University of Groningen

Delivery of the anti-HIV drug azidothymidine (AZT) to T-lymphocytes with neoglycoprotein carriers

Molema, Grietje

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1992

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Molema, G. (1992). *Delivery of the anti-HIV drug azidothymidine (AZT) to T-lymphocytes with neoglycoprotein carriers*. s.n.

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Download date: 13-02-2023

Chapter 4. Simultaneous analysis of azidothymidine and its mono-, di- and triphosphate derivatives in biological fluids, tissue and cultured cells by a rapid high-performance liquid chromatographic method

Grietje Molema, Robert W. Jansen, Jan Visser and Dirk K.F. Meijer

Abstract

A rapid HPLC method for the simultaneous analysis of the antiviral drug azidothymidine (AZT), AZT-monophosphate, AZT-diphosphate and AZT-triphosphate, with UV detection in the nanomolar range, is described. Determination of these compounds *in vitro* in the human MT-4 lymphocyte cell line did not require a prior extraction and AZT and its phosphorylated derivatives could be accurately analyzed in one HPLC run. However, plasma, bile, liver homogenate and urine samples could not be injected directly into the chromatograph. Therefore, a solid phase extraction procedure was developed, using azidodideoxyinosine as internal standard. The extractions of the compounds of interest from all but urine samples were reproducible, with recoveries between 65% (AZTTP from plasma) and 100% (AZT from plasma).

Introduction

To date, the most commonly used drug for the treatment of the acquired immune deficiency syndrome (AIDS[1]) and AIDS related symptoms, is AZT (3'-azido-3'-deoxythymidine, Retrovir[R], fig. 1). AZT was reported to be a very potent agent *in vitro* against HIV-1 by Mitsuya et al. in 1985 [1]. Although it has shown definite clinical effects in AIDS patients [2-4], AZT also induces toxic side effects [2,5], the most severe being bone marrow depression [6].
Once inside the cell, the drug is phosphorylated into its active triphosphate form (AZTTP) [7], which interacts with the human immunodeficiency virus (HIV) associated reverse transcriptase (RT). The triphosphate derivative of AZT acts either as a competitive inhibitor of RT or as an alternative substrate instead of thymidine-triphosphate, leading to integration into the newly formed DNA and subsequent inhibition of DNA chain elongation [8].
The covalent attachment of AZT to a macromolecular carrier may result in a lower systemic concentration of the drug and may therefore reduce the toxic side effects.

[1] Abbreviations used: AIDS: acquired immune deficiency syndrome; AZT: 3'-azido-3'-deoxythymidine; AZTMP: AZT-monophosphate; AZTDP: AZT-diphosphate; AZTTP: AZT-triphosphate; RT: reverse transcriptase; NaH_2PO_4: sodiumhydrogenphosphate; Na_2HPO_4: disodiumhydrogenphosphate; TBAS: tetrabutylammoniumsulphate; aqua dest.: aqua destillata, single destilled water; ddI: 2',3'-dideoxyinosine; ddC: dideoxycytidine; azidoddI: 3'-azido-2',3'-dideoxyinosine; azidoddBrA: 3'-azido-2',3'-dideoxy-8-bromoadenosine; FddBrU: 3'-fluoro-2',3'-dideoxy-5-bromouridine.

This will also alter its pharmacokinetic behaviour *in vivo*, among others, resulting in a diminished susceptibility to metabolic inactivation and clearance by the liver and the kidneys [5]. Also, by employing a carrier specifically recognized by the main target cells of HIV-1 (CD4 molecule positive T-lymphocytes, monocytes and macrophages [9-16]) the selectivity of the drug can be increased.
Covalent binding of the 5'-monophosphate derivative of AZT (AZTMP) to some neoglycoproteins (human serum albumin chemically modified with various sugar derivatives) resulted in potent conjugates against HIV-1 cytopathicity *in vitro* in the human CD4 positive T-lymphocyte cell line MT-4 [17].
To unravel the mechanisms of anti-HIV-1 activity of these conjugates, it is of great importance to learn more about the intracellular kinetics of the drug, administered to the cells in its free form and in its conjugated form.
The present study describes the development of a stable, rapid and sensitive HPLC method for the simultaneous analysis of AZT and its mono-, di- and triphosphate derivatives (AZTMP, AZTDP and AZTTP, respectively).

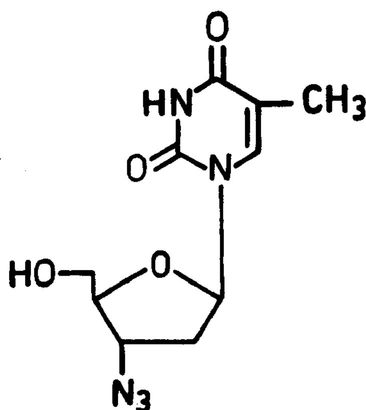

Figure 1. Structure of 3'-azido-3'-deoxythymidine (AZT, Retrovir[R]).

Materials and methods

Materials. NaH_2PO_4, Na_2HPO_4 and TBAS (Merck, Darmstadt, Germany) were of analytical grade. Acetonitrile (Labscan Ltd., Dublin, Ireland) was of HPLC-grade. The water used was Milli-Q quality. AZT, AZTMP and AZTTP were kindly provided by dr. P. Herdewijn, Rega Institute, Leuven, Belgium. ddI, ddC, azidoddI, azidoddBrA and FddBrU were kindly provided by dr. J. Balzarini, Rega Institute, Leuven, Belgium. Alkaline phosphatase (type I-S, from bovine intestinal mucosa) was purchased from Sigma Chemical Co., St. Louis MO.
All other chemicals used were the best grade available.

Chromatographic method. The HPLC analyses were performed on a Waters liquid chromatograph (Waters Assoc., Milford, MA) consisting of a 510 pump, an U6K injector and a Model 440 UV detector set at 254 nm and mostly operating at 0.02 AUFS. A Novapak C18 column, 15cm x 3.9mm I.D., 4 μm (Waters Assoc.) was used guarded with a μBondapak C18 Guard-pak precolumn (Waters Assoc.). The eluent consisted of a 0.2 M NaH_2PO_4/Na_2HPO_4 buffer supplemented with 8 mM TBAS, set at pH 7.5, and acetonitrile. **Method I** consists of a volume ratio buffer : acetonitrile of 95 : 5, **method II** of 96.5 : 3.5. The flow-rate was 1.5 ml/min. Both methods are based on the method described by Ryll et al. [18], but adjusted due to the lipophilic character of the azido group present in the compounds of interest.

Reproducibility of the HPLC method. The day to day reproducibility of the HPLC method was determined by making a calibration curve of AZT, AZTMP and AZTTP on various days, using freshly made buffer and eluent. Known amounts of the compounds were analyzed using these daily calibration curves. Detection limits were defined as the concentrations at which the detector response was approximately equal to three times the detector noise.

Cell cultures. MT-4 cells were kindly provided by dr. R. Pauwels, Rega Institute, Leuven, Belgium. The cells were grown in RPMI 1640 DM medium (Life Technologies Ltd, Paisley, Scotland), supplemented with 10% (v/v) heat inactivated fetal calf serum, 0.1% $NaHCO_3$, 2 mM glutamine (all purchased from Life Technologies) and 20 μg/ml gentamycin (Merck, Darmstadt, Germany). Medium supplemented with the above described ingredients will be referred to as 'complete RPMI 1640 DM'. The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air. Every 3-4 days, the cell suspension was homogenized and seeded at 3 * 10^5 cells/ml.

Incubation experiments. The intracellular amounts of AZT and its derivatives were determined according to the following protocol: MT-4 cells were split 24-48 hrs before use at 3 * 10^5 cells/ml in complete RPMI 1640 DM, achieving cells in exponential growth at the beginning of the incubation. Exact determination of the cell concentration and viability (using Trypan Blue exclusion) were performed just before the incubation started. Cells were incubated with known concentrations of AZTMP using the conditions described above in 'Cell cultures'. After 6 hrs, 6 ml of the cell suspensions were pelleted at 380g for 5 min at 4 °C and the supernatant was discarded. Cell pellets were washed twice with 1 ml of cold PBS and centrifuged at 380g, 5 min, 4 °C. Cell lysis took place by resuspending the pellet in 200 μl PBS and freeze-thawing 5 times, using liquid nitrogen. Pelleting of cell debris was performed at 3200g, 4 °C for 10 min. 20 - 50 μl of the supernatant was analyzed by HPLC.

Alkaline phosphatase incubation. Cell lysis supernatant was incubated with alkaline phosphatase to confirm that the detected peaks in various chromatograms were AZT-metabolites: a decrease in peak height of the metabolite accompanied by an increase in AZT peak height upon incubation with this enzyme strongly indicates that the peak under investigation is an AZT-metabolite. 0.75 mg/ml alkaline phosphatase was dissolved in a 0.2 M Tris/1 mM $MgCl_2$ buffer pH 9.5. 200 μl of this alkaline phosphatase solution was mixed with 100 μl cell lysis supernatant. The mixture was

incubated for 1 hr at 37 °C and analyzed. AZT, AZTMP and AZTTP were incubated in the same way as a control experiment.

Internal standard. Based on the knowledge about lipophilicity of the nucleosides and nucleotides analyzed by methods I and II, the following compounds were proposed for internal standard use: ddI, ddC, azidoddI, azidoddBrA and FddBrU. The compounds were dissolved at 1 mg/ml in aqua dest. and analyzed using method I. Results are shown in table Ia. Based on these results, we chose azidoddI as internal standard for the extraction.

Extraction procedure. For the determination of AZT and its phosphorylated derivatives in blood samples, urine, bile and tissue homogenates, an extraction procedure on an SPE system (Baker, Phillipsburg, NJ) was developed.
To water, plasma (human, cat, rat), bile (rat), urine (human, rat) and liver homogenate (rat), known amounts of the compounds of interest and azidoddI as internal standard were added. The C18 disposable extraction columns were prepared by treatment with successive solvent washes of 3 * 1 ml methanol, 1 ml aqua dest. and 3 * 1 ml 0.2 M sodiumphosphate buffer supplemented with 8 mM TBAS, pH 7.5. 200 μl of the biological samples were diluted with 200 μl buffer and loaded on the SPE column. After washing with 2 * 200 μl buffer, the SPE column was allowed to dry. Elution took place with 2 * 100 μl methanol and the combined methanol eluates were mixed with an equal amount of aqua dest. to prevent the formation of precipitate. 40 μl of the mixture was injected into the chromatograph.

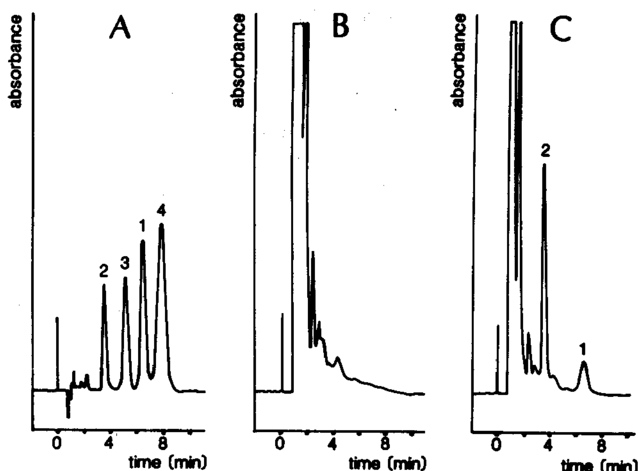

Figure 2. A typical chromatogram of a mixture of AZT and its mono-, di- and triphosphate derivatives in aqua dest. is presented in figure a. Figure b. shows a chromatogram of MT-4 cell lysis supernatant and figure c. a chromatogram of MT-4 cell lysis supernatant after incubation with AZTMP, analyzed using method I. 1 = AZT, 2 = AZTMP, 3 = AZTDP and 4 = AZTTP.

Results and Discussion

Figures 2 and 3 show typical chromatograms of AZT and its mono-, di- and triphosphate derivatives using method I and II, respectively. The use of a ratio buffer to acetonitrile of 95 : 5 resulted in a separation of the four compounds within 10 min. Lowering the amount of acetonitrile to 3.5% gave rise to a better separation between the solvent front and the first eluting nucleotide (AZTMP), which may be an advantage in case of early eluting contaminants (fig. 3a). However, running time was prolonged to about 22 min.

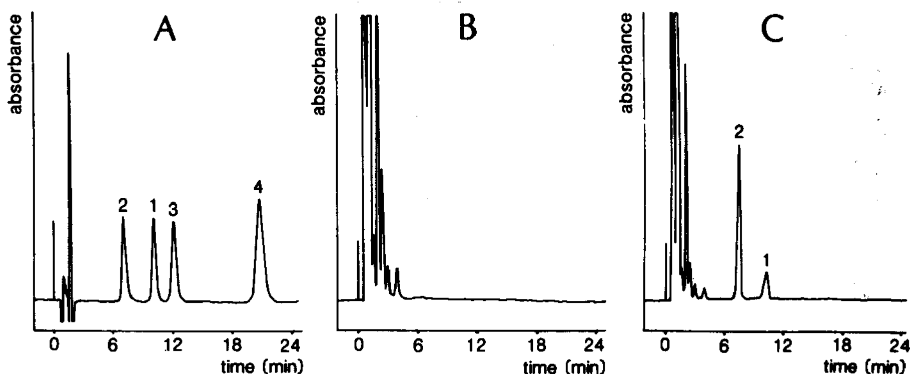

Figure 3. A typical chromatogram of a mixture of AZT and its mono-, di- and triphosphate derivatives in aqua dest. is presented in figure a. Figure b. shows a chromatogram of MT-4 cell lysis supernatant and figure c. a chromatogram of MT-4 cell lysis supernatant after incubation with AZTMP, analyzed using method II. 1 = AZT, 2 = AZTMP, 3 = AZTDP and 4 = AZTTP.

Table Ia and Ib show the results of the day to day reproducibility of both methods. Noteworthy is the change in eluting sequence, comparing method I and II, with regard to AZT and AZTDP: retention times of AZT and AZTDP were 6.5 ± 0.3 min and 5.3 ± 0.2 min with method I, and 11.3 ± 1.0 min and 13.0 ± 1.0 min with method II (mean values ± S.D., n=5). From these tables it can be concluded that both methods are reliable and give reproducible results during time. Linear calibration curves for the compounds can be obtained in the nanomolar range (correlation coefficients varied between 0.99822 ± 0.00101 and 0.99924 ± 0.00065 (mean values ± S.D., n=5)). We commonly observed some variation in the slope of the calibration curves from day to day. However, this only implies the need for a daily calibration curve. As can be seen from tables Ia and Ib, daily determination of

known amounts of the compounds can be carried out accurately. In the case of method II it should be stressed that eluting the column overnight with methanol is essential to maintain the (longer) retention times and separation as in figure 3a. Omitting this re-equilibration step will lead to slowly decreasing retention times of all compounds and nonreproducible results during the analysis.

Table Ia. Chromatographic features of AZT, its phosphorylated derivatives and the proposed internal standard compounds using method I. Values represent mean values of 5 experiments ± S.D. Each experiment was performed on a different day, as described in 'Materials and methods'.

	retention time(min)	detection limit(nM)	spiked amount(ng)	calculated amount(ng)	% of theor.amount
AZT	6.5 ± 0.3	56 ± 6	136	141 ± 3	103
AZTMP	3.6 ± 0.1	90 ± 8	109	110 ± 6	101
AZTDP	5.3 ± 0.2	70 ± 10	ND[a]	ND	ND
AZTTP	8.2 ± 0.2	69 ± 6	168	169 ± 3	101
ddI	1.4 ± 0.1	107 ± 13	ND	ND	ND
ddC	1.1 ± 0.1	120 ± 15	ND	ND	ND
azidoddI	4.0 ± 0.2	36 ± 4	200	190 ± 8	95
azidoddBrA	> 30	ND	ND	ND	ND
FddBrU	8[b]	ND	ND	ND	ND

[a] ND = not determined
[b] the peak of FddBrU displayed a large degree of tailing

Table Ib. Chromatographic features of AZT and its phosphorylated derivatives using method II. Values represent mean values of 5 experiments ± S.D. Each experiment was performed on a different day, as described in 'Materials and methods'.

	retention time(min)	detection limit(nM)	spiked amount(ng)	calculated amount(ng)	% of theor.amount
AZT	11.3 ± 1.0	150 ± 8	242	254 ± 1	105
AZTMP	7.6 ± 0.5	140 ± 10	223	216 ± 8	97
AZTDP	13.0 ± 1.0	115 ± 7	ND	ND	ND
AZTTP	22.5 ± 1.9	200 ± 15	179	185 ± 9	103

Incubation of MT-4 cells with AZTMP resulted in an intracellular amount of AZTMP dependent on the concentration present extracellularly (fig. 4). Very small amounts of AZT could be detected (less than 10 ng/10^6 cells). Within 2 hrs of incubation in the complete RPMI 1640 DM medium, AZTMP was completely

hydrolysed into AZT. AZT apparently diffused passively into the cells and was phosphorylated into AZTMP by the cytosol thymidine kinase [7,19,20]. Accumulation of AZTMP, as can be seen from figure 4, indicates that the thymidilate kinase is the rate limiting step in the anabolism of AZT to AZTTP, as has been shown for other cell types [7]. The identity of the peak at the retention time of 3.6 min was established using incubation with alkaline phosphatase (fig. 5).

The fact that no interference by endogenous compounds occurred, can be explained by the fact that all endogenous nucleosides/tides possess a 3'-hydroxyl group, whereas AZT and its derivatives have a 3'-azido substituent (see fig. 1). This makes the latter compounds far more lipophilic (endogenous compounds, e.g. adenosine, thymidine, cytidine, guanosine and uridine, did not have any retention in the methods used). Even the antivirals ddI and ddC (compounds lacking the hydrophilic 3'-OH moiety) eluted within 2 min.

Intracellular AZTDP and AZTTP could not be detected with the present method due to the extremely low levels achieved in the cells, as shown by others [20-22]. Although each sample contained 3 - 6 * 10^6 cells, the total AZTDP and AZTTP amounts remained below the UV-absorbance detection limits. Performing the experiments with radiolabeled AZT could in principle lower this detection limit considerably [7,20,22].

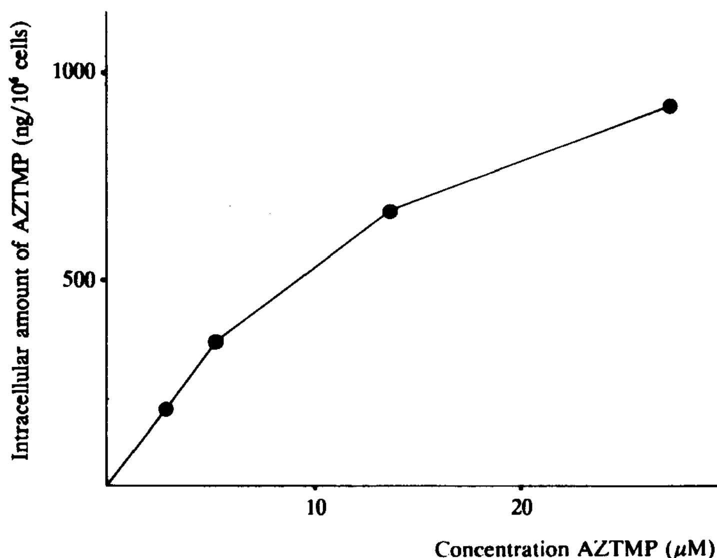

Figure 4. Intracellular accumulation of AZTMP after 6 hrs incubation of MT-4 cells under conditions described in 'Materials and methods' with increasing concentrations of AZTMP.

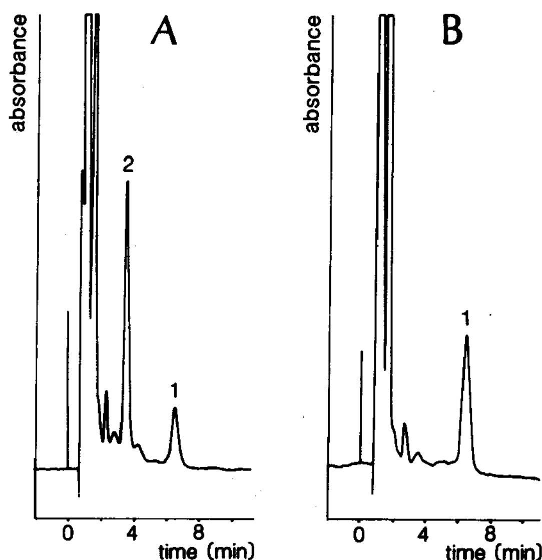

Figure 5. Identification of AZTMP, present in MT-4 cells after incubation with AZTMP, using alkaline phosphatase (1 = AZT, 2 = AZTMP):
A: Chromatographic pattern of the cell lysis supernatant of MT-4 cells.
B: Chromatographic pattern after incubation of the cell lysis supernatant with alkaline phosphatase.
(The HPLC method used is method I)

Table II. Recoveries of extraction of AZT, AZTMP, and AZTTP from various biological samples, using the 'Baker'-10 SPE system. The recovered amounts are related to the internal standard azidoddI. Values represent mean values of 5 experiments ± S.D.

	Recovery (%)		
Biological sample	AZT	AZTMP	AZTTP
200 µl H_2O	104.2 ± 3.9	100.1 ± 2.7	79.1 ± 3.1
200 µl plasma	106.0 ± 5.4	83.6 ± 3.2	65.3 ± 9.1*
200 µl liver homogenate	100.9 ± 1.3	77.8 ± 2.9	71.6 ± 8.4*
100 µl bile	95.5 ± 4.1	87.4 ± 2.2	73.3 ± 8.7*
100 µl urine	-	-	-

* AZTTP was unstable in these biological samples. Due to this, the recovery was inferred from the recoveries of AZTMP, AZTDP and AZTTP.

Whereas the MT-4 cell lysis supernatants presented a relative clean chromatographic pattern, in the other biological samples such as tissue homogenates, plasma and bile, AZT and its derivatives could only be studied after extraction. Using method I, of the compounds tested for internal standard use, FddBrU and azidoddBrA had retention times of 8 min and > 30 min, respectively (table Ia). Furthermore, the peak of FddBrU was broad and showed a high degree of tailing. These characteristics made them both not suitable for internal standard use. However, the compound azidoddI showed appropriate retention time, good peak shape and extraction characteristics. Table II shows the recoveries of the extractions of AZT and its derivatives from the biological samples. Percentages of recovery were related to azidoddI, the latter one having a recovery of more than 95% from all but the urine samples. AZT showed a good and very reproducible recovery from all biological fluids as well as aqua dest. Although the recoveries of AZTMP were slightly less in the biological samples, they were highly reproducible. AZTTP could only be extracted in its original triphosphate form from aqua dest. The latter compound was unstable in plasma, tissue homogenate and bile. Therefore, the recovery values were a summation of recovered AZTMP, AZTDP and AZTTP. Furthermore, it was impossible to extract the compounds from (human and rat) urine, due to the interference of several endogenous compounds in the chromatograms.
Experiments are now in progress to study the behaviour of some of the AZTMP-neoglycoprotein conjugates in MT-4 cells with regard to the mechanism(s) underlying the anti-HIV-1 activity, using the methods described above.
In conclusion it can be said, that, compared to the analyses published so far (e.g. [7,20,21,23,24]), the HPLC methods described above have three major advantages. First, AZT and its phosphorylated derivatives can be well separated within 10 min. Second, the methods described utilize relative simple isocratic elution, and third, the compounds can be detected in the nanomolar range using UV detection. Furthermore, extraction of AZT and its metabolites from bile, plasma and tissue homogenates can be easily and reproducibly performed using a solid phase method.

References

1. Mitsuya H, Weinhold KJ, Furman PA, St.Clair MH, Nusinoff-Lehrman S, Gallo RC, Bolognesi D, Barry DW and Broder S, 3'-azido-3'-deoxythymidine (BW A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus in vitro. *Proc Natl Acad Sci USA* **82**: 7096-7100, 1985.
2. Hirsch MS, Azidothymidine. *J Infect Dis* **157**: 427-431, 1988.
3. Fischl MA, Richman DD, Grieco MH, Gottlieb MS, Volberding PA, Laskin OL, Leedom JM, Groopman JE, Mildvan D, Schooley RT, Jackson GG, Durack DT, Phil D and King D, The efficacy of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. *N Engl J Med* **317**: 185-191, 1987.
4. Hirsch MS and Kaplan JC, Treatment of Human Immunodeficiency Virus Infections. *Antimicrob Agents Chemother* **31**: 839-843, 1987.
5. Fischl MA, State of Antiretroviral Therapy with Zidovudine. *AIDS* **3**: S137-S143, 1989.
6. Biglino A, Pugliese A, Forno B, Pollono AM, Busso M and Gioannini P, Effects of Long-Term Zidovudine Treatment on Cell-Mediated Immune Response and Lymphokine Production. *J Acquir Immune Defic Syndr* **4**: 261-266, 1991.
7. Furman PA, Fyfe JA, St.Clair MH, Weinhold K, Rideout JL, Freeman GA, Nusinoff-Lehrman

S, Bolognesi DP, Broder S, Mitsuya H and Barry DW, Phosphorylation of 3'-azido-3'-deoxythymidine and selective interaction of the 5'-triphosphate with human immunodeficiency virus reverse transcriptase. *Proc Natl Acad Sci USA* **83**: 8333-8337, 1986.

8. De Clercq E, Basic approaches to anti-retroviral treatment. *J Acquir Immune Defic Syndr* **4**: 207-218, 1991.
9. Gendelman HE, Orenstein JM, Baca LM, Weiser B, Burger H, Kalter DC and Meltzer MS, The Macrophage in the Persistence and Pathogenesis of HIV Infection. *AIDS* **3**: 475-495, 1989.
10. Schmitt MP, Steffan AM, Gendrault JL, Jaeck D, Royer C, Schweitzer C, Beyer C, Schmitt C, Aubertin AM and Kirn A, Multiplication of Human Immunodeficiency Virus in Primary Cultures of Human Kupffer Cells - Possible Role of Liver Macrophage Infection in the Physiopathology of AIDS. *Res Virol* **141**: 143-152, 1990.
11. Perno CF, Yarchoan R, Cooney DA, Hartman NR, Webb DSA, Hao Z, Mitsuya H, Johns DG and Broder S, Replication of human immunodeficiency virus in monocytes. Granulocyte/Macrophage Colony-stimulating factor (GM-CSF) potentiates viral production yet enhances the antiviral effect mediated by 3'-azido-2'3'-dideoxythymidine (AZT) and other dideoxynucleoside congeners of thymidine. *J Exp Med* **169**: 933-951, 1989.
12. Gartner S, Markovits P, Markovitz DM, Kaplan MH, Gallo RC and Popovic M, The role of mononuclear phagocytes in HTLV-III/LAV infection. *Science* **233**: 215-219, 1986.
13. Klatzmann D, Barré-Sinoussi FB, Nugeyre MT, Dauguet C, Vilmer E, Griscelli E, Brun-Vezinet F, Rouzioux C, Gluckman JC, Chermann JC and Montagnier L, Selective tropism of lymphadenopathy associated virus (LAV) for helper-inducer T lymphocytes. *Science* **225**: 59-63, 1984.
14. Mcdougal JS, Kennedy MS, Sligh JM, Cort SP, Mawle A and Nicholson JKA, Binding of HTLV-III/LAV to T4+ T cells by a complex of the 110 K viral protein and the T4 molecule. *Science* **231**: 382-385, 1986.
15. Dalgleish AG, Beverley PCL, Clapham PR, Crawford DH, Greaves MF and Weiss RA, The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature* **312**: 763-767, 1984.
16. Barré-Sinoussi F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, Gruest J, Axler-Blin C, Brun-Vezinet F, Rouzioux C, Rozenbaum W and Montagnier L, Isolation of a T-lymphotropic retrovirus from a patient at risk for the Acquired Immune Deficiency Syndrome (AIDS). *Science* **220**: 868-871, 1983.
17. Molema G, Jansen RW, Pauwels R, De Clercq E and Meijer DKF, Targeting of antiviral drugs to T-4 lymphocytes. Anti-HIV activity of neoglycoprotein-AZTMP conjugates in vitro. *Biochem Pharmacol* **40**: 2603-2610, 1990.
18. Ryll T and Wagner R, Improved ion-pair high-performance liquid chromatographic method for the quantification of a wide variety of nucleotides and sugar-nucleotides in animal cells. *J Chromatogr* **570**: 77-88, 1991.
19. Zimmerman TP, Mahony WB and Prus KL, 3'-azido-3'-deoxythymidine. An unusual nucleoside analogue that permeates the membrane of human erythrocytes and lymphocytes by nonfacilitated diffusion. *J Biol Chem* **262**: 5748-5754, 1987.
20. Balzarini J, Herdewijn P and De Clercq E, Differential patterns of intracellular metabolism of 2',3'-didehydro-2',3'-dideoxythymidine and 3'-azido-2',3'-dideoxythymidine, two potent anti-human immunodeficiency virus compounds. *J Biol Chem* **264**: 6127-6133, 1989.
21. Dhawan RK, Kharbanda S, Nakamura M, Ohno T and Kufe D, Effects of Granulocyte-Macrophage Colony-Stimulating Factor on 3'-Azido-3'-Deoxythymidine Uptake, Phosphorylation and Nucleotide Retention in Human U-937 Cells. *Biochem Pharmacol* **40**: 2695-2700, 1990.
22. Fridland A, Connelly MC and Ashmun R, Relationship of Deoxynucleotide Changes to Inhibition of DNA Synthesis Induced by the Antiretroviral Agent 3'-Azido-3'-Deoxythymidine and Release of its Monophosphate by Human Lymphoid Cells (CCRF-CEM). *Mol Pharmacol* **37**: 665-670, 1990.
23. Balzarini J, Pauwels R, Baba M, Herdewijn P, De Clercq E, Broder S and Johns DG, The in vitro and in vivo anti-retrovirus activity, and intracellular metabolism of 3'-azido-2',3'-dideoxythymidine and 2',3'-dideoxycytidine are highly dependent on the cell

species. *Biochem Pharmacol* **37**: 897-903, 1988.
24. Toyoshima T, Kimura S, Muramatsu S, Takahagi H and Shimada K, A sensitive nonisotopic method for the determination of intracellular azidothymidine 5'-mono-,5'-di- and 5'-triphosphate. *Anal Biochem* **196**: 302-307, 1991.