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In vitro antiviral activity of four isothiazole derivatives against poliovirus type 1

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Summary

The in vitro effects of four isothiazoles [5,5′-diphenyl-3,3′-diisothiazole disulfide, 5-phenyl-3-mercapto-isothiazole, 5,5′-(4-chlorophenyl)-3,3′-diisothiazole disulfide, and 5-(4-chlorophenyl)-3-mercapto-isothiazole] on poliovirus type 1 were studied. The derivatives tested demonstrated remarkable viral inhibition, with a higher selectivity index than the previously studied iminodithiole precursors. Under one-step growth conditions, all the isothiazole derivatives caused the greatest activity if added during or after (within 1 h) poliovirus adsorption. These data suggest interference with early events of viral replication. [5-^{3}H]Uridine incorporation into RNA showed that the compounds tested reduced poliovirus RNA synthesis, which was completely shut off after 2 h of incubation and reduced by 50–60% after 4 h. Also, pretreatment of the cell cultures with the compounds for 24 h caused a substantial inhibition of viral replication. The data suggest that the four isothiazole derivatives may have a multi-step antiviral mode of action different from their iminodithiole precursors.

Isothiazole derivative; Poliovirus type 1; RNA synthesis

Introduction

In previous studies marked in vitro antiviral activity of 3-imino-5-phenyl-

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3H-1,2-dithiole hydrochloride (PDTI) and 3-imino-5-(4-chlorophenyl)-3H-1,2-dithiole against poliovirus type 1, echovirus type 9 and adenovirus type 17 was demonstrated (Pinizzotto et al., 1990).

Further experiments on poliovirus type 1 replication showed that PDTI inhibits the viral RNA synthesis, suggesting an early inhibitory effect on the transcriptional and/or replicative functions of the poliovirus genome (Garozzo et al., 1990). The identification of these new antiviral agents led to the synthesis of corresponding structural isomers [5-phenyl-3-mercapto and 5-(4-chlorophenyl)-3-mercapto isothiazoles] (Wooldridge, 1972) and their respective disulfides (see Fig. 1), and to the testing of their antiviral activities in cell cultures. These compounds demonstrate an in vitro antiviral activity similar to that of their precursors (Pinizzotto et al., 1991a).

In the present study we have investigated some aspects of the effects of four isothiazole derivatives on replication of poliovirus type 1.

Materials and Methods

Cells

Human aneuploid HEp-2 cells were grown at 37°C in Falcon 75 cm^2 tissue culture flasks (Falcon Plastic, Oxnard, CA) using Eagle's Minimum Essential Medium (MEM) supplemented with 200 μg of streptomycin and 200 U of penicillin G per ml.

Six percent heat-inactivated fetal calf serum (FCS, Gibco) was added to the medium for cell growth (growth medium); the concentration was reduced to 2% for cell maintenance (maintenance medium).

Virus

Poliovirus type 1 (Brunhilde strain) was grown in HEp-2 cells maintained in MEM supplemented with 2% FCS. Virus stocks were prepared by inoculating HEp-2 monolayers at low multiplicity at 37°C. When the cytopathic effect (CPE) involved most of the cell monolayer, the cultures were freeze-thawed 3 times and titered by plaque assay. The virus was stored in aliquots at −20°C until used.

For the plaque assay method, HEp-2 cells growing in plastic trays containing 96 wells (tissue culture plates Flow Laboratories, VA) were infected with 10-fold dilutions of each sample (4 wells for each dilution), and after the adsorption period the monolayers were washed with phosphate-buffered saline (PBS) to remove the unadsorbed virus and overlaid with MEM containing 1% methylcellulose.

The monolayers were examined microscopically, and when plaques appeared clearly (usually after 24–48 h), the solid overlay was removed, the cultures were stained with 1% crystal violet in methanol and the plaques were counted.

Compounds

The structural formulae of the four isothiazole derivatives are shown in Fig. 1. The synthesis and chemical properties of compounds **1** and **2** were previously reported (Tornetta, 1960; Condorelli et al., 1967; Grandin and Vialle, 1967). They were prepared with required modifications to improve their yield. A similar procedure was used to prepare compounds **3** and **4**. The melting points, yields and analytical data are as follows:(**1**) 5,5′-diphenyl-3,3′-diisothiazole disulfide, m.p: 72°C;(**2**) 5-phenyl-3-mercapto-isothiazole, m.p. 67°C;(**3**) 5,5′-(4-chlorophenyl)-3,3′-diisothiazole disulfide, m.p. 155°C, from C_2H_5OH (90%). Analysis for $C_{18}H_{10}Cl_2N_2S_4$: Calcd. %: C,47.68: H,2.22: N,6.17: S,28.28. Found: C,47.75: H,2.21: N,6.19: S,28.23.(**4**)5-(4-chlorophenyl)-3-mercapto-isothiazole, m.p.88°C (70%). Analysis for $C_9H_6ClNS_2$: Calcd. %: C,47.57:

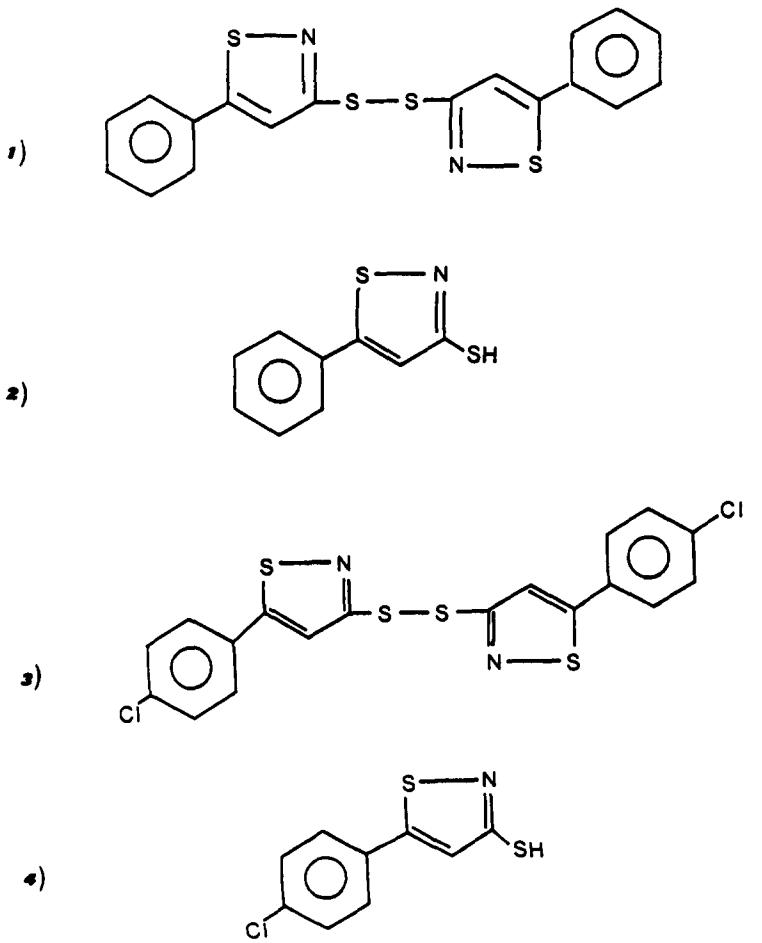

Fig. 1. Structural formulae of isothiazole derivatives: (1) 5,5′-diphenyl-3,3′-diisothiazol disulfide; (2) 5-phenyl-3-mercapto-isothiazole; (3) 5,5′-(4-chlorophenyl)-3,3′-diisothiazol disulfide; (4) 5-(4-chlorophenyl)-3-mercapto-isothiazole.

H,2.65: N,6.14: S,28.15. Found: C,47.69: H,2.70: N,6.08: S,28.10.

All the compounds were initially dissolved in dimethyl sulfoxide (DMSO, Merck) at 0.1 M and further diluted in maintenance medium before use to give concentrations ranging between 0.05 and 150 μM.

Cellular toxicity

HEp-2 monolayers were prepared in 24-well tissue culture plates (16 mm diameter, Linbro, Flow Laboratories, VA, USA) and when the cells were confluent, monolayers were washed with PBS and exposed to various concentrations (150, 100, 75, 50, 25, 12.5 and 0 μM) of the compounds (4 wells were used for each concentration). Plates were checked by light microscopy after 12, 24 and 48 h. Cytotoxicity was scored as morphological alterations (e.g. rounding up, shrinking, detachment).

The effect of the compounds on cell proliferation was also evaluated by both a quantitative colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, (MTT, Sigma cat. no. M2128) and incorporation of [*methyl*-^{3}H]thymidine into DNA (dpm/mg DNA), as previously described (Garozzo et al., 1990).

The concentration of compounds that inhibited at 48 h the incorporation of thymidine to 50% of the control value was estimated as the 50% cytotoxic concentration (CC_{50}).

Antiviral activity

(a) Virus yield reduction assay. HEp-2 cells were grown in 24 well-plates in growth medium and incubated at 37°C. When the cell cultures became confluent (3×10^5 cells/well), growth medium was removed and the cells were infected with the virus at varying multiplicities of infection (MOI) of 1, 0.1, 0.01 and 0.001 plaque-forming unit (PFU) per cell.

After 30 min at 37°C of adsorption, the inoculum was removed, the monolayers were washed with PBS, and maintenance medium containing various concentrations of compounds was added (using 3 wells per sample). Cell control (no drug or virus), drug control (no virus) and virus control (no drug) were included in each plate. When 100% cytopathic effect (CPE) was observed in the virus control, usually after 24–48 h, the samples were freeze-thawed 3 times and titered by plaque assay as described above.

(b) Plaque reduction assay. Confluent HEp-2 cells (1×10^6) were grown in 3.5 cm diameter 6-well tissue culture plates (NUNC) and infected with 300 PFU of the virus stock per well.

After 30 min of virus adsorption at 37°C, overlay medium containing 2% methylcellulose without (control) or with the test compounds ranging between 50 and 0.05 μM were added to the infected cell monolayers.

After 24–48 h of incubation at 37°C, when the plaques appeared clearly in virus controls, the overlay was removed and cells stained with 1% crystal violet

in methanol. The number of visible plaques was then counted under light microscopy.

The anti-poliovirus activity of the compounds was determined as % decrease in number of plaques according to the following formula:

$$\% \text{ decrease of plaques} = \frac{\text{No. of plaques (control)} - \text{No. of plaques (test)}}{\text{No. of plaques (control)}} \times 100$$

The compound concentration required to inhibit poliovirus plaque formation by 50% is expressed as IC_{50}.

Culture pretreatment

Pretreatment of cultures was performed by exposing the cell monolayers to 25 and 50 μM of the compound in maintenance medium for 2, 6, 12 and 24 h at 37°C. After treatment the cells were washed thoroughly with PBS and inoculated with virus at an MOI of 0.001. Virus titration was performed as described above for the antiviral activity assay.

Virucidal activity

To test possible virucidal activity, equal volumes (0.5 ml) of virus stock suspension (containing 10^7 PFU/ml) and MEM containing 50 and 100 μM of the 4 compounds were mixed in test tubes and incubated for 2 h at 37°C.

After incubation each virus mixture was diluted 10-fold serially and assayed for any virus remaining in HEp-2 cells, where the concentration of the compounds should be less than 0.1 μM so as not to interfere with virus replication. Infectious virus was then titered by plaque assay as described above.

Addition at different time intervals

Monolayers of HEp-2 cells were grown in 24-well plates (inoculum 2×10^5 cells/well). Cells were synchronized first by giving a cold shock (4°C) to the cultures and then bringing them back to 37°C (Newton and Wildy, 1959). Plates were then inoculated with the virus at an MOI of 0.01. The compounds (50 μM) were added during virus adsorption and discarded at the end of the adsorption period or added at various times (0, 0.5, 1, 2, 3, 4, 5 and 6 h) after the adsorption period.

After 8 h, cultures were frozen and virus yield was determined by plaque assay.

Isothiazole derivatives and RNA synthesis

HEp-2 cells grown in 6-well plates (inoculum 5×10^5 cells/well) were pretreated for 2 h before infection and throughout the cycle with maintenance medium plus 2.5 μg/ml of actinomycin D (Sigma) to inhibit cellular RNA

synthesis. Cells were infected with the virus (MOI 0.01) and incubated for 30 min at 37°C with or without compounds **1** and **2** (50 μM). After this time the cells were washed with PBS and incubated for 2 or 4 h with 1 μCi/ml of [5-^{3}H]uridine (specific radioactivity 46 Ci/mmol, Amersham) in the presence or absence of the compounds (50 μM). At the end of the incubation period, cells were washed thoroughly with cold 0.9% NaCl. Nucleoproteins were precipitated with 1 N PCA for 30 min at 4°C and then washed with 0.5 N PCA. The precipitate was treated with cold absolute ethanol and then dried. The resulting powders were resuspended in 0.3 N KOH and incubated at 37°C for 18 h to hydrolyze RNA to mononucleotides. Aliquots were used for the spectrophotometric and radioactivity measurements. RNA was determined by UV absorption using the following equivalence: 1 μg/ml of RNA is equal to 0.03 OD/cm at λ = 260 nm (Løvtrup-Rein, 1970). The results are expressed as specific radioactivity (dpm of [5-^{3}H]uridine incorporated into total RNA/mg of total RNA).

Results

Cytotoxicity

No significant cytotoxicity (inhibition of cell growth using both MTT assay and incorporation of [*methyl*-^{3}H]thymidine) was observed for either compound at the concentration of 50 μM after 48 h of treatment. Therefore this concentration was employed for antiviral activity determinations. The values of cytotoxic concentration (CC_{50}) for the 4 compounds are shown in Table 1.

Antiviral activity against poliovirus type 1

Virus yield reduction. All isothiazole derivatives inhibited poliovirus replication in the virus yield reduction assay in HEp-2 cells. The sensitivity of poliovirus to the compounds was not affected by the host-cell system used.

TABLE 1

Antiviral indexes of the four isothiazole derivatives in HEp-2 cells

[a]50% inhibitory concentration: concentration of isothiazole derivatives required to inhibit plaque formation by 50%. The IC_{50} values were determined from dose-response curves presented in Fig. 3. [b]50% cytotoxic concentration: concentration of compounds that inhibited at 48 h the incorporation of [*methyl*-^{3}H]thymidine into DNA to 50% of the control value. [c]Selectivity index determined by dividing CC_{50} by IC_{50}.

Compounds	Inhibitory[a] concentration (μM) (IC_{50})	Cytotoxic[b] concentration (μM) (CC_{50})	Selectivity[c] index
1	0.35	89.28	255
2	0.42	90.75	216
3	1.10	88.39	80
4	1.34	87.50	65

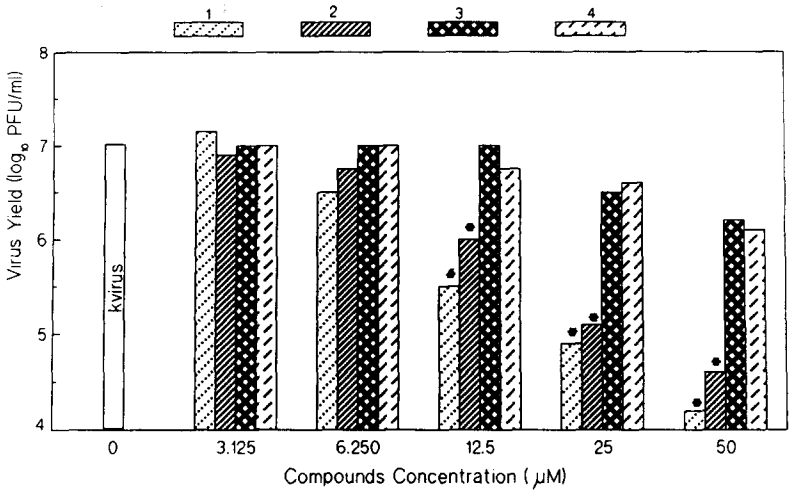

Fig. 2. Effect of various compound concentrations on poliovirus yield. Poliovirus (MOI 0.001) was incubated with various concentrations of the four compounds. Student's *t*-test was used to assess the significance of the differences between non-treated and compound-treated virus-infected cells. $^{*}P < 0.001$.

In fact, when African green monkey kidney (Vero) cells were employed, there was no difference in the virus yield reduction observed (data not shown). The effect of various concentrations of the 4 compounds on poliovirus replication in HEp-2 cells at an MOI of 0.001 is shown in Fig. 2. The extent of inhibition was related to the concentration of the compounds. We observed that within the concentration range of 3.125–50 μM the compounds (particularly compounds **1** and **2**) produced a concentration-dependent decrease in virus yield. Table 2 demonstrates that the antiviral effect of the 4 isothiazole derivatives depends on the MOI. When HEp-2 cells were infected at different MOIs and 50 μM of the

TABLE 2

Effect of the four isothiazole derivatives (50 μM) on poliovirus replication in HEp-2 cells at different multiplicities of infection

[a]Antiviral activity is expressed as $\log_{10}$ units of virus yield reduction as compared with the virus control (i.e. infected cultures without compounds). Data represent the mean ± S.E.M. of five different experiments.

Isothiazole derivative (50 μM)	MOI			
	1	0.1	0.01	0.001
	Antiviral activity[a]			
1	0	0	1.4 (±0.07)	2.70 (±0.1)
2	0	0	1.1 (±0.06)	2.63 (±0.1)
3	0	0	0.3 (±0.01)	0.78 (±0.03)
4	0	0	0.38 (±0.01)	0.95 (±0.04)

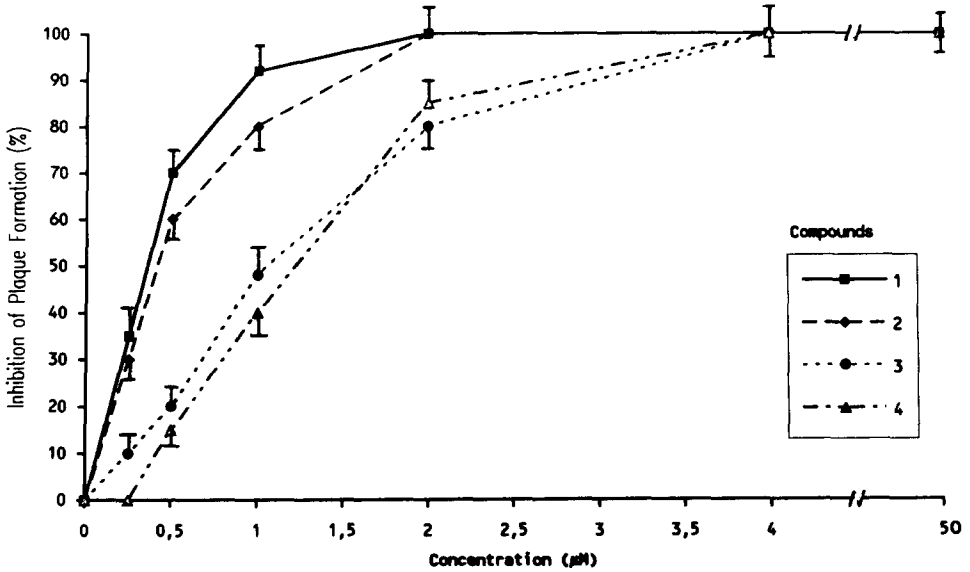

Fig. 3. Concentration–response curves for the four isothiazole derivatives against poliovirus type 1 replication in HEp-2 cells, as monitored by a plaque reduction assay. Data represent the mean ± S.E.M. of five different experiments.

various compounds were added, increasing MOIs reduced the inhibitory activity of the compounds. In fact, the concentration that was effective at the multiplicity of 0.001 produced little or no virus yield reduction at an MOI of 0.1 or greater.

Inhibition of plaque formation. Concentration–response curves for the plaque number obtained in the virus-infected cells exposed to varying concentrations of the compounds are presented in Fig. 3. The concentrations required to reduce plaque formation by 50%, i.e. IC_{50} values, are listed in Table 1.

TABLE 3

Effect of pretreatment of HEp-2 cells with the four isothiazole derivatives on inhibition of poliovirus type 1 replication

[a]Cells were incubated with 50 μM of the compounds for 24 h at 37°C. The cells were washed with PBS before infection at an MOI of 0.001. Student's *t*-test was used to assess significance of the difference between non-pretreated and pretreated cells: $P < 0.001$.

Sample	Isothiazole derivative (50 μM)	Virus yield (pfu/ml)	$\log_{10}$ reduction in virus titer
Virus control	0	2.5×10^7	–
Compounds added[a] before infection	1	1×10^4	3.39
	2	7.5×10^3	3.52
	3	9×10^4	2.44
	4	3.2×10^4	2.89

Compounds **1** and **2** had an equivalent and significant antiviral activity (mean IC_{50} values for **1** and **2** were 0.35 and 0.42 μM, respectively), whereas compounds **3** and **4** showed IC_{50} values of 1.1 and 1.34 μM, respectively. The selectivity index (ratio of the CC_{50}/IC_{50}) for the compounds reflects their activity and ranged from 65 to 255 (Table 1).

Effect of cell culture pretreatment

Pretreatment of the cell cultures for 24 h with the compounds resulted in substantial inhibition of virus replication. The effects of 50 μM pretreatment on poliovirus replication are presented in Table 3. The highest reduction of virus yield was observed with compounds **1** and **2** (3.39 and 3.52 $\log_{10}$ units, respectively). Compounds **3** and **4** showed a lower activity causing a $\log_{10}$ yield reduction of 2.44 and 2.89, respectively. If the cell pretreatment period was shortened to 12, 6, or 2 h, no significant inhibition of virus yield was noted (data not shown).

Virus inactivation

No loss of infectivity was observed when poliovirus was incubated with the four compounds at concentrations of 50 and 100 μM for 2 h at 37°C.

Effect of time addition of compounds

In order to determine whether the compounds inhibited the yield of infectious virus during a specific period in the virus cycle, 50 μM of the various

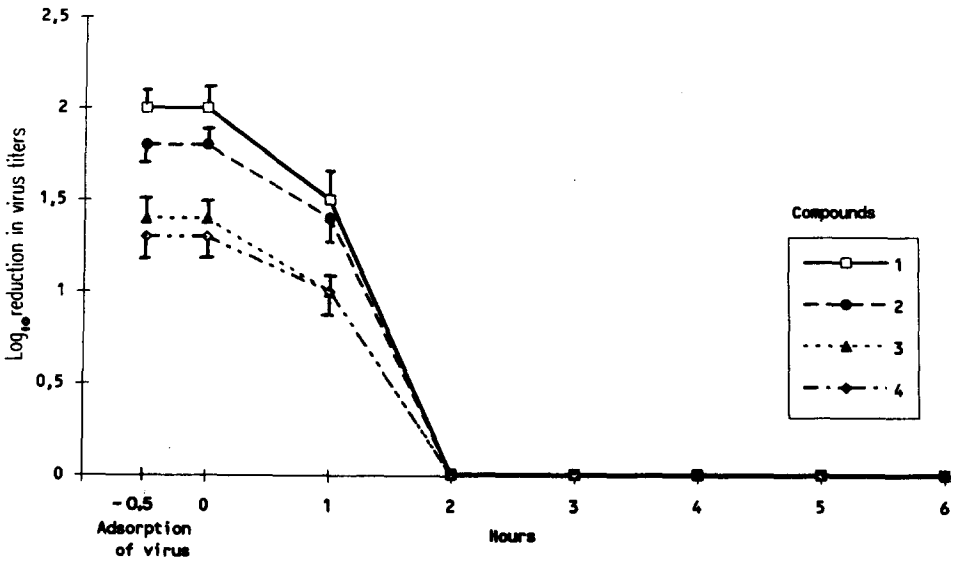

Fig. 4. Effect of addition of four isothiazole derivatives (50 μM) at various times during and after adsorption period to infected HEp-2 cells with poliovirus. Data represent the mean ± S.E.M. of five different experiments.

compounds were added simultaneously or at various times after poliovirus adsorption (30 min). In all cases incubation with the compounds was continued until 8 h after virus adsorption when the total virus yield was measured. The results reported in Fig. 4 show that all the compounds had their greatest effect when added simultaneously with the virus, and maximal inhibition (2 $\log_{10}$ units) of virus yield was observed for compound **1**. However, there was still a

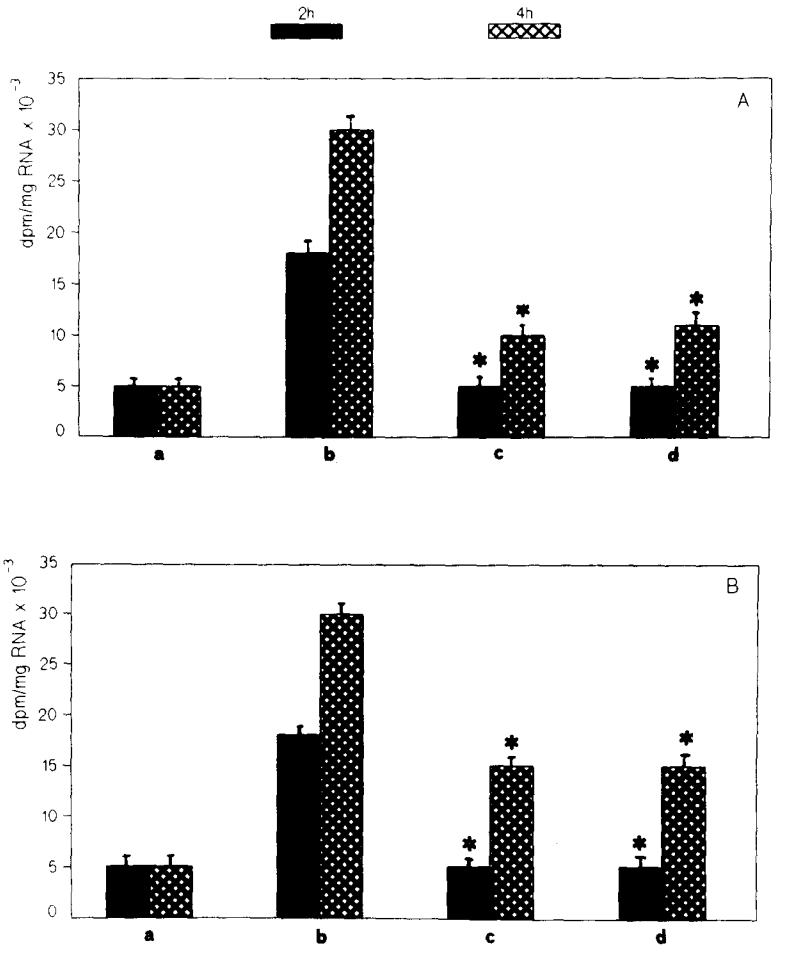

Fig. 5. Incorporation of [5-^{3}H]uridine into total RNA of poliovirus infected and mock-infected cells in the presence and absence of the compounds (50 μM) at 2 and 4 h post-infection. Cellular RNA synthesis was inhibited by actinomycin D (2.5 μg/ml) added 2 h before infection. Actinomycin D was present throughout the growth cycle. (a) Mock-infected HEp-2 cells + Act.D; (b) virus-infected HEp-2 cells + Act. D; (c) virus-infected HEp-2 cells + Act. D + compound: virus and compound were added simultaneously and discarded after the adsorption period (30 min); (d) virus-infected HEp-2 cells + Act. D + compound: compound was added at the end of adsorption period. (A) Data for compound **1**. (B) Data for compound **2**. Data represent the mean ± S.E.M. of five different experiments. Student's *t*-test was used to assess the significance of the difference between non-treated (b) and compound-treated (c and d) virus-infected cells. $^*P < 0.001$.

reduction in virus yield even when the compounds were added within 1 h after the adsorption period and maintained until the end of the replicative cycle. In all cases, addition of the compounds 2 h after poliovirus adsorption was completely ineffective.

Effect on poliovirus RNA synthesis

Incorporation of [5-^{3}H]uridine into total RNA of virus-infected and mock-infected cells was studied for the more active compounds (**1** and **2**).

In our experimental conditions actinomycin D inhibited cellular RNA synthesis with an efficiency of 92%, and none of the compounds had any effect on cellular RNA synthesis at the concentrations tested (data not shown).

Fig. 5 shows the specific activity of [5-^{3}H]uridine incorporated into total RNA in mock-infected (a) and virus-infected cells (b). As can be seen, labeled precursor incorporation is higher in virus-infected cells than in the former (a). Therefore, the difference between the (a) and (b) values can be attributed to viral RNA synthesis. Addition of the two compounds tested led to inhibition of viral RNA synthesis. In fact, in the presence of the two compounds no viral RNA synthesis occurred at 2 h of the virus growth cycle. At 4 h, poliovirus RNA synthesis was reduced by 60% for compound **1** and by 50% for compound **2** (d). A similar inhibition was observed if the compounds were added simultaneously with the virus and discarded at the end of the adsorption period (c).

Discussion

The four isothiazole derivatives are selective inhibitors of poliovirus replication, as shown by the great difference between the concentrations which affected virus replication and cell metabolism. Higher selectivity indexes than observed for their precursor PDTI (79), were demonstrated for compounds **1** and **2** (255 and 216, respectively). This indicates that starting from PDTI new derivatives can be obtained that are endowed with lower cytotoxicity and higher antiviral activity.

The antiviral activity of the compounds was monitored by both virus plaque reduction and virus yield reduction. Previous studies have shown that these two test methods may provide contradicting results, since they detect different aspects of viral infection in cultured cells (Hu and Hsiung, 1989; Boyd et al., 1987). In addition, the diverse test conditions (i.e. virus harvesting time and virus challenge dose) in these two methods may contribute to a discrepancy in the antiviral evaluation results, as reported by other authors (Hu and Hsiung, 1988). In our assays, the two compounds (**1** and **2**) that showed the highest activity in the plaque reduction assays (Table 1), were also the most active in the virus yield reduction assays (Table 2). Moreover, our data demonstrated that the antiviral effect was MOI-dependent: increasing MOIs reduced the inhibitory activity of the compounds (Table 2).

From experiments whereby the compounds were added at various times during and after virus adsorption, it is clear that the antiviral effect is achieved very early in the poliovirus replicative cycle. In fact, all the compounds were most effective when added during or within 1 h after poliovirus adsorption. Moreover, as previously demonstrated for the PDTI precursor (Garozzo et al., 1990), experiments on [5-^{3}H]uridine incorporation into RNA showed that both compounds **1** and **2** inhibited poliovirus RNA synthesis. Viral RNA synthesis was completely shut off after two hours of incubation.

Our preliminary experiments (Pinizzotto et al., 1991b), using radiolabeled poliovirus and evaluating the sensitivity to ribonuclease treatment, have shown that viral attachment and uncoating are unaffected by compound **1**.

The data obtained from culture pretreatment experiments have shown that all the derivatives when added 24 h before viral infection have a remarkable inhibitory effect on viral replication. In fact, the greatest reduction in poliovirus yield was observed for compounds **1** and **2** (3.39 and 3.52 $\log_{10}$ reduction, respectively) (Table 3). This effect was not observed for the PDTI precursor.

Further studies are now under way to elucidate the precise mode of action of the compounds and to verify whether the antiviral activity observed with cell culture pretreatment is due to the induction of natural antiviral substances (i.e. interferon) or to an intracellular accumulation of the derivatives.

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