Thesis of PhD Dissertation

SYNTHESIS AND BIOLOGICAL CHARACTERISATION OF A NEW OLIGONUCLEOTIDE INHIBITING HIV-REPLICATION

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1 INTRODUCTION

The development and discovery of inhibitors of Human Immunodeficiency Virus Type 1 (HIV-1), the etiological agent for HIV/AIDS, has been dominated by inhibitors of the HIV enzymes reverse transcriptase (RT) and protease. However, as issues of patient compliance, cost and access to therapies and the emergence of single- and multi-drug resistant strains have come to the forefront of HIV/AIDS therapy the need for more potent and novel antivirals as well as new antiviral targets has arisen. Part of the need for new antiviral targets has been met by the identification of many of the processes involved in HIV entry into cells.

The HIV-1 envelope protein is a trimer of gp120-gp41 heterodimers on the virion surface. The multi-step HIV entry process is begun by the highly specific interaction of the gp120 HIV envelope glycoprotein and its primary receptor on cells, CD4. This interaction induces conformational alterations in gp120, enabling it to subsequently interact with the co-receptor (CCR5 or CXCR4). Following binding of gp120 to CD4 and a chemokine receptor, a further conformational change occurs in the gp120-gp41 oligomer that leads to insertion of the hydrophobic N-terminal peptide sequence of gp41 into the membrane of the host cell bringing about the fusion process. Very recently, Enfuvirtide (T20), an inhibitor of gp41 and virus entry, has been approved for use in combination with other anti-HIV medications, providing access to a new drug class of antiviral targets for the treatment of HIV/AIDS.

In addition to the above conformational changes, a chemical process, i.e., redox changes of cell surface proteins and viral envelope glycoprotein (gp120) are required for successful entry of HIV-1 into cells. It was shown recently that the 2^{nd} domain (D2) disulfide bond of CD4 is redox-active and the redox process is controlled by cell surface thioredoxin, secreted by the T cell. Another cell surface protein catalysing redox changes, protein-disulfide isomerase (PDI), may also be involved in the control of cell surface redox chemistry required for HIV-1 entry. It was reported that purified PDI can cleave disulfide bonds in recombinant gp120 and two of the nine disulfides of gp120 are reduced during interaction with the lymphocyte surface on $CD4^+$ cells and anti-PDI agents effectively block CXCR4 Env-mediated fusion. Localization of CD4 receptors in cholesterol and glycosphingolipid rich lipid rafts, present in the external leaflet of the plasma membrane, and also appear indispensable for successful HIV-1 entry, although contradictory data have also been published. Further studies are needed to elucidate the detailed functional connection between the well-established conformational changes and disulfide exchange processes in the entry of HIV-1

into target cells. However, these two findings, the cell surface reducing power, i.e., thioredoxin and perhaps PDI, as well the localization of the CD4 receptors in lipid rafts, together may represent new information which can be exploited as new targets in the inhibition of HIV-1 entry.

Although oligonucleotides with demonstrated activity against various viral structural and enzyme targets have been reported, no oligonucleotide drug has yet been approved for routine therapeutic use against HIV infection. Both antisense and triple-helix forming oligonucleotides exert their inhibitory activities by sequence specific nucleic acid-nucleic acid interactions. *In vitro* oligodeoxycytidine containing a phosphorothioate backbone binds to CD4, blocking the interaction of gp120/CD4 and can inhibit the polymerase activity of purified RT enzyme. Guanine-rich oligonucleotides with phosphorothioate internucleotide linkages also exert anti-HIV activity. We have previously shown that 5-mercaptopyrimidine-containing oligo- and polynucleotides are potent inhibitors of RT enzyme activity and HIV-1 replication *in vitro*. In the studies described here, we have characterised anti-HIV activity of a 35-mer homo-oligonucleotide composed of 4-thio-deoxyuridylates $(s^4dU)_{35}$, which was previously shown to be a potent inhibitor of HIV-1 RT and telomerase. Suligovir was found to be a potent inhibitor of virus entry.

2 SCOPE OF THE THESIS

Our working group showed earlier that the 35mer homo-oligonucleotide containing 4-thio-deoxyuridilate, $(s^4dU)_{35}$ is a potent inhibitor of the HIV-1 reverse transcriptase (K_i= 3 nM). We had studied the anti-HIV activity of this oligonucleotide and after the promising results we have synthesized the compound by two methods and we have studied its antiviral mechanism. For our studies we have obtained experimental and financial assistance from NIH and OmniPharm Research Inc. During my PhD studies I have been working with the following aims:

1. Working out two methods for the synthesis of $(s^4dU)_{35}$: *i)* thiolation of $(dC)_{35}$ and *ii)* chemical coupling of thiolated monomers.
2. Working out a chromatographical method for the purification of $(s^4dU)_{35}$.
3. Chemical analysis of $(s^4dU)_{35}$.
4. Biochemical and biological characterisation of $(s^4dU)_{35}$.
5. Studying the anti-HIV activity of $(s^4dU)_{35}$.
6. Examining the anti-HIV mechanism of $(s^4dU)_{35}$.

The name of $(s^4dU)_{35}$ (35mer oligonucleotide containing 4-thio-deoxyuridilate) is very complicated that we give the name Suligovir to the compound. I use this name in this Thesis as well.

The name of $(s^4dU)_{35}$ is NSC-722038 in the NIH project.

3 MATERIALS AND METHODS

3.1. Chemical methods

3.1.1 Study of the applicability of universal supports for the synthesis of Suligovir A

From each of the supports (Universal Support 500 (US 500) and Universal Support II (US II)) 25 mg were packed in a column. The synthesis was completed with a Gene Assembler Plus oligonucleotide synthesizer, using the standard phosphoramidite protocol for column with 1.3 μmol capacity. The deprotection and cleavage were done according to the protocol of Glen Research for 4-thio-deoxyuridine phoshoramidite (incubation in 50 mM NaSH/cc. NH_3 for 24 hours at room temperature). After the deprotection step, the $(dT)_{16}$ samples were precipitated with 14 volume of butanol, centrifuged, redissolved in water and precipitated with three volume of ethanol in the presence of 2 M NH_4-acetate and 10 mM $MgCl_2$. Then the products were dissolved in 1 ml of water. To determine the 3' phosphate left on the oligonucleotide after cleavage, 60 nmol of oligonucleotide were digested by phosphomonoesterase, in 100 mM TrisHCl, pH=8.1, containing 5 mM $MgCl_2$ in a final volume of 100 μl at 37°C for two hours. The reaction mixture was cooled in an ice-bath and mixed with 100 μl of 10% perchloric acid. The precipitate was removed by centrifugation and the inorganic phosphate content of the supernatants were measured.

3.1.2 Synthesis of Suligovir A

The synthesis of Suligovir A was performed on a Pharmacia Gene Assembler Plus oligonucleotide synthesizer, equipped with a 10 μmol reactor column. We used 330 mg support (4-thio-deoxiuridine-CPG) for each synthesis cycle. The standard phosphoramidite chemistry was used; there was only one difference from the usual method: instead of the regular capping solution (Capping A and B), acetonitrile was used.

Since the Suligovir is a homo-oligonucleotide, there was no need to block the unreacted 5’ -OH groups after each synthesis cycle to avoid the formation of false sequences. This modification resulted in an increased overall yield. The deprotection step was performed according to the protocol proposed by Glen Research: the support was kept in concentrated NH_3 solution containing 50 mM NaSH for 24 hours at room temperature, followed by the removal of the support by centrifugation. The clear supernatant was mixed with 600 μl 3 M Na-acetate (pH 5.2) and 30 μl of 1 M $MgCl_2$; then the oligonucleotide was precipitated by adding 17 ml of cold (-20°C) ethanol. After keeping the mixture at -20°C for 30 minutes, the oligonucleotide was collected by centrifugation, dried in vacuum and stored at -20°C. The

precipitated oligonucleotide was washed twice with 80% ethanol and the ethanol was removed in vacuum.

3.1.3 Synthesis of Suligovir B by reacting $(dC)_{35}$ with H_2S

80 mg $(dC)_{35}$ was dissolved in 5 ml of 50% pyridine and it was transferred to a stainless steal container with Teflon lining. The container was cooled to -70°C and 8 ml H_2S was added to the reaction mixture. Then, the high-pressure container was carefully sealed and it was kept at 55°C for 10 days. The obtained cloudy yellow solution was washed with distilled water and the solution was kept at 4°C for 1 hour. Afterwards, the formed fine sulphur precipitate was removed by filtration and the filter was washed with water. Suligovir B was precipitated in the same way as Suligovir A.

3.1.4 Purification of Suligovir A and B by ion exchange chromatography

The products were dissolved in water and they were purified using anion exchange chromatography. RESOURCE® Q anion exchange column was used and the separation were followed continuously by UV detection at 330 nm. In these experiments, the salt concentration was increased by a rate of 1 mM/min at a flow rate of 1 ml/min. The appropriate fractions were combined and the volume was decreased to one-third by evaporation. The oligonucleotides were precipitated with three volume of acetone. After incubation for 5 min at -20°C, the oligonucleotides were collected by centrifugation and washed three times with cold acetone. Then the precipitated oligonucleotides were dried and dissolved in water.

3.1.5 Analysis of Suligovir A and B by PAGE

The Suligovir samples were analysed by polyacrylamide gel electrophoresis (PAGE). 15% polyacrylamide gel (7 M urea) was used and the samples were run at 500 V in 1X TBE buffer (89 mM Tris base, 89 mM boric acid and 2 mM EDTA). The oligonucleotide bands were stained by PlusOne® Amersham-Pharmacia silver staining kit.

3.2. Biochemical, cell biological and virological methods

3.2.1 Cells and viruses

The CEM-SS, HL2/3 and HeLa CD4 LTR β-gal cell lines were obtained from the NIH AIDS Research and Reference Reagent Program (Bethesda, Maryland). CEM-SS cells were maintained in RPMI 1640 medium supplemented with 10% Fetal Calf Serum (FCS), 2 mM L-glutamine, and antibiotics. HL2/3 and HeLa CD4 LTR β-gal were maintained in DMEM 10% FCS, 2 mM L-glutamine, and antibiotics. Human immunodeficiency virus type 1 (HIV-1)

strains RF, HIV IIIB, and the molecular clone NL4-3 were obtained from the NIH AIDS Research and Reference Reagent Program.

Kit225 K6 human T lymphoma cells were cultured in RPMI 1640 medium supplemented with 10 % FCS and antibiotics in the presence of human recombinant IL-2. N87 gastric carcinoma cells were cultured in RPMI 1640 medium with 10 % FCS and antibiotics.

3.2.2 Effect of Suligovir A and B on HIV-1 replication

HIV-1(IIIB) strain was used, prepared from cell-free supernatant of chronically infected H9 cultures. Antiviral activity was determined by measuring the RT activity in the culture fluid of infected MT-4 cells after 96 hours of infection. $2.5x10^5$ MT-4 cells were infected with virus stock containing 100 $TCID_{50}$ (50% tissue culture infective dose) per ml. After 96 hours of growing, the cells were removed by centrifugation and the RT activity was determined in 50 µl aliquots, using poly(A)·$(dT)_{16}$ and [^{3}H]dTTP as we describe below in the RT assay. The oligonucleotides were added to MT-4 cells 30 minutes before infection.

3.2.3 Reverse transcriptase studies of Suligovir A and B

Reaction mixtures were incubated for 1 hour at 37°C. In a final volume of 100 µl they contained 100 mM TrisHCl (pH 8.0), 50 mM KCl, 6 mM $MgCl_2$, 100 µg/ml bovine serum albumin, 5 mM DTT, 10 µM [^{3}H]dTTP (specific activity 30 Ci/mmol), 0.1 µM poly(A)·$(dT)_{16}$ as template-primer and 0.1 U of RT enzyme (Amersham-Pharmacia). The reaction was terminated by addition of 10% cold TCA, containing 100 mM Na-PP_i. The radioactive product was collected by filtration on Whatman GF/C filter, washed three times with 1 ml of 5% TCA containing 50 mM Na-PP_i and finally with ethanol. The filter disks were dried and the nucleotide incorporation was quantified by scintillation counting.

3.2.4 Formation of covalent interaction between Suligovir and thioredoxin

4 µg of thioredoxin (Sigma) and 2 µg biotin-labelled Suligovir were incubated in 12 µl of 10 mM TrisCl (pH 7.5 or 8.5) for 30 min at 37 ºC, then 3 µl samples of the mixtures were separated by PAGE on 15% polyacrylamide gel containing 7 M urea. The products were blotted to PVDF membrane and the biotin-containing bands were visualized by streptavidin-conjugated horseradish-peroxidase and ECL™.

3.2.5 Flow cytometric measurements, competition experiments and FRET analysis

. Flow cytometric measurements were carried out using a Becton Dickinson FACScalibur flow cytometer. For the competition assays, cells were incubated with the mixture of fluorescently conjugated monoclonal antibody and unlabelled Suligovir. The

degree of competition was calculated from the fractional decrease of the fluorescence of the antibody in presence of Suligovir.

For FRET measurements, the cells were labelled simultaneously with the mixture of donor (Cy3) labelled monoclonal antibody and with acceptor (Cy5) labelled Suligovir. Four fluorescent intensities were detected. Three were excited at 488 nm and detected at 530±30 nm, 585±42 nm and above 670 nm, respectively, and the fourth was excited at 635 nm and detected at 661±16 nm. Correction factors for the spectral overlap between the different fluorescence channels were obtained from data measured on unlabelled and single-labelled cells. Forward and side angle light scatterings were used for gating out debris and dead cells. We calculated the energy transfer efficiency on a cell-by-cell basis from the four fluorescence intensities on 10,000 cells using list mode data.

3.2.6 Determination of nuclease stability of Suligovir A and B

The reaction mixture contained, in a final volume of 500 µl, 100 mM TrisCl (pH 8.0), 5 mM $MgCl_2$, 1.250 µg/ml Suligovir 50 µg/ml phophodiesterase I (0.16 U/mg, Sigma), 25 µg/ml deoxyribonuclease I (3000 Kunitz U/mg protein, Sigma) and 0.025% NaN_3. The reaction mixtures were incubated at 37°C, and 40 µl aliquots were sampled in every hour. After stopping the reaction, the terminal phosphate groups formed by the nuclease enzyme reaction were split off and determined as inorganic phosphate.

3.2.7 Toxicity of Suligovir on human bone marrow granulocyte-macrophage progenitor cells

$2x10^5$ mononuclear bone marrow cells were grown in the presence of Suligovir (highest concentration: 180 µg/ml, 15.8 µM) incubated for 14 days at 37EC at 100% relative humidity in an atmosphere containing 5% CO_2. Colonies, defined as groups of at least 50 cells, were counted under a dissecting microscope at the end of the incubation period. The protocol was approved by the Regional and Institutional Ethics Committee of University of Debrecen, Hungary (DEOEC RKEB/IKEB-2055/2003).

3.2.8 Antibodies and labelling of cell surface molecules, CLSM analysis

MEM115 antibody against CD4 molecule, and MEM102 antibody against CD48 molecule was a kind gift from Vaclav Horejsi (Institute of Molecular Genetics, Prague, Czech Republic); anti-Tac antibody against IL2Rα chain was a kind gift from Thomas Waldmann (NIH, Washington, USA). For labelling of the antibodies with fluorescent probes, purified mAbs were conjugated with succinimidyl ester of Alexa 546 or with sulfoindo-cyanin

succinimidyl bifunctional ester derivative of Cy3 and Cy5. The labelled antibodies were utilized in the CLSM experiments.

For labelling of cell-surface antigens, about 10^6 cells in 50 µl of PBS were incubated with fluorescently tagged labelling molecules for 30 min on ice, in the dark. The applied antibody concentration (50-100 µg/ml) saturated all available binding sites. The excess mAbs were removed by washing the cells twice with PBS. Antibodies were centrifuged at 100,000 g for 30 min before use to remove aggregated molecules.

4 RESULTS AND DISCUSSION

4.1. Measuring the anti-HIV activity of 4-thio-deoxuridilate-containing oligonucleotides

We have studied the anti-HIV activity of various chain-length 4-thio-deoxyuridilate containing oligonucleotides. The efficacy of inhibition increased with the length non-linearly. The 35mer (Suligovir) had the highest antiviral activity; therefore we used this Suligovir for the further antiviral activity studies.

If the Suligovir was added to MT4 cells in 1 μg/ml concentration prior to or in the same time as virus infection, it inhibited the virus replication with efficacy about 90-98%. The results demonstrate that the Suligovir inhibits the virus replication cycle in an early phase, most likely the virus entering into the cell.

4.2. The synthesis of Suligovir

We have worked out two methods for the Suligovir synthesis:

A. By automatic oligonucleotide synthesizer, using protected 4-thio-deoxyuridine-phosphoramidite (method A).

B. By chemical thiolation of $(dC)_{35}$ (method B)

4.2.1 The synthesis of 4-thio-deoxyuridine phosphoramidite

The Suligovir A was synthesised by using standard phosphoramidite chemistry. The key compound for this method was the 5'-dimethoxytritil-2'-deoxy-4-(2-cyanoethyl-thio)-uridine-3'-[(2-cyano-ethyl)-(N,N-diisopropyl)]-phosphoramidite (briefly: 4-thio-deoxyuridine phosphoramidite). The 4-thio-deoxy-uridine phosphoramidite is commercially available; however, it is extremely expensive ($2,700/g). Although the synthesis of this compound was known from the literature, we worked out a more advantageous method.

2'-deoxyuridine was treated by dimethoxytrityl-chloride in pyridine; then the obtained 5'-dimethoxytrityl-2'-deoxyuridine was acetylated by acetic-anhydride. The benefit of this acetyl protecting group compared to the acid-labile *tert*-butyl-silyl group that the former can cleave in a low alkaline media (pH=7.5) while the cyano-ethyl group stays intact. In the following *one-pot* reaction we synthesised the 5'-O-(Bis-(4-methoxyphenyl)-phenylmethyl)-S-(2-cyanoethyl)-4-thio-2'-deoxyuridine. The desired 4-thio-deoxyuridine phosphoramidite was obtained by Zemplén's deacetylation reaction.

4.2.2 Synthesis of 4-thio-deoxyuridine support

When Suligovir is synthesised with Method A, the 3' end nucleoside is attached to a solid phase. It was necessary to study the applicability of the universal support for the synthesis because the 4-thio-deoxyuridine support is not available commercially. We synthesised $(dT)_{16}$ as model compound using universal supports 500 or II. Large amount of these oligonucleotides carried the 3' phosphate group (US 500: 57.2%; US II: 97.2%) as it was shown by inorganic phosphate determination after digestion with phosphomonoesterase. Therefore we concluded that the commercially available universal supports couldn't be used for synthesis of Suligovir. We decided to develop the synthesis using CPG (Controlled Pore size Glass). We completed the synthesis step by step, characterising only the wanted final product, the 4-thio-deoxyuridine-CPG.

4.2.3 The synthesis of Suligovir A and B

We used the 4-thio-deoxyuridine phosphoramidite and the 4-thio-deoxyuridine-CPG support for the synthesis of Suligovir A. 30 mg of Suligovir A were synthesised in 10 hours. The advantage of Method A that we can mark the Suligovir with various chemical labels (Cy-5, Cy-3, biotin, etc.), but this method is highly expensive and very laborious.

Method B involves thiolation of $(dC)_{35}$ with liquid H_2S. The full thiolation required 10 days. The cost of production is ten times lower and the thiolation rate is highly better than with Method A but labelling of Suligovir is not possible with this method.

4.3. Chemical, biochemical and cell-biological characterisation of Suligovir A and B

4.3.1 The chemical characterization of Suligovir A and B

UV spectrum. We compared the UV absorbance of Suligovir A and B at 247 nm, 270 nm and 327 nm wavelengths because cytidilate has an absorption maximum at 270 nm. The thiolation degrees estimated from the absorbances at the wavelengths above were the same for Suligovir A and B.

Chromatography. The Suligovir samples were analysed by PAGE and by anion exchange chromatography. Oligonucleotides were detected with DNA silver staining kit when using PAGE or by measuring the UV absorbance at 330 nm when using anion exchange chromatography. The analysis of Suligovir A and B showed that Suligovir A contains large quantity of shorter length oligonucleotides than Suligovir B, most likely because the 4-thio-dU-phosphoramidite gives lower coupling yield than the common phosphoramidites. In the

Suligovir B sample much lower quantity of shorter length oligonucleotides could be detected, demonstrating that the starting $(dC)_{35}$ was not degraded by H_2S treating.

NMR studies. The ^1H NMR spectrum of Suligovir exhibits eight signals at room temperature. The assignation was made by the help of two-dimensional correlation spectroscopy (2D COSY) and by measuring the coupling constants, where available (data can be found in the Experimental Section). In the case of Suligovir A, other, additional ^1H NMR peaks were detected, indicating that a by-product is present. Separated, visible peaks of this by-product were present at 6.05, 6.27 and 7.79 ppm, which were assigned as the ^1H signals of 5_O, $1'_O$ and 6_O hydrogens, according to their coupling pattern. From the integral ratios we can estimate that the ratio between this by-product and the preferred compound is 1 to 10. Other signals of the by-product could not be detected because of signal overlapping with Suligovir but the integral values clearly indicated the presence of them, folded by the larger signals of Suligovir. In the case of Suligovir B, we did not detect the presence of this by-product in the ^1H NMR spectrum.

LC-MS studies. In order to determine the differences between Suligovir A and B, we studied their mass spectra. We worked with Suligovir samples purified by anion exchange chromatography. In the case of Suligovir A, large percentage of partially thiolated 35-mers was detected, indicating a substantial sulphur loss during the deprotection step. The differences between the appropriate peaks were always 16 mass units, since a sulphur atom was exchanged into an oxygen atom. The MS studies verified the preliminary NMR results; since they showed that Suligovir A contains 11.7% deoxyuridine, while the thiolation of $(dC)_{35}$ resulting Suligovir B was complete with less than 1% deoxyuridine.

4.3.2 The biological studies of Suligovir A and B

Anti-HIV-1 Reverse Transcriptase activity of Suligovir A and B. It was previously shown in our laboratory that the Suligovir is a potent inhibitor of HIV-1 reverse transcriptase. Therefore, we determined the inhibitor activity of Suligovir A and B on recombinant HIV-1 RT. Both Suligovir A and B in an equal proportion inhibited the HIV-1 reverse transcriptase with inhibitor concentration 50% (IC_{50}) of 6.2 nM.

Inhibition of HIV-1 replication with Suligovir A and B. Suligovir A and B were assessed for inhibition of HIV-1 replication. The oligonucleotide inhibitors were added to the cultures of MT-4 cells 30 minutes before infection. The results were obtained from a single representative antiviral assay with individual determinations in triplicate. The Suligovir A and B samples are strongly similar, they were inhibited the replication of HIV-1 by IC_{50} of 0.35 μg/ml.

Nuclease stability of Suligovir A and B. Preliminary experiments with Suligovir in serum containing tissue culture media and cell extracts showed this compound to be highly stable. The degradation of Suligovir samples was very slow, unsuitable for comparison. Therefore, nuclease mixture was used for this study containing snake venom diesterase (50 μg/ml) and DNase I (25 μg/ml). The stability of Suligovir A & B was compared to the stability of the parent oligodeoxycytidilate [$(dC)_{35}$]. After digestion with nucleases the enzymes were inactivated by heating and the phospho-monoester groups of the oligonucleotides, formed on the action of nucleases, were split off by phospho-monoesterase and the released inorganic phosphate was determined as a measure of the oligonucleotide degradation. Both Suligovir A and B are equally stable against nucleases and their stability are about 40 times more then $(dC)_{35}$.

Toxicity of Suligovir. We determined the effect of Suligovir on the colony formation of human granulocyte-macrophage progenitor cells, which are supposed to be the major targets of agents damaging the bone marrow. Colony formation of granulocyte-macrophage progenitor cells was not considerably affected by Suligovir, even at doses as high as 180 μg/ml.

4.4. Mechanism of anti-HIV action of Suligovir

4.4.1 The inhibition effect of Suligovir to virus-cell interaction

Our cooperation partners assessed the direct effects of Suligovir on virus entry. Inhibition of virus binding to cells was estimated by ELISA quantitation of cell-associated p24, while inhibition of virus entry was assessed after 48 h by β-galactosidase reporter expression. Inhibition of both virus binding and entry by Suligovir were equivalent (IC_{50}: 0.003 and 0.002 μg/ml, respectively). They next determined the effect of Suligovir on cell fusion using co-culture of HeLa CD4 LTR β-gal (providing CD4 and LTR-β-galactosidase reporter) and HL2/3 cells (providing HIV Tat and Env). The two cell lines were co-cultured in the continuous presence of Suligovir for 48 h and β-galactosidase reported activity determined. Gp120-CD4 mediated fusion was inhibited with an IC_{50} of 8.75 μg/ml. Chicago Sky Blue was used as a positive control. The results demonstrate that the antiviral activity of Suligovir is associated with early virus-cell binding

4.4.2 Interaction of Suligovir with thioredoxin

The chemical structure of Suligovir suggests the possibility of the formation of mixed disulfide linkage with -SH containing proteins. Cell surface thioredoxin is required for the entry process and represents a likely target for Suligovir. The analysis of a mixture of purified

thioredoxin and biotin-labelled Suligovir indicates that a covalent linkage was readily formed between the two molecules.

4.4.3 Interaction of Suligovir with cell surface

To get more information on the binding specificity of Suligovir, titration was performed on $CD4^+$ Kit225 K6 T lymphoma and $CD4^-$ N87 gastric carcinoma cell lines. Fluorescence intensities followed by subtraction of the mean fluorescence arising from unlabelled cells were plotted versus the corresponding Suligovir concentrations. Suligovir shows nearly saturating binding on $CD4^+$ cells, although we could detect a low level of non-specific binding on $CD4^-$ cells. It should be noted that the signal from $CD4^-$ cells was still at least 10-fold lower at each concentration than the signal from $CD4^+$ cells.

Since the number of IL2Rα molecules was known on Kit225 K6 cells (~10^5 IL2Rα/cell), we could use its fluorescence intensity after labelling with Cy5-conjugated monoclonal antibodies as a standard to determine the amount of Cy5-conjugated Suligovir molecules bound to the cell surface. Only about 20,000 anti-CD4 mAb (MEM115) molecules were bound on the cell surface. The numbers of bound Suligovir molecules were much higher, around 170-350,000, depending on its concentration.

Since the primary receptor for HIV-1 gp120 protein is the CD4 antigen we performed binding competition and flow cytometric FRET studies to determine whether the binding site of Suligovir is physically located on the CD4 molecule as it is suggested by the above-presented results.

When cells were labelled with fluorescently tagged anti-CD4 mAb (MEM115) and unlabeled Suligovir, simultaneously, we could detect a partial competition, i.e., a concentration dependent decrease in CD4 fluorescence compared to cells labelled only with anti-CD4 mAb. When performing the assay with anti-CD48 mAb (MEM102), the competition was negligible. It should be pointed out that CD48 is a typical glycosylphosphatidylinositol (GPI) anchored protein, located in lipid rafts.

We then measured the association of Suligovir with CD4 molecules at the cell surface by FRET analysis. With this technique we could detect if the donor (Cy3-labeled anti-CD4 mAb) and the acceptor (Cy5 labelled Suligovir) were within close molecular distance since the calculated energy transfer efficiency has an extremely high sensitivity to changes in distance within the range of 1-10 nm. Large energy transfer (ET) efficiencies (above 5 %) may reflect physical association of the donor and acceptor labelled molecules. We found high ET values (10-25 %) between donor (anti-CD4 mAb) and the acceptor (labelled Suligovir) indicating that some of the donor and acceptor molecules are in close proximity. In order to

prove the specificity of interaction of CD4 and Suligovir and to rule out the role of so-called "chance" transfer we performed FRET analyses between Cy3 labelled CD48 mAb and Cy5 labelled Suligovir. Since the number of bound anti-CD48 antibody was comparable to that of anti-CD4, FRET values were not affected by any differences in donor to acceptor ratios. Low energy transfer (1-5%) efficiencies were found between CD48 mAb and Suligovir, indicating no significant physical association between them. Our findings together with competition data indicate that Suligovir binds specifically to CD4 molecules but not to CD48. However, we cannot exclude the possibility that Suligovir binds to other cell surface molecules.

4.4.4 Localization of Suligovir on the cell surface with CLSM

The low toxicity and the above-presented results together suggest that Suligovir acts on the cell surface. Therefore, we used fluorescently labelled Suligovir to prove its localization on the cell surface or to detect its internalisation by CLSM. Two significant recent findings, the role of cholesterol rich lipid rafts and the participation of cell surface thioredoxin (csTrx) in the entry process were also reasons for the extended CLSM studies.

In the first CLSM experiment we examined the effect of two different labels (Cy3 and Cy5) on the localization of Suligovir. We could demonstrate that these fluorescent dyes do not modify the binding site of the Suligovir. Suligovir was found only on the cell surface and formed well-defined clusters and patches. Then we examined the colocalization of the Suligovir with CD4 and lipid rafts. We found that Suligovir is attached to the areas of the cell surface enriched in lipid rafts and enriched in CD4 receptors with considerable overlapping. In the next experiment we could demonstrate a high colocalization of csTrx and Suligovir. To study the relationship between the lipid rafts and csTrx we examined their localization. We showed that almost all of the thioredoxin signals were located in the cholera toxin labelled lipid rafts, although the signal ratios were not the same in all of the labelled spots.

It was proved by CLSM studies that Suligovir is attached to the cell surface in well defined clusters and patches, suggesting specific interactions. This finding was rather surprising, although the results were not contradictory to the binding and competition studies as well as FRET analysis. The labels (Cy3, Cy5) did not modify the sites of the attachment of Suligovir.

Most of the lipid-raft-localized CD4 receptors were labelled with Suligovir. It should be noted that only weak ET was detected between Suligovir and the GPI-anchored-protein, CD48, located exclusively in lipid rafts. This indicates that the lipid raft localization of a receptor is not sufficient for preferential accumulation of Suligovir in close vicinity of that receptor.

4.4.5 The antiviral activity of Suligovir

Suligovir was assessed for inhibition of HIV-1 replication in a standard cell-based assay using both wild-type and viruses expressing mutations engendering resistance to nucleoside (NRTI) and non-nucleoside (NNRTI) reverse transcriptase inhibitors. Inhibitory concentration 50% (IC_{50}s) ranged from 0.8 to 25.4 μg/ml. Minor 0.9 to 3.3-fold variations in Suligovir IC_{50}s were noted for non-nucleoside reverse transcriptase inhibitor (NNRTI) and nucleoside reverse transcriptase inhibitor (NRTI) resistant viruses compared to corresponding wild type strains (NL4-3 and IIIB); but these changes are not significant in the bioassay. There was no evidence at the high-test concentration of 100 μg/ml of cytotoxicity by Suligovir. AZT and nevirapine were included as positive controls in all assays and showed the expected fold- resistance (16-333-fold) for the mutant viral strains. Thus, Suligovir is antiviral inhibiting both wild type and RT resistant viruses. These results showed that the Suligovir is a non-toxic anti HIV agent, which can inhibit the virus entry.

Chemically, at least three features of Suligovir could be important for its antiviral activity. *i)* the 4-thiono-group increases the hydrophobic character of the base. *ii)* the 4-thiono group has a propensity toward tautomeric conversion to form reactive -SH groups at position 4. *iii)* the 4-thiolation converts the natural oligonucleotide to a highly nuclease resistant molecule.

The dependence of antiviral activity on the length of the thiolated oligonucleotides indicates that the presence of the 4-thiono or 4-mercapto group alone was not sufficient to mediate inhibition of virus entry. Chain-length dependency further suggests an aptamer-like effect or a size dependent affinity for the antiviral target(s) requiring hydrophobic and/or ionic interaction. A similar chain-length dependent effect was observed for other oligonucleotide-based entry inhibitors.

Suligovir was shown not to cross the cell membrane and penetrate or concentrate in significant amounts within cells. This is a significant difference between Suligovir and many other oligonucleotides with anti-HIV activity and may be responsible for its low toxicity. On the other hand, the non-toxic nature of Suligovir suggests that the cell surface interaction is specific, and is confined to cellular targets without apparent in vitro cellular toxicity.

5 SUMMARY

The future goal of our working group is to develop oligonucleotide HIV inhibitors. In this Thesis I present our results on the synthesis, the chemical and biochemical characterization and anti-HIV activity of a 35-mer deoxyoligonucleotide composed exclusively of 4-thio-deoxyuridylate [$(s^4dU)_{35}$, Suligovir].

1. We have worked out two methods for the synthesis of Suligovir:
    a. Method A: Suligovir synthesis from 4-thio-deoxyuridin-phosphoramidite on CPG (Controlled-Pore Glass)
    b. Method B: Suligovir synthesis through liquid H_2S treatment of $(dC)_{35}$
2. We have determined the chemical structure of Suligovir A and B by ^1H-, ^{13}C- and ^{31}P-NMR spectroscopy and mass spectrometry. It was shown that the Suligovir A is less thiolated than Suligovir B. As an average, Suligovir A contains 31-32, while Suligovir B contains the desired 35 sulphur atoms per molecule.
3. The biological activities of Suligovir A and B do not differ significantly. We have shown that the structural inhomogeneity and the high cost of production do not make the Suligovir A suitable for therapy. However, it can be used for the *in vitro* test systems because its biologically activity is the same as the Suligovir B's.
4. We have shown that Suligovir A and B are equally stable in biological media and they are forty times more stable against nucleases than the starting compound, the unmodified $(dC)_{35}$ oligonucleotide. The cellular toxicities of both Suligovirs are very low (Suligovir had negligible effect even until 180 μg/ml concentration on the colony formation of human macrophage granulocite progenitor cells).
5. The Suligovir binds strongly to $CD4^+$ T lymphoma cells until saturation, while its binding is less to $CD4^-$ gastric carcinoma cells.
6. Our CLSM (Confocal Laser Scanning Microscopy) studies showed that the Suligovir can't penetrate into the cell but it binds to the CD4 molecules localized in the cholesterol rich lipid rafts on the cell surface.
7. The Suligovir co-localizes with and it forms covalent binding to thioredoxin, therefore it inhibits the redox power of the cell surface. This redox change would be necessary for successful HIV-1 entry.

8. The Suligovir inhibits the replication of various laboratory strains of HIV, including resistant mutants to NRTI and NNRTI, with IC_{50} values in the range from 0.8 to 25.4 μg/ml. It also inhibits the replication of various subtypes of HIV-1 (IC_{50} = 0.02-4.77 μg/ml) and SIV (IC_{50} (0.17-11.5 μg/ml).

As a conclusion, Suligovir is a new member of HIV-1 entry inhibitors, which acts through non-sequence specific mode of action. It inhibits the replication of HIV-1 in an early phase of the virus life cycle; apparently it can be used for the inhibition of HIV-1 infection.

6 PUBLICATIONS

In extenso publications involved in the Thesis:

1. Horváth A. and Aradi J. (2005): Advantages of sodium-perchlorate solution as mobile phase for purification of synthetic oligonucleotides by anionexchange chromatography *Analitical Biochemistry* **338**(2), 341-343. IF: 2.174

2. Horváth, A., Tőkés S., Hartman, T., Watson, K., Turpin, J.A., Buckheit, R.W., Jr., Sebestyén, Z., Szöllősi, J., Benkő, I., Bardos, T.J., Dunn, J.A., Tóth, F.D., Fésüs, L., and Aradi, J. (2005): Potent inhibition of HIV-1 entry by $(s^4dU)_{35}$ *Virology* (*megjelenés előtt*) IF: 3.391

3. Horváth, A., Tőkés S., Beck, Z., and Aradi, J. (2005) Inhibition of HIV-1 replication by 4-thio-oligodeoxyuridylates *(közlésre beküldve)*

In extenso publications not involved in the Thesis:

1. Sztaricskai, F., Csorvási, A., Horváth, A., Batta, Gy. and Dinya, Z. (2000) Synthesis and conformational studies of unnatural pyrimidine nucleosides. *J. Carbohydrate Chemistry* **19**(9), 1223-1233. IF: 0.974

2. Tarkanyi, I., Horváth, A., Szatmari, I., Eizert, H., Vámosi, Gy., Damjanovich, S., Segal-Bendirdjian, E., Aradi, J., (2005) Inhibition of human telomerase by oligonucleotide chimeras, composed of an antisense moiety and a chemically modified homo-oligonucleotide *FEBS Lett.* **579**(6), 1411-1416. IF: 3.609

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