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Anti-human immunodeficiency virus 1 (HIV-1) activities of 3-deazaadenosine analogs: Increased potency against 3′-azido-3′-deoxythymidine-resistant HIV-1 strains

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ABSTRACT **3-Deazaadenosine (DZA), 3-deaza-(±)-aristeromycin (DZAri), and 3-deazaneplanocin A (DZNep) are powerful modulators of cellular processes. When tested against H9 cells infected acutely with two different strains of human immunodeficiency virus 1 (HIV-1) and in the chronically infected monocytoid cell lines U1 and THP-1, the 3-deazanucleosides caused a marked reduction in p24 antigen production. Similar reductions in p24 antigen were seen in phytohemagglutinin-stimulated peripheral blood mononuclear cells infected with clinical HIV-1 isolates. Strikingly, in comparing the therapeutic indices between the paired pre- and post-3′-azido-3′-deoxythymidine (AZT) treatment HIV-1 isolates, DZNep and neplanocin A showed an increase of 3- to 18-fold in their potency against AZT-resistant HIV-1 isolates. In H9 cells treated with DZNep and DZAri, the formation of triphosphate nucleotides of DZNep and DZAri was observed. The mode of action of DZNep and DZAri appears complex, at least in part, at the level of infectivity as shown by decreases in syncytia formation in HIV-1-infected H9 cells and at the level of transcription as both drugs inhibited the expression of basal or *tat*-induced HIV-1 long terminal repeat chloramphenicol acetyltransferase activity in stably transfected cell lines. Since DZNep induced in H9 cells a rapid expression of nuclear binding factors that recognize the AP-1 transcription site, the anti-HIV-1 activity of the DZA analogs could partly be the induction of critical factors in the host cells. Thus, the 3-deazanucleoside drugs belong to an unusual class of anti-HIV-1 drugs, which may have therapeutic potential, in particular against AZT-resistant strains.**

3-Deazaadenosine (DZA), 3-deaza-(±)-aristeromycin (DZAri; carbocyclic 3-deazaadenosine), and 3-deazaneplanocin A (DZNep) (Fig. 1) exert a variety of biological effects, such as highly potent inhibition of *S*-adenosylhomocysteine hydrolase (1–3), gene activation (4), cellular differentiation (5, 6), immunological modulation (7, 8), and antiviral activity (2, 9–11). The DZA analogs have been shown to inhibit methylation of DNA (5), RNA (12), lipid (1, 13, 14), and protein (14). A unique advantage to the DZA analogs is their resistance to deamination by adenosine deaminase (1).

Although their antiviral activity is one of the most significant and studied effects of DZA analogs (2, 9–11), their mode of action as antiviral compounds remains largely undefined. Two relevant observations suggest possible antiviral mechanisms. First, there is a strong correlation between the inhibition of *S*-adenosylhomocysteine hydrolase and the antiviral activity of the DZA analogs (2, 11), suggesting that the DZA analogs may inhibit methylation. Second, a triphosphate analog of DZA (15, 16) may act as an inhibitor of DNA or RNA synthesis. Alternatively, a combination of these or other mechanisms is possible.

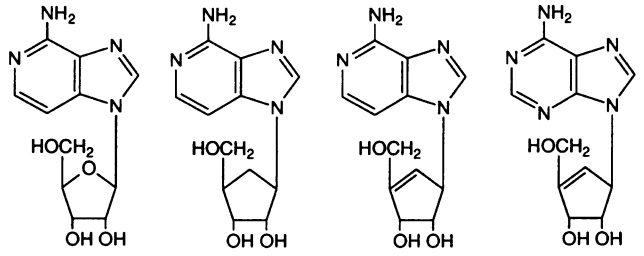

FIG. 1. Chemical structures of DZA, DZAri, DZNep, and neplanocin A (NepA).

Most of the potential drugs against HIV-1 are nucleoside analogs that inhibit the reverse transcriptase (RT) of HIV-1 after anabolic phosphorylation (17). Preliminary reports have indicated anti-HIV activity of DZA (18, 19), but the potential use and mechanism of action of the DZA analogs as anti-HIV drugs have not been explored in detail. Furthermore, in view of the rise of 3′-azido-3′-deoxythymidine (AZT)-resistant strains of HIV-1 in AIDS patients (20–22), it is important to determine whether the AZT-resistant strains of HIV-1 are susceptible to the DZA analogs. We show here that when tested against different HIV-1 strains in different cell cultures, the DZA analogs exhibited potent anti-HIV-1 activities.

MATERIALS AND METHODS

Inhibition of HIV-1 p24 Antigen Production in Peripheral Blood Mononuclear Cells (PBMCs). All clinical HIV-1 isolates were obtained by cocultivation of phytohemagglutinin (PHA)-stimulated uninfected PBMCs with fresh patient PBMCs obtained by Ficoll/Hypaque separation of heparinized blood (22). The HIV-1 tissue culture infectious dose 50% ($TCID_{50}$) was determined as described (22). PHA-stimulated PBMCs were incubated with cell-free HIV-1 stock at 200 $TCID_{50}$ per 2×10^5 cells for 1 h at 37°C. After washings with RPMI 1640 medium, the cells were grown in microtiter

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Abbreviations: AZT, 3′-azido-3′-deoxythymidine; CAT, chloramphenicol acetyltransferase; DZA, 3-deazaadenosine; DZAri, 3-deaza-(±)-aristeromycin; DZNep, 3-deazaneplanocin A; HIV-1, human immunodeficiency virus 1; LTR, long terminal repeat; NepA, neplanocin A; RT, reverse transcriptase; PHA, phytohemagglutinin; PBMC, peripheral blood mononuclear cells.

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plates with different drug concentrations at 2×10^5 cells per well. On day 4, the cells were resuspended and split 1:3. Medium with the appropriate drug concentration was then replaced in each well. Supernatant p24 antigen was determined on day 7 by ELISA (Coulter).

Inhibition of HIV-1_{MN} in H9 Human Lymphoma T Cells. H9 cells in logarithmic phase, grown in RPMI 1640 medium with 10% fetal bovine serum (FBS), were infected with HIV-1_{MN} at a ratio of 10 ng of p24 per 10^8 cells. After 60 min at 37°C, the cells were washed with phosphate-buffered saline (PBS) removing unadsorbed virus. The cells were then diluted 1:5 with RPMI 1640 medium with serum plus drugs. After 8 days, the number of syncytia formed was counted (23), and viral RT released into the supernatant was assayed (24).

Inhibition of HIV-1 p24 Antigen Production in Chronically Infected Cell Lines. Chronically HIV-1-infected U1 (25) and high level HIV-1-producing cells THP-1-ADA (monocytes) and HUT 78-BP-1 (T cells) (26) were cultured in RPMI 1640 medium with 10% FBS at 2×10^5 cells per ml in the presence of DZNep and DZAri. After 4 days, cell viability was determined and cultures were assayed for p24 antigen.

HIV-1 Long Terminal Repeat Chloramphenicol Acetyltransferase (LTR–CAT) Assay. H938 (H9 stably transfected with HIV LTR) and BF24 (THP-1 stably transfected with HIV LTR) cell lines (27) were cultured at 3×10^5 cells per ml in RPMI 1640 medium with 10% fetal calf serum for 2–4 days with various concentrations of DZNep and DZAri. Cell extracts were prepared by three cycles of freezing and thawing, and the CAT assay was performed with 16 mM acetyl-CoA for 8 h as described (4).

Electrophoretic Mobility-Shift Assay (EMSA) of Transcription Factors. After each treatment, H9 cells were centrifuged and washed with PBS. The cell pellet was resuspended in 400 μl of ice-cold buffer containing 10 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride and placed on ice. The EMSA was subsequently performed using the double-stranded oligonucleotide for AP-1 (Stratagene), which was end-labeled with [γ-^{32}P]ATP using T4 kinase.

Identification of DZAri and DZNep Triphosphates in H9 Cells. Logarithmic phase H9 cells were incubated with 1 μM [^{3}H]DZAri (14 Ci/mmol; 1 Ci = 37 GBq) or [^{3}H]DZNep (1.6 Ci/mmol) (Moravek Biochemicals, Brea, CA). After 18 h, the cells were centrifuged and washed twice with PBS minus calcium and magnesium. Next, 0.5 M $HClO_4$ was added, sonicated on ice for 30 sec, and neutralized with K_2CO_3. After centrifugation, the supernatant was lyophilized, dissolved in water, and filtered. The extracted nucleotides were chromatographed on a Whatman Partisil 10 SAX anion-exchange column (28).

The triphosphates of DZAri and DZNep collected from the SAX column were desalted over activated charcoal (16). The samples were dissolved in 0.25 ml of 50 mM Tris, adjusted to pH 8 with NaOH, and incubated with 90 units of calf alkaline phosphatase and 2.5 units of *Escherichia coli* alkaline phosphatase for 24 h at 37°C (28). The reaction was stopped by boiling for 1 min and the mixture was filtered. The nucleosides were chromatographed with a Waters μBondapak C_{18} 125-Å 10-μm column (3.9 × 300 mm) with a C_{18} Guard-Pak insert. The buffer used for isocratic elution at 1 ml/min was 98% 5 mM $(NH_4)H_2PO_4$ (pH 2.8) and 2% acetonitrile.

Measurement of Cellular Toxicity. Cellular toxicity of the drugs was assessed by (*i*) trypan blue exclusion; (*ii*) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to measure growth by cell-mediated reduction of tetrazolium; (*iii*) and Alamar Blue, a redox potential indicator of metabolic activity (Biosource International, Camarillo, CA).

RESULTS

Inhibition of p24 Antigen Production by HIV-1 Clinical Isolates in PHA-Stimulated PBMC. DZNep, DZAri, and DZA were tested against the HIV-1 isolates in PHA-stimulated PBMCs and compared to AZT. The ability of the drugs to inhibit the production of p24 antigen, expressed as IC_{50} values, by clinical HIV-1 strains isolated from patients with AIDS or ARC (AIDS-related complex) is shown in Table 1. An IC_{50} value of <0.2 μM AZT is the normal value for HIV-1 isolates from AZT-naive patients, while an IC_{50} value of >1.0 μM AZT suggests AZT resistance (20–22). For each of the three paired HIV-1 isolates pre- and post-AZT treatment (A012, A018, and 14a), DZNep was consistently more potent than AZT in inhibiting p24 production. Strikingly, when DZNep was tested against the post-AZT treatment (AZT resistant) HIV-1 isolates, the IC_{50} value decreased in each case, indicating an increased sensitivity of the AZT-resistant HIV-1 isolates to DZNep. Specifically, the IC_{50} values of DZNep decreased in the HIV-1 isolates resistant to AZT from about 10 to 1 nM for the A012 isolates, from 16 to 5 nM for the A018 isolates, and from 10 to 1 nM for the 14a-4/87 and 14a-6/89 HIV-1 isolates pre- and post-AZT treatment, respectively (Table 1). Similar trends were observed for NepA, the parent compound of DZNep (Table 1). Thus, there was unequivocally a heightened sensitivity of the AZT-resistant HIV-1 isolates to both DZNep and NepA.

In comparison, DZAri and DZA were less effective than DZNep or NepA in the inhibition of p24 antigen production by the paired pre- and post-AZT treatment HIV-1 isolates. For all the HIV-1 isolates, the IC_{50} values ranged from 8.0 nM to 0.4 μM for DZAri and from 7 nM to 0.9 μM for DZA. The therapeutic indices ranged from 600 to ≈15,400 for both DZNep and NepA (Table 1). There was an increase of 3- to 18-fold in the therapeutic index of DZNep or NepA from the pre-AZT isolates to the post-AZT isolates. None of the post-AZT treatment HIV-1 isolates (A012, A018, and 014a-6/89) showed any cross-resistance to the drugs. With respect to the five unpaired clinical HIV-1 isolates (series 18000), DZNep remained consistently more potent than AZT, DZAri, or DZA, with a therapeutic index ranging from about 200 to 4000 (Table 1).

Inhibition of HIV-1_{MN} Infectivity in H9 Cells. H9 cells were infected with HIV-1_{MN} strain and then treated with DZNep, DZA, and DZAri. The IC_{50} values obtained for DZNep in the inhibition of (*i*) syncytia formation, (*ii*) RT released into the cell supernatant, and (*iii*) p24 antigen production by HIV-1_{MN} in the H9 cells were all ≈1 nM (Fig. 2). Both DZA and DZAri inhibited syncytia formation with IC_{50} values also at ≈1 nM (data not shown).

Formation of DZAri Triphosphate and DZNep Triphosphate in H9 Cells. Fig. 3*A* identifies ADP and ATP extracted from the H9 cells with retention times of 20.4 and 38 min, respectively. Two radioactive peaks (Fig. 3*B*) were tentatively identified as [^{3}H]DZAri diphosphate ([^{3}H]DZAriDP; 16.6 min) and [^{3}H]DZAri triphosphate ([^{3}H]DZAriTP; 38 min) because they were eluted with retention times characteristic of diphosphates (14–21 min) or triphosphates (31–43 min). The peak observed in the void volume (Fig. 3*B*, arrow) was unphosphorylated [^{3}H]DZAri, which might also obscure a [^{3}H]DZAri monophosphate peak. A similar profile was observed for [^{3}H]DZNep (Fig. 3*C*). The two new radioactive peaks, [^{3}H]DZNep diphosphate ([^{3}H]DZNepDP) and [^{3}H]DZNep triphosphate ([^{3}H]DZNepTP), were eluted at 17 and 39 min, respectively.

To verify whether the observed peaks were the 3-deaza triphosphates, the peaks were collected from the anion-exchange column and digested with alkaline phosphatase. Fig. 3*E* shows that when the putative [^{3}H]DZNepTP was treated with alkaline phosphatase, it was converted to [^{3}H]DZNep

Table 1. Inhibition of HIV-1 p24 antigen production in PBMCs stimulated by PHA

HIV-1 isolate	Mean IC_{50}, $\mu M \pm SEM$ (n) [therapeutic index]				
	AZT	DZNep	NepA	DZAri	DZA
Paired isolates					
A012 pre-AZT*	0.020 (5)	0.0104 ± 0.0004 (3) [962]	0.0109 (2) [917]	0.141 ± 0.035 (3) [71]	0.144 ± 0.037 (3) [69]
A012 post-AZT*	2.101 (5)	0.0013 ± 0.0004 (3) [7692]	0.0028 (2) [3571]	0.365 (2) [27]	0.110 (2) [91]
A018 pre-AZT*	0.029 (5)	0.0161 ± 0.005 (4) [621]	0.0178 (2) [562]	0.217 (2) [46]	0.226 (2) [44]
A018 post-AZT*	2.362 (5)	0.0048 ± 0.002 (4) [2083]	0.001 (2) [10,000]	0.086 ± 0.010 (3) [116]	0.683 (2) [15]
14a-4/87 pre-AZT†	0.017 (2)	0.0105 (2) [952]	ND	0.0886 (1) [113]	0.122 (1) [82]
14a-6/89 post-AZT†	1.607 (2)	0.00065 (2) [15,385]	ND	0.0648 (2) [154]	0.059 (2) [169]
Unpaired isolates					
18431	0.422 (3)	0.0027 ± 0.001 (3) [3074]	ND	0.009 ± 0.001 (3) [1111]	0.0069 ± 0.001 (3) [1449]
18453	0.005 (2)	0.0034 (2) [2941]	ND	0.008 (2) [1250]	0.0217 (2) [461]
18190	0.047 (5)	0.0058 ± 0.0023 (3) [1724]	0.0251 (2) [398]	0.062 ± 0.003 (3) [161]	0.127 (2) [79]
18199	1.855 (5)	0.015 ± 0.003 (3) [667]	0.010 (2) [1000]	0.161 ± 0.009 (3) [62]	0.908 (2) [11]
18601	2.064 (4)	0.046 ± 0.005 (3) [217]	0.0534 (1) [187]	0.189 ± 0.058 (3) [53]	0.254 (2) [39]

Number of experimental replicates is indicated in parentheses. Therapeutic index is defined as CC_{50}/IC_{50}, where CC_{50} is the concentration of the drug that kills 50% of the cells and IC_{50} is the concentration of drug that reduces viral p24 production by 50%. ND, not determined.
*Described in ref. 20 and obtained from National Institutes of Health AIDS Research and Reference Reagent Program.
†Obtained from Johnson *et al.* (21).

since it coeluted with the standard [^{3}H]DZNep at 10.3 min (Fig. 3*D*). Similarly, Fig. 3*G* shows that after the putative [^{3}H]DZAriTP was treated with alkaline phosphatase, it coeluted with standard [^{3}H]DZAri (Fig. 3*F*) at 14.5 min. Therefore, these results were strongly suggestive of the intracellular formation of DZAriTP and DZNepTP in H9 cells.

Inhibition of HIV-1 p24 Antigen Production in Chronically Infected Cell Lines by DZNep. The IC_{50} of DZNep was 20–200 nM for inhibition of p24 antigen production by HIV-1 in U1, a chronically HIV-1-infected low-level virus-producing monocytoid cell line. For the high-level virus-producing monocytoid cell lines, THP-1-ADA and THP-1-BP1, the IC_{50} values of DZNep were 2 μM and 20–200 nM, respectively. For the human T-cell line HUT 78 producing BP-1, the IC_{50} values of DZNep were 2–20 nM. The lymphoid cell lines, CEM, THP-1, and U1 were also tested, and the cytotoxicity (CC_{50}) of all three drugs in the three cell lines was about equal (10–25 μM) (data not shown). Cytotoxicity due to DZNep was not seen until 20–100 μM (data not shown).

Inhibition of HIV-1 LTR-CAT Activity. Fig. 4 shows that the HIV-1 LTR-CAT activity was down-regulated (5-fold) in both H938 and BF24 cells after a 3-day incubation with either DZNep (2–20 nM) or DZAri (2 nM to 2 μM). As observed above, little cytotoxicity was observed until 100 μM (data not shown). When the cell lines were infected with HIV-1 as a source of the transcriptional activator *tat*, the HIV LTR-mediated CAT activity increased 6-fold and DZNep inhibited this activity 5-fold (data not shown), which was similar to the inhibition observed for basal transcription (Fig. 4). DZNep was found to down-regulate the basal expression of the HIV-1 LTR in H938 cells as early as 8 h after treatment, and no effect of DZNep was seen on human T-cell leukemia virus type 1-LTR-mediated CAT activity (data not shown). Since DZNep or DZAri inhibited the transcription of the LTR in cell lines not infected with HIV-1, the results suggested that cellular factor(s) that modulate the expression of HIV-1 LTR might be affected. This possibility was supported by the following experiment.

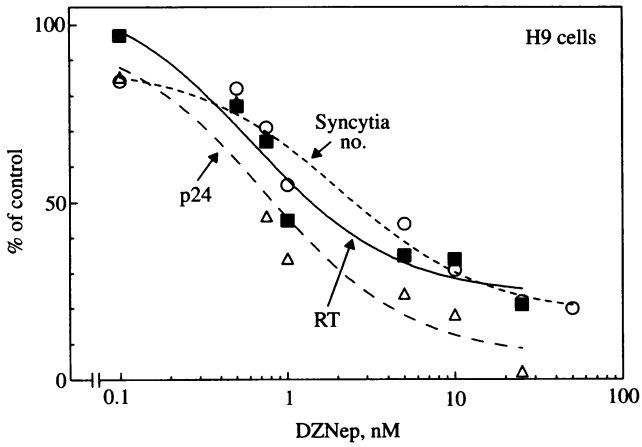

FIG. 2. Inhibition by DZNep of syncytia formation, p24 antigen production, and RT released in H9 cells infected with HIV-1_{MN}.

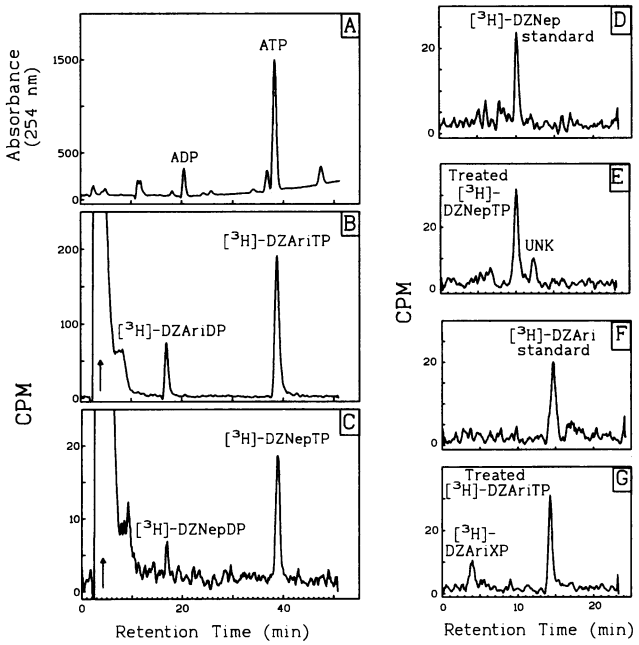

FIG. 3. (*A–C*) Anion-exchange chromatography of nucleotides extracted from H9 cells. (*A*) UV at 254 nm showing ATP and ADP. (*B*) Elution of [^{3}H]DZAriDP and [^{3}H]DZAriTP; arrow, elution of unreacted [^{3}H]DZAri. (*C*) Elution of [^{3}H]DZNepDP and [^{3}H]DZNepTP; arrow, elution of unreacted [^{3}H]DZNep. (*D–G*) Reverse-phase chromatography of isolated triphosphate peaks treated with alkaline phosphatase. (*D*) [^{3}H]DZNep standard. (*E*) [^{3}H]DZNepTP treated with alkaline phosphatase; UNK, unknown peak. (*F*) [^{3}H]DZAri standard. (*G*) [^{3}H]DZAriTP treated with alkaline phosphatase. [^{3}H]DZAriXP, nucleotide unhydrolyzed or partially hydrolyzed by the alkaline phosphatase.

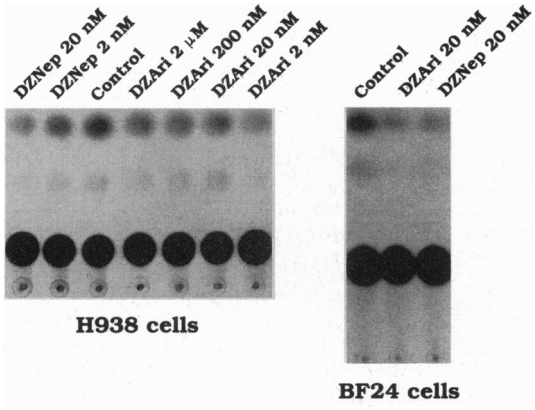

FIG. 4. Inhibition of HIV-1 LTR-CAT. H938 cells and BF24 were treated for 3 days with DZNep or DZAri. CAT was assayed with 16 mM [^{14}C]chloramphenicol for 8 h.

Induction of the Transcription Factor AP-1 in H9 Cells by DZNep. When the H9 cells were incubated with DZNep, there was a rapid expression of the transcription factor AP-1 (Fig. 5). The AP-1 formed was evident after 30 min, reaching its peak at 90 min, and it started to diminish at 120 min. Moreover, the induction of AP-1 by DZNep was dose dependent. The induction of AP-1 expression was in accord with an earlier observation that all three DZA analogs can induce gene transcription (5).

DISCUSSION

The results presented here demonstrate that the DZA analogs belong to a new class of drugs that are potent inhibitors of HIV-1 *in vitro.* Furthermore, in the human CD4$^+$ cell line H9, the IC_{50} values of DZNep, DZAri, and DZA for the inhibition of p24 antigen production by HIV-1, RT released, and inhibition of syncytia formation were all 1 nM. Importantly, the 3-deazanucleosides were more effective against AZT-resistant HIV-1 isolates, which unexpectedly showed increased sensitivity to both DZNep and NepA. In the paired isolates (pre-AZT and post-AZT), the therapeutic index increased 3- to 18-fold for DZNep and NepA, in comparison to the same pre-AZT isolates. The latter finding is of clinical significance in view of increases in the number of AZT-resistant HIV-1 isolates after prolonged therapy with AZT (20–22). Because the unpaired HIV-1 isolates (18000 series) were from patients of unknown treatment history, the therapeutic indices might not respond as markedly because of unknown prior drug therapies. Viruses with resistant and sensitive mutations derived from molecular clones will further clarify this issue. Because there are only small differences between the enzymatic activities of AZT-resistant and AZT-sensitive RT, the mechanism of HIV-1 resistance to AZT remains to be elucidated (29). However, the DZA analogs were not very cytotoxic until ≈100 μM, in agreement with other reports (9, 11, 18, 19, 30).

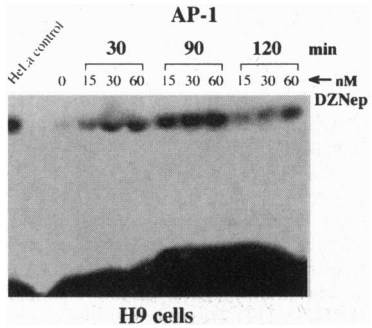

FIG. 5. Induction of AP-1 transcription factor in H9 cells.

In comparing the different IC_{50} values for inhibition of p24 antigen production in the chronically infected cell lines, a more complex situation emerged. There was a 3 orders of magnitude difference between the IC_{50} of DZNep for the anti-HIV-1 activity in BP1-HUT 78 (T cells) and the activity in THP-1-ADA cells (monocytes)—i.e., 2 nM vs. 2 μM, respectively. Among the possible explanations for the observed variations in IC_{50} values are differences in drug uptake or phosphorylation efficiencies. Moreover, the difference in the IC_{50} of DZNep for the anti-HIV-1 activity might be due to the variations in transcription factors, such as the NF-κB binding proteins between these two cell types (31).

Part of the anti-HIV-1 effect of the drugs could be the inhibition of methylation reaction(s). The DZA analogs might modulate methylation of the viral genome or posttranslational events. Possibly, changes in methylation could occur in host cells leading to the anti-HIV-1 effects. Or a nucleotide of any of the DZA analogs may act as an inhibitor of DNA or RNA synthesis. It has been shown here and by others (15, 16, 28) that DZA analogs can be converted to nucleotide derivatives in cells. As expected, the nucleoside (unphosphorylated) forms of the DZA analogs by themselves were unable to inhibit the activity of RT *in vitro* (data not shown). The effect of DZA nucleotides on HIV-1 RT awaits a feasible synthetic procedure.

The fact that DZNep and DZAri inhibited the expression of basal, or *tat*-induced, HIV-1 LTR-CAT activity in T cells (H938) and monocytes (BF24) suggests that these drugs may act at the transcription level. In addition, DZNep and DZAri showed HIV-1 specificity since they had no effect on the human T-cell leukemia virus type 1 LTR-CAT. Since DZNep and DZAri inhibited the transcription of the LTR in cell lines not infected with HIV-1, cellular factor(s) that modulate the expression of HIV-1 LTR might be affected by the drugs. It was interesting that DZNep rapidly increased the expression of AP-1, a protein product of the protooncogenes c-*fos* and c-*jun*, which may be involved in cell proliferation, differentiation, and viral expression (31–33). The down-regulation of HIV-1 LTR-CAT activity in H938 and BF24 cells by DZA analogs as early as 8 h supports the possible induction of cellular factor(s) that may interact with HIV-1 LTR, leading to the inhibition of HIV-1 replication. Moreover, NF-κB is down-regulated when AP-1 is induced (34), an effect that could contribute to the anti-HIV activity of these drugs because NF-κB is critical for the LTR-directed HIV-1 transcription (34). The induction of AP-1 expression agreed with the findings that DZA analogs can induce gene transcription (4) and modulate cytokine production (8).

In summary, DZNep, DZAri, and DZA belong to an unusual class of anti-HIV-1 drugs. The mode of action of this class of drugs is most likely multifactorial. Since there are clinical data on the lack of toxicity of DZA in humans (35), the use of this class of drugs for AIDS therapy warrants further investigation. A thorough elucidation of the mechanism of action of the DZA analogs may reveal new means to control HIV-1 infectivity.

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