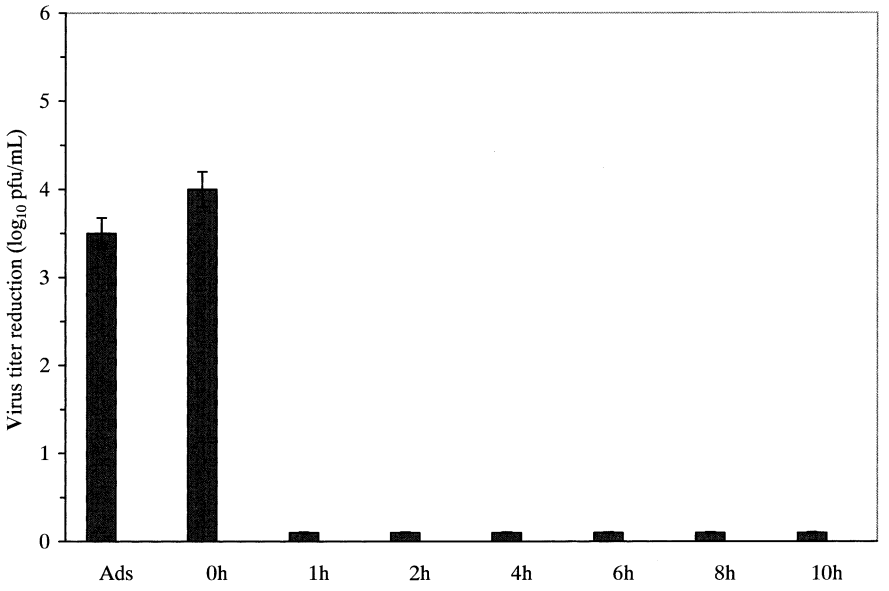


Fig. 2. Effect of time addition of compound **3a** (5 μM) on virus yield from a single-round replication of HRV 89. Ads, adsorption period (2 h) at 4 °C. Time 0 h = post 2 h adsorption period. Time 1 h, 2 h, ... correspond to addition of compound at 1, 2, ... h after the 2 h adsorption period. All values are mean ± S.D. for three separate assays.

Fig. 2. The plates were incubated at 33 °C for 12 h, cultures were then frozen and virus yield was determined by plaque assay.

2.8. Virucidal activity

To test possible virucidal activity, equal volumes (0.5 ml) of HRV 89 suspension (containing 10^6 PFU/ml) and MEM containing compound **3a** (10 × and 50 × 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.

2.9. Thermal inactivation

HRV 89 (10^6 PFU/ml) was incubated for 1 h at 33 °C with or without compound **3a** (10 × and 50 × 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.

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

2.10. Extraction with chloroform

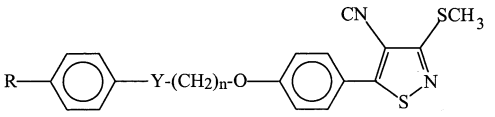
For the extraction of the test compound after pre-incubation with HRV 89, an equal volume of 100% $CHCl_3$ was added, the sample was vortexed for 1 min at room temperature, and centrifuged at 700 × *g* for 5 min. The aqueous phase, which contained the virus, was collected and the titer was determined by plaque assay.

3. Results

3.1. Effect of 3-methylthio-5-aryl-4-isothiazolecarbonitriles on cell proliferation and on virus replication

Tables 1–3 show the CC_{50} and IC_{50} values of the test compounds: Tables 1 and 2 report the CC_{50} values on HeLa-Ohio cells and IC_{50} values against group A (HRVs 14, 42, 45, 70, 72, and 86)

Table 1
Antirhinovirus activity of **IS-50** and isothiazoles **3a**–**n** against group A serotypes



Compound	*n*	Y	R	CC_{50} (μM)[a,c]	IC_{50} (μM)[b,c]					
				HeLa-Ohio	HRV 14	HRV 42	HRV 45	HRV 70	HRV 72	HRV 86
IS-50	1	–	H	> 50	25	7	> 50	25	> 50	2
3a	1	CH_2	H	> 20	> 20	> 20	> 20	> 20	> 20	2
3b	2	CH_2	H	> 20	> 20	> 20	> 20	> 20	> 20	2
3c	2	O	H	> 20	> 20	> 20	> 20	> 20	> 20	0.3
3d	3	O	H	> 20	10	> 20	> 20	> 20	> 20	0.6
3e	4	O	H	> 20	2.37	> 20	> 20	1	> 20	0.45
3f	2	O	NO_2	2	> 2	> 2	> 2	> 2	> 2	0.4
3g	3	O	NO_2	1	> 1	> 1	> 1	> 1	> 1	0.4
3h	4	O	NO_2	0.5	> 0.5	> 0.5	> 0.5	> 0.5	> 0.5	> 0.5
3i	2	O	COOEt	2	> 2	> 2	> 2	> 2	> 2	> 2
3j	3	O	COOEt	2	0.5	> 2	> 2	0.5	> 2	0.4
3k	4	O	COOEt	2	> 2	> 2	> 2	> 2	> 2	> 2
3l	2	O	CN	2	> 2	> 2	> 2	> 2	> 2	> 2
3m	3	O	CN	2	> 2	> 2	> 2	> 2	> 2	> 2
3n	4	O	CN	2	> 2	> 2	> 2	> 2	> 2	> 2
R77975				19	0.04	> 19	> 19	0.005	0.4	0.087

[a] CC_{50}, concentration which inhibited HeLa-Ohio cell growth by 50% as 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.

and B (HRVs 2, 9, 15, 29, 39, 41, 51, 59, 63, 85, and 89) rhinoviruses, respectively; Table 3 shows the CC_{50} values on HEp-2, L-929 and Vero cell monolayers and the IC_{50} values against viruses polio 1, ECHO 9, Coxsackie B1, EMC, measles, and HSV-1. The data of the previously published compound **IS-50** (Cutrì et al., 1998; Garozzo et al., 2000) are also reported. Pirodavir (Andries et al., 1992) was included for comparison.

Compound **IS-50** exhibited the broadest antirhinovirus spectrum among the isothiazole derivatives studied (Tables 1 and 2), although it was only weakly effective against Coxsackie B1, EMC, and measles viruses and inactive against Polio 1, ECHO 9 and HSV-1 (Table 3). The new series of 3-methylthio-5-aryl-4-isothiazolecarbonitriles were prepared and tested in comparison with **IS-50**.

The presence of a voluminous group in the chemical structure of the compounds **3a**–**e** probably caused a lower solubility than for **IS-50**; their CC_{50} values were found to be > 20 μM for all the cell lines used (Tables 1 and 3). The introduction of some substituents, such as NO_2 (compounds **3f**–**h**), COOEt (compounds **3i**–**k**) or CN (compounds **3l**–**n**) groups, in the *para* position of the alkoxyphenyl group yielded compounds that were more toxic (0.5–2 μM, Tables 1 and 3).

As far as the antirhinovirus activity was concerned, a structure–activity study was initially performed by varying the length of the alkyl chain between the phenyl rings. When compared to **IS-50**, compounds **3a** and **3b**, with two and three methylene groups, respectively, demonstrated a limited antirhinovirus spectrum. In fact, **3a** was effective against 47% (eight out of 17) and **3b** against 29% (five out of 17) of the 17 serotypes screened. These compounds were active against one of the six group A serotypes (HRV 86), with similar IC_{50} values as for **IS-50** (Table 1). On the contrary, compounds **3a** and **3b** showed lower IC_{50}

Table 2
Antirhinovirus activity of **IS-50** and isothiazoles **3a**–**n** against group B serotypes

Compound	IC_{50} (μM)[a,b]										
	HRV 2	HRV 9	HRV 15	HRV 29	HRV 39	HRV 41	HRV 51	HRV 59	HRV 63	HRV 85	HRV 89
IS-50	20	3	3	20	3	32	19	12	10	1	2
3a	3.9	5	10	10	2.5	> 20	> 20	> 20	> 20	1.25	0.4
3b	> 20	5	> 20	> 20	1.8	> 20	> 20	> 20	> 20	5	1.2
3c	> 20	> 20	> 20	> 20	> 20	> 20	> 20	> 20	> 20	> 20	3.5
3d	> 20	> 20	> 20	> 20	3.75	> 20	> 20	> 20	> 20	> 20	2.5
3e	> 20	> 20	> 20	> 20	3	> 20	> 20	> 20	> 20	> 20	2.5
3f	> 2	> 2	> 2	> 2	0.25	> 2	> 2	> 2	> 2	> 2	> 2
3g	> 1	> 1	> 1	> 1	0.5	> 1	> 1	> 1	> 1	> 1	> 1
3h	> 0.5	> 0.5	> 0.5	> 0.5	> 0.5	> 0.5	> 0.5	> 0.5	> 0.5	> 0.5	> 0.5
3i	> 2	> 2	> 2	> 2	> 2	> 2	> 2	> 2	> 2	> 2	> 2
3j	> 2	> 2	> 2	> 2	0.5	> 2	> 2	> 2	> 2	> 2	> 2
3k	> 2	> 2	> 2	> 2	> 2	> 2	> 2	> 2	> 2	> 2	> 2
3l	> 2	> 2	> 2	> 2	> 2	> 2	> 2	> 2	> 2	> 2	> 2
3m	> 2	> 2	> 2	> 2	> 2	> 2	> 2	> 2	> 2	> 2	> 2
3n	> 2	> 2	> 2	> 2	> 2	> 2	> 2	> 2	> 2	> 2	> 2
R77975	0.01	0.04	0.09	0.02	0.02	0.7	0.01	0.03	0.01	0.005	0.005

[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.

Table 3
Antiviral activity of **IS-50** and isothiazoles **3a–n**

Compound	CC_{50} (μM)[a,c]	IC_{50} (μM)[b,c]					
	HEp-2\L-929\Vero	Polio 1	ECHO 9	Cox B1	EMC	Measles	HSV-1
IS-50	> 50	> 50	> 50	10	20	10	> 50
3a	> 20	0.25	0.3	10	10	10	> 20
3b	> 20	0.12	0.2	10	10	10	> 20
3c	> 20	5	> 20	> 20	> 20	> 20	> 20
3d	> 20	5	0.2	2.5	> 20	5	> 20
3e	> 20	5	0.5	0.3	> 20	0.5	> 20
3f	1	> 1	> 1	> 1	> 1	> 1	> 1
3g	0.5	> 0.5	> 0.5	> 0.5	> 0.5	> 0.5	> 0.5
3h	0.5	> 0.5	> 0.5	> 0.5	> 0.5	> 0.5	> 0.5
3i	2	> 2	> 2	> 2	> 2	> 2	> 2
3j	2	> 2	> 2	> 2	> 2	> 2	> 2
3k	2	> 2	> 2	> 2	> 2	> 2	> 2
3l	2	> 2	> 2	> 2	> 2	> 2	> 2
3m	2	> 2	> 2	> 2	> 2	> 2	> 2
3n	2	> 2	> 2	> 2	> 2	> 2	> 2
R77975	19	0.23	0.05	2	nd	nd	> 19

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

values than **IS-50** against HRVs 39 (2.15 and 1.8 μM, respectively) and 89 (0.4 and 1.2 μM, respectively). Compound **3a** was more active than **IS-50** against HRV 2 (IC_{50}: 3.9 μM) (Table 2).

Compounds **3c–e**, characterised by the presence of another oxygen atom in the intermediate chain, were particularly active against HRV 86, with a range of IC_{50} values of 0.3–0.6 μM. Moreover, compound **3e** showed inhibitory activity against HRVs 14 and 70, whereas **3d** was only weakly active against HRV 14 (Table 1). IC_{50} values similar to those obtained for **IS-50** were observed for compounds **3c–e** against HRVs 39 and 89; the sole exception was compound **3c**, which was inactive against HRV 39 (Table 2).

Our next chemical approach was to examine the effect of the introduction of some substituents at the *para* position of the second phenyl ring with respect to antiviral activity. Almost all the compounds were inactive. Surprisingly, compound **3j** was active against HRVs 14, 70 and 86 (group A) and HRV 39 (group B); compounds **3f** and **3g** were effective against HRVs 86 and 39 (Tables 1 and 2).

Regarding the antienterovirus activity, the lowest IC_{50} values were observed for compounds **3a** and **3b** against polio 1, for compounds **3a**, **3b**, **3d** and **3e** against ECHO 9 and for compound **3e** against Coxsackie B1. None of the compounds of the series **3f–n** was active against enteroviruses (Table 3).

Only compounds **3a** and **3b** showed marginal activity against EMC (Table 3).

The most potent inhibitor of measles virus was compound **3e**, which exhibited an IC_{50} value of 0.5 μM, 20 times lower than that observed for **IS-50**, whereas compounds **3a**, **3b** and **3d** demonstrated only weak activity. No inhibitory effect was observed for the other isothiazole derivatives tested (Table 3).

Finally, all the new compounds synthesised were ineffective against HSV-1 (Table 3).

3.2. Effect of time addition of compound **3a**

In order to determine whether compound **3a** inhibited the virus yield during a specific period in the virus cycle, the effect of time addition of this compound was studied for HRV 89. Results obtained from these experiments clearly demonstrated maximal inhibition of the virus when the compound was added during or at the end of the adsorption period (3.5 and 4 $\log_{10}$ virus yield reduction, respectively). The inhibitory effect on rhinovirus replication was completely lost when the compound was added after the virus adsorption period (Fig. 2).

3.3. Effect on virus infectivity and on heat inactivation of HRV 89

The binding of compound **3a** to HRV 89 was not virucidal. In fact, the test compound did not significantly reduce the virus titers after a 1 h incubation period at 10 or 50 times the IC_{50}. Moreover, the same concentrations of compound **3a** were insufficient to protect HRV 89 from heat inactivation (data not shown).

4. Discussion

When antiviral activity was discovered for a series of 3-methylthio-5-aryl-4-isothiazolecarbonitriles (Cutrì et al., 1998, 1999; Garozzo et al., 2000), we set up a synthesis program to optimise activity and to broaden the spectrum of action by preparing and testing new isothiazole derivatives. As compound **IS-50** was effective against several rhinovirus serotypes and was also active against Coxsackie B1, EMC and measles virus, we chose it as the lead molecule for some chemical modifications to establish a structure–activity relationship. In the past, the development of WIN compounds as broad-spectrum antipicornavirus agents led to the synthesis of a series of molecules differing in the length of the aliphatic chain connecting the oxazolylphenoxy and isoxazole moieties and in the variety of substituents introduced on the rings. Extensive structure–activity studies revealed that these chemical modifications had significant effects on both potency and spectrum of activity (Diana et al., 1985, 1989). Further studies reported the variation in antirhinovirus activity of a class of WIN compounds, where the oxazoline ring was replaced with different heterocyclic rings. Among these, the isothiazole nucleus was not included (Bailey et al., 1992).

As a result, in our chemical approach, we considered **IS-50** as the prototype of a new series of compounds characterised by the presence of an isothiazolylphenoxy group and a phenyl ring. As first chemical modification we replaced the methylene in the benzyl group of **IS-50** with a short alkyl chain. Then, we examined the effect of an isosteric substitution by introducing an oxygen atom in place of a CH_2 group and, after this, we retained the second oxygen atom in the general structure and varied the aliphatic chain length. Finally, we studied the effect of the introduction of some substituents on the *para* position of the phenoxy ring, with respect to antiviral activity.

On the basis of the results obtained, we observed that some variations in the alkyl chain length and the presence of the second oxygen atom could determine a different inhibitory action against the two rhinovirus groups.

In fact, compounds **3a** and **3b**, with two- and three-carbon atoms, respectively, were more active than **IS-50** against HRVs 39 and 89. On the contrary, only the alkyl chain extension did not provide a variation in activity against HRV 86, whereas the simultaneous introduction of an oxygen atom improved antiviral activity against this serotype; in fact, alkoxy derivatives **3c**–**e** were more active than **IS-50** and compounds **3a** and **3b**. Nevertheless, compounds **3c**–**e** were weakly active or ineffective against HRVs 39 and 89 (Tables 1 and 2).

The presence of particular substituents (NO_2, COOEt and CN) at the *para* position of the phenoxy ring produced uniformly toxic molecules (**3f**–**n**). In our opinion, the electronic properties of these groups could be responsible for the cytotoxicity of these compounds. In fact, σ values used to describe the electronic characteristics of *para*-NO_2, *para*-COOEt and *para*-CN substituents are very similar (Skagerberg et al., 1989). On the contrary, it was not possible to establish a direct relationship

between Hansch substituent parameters (e.g. σ, π, MR) and corresponding antiviral activity, since only three *para*-substituted compounds (**3f**, **3g** and **3j**) were active.

Results obtained from this screening demonstrated a wide variation in sensitivity of the different HRV serotypes. The rhinovirus serotypes that were more susceptible to our isothiazole derivatives were HRV 86 (group A) and HRVs 39 and 89 (group B). In particular, HRV 86 was sensitive against 60% (nine out of 15) of the compounds tested.

Our next goal was to evaluate if chemical modifications made in the new isothiazoles provided an improvement in antiviral activity against polio 1, ECHO 9, Coxsackie B1, EMC and measles virus.

Although **IS-50** was inactive against polio 1 and ECHO 9, the addition of methylene groups made compounds **3a** and **3b** effective. On the contrary, the inhibitory effect against Coxsackie B1, EMC and measles virus did not depend on the length of the alkyl chain, as demonstrated by the similar IC_{50} values for **IS-50** and compounds **3a** and **3b** (Table 3).

The presence of an aliphatic chain between two phenoxy rings (compounds **3c**–**e**) caused a variation in activity against the different viruses tested. In fact, while **3c** was ineffective, interesting inhibitory effect against ECHO 9, Coxsackie B1 and measles virus was obtained upon extension of the chain to propoxy (compound **3d**) and butoxy (compound **3e**). Moreover, the different length of the chain did not influence antipoliovirus activity, whereas the introduction of the second oxygen atom resulted in a loss of activity against EMC.

It is noteworthy that small modifications in the chemical structure had a pronounced effect on antiviral activity, as already observed with other antipicornavirus compounds. For example, [[(4,5-dihydro-2-oxazolyl) phenoxy]alkyl] isoxazoles bearing a five- and seven-carbon chain exhibited the greatest activity against polio 2 and HRV 2, whereas the four- and eight-carbon homologues were considerably less effective against both viruses (Diana et al., 1985).

The introduction of any electron-withdrawing substituents on the *para* position of the second phenyl ring is generally not favourable for antiviral activity. Therefore, further compounds with electron-releasing groups in this position will be synthesised in order to elucidate if an electron-rich phenyl ring or an unsubstituted benzene might improve antiviral activity.

Our results indicate that new isothiazole derivatives endowed with a broader antiviral spectrum can be obtained starting from the lead compound **IS-50**. In fact, although the new compounds **3a**–**b** and **3d**–**e** showed a more limited antirhinovirus spectrum, they are effective against polio 1, ECHO 9, Coxsackie B1, and measles viruses, often with lower IC_{50} values than those obtained for **IS-50**.

Mode of action studies have shown that many antipicornavirus compounds inhibit virus replication by binding to a hydrophobic pocket on the virion surface: binding within this 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).

As compound **3a** exhibited the broadest antiviral spectrum, we set up some experiments to investigate its mode of action. In our previous studies (Garozzo et al., 2000), we hypothesised a capsid-binding activity for 3-methylthio-5-(4-OTs-phenyl)-4-isothiazolecarbonitrile, coded **IS-44**, a specific inhibitor of group B serotypes. The binding of **IS-44** resulted in a neutralisation of the viral infectivity that was restored to the original value by organic solvent extraction. However, this binding did not protect the viral infectivity against heat inactivation. Moreover, it was demonstrated that this compound interfered with rhinovirus cellular attachment, suggesting a direct interaction with the virion, which is typical of capsid-binder compounds (Pevear et al., 1989).

The effect of **3a** under different experimental conditions was determined to investigate if the mechanism of inhibition was similar to the previously published compound **IS-44**. Our studies

showed that this compound did not inactivate HRV 89 infectivity and did not stabilise the virion against thermal degradation. Moreover, results obtained from studies of compound addition at different time intervals suggested that **3a** interferes with an early step of the HRV 89 replicative cycle. In fact, addition of compound **3a** during or immediately after the adsorption period resulted in a significant antiviral activity (Fig. 2). Addition of **3a** later than 30 min after the virus adsorption period did not result in any virus yield reduction. This suggests that the compound may act at an early step of the virus replicative cycle, demonstrating a different mechanism of action from compound **IS-44** that was active only during the adsorption period. Since we suppose that these isothiazoles have a capsid-binding activity, the extent of conformational changes induced in the binding site may be responsible for the observed differences in the mode of action. However, further studies are necessary to understand the precise mode of action of compound **3a**.

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