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Mechanism of Antiviral and Cytotoxic Action of (±)-6′β-Fluoroaristeromycin, a Potent Inhibitor of S-Adenosylhomocysteine Hydrolase

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SUMMARY

(±)-6′β-Fluoroaristeromycin (F-C-Ado) is a potent and competitive inhibitor of purified S-adenosylhomocysteine (AdoHcy) hydrolase isolated from murine L929 cells (K_i = 3.1 nM). It also inhibits vaccinia virus and vesicular stomatitis virus replication in L929 cells, at a 90% inhibitory dose (ID_{90}) of 3.5 and 13 μM, respectively. Considering the close correlation that has been found between K_i and ID_{90} for other AdoHcy hydrolase inhibitors [*Biochem. Pharmacol.* **38**:1061–1067 (1989)], F-C-Ado is a weaker antiviral agent than expected from its K_i value. Nevertheless, the antiviral action of F-C-Ado appears to be targeted at AdoHcy hydrolase. The fact that F-C-Ado is less antivirally active than expected may be due to its further metabolism to its ATP and GTP derivatives. The cytotoxicity of F-C-Ado may be attributed to both its inhibitory effect on AdoHcy hydrolase and the inhibitory effect of its phosphorylated products on host cell RNA synthesis.

Several acyclic and carbocyclic adenosine analogues exert a broad-spectrum antiviral activity, which can be attributed to their inhibitory effect on AdoHcy hydrolase (1, 2). AdoHcy hydrolase catalyzes the hydrolysis of AdoHcy to Ado and homocysteine. AdoHcy is a product and feedback inhibitor of AdoMet-dependent methylation reactions. Several viruses rely on a viral methyltransferase for the 5′-capping of their mRNA (3–5). This 5′-cap formation is essential for an efficient translation (6). Inhibition of AdoHcy hydrolase leads to the intracellular accumulation of AdoHcy, which is assumed to inhibit viral mRNA capping and viral replication.

The mechanism of action of AdoHcy hydrolase has been elucidated by Palmer and Abeles (7, 8). The enzyme avoids direct elimination of the homocysteinyl moiety of AdoHcy by using a tightly bound NAD^+ to oxidize the 3′-hydroxyl group of AdoHcy. As a consequence, AdoHcy is converted to 3′-keto-AdoHcy and NAD^+ to NADH. A base at the active site of the enzyme removes the C-4′ proton to form a carbanion, which eliminates homocysteine to form 3′-keto-4′,5′-dehydroadenosine. This intermediate adds water to form 3′-keto-Ado, which is then reduced by the enzyme-bound NADH to Ado. Recently, some mechanism-based inhibitors of AdoHcy hydrolase have been synthesized, in which a fluorine atom was introduced at a position adjacent to the carbanion (9). From this series of compounds, F-C-Ado emerged as the most potent AdoHcy hydrolase inhibitor. This compound has now been evaluated for its inhibitory effect on the AdoHcy hydrolase isolated from L929 cells, its antiviral activity against VV and VSV, its cytotoxic action, and its metabolism in L929 cells. The mechanism of the antiviral and cytotoxic action of F-C-Ado has also been addressed in the present study.

Materials and Methods

Compound. (±)-3β-Adenin-9-yl-4β-fluoro-5β-(hydroxymethyl)-1α,2α-cyclopentanediol was synthesized by G. V. B. Madhavan (Syntex Research, Palo Alto, CA) and provided by E. J. Prisbe (9). The structural formula of the compound, designated by these authors as F-C-Ado, is presented in Fig. 1.

Cells and viruses. Murine L929 cells, VV, and VSV were obtained from the American Type Culture Collection (Rockville, MD).

AdoHcy hydrolase assay. AdoHcy hydrolase was purified from murine L929 cells to apparent homogeneity, using affinity chromatography (10). The AdoHcy hydrolase activity was measured in the direction of AdoHcy synthesis, using [8-^{14}C]Ado (60 mCi/mmol; Amersham, Buckinghamshire, England) and 2 mM D,L-homocysteine as substrates (1). The specific enzyme activity was 1.5 μmol of product (AdoHcy) formation/mg of protein/min. The amount of 15 ng of protein (final

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ABBREVIATIONS: AdoHcy, S-adenosylhomocysteine; HPLC, high pressure liquid chromatography; F-C-Ado, (±)-6′β-fluoroaristeromycin; VV, vaccinia virus; VSV, vesicular stomatitis virus; TCA, trichloroacetic acid; AdoMet, S-adenosylmethionine; Ado, adenosine; Ino, inosine; PBS, phosphate-buffered saline; F-C-Ino, inosine analogue of 6′β-fluoroaristeromycin; dCK, deoxycytidine kinase; AK, adenosine kinase; C-c^3-Ado, carbocyclic 3-deazaadenosine; ID_{90}, 90% inhibitory dose; C-Ado, aristeromycin; F-C-Guo, guanosine analogue of 6′β-fluoroaristeromycin; C-Guo, guanosine analogue of aristeromycin; C-IMP, inosine analogue of aristeromycin monophosphate.

concentration, 0.2 pmol of enzyme/ml) was used in each assay. The incubation time of the assay was 5 min. Unreacted Ado was converted to Ino with Ado deaminase (Sigma Chemical Co., St. Louis, MO). AdoHcy and Ino were separated on SP-Sephadex C-25 columns (Pharmacia Fine Chemicals, Uppsala, Sweden) with 0.1 N formic acid (Ino) and 0.5 N NaOH (AdoHcy). The amount of AdoHcy formed was calculated from the ratio of the radioactivity found in the two eluates.

Virus yield reduction assay. To determine the inhibitory effect of the compound on VSV or VV replication, confluent L929 cells were inoculated with VSV (multiplicity of infection, 0.001) or VV (multiplicity of infection, 0.1). After a 1-hr adsorption period, cells were refed with fresh medium containing various concentrations of the test compound. For VSV, cell cultures were frozen at 12 hr and 24 hr. For VV, cell cultures were frozen at 24 hr and 48 hr. Subsequently, the virus titer of the supernatant was determined by a virus plaque assay (1).

Inhibition of virus-induced cytopathogenicity. Confluent L929 cell monolayers (60,000 cells/well) in 96-well microtiter plates (Falcon; Becton-Dickinson, Lincoln Park, NJ) were inoculated with 100 $CCID_{50}$ of VV (1 $CCID_{50}$ being the 50% cell culture infective dose). After 1 hr of virus adsorption to the cells, virus was removed and replaced by fresh medium containing various concentrations of the test compound. Viral cytopathogenicity was recorded as soon as it reached completion in the untreated virus-infected cell cultures, i.e., after 2 or 3 days. The antiviral activity of the compound is expressed as the concentration required to inhibit viral cytopathogenicity by 50%. Simultaneously, confluent cell cultures that were not infected but that were treated with the same concentration of test compound were examined microscopically to record any alteration of cell morphology at the same time as virus cytopathogenicity. Rounding up or detachment of the cells was considered evidence for cytotoxicity.

HPLC analysis of intracellular AdoHcy and AdoMet levels in L929 cells. L929 cells (3×10^6 cells/6-cm dishes) were seeded 24 hr before analysis. At different time intervals after treatment with the test compound, cells were washed with PBS, trypsinized, and washed with PBS. The cells were extracted with 10% TCA. The TCA-soluble fraction was analyzed by reverse phase HPLC, using a Superspher RP-8 column (Merck, Darmstadt, FRG) with acetonitrile and a buffer containing 50 mM NaH_2PO_4 and 5 mM heptane sulfonate, pH 3.2, as solvents. AdoHcy and AdoMet were eluted from the column with a linear gradient from 3% acetonitrile to 20% acetonitrile in 15 min, followed by a gradient to 25% acetonitrile in 5 min (flow rate, 1 ml/min). The retention times of AdoHcy and AdoMet were 10.5 and 11.5 min, respectively. The acid-insoluble pellets were redissolved in 0.1 N NaOH, and the protein content was measured using the Bio-Rad protein assay (Bio-Rad, München, FRG), with bovine serum albumin as standard.

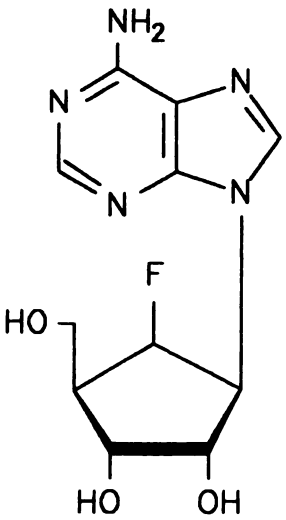

Fig. 1. Structural formula of F-C-Ado.

Inhibition of L929 cell growth. L929 cells were seeded in 96-well microtiter plates (Falcon), at a density of 10,000 cells/well. After 4 hr, the test compound was added at various concentrations. After incubation for 24, 48, 72, 96, or 120 hr, cells were trypsinized and counted using a Coulter counter (Herpenden Herts, England).

Inhibition of CEM cell growth. All assays were performed in flat-bottomed Microtest III plates (96 wells) (Falcon), as previously described (11). CEM/dCK^- represents a dCK-deficient CEM cell line. CEM/AK^- represents an AK-deficient CEM cell line. CEM/dCK^- AK^- represents a CEM cell line deficient in both dCK and AK.

Inhibition of macromolecule synthesis in L929 cells. L929 cells were seeded in 96-well microtiter plates (50,000 cells/well). Simultaneously or 21 hr later, test compound at the appropriate dilution was added. After 21.5 hr, cells were pulse-labeled for 3 hr with [*methyl*-^{3}H]thymidine, [5-^{3}H]uridine, or L-[4,5-^{3}H]leucine (46, 30, and 70 Ci/mmol, respectively; Amersham), at 0.5 μCi/well. After the pulse-labeling, the cells were washed with PBS and fixed with methanol. The macromolecules (DNA, RNA, and proteins) were precipitated with ice-cold 10% TCA. The TCA pellet was solubilized overnight with 1 N NaOH (modified from Ref. 12). The solubilized samples were then transferred to scintillation vials and, upon addition of scintillation fluid (Instafluor and Soluene; Packard Instruments, Groningen, The Netherlands), analyzed for radioactivity.

Treatment of L929 cell lysate with alkaline phosphatase. Confluent L929 cells (7×10^6 cells/10-cm dish) were treated for 4 hr with 100 μM F-C-Ado. Cells were washed with PBS, trypsinized, and lysed in 200 μl of 50 mM Tris·HCl, pH 9.5. Ten units of alkaline phosphatase from calf intestine (Boehringer, Mannheim, FRG) were added. After a 30-min treatment at room temperature, proteins were removed and 100 μl of the partially dephosphorylated cell lysate were analyzed by HPLC (column, buffers, and elution profile as described above for the determination of AdoHcy and AdoMet).

Analysis of nucleotide pools in L929 cells. L929 cells (7×10^6 cells/10-cm dishes) were seeded 24 hr before analysis. After treatment with F-C-Ado, cultures were pulse-labeled with [8-^{14}C]hypoxanthine (51 mCi/mmol, 25 nCi/ml) for 1 hr. Cell cultures were washed in PBS, trypsinized, and washed with PBS. Intracellular nucleotides were extracted from the cell pellet with 10% TCA. The TCA-soluble fraction was neutralized with tri-*n*-octylamine in Freon (1:4). It was then analyzed by ion exchange HPLC, using a Partisil-10 SAX column (Whatman, Springfield Mill, Kent, England) with buffer A containing 7 mM $NH_4H_2PO_4$, pH 3.8, and buffer B containing 250 mM $NH_4H_2PO_4$ and 500 mM KCl, pH 4.5 (flow rate, 2 ml/min). The nucleotides were eluted isocratically for 6 min with 100% buffer A, followed by a 20-min linear gradient from 0% buffer B to 100% buffer B and 20-min isocratic elution with 100% buffer B. Radioactivity was determined in the 1-min fractions. The retention time values for UTP, CTP, ATP, and GTP were 30.5, 32.7, 36.9, and 46.5 min, respectively. In the extracts of F-C-Ado-treated cell cultures, two novel peaks, X_1 and X_2, eluted at 40.7 and 47.4 min, respectively.

Alternative ion exchange HPLC method for the separation of natural GTP from the satellite peak found in extracts from L929 cells treated with F-C-Ado. HPLC samples were prepared as described in the previous section. They were analyzed using the same ion exchange column (Partisil-10 SAX; Whatman), but with different buffers for a better separation of triphosphate pools (13). Buffer A contained 150 mM $NH_4H_2PO_4$, pH 2.8, and buffer B contained 750 mM $NH_4H_2PO_4$, pH 3.5. The nucleotides were eluted with a linear gradient from 40% buffer B to 70% buffer B in 15 min, followed by a linear gradient from 70% buffer B to 80% buffer B in 10 min.

Results

Effect of F-C-Ado on purified AdoHcy hydrolase. The inhibitory effect of F-C-Ado on the purified murine L929 cell AdoHcy hydrolase was monitored by measurement of the enzyme activity in the direction of AdoHcy synthesis. The com-

pound exerted an inhibitory effect on AdoHcy hydrolase, which was competitive with respect to the natural substrate Ado. From the Lineweaver-Burk plot, a K_i value of 3.1 ± 1.3 nM was calculated (data not shown).

Antiviral activity of F-C-Ado. The antiviral potency was determined using a virus yield reduction assay. The VSV and VV yields obtained 12 hr (VSV) or 24 hr (VV) after incubation of the virus-infected cells in the presence of various concentrations of F-C-Ado are presented in Fig. 2. The ID_{90} of F-C-Ado was 3.5 μM and 13 μM for VV and VSV replication, respectively.

Intracellular AdoHcy levels and AdoHcy/AdoMet ratio after treatment of L929 cells with F-C-Ado. Treatment of murine L929 cells with F-C-Ado (1, 5, 10, or 50 μM) resulted in a rapid and dose-dependent increase in AdoHcy pool levels (Fig. 3). Within 4 hr, the AdoHcy pool levels reached their peak values, and they slowly declined thereafter. The AdoMet pool levels remained unchanged after F-C-Ado treatment. Thus, the AdoHcy/AdoMet ratio followed the same pattern as the AdoHcy pool levels.

Antiviral activity of F-C-Ado when combined with homocysteine. De Clercq *et al.* (15) demonstrated that the addition of homocysteine increases the antiviral activity of those Ado analogues that are targeted at AdoHcy hydrolase but does not affect the antiviral activity of those compounds that are targeted elsewhere. The activity of F-C-Ado against VV was markedly enhanced upon addition of exogenous homocysteine. Also, the addition of Ado caused an increase in the anti-VV activity of F-C-Ado (Table 1).

Effect of F-C-Ado on L929 cell growth. At a dose of 0.5 or 1 μM, F-C-Ado reduced L929 cell growth after 6 days by 67 or 73%, respectively (Fig. 4A). To distinguish between the cytostatic and cytotoxic actions of F-C-Ado, the cells were treated for 24 hr with F-C-Ado at 1 or 10 μM and, after removal of the compound, cell growth was followed for the next 4 days (Fig. 4B). Under these experimental conditions, F-C-Ado (at concentrations of 1 and 10 μM) effected a complete inhibition of L929 cell growth.

Effect of F-C-Ado on cellular DNA, RNA, and protein synthesis. To unravel the mechanism of the cytotoxicity of F-C-Ado, the compound was examined for its inhibitory effects on the synthesis of L929 cell DNA, RNA, and proteins. After exposure of the cells to varying concentrations of the test compound, the cells were pulse-labeled with [*methyl*-^{3}H]thymidine, [5-^{3}H]uridine, or [4,5-^{3}H]leucine (Fig. 5). Cells grown for 24 hr in the presence of F-C-Ado showed a marked reduction of the synthesis of DNA, RNA, and proteins. When the cell cultures were exposed to F-C-Ado for only 3 hr, only RNA

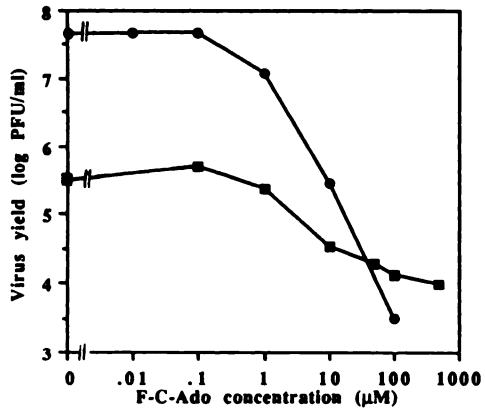

Fig. 2. VSV yield (■) and VV yield (●), measured at 12 hr (VSV) and 24 hr (VV) after exposure of the virus-infected cells to different concentrations of F-C-Ado. Data shown are average values of an assay done in duplicate. Similar data were obtained in at least two independent experiments. *PFU*, plaque-forming units.

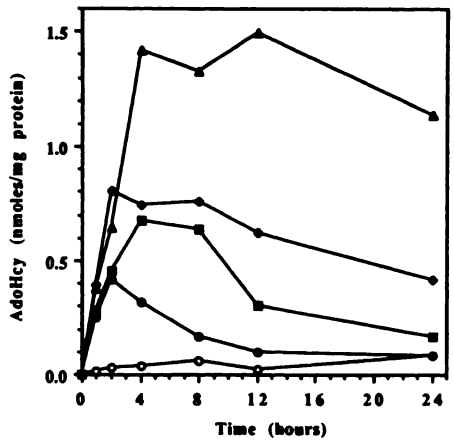

Fig. 3. Intracellular AdoHcy pool levels in L929 cells treated with F-C-Ado [at 0 μM (○), 1 μM (●), 5 μM (■), 10 μM (♦), or 50 μM (▲)]. Data shown are the mean of at least three independent experiments. Standard deviations are within 15%.

TABLE 1

Antiviral activity of F-C-Ado when combined with homocysteine or Ado

Homocysteine or Ado alone did not show any antiviral activity at the concentrations used (1 mM and 0.5 mM, respectively).

Compounds	Minimum inhibitory concentration[a]	Minimum cytotoxic concentration[b]	Selectivity index[c]
	μg/ml	μg/ml	
F-C-Ado	0.1	>100	>1,000
F-C-Ado + 1 mM homocysteine	0.002	100	50,000
F-C-Ado + 0.5 mM Ado	0.01	100	10,000

[a] Concentration required to reduce VV-induced cytopathogenicity by 50%.
[b] Concentration required to cause a microscopically detectable alteration of normal cell morphology.
[c] Ratio of minimum cytotoxic concentration to minimum inhibitory concentration.

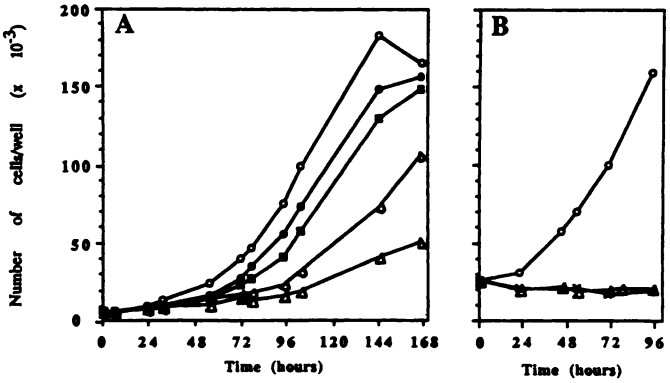

Fig. 4. Inhibitory effect of F-C-Ado on L929 cell growth. A, Cells were permanently grown in the presence of F-C-Ado [at 0 μM (○), 0.05 μM (●), 0.1 μM (■), 0.5 μM (◇), or 1 μM (△)]. At various time intervals, the cell number was determined with a Coulter counter. B, Cells were grown for 24 hr in the presence of F-C-Ado [at 0 μM (○), 1 μM (△), or 10 μM (×)]. The medium was removed, and the cells were washed and refed with fresh medium without F-C-Ado. After 1, 2, 3, or 4 days, the cells were counted with a Coulter counter. Data shown are average values of an assay done in triplicate. Similar data were obtained in at least two independent experiments.

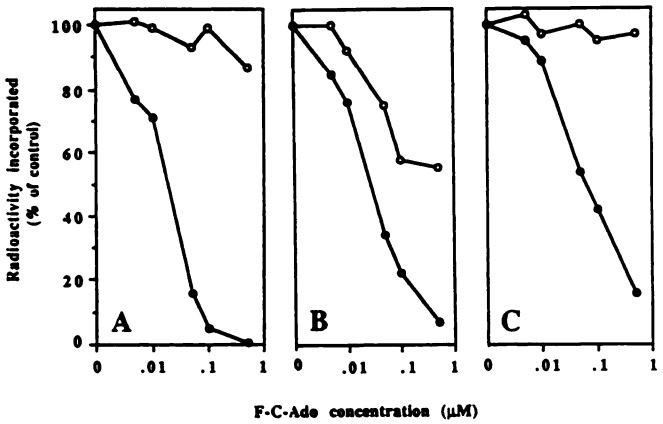

Fig. 5. Effect of F-C-Ado on L929 synthesis of DNA (A), RNA (B), or proteins (C). L929 cells were treated for 24 hr (●) or 3 hr (○) with different concentrations of F-C-Ado. The cell cultures were pulse-labeled with [*methyl*-^{3}H]thymidine (A), [5-^{3}H]uridine (B), or [4,5-^{3}H]leucine (C) during the last 3 hr of the drug treatment, and then the macromolecules were precipitated with TCA. Radioactivity incorporated into the macromolecules was determined using a liquid scintillation counter. Data shown are the mean of at least three independent experiments. Standard deviations are within 15%.

synthesis was inhibited, which indicates that F-C-Ado is primarily inhibitory to RNA synthesis.

Phosphorylation of F-C-Ado in L929 cells. When L929 cells were grown in the presence of 100 μM F-C-Ado and analyzed by ion exchange HPLC, the ATP peak (retention time, 36.9 min) was reduced, the GTP peak (retention time, 46.5 min) completely disappeared, and two new peaks, with retention times of 40.7 min (X_1) and 47.4 min (X_2), appeared. Decreased CTP and increased UTP levels were also noted (data not shown).

When the L929 cells were pulse-labeled with [8-^{14}C]hypoxanthine, F-C-Ado at 100 μM reduced the incorporation of hypoxanthine into ATP by 16% and the incorporation of hypoxanthine into GTP by almost 100% (data not shown).

By modifying the buffers used for the ion exchange chromatography for the determination of intracellular nucleotide pools (13), we could separate the GTP peak from the X_2 peak (Fig. 6). In these experiments, L929 cells were treated with lower concentrations of F-C-Ado (5 and 20 μM). Both the X_1 and the X_2 peaks (retention time, 13.3 min and 19.2 min, respectively) increased as a function of time and dose. The X_2 peak (retention time, 19.2 min) was larger than the X_1 peak (Fig. 6).

To obtain evidence for the presence of the 5′-triphosphate derivatives of F-C-Ado and F-C-Guo, a sample of the entire cell extract was treated with alkaline phosphatase and, after dephosphorylation and deproteination, the cell extract was subjected to reverse phase chromatography. The chromatogram of the extract from drug-treated cells not only showed the presence of a new peak (Y_1) with exactly the same retention time as F-C-Ado (9.61 min), but also revealed the presence of a new peak (Y_2) with a retention time of 4.74 min (Fig. 7). This peak eluted in the same region as guanosine but was clearly different from it, as revealed by spiking experiments with guanosine (data not shown). X_1 has been tentatively identified as the guanosine analogue of F-C-Ado.

Biological activity of deaminated F-C-Ado. In the presence of Ado deaminase (Sigma), F-C-Ado was completely deaminated to F-C-Ino, as shown by reverse phase HPLC and spectrophotometry. F-C-Ino did not exert any antiviral or

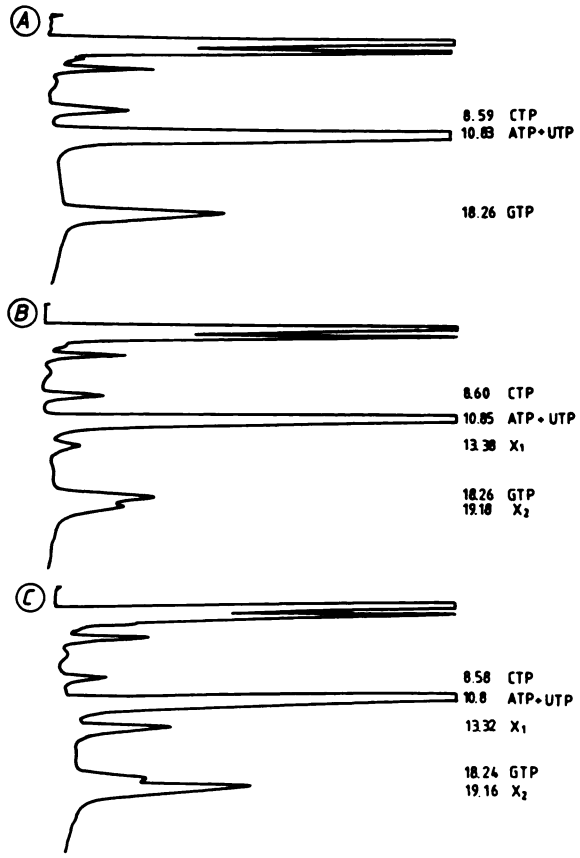

Fig. 6. Alternative ion exchange chromatography for the separation of the natural GTP peak and its satellite peak in lysates from L929 cells treated with F-C-Ado. L929 cells were treated for 2 hr with F-C-Ado at a concentration of either 0 μM (A), 5 μM (B), or 20 μM (C). HPLC samples were prepared and analyzed by anion exchange chromatography, using the alternative buffer system, as described in Materials and Methods.

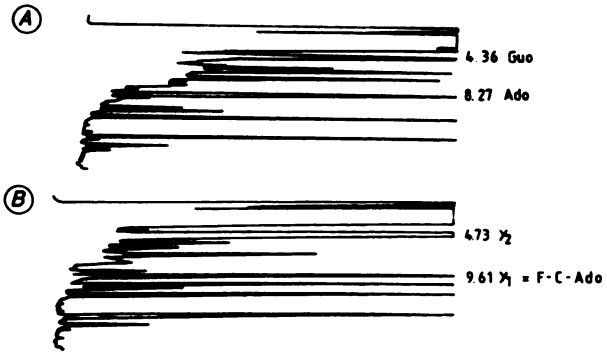

Fig. 7. Reverse phase chromatography of phosphatase-treated cell lysate from L929 cells that were either not treated (A) or treated with F-C-Ado at 100 μM (B). Cell lysates from untreated and treated cells were incubated with alkaline phosphatase, and the resulting mixture was subjected to reverse phase HPLC, as used for the determination of AdoHcy and AdoMet pools. *Guo*, guanosine.

cytotoxic activity at concentrations up to 10 μM (data not shown). Ion exchange analysis of L929 cells treated for 10 hr with 10 μM F-C-Ino did not reveal any new peaks in the ATP or GTP region, indicating that the F-C-Ino was not further phosphorylated to a marked extent.

Inhibitory effect of C-Ado and F-C-Ado on the proliferation of different mutant CEM cell lines. C-Ado and F-C-Ado were evaluated for their inhibitory effects on wild-type

CEM/0 cells and CEM cells deficient in dCK (CEM/dCK$^-$), AK (CEM/AK$^-$), or both dCK and AK (CEM/dCK$^-$AK$^-$) (Table 2). F-C-Ado proved 10-fold more inhibitory to CEM/0 and CEM/dCK$^-$ cells than did C-Ado. This difference went up to almost 1000-fold for the CEM/AK$^-$ and CEM/dCK$^-$AK$^-$ cells; C-Ado was about 6-fold less effective in inhibiting the growth of CEM/AK$^-$ and CEM/dCK$^-$AK$^-$ cells, whereas F-C-Ado was 15-fold more inhibitory to the growth of CEM/AK$^-$ and CEM/dCK$^-$AK$^-$ cells than to the growth of CEM/0 cells.

AdoHcy/AdoMet ratio in CEM/0 and CEM/dCK$^-$AK$^-$ cells treated with F-C-Ado. CEM/0 and CEM/dCK$^-$AK$^-$ cells showed marked differences in the increase of the AdoHcy/AdoMet ratio upon F-C-Ado treatment (Table 3). In the CEM/dCK$^-$AK$^-$ cells treated with 0.01 μM F-C-Ado, the AdoHcy/AdoMet ratio rose to 40-fold the ratio for untreated CEM/dCK$^-$AK$^-$ cells, whereas in CEM/0 cells only a 4-fold increase of the AdoHcy/AdoMet ratio was noted after F-C-Ado treatment. A 40-fold increase of the AdoHcy/AdoMet ratio in CEM/0 cells was noted only at a F-C-Ado concentration of 0.1–0.5 μM.

Discussion

F-C-Ado is a potent competitive inhibitor of AdoHcy hydrolase from L929 cells, with a K_i of 3.1 nM. In the same assay system, neplanocin A, Ado dialdehyde, and C-c^{3}Ado had K_i values of 2.6, 4.3, and 18 nM, respectively (1). F-C-Ado is also a potent inhibitor of VV and VSV replication (ID_{90}, 3.5 and 13 μM, respectively). In comparison, neplanocin A, Ado dialdehyde, and C-c^{3}Ado achieve 90% inhibition of VV and VSV at concentrations of 0.1, 0.2, and 3 μM (VV) and 0.25, 0.34, and 16 μM (VSV), respectively (1).

Previously, we have demonstrated that the K_i of several Ado analogues (including neplanocin A, Ado dialdehyde, and C-c^{3}Ado) for murine L929 cell AdoHcy hydrolase correlates well with their inhibitory effect on VSV and VV replication in L929 cells (1). Based on this correlation, we assumed that F-C-Ado would have an antiviral activity comparable to that of neplanocin A and Ado dialdehyde. However, F-C-Ado is a much weaker antiviral agent than the other AdoHcy hydrolase inhibitors, despite their comparable inhibitory effect on the purified AdoHcy hydrolase.

Although the compound is metabolized intracellularly to phosphorylated products, we believe that the antiviral activity of F-C-Ado is due to the inhibitory effect of the unmetabolized nucleoside analogue on AdoHcy hydrolase. Evidence for this hypothesis stems from the following observations. (i) Treatment of L929 cells with a concentration of F-C-Ado that reduced VV growth by approximately 90% elevated the intracellular AdoHcy levels to levels comparable to those obtained after treatment of L929 cells with other AdoHcy hydrolase inhibitors at their ID_{90} (14). For example, treatment of L929 cells for 12 hr with Ado dialdehyde or C-c^{3}Ado at their ID_{90} for VV increased the AdoHcy pool levels from 0.03 to 0.3 nmol/mg of protein. Treatment of L929 cells with 5 μM F-C-Ado for 12 hr also increased the AdoHcy levels to approximately 0.3 nmol/mg of protein, a level sufficient to reduce VV growth by 90%. These observations have led to the conclusion that the antiviral activity of these compounds, including F-C-Ado, is directly related, and probably attributed, to the intracellular increase in AdoHcy and the AdoHcy/AdoMet ratio. (ii) Homocysteine has a marked potentiating effect on the antiviral activity of F-C-Ado. De Clercq *et al.* (15) recently demonstrated that homocysteine increases the antiviral potency of those analogues that act as AdoHcy hydrolase inhibitors, but not of those Ado analogues that act through other molecular mechanisms. In this respect, F-C-Ado behaves similarly as the well known AdoHcy hydrolase inhibitors. F-C-Ado behaves as a reversible inhibitor of AdoHcy hydrolase but not as an inactivator of the enzyme. Indeed, its effect on cellular metabolism is comparable to that of the parent compound C-Ado. In addition, F-C-Ado causes a relatively rapid decline of the increased AdoHcy/AdoMet ratios, whereas potent inactivators such as neplanocin A afford increased cellular AdoHcy/AdoMet pool levels that are considerably sustained for at least 24 hr (14).

The metabolism of F-C-Ado shares many similarities with that of C-Ado. C-Ado is also a strong competitive inhibitor of AdoHcy hydrolase. Guranowski *et al.* (16) reported a K_i of 5 nM for AdoHcy hydrolase from bovine liver. Houston *et al.* (17) found a K_i of 110 nM for AdoHcy hydrolase from bovine liver. Although C-Ado is a potent inhibitor of purified AdoHcy hydrolase, the compound exerts only a limited effect on the enzyme of intact hepatocytes and does not increase the AdoHcy pools by more than 2-fold (18). These authors attributed the weak stimulatory effect of C-Ado on intracellular AdoHcy pools to the fact that the compound is extensively phosphorylated and that the intracellular concentration of the remaining unmetabolized C-Ado is rather low. The extent of phosphorylation of C-Ado may differ from one cell type to another, which, in turn, may explain why C-Ado may achieve a more pronounced increase in AdoHcy pool levels in some cell lines than in other cells. For example, treatment of murine embryo 3T3-L1 fibroblasts with 10 μM C-Ado caused a 10-fold increase in AdoHcy pool levels (16), compared with a 2-fold increase in AdoHcy pool levels in intact hepatocytes (see above).

Bennett *et al.* (19) demonstrated that inside the cells C-Ado can be converted not only to C-Ado triphosphate but also to C-Guo triphosphate. The starting material (C-Ado) could be

TABLE 2

Inhibitory effect of C-Ado and F-C-Ado on the proliferation of different mutant CEM cell lines

Compound	Proliferation			
	CEM/0[a]	CEM/dCK$^-$[b]	CEM/AK$^-$[c]	CEM/dCK$^-$AK$^-$[d]
F-C-Ado (μM)	0.15 ± 0.04	0.16 ± 0.02	0.01 ± 0.002	0.01 ± 0.003
C-Ado (μM)	1.4 ± 0.04	1.7 ± 0.73	9.1 ± 1.6	9.3 ± 2.3

[a] Wild-type human lymphocyte CEM cells.
[b] CEM cells deficient for dCK.
[c] CEM cells deficient for AK.
[d] CEM cells deficient for both dCK and AK.

TABLE 3

AdoHcy/AdoMet ratio in CEM/0 and CEM/dCK$^-$AK$^-$ cells treated with F-C-Ado for 4 hr

Concentration of F-C-Ado	AdoHcy/AdoMet	
	CEM/0	CEM/dCK$^-$AK$^-$
μM		
0	0.02 ± 0.01	0.02 ± 0.01
0.01	0.08 ± 0.03	0.81 ± 0.09
0.05	0.26 ± 0.04	1.65 ± 0.04
0.1	0.58 ± 0.07	1.88 ± 0.05
0.5	1.39 ± 0.11	1.98 ± 0.06

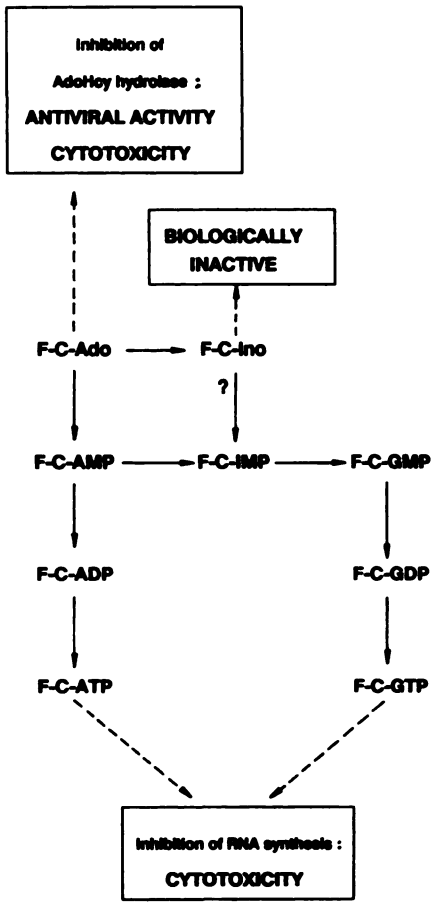

Fig. 8. Metabolic conversions of F-C-Ado.

either deaminated to the biologically inactive C-Ino or phosphorylated to C-Ado monophosphate. The latter could then be phosphorylated to C-Ado diphosphate and C-Ado triphosphate or it could also be deaminated by AMP deaminase to C-IMP, which may subsequently be converted to C-Guo monophosphate, C-Guo diphosphate, and C-Guo triphosphate. C-Ado inhibits the utilization of hypoxanthine and guanosine for purine nucleotide synthesis, because both C-IMP and C-Guo monophosphate are potent inhibitors of hypoxanthine-guanine phosphoribosyltransferase (19).

Our findings clearly indicate that F-C-Ado is phosphorylated to F-C-Ado triphosphate and F-C-Guo triphosphate. The latter triphosphate must have originated from F-C-Ado after deamination to F-C-Ino, conversion to F-C-Ino monophosphate and F-C-Guo monophosphate, and phosphorylation of F-C-Guo monophosphate to F-C-Guo diphosphate and F-C-Guo triphosphate. As in the case of C-Ado (19), no triphosphate analogue of F-C-Ino could be detected in cell extracts treated with F-C-Ado. These findings are in agreement with the lack of biological activity of F-C-Ino.

Although the metabolism of F-C-Ado resembles that of C-Ado, the two compounds behave clearly differently with respect to their antiproliferative effects on AK-deficient cell lines. Indeed, whereas C-Ado shows reduced cytotoxic activity against AK^- cells, compared with wild-type cells, F-C-Ado is markedly more inhibitory to AK^- cells (Table 2). From our antimetabolic data obtained for the CEM cells, it has become evident that the increased cytotoxity of F-C-Ado for AK^- cells is related to a marked increase in the AdoHcy/AdoMet ratio. At the IC_{50} values of F-C-Ado for CEM/0 and CEM/dCK^-AK^- cells (0.15 and 0.01 μM, respectively), intracellular AdoHcy concentrations reached a similar level. This suggests that the cytotoxicity of F-C-Ado in CEM cells may result from the intracellular accumulation of the unmetabolized nucleoside analogue and not its phosphorylated products (i.e., F-C-Ado triphosphate or F-C-Guo triphosphate). In fact, the triphosphate metabolites of F-C-Ado could not be detected in CEM/dCK^-AK^- cells (data not shown). Presumably, F-C-Ado is not phosphorylated in AK-deficient cells. Moreover, the cytotoxic action of F-C-Ado against CEM cells could not be reversed by the addition of Ado or guanosine, which corroborates our hypothesis that the toxicity of F-C-Ado for CEM cells is due to the nucleoside itself and not its phosphorylated products.

Taking all data together, the metabolic conversion of F-C-Ado and its mode of antiviral and cytotoxic action can be summarized as follows (Fig. 8). F-C-Ado as such is targeted at AdoHcy hydrolase and, thus, capable of inhibiting virus growth. Deamination of F-C-Ado (by Ado deaminase) to F-C-Ino makes it biologically inert. If initially phosphorylated to F-C-Ado monophosphate, F-C-Ado may eventually give rise to both F-C-Ado triphosphate and F-C-Guo triphosphate. The conversion of the C-Ado metabolites to C-Guo metabolites would be accomplished at the monophosphate level, with F-C-Ino monophosphate as the intermediate (formed through the action of AMP deaminase on F-C-Ado monophosphate). Our results obtained from CEM/0 and CEM/dCK^-AK^- cells suggest a close correlation between the cytotoxicity of F-C-Ado and an increased AdoHcy/AdoMet ratio. In other cell systems, inhibition of RNA synthesis by phosphorylated metabolites of F-C-Ado may act solely or in concert with AdoHcy hydrolase inhibition in achieving the cytotoxic action of F-C-Ado.

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