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Antipoliovirus activity and mechanism of action of 3-methylthio-5-phenyl-4-isothiazolecarbonitrile

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ABSTRACT

Our previous studies described the synthesis and the antiviral activity of 3,4,5-trisubstituted isothiazole derivatives that were found to be particularly effective against enteroviruses. Compound *3-methylthio-5-phenyl-4-isothiazolecarbonitrile* (IS-2) exhibited an interesting anti-poliovirus activity with a high selectivity index.

In the present study we investigated the mechanism of action of this compound. Studies on the time of IS-2 addition to poliovirus type 1 infected cells suggested that the compound may inhibit some early process of viral replication. In order to determine its mechanism of action, we evaluated the rate of attachment and internalization of purified [^{3}H]uridine-labeled poliovirus to HEp-2 cells in the presence or absence of IS-2. No effect on poliovirus adsorption and internalization to host cells was detected. We also investigated the influence of the compound on virus uncoating using labeled poliovirus and measuring the radioactivity of oligoribonucleotides formed from viral RNA susceptible to ribonuclease. These experiments demonstrated that poliovirus uncoating is influenced by IS-2 action.

1. Introduction

Isothiazole compounds, including their analogs, play an important role in pharmaceutical research. The isothiazole ring has been incorporated into several compounds possessing a wide range of activities: e.g. protein kinase (MEK) (Varaprasad et al., 2006), histone acetyltransferase (Stimson et al., 2005), and vascular endothelial growth factor receptors I and II (VEGFR-1/2) (Kiselyov et al., 2009) inhibitors.

Moreover, 3,5-di-substituted 4-isothiazolecarbonitriles are known as herbicidal, insecticidal and fungicidal agents (Walsh and Wooldridge, 1972), and the fusion of isothiazole to pyrimidine gave derivatives with antitumor or sedative properties (Wooldridge, 1972).

Few research groups have investigated isothiazoles as antiviral inhibitors. Kuczynski et al. (1984) demonstrated for isothiazolehydrazides a weak antiviral activity against polio and herpes simplex viruses.

During the course of routine screening of compounds for antiviral activity, it was discovered that some 3,4,5-trisubstituted-isothiazoles demonstrated significant inhibitory activity against enteroviruses (Cutrì et al., 1998, 1999). Structure–activity relationship studies revealed that some chemical modifications at three different positions in the isothiazole nucleus had significant effects on both potency and spectrum of activity (Cutrì et al., 2002, 2004; Garozzo et al., 2000, 2007).

In particular, we demonstrated that the presence of a short thioalkyl chain in the 3-position, a cyano group or methylester group in the 4-position, and an unsubstituted phenyl ring in the 5-position of the isothiazole ring are the structural features which seem to ensure the best activity profile against polioviruses and Echoviruses (Cutrì et al., 1999).

Currently, there are no specific antipicornavirus agents approved by the US Food and Drug Administration (FDA) even if a considerable number of compounds have been reported as potent and selective inhibitors of picornavirus replication (De Palma et al., 2008a, 2008b). Among these compounds there are the capsid binding agents and the inhibitors that target the enteroviral non structural protein 2C and/or 3A, and the virus encoded protease 2A and/or 3C. Some of these have been close to clinical approval for the treatment of rhinovirus and non-polio enterovirus infections (De Palma et al., 2008b).

Several drugs, some of which have been (rupintrivir and its analog compound 1, pirodavir, valopicitabine) or are being (pleconaril) studied in the clinical setting, have shown a potent anti-poliovirus activity *in vitro* (Barnard et al., 2004; Collett et al., 2008; De Palma et al., 2008a; Florea et al., 2003; Patick et al., 1999, 2005). Unfortunately, none of these has yet been approved for the treatment of poliovirus infections.

In the present study, compound *3-methylthio-5-phenyl-4-isothiazolecarbonitrile*, coded IS-2, was selected from the series of 4 isothiazole derivatives, since it exhibited the highest *in vitro* activ-

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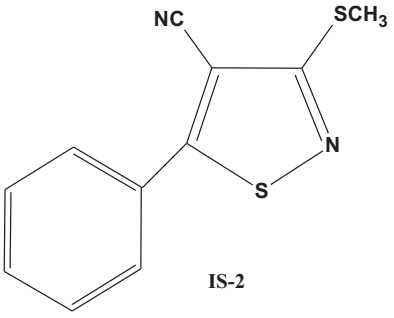

Fig. 1. Structural formula of 3-methylthio-5-phenyl-4-isothiazolecarbonitrile (IS-2).

ity against poliovirus type 1 compared with the previously studied compounds. In fact, IS-2 had no adverse effect on HEp-2 cell cultures up to a concentration of 20 μM, which is much higher than the 50% inhibitory concentration of poliovirus type 1 (IC_{50} = 0.045 μM) (Cutrì et al., 1999).

The aim of the present study was to determine the mechanism whereby IS-2 inhibits poliovirus type 1 replication.

2. Materials and methods

2.1. Compound

Fig. 1 shows the structural formula of the isothiazole derivative 3-methylthio-5-phenyl-4-isothiazolecarbonitrile (coded IS-2). The synthesis and chemical properties of this compound were previously reported (Cutrì et al., 1998).

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

2.2. Viruses and cells

Poliovirus type 1 Sabin strain was propagated in HEp2 cells at 37 °C. Cells were kept in a humidified 5% carbon dioxide atmosphere at 37 °C and grown in D-MEM supplemented with 10% heat inactivated foetal calf serum (FCS), 200 μg/ml of streptomycin and 200 units/ml of penicillin G. The working stock solution was prepared as cellular lysates using D-MEM with 2% FCS (maintenance medium).

2.3. Effect of time of addition

Monolayers of cells were grown to confluence in 24-well plates and inoculated with virus 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 IS-2 compound for the adsorption period. The inoculum was then removed and the medium, with or without the compound, was added at various times after the adsorption period, as indicated in Fig. 2. The plates were incubated at 37 °C for 8 h, the cultures were then frozen and virus yield was determined by plaque assay as previously described (Cutrì et al., 1999).

2.4. Preparation of purified radiolabeled poliovirus

Virus stocks were prepared in HEp-2 cells infected at a MOI of 10 and incubated at 37 °C in the presence of Actinomycin D (2 μg/ml). After 3 h from the addition of the virus, 25 μCi/ml of [5,6-^{3}H] uridine (specific radioactivity 43 Ci/mmol) was added to the cell cultures.

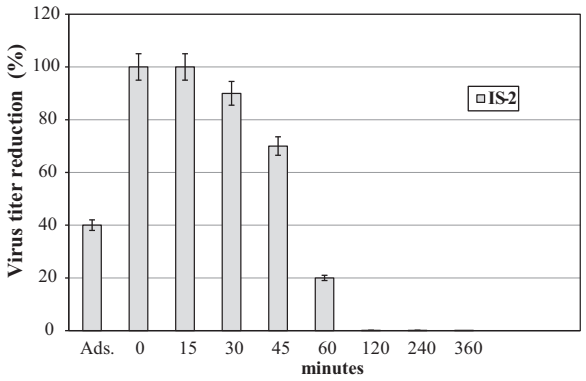

Fig. 2. Effect of addition of compound IS-2 (10× IC_{50}) at various times during the replicative cycle of poliovirus type 1. Time 0 = post 2 h adsorption period at 4 °C. The concentrations used are ratios with respect to the IC_{50} (e.g. 10× the IC_{50} is 10 times the IC_{50} of the compound). Each value represents the mean ± S.E.M. of three separate assays.

The virus was harvested when the cells showed an extensive cytopathic effect.

To purify the virus, the culture medium was clarified by centrifugation at 700 × *g* for 15 min at 4 °C, then the virus was sedimented at 100,000 × *g* for 6 h at 4 °C in a Beckman SW28 rotor. The pellet was treated with 1% Nonidet P 40 (Sigma) and then layered onto a 45–15% linear sucrose gradient and centrifuged at 80,000 × *g* for 4 h at 4 °C.

The radiolabeled virus was detected between the 10th and 14th fraction. These fractions were diluted with PBS, pelleted, and suspended in the maintenance medium. 1 ml of purified poliovirus gave 2.9×10^4 dpm/2×10^8 PFU.

2.5. Assay for inhibition of virus adsorption and internalization

Infective center assay was used to study the effect of the compound on the virus adsorption step. A cell suspension (10^6 cells/ml) was cooled to 4 °C for at least 1 h. Polio 1 (10^6 PFU/ml) was incubated for 60 min at 37 °C with different concentrations of the test compound (1×, 10× and 100× the IC_{50}), cooled to 4 °C, and subsequently added to the cell suspension. Cells were incubated with the virus-drug mixtures for 120 min at 4 °C to prevent the virus from entering the cells. After the adsorption period, unadsorbed virus and free compound were removed by washing three times with cold D-MEM. The cells were then diluted serially and plaque assayed for cell-associated viral activity (Dewindt et al., 1994).

This step was also investigated with purified [^{3}H]uridine-labeled virus. Briefly, HEp-2 cells were infected with purified radiolabeled virus (2×10^4 dpm/sample) and incubated for 2 h at 4 °C with or without IS-2 (1×, 10× and 100× the IC_{50}). For the experiment of virus internalization, infected cells were incubated for another 1 h at 37 °C, washed in cold PBS and treated for 1 h at 0 °C with 5 mM EDTA to remove non internalized virions. Cells were then washed in cold PBS, dissolved in 1 ml of 0.3 M NaOH and cell-bound radioactivity was determined in a liquid scintillation counter.

2.6. Assay for inhibition of viral uncoating

To examine the effect of the compound on virus uncoating we evaluated the sensitivity of internalized [^{3}H]uridine-labeled virions to ribonuclease A: cell monolayers were infected with purified radiolabeled poliovirus (2×10^4 dpm/sample) for increasing lengths of time (15, 30, 45, 60, 75, and 90 min) at 37 °C with or without the compound (1×, 10× and 100× the IC_{50}). The virus inoculum was then removed; the cells were washed in cold PBS, freeze-thawed

three times and incubated with ribonuclease A (50 μg/ml) in 0.02 M Tris buffer (pH 7.5) for 60 min at 37 °C. The reaction was stopped by adding TCA (5% final concentration). After 5 h at 4 °C nucleoproteins were collected by low speed centrifugation (400 × *g* for 10 min a 4 °C). The precipitates were washed with 5% TCA (acid-precipitable fraction), resedimented and dissolved in 1 ml of 0.3 M NaOH. The supernatant fluid (acid-soluble fraction) was used to estimate the amount of viral RNA that had become uncoated and susceptible to RNase treatment. The radioactivity of acid-soluble and -precipitable fractions was measured. Uncoating virus data are expressed as the percentage of acid-soluble radioactivity with respect to the total measured radioactivity (De Sena and Mandel, 1976).

3. Results and discussion

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

Compound *3-methylthio-5-phenyl-4-isothiazolecarbonitrile*, coded IS-2, exhibited remarkable viral inhibition against poliovirus type 1, with a selectivity index of 444, which was higher than those obtained using the previously studied isothiazole derivatives (Cutrì et al., 1999; Garozzo et al., 2000).

In the present study, we explored the antiviral mechanism of action of IS-2 against poliovirus type 1.

In order to determine whether IS-2 inhibited the virus yield during a specific period in the poliovirus cycle, the effect on compound addition at different time intervals using HEp2 cells was studied. Results obtained from these experiments clearly demonstrate that IS-2 interferes with an early step of the viral replicative cycle of poliovirus type 1. In fact, it was most effective when it was added at the end of the adsorption period. Addition later than 45 min after the virus adsorption period did not cause any virus yield reduction. Only a slight reduction (40%) was observed if the compound was added during the adsorption period (Fig. 2).

As IS-2 exerted its virus-specific activity through the inhibition of an early event in poliovirus type 1 replication, we set up some experiments in order to determine its mechanism of action.

The effect of the compound on the virus adsorption was studied by the infective center assay. Results obtained from this experiment demonstrated that IS-2, at concentrations up to 100 times the IC_{50}, did not inhibit adsorption of poliovirus 1 (Fig. 3).

In order to obtain confirmatory results, we adopted another experimental condition using purified [^{3}H]uridine labeled poliovirus. We also used this method to determine the effect of IS-2 on internalization of the virus in the cells.

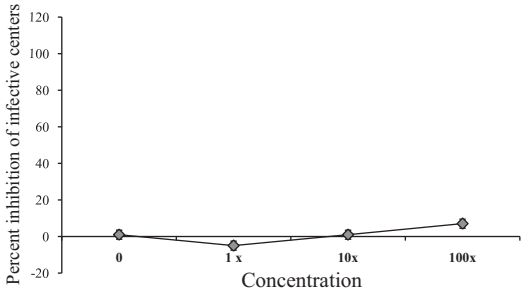

Fig. 3. Effect of compound IS-2 (1×, 10×, 100× the IC_{50}) on the adsorption of poliovirus type 1. Infective center assay data are plotted as percentage inhibition relative to the no-drug controls. The concentrations used are ratios with respect to the IC_{50}. Each value represents the mean ± S.E.M. of three separate assays.

Table 1
Effect of compound IS-2 on the adsorption and internalization of radiolabeled virus in the cells.

IS-2 (μM)[b]	Cell-bound radioactivity (dpm/sample)[a]	
	Adsorption (2 h, 4 °C)	Internalization (2 h, 4 °C plus 1 h, 37 °C + EDTA)
0	1976 ± 124	2056 ± 104
1× IC_{50}	1930 ± 99	2032 ± 102
10× IC_{50}	1956 ± 113	2132 ± 122
100× IC_{50}	1972 ± 108	2044 ± 117

[a] Each value represents mean ± S.E.M. of 3 different experiments.
[b] The concentrations used are ratios with respect to the IC_{50} (e.g. 10× IC_{50} is 10 times the IC_{50} of the compound).

The results obtained with this experiment (Table 1) demonstrated that the virus adsorbed at the same rate in the presence or absence of IS-2, indicating that this compound had no detectable inhibitory effect on virus adsorption. Similarly, the compound did not appear to affect internalization as demonstrated by the radiolabeled virus count after incubation for an additional 1 h at 37 °C and further treatment with EDTA. Therefore, the slight reduction of virus yield observed during the adsorption period in the experiments of time of addition was, probably, due to residual compound that could interfere with the following steps.

Since poliovirus was shown to be adsorbed in the presence of the compound, the next step was to investigate the effect of IS-2 on poliovirus uncoating.

The uncoating of poliovirus RNA, a step in the poliovirus replication cycle after the attachment of the virus to a cell surface receptor, is the target of capsid-binding anti-picornavirus compounds. It has been suggested that WIN compounds may interact directly with the viral capsid to stabilize it or to prevent changes which are required for the uncoating process. It is known that the interaction of poliovirus with its receptor leads to the loss of the VP4 protein and the extrusion of the amino-terminus of VP1; this causes the conversion into an altered A particle, which is believed to be an essential intermediate in cell entry. Although the uncoating process is not well understood, experimental evidence suggests that many A particles elute from the cell, while a small amount remains bound and releases the RNA into the cytoplasm (Fricks and Hogle, 1990; Hogle, 2002; Racaniello, 1996).

The conformational changes in the poliovirus particle are crucial in the uncoating process and consequently in viral infectivity. In fact, the antiviral WIN compounds inhibit virus replication by binding tightly in the hydrophobic pocket located within VP1 on the virion surface: filling this pocket they make some viral proteins more rigid, reducing capsid flexibility and making the virus more resistant to uncoating (McKinlay et al., 1992; Phelps and Post, 1995; Rotbart et al., 1998).

The results obtained with the experiments performed to evaluate the sensitivity of internalized [^{3}H]uridine-labeled poliovirus to ribonuclease A demonstrated that compound IS-2 may exert its effect by the inhibiting uncoating step. As can be seen in Fig. 4, the percentage of uncoating virus calculated by the radioactivity of oligoribonucleotides obtained from viral RNA susceptible to ribonuclease A (acid-soluble fraction) with respect to total radioactivity, was significantly lowered in the presence of the compound.

Maximal inhibition was observed with a concentration of 100 times the IC_{50} of the compound.Our previous studies revealed that the presence of a particular substituent at three different positions in the isothiazole nucleus had significant effects on spectrum of activity (Cutrì et al., 2002, 2004; Garozzo et al., 2000, 2007); as an example, the presence of bulky substituents in the 5-position caused a loss of antiviral activity against polio 1 and Echo 9 and was

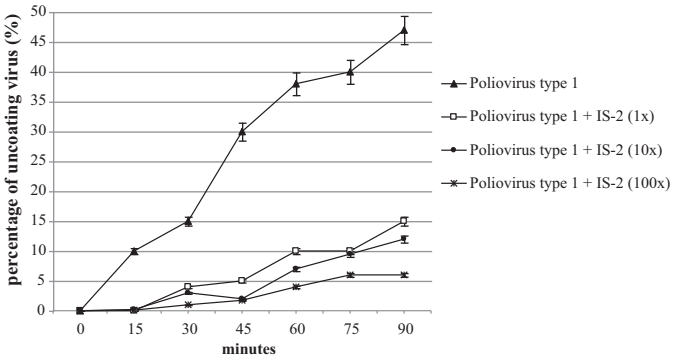

Fig. 4. Percentage of uncoating poliovirus type 1 (dpm of acid-soluble RNA/total dpm) in HEp2 cells in the presence or absence of IS-2 (1×, 10×, 100× the IC_{50}) at various time intervals. The concentrations used are ratios with respect to the IC_{50}. Each value represents the mean ± S.E.M. of three separate assays.

responsible for a good anti-rhinovirus activity. Moreover, mode of action studies showed that the compounds with anti-rhinovirus activity inhibit viral replication via inhibition of attachment of the virions to the cells, suggesting a potential capsid-binding activity for these compounds (Garozzo et al., 2000).

The preliminary results on the mechanism of action presented in this report indicate that poliovirus uncoating is influenced by IS-2 action.

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