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Inhibitory effects of 3-imino-5-phenyl-3H-1,2-dithiole on poliovirus type 1 replication in vitro

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Summary

The effect of 3-imino-5-phenyl-3H-1,2-dithiole (PDTI) on different steps of the replicative cycle of poliovirus type 1 in HEp-2 cells was studied. This compound inhibited the replication of poliovirus type 1 as shown by cytopathic effect and virus yield reduction. This inhibitory action was not due to a virucidal effect, nor did the cells to have been pretreated. Under one-step growth conditions 3-imino-5-phenyl-3H-1,2-dithiole caused the greatest inhibition if added within 1 h after poliovirus adsorption. [5-^{3}H]uridine incorporation into RNA showed that PDTI reduced poliovirus RNA synthesis. In fact, in the presence of PDTI viral RNA synthesis was shut off completely at 2 h post infection, and at 4 h post infection viral RNA synthesis was reduced by 50%. The compound may have an inhibitory effect on the early transcriptional and/or replicative functions of the poliovirus genome.

3-Imino-5-phenyl-3H-1,2-dithiole; Poliovirus type 1; Antiviral activity

Introduction

The synthesis and biological activities of N-substituted 3-imino-3H-1,2-dithiole salts have been extensively studied (Mollier and Lozac'h, 1961; Biton et al., 1968; Montagne, 1968). Although compounds such as 3-(arylimino)-4,5-dichloro-3H-1,2-dithioles show pronounced activity against phytopathogenic fungi, e.g. *Sphaerotheca pannosa* and *Phytophthora infestans* (Bader and Gaetzi, 1970), little is known about the biological activities of the 3-imino-5-aryl-3H-1,2-dithioles. The chemical properties of the latter compounds were previously reported (Tornetta,

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1960; Behringer and Bender, 1967; Condorelli et al., 1967; Grandin and Vialle, 1967, 1971).

In a preliminary antiviral screening program using 3-imino-5-aryl-3H-1,2-dithioles we found that 3-imino-5-phenyl-3H-1,2-dithiole and 3-imino-5-(4-chlorophenyl)-3H-1,2-dithiole possess remarkable antiviral activity (M.R. Pinizzotto, personal communication).

Therefore, in the present study we have investigated the effect of 3-imino-5-phenyl-3H-1,2-dithiole chloridrate (PDTI) on in vitro 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 titred by plaque assay. 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, U.S.A.) were infected with ten-fold dilutions of each sample (four wells for each dilution) and after the adsorption period the monolayers were washed with phosphate-buffered saline (PBS) to remove unadsorbed virus and overlaid with MEM containing 1% methylcellulose.

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

Compound

The structural formula of PDTI is shown in Fig. 1. The compound has been prepared and purified by methods used for the synthesis of imino-dithiole salts, as previously reported [Condorelli et al., (1967); Tornetta (1960)]. It is a yellowish crystalline solid, m.p. 198°C. The compound was initially dissolved in dimethyl sulfoxide (DMSO, Merck) at 10 mg/ml (43.5 mM) and further diluted in maintenance medium before use to give concentrations ranging between 0.5 and 100 μg per ml.

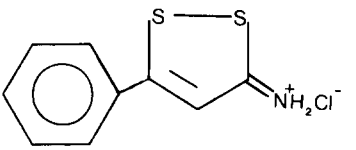

Fig. 1. Structural formula of 3-imino-5-phenyl-3H-1,2-dithiole (PDTI).

Cellular toxicity

The effect of PDTI on cell proliferation was measured as follows: HEp-2 monolayers were prepared in 24-well tissue culture plates (16 mm diameter, Linbro, Flow Laboratories, VA, U.S.A.), and when the cells were confluent, monolayers were washed with PBS and exposed to various concentrations (100, 50, 20, 10, 5, 1 and 0 μg/ml) of PDTI (4 wells were used for each concentration). Plates were checked by light microscopy after 6, 12, 24 and 48 h. Cytotoxicity was scored as morphological alterations (e.g. rounding up, shrinking, detachment). Cytotoxicity 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 inhibition of host cell DNA synthesis.

MTT. Briefly, HEp-2 cells were prepared in 96-well plates and serial concentrations of PDTI were added and incubated for 6, 12, 24 and 48 h at 37°C. Five mg/ml of MTT was dissolved in PBS and filtered to sterilize and remove the small amount of insoluble residue present. This MTT solution (10 μl × 100 μl medium) was added to all wells and plates were incubated at 37°C for 4 h. Acid-isopropanol (100 μl of 0.04 N HCl in isopropanol) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. The optical density (OD) was read at 570 nm on a Titertek Multiskan MCC/340, within 1 h of adding the isopropanol (Mosmann, 1983).

DNA synthesis. HEp-2 cells were prepared (5×10^5 cells per well) in 3.5 cm diameter six-well tissue culture plates (NUNC); after 24 h of incubation at 37°C serial concentrations of PDTI were added and maintained for 6, 12, 24 and 48 h. [Methyl-^{3}H]thymidine (specific radioactivity 91 Ci/mmol, Amersham) was added at 1 μCi/ml in the presence or absence of the compound for 2 h before the end of incubation. Then, DNA was extracted and assayed according to the procedure of Schmidt and Tannhauser (1945). Briefly, cells were washed several times with cold 0.9% NaCl. Nucleoprotein and protein were precipitated with 1 N perchloric acid (PCA) × 30 min at 4°C. The precipitates were washed in 0.5 N PCA, treated with cold absolute ethanol and dried. The resulting powders were resuspended in 0.3 N KOH and incubated at 37°C for 18 h in order to hydrolyze RNA to mononucleotides. After hydrolysis, DNA and protein were precipitated by addition of cold 60% PCA until pH was acid. DNA was extracted twice with 0.5 N PCA at 70°C × 20 min. Aliquots of nucleic acid hydrolysates were employed for

spectrophotometric assay and radioactivity measurement. DNA was determined by UV absorption using the following equivalence: 1 μg/ml of DNA is equal to 0.027 OD/cm at λ = 260 nm (Ceriotti, 1955). All radioactive measurements were made with a Beckman scintillation counter model LS 7500. The counts were corrected for 100% efficiency and expressed as dpm/mg DNA. The concentration of compound which inhibited at 48 h the incorporation of thymidine to 50% of the control value was estimated as the 50% inhibitory dose (ID_{50}).

Antiviral activity

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 compound was added (using three 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 three times and titered by plaque assay as described above.

Plaque reduction assay. Confluent HEp-2 cells (1×10^6) were grown in 3.5 cm diameter six-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, an overlay medium containing 2% methylcellulose without (control) or with concentration of PDTI ranging between 10 and 0.1 μg/ml was added to the infected cell monolayers.

After 48 h of incubation at 37°C, when the plaques appeared clearly in virus controls, the overlay was removed and the cells were stained with 1% crystal violet in methanol. The number of visible plaques was then counted under light microscopy. The anti-poliovirus activity of PDTI was determined as percent 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 PDTI concentration required to inhibit poliovirus plaque formation by 50% is expressed as ED_{50}.

Culture pretreatment

Pretreatment of cultures was performed by exposing the cell monolayers to various concentrations (50, 40, 30, 20, 10 μg/ml) of PDTI 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 μg of PDTI were mixed in test tubes and incubated for 2 h at 37°C. After incubation each virus mixture was diluted 10-fold serially. Aliquots of each dilution were inoculated to cell monolayers and incubated for 48 h at 37°C. Infectious virus was then titered by plaque assay as described above.

PDTI 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. PDTI (50 μg/ml) was 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 during the virus growth cycle. After 8 h, cultures were frozen and virus yield was determined by plaque assay.

PDTI 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 PDTI (50 μg/ml). After this time the cells were washed with PBS and incubated for 2 and 4 h with 1 μCi/ml of [5-^{3}H]uridine (specific radioactivity 46 Ci/mmol, Amersham) in the presence or absence of PDTI (50 μg/ml). 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 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 employed for spectrophotometric assay and radioactivity measurement. 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/mg RNA).

Results

Cytotoxicity

Data obtained on the cytotoxicity of PDTI (at 10, 50, and 100 μg/ml) are shown in Fig. 2. At a concentration of 10 μg/ml PDTI did not inhibit DNA synthesis or affect cell survival (MTT assay) after 48 h of treatment. Therefore, the maximum non-toxic dose was estimated at 10 μg/ml and employed for antiviral activity determinations. The ID_{50} was 37.2 μg/ml. The data obtained at 24 h of treatment were the same as observed at 48 h.

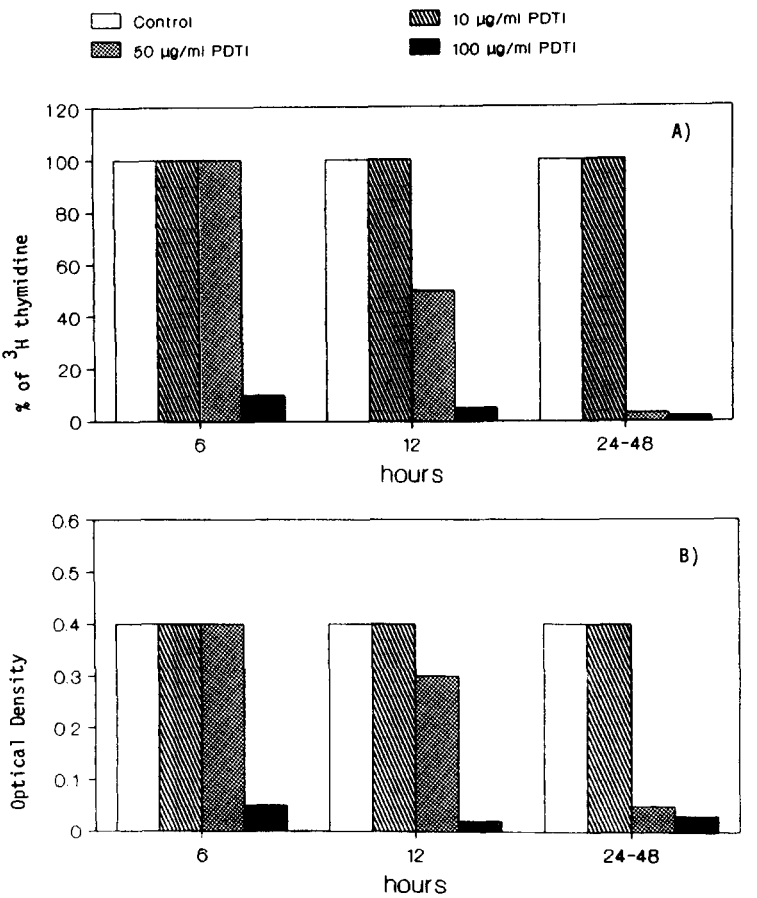

Fig. 2. Cytotoxicity of PDTI for uninfected HEp-2 cells after 6, 12, 24 and 48 h of incubation. (A) Percentage of in vitro incorporation of [methyl-^{3}H]thymidine into DNA (dpm/mg DNA). Control value was 100%. (B) Data are expressed in optical density (O.D.) values of living cells (MTT test). The data obtained at 24 h were identical to those obtained at 48 h.

When the incubation time was reduced to 6 h, PDTI concentrations up to 50 μg/ml did not cause any toxicity. Consequently, tests with short incubation time may allow the use of higher concentrations of the compound (50 μg/ml).

Activity of PDTI against poliovirus type 1

Virus yield reduction. The effect of various PDTI concentrations on the replication of poliovirus in HEp-2 cells infected at different multiplicities of infection (1, 0.1, 0.01 and 0.001) is shown in Fig. 3. The most remarkable activity of PDTI was obtained at the multiplicity of 0.001; in fact concentrations of 0.5–10 μg/ml produced a dose-dependent increase in virus yield reduction.

Virus yield reduction at 5 and 10 μg/ml was 4 log, while 1 μg/ml of PDTI produced a 2 log reduction in virus titer. A concentration of 0.5 μg/ml was ineffective. The increase of virus yield reduction depends on the MOI: a concentration of 10 μg/ml PDTI, at the multiplicity of 0.01, reduced infectious virus production by only 2 log and little or no virus yield reduction was seen at a MOI of 0.1 or greater.

Inhibitory effect of PDTI on plaque formation. The inhibitory effect of PDTI on poliovirus plaque formation in HEp-2 cells is shown in Fig. 4. From the dose-response curve, the ED_{50} (or dose that inhibited plaque formation by 50%) was determined graphically. It was 0.47 μg/ml. The therapeutic index of PDTI was calculated from the ID_{50}/ED_{50} ratio and was 79.

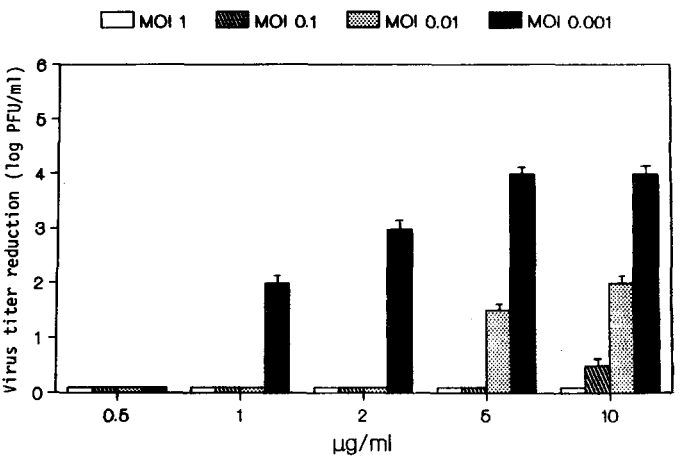

Fig. 3. Effect of various PDTI concentrations (0.5, 1, 2, 5 and 10 μg/ml) on poliovirus replication in HEp-2 cells infected at different multiplicities of infection. Antiviral activity is expressed as $\log_{10}$ units of virus yield reduction as compared with the virus control (i.e. virus-infected cultures without PDTI). Data represent the mean ± SEM of five different experiments.

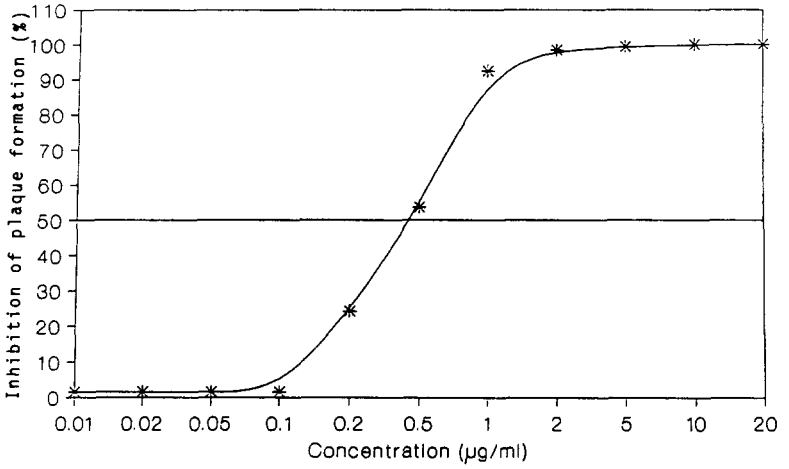

Fig. 4. Inhibitory effect of PDTI on poliovirus type 1 plaque formation in HEp-2 cells. HEp-2 cells were infected with poliovirus and treated with the indicated concentrations of PDTI. Data represent the mean ± SEM of five different experiments.

Effect of cell culture pretreatment

No reduction in virus yield was observed when HEp-2 monolayers were pretreated with various concentrations of PDTI for 2, 6 and 12 h (data not shown).

Virus inactivation

No loss of infectivity was observed when poliovirus was incubated with PDTI at concentrations of 50 and 100 µg/ml for 2 h at 37°C.

Effect of time of PDTI addition

In order to determine whether PDTI inhibited the yield of infectious virus during a specific period in the virus cycle, the compound was added simultaneously or at various times after poliovirus adsorption (30 min) using a MOI of 0.01. In all cases incubation with PDTI was continued until 8 h after virus adsorption when the total virus yield was measured.

As shown in Fig. 5, addition of PDTI to poliovirus-infected HEp-2 cells within 1 h after poliovirus adsorption caused maximal inhibition (2 log) of virus yield. The compound was ineffective when added during the virus adsorption period. Since PDTI was active only when added during the early phase of the viral replication cycle, its effect on viral RNA synthesis was investigated.

Effect of PDTI on poliovirus RNA synthesis

The incorporation of [5-^{3}H]uridine into viral RNA in the presence of actinomycin D is depicted in Fig. 6. Under our experimental conditions, actinomycin D inhibited

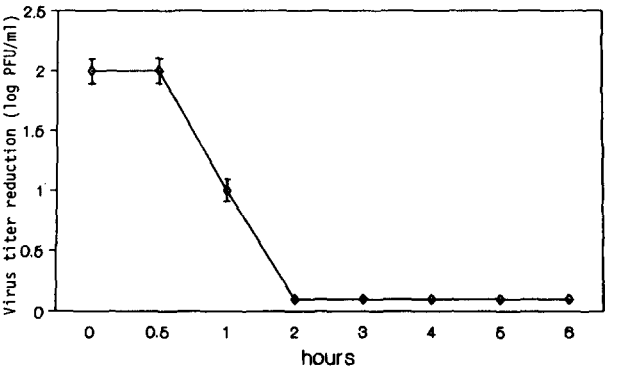

Fig. 5. Effects of addition of PDTI (50 μg/ml) at various times after the virus adsorption period to HEp-2 cells infected with poliovirus (MOI: 0.01). Data represent the mean ± SEM of five different experiments.

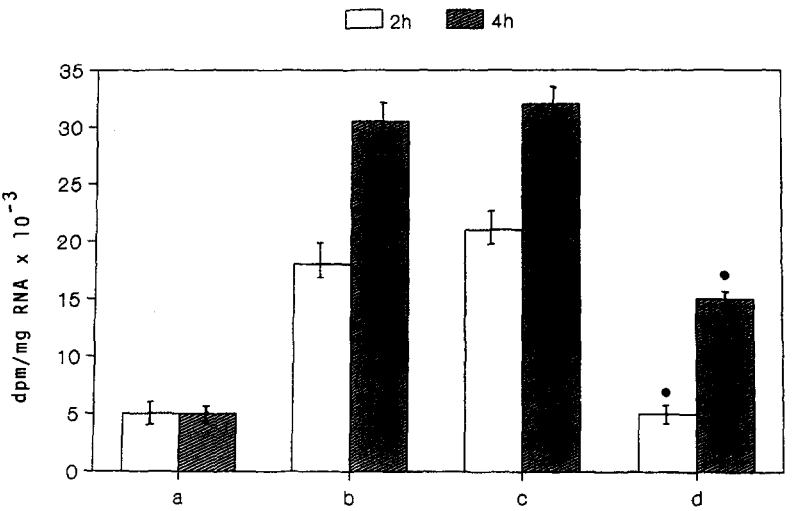

Fig. 6. Incorporation of [5-^{3}H]uridine into RNA of poliovirus type 1 in the presence and absence of PDTI (50 μg/ml) at 2 and 4 h post infection. Cellular RNA synthesis was inhibited by actinomycin D (2.5 μg/ml) added 2 h before infection. Actinomycin D was present throughout the growth cycle. (a) Mock-infected HEp-2 cells + Act. D; (b) Virus-infected-HEp-2 cells + Act. D; (c) Virus-infected HEp-2 cells + Act. D + PDTI: virus and PDTI were added simultaneously and discarded after the adsorption period (30 min); (d) Virus-infected-HEp-2 cells + Act. D + PDTI: PDTI was added at the end of the virus adsorption period. Data represent the mean ± SEM of five different experiments. Student's *t*-test was used to assess the significance of the difference between non-treated (b) and PDTI-treated (d) virus-infected cells. (•) $P < 0.001$.

cellular RNA synthesis with an efficiency of 92% and PDTI had no effect on cellular RNA synthesis at the concentrations tested (data not shown). Our data show that PDTI inhibited viral RNA synthesis when added after poliovirus adsorption. In fact, at 2 h of the virus growth cycle no viral RNA synthesis had occurred; at 4 h

viral RNA synthesis was reduced by 50%. It must also be noted that when PDTI was added simultaneously with the virus and discarded at the end of adsorption period, no detectable inhibition of viral RNA synthesis was observed.

Discussion

The results described here demonstrate that 3-imino-5-phenyl-3H-1,2 dithiole exerts a significant inhibitory effect in vitro on poliovirus type 1 at concentrations below the cytotoxic doses. Its 50% effective dose (ED_{50}) was found to be 0.45 μg/ml and was very much lower than its 50% cytotoxic dose (37.2 μg/ml). This finding points to a rather selective activity of PDTI against the virus tested.

We used two methods to obtain information on the antiviral activity of PDTI: plaque reduction and virus yield reduction. The former test revealed higher activity. It has been previously shown that these two assays may provide contradicting results, since they might detect different aspects of viral infection in cultured cells (Hu and Hsiung, 1989; Boyd et al., 1987). Furthermore, the activity obtained in the virus yield reduction assay was dependent on the virus multiplicity of infection (Fig. 3). Hu and Hsiung (1989) have reported that the MOI can substantially influence the evaluation of antiviral activity by both the plaque reduction method and yield reduction assay. Moreover, the sensitivity of poliovirus to PDTI was not affected by the host cell system used, as previously noted in our preliminary screening experiments, where also African green monkey kidney (VERO) cells were employed (Pinizzotto et al., in press, 1990).

Since PDTI does not exert any virucidal action, it is believed that a specific step in the viral replicative cycle must be inhibited. The effect of PDTI under different experimental conditions was studied in order to understand the mechanism of inhibition. Results obtained from studies of culture pretreatment and compound addition at different time intervals suggest that PDTI does not exert its antiviral effect via inhibition of virus attachment but interferes with a subsequent step of the virus replicative cycle.

In fact, addition of PDTI within 1 h after the end of the adsorption period resulted in significant antiviral activity. Addition of PDTI during virus adsorption and later than 1 h after the virus adsorption period did not result in any virus yield reduction. This suggests that the compound may act at an early step of the virus replicative cycle (following virus adsorption).

Results on [5-^{3}H]uridine incorporation into RNA showed that PDTI reduced poliovirus RNA synthesis pointing to an effect of PDTI on early transcriptional or replicative functions of the poliovirus genome. However, our data did not reveal which step(s) of the viral RNA synthesis process are blocked by PDTI. Nevertheless, we have previously reported (Pinizzotto, personal communication) that imino-dithiole derivatives act against some RNA-plus (+) strand viruses, while they are ineffective against some RNA-minus (−) strand viruses, such as measles or vescicular stomatitis virus. We suggest that PDTI may play a role as inhibitor of the transcription and/or synthesis of the virus-coded RNA polymerase. Therefore,

its mechanism of action remains the subject of further studies.

In conclusion, PDTI can be considered a compound endowed with a selective antiviral activity against poliovirus type 1, as demonstrated by its therapeutic index of 79. Although this value does not indicate a very high selectivity of PDTI as antiviral agent, additional studies with a series of PDTI derivatives with a higher therapeutic index (data not shown) encourage further studies with this class of compounds from an antiviral activity viewpoint.

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