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Synthesis and Antiviral Activity of a New Series of 4-Isothiazolecarbonitriles

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Abstract—A series of 4-isothiazolecarbonitriles was synthesized and screened for in vitro antiviral activity. The effect of various substituents on the phenyl ring, as well as the substitution of the phenyl for other aromatic and heteroaromatic rings, was examined to establish the requirements for optimum activity. The most active member of the series, 3-methylthio-5-phenyl-4-isothiazolecarbonitrile, exhibited a high level of activity against enteroviruses polio 1 and ECHO 9. Preliminary studies on its mechanism of action indicated that this compound had an effect on an early event in the replication of poliovirus type 1.

Introduction

Picornavirus infections are among the most common viral infections in man. The main genera of the picornavirus family are the rhinoviruses, of which over 100 different serotypes have been isolated, and the enteroviruses including polio, ECHO, Coxsackie A and B, and other human enteric viruses. The rhinoviruses are above all responsible for the highest incidence of the common cold, whereas the enteroviruses are implicated as the causative agents of several human diseases ranging from mild upper respiratory tract ailments to more severe illnesses such as aseptic meningitis, myocarditis and poliomyelitis. Enterovirus infections of the pediatric population may even result in long term neurological sequelae and even death. Moreover, the only effective enterovirus vaccines available at present are those for the polioviruses, although there remain a number of developing countries, chiefly in tropical areas, in which vaccination does not cover the entire susceptible population. Considering the world-wide diffusion of enterovirus diseases and the relevant socio-economic consequences, many efforts have been directed toward the identification of agents useful in the prophylaxis and therapy of these infections. However, only a few compounds have been shown to be efficacious in humans in the absence of significant adverse effects. Pirodavir, a substituted phenoxypyridazinamine, represents one of the most broad-spectrum antipicornaviral drugs, with high in vitro selectivity against enteroviruses (polio 1, ECHO 9, Coxsackie A15 and Coxsackie B1) and rhinoviruses belonging to both A and B groups.[1,2] Nevertheless, pirodavir is hydrolyzed to an inactive metabolite after intravenous or oral administration and only frequent intranasal sprays of drug (six times daily) provide protection against experimental rhinovirus colds.[3] Moreover, no clinical benefit was observed in treating naturally occurring rhinovirus colds.[4]

As a result, we initiated a systematic synthesis program to obtain new antiviral agents effective against picornaviruses. During the course of routine screening of compounds for antiviral activity in our laboratory it was discovered that several isothiazole derivatives selectively inhibited in vitro enteroviruses polio 1 and ECHO 9. Studies on their mechanism of action indicated that these compounds affect some early processes of the poliovirus growth cycle.[5–7] Among the various compounds, 3-mercapto-5-phenyl-isothiazole **1** (Fig. 1) demonstrated significant inhibitory activity against polio 1.[5,6]

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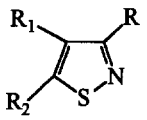


Compound	R	R_1	R_2
1	SH	H	C_6H_5
2	Cl	CN	Cl
3	SCH_3	CN	SCH_3
4	CH_3	CN	CH_3
5	OCH_3	CN	C_6H_5
6	C_6H_5	CN	Cl
7	C_6H_5	CN	H
8	SCH_3	CN	CH_3

Figure 1. Structure of compounds **1–8**.

In view of the novelty of this structural class as an antiviral agent, in this paper we examine the antiviral activity of known 4-isothiazolecarbonitriles **2–8**[8–13] (Fig. 1) and report on the synthesis and the antiviral activity of a new series of analogues **12** (Table 2) against both RNA and DNA viruses.

In an effort to establish a structure–activity relationship, our chemical approach examined the effect of some structural modifications at three different positions in the isothiazole nucleus, with respect to antiviral activity.

Results

Chemistry

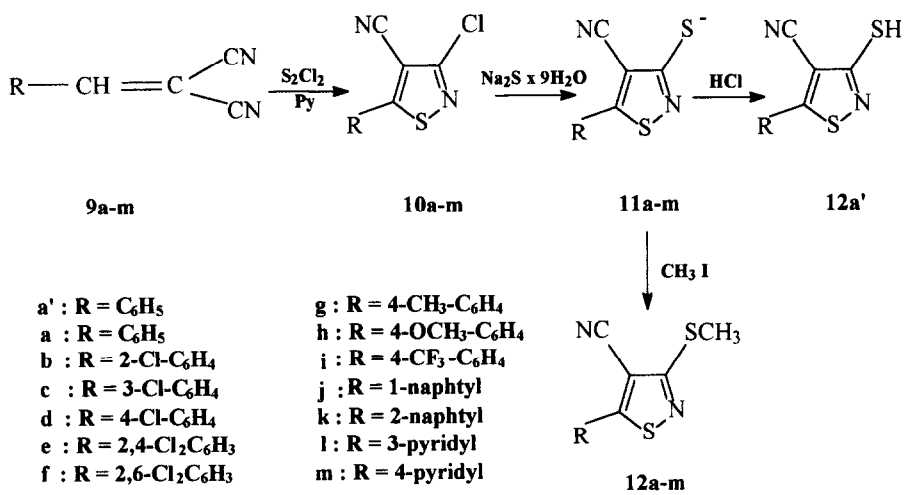
3-Chloro-5-aryl-4-isothiazolecarbonitriles **10a–k** and 3-chloro-5-pyridyl-4-isothiazolecarbonitriles **10l** and **10m** were prepared by cyclization of arylmethylene-malononitriles[14–16] **9a–k** and pyridylmethylene-malononitriles[17,18] **9l** and **9m** with sulfur chloride in the presence of pyridine.[19] The subsequent reaction with $Na_2S \times 9H_2O$ led to the corresponding sodium salts **11a–m**, which were converted to the desired 3-methylthio derivatives **12a–m** by alkylation with CH_3I, while the treatment of intermediate **11a** with HCl gave 3-mercapto-5-phenyl-4-isothiazolecarbonitrile **12a'**, as shown in Scheme 1. The reaction proceeded favourably in an aqueous alcoholic solution with an H_2O:MeOH ratio of 1:14, while a ring-opening reaction occurred with excess water. In fact, in the case of 3-chloro-5-phenyl-4-isothiazolecarbonitrile[19] **10a**, an excess of water yielded a mixture of 3-methylthio-5-phenyl-4-isothiazolecarbonitrile **12a** and α-(α-methylthiobenzylidene) malononitrile.[20]

As reported in Scheme 2, 4-isothiazolecarbonitriles **12p–s** were prepared from esters **13p–s** by known reactions.[13,21,22]

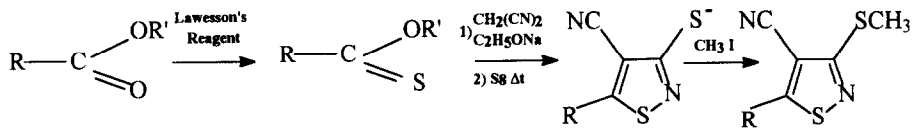
By alkylation of **12l** and **12m** with CH_3I, the corresponding pyridinium salts **12n** and **12o** were readily obtained. Whereas, by reacting **12r** with CH_3I the desired salt was not achieved. Complete cleavage of the benzyl group in **12s** by the thioanisole–TFA system gave compound **12t** without any side reaction.[23] Reaction with equimolar quantities of **12t**, 1-bromobutane and potassium carbonate in acetone yielded the corresponding ether **12u**. By heating **12t** with *p*-toluenesulfonyl chloride in pyridine, the ester **12v** was obtained. Chemical and physical data of new compounds are reported in the Experimental section.

Biology

Tables 1 and 2 show the values of test compounds CC_{50} and IC_{50}. The 50% cytotoxic dose (CC_{50}) was expressed



Scheme 1. Synthesis of compounds **12a'** and **12a–m**.



13p-s **14p-s** **11p-s** **12p-s**

13-14 p : R' = C_2H_5 **11,12,13,14 p : R = cyclohexyl**
13-14 q : R' = C_2H_5 **11,12,13,14 q : R = 2-thienyl**
13-14 r : R' = CH_3 **11,12,13,14 r : R = 2-pyridyl**
13-14 s : R' = C_2H_5 **11,12,13,14 s : R = 4-OBn-C_6H_4**

Scheme 2. Synthesis of compounds **12p–s**.

Table 1. Antiviral activity of isothiazole derivatives **1–8**

	CC_{50} (μM)[a,c]	IC_{50} (μM)[b,c]						
Compd	HEp-2 L-929 Vero	Polio	ECHO	Coxs.	EMC	Measles	Adeno	HSV
1	90	0.42	0.5	>90	>90	>90	>90	>90
2	4	>4	>4	>4	>4	>4	>4	>4
3	12	>12	>12	>12	>12	>12	>12	>12
4	1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
5	25	0.5	0.6	>25	>25	>25	>25	>25
6	20	>20	>20	>20	>20	>20	>20	>20
7	100	>100	>100	>100	>100	>100	>100	>100
8	100	12.5	>100	>100	>100	>100	>100	>100

[a]CC_{50}: concentration which inhibited cell growth by 50% as compared with control cultures.
[b]IC_{50}: concentration which inhibited virus plaque formation by 50%.
[c]Values are mean ± 0.5 SD (maximal standard deviation estimated) of three separate assays.

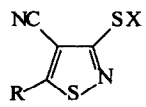
as the concentration that inhibited cell growth by 50% as compared with the control cultures. The compound concentration required to inhibit virus plaque formation by 50% was expressed as IC_{50}. The selectivity index (SI) was determined for the effective compounds dividing CC_{50} by IC_{50} (Table 3).

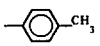
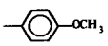
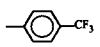
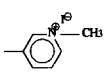
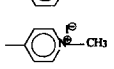
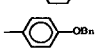
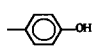
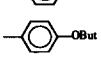
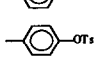
All the compounds were tested against RNA (polio 1, ECHO 9, Coxsackie B1, EMC, measles) and DNA (adeno 2, HSV-1) viruses. The lead compound **1** (Fig. 1) was effective against polio 1 and ECHO 9[5,6] (Table 1). Some isothiazole analogues, **12a′** and **12a**, were initially prepared and tested for a comparison with **1**. The presence of a –CN group in position 4 seemed to optimize the antiviral activity, since the new compounds **12a′** and **12a** showed a spectrum of activity similar to **1**, but exhibited higher selectivity indexes for polio 1 (Tables 2 and 3). Therefore, we evaluated the antiviral activity of known 4-isothiazolecarbonitriles **2–8** (Fig. 1) with other substituents in positions 3 and/or 5. When compared to **12a′** and **12a**, none of the compounds **2–8** was more effective against polio 1 and ECHO 9 viruses (Table 1).

Our next chemical approach was to examine the effect of various substituents on the phenyl ring of the most active compound (**12a**) with respect to antiviral activity. In the chloro-substituted series, we prepared 2-Cl (**12b**), 3-Cl (**12c**), 4-Cl (**12d**), 2,4-Cl_2 (**12e**) and 2,6-Cl_2 (**12f**) derivatives, which were weakly active (**12c** and **12d**) or completely inactive (**12b, 12e** and **12f**). The introduction of other substituents on the *para* position of the phenyl ring resulted in a diminished antiviral activity (**12g, 12h, 12i, 12t** and **12u**). The increased bulk of the substituents (O-Bn and O-Ts group for **12s** and **12v**, respectively) did not improve drug inhibitory activity; in fact, **12s** and **12v** were completely ineffective against screening viruses (Table 2).

We prepared analogues of **12a**, where the phenyl ring was replaced with some aromatic and heteroaromatic rings. In the pyridine series, antiviral activity depended on the position of substitution, decreasing as the isothiazole ring shifted from position 2 to position 4. In fact, compound **12r** was more active than **12l** and **12m** and displayed the highest selectivity index for polio 1 (Tables 2 and 3). Despite their low cytotoxicity,

Table 2. Antiviral activity of isothiazole derivatives **12a′** and **12a–v**



			$CC_{50}(\mu M)^{a,c}$	$IC_{50}(\mu M)^{b,c}$						
Compd	X	R	HEp-2 L-929 Vero	Polio	ECHO	Coxs.	EMC	Measles	Adeno	HSV
12a′	H		60	0.25	0.3	> 60	> 60	> 60	> 60	> 60
12a	CH_3		20	0.045	0.25	> 20	10	> 20	> 20	> 20
12b	CH_3		5	> 5	> 5	> 5	> 5	> 5	> 5	> 5
12c	CH_3		10	0.2	1	> 10	> 10	> 10	> 10	> 10
12d	CH_3		5	0.3	0.3	> 5	> 5	> 5	> 5	> 5
12e	CH_3		8	> 8	> 8	> 8	> 8	> 8	> 8	> 8
12f	CH_3		5	> 5	> 5	> 5	> 5	> 5	> 5	> 5
12g	CH_3		18	0.25	0.7	> 18	1	> 18	> 18	> 18
12h	CH_3		10	0.6	0.5	> 10	> 10	> 10	> 10	> 10
12i	CH_3		14	1	1.5	> 14	> 14	> 14	> 14	> 14
12j	CH_3		4	> 4	> 4	> 4	> 4	> 4	> 4	> 4
12k	CH_3		20	> 20	> 20	> 20	> 20	> 20	> 20	> 20
12l	CH_3		20	0.75	1.25	> 20	> 20	> 20	> 20	> 20
12m	CH_3		2.5	> 2.5	> 2.5	> 2.5	> 2.5	> 2.5	> 2.5	> 2.5
12n	CH_3		1250	250	> 1250	> 1250	> 1250	> 1250	> 1250	> 1250
12o	CH_3		1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
12p	CH_3		50	> 50	> 50	> 50	> 50	> 50	> 50	> 50
12q	CH_3		6	0.1	0.2	> 6	> 6	> 6	> 6	> 6
12r	CH_3		50	0.1	0.8	> 50	10	> 50	> 50	> 50
12s	CH_3		> 50	> 50	> 50	10	20	10	> 50	> 50
12t	CH_3		2	0.4	1	> 2	> 2	> 2	> 2	> 2
12u	CH_3		> 50	0.3	1	10	3	10	> 50	> 50
12v	CH_3		25	> 25	> 25	> 25	> 25	> 25	> 25	> 25

[a]CC_{50}: concentration which inhibited cell growth by 50% as compared with control cultures.
[b]IC_{50}: concentration which inhibited virus plaque formation by 50%.
[c]Values are mean ± 0.5 SD (maximal standard deviation estimated) of three separate assays.

Table 3. Selectivity index (SI) of effective compounds for polio 1 and ECHO 9

Compd	SI[a]	
	Polio	ECHO
1	214	180
5	50	42
8	8	1
12a′	240	200
12a	444	80
12c	50	10
12d	17	17
12g	72	26
12h	17	20
12i	14	9
12l	27	16
12n	5	1
12q	60	30
12r	500	63
12s	1	1
12t	5	2
12u	167	50

[a]Selectivity index (SI) was determined for the effective compounds dividing CC_{50} by IC_{50}.

pyridinium salts showed only slight activity (**12n**) or no activity (**12o**) against screening viruses. When the phenyl ring was substituted by thiophene (**12q**), a reduction of antiviral activity was observed. Replacement with a naphthyl group, compounds **12j** and **12k**, resulted in the loss of antiviral activity. Moreover, the presence of a cyclohexyl ring in the 5-position (**12p**) eliminated antiviral activity (Table 2).

As far as the anti-EMC activity was concerned, few compounds (**12a, 12g, 12r, 12s** and **12u**) were slightly active. A marginal activity was observed for **12s** and **12u** against Coxsackie B1 and measles viruses. No inhibition of adeno 2 and HSV-1 was detected for all compounds tested (Table 2).

Only slight or no activity against all screening viruses (data not shown) was exhibited by 3-chloro-5-aryl-4-isothiazolecarbonitriles **10a–k** and 3-chloro-5-pyridyl-4-isothiazolecarbonitriles **10l** and **10m**. Compound **12a** did not show virucidal activity.

In order to determine whether **12a** inhibited the virus yield during a specific period in the virus cycle, the effect of time addition of this compound was studied for poliovirus type 1. Results obtained from these experiments clearly demonstrated maximal inhibition of viruses when the compound was added at the end of the adsorption period. However, there was still a reduction in virus yield even when **12a** was added 30 min after the adsorption period. Addition during virus adsorption,

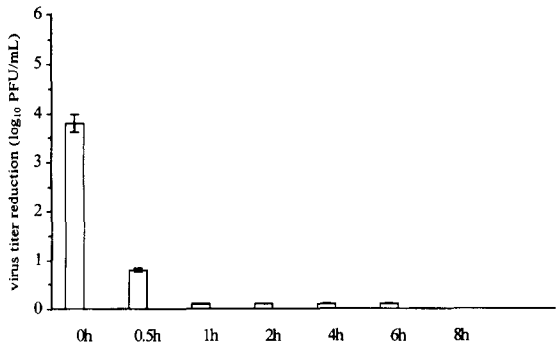


Figure 2. Effect of time of **12a** (5 μM) addition on virus inhibition from single-round replication of polio 1. Time 0 = post 2 h adsorption period at 4 °C. All values are mean ± SD of three separate assays.

and more than 1 h after the virus adsorption period, did not cause any virus yield reduction (Fig. 2).

Discussion

When antiviral activity against polio 1 and ECHO 9 viruses was discovered in a series of 3-mercapto-5-aryl-isothiazoles,[5–7] we established a synthesis program to optimize activity by preparing and testing new isothiazole derivatives. Our chemical approach was to investigate the effect of some structural modifications at different positions in the isothiazole nucleus to establish the requirements for optimum activity.

Structure–activity relationships revealed that the presence of a cyano group in the 4-position enhanced antiviral activity, since compound **12a′** exhibited a better antiviral activity than that observed for lead compound **1** (Tables 1 and 2). Moreover, the methylation of the mercapto group in the 3-position optimized antiviral activity and led to the most active member of this series, 3-methylthio-5-phenyl-4-isothiazolecarbonitrile **12a**, with selectivity indexes of 444 for polio 1 (Tables 2 and 3), which was higher than those obtained using the previously studied isothiazoles.

Therefore, we examined the effect of the introduction of various substituents on the phenyl ring and when compared to the unsubstituted phenyl analogue **12a**, all modifications in this series caused a diminished (compounds **12c, 12d, 12g, 12h, 12i, 12s** and **12t**), or a loss of antiviral activity (compounds **12b, 12e, 12f** and **12v**), as reported in Table 2.

As the replacement of the phenyl ring with other aryl groups did not improve antiviral activity, we synthesized a new series of analogues of **12a**, where the phenyl ring was substituted with some aromatic and heteroaromatic groups. Therefore, we prepared naphthyl deri-

vatives (**12j** and **12k**) and pyridyl (**12l, 12m** and **12r**) and thienyl (**12q**) derivatives to evaluate the effect of a major bulk and lipophilicity in the 5-position, and of isosteric substitution respectively. These chemical modifications generally reduced (compounds **12l** and **12q**) or eliminated (compounds **12j, 12k** and **12m**) antiviral activity (Table 2). Interestingly, the presence of a 2-pyridyl ring in the 5-position (**12r**), led to the highest selectivity index observed for polio 1 (Table 3). Moreover, treatment of compounds **12l** and **12m** with CH_3I gave water-soluble pyridinium salts **12n** and **12o**, producing a reduction of cytotoxicity values, as we expected, but without improving antiviral activity (Table 2).

As the presence of an O-Bn group (compound **12s**) resulted in a low activity against Coxsackie B1 and measles viruses, we synthesized compounds **12u** and **12v**, bearing bulky substituents on the *para* position of the phenyl ring, by trying to achieve new derivatives more effective against the above mentioned viruses. Unfortunately, **12u** (O-But derivative) exhibited IC_{50} values equivalent to those obtained for **12s**, whereas **12v** (O-Ts derivative) was inactive (Table 2). Only a few compounds (**12a, 12g, 12r, 12s** and **12u**) were weakly active against EMC virus (Table 2).

In conclusion, the spectrum of activity of most 4-isothiazolecarbonitriles was limited to polio 1 and ECHO 9. As peak activity against polio 1 was reached with the unsubstituted phenyl compound **12a**, we studied the effect under different experimental conditions in order to understand its mechanism of inhibition.

Since **12a** did not exert any virucidal action, it was believed that a specific step in the viral replicative cycle had to be inhibited. Results obtained from experiments of time of addition of compound on inhibition of polio 1 demonstrated that **12a** did not exert its antiviral effect via inhibition of virus attachment but interfered with a subsequent step of the replicative virus cycle. In fact, **12a** was effective when added within 1 h after the adsorption period. When this compound was added during virus adsorption, and more than 1 h after the virus adsorption period, no virus yield reduction was observed (Fig. 2). As **12a** is structurally an analogue of **1**, it is most likely to possess a similar mode of action. Previous experiments[6] on [5-^{3}H]uridine incorporation into RNA showed that **1** inhibited poliovirus RNA synthesis after 2 h incubation, so it is possible that **12a** exerts an interference with RNA synthesis of polio 1.

Experimental

Melting points were determined on a Büchi 510 apparatus and are uncorrected. 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 ±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.

Chemistry

General procedure for synthesis of 3-chloro-5-aryl-4-isothiazolecarbonitriles (10a–k) and 3-chloro-5-pyridyl-4-isothiazolecarbonitriles (10l and 10m) (Scheme 1). A mixture of **9a–m**[14–18] (50 mmol), pyridine (10 mmol) and sulfur chloride (150 mmol) was heated at 140–150 °C for 6–8 h and poured into ice-water. The resulting precipitate was filtered off, washed with water, dried, and then dissolved in hot ethanol to remove insoluble sulfur. After cooling, the supernatant gave 3-chloro-5-aryl-4-isothiazolecarbonitriles[19] **10a–k**. On the contrary, after filtration of the insoluble material and neutralization of the filtrate with $NaHCO_3$, **10l** and **10m** were obtained. The compounds **10a–m** were purified by column chromatography on silica gel (0.063–0.200 mm), eluting with benzene:cyclohexane (6:4).

The following compounds were obtained:

3-Chloro-5-phenyl-4-isothiazolecarbonitrile[19] (10a), 3-chloro-5-(2-chlorophenyl)-4-isothiazolecarbonitrile[19] (10b) and 3-chloro-5-(3-chlorophenyl)-4-isothiazolecarbonitrile (10c). Yield 73%; mp 99.5–101 °C (ligroin); IR (KBr) 2203 (CN) cm^{-1}.

3-Chloro-5-(4-chlorophenyl)-4-isothiazolecarbonitrile[19] (10d) and 3-chloro-5-(2,4-dichlorophenyl)-4-isothiazolecarbonitrile (10e). Yield 73%; mp 119.5–120.5 °C (ligroin); IR (KBr) 2225 (CN) cm^{-1}.

3-Chloro-5-(2,6-dichlorophenyl)-4-isothiazolecarbonitrile[19] (10f) and 3-chloro-5-(4-methylphenyl)-4-isothiazolecarbonitrile (10g). Yield 70%; mp 81–84 °C (cyclohexane); IR (KBr) 2208 (CN) cm^{-1}.

3-Chloro-5-(4-methoxyphenyl)-4-isothiazolecarbonitrile (10h). Yield 60%; mp 122–126 °C (ethanol); IR (KBr) 2213 (CN) cm^{-1}.

3-Chloro-5-(4-trifluoromethylphenyl)-4-isothiazolecarbonitrile (10i). Yield 72%; mp 84–85 °C (ligroin); IR (KBr) 2210 (CN) cm^{-1}.

3-Chloro-5-(1-naphthyl)-4-isothiazolecarbonitrile (10j). Yield 70%; mp 105–107 °C (ethanol); IR (KBr) 2208 (CN) cm^{-1}.

3-Chloro-5-(2-naphthyl)-4-isothiazolecarbonitrile (10k). Yield 70%; mp 118–120 °C (ethanol); IR (KBr) 2210 (CN) cm^{-1}.

3-Chloro-5-(3-pyridyl)-4-isothiazolecarbonitrile (10l). Yield 68%; mp 122–126 °C (cyclohexane); IR (KBr) 2222 (CN) cm^{-1}.

3-Chloro-5-(4-pyridyl)-4-isothiazolecarbonitrile (10m). Yield 69%; mp 90–92 °C (cyclohexane); IR (KBr) 2231 (CN) cm^{-1}.

General procedure for synthesis of isothiazoles 12a′ and 12a–m (Scheme 1). A solution of $Na_2S \times 9H_2O$ (4.54 mmol) in a mixture of methanol (50 mL) and water (5 mL) was refluxed for 15 min. A solution of **10a–m** (4.5 mmol) in methanol (20 mL) was added dropwise and the mixture was refluxed for 3 h. After cooling, the solution was partitioned between water and ether and an excess of CH_3I was added to the aqueous layer. The mixture reaction was stirred at room temperature for 12 h to yield **12a–m**. The products obtained were filtered, washed with H_2O and purified by crystallization. Whereas, **12a′** was prepared by the addition of HCl to the aqueous phase.

The following compounds were obtained:

3-Mercapto-5-phenyl-4-isothiazolecarbonitrile (12a′). Yield 68%; mp 143–145 °C (ethyl acetate); IR (KBr) 2217 (CN) cm^{-1}; MS *m/e* 219 (M + 1), 159, 77.

3-Methylthio-5-phenyl-4-isothiazolecarbonitrile (12a). Yield 74%; mp 81–82 °C (cyclohexane); IR (KBr) 2214 (CN) cm^{-1}; MS *m/e* 233 (M + 1), 156, 77.

3-Methylthio-5-(2-chlorophenyl)-4-isothiazolecarbonitrile (12b). Yield 70%; mp 121 °C (ligroin); IR (KBr) 2210 (CN) cm^{-1}; MS *m/e* 266 (M), 231, 155.

3-Methylthio-5-(3-chlorophenyl)-4-isothiazolecarbonitrile (12c). Yield 72%; mp 124–125 °C (ligroin); IR (KBr) 2205 (CN) cm^{-1}; MS *m/e* 266 (M), 231, 155.

3-Methylthio-5-(4-chlorophenyl)-4-isothiazolecarbonitrile (12d). Yield 70%; mp 112–114 °C (ligroin); IR (KBr) 2202 (CN) cm^{-1}; MS *m/e* 267 (M + 1), 232, 111.

3-Methylthio-5-(2,4-dichlorophenyl)-4-isothiazolecarbonitrile (12e). Yield 68%; mp 135–136 °C (ligroin); IR (KBr) 2225 (CN) cm^{-1}; MS *m/e* 301 (M + 1), 266, 32.

3-Methylthio-5-(2,6-dichlorophenyl)-4-isothiazolecarbonitrile (12f). Yield 70%; mp 125–126 °C (ligroin); IR (KBr) 2225 (CN) cm^{-1}; MS *m/e* 301 (M + 1), 266, 45.

3-Methylthio-5-(4-methylphenyl)-4-isothiazolecarbonitrile (12g). Yield 73%; mp 92–93 °C (cyclohexane); IR (KBr) 2210 (CN) cm^{-1}; MS *m/e* 246 (M), 231, 155.

3-Methylthio-5-(4-methoxyphenyl)-4-isothiazolecarbonitrile (12h). Yield 71%; mp 104–106 °C (ligroin); IR (KBr) 2213 (CN) cm^{-1}; MS *m/e* 262 (M), 247, 185.

3-Methylthio-5-(4-trifluoromethylphenyl)-4-isothiazolecarbonitrile (12i). Yield 70%; mp 93–95 °C (hexane); IR (KBr) 2212 (CN) cm^{-1}; MS *m/e* 300 (M), 231, 69.

3-Methylthio-5-(1-naphthyl)-4-isothiazolecarbonitrile (12j). Yield 68%; mp 108–110 °C (ethanol); IR (KBr) 2208 (CN) cm^{-1}; MS *m/e* 282 (M), 235, 82.

3-Methylthio-5-(2-naphthyl)-4-isothiazolecarbonitrile (12k). Yield 69%; mp 118–121 °C (ethanol); IR (KBr) 2208 (CN) cm^{-1}; MS *m/e* 282 (M), 127, 82.

3-Methylthio-5-(3-pyridyl)-4-isothiazolecarbonitrile (12l). Yield 84%; mp 119–122 °C (cyclohexane); IR (KBr) 2225 (CN) cm^{-1}; MS *m/e* 233 (M), 156, 78.

3-Methylthio-5-(4-pyridyl)-4-isothiazolecarbonitrile (12m). Yield 80%; mp 123–124 °C (cyclohexane); IR (KBr) 2230 (CN) cm^{-1}; MS *m/e* 233 (M), 160, 129.

Synthesis of 3-[(4-cyano-3-methylthio)isothiazol-5-yl] pyridinium iodide (12n) and of 4-[(4-cyano-3-methylthio)-isothiazol-5-yl] pyridinium iodide (12o). Compounds **12l** and **12m** were dissolved in acetone and an excess of CH_3I was added. After stirring at room temperature, yellow pyridinium salts **12n** and **12o** were obtained.

The following compounds were obtained:

3-[(4-Cyano-3-methylthio)isothiazol-5-yl] pyridinium iodide (12n). Yield 90%; mp 194–196 °C (cyclohexane); IR (KBr) 2221 (CN) cm^{-1}; MS *m/e* 234, 78, 32.

4-[(4-Cyano-3-methylthio)isothiazol-5-yl] pyridinium iodide (12o). Yield 90%; mp 200–201 °C (cyclohexane); IR (KBr) 2229 (CN) cm^{-1}; MS *m/e* 248, 233, 82.

General procedure for synthesis of thionoesters 14p–s (Scheme 2). Thionoesters **14p–s** were readily prepared from esters **13p–s** according to methods previously described in literature.[21]

The following compounds were obtained:

Cyclohexanecarbothioic acid O-ethyl ester[24] (14p), 2-thiophenecarbothioic acid O-ethyl ester[25] (14q), 2-pyridinecarbothioic acid O-methyl ester[26] (14r) and 4-(phenylmethoxy)-benzenecarbothioic acid O-ethyl ester (14s). Yield 63%; mp 72–73 °C (cyclohexane); IR (KBr) 1600 (C = S) cm^{-1}.

General procedure for synthesis of isothiazoles 12p–s (Scheme 2). A solution of sodium ethoxide, prepared from sodium (11 mmol) and absolute ethanol (5 mL), and malononitrile (11 mmol) was added to the solution of **15p–s** (7.3 mmol). The reaction mixture was stirred at room temperature for 1 h.[22] Then, sulfur (12 mmol) was added and the mixture was refluxed for 6 h. After cooling, the excess of sulfur was removed by filtration and an excess of CH_3I was added to the filtrate.[13] After stirring at room temperature, the products **12p–s**, which separated out, were filtered, washed with water and crystallized from suitable solvent.

The following compounds were obtained:

3-Methylthio-5-cyclohexyl-4-isothiazolecarbonitrile (12p). Yield 35%; mp 76–77 °C (cyclohexane); IR (KBr) 2210 (CN) cm^{-1}; MS *m/e* 239 (M + 1), 84, 32.

3-Methylthio-5-(2-thienyl)-4-isothiazolecarbonitrile (12q). Yield 50%; mp 120–123 °C (cyclohexane); IR (KBr) 2212 (CN) cm^{-1}; MS *m/e* 239 (M + 1), 109, 32.

3-Methylthio-5-(2-pyridyl)-4-isothiazolecarbonitrile (12r). Yield 48%; mp 140–142 °C (ethanol); IR (KBr) 2216 (CN) cm^{-1}; MS *m/e* 233 (M), 154, 78.

3-Methylthio-5-(4-OBn-phenyl)-4-isothiazolecarbonitrile (12s). Yield 56%; mp 126–128 °C (cyclohexane); IR (KBr) 2207 (CN) cm^{-1}; MS *m/e* 338 (M), 247, 91.

Synthesis of 3-methylthio-5-(4-hydroxyphenyl)-4-isothiazolecarbonitrile (12t). A mixture of **12s** (34 mmol), thioanisole (1.5 mL) and TFA (5 mL) was stirred at room temperature for 24 h.[23] Then, the suspension was dissolved in hot ethanol and poured into ice-water. The white precipitate was filtered off, washed with a solution of $NaHCO_3$ and crystallized from ethanol to give **12t**. Yield 80%; mp 211–214 °C; IR (KBr) 3337 (broad, OH), 2220 (CN) cm^{-1}; MS *m/e* 248 (M), 155, 93.

Synthesis of 3-methylthio-5-(4-butoxyphenyl)-4-isothiazolecarbonitrile (12u). A mixture of equimolar quantities (2 mmol) of **12t**, 1-bromobutane, and potassium carbonate in acetone was refluxed for 48 h. The mixture was concentrated and then partitioned between water and ether. The combined ether fractions were washed with 2 M NaOH and dried on Na_2SO_4. Removal of the solvent gave **12u**, which was crystallized from cyclohexane. Yield 55%; mp 91–94 °C; IR (KBr) 2213 (CN) cm^{-1}; MS *m/e* 304 (M), 248, 231.

Synthesis of 3-methylthio-5-(4-OTs-phenyl)-4-isothiazolecarbonitrile (12v). Equimolar quantities (5 mmol) of **12t** and *p*-toluenesulfonyl chloride were dissolved in pyridine and heated for 6 h. The mixture was then poured into cold water and acidified with 2 M HCl. The white precipitate was filtered and washed successively with 2 M NaOH and water. After drying, the product **12v** was crystallized from ligroin. Yield 63%; mp 155–156 °C; IR (KBr) 2222 (CN), 1352, 1160 (S = O) cm^{-1}; MS *m/e* 402 (M + 1), 155, 32.

Biology

Viruses and cells. Poliovirus 1 (Brunhilde strain), ECHO virus 9 (Hill strain), Coxsackievirus B1, measles (Edmonston strain) and adenovirus type 2 were purchased 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 purchased from the ATCC and propagated in mouse connective tissue cells (L-929) and African green monkey kidney cells (Vero), respectively. Cells were kept in a humidified 5% carbon dioxide atmosphere at 37 °C and grown in Dulbecco 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. For all viruses tested working stocks were prepared as cellular lysates using DMEM without FCS (maintenance medium).

Test compounds. Compounds were dissolved in DMSO and diluted in maintenance medium to achieve the final concentration needed. Dilution of test compounds contained a maximal concentration of 0.01% DMSO, which was not toxic to our cell lines. Pyridinium salts (**12n** and **12o**) were directly diluted in maintenance medium.

Cell viability. The cytotoxicity of the test compounds was evaluated by measuring the effect produced on cell morphology and cell 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 12, 24 and 48 h. Cytotoxicity was scored as morphological alterations (rounding up, shrinking and detachment). The viability of the cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method. Briefly, HEp-2, L-929 and Vero cells were prepared in 96-well tissue culture plates and serial concentrations of the compounds were added. After incubation for 48 h at 37 °C, MTT (0.5 mg/mL) in DMEM without phenol red was replaced in each well. After 90 min incubation at 37 °C the overlay was removed and isopropanol (100 μL) was added; plates were then mixed twice to dissolve the dark blue crystals. The optical density (OD) was read at 540 and 690 nm on a Titertek Multiscan MCC/340, within 15 min of adding the isopropanol.[27,28] The absorbance at 690 nm was

automatically subtracted from the absorbance at 540 nm, so as to eliminate the effect of non specific absorption. Cell viability values obtained in the presence of the compounds were expressed as the percentage of those obtained in untreated controls and were calculated by the following formula:

$$\frac{(OD)_c-(OD)_t}{(OD)_c} \times 100$$

where $(OD)_c$ and $(OD)_t$ indicated the absorbances of the untreated cell control and of the test sample respectively. The 50% cytotoxic dose (CC_{50}), calculated by dose–response curves and linear regression, was expressed as the concentration of the compound that reduced the absorbance of the control sample by 50%.

Antiviral activity. The antiviral activity was assessed using a plaque reduction assay. Confluent cells were grown in 6-well tissue culture plates and infected with 300 plaque forming units (PFU) of the virus stock per well. During and after 1 h of virus adsorption at 37 °C (30 min for picornavirus), overlay medium containing 1% of methylcellulose with or without the test compound at doses below CC_{50} was added. After 24 h of incubation at 37 °C, when the plaques appeared clearly in virus controls, the overlay was removed and cells were stained with 1% crystal violet in methanol. The number of visible plaques was then counted under light microscopy. The antiviral activity of each compound was determined as the percentage decrease in the number of plaques, which was calculated by the following formula:

$$\frac{\text{no. of plaques (control)} - \text{no. of plaques (test)}}{\text{no. of plaques (control)}} \times 100$$

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

Virucidal activity. To test possible virucidal activity, equal volumes (0.5 mL) of poliovirus suspension (containing 10^7 PFU/mL) and DMEM containing compound **12a** (50 and 100 μM) were mixed and incubated for 2 h at 37 °C. Infectivity was determined by plaque assay after dilution of the virus below the inhibitory concentration.

Effect of time of addition of 12a on virus inhibition. Monolayers of HEp-2 cells were grown to confluence in 24-well plates and inoculated with polio 1 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 **12a** (5 μM), for the adsorption period. The compound was removed or added at various times after the adsorption period respectively, as indicated in Figure 2. The plates were incubated at 37 °C for 8 h and were then frozen. Virus yield was determined by plaque assay.

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