ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 1996, p. 433–436 Vol. 40, No. 2
0066-4804/96/$04.00+0

Anticytomegaloviral Activity of Methotrexate Associated with Preferential Accumulation of Drug by Cytomegalovirus-Infected Cells

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Received 22 June 1995/Returned for modification 20 September 1995/Accepted 14 November 1995

We extend the observation that inhibitors of pyrimidine biosynthesis are active against human cytomegalovirus by demonstrating that methotrexate (MTX) has preferential activity against cytomegalovirus replication. The 50% and 90% inhibitory concentrations of MTX for inhibition of cytomegaloviral DNA replication at 3 days postinfection in MRC-5 cells were 0.05 and 0.2 μM, respectively. No cell toxicity was observed in uninfected confluent cells at the highest concentration tested (1 μM). Under similar conditions (3 days of treatment with 0.2 μM MTX), intracellular dTTP pools were diminished in cytomegalovirus-infected cells (87% decrease relative to untreated infected cells, $P < 0.001$) but were not reduced in uninfected cells. A potential explanation for the preferential antiviral effect of MTX was that human cytomegalovirus-infected cells preferentially accumulated MTX. Increased intracellular accumulation and increased polyglutamation of MTX were observed in cytomegalovirus-infected cells compared with uninfected cells. Increased uptake of [^{3}H]MTX by cytomegalovirus-infected cells was first observed at 48 h postinfection, with threefold-higher accumulation within infected cells. By 96 h, accumulation had increased to approximately fourfold in comparison with uninfected cells. The uptake of [^{3}H]MTX was saturable and was blocked by addition of unlabelled MTX. Intracellular MTX in infected cells was almost entirely in the polyglutamated form, as demonstrated by thin-layer chromatography, whereas intracellular MTX was almost exclusively in the parent form in uninfected cells.

Cytomegalovirus is the leading cause of blindness among those with AIDS (7) and may also be the most common secondary infection of this group (3, 7, 9, 22). Treatment options for cytomegaloviral retinitis remain limited, and the currently available therapies have significant toxicities (2). Large DNA herpesviruses such as cytomegalovirus and the closely related herpes simplex virus (HSV) require a supply of deoxynucleoside triphosphate (dNTP) precursors in order to synthesize viral DNA (21). In the case of HSV, these dNTPs are partially supplied by virally encoded enzymes such as the deoxynucleoside or "thymidine" kinase of HSV (20). Nucleoside analogs specifically metabolized by the virally encoded thymidine kinase are effective against HSV infections and have low toxicity (19). However, human cytomegalovirus (HCMV) has no analog to the virally encoded deoxynucleoside kinase of HSV (5) and depends upon increasing the host biosynthesis of DNA precursors (15, 17). Consequently, there is no comparable virus-specific enzyme against which to direct therapy. The HCMV-encoded kinase UL97, although capable of phosphorylating ganciclovir, does not appear to be involved in supplying dNTPs. An HCMV mutant with defective UL97, while unable to phosphorylate ganciclovir, was not different from wild-type virus in its ability to grow and to induce an increase in dNTP pools (4).

The ability of HCMV to increase host dNTP pools appears to be critical to viral growth, since we and others have demonstrated that inhibitors of the de novo pathway of pyrimidine biosynthesis, such as azaserine and pyrazofurin, inhibit cytomegalovirus production (12). Methotrexate (MTX), which is an analog of folic acid, also inhibits the de novo biosynthesis of thymidine (1). Folic acid is a required cofactor for dihydrofolate reductase, a key enzyme in the de novo biosynthesis of thymidine. Through the addition of charged glutamyl groups (polyglutamation), cells actively accumulate both folic acid and MTX. By substituting for folic acid, MTX inhibits dihydrofolate reductase and selectively affects cells that are actively synthesizing DNA (6). Reasons proposed for the specificity of MTX against rapidly growing cells include increased (i) cellular uptake of the drug, (ii) polyglutamation and retention of the drug, and (iii) sensitivity to the drug (6). MTX, like folate, is polyglutamated, and this increases both the half-life of the drug inside the cell and its ability to bind to enzymes in the place of folate (10). Actively metabolizing cells have increased polyglutamation and accumulation of intracellular MTX, which could account for the drug's specificity for cancer cells (1).

Cytomegalovirus-infected cells, like malignant cells, have an increased rate of DNA synthesis (23). For this reason, we postulated that MTX would also have a preferential effect on DNA synthesis in virus-infected cells compared with that in uninfected host cells. We demonstrate here that MTX has preferential activity against cytomegalovirus replication and that this correlates with increased intracellular accumulation and polyglutamation of MTX in cytomegalovirus-infected cells.

MATERIALS AND METHODS

Cells and virus. Human embryonic lung fibroblast cells (MRC-5) were obtained from the American Type Culture Collection (Rockville, Md.). Cells were maintained in 5% CO_2 at 37°C in Eagle's minimal essential medium (Gibco) supplemented with glutamine (2 mM) and 10% fetal calf serum (complete medium). These cells were used for propagation of the Towne strain of HCMV (4).

Drugs. MTX was obtained from Sigma Chemical Company. Radiolabelled MTX was obtained from Moravic Chemicals (Brea, Calif.).

Viral infection of cell monolayers. Confluent monolayers of MRC-5 cells in 75-cm^2 flasks (T75; Corning) were infected with HCMV at a multiplicity of infection of 3. Virus was allowed to adsorb for 1 h at 37°C with periodic agitation. At that time, unadsorbed virus was removed and complete medium was added until the time indicated in kinetic studies, routinely at 72 h postinfection (p.i.) for HCMV unless otherwise indicated. Cell monolayers were washed extensively

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with phosphate-buffered saline (PBS) and then trypsinized. For DNA hybridization experiments, the cells were washed twice with PBS and counted, and 10^6 cells were added to individual wells of a dot blot apparatus (Schleicher & Schuell, Inc., New York, N.Y.). For dNTP extraction, the trypsinized cells were centrifuged at 1,000 × *g* for 10 min and then extracted with 60% methanol at −20°C overnight. The cells were then centrifuged at 30,000 × *g* for 35 min, and the supernatant fraction was removed. This extract was lyophilized with a Sorvall concentrator at 4°C and reconstituted with distilled water immediately before the dNTP assay.

Radiolabelling with [^{3}H]MTX. Confluent monolayers of MRC-5 cells in 75-cm^2 flasks (T75; Corning) were infected with HCMV as outlined above. Immediately after HCMV infection, 10 ml of medium with 25 μCi of [^{3}H]MTX, with or without additional unlabelled MTX, was added to the flask. At various times after infection and labelling, the cell monolayers were rinsed twice with PBS and trypsinized. The trypsinized cells were then washed again with PBS and extracted with 60% methanol at 0°C overnight. Cell debris was removed by centrifugation at 35,000 × *g* for 35 min. The supernatant fraction was lyophilized and reconstituted with a small volume of water for further analysis.

Thin-layer chromatography. Cell extracts were analyzed by thin-layer chromatography to determine the polyglutamation state of the [^{3}H]MTX. A 0.005-ml sample was spotted onto polyethyleneimine cellulose (Whatman) for thin-layer chromatography, with solvent 0.5 N ammonium formate brought to pH 3.4 with the addition of formic acid. To quantify the tritiated MTX, individual squares of the chromatograms were cut out and radioactivity was counted with a Beckman LS-233 scintillation spectrometer and Hydrofluor scintillation cocktail (National Diagnostics, Atlanta, Ga.).

Dot blot. HCMV DNA replication was measured by dot blot DNA-DNA hybridization, with a cloned *Bam*HI-Q fragment from the end of the HCMV genome as a probe (15). Total cellular and viral DNA was processed for dot blot DNA-DNA hybridization as previously described (13, 14). The *Bam*HI-Q fragment from HCMV was prepared as previously described (13, 14) and was a gift from G. Hayward. Briefly, the HCMV-infected monolayers were treated with trypsin at 72 h and the cells were counted. Cells (2×10^5/ml) were prepared for dot blot hybridization without DNA extraction by adding sodium hydroxide to a final concentration of 0.5 M and incubating for 1 h at 37°C. At the end of the incubation period, the samples were neutralized with an equal volume of 3 M sodium acetate (pH 5.2) and applied to nylon membranes (Biodyn A; Pall Corp.) under a vacuum by using a 96-well apparatus (Schleicher & Schuell, Inc.). The membranes were dried at 80°C for 3 h and then stored in a closed plastic bag. The membranes were prehybridized for 2 h at 67°C in sealed bags containing 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.4]), 5× Denhardt solution (0.1% Ficoll, 0.1% polyvinylpyrollidone, 0.1% bovine serum albumin [BSA]), 0.2% sodium dodecyl sulfate (SDS), and 100 μg of salmon sperm DNA per ml (all reagents from Sigma). After prehybridization, the membranes were hybridized with 10^7 cpm of a radioactive HCMV DNA probe in a mixture of the same composition as the prehybridization buffer. The probe was labelled with [^{32}P]dCTP by nick translation with a commercial nick translation kit (Bethesda Research Laboratories) or by random priming as recommended by the manufacturer of the oligolabelling kit (Pharmacia). At the end of the hybridization period, each membrane was washed in at least 250 ml of each of the following solutions: 0.5% SDS–2× SSC for 5 min, 0.1% SDS–2× SSC for 5 min, 0.5% SDS–0.1× SSC at 65°C for 2 h with gentle agitation, and finally 0.5% SDS–0.5× SSC for 30 min at 65°C. The membranes were dried at 80°C for 2 h and then exposed to X-ray film (Kodak X-Omat). To quantify bound probe, individual dots were cut out and radioactivity was counted with a Beckman LS-233 scintillation spectrometer and Hydrofluor scintillation cocktail.

Toxicity studies. MRC-5 monolayers were treated with drug for 72 h in medium with 10% fetal calf serum, after which cells were counted and viability was determined by trypan blue exclusion.

Assay of mitochondrial respiration. The MTT (3-[4,5-dimethylthiazol-2-y]-2,5-diphenyltetrazolium bromide) assay was used as a measure of an intact mitochondrial respiratory chain (11). Cells were plated in microtiter plates for 3 days in the presence or absence of drug as outlined above; then 15 μl of a 5-mg/ml MTT solution was added and the mixture was incubated for 2 h at 37°C. At that time, 1.0 ml of lysing buffer (consisting of 1% SDS–80% 0.04 N HCl isopropanol) was added and the solution was mixed to dissolve the blue formazan crystals. After 15 min, the A_{570} nm was determined in a Titertek Multiskan microtiter plate reader.

dNTP assay. A bioassay for dNTP involved incorporation of radioactive dNTP into DNA as described by Hunting and Henderson (16). Briefly, the following components were mixed in a total volume of 80 μl: 0.02 A_{260} unit of poly(A) · poly(T) or poly(I) · poly(C) (Pharmacia), 25 mM $MgCl_2$, 10 mM Tris (pH 8.3), 0.22 mg of BSA (fraction V; Sigma) per ml, 2.5 mM dAMP (Sigma), 50 μM 2-mercaptoethanol, and 50 pmol of complementary dNTP containing 1 μCi of tritiated dNTP complementary to that to be assayed (ICN). The sample to be assayed was serially diluted, and 10 μl of the desired dilution was added to the reaction mixture; the reaction was started by the addition of 10 μl of a solution containing 1 U of the Klenow fragment of *Escherichia coli* DNA polymerase (Boehringer Mannheim). The reaction was allowed to proceed to completion (40 min for dATP and dTTP, 120 min for dCTP and dGTP), with the amount of the particular dNTP in the sample to be assayed as the limiting determinant of the quantity of labelled DNA produced by the reaction. The resultant labelled DNA was spotted onto no. 3 filter paper disks (Whatman), and unreacted dNTP was removed by repeated washing with DNA precipitation solution (10% trichloroacetic acid [Sigma], 1% sodium PP_i [Sigma]). The amount of dNTP in the sample was determined by comparison with a standard curve of known concentrations of a dNTP run in parallel.

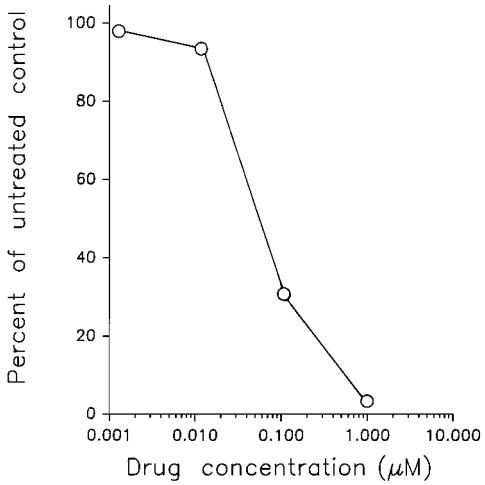

FIG. 1. Inhibition of cytomegalovirus replication by MTX. Cultures of confluent human fibroblasts (MRC-5 cells) were infected with human cytomegalovirus at a multiplicity of infection of 3 and treated for 72 h with different concentrations of MTX. At the end of the 72-h treatment period, viral DNA was quantified by DNA dot blot.

RESULTS

Inhibition of cytomegalovirus replication by MTX. We examined the ability of MTX to inhibit the replication of cytomegalovirus in human fibroblasts as quantified by viral DNA dot blots (Fig. 1). MTX inhibited DNA replication of cytomegalovirus at concentrations well below those producing host cell toxicity as measured by the MTT assay. The 50% inhibitory concentration (IC_{50}) for production of cytomegaloviral DNA was 0.05 μM, whereas no toxicity was observed at the highest dose of MTX examined (1 μM) (Fig. 1).

We examined the duration of MTX exposure required to inhibit HCMV. As in the previous experiment, confluent human fibroblasts were infected with HCMV, and MTX (0.2 μM, the IC_{90} from the previous experiment) was added immediately after adsorption of HCMV. The medium was replaced with fresh medium without MTX at various intervals after infection. All cultures were collected at 96 h p.i., and viral DNA was quantified by DNA dot blots. We found that decreasing the length of exposure to the drug markedly diminished the effectiveness of MTX at inhibiting HCMV DNA replication (Fig. 2). If MTX was added for the initial 24 or 48 h after HCMV infection and then replaced with fresh medium for the remainder of the 96-h infection interval, minimal inhibition occurred. The full effect of this concentration of MTX on inhibition of HCMV DNA was observed only when MTX was present up to the time of harvesting the cells for dot blot (Fig. 2).

Accumulation of MTX by HCMV-infected human fibroblasts. A potential explanation for both the prolonged exposure to drug needed to inhibit HCMV replication and the selective action of MTX on HCMV-infected cells is that the drug accumulates over time in HCMV-infected cells. To assess accumulation of MTX, we added [^{3}H]MTX with cold MTX to a total concentration of 0.05 μM to cultures with and without prior HCMV infection. At various times after HCMV infection, HCMV-infected and control cultures were washed, cells were collected, and intracellular [^{3}H]MTX was extracted. We

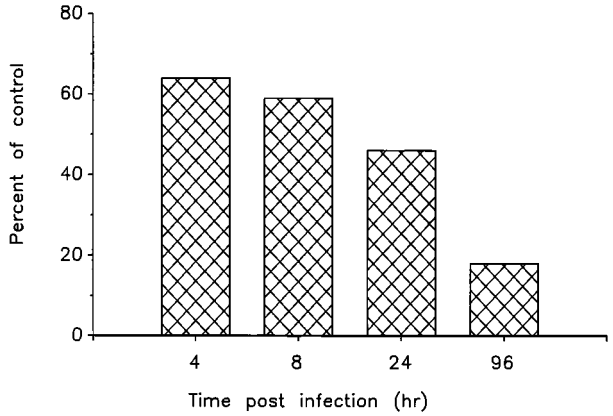

FIG. 2. Effect of duration of MTX exposure on inhibition of viral replication. Cells were infected with HCMV and treated with MTX (IC_{90}) for the time indicated; then cells were washed and put in medium without drug. All cells were then examined for HCMV DNA by dot blot at 96 h p.i.

found that, indeed, MTX accumulated intracellularly over time (Fig. 3). Moreover, MTX accumulated to much higher levels in HCMV-infected cells than in uninfected cells (Fig. 3). This preferential accumulation was first observed at 48 h p.i. with threefold-higher accumulation in infected cells, and it increased to approximately fourfold at 96 h p.i. (the last time point examined). The uptake of [^{3}H]MTX in cells infected by HCMV for 72 h was saturable and was blocked by addition of unlabelled MTX (Fig. 4) along with the label throughout the period of infection.

We examined the polyglutamation state of intracellular MTX in HCMV-infected and uninfected cells. Monolayers were infected and radiolabelled with [^{3}H]MTX as described in Materials and Methods. At 96 h p.i., HCMV-infected and control cultures radiolabelled with MTX were washed extensively and extracted, and these extracts were then subjected to thin-layer chromatography as described previously. The label was found almost exclusively in the polyglutamated fraction in the HCMV-infected cells but almost exclusively in the parent form in uninfected cells (Fig. 5).

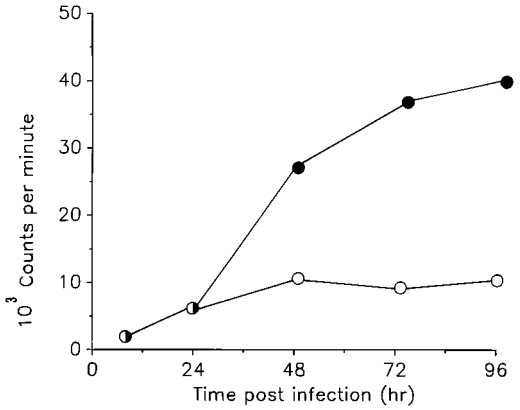

FIG. 3. Accumulation of MTX in HCMV-infected fibroblasts. MRC-5 cells were infected with HCMV and labelled with [^{3}H]MTX (25 μCi per culture). Uninfected cells were treated in parallel. At the indicated times, cultures were extensively washed and extracted with 60% methanol to obtain the intracellular [^{3}H]MTX. Intracellular [^{3}H]MTX in MRC-5 cultures with (●) and without (○) HCMV infection was quantified by scintillation counting as described in Materials and Methods.

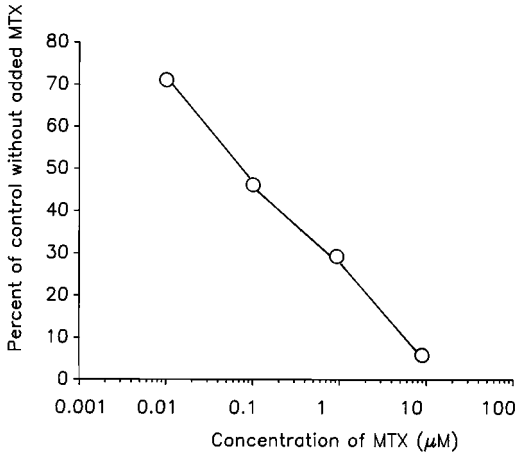

FIG. 4. Inhibition of [^{3}H]MTX transport by the addition of unlabelled MTX. Cells infected in the presence of [^{3}H]MTX (25 μCi per culture) as for Fig. 3 were incubated with additional cold MTX. At 96 h p.i., the level of accumulated intracellular [^{3}H]MTX was determined as described for Fig. 3.

Effect of MTX on intracellular dTTP pools. We then examined the effect of MTX on intracellular pools of dTTP in HCMV-infected and uninfected cells. Cells were infected with HCMV and treated with MTX at the IC_{90} (0.2 μM). At 72 h p.i., the cells were washed and the intracellular dTTP was extracted and analyzed. As in our previous studies (14) and those of others (4), HCMV infection elevated intracellular dTTP pools. The presence of MTX reduced dTTP pools only in HCMV-infected cells and had little effect on the dTTP pools in uninfected cells (Table 1).

DISCUSSION

Cytomegalovirus is a large DNA virus that does not encode enzymes to supply the dNTPs needed for its DNA replication (5). Consequently, for HCMV replication to occur, the host cell must supply these nucleotides. Indeed, HCMV infection of the host is known to result in significant increases in the intracellular pools of dNTPs, especially dTTP (4). This process appears to involve induction of the relevant host enzymes of

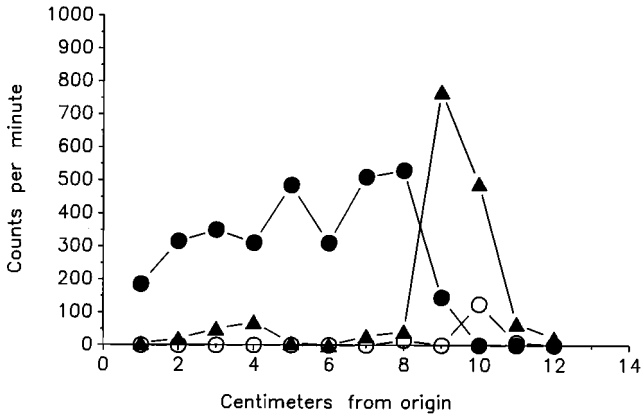

FIG. 5. Thin-layer chromatography of intracellular [^{3}H]MTX. As for the previous figures, MRC-5 cultures with (●) and without (○) HCMV infection were labelled with [^{3}H]MTX and extracted with methanol. The extracts were lyophilized, reconstituted with water, and subjected to polyethyleneimine-cellulose thin-layer chromatography with [^{3}H]MTX as a standard (▲).

TABLE 1. Effect of drugs on dTTP levels with and without HCMV infection

HCMV infection[a]	Drug treatment	dTTP level[b]
−	None	0.38 ± 0.11
+	None	3.14 ± 0.44
−	MTX	0.52 ± 0.04
+	MTX	0.39 ± 0.05

[a] MRC-5 monolayers were infected with HCMV at a multiplicity of infection of 3. Uninfected cell cultures were run in parallel with and without MTX (0.2 μM—the IC_{90}). After 72 h, cells were extracted with methanol to obtain the intracellular dTTP. Intracellular pools of dTTP were assayed by measuring their ability to direct incorporation of labelled dATP into DNA.

[b] Results are in picomoles per 10^6 cells and are expressed as means ± standard deviations.

the de novo biosynthetic pathway; inhibition of these enzymes results in inhibition of HCMV DNA replication (12, 15, 17). However, since host DNA synthesis is also inhibited, this approach does not necessarily imply that the drug will be effective at concentrations that do not affect the host. Nevertheless, we have shown that, at least for MTX, HCMV infection can induce increased sensitivity to the inhibitor.

We found that MTX inhibits HCMV DNA replication with a 50% effective dose of 0.05 μM and a toxic dose (determined as the dose of MTX inhibiting MTT by 50%) of greater than 1 μM (therapeutic index > 20-fold). At the 90% effective dose for MTX inhibition of HCMV DNA replication, MTX reduced intracellular dTTP pools only in HCMV-infected cells and not in uninfected cells (Table 1).

MTX is known to be preferentially toxic to rapidly metabolizing cells. The mechanism for this is controversial. One postulated mechanism for which there is much support is that polyglutamated MTX accumulates more rapidly in rapidly metabolizing cells than in resting cells (1, 6, 10). These studies have shown that polyglutamated MTX is a more potent inhibitor of dihydrofolate reductase and has a much longer intracellular half-life (1) than intracellular MTX which is not polyglutamated.

We found that HCMV infection was associated with increased intracellular accumulation of MTX (Fig. 3). This was not due to a nonspecific increase in permeability of the cell membrane, because MTX uptake was saturable (Fig. 4). We found that MTX in HCMV-infected cells was virtually exclusively in the polyglutamated form (Fig. 5). The alterations associated with rapid cell growth that make MTX selectively toxic to cancer cells (increased MTX transport through the plasma membrane and increased polyglutamation) are recapitulated by HCMV infection. By inducing the host cell to enter a DNA-replicating state (23), HCMV infection also induces increased sensitivity to MTX.

HCMV has been shown to induce the de novo synthesis of host biosynthetic enzymes involved in DNA replication (8, 18, 24, 25). These enzymes include the dihydrofolate reductase enzyme of which MTX is an inhibitor (24). It is possible that HCMV infection could also induce the MTX polyglutamation enzyme and produce preferential polyglutamation in HCMV-infected cells.

In conclusion, we demonstrated that MTX preferentially inhibits HCMV DNA replication. Although MTX is an inhibitor directed against a host cell function, viral perturbation of the host cell can increase its action sufficiently to produce a virus-specific effect.

ACKNOWLEDGMENTS

We thank Pamela Tallalay for helpful discussions.

This work was supported by AIDS Clinical Trials Group (ACTG) developmental funds AI-27565 and fellowship funds from Sandoz Pharmaceuticals.

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