ANTIRETROVIRAL EFFECT OF 4-THIO-URIDYLATE AGAINST HUMAN IMMUNODEFICIENCY VIRUS TYPE 1

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Antiretroviral effect of thiolated nucleotide 4-thio-uridylate (S^4UMP, designated as UD29) against human immunodeficiency virus type 1 (HIV-1) have been quantitatively determined in cell-based viral infectivity assays. In syntitium inhibition assay on MT-2 human T-cell line UD29 prevented cell fusion and formation of syntitia induced by HIV-1$_{IIIB}$ with IC_{50} values of 11.7 µg/ml. In a single-cycle viral infection assay (MAGI assay) UD29 proved to have a potent inhibitory effect against HIV-1$_{IIIB}$ on HeLaCD4-LTR/β-gal cells, which was dose dependent with IC_{50} values of 4.75 µg/ml and IC_{90} of 39.7 µg/ml. UD29 showed a most prominent antiviral effect when administered 30 min prior HIV-1 infection. As HIV entry requires thiol/disulfide exchange process, results suggest that reactive –SH group of enol-form of the thiolated nucleotide may interfere with the function of cell surface proteins. UD29 cannot penetrate into cells and may have an interactive role in redox processes active in viral entry.

Keywords: HIV infection, thiolated nucleosides, redox system, entry inhibitor, infectivity assays

Introduction

Human immunodeficiency virus type 1 (HIV-1) infection and AIDS are serious public health issues, as there are more than 35 million people worldwide with HIV-1 infection or AIDS, according to estimates by the Joint United Nations Programme on HIV/AIDS (UNAIDS) [1].

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The introduction of highly active antiretroviral therapy (HAART) has dramatically reduced morbidity and mortality of patients infected with HIV-1 [2]. The success of antiretroviral treatment, however, is limited by the emergence of HIV-1 drug resistance, and by the fact that the drugs (which are inhibitors of virally coded enzymes) used so far act only while the virus already infected the cells.

The effort to identify therapeutic targets and develop effective treatment [3] has led to extensive characterization of HIV and a better understanding of its replication cycle. The life cycle of retroviruses, including HIV is divided into two distinct phases: the early phase refers to the steps of infection from cell binding to the integration of the viral cDNA into the cell genome, whereas the late phase begins with the expression of viral genes and continues through to the release and maturation of progeny virions.

The first clinically used effective inhibitor of HIV replication was the nucleoside analogue, azido-thymidine (AZT) inhibiting the virus coded reverse transcriptase (RT); later several other nucleoside analogues were developed with the same mode of action. For sensitive detection of AZT, we have developed a specific radioimmuno-assay [4]. A new class of therapeutic HIV inhibitor was developed, the non-nucleoside RT inhibitors (NNRTI). Later, another HIV enzyme, protease (PR) paved the way for the development of new class of antiviral agents: protease inhibitors [5]. Today these classes of inhibitors are the bases of the HAART that has considerably extended the lifespan of many HIV patients. However, the frequent development of resistance towards these drugs limits their therapeutic application, as we analyzed it recently in Hungary [6, 7].

RT and PR inhibitors target HIV enzymes while virus infection has occurred, and HIV already entered into the susceptible cells. The most effective anti-HIV compounds should inhibit HIV entry into cells [8]. This initiated a more extensive study on the early steps of HIV infection and the virus-cell interactions. However, in comparison with the later events of HIV infection, early steps: HIV entry, the mechanism of receptor binding, conformational changes and fusion are still poorly understood [9]. Fully elucidating the early steps of HIV replication is therefore crucial, not only for identifying new antiretroviral drugs, but also for improving the design of retroviral vectors for gene therapy.

The first step in the primary infection is HIV-1 entry into host cells mediated by its envelope glycoprotein (Env). The mature HIV envelope is composed of the surface glycoprotein (gp)120 and transmembrane gp41 subunits [10]. HIV binding to CD4$^+$ lymphocytes is initiated by gp120 interaction with cell surface CD4 (the primary receptor for HIV), which creates a high affinity binding site on

gp120 for the chemokine coreceptor CXCR4 (or in the case of macrophages, CCR5) [11]. Interactions between gp120 and the receptors lead to extensive conformational changes within Env that eventually promote the unmasking of the fusion peptide present on gp41. These conformational changes lead to the fusion of viral and cell membranes allowing HIV to get into cytoplasm [12]. An inhibitor of gp41 (Enfuvirtide/T20) has recently been approved for therapeutic use in combination therapy as HIV entry inhibitor [13].

In addition to conformational changes in cell surface receptors, coreceptors of HIV and viral envelope proteins, redox changes in these proteins are also required for successful HIV-1 entry and infection. Several inhibitors of protein disulfide isomerase (PDI) as well as cell-surface-thiol interacting agents including thioredoxin, influence HIV infection [14, 15] indicating that redox processes active in viral entry could be potential targets for treatment of HIV infection, as we and others reported earlier [14–18].

In this paper we extend the earlier study on the antiretroviral activity of 4-thio-uridylate (designated as UD29) [16] measuring its inhibitory activity in cell infectivity assays.

Materials and Methods

Preparation of thiolated uridylate (S^4UMP, UD29)

The 4-thio uridylate (designated as UD29) was prepared by H_2S treatment of cytidine 5'-monophosphate, as described for oligonucleotides [18], and was provided by Dr. Janos Aradi, University of Debrecen. A 20 mM (6.66 mg/ml) aqueous stock solution of UD29 was prepared and kept at –20º C. It was diluted to the required concentration with tissue culture media before use. In UD29 the 4-thiono group has a propensity toward tautomeric conversion to form a reactive –SH, which may affect redox processes [16] (see Fig. 1). It is soluble in water, tissue culture fluid, and the solution is stable at 4ºC for 15 days. Stock solution has been sterilised by filtration. The UD29 solutions are slightly light sensitive.

Cells and viruses

H9 human T-cell line, MT-2 human T-cell line and HeLaCD4-LTR/β-gal cell lines were obtained from the NIH AIDS Research and Reference Reagent Pro-
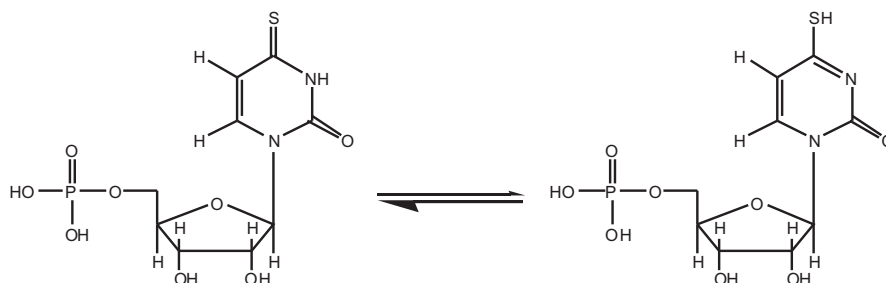
Figure 1. Structure of 4-thio-uridylate (s4UMP, UD29); keto- and enol-tautomers

gram (Bethesda, MD). H9 and MT-2 cell were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM l-glutamine and antibiotics. HeLaCD4-LTR/β-gal cells were cultured in DMEM, 10% fetal calf serum, 2 mM L-glutamine and antibiotics.

Human immunodeficiency virus type 1 (HIV-1$_{IIIB}$) was obtained from R. Weiss (London University, London, UK).

HIV-1$_{IIIB}$ stocks

The HIV-1$_{IIIB}$ strains were propagated in H9 human permanent T lymphocyte cells. HIV-1$_{IIIB}$ stocks were prepared from 24-h supernatants of freshly infected H9 cells at 4 to 6 days after infection. Infectious titer was determined by infectivity assays on MT-2 human T cells (see below). Supernatants with titers of 2.1×10^5 IU/ml were filtered through a 0.45 µ pore size filter (Millipore) and stored at –70°C.

HIV-1 infectivity assays

Syncytium induction and inhibition assays on MT-2 cells

The infectious units of biologically active HIV-1 were determined by their ability to induce syncytia (multinucleated giant cells or big balloon cells) [19, 20] on MT-2 human T-cell line. Cells were cultured in a completed RPMI 1640 medium and seeded in a 24-well plate (8×10^4 cell/well) , and various dilutions of HIV-1 stock (prepared according to our published method [5]) were added. Appearance of syncytia was counted 48 h post infection. The possible inhibitory ac-

tivity on syncytia formation of the thiolated compounds (UD29) was quantitatively determined by adding the compounds in various concentrations (1, 2.5, 5, 10, 20, 40 µg/ml, respectively) to the cells 30 min before, at the time and 30 min after virus infection. Reduction in the numbers of syncytia compared to the one without UD29 treatment was calculated.

Quantitative HIV-1 p24 core antigen assay

Presence of HIV-1 core protein p24 in the supernatant of MT-2 cells upon infection with HIV-1 stock, and the reduction of the concentration of HIV-1 p24 upon addition of the thiolated nucleotide (UD29) in various concentrations (see above) before, at the time and after HIV-1 infection was quantitatively determined by a HIV-1 p24 core antigen ELISA (BioMérieux, France).

Multinuclear activation of galactosidase inhibition (MAGI) assay

Infectious titer of HIV-1 has also been determined by the MAGI assay, as we previously published [5]. Basically HeLaCD4-LTR/β-gal cells was seeded in 24-well plates and infected with a multiplicity of infection (moi) of 0.02, 0.2, and 0.4, respectively, of HIV-1 stock virus in the presence or in the absence of various concentrations (1, 2.5, 5, 10, 20, 40 µg/ml, respectively) of UD29 . UD29 was added to the cells 30 min before, at the time and 30 min after virus infection. Forty hours after infection, infected cells were counted *in situ* with light microscope by virtue of their blue color after incubation with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Per cent inhibition was calculated as mean numbers of blue cells of inhibitor-treated versus non-treated cultures using Hill analysis.

Statistical methods

For the determination of UD29 inhibitory concentrations IC_{50} and IC_{90} values Hill analysis with 95% confidence limit was used. For other biostatistical analysis two-tailed Student's test was used.

XTT Assay measuring cytotoxicity

Prior to infectivity assays, the potential toxic effect of UD29 compound on HeLaCD4-LTR/β-gal cells has been determined quantitatively *in vitro* by XTT

based Toxicology Assay Kit (Sigma-Aldrich), which is a spectrophotometric measurement of cell viability based on mitochondrial dehydrogenase activity in living cells. The mitochondrial dehydrogenases of viable cells reduce the terazolium ring of XTT (2,3-bis/2-Mthoxy-4-nitro5-sulphophenyl/-2H-terazo-lium-5-carboxyanilide inner salt) yielding an orange formazane derivative. Absorbance of converted dye is measured at a wavelength of 450 nm. During XTT assay to measure cytotoxicity each well contained 125 µl (DMEM, 10%FCS, Pen/Str) volume. The incubation period was 48 hours. At test all of the media was removed and 100 µl DMEM was added. 20% of dissolved XTT reagent (Sigma-Aldrich) was added to the media. Incubation time prior to measuring was 2.5 hours. Assay was performed according to the protocol.

Results

UD29 effect on cell viability

For analyzing possible antiviral effect of the thiolated uridylate *in vitro,* its potential cytotoxicity on cell viability was determined on MT-2 human T lymphoid cells as well as quantitatively on HeLaCD4-LTR/β-gal cells (Fig. 2). The acute (24 h) and chronic (48 h) cytotoxicity of the UD29 compound was determined by XTT Assay (Sigma-Aldrich), that measures metabolic activity, and is
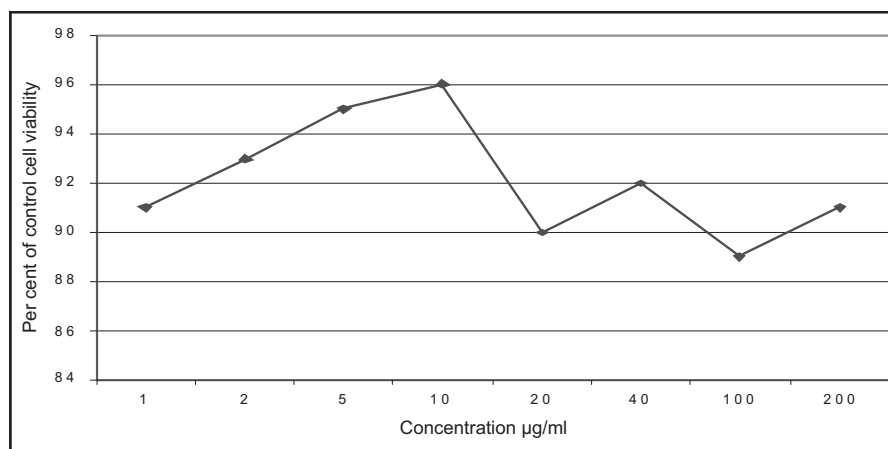
Figure 2. Toxicity of UD29 on HeLaCD4-LTR/β-gal cells
UD29 was administered at the indicated concentration to the monolayer cell cultures, and incubated
for 48 h. XTT assay, absorbance was read at 450 nm

suitable for analyzing proliferation, viability, and cytotoxicity. There was no detectable cytotoxicity (morphologically or by formazan dye reduction) when as high as 200 µg/ml doses were utilized demonstrating that UD29 is a relatively non-cytotoxic nucleotide analogue.

Table I

Inhibitory effect of UD29 on a single cycle infection of HeLaCD4-LTR/β-gal cells infected with HIV-1$_{IIIB}$

Concentration of UD29 µg/ml	% inhibition[a]		
	−30 min	at infection	+30 min
1	2	2	<1
2.5	40	19	2
5	46	26	5
10	81	32	7
20	85	39	8
40	91	48	9
IC$_{50}$	4.75 µg/ml	>40 µg/ml	>40 µg/ml
IC$_{90}$	39.7 µg/ml	>40 µg/ml	>40 µg/ml

[a] Inhibitor was added to the cultures at indicated time intervals before, at the time and after infection with virus. β-galactosidase enzyme expression was quantitatively determined (see Materials and Methods), and per cent inhibition was calculated using Hill analysis. Results are representative of multiple antiviral assays with values shown as means of triplicates. Standard deviation was ± 5.8%.

Antiviral activity of UD29 in cell based infectivity assays in vitro

Inhibition of cell fusion on MT-2 cells

When added to MT-2 human T-cells 30 min prior infection with HIV-1$_{IIIB}$ strain, UD29 prevented formation of syncytia (multinucleated giant cell and/or "balloon" cells without cell structures) (Fig. 3). Quantitative determination of HIV-1 p24 core protein in the supernatant of virus infected MT-2 cells with and without treatment of indicated UD29 concentrations demonstrated that UD29 showed an activity on inhibition of HIV-1$_{IIIB}$ induced cell fusion with IC$_{50}$ values of 11.7 µg/ml (Table II).

Inhibitory effect of UD29 on a single cycle infection with HIV-1$_{IIIB}$ of HeLaCD4-LTR/β-gal cells

Now inhibition of HIV-1 replication by UD29 was assessed in standard cell-based assay, such as the MAGI assay. HeLaCD4-LTR/β-gal cells have been

Table II

Antiviral activity of UD29 against HIV-1$_{IIIB}$: assessment for inhibition cell fusion and virus entry

|	IC$_{50}$[a]	TC$_{50}$[b]	TI[c]
Cell fusion	11.7 µg/ml	>200	> 17
Viral entry[d]	4.75 µg/ml	> 200 > 42	

[a] 50% inhibition of virus infection
[b] 50% reduction in cell viability
[c] *In vitro* therapeutic index
[d] In MAGI assay

treated with various concentration of UD29 (1–40 µg/ml) 30 min before, at the time and 30 min after infection of cells with HIV-1$_{IIIB}$ virus of 0.02, 0.2, and 0.4 moi. HeLa cell construct allows only a single cycle of virus infection, therefore it is suitable to test compounds with a potential of inhibiting viral entry. Forty hours after virus infection β-galactosidase enzyme expression was quantitatively determined (see Materials and Methods), and per cent inhibition as well as IC$_{50}$ and IC$_{50}$ values were calculated using Hill analysis. UD29 showed a most prominent antiviral effect when administered 30 min prior to HIV-1 infection (Table I). UD29 proved to have a potent inhibitory effect against HIV-1$_{IIIB}$ on HeLaCD4-LTR/β-gal cells, which was dose dependent with IC$_{50}$ values of 4.75 µg/ml and IC$_{90}$ of 39.7 µg/ml (Table I). It was noted, that its antiviral activity was more pronounced in the infectivity (MAGI) assay than UD29 inhibitory effect on cell fusion (Table II).
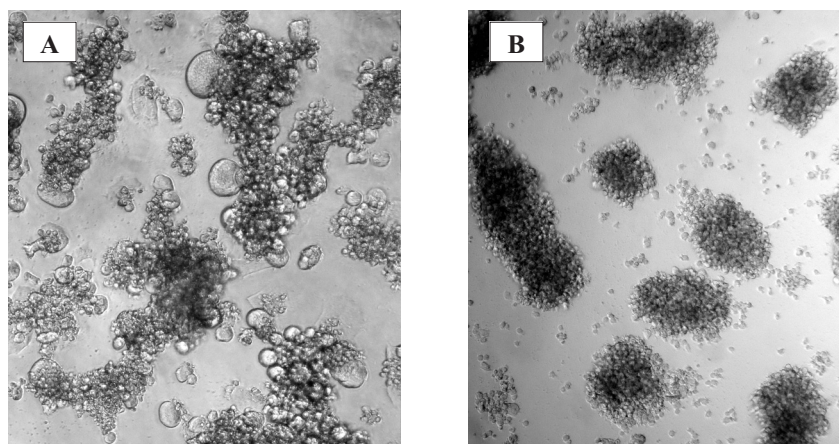
Figure 3. Inhibition of formation of syntitia (arrows) i.g. HIV-1$_{IIIB}$ virus infected MT-2 cells upon treatment with UD29. A: virus infected, untreated cells, B: MT-2 cells treated with UD29 (10 µg/ml) 30 min prior to viral infection. No syncyitia were formed

Discussion

To elucidate the exact inhibitory mechanism of UD29 a time-of-addition experiment was performed. This experimental approach was able to specifically identify the stages of viral life cycle targeted by thiolated uridylate, UD29. It was shown that maximal inhibitory activity was observed when the drug (UD29) was added prior to HIV-1 infection, in both syntitium, and in infectivity (MAGI) assays. When UD29 was added after virus infection its inhibitory activity was reduced significantly (Table I). This result suggests that the inhibitory activity on virus infection was exerted during early stage of viral life cycle, most likely, in the entry process. Moreover, as UD29 is a nucleotide, its cellular uptake is not likely, it cannot penetrate into the cells, therefore its activity should be associated with events on the cell surface, virus-cell binding, and/or the entry.

In addition to conformational changes in cell surface receptors, coreceptors of HIV (CD4, CCR5, and CXCR4) and viral envelope proteins (gp120, gp41), redox changes in these proteins are also required for successful HIV entry [21–28]. Specifically, (1) the reduction of the second domain of CD4 was reported to be an obligatory step in CD4-dependent fusion [25]; (2) two of the nine disulfide bonds of gp120 must be reduced in order to promote the successful HIV entry [23, 28]. UD29 is chemically a thiolated nucleotide, in which the enol form of the compound (Fig. 1) contains a reactive –SH group. It was shown earlier that UD29 is an inhibitor of glyceraldehyde-phosphate dehydrogenase (GAPDH) [16], likely interfering with the function of the essential –SH group in the active centre of the enzyme. The interfering effect of the –SH group of the nucleotide with protein –SHs could be the bases of the HIV entry inhibitory activity of UD29, verified by our time-of-addition experiment. UD29 may affect the redox changes of the second domain of CD4 and/or the proper reduction of the disulfide bonds of gp120. The proposed mode of action is in good agreement with results of the time-of-addition experiments and with the syncytia formation assay. The formation of syncytia requires active membrane reorganization in which cell membrane redox processes are likely to be also involved.

It must be noted that UD29 is a mononucleotide, however, its deoxyoligonucleotide derivatives are also active biologically inhibiting the HIV entry and the telomerase enzyme [18, 29, 30]. Preliminary results on the activity of some other derivatives of the thiolated nucleotide and polynucleotide have also been reported [31].

In summary, UD29 is a new HIV entry inhibitor acting on the cell surface, likely affecting the redox modifications of receptors and viral proteins. This mechanism has not been exploited yet for the treatment of HIV infection.

Aknowledgement

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