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# Structure-Activity Relationships of Fluorinated Nucleoside Analogs and Their Synergistic Effect in Combination with Phosphonoformate against Human Immunodeficiency Virus Type 1

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**One hundred nucleoside analogs with fluorine substitutions at various positions on the pentose ring were evaluated for inhibitory activity against human immunodeficiency virus type 1 (HIV-1). Nine compounds emerged as inhibitors of HIV-1 replication, with various degrees of selectivity; the most active of these was 3′-fluoro-3′-deoxythymidine, followed by 5′-amino-3′-fluoro-3′-deoxyadenosine. Substitution of fluorine at the 2′-deoxy or 3′-deoxy position resulted in increased antiviral activity of the thymidine analogs, whereas the activity of adenosine or cytidine analogs was not increased by fluorination at either position. The most potent inhibitor, 3′-fluoro-3′-deoxythymidine, was shown to give synergistic inhibition of HIV-1 replication in combination with the $PP_i$ analog phosphonoformate.**

Following the discovery of the retrovirus human immunodeficiency virus type 1 (HIV-1) as the causative agent of acquired immunodeficiency syndrome (3, 9), there has been an intense effort to identify and develop potent inhibitors of the replication of the virus for antiviral chemotherapy (7, 24). Most effort has concentrated on inhibitors of the viral reverse transcriptase (RT), which is a key enzyme in the replicative cycle of the virus and therefore an attractive target for chemotherapy.

The most active class of compounds described to date are the 2′,3′-dideoxynucleosides (24). The 5′-triphosphates of these compounds act as chain terminators of DNA synthesis since they lack the 3′-hydroxyl group required for further chain polymerization (8, 22). The most active of these are 3′-azido-3′-deoxythymidine (AZT) and 2′-3′-dideoxycytidine (20, 23). AZT is the only compound licensed for treatment of patients with acquired immunodeficiency syndrome. 2′-3′-Dideoxycytidine has recently entered clinical trials. However, both compounds show toxic side effects that limit their usefulness (26, 32). Also, the development of resistance to AZT has recently been described (16), which emphasizes the need for new compounds with anti-HIV activity.

The use of combinations of antiviral substances to treat patients with acquired immunodeficiency syndrome is an attractive proposal if such combinations show a synergistic or at least additive effect without any increased toxicity. The potential benefits of combination chemotherapy are an increased antiviral effect (resulting in the requirement of lower doses of compounds), a lowered toxicity, and a reduced risk of development of resistance. Recent studies show that several combinations of anti-HIV-1 compounds give synergistic effects, such as AZT with alpha interferon (12), with phosphonoformate (PFA) (15), and with acyclovir (21). However, combinations of antiviral compounds are not always successful, and marked antagonism between ribavirin and AZT has been reported (31), although ribavirin and PFA appear to act synergistically (30).

Several fluorinated nucleoside analogs have recently been described as inhibitors of HIV-1 replication, such as 5-fluoro-2′,3′-dideoxycytidine (14), 3′-fluoro-3′-deoxythymidine (1, 5, 11), 2′-fluoro-2′,3′-dideoxyadenosine arabinoside (19), 3′-fluoro-2′,3′-dideoxyguanosine (2), 2,6-diaminopurine-3′-fluoro-2′,3′-dideoxyribonucleoside (2), and 2′- and 3′-fluoro-2′,3′-dideoxyadenosine (25). These compounds show various toxicities toward uninfected cells, with concentrations that inhibit cell viability by 50% ranging from 15 to 500 μM or greater. However, the use of different cell lines and procedures makes direct comparison difficult.

We therefore investigated the anti-HIV-1 activities of 99 newly synthesized nucleoside analogs with fluorine substitutions at different positions on the pentose ring. The structures and activities of the most active and selective compounds are presented below. We also studied the inhibition of HIV-1 replication by 1-(3′-fluoro-3′-deoxy-β-D-erythropentofuranosyl)thymidine (FLT), the most potent and selective analog, both alone and in combination with the $PP_i$ analog PFA.

## MATERIALS AND METHODS

**Compounds.** Ninety-nine newly synthesized fluorinated nucleoside analogs were obtained from Y. Morizawa, The Research Centre, Asahi Glass Co. Ltd, Yokohama, Japan. Details of the synthetic methods will be presented elsewhere (Y. Morizawa, personal communication). The compounds were classified according to the type of base they contain and the position of the fluorine substitution (Table 1). FLT was a gift from B. Öberg, Medivir AB, Stockholm, Sweden. PFA (foscarnet) was a gift from Astra Alab AB, Södertälje, Sweden. AZT was obtained from Sigma Chemical Co., St. Louis, Mo.

**Cells and virus.** H9 cells (a human $CD4^+$ lymphocyte cell line) were used for the assay of antiviral activity. The cells were grown in suspension in RPMI 1640 medium with 10% heat-inactivated fetal calf serum at 37°C in a humidified atmosphere of 5% $CO_2$ in air.

HIV-1 was obtained from the supernatant of H9 cells persistently infected with human T-lymphotropic virus type $III_B$ (9) and was stored in medium with 20% fetal calf serum at −70°C.

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TABLE 1. Newly synthesized compounds classified according to the base and the position of the fluorine substitution

| Fluorination position | Compound no.[a] | | |
|---|---|---|---|
| | Purine | Pyrimidine | Modified |
| 3′ | **1**, **3**, 4, 7, 8, 12, 14, 25, **26**, **27**, 28, 41, 46, 50, 61, **83**, 88 | 2, **15**, **17**, 48, 49, 56, 58, 59, 62, 63, 64, 73, 79, 80, 81, 82, **84**, 85, 86, 87, 98, 99, **FLT** | 9, 10, 16, 18, 19, **20**, 21, 22, 23, 24, 31, 32, 33, **34**, 35, 36, 47, 51, 55, 57, 60, 65, **66**, **67**, **71**, **72**, 74 |
| 2′ | | 38, **39**, 40, 42, 45, 68, **70**, **75**, 76, 77, 78 | 69 |
| 2′,3′ | 5, 6 | **37** | |
| Other | 11, 13, **29**, 30, 43, 89, 90, 92, 93, 95, 96, **97** | 52, **53**, 54 | 44, 91, 94 |

[a] All compounds with an $IC_{50}$ of ≤10 μg/ml in any assay are in boldface.

**Antisera.** All antisera were obtained from the National Bacteriological Laboratory, Stockholm, Sweden.

**Assay for antiviral activity.** A total of $10^5$ H9 cells were seeded in 24-well microdilution plates (Costar, Cambridge, Mass.). A 0.5-ml volume of medium containing the test compound and 1 ml of virus were then added, and the cells were incubated at 37°C for 6 days (4, 15). Two different concentrations of virus were used: a high dose, with a multiplicity of infection of approximately 1.0, which in the absence of compound gave 60 to 70% infected cells after 6 days, and a low dose, with a multiplicity of infection of approximately 0.3, which gave 40 to 50% infected cells. The replication of HIV-1 was assayed after 6 days by measuring the antigen content of the infected cells (by immunofluorescence [IF]) and of the supernatant (by enzyme-linked immunosorbent assay [ELISA]).

**IF.** Infected cells were washed, fixed onto slides, and incubated for 30 min at 37°C with a human anti-HIV-1 antiserum (4). After a washing, the cells were incubated with a fluorescein isothiocyanate-labeled sheep anti-human immunoglobulin G antibody. The cells were counterstained with Evans blue, and the percentage of cells expressing HIV-1 antigens was assessed by using a fluorescence microscope. The concentration of drug which inhibits the replication of HIV-1 by 50% compared with the control ($IC_{50}$) was calculated.

**ELISA.** A 100-μl volume of supernatant from infected cells was assayed by ELISA by the method of Sundqvist et al. (V.-A. Sundqvist, J. Albert, E. Ohlsson, J. Hinkula, E. M. Fenyö, and B. Wahren, J. Med. Virol., in press), using microdilution plates coated with human anti-HIV-1 antibody. Two horseradish peroxidase-conjugated mouse monoclonal antibodies directed against the HIV p24 protein were used to detect bound antigen. An HIV-1 antigen standard was used each time to calibrate the assay. An $IC_{50}$ was calculated for each compound.

**Cytotoxicity.** H9 cells ($10^5$ cells per well) were seeded in 24-well microdilution plates (Costar). A 1-ml volume of medium containing various concentrations of the antiviral compounds was added, and the cells were incubated at 37°C. Untreated cells were cocultivated as a control. After 6 days, the growth of the cells was measured with a volume distribution analyzer (VDA 140; Analys Instrument AB, Stockholm, Sweden). A selectivity index was obtained by dividing the concentration of antiviral compound which inhibits cell growth by 50% compared with that of the control by the $IC_{50}$.

**Combined antiviral activity of FLT and PFA.** Various concentrations of FLT and PFA were used in the assays described above, both alone and in combination with each other. The fractional product method (29) was used to determine the combined antiviral effect. The observed inhibition of HIV-1 replication (measured as a reduction in IF or ELISA values) by the combination of FLT and PFA was compared with the inhibition expected (calculated by adding together the inhibitions observed when the same concentrations of FLT and PFA were used separately). The combination of FLT and PFA was considered synergistic when the observed inhibition was greater than the expected inhibition and antagonistic when it was less than the expected inhibition.

The median effect principle was also used to assess synergy (6). The gradient of a plot of log fraction affected/fraction unaffected versus log concentration of inhibitor/$IC_{50}$ indicates whether two inhibitors are mutually exclusive. At one given fractional inhibition, the dose of inhibitor in the combination can be compared with the dose of inhibitor alone. From this comparison, a combination index can be calculated. Combination index values of <1 indicate synergy, while values of >1 imply antagonism.

## RESULTS

**Inhibition of HIV-1 replication.** Compounds with antiviral activities at concentrations of ≤10 μg/ml in all assays are shown in Table 2. The compounds are classified according to the type of base (T, U, C, A, or G), the configuration of the sugar (pentofuranose, 2′,3′-unsaturated pentofuranose, or a carbocyclic sugar), and the position of the fluorine substitution (2′, 3′, or both). Seven compounds had bases which were also modified.

None of the uridine or guanosine analogs tested showed antiviral activity. FLT and compound 37 (2′,3′-difluoro-3′-deoxythymidine) were the only active thymidine analogs. Three cytidine analogs showed activity: 2′-fluoro-2′,3′-dideoxycytidine (no. 39), 2′-fluoro-2′,3′-dideoxydidehydrocytidine (no. 75) and the 3′-fluoro isomer of compound 75 (no. 84).

Carbocyclic 3′-fluoro-2′,3′-dideoxyadenosine (no. 3) and 3′-fluoro-2′,3′-dideoxydidehydroadenosine (no. 83) were the active adenosine analogs, in addition to 3′-fluoro-3′-deoxyadenosine (no. 1), 5′-amino-3′-fluoro-3′-deoxyadenosine (no. 26), and 3′-fluoro-2′,3′-dideoxyadenosine (no. 27). A total of 7 of the 31 analogs with modified bases were also active (nos. 20, 33, 34, 53, 66, 67, and 72).

The cellular toxicity was measured for every substance. The majority of compounds were toxic in concentrations similar to the $IC_{50}$. Eight new compounds had selectivity

TABLE 2. Anti-HIV-1 activity of fluorinated nucleoside analogs

| Base | Active compound with the following sugar configuration[a]: | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pentofuranose | | | | 2',3'-Unsaturated | | Carbocyclic | | | |
| | 2'F3'deoxy | 3'F2'deoxy | 3'F2'hydroxy | 2'3'diF | 2'F | 3'F | 3'F2'deoxy | 3'F2'hydroxy | 2'3'Unsat 2'F | 2'3'Unsat 3'F |
| T | — | FLT | — | 37 | — | — | — | — | — | — |
| U | — | NS | — | NS | — | — | — | — | — | — |
| C | 39 | NS | — | NS | 75 | 84 | — | — | NS | NS |
| A | NS | 27 | 1, 26[b] | — | NS | 83 | 3 | — | NS | — |
| G | NS | NS | NS | NS | NS | NS | — | — | NS | NS |
| Modified | | | 23, 33, 34, 66, 67, 72 | | | | 53 | | | |

[a] Compounds listed showed antiviral activity (irrespective of cytotoxicity). Fluorination is indicated as follows: 2'F3'deoxy, 2'-fluoro-3'-deoxy; 3'F2'deoxy, 3'-fluoro-2'-deoxy; 3'F2'hydroxy, 3'-fluoro-2'-hydroxy; 2'3'diF, 2',3'-difluoro; 2'3'Unsat, 2',3'-unsaturated. —, No antiviral activity; NS, not synthesized.
[b] 5' C was further modified.

indices of ≥5. The $IC_{50}$s, the toxic concentrations, and the selectivity indices of these eight compounds and of AZT and PFA are shown in Table 3. $IC_{50}$s obtained by IF and ELISA usually were similar. 5'-Amino-3'-fluoro-3'-deoxyadenosine (no. 26), 2'-fluoro-2',3'-dideoxycytidine (no. 39), and 3'-fluoro-2',3'-dideoxydidehydrocytidine (no. 84) were the most active and selective of the newly synthesized analogs (Fig. 1). FLT was the most active compound tested, with an $IC_{50}$ of 0.005 to 0.01 μM and a selectivity index of more than 500.

**Synergistic inhibition of HIV-1 replication by combinations of FLT and PFA.** The most active compound, FLT, was tested in combination with PFA, an inhibitor of HIV RT having a different mechanism of action, in order to assess any synergistic or antagonistic effect.

PFA inhibited the replication of HIV-1 in H9 cells in the concentration range of 10 to 160 μM, and FLT inhibited replication in the range of 0.001 to 0.025 μM. The addition of different concentrations of FLT to the various concentrations of PFA resulted in increased inhibition of production of HIV-1 antigens in the infected-cell supernatants (Fig. 2). In IF, the same effects were seen.

Figure 3 shows the inhibition observed by using 0.001 μM FLT and various concentrations of PFA, together with the expected inhibition for these combinations, as calculated by the fractional product method. These combinations gave greater inhibition of HIV-1 replication than expected, indicating synergy between FLT and PFA in both the IF assay and the ELISA. The higher concentrations of FLT shown in

TABLE 3. Comparative potency and selectivity of fluorinated nucleoside analogs as inhibitors of HIV-1 replication in H9 cells

| Compound | Base | $IC_{50}$[a] for HIV dose | | | | Toxicity[b] | SI[c] |
|---|---|---|---|---|---|---|---|
| | | High | | Low | | | |
| | | IF | ELISA | IF | ELISA | | |
| 39 | C | 0.5 | 1 | 0.4 | 1 | 90 | 90 |
| 75 | C | 0.7 | 1 | 0.2 | 0.5 | 6 | 6 |
| 84 | C | 1.4 | 0.1 | 1 | 1 | 100 | 71 |
| 3 | A | 4 | 5 | 5 | 10 | 50 | 5 |
| 26 | A | 0.03 | 1 | 0.02 | 1 | 100 | 100 |
| 83 | A | 6 | 5 | 1.5 | 1 | 50 | 8.3 |
| 20 | Purine | 7 | 10 | 10 | 10 | 60 | 6 |
| 67 | Purine | 0.5 | 1 | 1 | 1 | 33 | 33 |
| FLT[d] | T | 0.01 | 0.005 | 0.01 | 0.01 | 5 | 500 |
| AZT[d] | T | 0.05 | 0.02 | 0.05 | 0.01 | 90 | 1,800 |
| PFA[d] | | 10 | 10 | 15 | 5 | >1,000 | >66 |

[a] The average was obtained from at least two dose-response curves. Values are in micrograms per milliliter except where otherwise indicated.
[b] Concentration which inhibits cell growth by 50% compared with untreated cells. Values are in micrograms per milliliter except where otherwise indicated.
[c] SI, Selectivity index (ratio of toxicity to $IC_{50}$). The lowest values are shown.
[d] $IC_{50}$s and toxicity values are in micromolar.

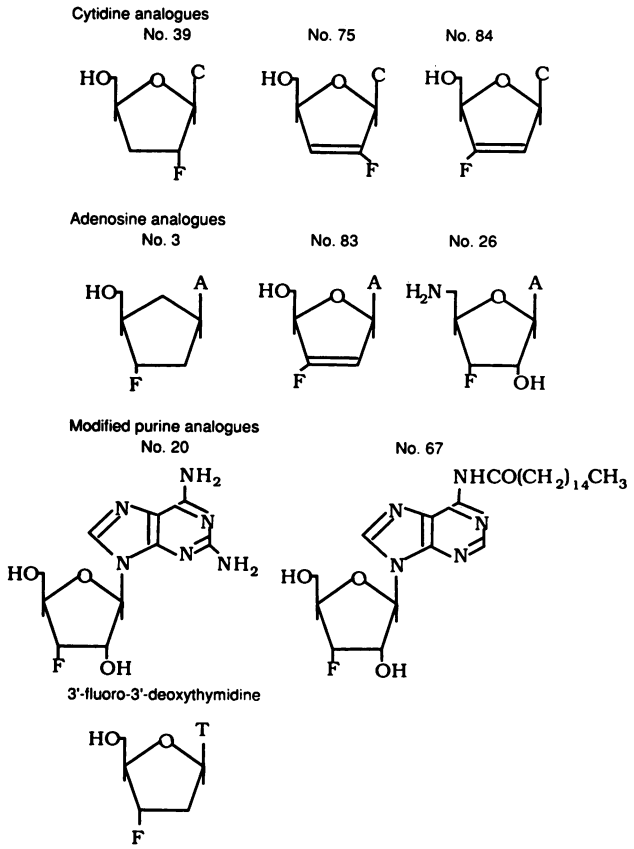


FIG. 1. Structures of the most active and selective fluorinated compounds. The optical isomer of compound 84 (compound 85) was not active against HIV-1. Compound 83 is a racemic mixture of alpha and beta isomers.

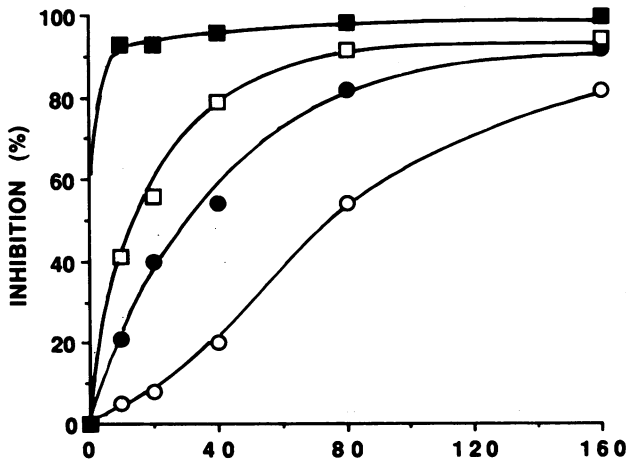


FIG. 2. Effect of PFA and FLT on HIV-1 replication in H9 cells, as measured by ELISA. Data represent the mean of two experiments. The FLT concentrations used are indicated as follows: ○, none; ●, 0.005 μM; □, 0.01 μM; ■, 0.025 μM.

Fig. 2 also gave greater inhibition than expected (data not shown).

Analysis of the data by using the median effect principle showed that PFA and FLT are mutually nonexclusive inhibitors. At the $IC_{50}$, the combination index values for the high and low doses of HIV-1 were 0.35 and 0.6, respectively, in the IF assay and 0.61 and 0.56, respectively, in the ELISA. Thus, the median effect principle also indicates synergy between FLT and PFA at both doses of virus and in both assays.

## DISCUSSION

Alterations at the 2′ and/or 3′ position of the pentose ring of nucleosides have served to create effective antiviral nucleoside analogs. Substitution with fluorine at these positions appears to be one of the most promising alterations. A series of 2′-fluoro-2′-deoxynucleoside analogs, comprising 2′-fluoro-2′-deoxy-5-methyl-arauridine (FMAU), 2′-fluoro-2′-deoxy-5-iodo-arauridine (FIAU), 2′-fluoro-2′-deoxy-5-methyl-aracytidine (FMAC), and 2′-fluoro-2′-deoxy-5-iodo-aracytidine (FIAC), were found to be very potent inhibitors of herpes simplex virus types 1 and 2 and cytomegalovirus (17, 18).

Recently, several fluorinated nucleoside analogs have been reported as inhibitors of HIV-1 (10, 11, 13, 14, 19, 25). However, modification with fluorine was not always advantageous but depended on the position of the fluorine and the combination of fluorination with other modifications of the pentose ring.

Fluorination of 3′-deoxythymidine to give FLT has been shown to result in increased antiviral activity (4, 11, 13). We tested several other fluorine-substituted thymidine analogs, but none showed greater antiviral activity than FLT.

Thus, for thymidine analogs, fluorination at the 3′ position seems to confer the greatest antiviral activity. Recent studies demonstrate that 3′-fluoro-2′,3′-dideoxyuridine (FLU) also has anti-HIV-1 activity (1, 4). We did not see this activity in our experiments. In our experiments, no fluorinated uridine analog had good antiviral activity. Three fluorinated cytidine analogs had good antiviral activities and low toxicities; they can be ranked in terms of effectiveness as 39 > 84 > 75. However, the selectivities of these compounds are all lower than that of the nonfluorinated parent compound, 2′,3′-dideoxycytidine, as shown by Kim et al. (14). Similarly, our fluorinated adenosine analogs were also less selective than the parent compound 2′,3′-dideoxyadenosine (20). The most potent adenosine analog was 5′-amino-3′-fluoro-3′-deoxyadenosine, with a selectivity index of 100. Thus, fluorination of cytidine or adenosine analogs does confer antiviral activity but less than that of the fluorinated thymidine analogs and with reduced selectivity. The activity and selectivity of compound 26 (5′-amino-3′-fluoro-3′-deoxyadenosine) are extremely interesting, since the compound has no 5′-hydroxyl group at which phosphorylation can occur. The mode of action of this compound therefore appears to be different from those of other dideoxynucleoside analogs and deserves further investigation.

We did not test enough guanosine analogs to assess the effects of fluorination. Modification of the bases of fluorinated nucleoside analogs seems to be of little significance; 7 of 31 compounds tested showed antiviral activities, and 2 of those had rather poor selectivities.

In this report, we have demonstrated synergistic inhibition of HIV-1 replication by combinations of FLT and PFA. These two compounds both inhibit the viral RT but with different mechanisms. FLT is phosphorylated by cellular

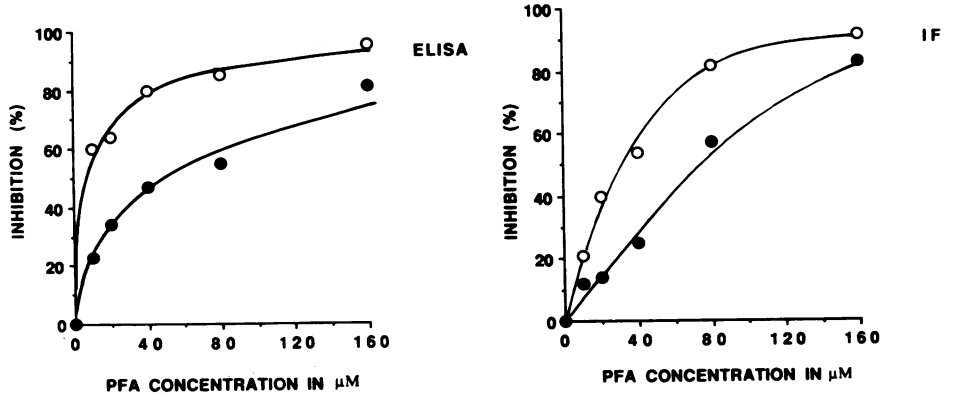


FIG. 3. Inhibition of HIV-1 replication by PFA and FLT, measured by IF and ELISA. A 0.001 μM concentration of FLT was used. Symbols: ○, observed inhibition; ●, expected inhibition calculated by the fractional product method.

kinases and, as a 5'-triphosphate, is incorporated into the viral DNA by RT and thus terminates chain elongation (7, 24), whereas PFA is a $PP_i$ analog and inhibits the RT by occupying the $PP_i$-binding site (27, 28). The mechanism of synergistic inhibition between FLT and PFA is unclear. Several possibilities exist. First, PFA may affect the intracellular metabolism of FLT through the alteration of cellular enzymes or natural deoxynucleoside pools. Second, the binding of PFA to RT may alter the conformation of the adjacent nucleoside-binding site such that FLT can bind with increased affinity at that site as an inhibitor, leading to increased inhibition of RT. Third, PFA may bind with increased affinity to RT in the complex between the enzyme and an FLT-terminated DNA chain, perhaps preventing the enzyme from leaving the terminated DNA chain.

We recently demonstrated synergistic inhibition of HIV-1 replication with the combination of PFA and AZT (15). This combination showed no increased toxicity; in fact, the toxicity of the combination was less than expected. These two factors, increased antiviral activity and decreased toxicity, make combination chemotherapy an attractive proposal.

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