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Synthesis of New 3,4,5-Trisubstituted Isothiazoles as Effective Inhibitory Agents of Enteroviruses

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Abstract—The synthesis and evaluation of 3,4,5-trisubstituted isothiazoles as antiviral agents led to the discovery of several compounds effective in vitro against enteroviruses polio 1 and ECHO 9. Structure–activity relationship studies revealed that a short thioalkyl chain in the 3-position and a methyl ester group in the 4-position are the structural components that, to a large extent, contribute to the positive biological profile in terms of both selectivity and low cytotoxicity. Under one-step growth conditions, methyl 3-methylthio-5-phenyl-4-isothiazolecarboxilate caused the greatest activity if added within 1 h after poliovirus adsorption. These data suggest interference with early events of viral replication.

Introduction

Isothiazole derivatives are known to possess many biological properties. The isothiazole ring has been incorporated into a wide range of established drugs including sulfonamides,[1] thiosemicarbazones,[2] amidines[3] and benzimidazoles,[4] although this approach does not appear to have resulted in any major improvements. However, many isothiazoles, not directly related to known drugs, have demonstrated biological activity: a derivative of an isothiazolo[5,4-*d*]pyrimidine has been reported to possess antitumoral activity,[5] 3,5-disubstituted 4-isothiazolecarbonitriles are known as herbicide, insecticide and fungicide agents[6,7] and isothiazolecarboxylic esters[8] have exhibited spasmolytic activities against acetylcholine and histamine as spasmogens.

So far, few research groups have investigated the potential antiviral properties of the isothiazole nucleus. Only isothiazolehydrazides have shown a weak antiviral activity against polio and herpes simplex viruses.[9] As a result, we have been studying antiviral activity of new isothiazole derivatives in order to obtain compounds that are effective as antiviral agents. Currently, no drugs are available for the treatment of diseases caused by enteroviruses, one of the main genera of the picornavirus family. The widespread nature of picornavirus illnesses, the economic consequences and the impracticality of vaccine development have stimulated our search for new selective antipicornaviral drugs. Our previous studies described the synthesis and antiviral activity of 3-mercapto-5-aryl-isothiazoles, which were active against enteroviruses polio 1 and ECHO 9.[10–12] Following works have demonstrated that the presence of a cyano group in the 4-position of isothiazole ring improved antiviral activity and broadened the spectrum of action; in fact, some new 4-isothiazolecarbonitriles were also effective against Coxsackie B1 and measles viruses.[13] The most active member of this series, 3-methylthio-5-phenyl-4-isothiazolecarbonitrile, coded **IS-2**, exhibited remarkable viral inhibition against polio 1, with selectivity index of 444, which was higher than those obtained using the previously studied 3-mercapto-5-aryl-isothiazoles.[11–13]

On the basis of these data, we selected **IS-2** as the lead compound for some chemical transformations to establish the requirements for optimum activity. As a result of our work, the introduction of substituents on the phenyl ring or the replacement of the phenyl for other aromatic and heteroaromatic groups in the 5-position did not improve antiviral activity.[13]

As an approach to more extensive structural modifications, in the present study we report the effect of replacement of the SCH_3 in the 3-position, as well as the CN conversion into $CONH_2$, COOH or COOMe groups in

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the 4-position. The new 3,4,5-trisubstituted isothiazoles were screened against both RNA viruses (polio 1, ECHO 9, Coxsackie B1, EMC, measles) and DNA viruses (adeno 2 and HSV 1).

Chemistry

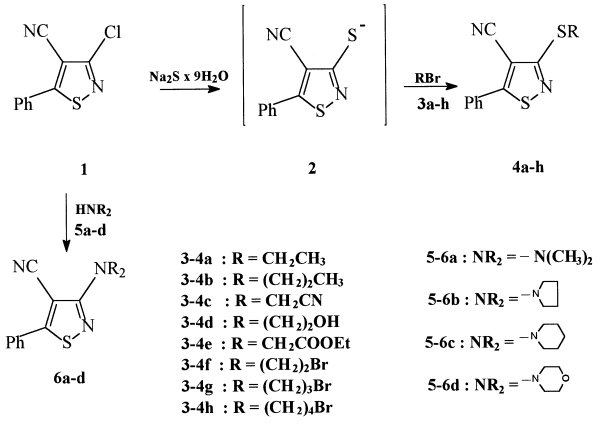
3-Alkylthio-5-phenyl-4-isothiazolecarbonitriles **4a–h** were obtained by the reaction of **1**[14] with $Na_2S\times9H_2O$ and suitable alkylating reagents **3a–h** (Scheme 1). Reaction of ethanol solutions of **1** with excess dimethylamine **5a**, pyrrolidine **5b**, piperidine **5c** or morpholine **5d** gave the corresponding 3-dialkylamino-5-phenyl-4-isothiazolecarbonitriles **6a–d** by replacement of the 3-chlorine atom (Scheme 1). A number of 3,5-disubstituted 4-isothiazolecarboxylic amides, acids and esters were prepared from the corresponding 4-isothiazolecarbonitriles. 4-Isothiazolecarboxamides **7a,b** were prepared by solution of the appropriate nitrile in sulfuric acid and subsequent reaction with water. 4-Isothiazolecarboxylic acids **8a,b** were prepared by hydrolysis with potassium hydroxide in aqueous ethylene glycol. Methyl esters **9a,b** were obtained by refluxing the respective alcoholic solutions of the acids **8a,b** with an excess of gaseous hydrochloric acid, as shown in Scheme 2.

Chemical and physical data of new compounds are reported in Experimental.

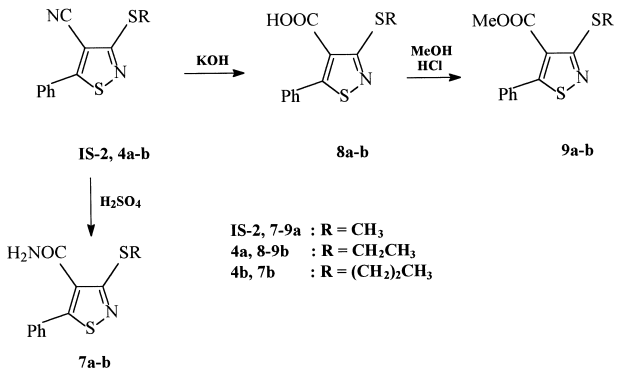
Results and Discussion

Table 1 shows the values of test compounds CC_{50} and IC_{50}. The 50% cytotoxic dose (CC_{50}) was expressed as the concentration which 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 2).

The lead compound **IS-2** demonstrated the highest level of activity against polio 1 and ECHO 9 viruses, with IC_{50} values of 0.045 and 0.25 μM, respectively (Table 1).



Scheme 1. Synthesis of compounds **4a–h** and **6a–d**.



Scheme 2. Synthesis of compounds **7a,b, 8a,b** and **9a,b**.

A structure–activity study was initially performed by maintaining the cyano group and the phenyl ring in the 4- and 5-positions, respectively, and varying the alkylthio chain in the 3-position.

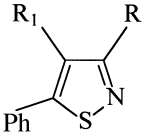
Compounds **4a** and **4b**, characterized by the replacement of the SCH_3 in the 3-position with a longer alkylthio chain, showed in vitro a reduced antiviral activity, but a cytotoxicity from five (**4a**) to ten (**4b**) times lower, when compared to **IS-2** (Table 1). The introduction of a cyano (**4c**), hydroxy (**4d**), carboethoxy (**4e**) group or a bromo atom (compounds **4f–h**) in the alkyl chain resulted in a diminished activity (compounds **4c,d**) or in the loss of antiviral activity (compounds **4e,f**), as reported in Table 1. More extensive structural modifications in the 3-position gave the 3-dialkylamino derivatives **6a–d**, which were less effective than **IS-2** against polio 1 and ECHO 9 (**6a,b**), only slightly active against polio 1 (**6c**) or completely inactive (**6d**), as reported in Table 1.

Our next chemical approach was to examine the effect of substitution of the cyano group in the 4-position with respect to antiviral activity (compounds **7–9a**). By far the greatest enhancement of selectivity was produced by the 4-carboxymethylester **9a** (Table 2), whereas, despite their low cytotoxicity, the amide and acid derivatives (**7a** and **8a**, respectively) were inactive against screening viruses (Table 1). Moreover, the simultaneous replacement of SCH_3 and CN groups, which led to the new alkylthio amide, acid and ester (compounds **7–9b**), caused a diminished (**9b**) or a loss of antiviral activity (**7–8b**), as reported in Table 1. Only few compounds (**4a,b, 4g,h** and **9a**) were weakly active against EMC (Table 1).

All the new trisubstituted isothiazoles were ineffective against Coxsackie B1, measles, adeno 2 and HSV 1 (Table 1).

Compound **9a** was not virucidal, since exposure of poliovirus to 100 and 500 μM drug concentrations had no effect on virion infectivity. In order to determine whether **9a** 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 that **9a** was effective to the highest degree

Table 1. Antiviral activity of isothiazoles **4a–h, 6a–d,** and **7–9a,b**



Compd	R	R_1	CC_{50}(μM)[a,c] HEp-2 L-929Vero	IC_{50}(μM)[b,c] Polio	ECHO	Coxs.	EMC	Measles	Adeno	HSV
IS−2	SCH_3	CN	20	0.045	0.25	> 20	10	> 20	> 20	> 20
4a	SCH_2CH_3	CN	100	0.1	0.5	> 100	20	> 100	> 100	> 100
4b	$S(CH_2)_2CH_3$	CN	200	15	5	> 200	5	> 200	> 200	> 200
4c	SCH_2CN	CN	10	0.5	0.5	> 10	> 10	> 10	> 10	> 10
4d	$S(CH_2)_2OH$	CN	5	0.3	0.6	> 5	> 5	> 5	> 5	> 5
4e	SCH_2COOEt	CN	200	> 200	> 200	> 200	> 200	> 200	> 200	> 200
4f	$S(CH_2)_2Br$	CN	25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
4g	$S(CH_2)_3Br$	CN	25	> 25	> 25	> 25	2.5	> 25	> 25	> 25
4h	$S(CH_2)_4Br$	CN	25	> 25	> 25	> 25	5	> 25	> 25	> 25
6a	$N(CH_3)_2$	CN	15	0.2	1	> 15	> 15	> 15	> 15	> 15
6b		CN	7	0.1	0.3	> 7	> 7	> 7	> 7	> 7
6c		CN	25	2.5	> 25	> 25	> 25	> 25	> 25	> 25
6d		CN	1	> 1	> 1	> 1	> 1	> 1	> 1	> 1
7a	SCH_3	$CONH_2$	250	> 250	> 250	> 250	> 250	> 250	> 250	> 250
7b	$S(CH_2)_2CH_3$	$CONH_2$	300	> 300	> 300	> 300	> 300	> 300	> 300	> 300
8a	SCH_3	COOH	500	> 500	> 500	> 500	> 500	> 500	> 500	> 500
8b	SCH_2CH_3	COOH	500	> 500	> 500	> 500	> 500	> 500	> 500	> 500
9a	SCH_3	COOMe	100	0.09	0.3	> 100	15	> 100	> 100	> 100
9b	SCH_2CH_3	COOMe	50	0.2	2	> 50	> 50	> 50	> 50	> 50

[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 S.D. (estimated maximal standard deviation) of three separate assays.

when it was added at the end of adsorption period, whereas the viral yield was already reduced when **9a** was added 30 min after the adsorption period. Addition during virus adsorption, and more than 1 h after the virus adsorption period, did not cause any virus yield reduction (Fig. 1).

Our results indicate that new derivatives endowed with lower cytotoxicity and a higher selectivity index can be obtained starting from the lead compound **IS-2**. In fact, 3-ethylthio-5-phenyl-4-isothiazolecarbonitrile **4a** and methyl 3-methylthio-5-phenyl-4-isothiazolecarboxilate **9a** were demonstrated to be the most potent inhibitors of members of the picornavirus family (polio 1 and ECHO 9), with a range of IC_{50} values of 0.09–0.5 μM, which were very much lower than CC_{50} values (100 μM), as reported in Table 1. In this way, we obtained selectivity indexes for **4a** (1000 and 200) and **9a** (1111 and 333) for polio 1 and ECHO 9, respectively, which were higher than those observed for **IS-2** (Table 2).

Table 2. Selectivity Index (SI) of effective compounds for polio 1 and ECHO 9

Compd	SI[a] Polio	ECHO
IS−2	444	80
4a	1000	200
4b	13	40
4c	20	20
4d	17	8
6a	75	15
6b	70	23
6c	10	1
9a	1111	333
9b	250	25

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

In conclusion, considering the general formula (Table 1) of the new antiviral agents described, the structural features which ensure the best biological profile seem to be: (1) a short thioalkyl chain in the 3-position, (2) a cyano or methylester group in the 4-position, and (3) a not substituted phenyl ring in the 5-position. In fact, the presence of an aryl group or of other aromatic (naphthyl) or heteroaromatic (pyridyl or thienyl) rings in the 5-position, as we previously reported,[13] the introduction of polar groups ($CONH_2$ or COOH) in the 4-position, as well as the presence of substituents in the alkyl chain of the 3-position or its replacement with a dialkylamino function have always reduced or eliminated antiviral activity.

On the basis of the results of these studies, **IS-2** and its derivatives (compounds **4a** and **9a**) are strong candidates for in vivo evaluation as systemic agents for the treatment of enterovirus infections.

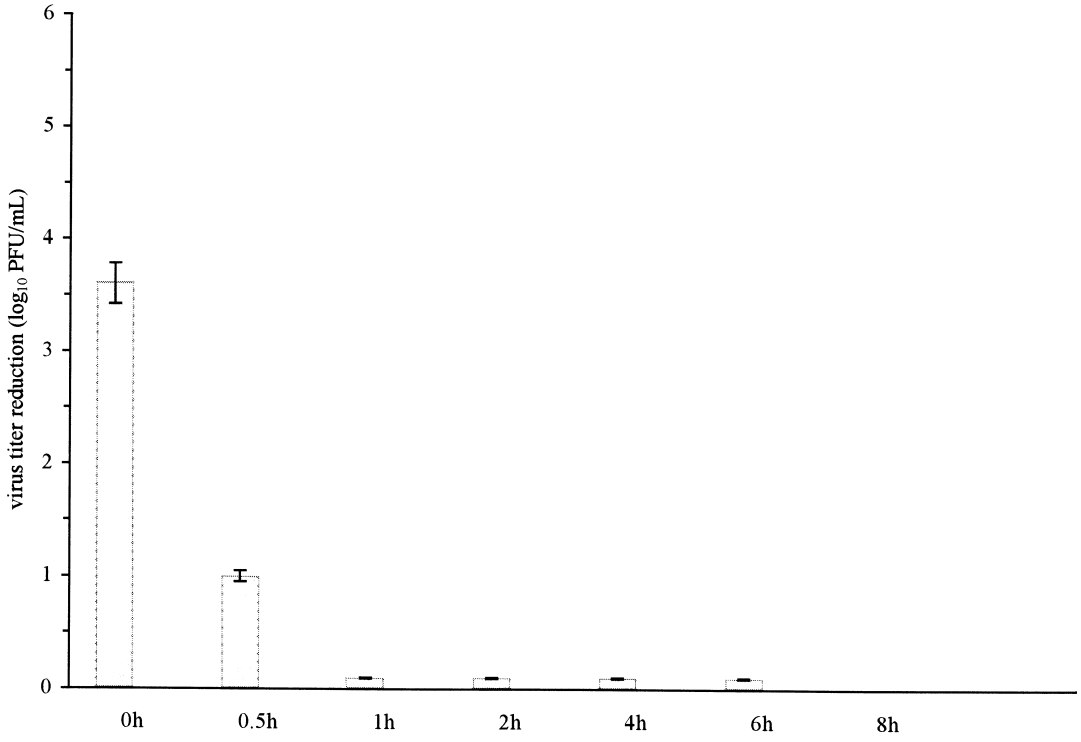


Figure 1. Effect of time of **9a** (10 μM) addition on virus inhibition from single-round replication of polio 1. Time 0 = post 2 h absorption period at 4 °C. All values are mean ± S.D. of three separate assays.

Experimental

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 ±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-alkylthio-5-phenyl-4-isothiazolecarbonitriles 4a–h (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 **1**[14] (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 diethyl ether and an excess of suitable alkylating reagents **3a–h** was added to the aqueous layer. The mixture reaction was stirred at room temperature for 12 h to yield the compounds **4a–h**. The products obtained were filtered, washed with H_2O and purified by crystallization. The following compounds were obtained:

3-Ethylthio-5-phenyl-4-isothiazolecarbonitrile (4a). Yield 67%; mp 78–79 °C (petroleumether 40–60 °C); IR (KBr) 2210 (CN) cm^{-1}; MS *m/e* 246 (M), 231, 77.

3-Propylthio-5-phenyl-4-isothiazolecarbonitrile (4b). Yield 52%; mp 66–68 °C (petroleumether 40–60 °C); IR (KBr) 2212 (CN) cm^{-1}; MS *m/e* 260 (M), 218, 77.

3-Cyanomethylthio-5-phenyl-4-isothiazolecarbonitrile (4c). Yield 55%; mp 160–162 °C (cyclohexane); IR (KBr) 2251 (CN), 2219 (CN) cm^{-1}; MS *m/e* 258 (M + 1), 231, 32.

3-(2-Hydroxy-ethylthio)-5-phenyl-4-isothiazolecarbonitrile (4d). Yield 65%; mp 107–110 °C (cyclohexane); IR (KBr) 3274 (br, OH), 2221 (CN) cm^{-1}; MS *m/e* 262 (M), 231, 218.

3-Carboxyethylmethylthio-5-phenyl-4-isothiazolecarbonitrile (4e). Yield 51%; mp 123–125 °C (ligroin); IR (KBr) 2218 (CN), 1738 (C=O) cm^{-1}; MS *m/e* 304 (M), 231, 77.

3-(2-Bromo-ethylthio)-5-phenyl-4-isothiazolecarbonitrile (4f). Yield 40%; mp 105–106 °C (hexane); IR (KBr) 2210 (CN) cm^{-1}; MS *m/e* 326 (M + 1), 245, 218.

3-(3-Bromo-propylthio)-5-phenyl-4-isothiazolecarbonitrile (4g). Yield 42%; mp 65–67 °C (hexane); IR (KBr) 2210 (CN) cm^{-1}; MS *m/e* 340 (M + 1), 259, 232.

3-(4-Bromo-butylthio)-5-phenyl-4-isothiazolecarbonitrile (4h). Yield 42%; mp 64–67 °C (hexane); IR (KBr) 2214 (CN) cm^{-1}; MS *m/e* 354 (M + 1), 273, 218.

General procedure for synthesis of 3-dialkylamino-5-phenyl-4-isothiazolecarbonitriles 6a–d (Scheme 1). An ethanol solution of **1** (1.3 mmol) was heated under reflux

with an excess of dialkylamine **5a–d** for 2–4 h and poured into water. The precipitated solid was collected on a filter, washed with ethanol and water to remove excess amine, and dried to yield the compounds **6a–d**. The following compounds were obtained:

3-*N*,*N*-Dimethylamino-5-phenyl-4-isothiazolecarbonitrile (6a). Yield 70%; mp 75 °C (petroleumether 40–60 °C); IR (KBr) 2205 (CN) cm^{-1}; MS *m*/*e* 229 (M), 77, 44.

3-(1-Pyrrolidinyl)-5-phenyl-4-isothiazolecarbonitrile (6b). Yield 84%; mp 106 °C (petroleumether 40–60 °C); IR (KBr) 2210 (CN) cm^{-1}; MS *m*/*e* 255 (M), 77, 70.

3-(1-Piperidinyl)-5-phenyl-4-isothiazolecarbonitrile (6c). Yield 75%; mp 74 °C (ethanol); IR (KBr) 2215 (CN) cm^{-1}; MS *m*/*e* 269 (M), 84, 77.

3-(4-Morpholinyl)-5-phenyl-4-isothiazolecarbonitrile (6d). Yield 75%; mp 129 °C (ethanol); IR (KBr) 2215 (CN) cm^{-1}; MS *m*/*e* 271 (M), 86, 77.

General procedure for synthesis of 3-alkylthio-5-phenyl-4-isothiazolecarboxamides 7a,b (Scheme 2). 3,5-Disubstituted 4-isothiazolecarboxamides **7a,b** were prepared from the corresponding nitriles (1.6 mmol), **IS-2** and **4b**, respectively, in concentrated sulfuric acid, warming the solution in a steam bath for 20 min and allowing the mixture to stand 18 h at room temperature. The products were precipitated by dilution in ice-water. The following compounds were obtained:

3-Methylthio-5-phenyl-4-isothiazolecarboxamide (7a). Yield 92%; mp 208–209 °C (ethyl acetate); IR (KBr) 3382, 3190 (br, NH),1645 (C=O) cm^{-1}; MS *m*/*e* 250 (M), 129, 44.

3-Propylthio-5-phenyl-4-isothiazolecarboxamide (7b). Yield 92%; mp 191–192 °C (cyclohexane); IR (KBr) 3380, 3187 (br, NH), 1645 (C=O) cm^{-1}; MS *m*/*e* 278 (M), 77, 44.

General procedure for synthesis of 3-alkylthio-5-phenyl-4-isothiazolecarboxylic acids 8a,b (Scheme 2). A mixture of 4-isothiazolecarbonitriles (1.6 mmol), **IS-2** or **4a**, of potassium hydroxide (3.6 mmol), 0.36 mL of water and 5 mL of ethylene glycol was refluxed for 48 h. After cooling, the reaction mixture was poured into 10 mL of water. A small amount of insoluble material was filtered off and the filtrate was acidified with dilute hydrochloric acid to separate the crude acids **8a,b**. The following compounds were obtained:

3-Methylthio-5-phenyl-4-isothiazolecarboxylic acid (8a). Yield 86%; mp 160 °C (cyclohexane); IR (KBr) 3450 (br, OH), 1693 (C=O) cm^{-1}; MS *m*/*e* 251 (M), 129, 45.

3-Ethylthio-5-phenyl-4-isothiazolecarboxylic acid (8b). Yield 86%; mp 134–136 °C (cyclohexane); IR (KBr) 3400 (br, OH), 1705 (C=O) cm^{-1}; MS *m*/*e* 265 (M), 77, 45.

General procedure for synthesis of methyl 3-alkylthio-5-phenyl-4-isothiazolecarboxylates 9a,b (Scheme 2). Dry gaseous hydrochloric acid was passed into a refluxing solution of 4-isothiazolecarboxylic acid **8a,b** (1.6 mmol) in absolute methanol for 12 h. Concentration to a small volume and dilution with diethyl ether gave the products **9a,b**. The following compounds were obtained:

Methyl 3-methylthio-5-phenyl-4-isothiazolecarboxylate (9a). Yield 56%; mp 62–63 °C (ligroin); IR (KBr) 1698 (C=O) cm^{-1}; MS *m*/*e* 265 (M), 129, 59.

Methyl 3-ethylthio-5-phenyl-4-isothiazolecarboxylate (9b). Yield 59%; mp 104–105 °C (cyclohexane); IR (KBr) 1702 (C=O) cm^{-1}; MS *m*/*e* 279 (M), 77, 59.

Biology

Viruses and cells. Poliovirus 1 (Brunhilde strain), echovirus 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^{-1} of streptomycin and 200 units mL^{-1} 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 maximum concentration of 0.01% DMSO, which was not toxic to our cell lines.

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, 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^{-1}) 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.[15,16] 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^{-1}) and DMEM containing compound **9a** (100 and 500 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 9a 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 **9a** (10 μM), for the adsorption period. The compound was removed or added at various times after the adsorption period, respectively, as indicated in Figure 1. The plates were incubated at 37 °C for 8 h and were then frozen. Virus yield was determined by plaque assay.

Acknowledgements

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