ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 1972, p. 261-266

Vol. 2, No. 4
Printed in U.S.A.

Mechanism of Action of Gliotoxin: Elimination of Activity by Sulfhydryl Compounds

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Received for publication 17 August 1972

Gliotoxin and two other compounds, with antiviral activity against a number of ribonucleic acid (RNA) viruses and structurally related via the epidithiapiperazinedione moiety, appeared to be equally active in their oxidized and reduced forms. However, the ability of the reduced forms to inhibit viral RNA synthesis was abolished when these compounds were maintained in the reduced state by the simultaneous presence of a large molar excess of dithiothreitol or reduced glutathione. The active form therefore appeared to be that containing a disulfide bridge, and the apparent activity of the dithiol was due to cellular oxidation. Possible mechanisms by which the compounds could interact with viral proteins, e.g., viral RNA-dependent RNA polymerase, are proposed.

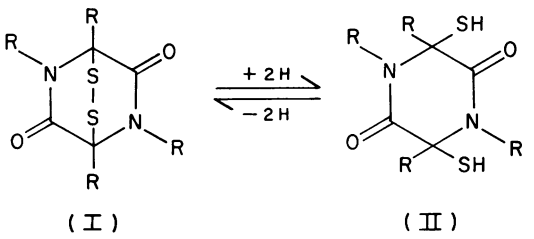
The microbial metabolites gliotoxin (18) and acetylaranotin (8, 10) and the synthetic compound *N*,*N*'-dimethylepidithiapiperazinedione (16) have in common the structural moiety I (Fig. 1; 1, 10, 16) and the ability to inhibit the multiplication of ribonucleic acid (RNA) viruses. In tissue culture, one or all of these compounds have been reported to inhibit picornaviruses of the poliovirus, echovirus, rhinovirus, and coxsackievirus type A subgroups; they also inhibit members of the myxovirus group, such as measles virus, influenza virus, and parainfluenza virus (5, 12, 16, 17). Antiviral activity against infections in animals has also been reported for gliotoxin against poliovirus type 1 and influenza B/Md (5) and for acetylaranotin against coxsackievirus A21 and influenza B/Md (17).

The three compounds appear to act by specifically inhibiting the synthesis of viral RNA (7, 16, 17), and this inhibitory activity has been attributed to the structural moiety I (16). Removal of the sulfur atoms results in complete loss of activity (16). Kinetic experiments have shown that the probable mode of action of these epidithiapiperazinediones is via inhibition of the action of viral RNA-dependent RNA polymerase rather than synthesis of this enzyme (7, 17). Because many viruses of different types are inhibited, the present studies were undertaken to define more precisely the mechanism of this inhibition.

During studies on the synthesis of *N*,*N*'-dimethylepidithiapiperazinedione and the preparation of derivatives of the naturally occurring epidithiapiperazinediones, it was discovered that the activity of the reduced form II (Fig. 1) of all members of the group tested was indistinguishable from that of the disulfide form I (9, 16). Indeed, even some derivatives of the reduced form, such as *S*-acetyl, that are potentially capable of biochemical conversion to form II, are active, whereas other derivatives, such as the thioether *S*-methyl (for which biochemical conversion is less likely), are inactive (16). Since intracellular conversion between form I and form II might be expected to occur, the question of which form(s) is the active one(s) arose. This is an important question because a likely mechanism by which the epidithiapiperazinediones could inhibit viral RNA-dependent RNA polymerase is via formation of a mixed disulfide with an essential sulfhydryl group on the enzyme. Only the native oxidized form I would be expected to participate in such an interaction, and in this paper we show that only this form is active.

MATERIALS AND METHODS

Cell culture. HeLa S_3 cells obtained in 1963 from Microbiological Associates, Inc., Bethesda, Md., were freed from mycoplasma contamination by a cloning method similar to that subsequently described by Robb (13). The cell line thus derived was designated U8; it has been periodically tested for mycoplasma contamination by three different test procedures (3, 14, 15) and has been found negative since isolation. Cells were grown at 37 C either as suspension cultures in Eagles's suspension culture medium containing 10% fetal calf serum (FCS), or as monolayer cultures in Eagle's minimal essential



(I) (II)

FIG. 1. *Structure of the oxidized (I) and reduced (II) forms of gliotoxin, acetylaranotin, and N,N'-dimethylepidithiapiperazinedione.*

medium (MEM) with Earle's salts base, and containing 5% FCS. Antibiotics were not included in the culture media. All media, medium components, and serum were obtained from Gibco, Grand Island, N.Y.

Virus preparation and purification. Poliovirus type I (Sabin) was grown in suspension cultures of HeLa S_3-U8 as follows. Cells from suspension culture were harvested by centrifugation, resuspended at 10^7 cells per ml in serum-free Eagle's suspension medium, and infected with poliovirus type I (Sabin) at 20 plaque-forming units (PFU) per cell. The infected cells were stirred for 30 min at 37 C and then diluted 10-fold with Eagle's suspension medium containing 5% FCS; the resulting suspensions were stirred at 37 C for 18 to 24 hr. Cells and cell debris were then removed by centrifugation at 10,000 × *g* for 30 min. Virus was concentrated from the supernatant fluid by polyethylene glycol 6000 (Union Carbide Inc., New York, N.Y.) "precipitation" (6) and further purified by isopycnic centrifugation in cesium chloride density gradients (SW 41 rotor, 150,000 × *g*, 48 hr, 4 C). The purified virus stock used in these studies was titrated on monolayers of HeLa S_3-U8 and contained 3×10^{12} PFU/ml.

Infection of cells and measurement of viral RNA and protein synthesis. In all kinetic experiments, a modified Eagle's medium (HEMA) similar to that described previously (7) was used, except that the bovine serum albumin concentration was 1 g/liter. The sodium bicarbonate concentration was 0.18 g/liter, and the medium contained penicillin, streptomycin, and neomycin (100 units, 100 μg, and 50 μg, respectively, per ml). Cells were infected with poliovirus and resuspended in HEMA; viral RNA and protein synthesis were then measured as described (7) except for the preparation of samples for scintillation counting. Trichloroacetic acid precipitates on wet membrane filters (Millipore Corp., Bedford, Mass.) were transferred to glass scintillation vials and dissolved in 5 ml of scintillation solution A (0.5% Butyl-PBD [Ciba Pharmaceutical Co., Summit, N.J.] in ethyleneglycol monomethyl ether-toluene, 1:1). A 5-ml amount of scintillation solution B (0.5% Butyl-PBD in toluene) was added, and the samples were counted in a liquid scintillation spectrometer. In earlier experiments, samples were counted in a Tri-Carb spectrometer (model 3300; Packard Instrument Co., Downers Grove, Ill.), and ^{14}C-spillover corrections were made with constant quenching assumed. Later, absolute activities of ^3H and ^{14}C were determined automatically by use of a Multi-Mat I liquid scintillation counter (Intertechnique Instruments Inc., Dover, N.J.) in the external standard mode.

Inhibitors. Acetylaranotin and gliotoxin were obtained from Lederle Laboratories, Pearl River, N.Y., and a further sample of acetylaranotin was obtained from Lilly Research Laboratories, Indianapolis, Ind. Acetylaranotin was converted to its dithiol form by reduction with sodium borohydride in solution in 95% ethanol. The product was extracted with chloroform, crystallized from absolute alcohol, and characterized by nuclear magnetic resonance and infrared spectroscopy (Trown, *unpublished data*). The dithiol form of gliotoxin was prepared by reduction of aqueous ethanolic solutions of the compound with dithiothreitol (DTT). The product was extracted with ethyl acetate, crystallized from alcohol, and characterized by infrared spectroscopy. However, this compound is unstable, particularly in aqueous solution, being partially reoxidized back to gliotoxin (Trown, *unpublished data*). Therefore, in experiments reported here, gliotoxin was reduced in situ with DTT, and was maintained in the reduced state by the continued presence of DTT.

Acetylaranotin dithiol was dissolved in dimethyl sulfoxide (DMSO), 15 mg/ml, and a 100-fold dilution was immediately made into rapidly stirred HEMA at 37 C. The final concentration of the compound in the infected cell suspension was 5 μg/ml. Fresh solutions were made for each experiment because acetylaranotin is relatively unstable in DMSO.

Gliotoxin was dissolved in warm absolute ethanol (1 mg/ml) and diluted 200-fold into HEMA at 37 C. A further 100-fold dilution was made at the time of addition to cell suspensions to give a final concentration of 0.05 μg/ml. Ethanolic solutions of gliotoxin are stable indefinitely at 4 C; solutions in HEMA are stable for about 1 week at 4 C.

1,4 - Dimethyl - 2,5 - piperazinedione - 3,6 - disulfide and the corresponding 3,6-dithiol were prepared by the method of Trown (16). Both compounds were dissolved in DMSO (1 mg/ml); the solution was immediately diluted 300-fold into HEMA, and the final concentration in the cell suspensions was 0.01 μg/ml.

Reducing and oxidizing agents. Reduced glutathione (GSH) was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio; DTT and oxidized DTT were obtained from Calbiochem, Los Angeles, Calif. Stock solutions were prepared in HEMA immediately before use and were added to cell suspensions to give final concentrations of 25 μg/ml (GSH) and 50 μg/ml (DTT and oxidized DTT).

RESULTS

The dithiol forms of the two epidithiapiperazinediones acetylaranotin and *N,N'*-dimethylepidithiapiperazinedione are stable, well-characterized substances, whereas that of gliotoxin is not (9, 16; see also Materials and Methods). Preliminary experiments were therefore carried

out with these compounds, in particular acetylaranotin dithiol, because it was more readily available.

When acetylaranotin dithiol was added to a suspension of poliovirus-infected HeLa cells during the linear phase of viral RNA and protein synthesis, it produced an effect indistinguishable from that of acetylaranotin itself (Fig. 2) or of gliotoxin (7). There was a rapid, complete inhibition of viral RNA synthesis with little or no effect on viral protein synthesis (Fig. 2). Similar results were obtained with the reduced and oxidized forms of the synthetic compounds *N,N'*-dimethylepidithiapiperazinedione (not shown). These results may be interpreted in two ways: either the reduced (dithiol) form and the oxidized (disulfide) form of these compounds are equally active, or only one form is active and the other is converted rapidly by the cells to that form under the conditions of the experiment. Therefore, conditions were sought under which the epidithiapiperazinedione under study would be maintained in only one form throughout the experiment.

Attempts were made, through the use of large excesses of biologically compatible thiol compounds, to maintain acetylaranotin in its dithiol form. GSH, when added simultaneously with acetylaranotin dithiol in approximately eightfold molar excess, prevented the immediate inhibition of viral RNA synthesis seen with acetylaranotin dithiol alone (Fig. 2). Viral RNA synthesis continued, but at a reduced rate; the amount of RNA synthesized approached 50% of the control value. Higher concentrations of GSH could not be used because they are toxic to the cells and caused inhibition of both viral RNA and protein synthesis.

The partial prevention of the inhibitory activity of acetylaranotin by GSH prompted us to examine other biologically compatible reducing agents in this system. DTT was shown to be very suitable for this purpose; it is less toxic than GSH, molar concentrations approximately four times those used for GSH being well tolerated by HeLa cells. In addition, it was found that DTT is capable of reducing aqueous solutions of epidithiapiperazinediones (see Materials and Methods). Therefore, it was not necessary to prepare and isolate the reduced form of the inhibitor being studied; instead, the reduction was carried out in situ by DTT added immedi-

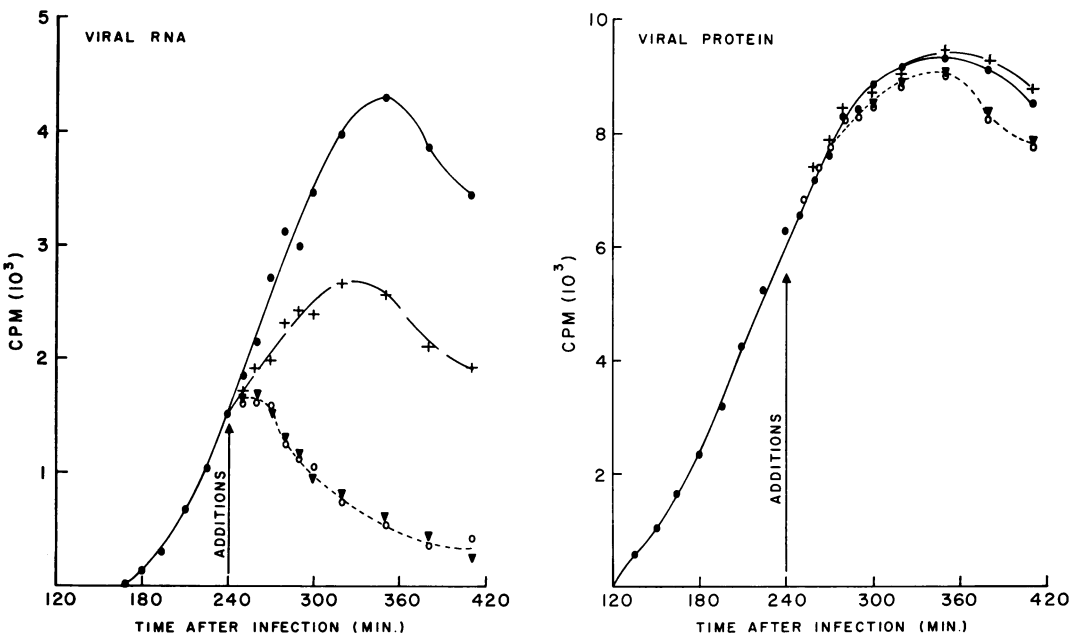


FIG. 2. *Effects on poliovirus RNA and protein synthesis of acetylaranotin alone and of acetylaranotin dithiol in the presence and absence of GSH. The preparation of poliovirus-infected HeLa cell suspensions containing actinomycin D, ^{3}H-uridine, and ^{14}C-labeled amino acids and the measurement of incorporation of ^{14}C and ^{3}H into trichloroacetic acid-insoluble materials are described in Materials and Methods. At 4 hr after infection, solutions of inhibitors and GSH were added to give the following final concentrations: ●, control, no additions; ○, acetylaranotin, 5 μg/ml; ▼, acetylaranotin dithiol, 5 μg/ml; +, acetylaranotin dithiol, 5 μg/ml, plus GSH, 50 μg/ml.*

ately before the inhibitor. Thus, it became possible to carry out all subsequent experiments with the relatively plentiful compound gliotoxin.

DTT, when added at a concentration of 50 μg/ml, completely prevented the inhibition of viral RNA synthesis by gliotoxin (Fig. 3). DTT alone appeared to be slightly stimulatory in this experiment, but this result was not consistently observed. Neither of the reducing agents alone or in combination with the inhibitors had a significant effect on the rate or amount of viral protein synthesis; results similar to those shown in Fig. 2 were obtained in every case.

These results clearly suggest that only the oxidized forms of gliotoxin, acetylaranotin, and probably all other epidithiapiperazinediones are active. This conclusion is further strengthened by the experiment illustrated in Fig. 4, in which gliotoxin was presumably maintained in its oxidized state by the simultaneous addition of the

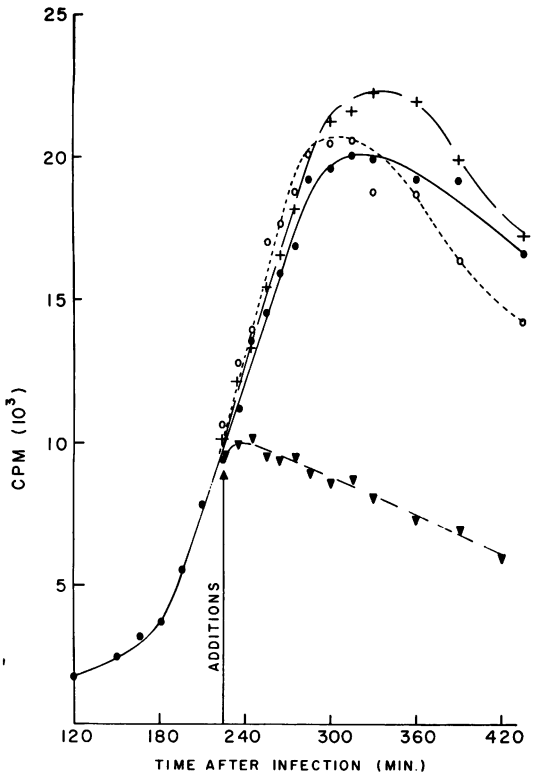


FIG. 3. *Elimination of the inhibition of poliovirus RNA synthesis by gliotoxin through simultaneous addition of DTT. At 3.75 hr after infection, solutions of inhibitors and DTT were added to give the following final concentrations: ●, control, no additions; ▼, gliotoxin, 0.05 μg/ml; +, DTT, 50 μg/ml; ○, gliotoxin, 0.05 μg/ml, plus DTT, 50 μg/ml.*

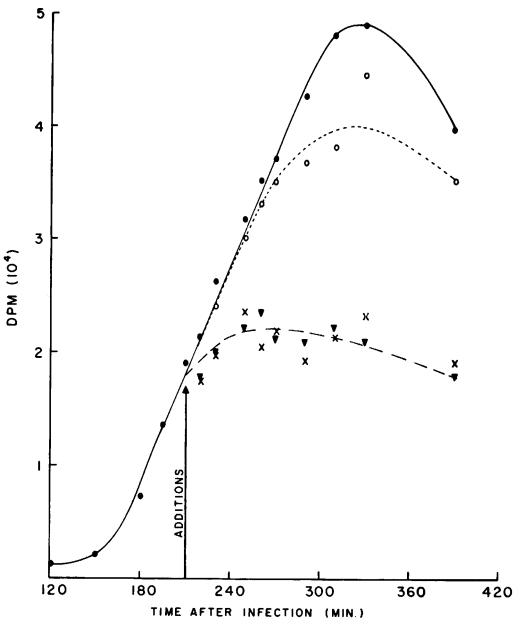


FIG. 4. *Effects of oxidized DTT and gliotoxin, alone and combined, on the synthesis of poliovirus RNA. Additions of the compounds were made 3.5 hr after infection, and the final concentrations were as follows: ●, control, no additions; ○, oxidized DTT, 50 μg/ml; ▼, gliotoxin, 0.05 μg/ml; × gliotoxin, 0.05 μg/ml, plus oxidized DTT, 50 μg/ml.*

oxidized form of DTT. The rates of inhibition of viral RNA synthesis by gliotoxin in the presence and absence of oxidized DTT were indistinguishable. Oxidized DTT alone was slightly inhibitory in this experiment. Again, none of these compounds had significant effects on the rate of viral protein synthesis (not shown).

Since GSH and DTT appear to prevent the inhibition of viral RNA synthesis by gliotoxin and acetylaranotin when added immediately before the inhibitors, attempts were made to reverse the inhibition after it had become fully established. A typical experiment is shown in Fig. 5. DTT was added 60 min after the addition of gliotoxin, when all viral RNA synthesis had ceased. The only effect observed was that the rate of degradation of viral RNA was decreased temporarily, as in this experiment, or permanently, as in other experiments not shown. Similar results were obtained when reversal was attempted 30 min after addition of gliotoxin.

DISCUSSION

The results of the experiments described in this paper clearly indicate that only the native (disulfide) forms of the antiviral compounds acetyl-

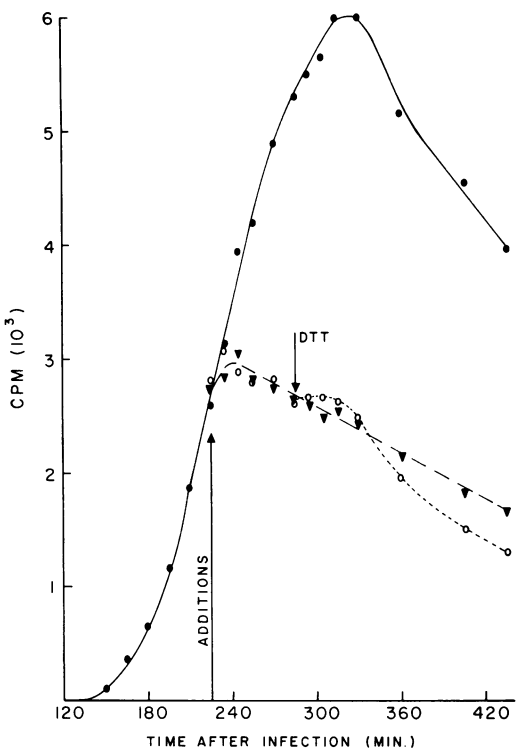


FIG. 5. *Attempt to reverse the inhibition of poliovirus RNA synthesis by gliotoxin through subsequent addition of DTT. Gliotoxin (0.05 μg/ml) was added to two suspension cultures of poliovirus-infected HeLa cells 3.75 hr after infection. One hour later, DTT (50 μg/ml) was added to one of these two cultures. (●) Control, no additions; (▼) gliotoxin added 3.75 hr postinfection; (○) gliotoxin added 3.75 hr postinfection, DTT added 4.75 hr postinfection.*

aranotin and gliotoxin are inhibitors of viral RNA synthesis. Previous reports (9, 16, 17) and present experiments (Fig. 2) indicated that the inhibition of viral RNA synthesis caused by the oxidized forms of these compounds is indistinguishable from that caused by their reduced forms, both quantitatively and kinetically. However, it has now been shown that the activity of the dithiol forms must be due to their rapid oxidation, under the experimental conditions, to the disulfide forms because, if a large excess of a reducing agent such as GSH or DTT is added simultaneously, antiviral activity is decreased or abolished. It is likely that similar arguments apply to other members of the epidithiapiperazinedione group of compounds, as the disulfide bridge seems to be essential for activity (16).

The effects of epidithiapiperazinediones on the kinetics of viral RNA and protein synthesis suggest that the primary target of inhibition is viral RNA synthesis (7). Complete inhibition of viral RNA synthesis can occur within 10 min of addition (e.g., Fig. 2), whereas viral protein synthesis continues almost unaffected until the normal shut-off occurs 300 min after infection. Thus, it appears likely that the epidithiapiperazinediones achieve their inhibitory effects through inhibition of the action of the viral RNA polymerase rather than its synthesis. The present results are consistent with this hypothesis, because an interaction between the inhibitors and a protein via formation of a mixed disulfide between the disulfide bridge of the inhibitor and a sulfhydryl group on a protein (possibly the polymerase) is possible, whereas such an interaction is unlikely with the dithiol forms of the inhibitors. However, the synthesis of poliovirus-specific proteins is a complex process, involving not only protein synthesis but also cleavage of what appears to be the translation product of the entire poliovirus genome into capsid and noncapsid viral proteins (4). Specific interference with this cleavage by the epidithiapiperazinediones would prevent production of active polymerase and, since the enzyme has been shown to be unstable in the cell (2), would lead to inhibition of viral RNA synthesis. However, the reported half-life for the enzyme (15 min) appears to be too long to account for the rapid cessation of RNA synthesis. It therefore appears more likely that the epidithiapiperazinediones inhibit the viral RNA polymerase directly. Studies are in progress to attempt to verify this conclusion.

ACKNOWLEDGMENT

Grateful acknowledgment is made to Mary Cormier for technical assistance during early phases of this work.

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