ANTIRETROVIRAL EFFECTS OF CERTAIN PHENOTHIAZINES AND STRUCTURALLY RELATED COMPOUNDS

PhD Thesis

János Nacsa

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List of publications related to the subject of the Thesis

Book chapters

I. J. Nacsa, I. Hewlett and J. Molnar: Antiretroviral Compounds Focused on Reverse Transcriptase. *„Non Antibiotics: A New Class of Unrecognised Antimicrobics", eds. by A.N. Chakrabarty, J. Molnár, S.G. Dastidar and N. Motohashi,* NISCOM, New Delhi, India, ISBN 81-7236-183-1, **1998**, *Chapter 30,* 357 -376

II. J. Molnár, D. Szabó, Cs. Miskolci, **J. Nacsa**, M. Kawase, S. Saito and N. Motohashi: Effect of some New 3-Benzazepine on Plasmid DNA, mdr P-glycoprotein and Reverse Transcriptase of Leukemia. *„Non Antibiotics: A New Class of Unrecognised Antimicrobics", eds. by A.N. Chakrabarty, J. Molnár, S.G. Dastidar and N. Motohashi,* NISCOM, New Delhi, India, ISBN 81-7236-183-1, **1998**, *Chapter 23,* 272 -280

III. J. Molnár, S. Földeák, M. Tanaka, **J. Nacsa**, M. J. Nakamura, T. Kurihara, H. Sakagami and N. Motohashi: Antibacterial Activity of Phenothiazine: Part I - Effects on Bacteria. *„Non Antibiotics: A New Class of Unrecognised Antimicrobics", eds. by A.N. Chakrabarty, J. Molnár, S.G. Dastidar and N. Motohashi,* NISCOM, New Delhi, India, ISBN 81-7236-183-1, **1998**, *Chapter 10,* 100-113

IV. J. Molnár, S. Földeák, M. Tanaka, **J. Nacsa**, M. J. Nakamura, T. Kurihara, H. Sakagami and N. Motohashi: Antibacterial Activity of Phenothiazine: Part II - Effects on Chromosomal DNA and Physicochemical Nature of Drug Binding. *„Non Antibiotics: A New Class of Unrecognised Antimicrobics", eds. by A.N. Chakrabarty, J. Molnár, S.G. Dastidar and N. Motohashi,* NISCOM, New Delhi, India, ISBN 81-7236-183-1, **1998**, *Chapter 11,* 114-138

V. J. Molnár, S. Földeák, M. Tanaka, **J. Nacsa**, M. J. Nakamura, T. Kurihara, H. Sakagami and N. Motohashi: Antibacterial Activity of Phenothiazine: Part III - Outer Layer Protein A, Endotoxin and Adhesion. *„Non Antibiotics: A New Class of Unrecognised Antimicrobics", eds. by A.N. Chakrabarty, J. Molnár, S.G. Dastidar and N. Motohashi,* NISCOM, New Delhi, India, ISBN 81-7236-183-1, **1998**, *Chapter 12,* 139-157

Full papers

VI. A. Varga, H. Nugel, R. Baehr, U. Marx, A. Hever, **J. Nacsa**, I. Ocsovszki and J. Molnar: Reversal of Multidrug Resistance by Amitriptyline *in Vitro. Anticancer Research,* **1996**, *16(1),* 209-11

VII. G. Timári, T. Soós, Gy. Hajós, A. Messmer, **J. Nacsa** and J. Molnár: Synthesis of Novel Ellipticines Analogues and their Inhibition of Moloney Leukemia Reverse Transcriptase. *Bioorganic & Medicinal Chemistry Letters,* **1996,** *6(23),* 2831-2836

VIII. J. Nacsa, J. Segesdi, Á. Gyuris, , T. Braun, R. Rausch, Á. Buvári-Barcza, L. Barcza, J. Minárovits, J. Molnár: Antiretroviral Effects of Nonderivatized C_{60} In Vitro. *Fullerene Science & Technology,* **1997,** *5(5),* 969-976

IX. N. Motohashi, T. Kurihara, M. Kawase, A. Hevér, M. Tanaka, D. Szabó, **J. Nacsa**, W. Yamanaka, A. Kerim and J. Molnár: Drug Resistance Reversal, Anti-mutagenicity and Antiretroviral Effect of Phthalimido- and Chloroethyl- Phenothiazines. *Anticancer Research,* **1997,** *17,* 3537-3544

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XI. **J. Nacsa**, L. Nagy, D. Sharples, A. Hevér, D. Szabó, I. Ocsovszki, A. Varga and J. Molnár: The Inhibition of SOS-Responses and MDR by Phenothiazine-Metal Complexes. *Anticancer Research,* **1998,** *18(4C),* 3093-3098

XII. **J. Nacsa**, L. Nagy, J. Molnár and J. Molnár: Trifluoperazine and its metal complexes inhibit the Moloney leukemia virus reverse transcriptase. *Anticancer Research,* **1998,** *18(3A),* 1373-1376

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XIII. **J. Nacsa**, L. Nagy, A. Hevér, I. Ocsovszki, J. Molnár: Multidrug resistance reversing effect of some metal complexes of tricyclic compounds. *Bulletin of Fifth International Conference of Anticancer Research,* **1996**

XIV. **J. Nacsa**, L. Nagy, J. Molnár and J. Molnár: Inhibitor effect of metal complexes of phenothiazines on reverse transcriptase enzyme. *Medical Microbiology Letters,* **1996,** *5,* Suppl.1, S1-S129, ISSN 1018-4627

XV. **J. Nacsa**, L. Nagy, J. Molnár: Effect of some metal complexes of tricyclic compounds on extrachromosomal genetic elements. *Acta Microbiol. Immunol. Hung.,* **1996,** *43,* 169

XVI. **J. Nacsa**, L. Nagy, J. Molnár and J. Molnár: Inhibition of reverse transcriptase activity by metal complexes of tricyclic compounds. *Acta Microbiol. Immunol. Hung.,* **1996,** *43(4),* 411

XVII. A. Hevér, **J. Nacsa**, P. Brouant, A. Mahamoud, J. Molnár, J. Barbe: Antiparasitic acridine related derivatives reverse multidrug resistance of tumors, inhibit cell proliferation and reverse transcription. *Tropical Medicine and International Health,* **1996,** *1(6),* A17-A39

Contents

Abbreviations

AIDS - acquired immuno-deficiency syndrome
AZT - azidotimidine
CPZ - chlorpromazine
C_{60} - nonderivatized fullerene molecule containing 60 carbon atoms
ddI - dideoxiinosine
dNTP - deoxy-nucleoside triphosphate
γCD - gamma-cyclodextrin
HERV - human endogenous retrovirus
HIV - human immuno-deficiency virus
HTLV - human T-cell leukemia virus
LTR - long terminal repeat
NI - nucleoside inhibitor
NNI - non-nucleoside inhibitor
Pz - promethazine
RNase - ribonuclease
RT - reverse transcriptase
SIV - simian immuno-deficiency virus
SOS gene -"save our soul" gene, stress response genetic element
TFP - trifluoperazine
TSP - tropical spastic paraparesis

1. Introduction

1.1. Retroviruses

Retroviruses are distinct from other viruses in their ability to encode an enzyme called reverse transcriptase (RT). Some of these viruses cause a variety of diseases such as cancer, AIDS, autoimmunity and diseases of central nervous system, bone and joints. The cell interactions of retroviruses largely determine their pathogenic role.

Other retroviruses are apparently harmless. In humans, not only exogenous retroviruses which are spread horizontally in their natural hosts are found, but also endogenous retroviruses which are transmitted genetically and can therefore be detected in all cell genomes of the host species. In human DNA, endogenous retrovirus sequences (HERV) are well known genetic elements and they all seem to be defective. Replication competent human endogenous retroviruses have not yet been isolated. Retroviruses are being exploited therapeutically as vectors for gene therapy too.

Retroviruses are broadly classified into three subfamilies, oncoviruses, lentiviruses and spumaviruses, based on electron microscopic morphology of virions and analysis of genome sequences (Table 1.). Oncoviruses are further classified into B- C- and D-type particles morphologically (A-type particles are cytoplasmic cores of B- and D-type particles). Whereas oncoviruses generally have a low virus load, lentiviruses like HIV have high load and turnover. Human T-cell leukemia virus type-I (HTLV-I) was first identified in 1980 and cases adult T-cell leukemia/lymphoma and is associated with tropical spastic paraparesis (TSP). It is also associated with some other less well defined malignancies and with a variety of other neurologic diseases. HTLV-II was first isolated in 1982 from a T-cell line of a patient with hairy cell leukemia. Despite the extensive similarity in genomic organization (with about 40% sequence similarity) of the viruses, HTLV-II has not been firmly established as the causative agent of malignant or neurological disease.

Human immuno-deficiency virus type 1 (HIV-1) was first isolated in 1983 (Barré-Sinoussi et al. 1983) and rapidly became established as a cause of acquired immuno-deficiency syndrome (AIDS). HIV-1 isolates can be classified genetically into two divergent groups: most HIV-1 isolates belong to the M group containing at least ten subtypes (A-J). HIV-1 group O strains are considerably distinct from group M strains based on the aminoacid homology in the *env* region

(55%). HIV-2 which shares a certain homology with HIV-1 was isolated from patients with AIDS-like symptoms in 1986 in West Africa (Clavel et al. 1986) but is more closely related to simian immuno-deficiency viruses (SIVs) than the previously described HIV-1, despite a similar biology.

Table 1. Classification of retroviruses

Subfamily	Examples	
Oncovirus		
B-type	Murine mammary tumor virus	(MMTV)
C-type	Human T-cell leukemia virus I, II	(HTLV-I, -II)
	Avian leukosis and sarcoma viruses	(ALSV)
	Salmon lymphoma virus	
D-type	Mason-Pfizer monkey virus	(MPMV)
Lentivirus	Human immuno-deficiency viruses	(HIV-1, -2)
	Simian immuno-deficiency viruses	(SIVs)
	Ovine maèdi-visna virus	(MVV)
	Equine infectious anaemia virus	(EIAV)
Spumavirus	Simian foamy viruses	(SFV)

1.1.1. Retrovirus replication

Retroviruses acquired their name due to the reverse transcriptase enzyme which copies an RNA genome into DNA (Temin and Mizutani and independently Baltimore, 1970). This RNA to DNA conversion was the first example of a 'backwards' direction of genetic information in biological systems. When the virions enter the host cell, the virus particle is diploid, it carries two genome copies in its core. After binding of the *env* glycoprotein of the virus to the cellular receptor (CD4 and other co-receptors in case of HIV, but HIV-1 also can enter cells that lack of CD4) the envelope protein complex mediates fusion of the viral lipid envelope with the cellular membrane, and the virion gets into the cell by endocytosis (Fig. 1.). In addition to CD4, other cell surface molecules that act as receptors for chemokines have been shown to function as co-receptors for HIV in target T cells and monocytes (Weiss 1996). Reverse transcriptase is also packaged in the core but does not become enzymatically active until penetration and uncoating of the virion takes place.

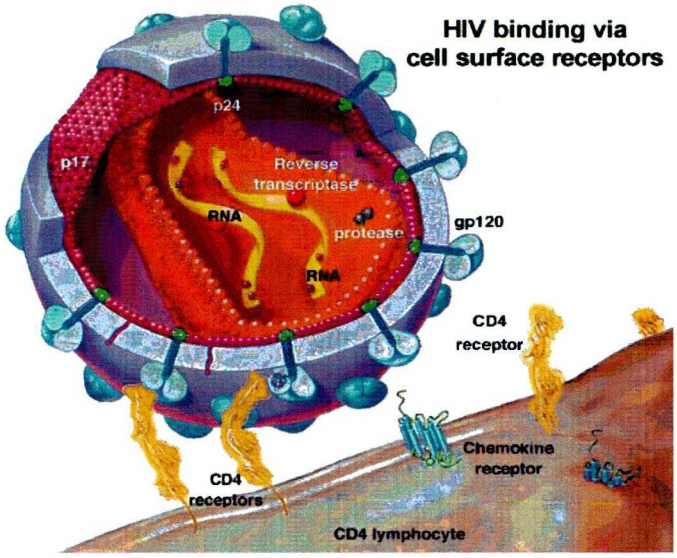

Figure 1/a.
The HIV and the initial phase of its life cycle binding via cell surface receptors. The virus attaches to the host cell, in this case a CD4 Lymphocyte (or T-4 cell), by binding to the various cell surface receptors available to it. These include the well-known CD4+ cell surface marker and a number of chemokine. (Picture based on ARIC's AIDS Image Gallery.)

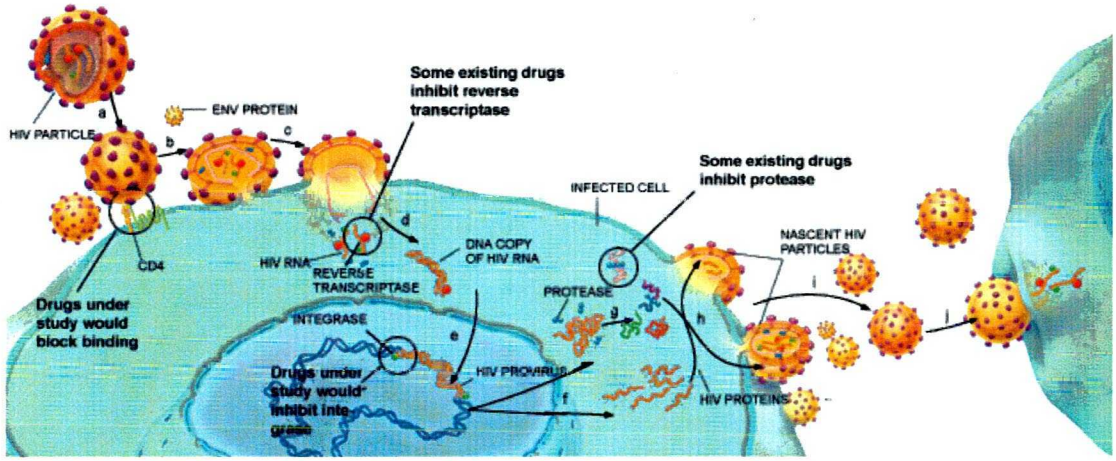

Figure 1/b.
HIV replication and targets. HIV life cycle begins when the virus binds to the cell surface (a), fuses with the cell membrane (b) and empties its contents into the cell (c). Next, the HIV enzyme reverse transcriptase copies the viral genetic material from RNA into double-strand DNA (d), which integrase splices into the cellular DNA (e). Using the integrated DNA, or provirus, as a blueprint, the cell makes viral proteins and RNA (f). A third enzyme, HIV protease, cleaves the new proteins (g), enabling them to join the RNA in new viral particles (h) that bud from the cell (i) and infect others (j). (Picture based on database of Scientific American.)

The primers for reverse transcription are cellular transfer RNA molecules that hybridize with the viral RNA genome. From these primers nascent DNA chains are formed. Reverse transcriptase also has an RNase-H activity that removes the parental RNA from the DNA, which

then forms the template for second strand DNA synthesis to form a double-stranded DNA provirus. This provirus is haploid but has sequences derived from both RNA strands through a copy-choice mechanism to provide long terminal repeats (LTR) at each end of the DNA genome (Figure 1., 2.).

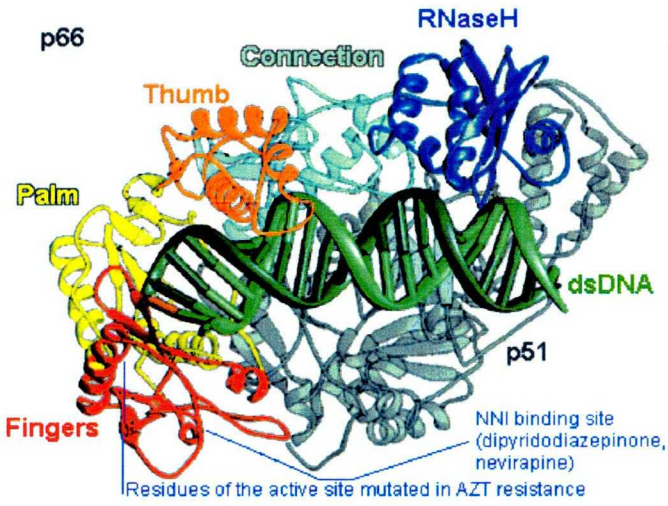

Figure 2. The structure of HIV reverse transcriptase. Functional HIV1-RT is a heterodimer containing subunits of 66 kDa (p66) and 51 kDa (p51). Portions of both p51 and the polymerase domain of p66 can be described as a "right hand" that contains three subdomains: fingers, palm, and thumb. The connection subdomain connects the hand of the polymerase domain and the RNase-H domain in p66. (Picture based on database of Science magazine.)

Functional HIV1-RT is a heterodimer containing subunits of 66 kDa (p66) and 51 kDa (p51). p66 contains two domains, the N-terminal polymerase domain (440 residues) and the C-terminal RNase-H domain (120 residues). p51 is processed by proteolytic cleavage of p66 and corresponds to the polymerase domain of the p66 subunit. Portions of both p51 and the polymerase domain of p66 can be described as a "right hand" that contains three subdomains: fingers, palm, and thumb. The connection subdomain connects the hand of the polymerase domain and the RNase-H domain in p66, which provides the ribonuclease activity of HIV-RT. Interestingly, although the two subunits are identical in their primary amino acid sequence (except for length), they are structurally very different. The majority of the template/primer-RT interactions are thought to occur between the sugar-phosphate backbone of the DNA/RNA and p66. The two alpha helices of the thumb, in combination with the fingers, serve as a clamp, holding the nucleic acid in place over the palm, which contains the polymerase active site. Helix H of the thumb of p66 partially embeds itself in the minor groove of the DNA and the thumb of p51 plus the connection domains of p66 and p51 form the floor of the binding cleft. The active site of p66 contains three catalytic residues in the palm subdomain that may bind metal ions (Asp185, Asp186 and Asp110). This active triad is positioned close to the 3' - OH terminus of the DNA primer. Non-nucleoside inhibitors (NNI) have been shown to bind in a pocket formed between two ß-sheets of the p66 palm, some 10 Å from the polymerase active site (Ren et al. 1995, Esnouf et al. 1995). The internal surface of this pocket is predominantly hydrophobic, being constructed primarily from leucine, valine, tryptophan and tyrosine residues. Although the NNIs are chemically diverse compounds, the crystal structures (e.g. Ren et al. 1995) reveal a common mode of binding. Depending on the NNI bound, the volume of the pocket varies between ~600 and ~700 Å3, with the inhibitors

occupying ~250-350 Å3. There is a clear matching of NNI shape to fit in this volume and in some cases this is achieved by conformational rearrangement of the compound from its lowest energy structure in solution (Figure 2.).

The preintegration complex targets to the nucleus where the completed provirus then integrates into host genome, catalysed by another viral enzyme, integrase. Integration is not host site specific but occurs preferentially in euchromatine. Expression of the provirus - depending on cellular transcriptional regulators - however, provides mRNA for packaging into virions. Complex retroviruses like HIV also require viral regulator proteins, such as *tat* for efficient RNA transcription, and *rev* which aids transport of the larger viral transcripts from nucleus to cytoplasm (Figure 1.).

In virion maturation the core proteins and enzymes are synthesized as two high molecular weigh precursor proteins in the cytoplasm which are cleaved by viral protease at a late stage of virion budding into the major structural proteins. The envelope glycoproteins, (gp41 transmembrane and gp120 surface proteins in case of HIV) are synthesized in the endoplasmatic reticulum and are cleaved by cellular protease during processing through the Golgi to the cell surface, where they are incorporated before virions release (Figure 1.).

1.1.2. Drug combinations as current antiretroviral therapy and perspectives

Retrovirus (e.g. HIV) inhibitor drugs targeted different steps of the replicative cycle (Figure 1.). Although several new therapies for HIV disease have been approved the development of drug resistant strains has been a universal problem and current research is focused on strategies that would be effective in reducing viral load to undetectable levels to minimize the potential for drug resistance. One possible way is the combination therapy offered significant clinical benefits over monotherapy: the simultaneously applied drugs on different viral targets reduce the risk of appearance of resistant mutant. The currently applied anti-HIV drugs can be subdivided according to the mechanisms of action into two main groups: reverse transcriptase (RT) inhibitors and protease inhibitors; the former may be subdivided into nucleoside inhibitors (NI) and non-nucleoside inhibitors (NNI) of reverse transcriptase (Table 2.). The currently most available combination consists of two RT inhibitors plus one protease inhibitor. This combinations of inhibitors may slow viral replication sufficiently to prevent generation of resistant virus, to extend the duration of antiviral activity and increase the benefit to patients.

Table 2. **Antiretroviral inhibitors**

Abbreviation	Common name
Inhibitors of reverse transcriptase	
1) Nucleoside analogues	
AZT	zidovudine
ddI	didanosine
ddC	zalcitabine
3TC	lamivudine
d4T	stavudine
2) Non-nucleoside analogue inhibitors	
	nevirapine
TIBO	thiobenzimidazolone
Inhibitors of protease	
SQV	saquinavir
RTV	ritonavir
IDV	indinavir
	nelfinavir

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1.1.2.1. Inhibition of reverse transcription

RT inhibitors can be divided into two groups, depending on whether they are targeted at the substrate or non substrate binding site. To the first group belong nucleoside analogs such as 3'-azido-2',3'-dideoxythymidine - azidotimidine (AZT) - and other dideoxy-nucleoside analogues (Mitsuya et al 1990). While nucleoside analogues such as AZT and 2',3'-dideoxiinosine (ddI) have exhibited therapeutic value in slowing down the rate of AIDS progression, the development of *in vivo* resistance by HIV-1 RT in response to these inhibitors has been reported. Many of the latter mutations are located on the surface of the DNA-binding cleft and may lead to altered template-primer positioning or conformation, causing a distortion of the geometry of the polymerase active site and consequent discrimination between normal and altered deoxy-nucleoside triphosphate (dNTP) substrates. Other nucleoside analog-resistance mutations located on the periphery of the dNTP-binding site may exert their effects via altered interactions with dNTP-binding site residues (Tisdale et al. 1997 and Krebs et al. 1997).

Non-nucleoside reverse transcriptase inhibitors which differs mechanistically from the chain terminator AZT and ddI include several components called nevirapine, thiobenzimidazolone (TIBO) derivatives, delavirdine, atevirdine, pyridinones and others (Maruenda and Johnson 1995,

Wu et al. 1991, De Clercq 1992, Loya et al. 1995). These compounds interact non competitively, with a specific allosteric binding site of HIV-1 RT (Schäfer et al. 1993). The therapeutic non nucleoside analogue drugs have the advantage of good tolerance but resistance may develop early when they are given alone (Sereni et al. 1996). Nevirapine and TIBO has been analyzed in the absence of bound ligand. The pocket that is present when non nucleoside inhibitors are bound is not observed in the inhibitor-free structure of HIV-1 RT with dsDNA. In particular it is filled by Tyr181 and Tyr188, suggesting that the pocket is formed primarily by rotation of these large aromatic side-chains. Existing biochemical data, taken together with the three-dimensional structure of HIV-1 RT, makes it possible to propose potential mechanisms of inhibition by non-nucleoside inhibitors.

One such mechanism is local distortion of HIV-1 RT structural elements thought to participate in catalysis: the beta 9-beta 10 hairpin (which contains polymerase active site residues) and the beta 12-beta 13 hairpin ("primer grip"). An alternative possibility is restricted mobility of the p66 thumb subdomain, which is supported by the observation that structural elements of the non-nucleoside inhibitor-binding pocket may act as a "hinge" for the thumb (Schäfer et al. 1993 and Tantillo et al. 1994).

1.1.2.2. Protease inhibitors and some potential antiretrovirals (except subjects of present study)

HIV protease required for post-translational cleavage of *gag* and *gag-pol* precursor polyproteins into functional products needed for viral assembly at the end of the replication. HIV protease is an aspartic protease encoded by the amino terminus of the *pol* gene. Anti-protease drugs such as substrate competitive peptide derivative saquinavir inhibit the formation and release of new infecting of the human immuno-deficiency virus. Ritonavir and indinavir are also among the most widely studied drugs (Hammer et al. 1994). Other retroviral enzymes can be also targeted: RNA:DNA hybrid cleaving RNase-H (e.g. by ribonucleoside vanadyl complexes, dAMP, monoclonal antibodies (Smith and Roth 1993)) or integrase (e.g. zintinavir - AR177 (Bishop et al. 1996), flavones, caffeic acid phenethyl ester, curcumin (Fesen et al 1993)).

There are several other targets to be an object of effective blocking of virus replication including virus entry (e.g. T20, an HIV-1 gp41 derivative (Lawless et al. 1996), soluble CD4, co-receptor blockers), nucleocapsid NCp7 (e.g. disulfide-substituted benzamides (Rice et al. 1995)), cell proteins, viral regulator proteins (e.g. protein kinase C (PKC) or *nef, vpr, vpx, vif*, *tat* (Coates et al. 1997, Yang et al 1996, Guy et al. 1991, Wu et al. 1996)) and cytokines (Poli and Fauci,

1993). Some sequence specific effectors also promise spectacular result: the short fragments of DNA or RNA - oligonucleotide antisens - complementary to different regions of HIV RNA can block replication of the virus (Lisziewicz et al. 1992 and Bordier et al 1995). Short complementer peptides - peptide antisens - can be designed based on the hydropathic characteristic of amino acids against certain regions of proteins resulting inhibition in its function. HIV enzymes and regulator proteins are targeted recently following this idea (Baranyi et al. 1995 and unpublished data). Certain RNA molecules, called ribozymes, possess enzymatic self-cleaving activity and cleave HIV-1 sequences (Sarver et al. 1990).

1.2. Possible targets of phenothiazines, ellipticines and fullerenes on retroviruses focused on reverse transcription and preintegration stage

After the complex series of events: including duplication, circularisation of the proviral strands and digestion of the viral RNA strand by the viral RNase-H the double-stranded DNA copy, or provirus is subsequently integrated into the host chromosomal DNA, or it may remain as a cytoplasmic plasmid (Sandström 1989, Sandström and Öberg 1993). In these stages the proviral dsDNA can be respected as a potential target of DNA binding compounds such as phenothiazines or ellipticines.

Phenothiazine drugs have been used in human and veterinary therapy for more than 40 years, predominantly as psychotropic agents (Figure 3.). In addition to the effect of phenothiazines on membrane they interact with the DNA in variable ways too. (Kantesaria and Marfey 1975). Some chlorpromazine derivatives show intercalation by fluorescence polarization and circular dichroism methods (Barabas et al. 1980). The electrostatic binding of the amino group of phenothiazine sidechain with the negative phosphate groups of the DNA and/or a partial insertion of either of its two phenyl rings between the nucleotide base pairs are probable mode of the binding (Gocke 1996). Chlorpromazine was shown to have antiviral effects but this ability was not thought to be necessarily related to its intercalation ability (Hahn 1979 and Barabas et al. 1980).

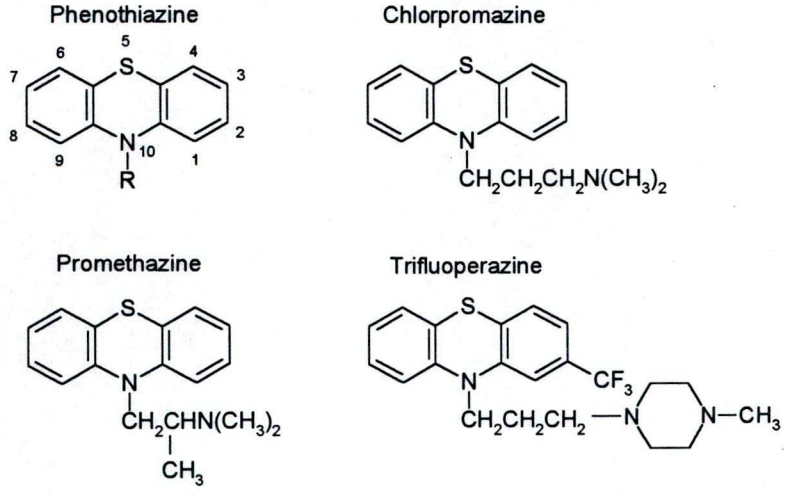


Figure 3.
Structure of phenothiazine frame, chlorpromazine, promethazine and trifluoperazine.

Most experimental data clearly suggest that ellipticine series, planar polycyclic aromatic molecules exert their cytotoxic and antitumor activity by stabilizing DNA-Topoisomerase II complexes (Figure 4., Vilarem et al. 1987). This multimodal - direct and indirect - mechanism of action at the DNA level is related to their intercalating activity and topoisomerase II binding property stabilizing the enzyme-DNA complex (Auclair 1987, Acton et al. 1994, Kayitalire et al. 1992, D'Incalci 1993, Fosse et al. 1990, 1994, Pognan et al. 1992)

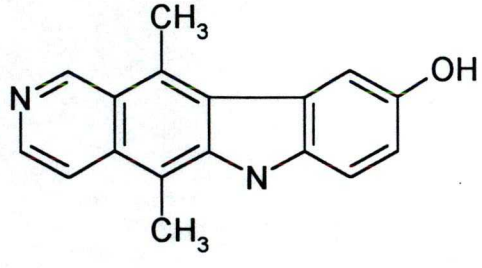


Figure 4.
Ellipticine analogue which has topoisomerase II-DNA complex stabilizing (antitumor) activity.

The reverse transcriptase can be inhibited by derivatized fullerene therefore targeted by water soluble non-derivatized C_{60} also (Sijbesma et al. 1993).

Some reports have described the anti-HIV activity of water soluble *fullerene* derivatives (Schinazi et al. 1993 and Friedman et al. 1993), but it was not clarified, however, whether the biological effects were due to the derivatized compound or to the C_{60} skeleton itself.

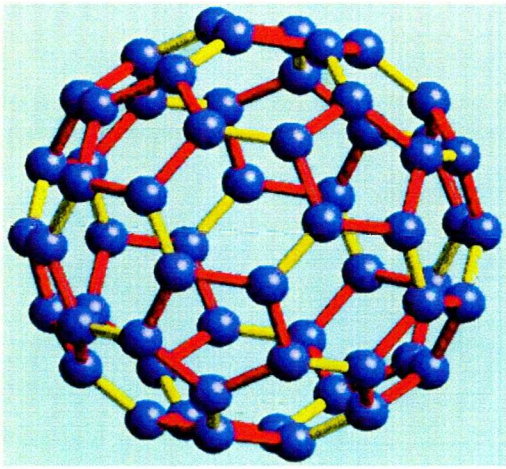


Figure 5.

The framework of C_{60}. The "hollow molecule" constructed of 12 pentagons and 20 hexagons is called as "Buckminsterfullerene" or "Buckyball". In the case of less associating pentagons the stability of C_{60} is higher. This izomer (only one of 1812 ones) does not contain pentagon adjoining to another one, corresponding to the structure of soccer ball.

The Buckminsterfullerene (C_{60}) is characterized by its insolubility in water and extreme hydrophobicity (Figure 5.)(Schinazi et al. 1995, Scrivens and Tour 1994). Therefore a water-soluble derivative of the "Buckyball", diamino diacid diphenyl fulleroid, was synthesized earlier to inhibit the HIV protease and reverse transcriptase, but the negatively charged functional groups were thought to be desirable to inhibit these enzymes (Sijbesma et al. 1993).

The DNA integration step requires the integration machinery ('preintegration complex') to bind to the host DNA before connecting the viral and host DNAs. The integrase sequence-specific DNA-binding domain binds quickly and tightly to the proviral strands. Target-DNA capture by HIV-1 preintegration complexes proceeds without subsequent sliding and target-site capture in vivo is reversible as a result of the action of cellular factors. The mechanism of target-DNA capture probably has implications mostly for the design of gene therapy methods, and influences the interpretation of results on the selection of integration target sites in vivo (Miller et al. 1995). Some former observations also support the antiviral effects of phenothiazines, ellipticines or fullerenes suggesting their other direct or indirect antiretroviral mechanisms as well (Lambrecht et al. 1991, Hewlett et al. 1997, Antonelli et al. 1992, Lewis et al. 1986, Pan et al. 1996, Kidd et al. 1997, Mathe et al. 1990, 1993, 1994, Ding et al. 1992).

4. Aims of the study

(i) To prepare and study some new or previously not tested phenothiazines and phenothiazine-metal complexes in order to find increased inhibiting effect on reverse transcription comparing to the parent compounds in Moloney murine leukemia reverse transcriptase enzyme assay. The idea is based on the interaction between phenothiazines and DNA on one hand, which is supported by their other more complex actions *in vitro*, and on the other hand on their structural similarities to other non nucleoside reverse transcriptase inhibitors affecting the enzyme activity on NNI binding site.

(ii) To investigate the interaction between newly prepared phenothiazine-metal complexes and extrachromosomal or chromosomal DNA, in stress gene inducing system studying their effect on protein - DNA interaction and by thermal stability measurement on calf thymus DNA testing their intercalative capability. These results can explain some increased inhibition in function of reverse transcriptase due to blocking of nascent DNA on the RT.

(iii) To test some novel ellipticine analogues in reverse transcriptase enzyme assay. The possible inhibition of RT is supported by the feature of ellipticines, that they are able to bind to the DNA and fix the DNA and interacting enzyme too, which can accept DNA as a substrate resulting its inhibition and that the non-nucleoside binding site of RT has conformational plasticity with accommodating ability to structurally different and unique molecules.

(iv) To clarify whether the non-derivatized fullerene (fullerene-cyclodextrin inclusion supermolecule complex - which is water soluble - and colloidal C_{60}) molecule has antiretroviral effect alone in enzyme assay and in replicating virus system too. This will be an important additional observation if one of the fullerene molecules acts as a frame in target orientated drug delivery system. The basically stable Buckminsterfullerene represents neutral surface of molecule group without disorganization suggesting some not very specific effects of the molecule. Therefore considering the hydrophobic site(s) of the reverse transcriptase and its membrane affinity the fullerene can show antiretroviral action primarily *in vitro*.

3. Materials and methods

3.1. Compounds studied

3.1.1. Phenothiazines and metal complexes

Four (phthalimido and chloroethyl) phenothiazine derivatives, i.e., [1]: 10-(3-phthalimido)propyl-2-chloro-10H-phenothiazine, [2]: 10-(4-phthalimido) butyl-2-chloro-10H-phenothiazine, [3]: 1-(2-chloroethyl)-3-(2-chloro)-10H-phenothiazin-10-yl)butyl-1-urea, [4]: 1-(2-chloroethyl)-3-(2-trifluoromethyl)-10H-phenothiazin-10-yl)propyl-1-urea tested were prepared as described previously (Motohashi et al. 1996).

Promethazine (Pz), chlorpromazine (CPZ), trifluoperazine (TFP) and the dimethyltin(IV)-dichloride were purchased from Aldrich, the di-n-butyltin(IV) oxide and the metal salts used were purchased from Fluka. The CPZ complexes with di-n-butyltin(IV) oxide and platinum(II) and the TFP complexes with di-n-butyltin(IV) oxide, copper(II), nickel(II), palladium(II) and vanadium(IV) in 1:1 ratio of the components were prepared by Nagy et al.

3.1.2. Ellipticines

Several novel ellipticine analogues were synthesized by Timári et al but the action of only two of them are interpreted in this study (Figure 6.).

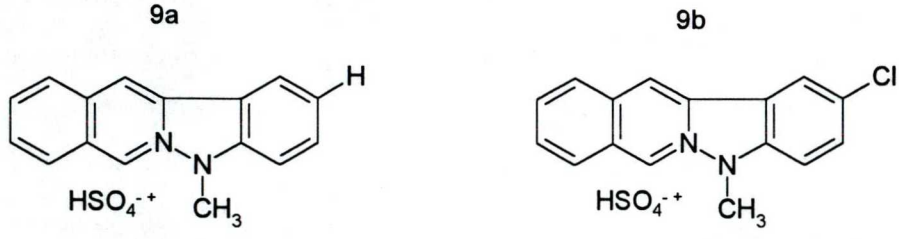


Figure 6.
Two novel ellipticine analogues: 9a and 9b as hydrogensulfate salt.

3.1.3. Fullerenes

C_{60} was of Gold Grade purity (Hoechst AG, Frankfurt/M, Germany. A stock solution of C_{60}-gamma-cyclodextrin supermolecule complex (C_{60}-γCD) was prepared as follows: a mixture of C_{60} and γ-cyclodextrin have been ball-milled and then dissolved in redistilled water to reach a final concentration of $2x10^{-4}$ M and $1x10^{-2}$ M for C_{60} and γCD, respectively.

A colloidal C_{60} suspension was prepared as follows: 100 µl of a saturated C_{60} solution in dichloro-benzene was added to 10 ml tetrahydro-furane at room temperature, by mixing the resulting solution dropwise in 100 ml acetone under rigorous stirring; then 150 ml water was slowly added while the dissolved C_{60} began to precipitate as a mustard yellow fine dispersed colloidal suspension. The organic solvents were removed by distillation. A $2.1x10^{-4}$ M stock solution of colloidal C_{60} was diluted to the end concentrations as indicated (Table 6. and Fig. 11.).

3.2. Detection of SOS signal by titer of induced megacine

The plasmid in *B. megaterium 216* encodes a bactericidal substance called megacine, but only when the plasmid DNA and the repressor protein complex are separated by mitomycin-*C*. YT (yeast extract tryptone) medium: tryptone (Oxoid), 10 g; yeast extract (Oxoid), 2.5 g; K_2HPO_4, 1 g; NaCl, 5 g; distilled water, 1000 ml; pH 7.5. YP (yeast extract peptone) medium was prepared as described previously [12]. From a standing culture in YT medium incubated overnight 0.2 ml was transferred to 10 ml YT medium pipetted into a 100 ml Erlenmeyer flask. The flask was shaken in water bath at 37 °C. When an OD value of 0.25 had been reached (number of colony formers, $5x10^6$/ml average chain length, 4-5), the cultures were supplemented with phenothiazines and after 10 minutes with mitomycin (0.5 µg/ml) and the change in OD on further incubation was recorded. YP broth culture of *B. megaterium KM* was diluted 1 : 4 with YP medium and 1 ml was mixed with 1 ml molten (47 °C) nutrient agar and poured on a basic layer of the same medium. The basic layer was prepared with 1.5 % agar. The upper soft layer contained half of this concentration. Serial dilutions of the megacine containing lysates were prepared with saline containing 20 % YP broth. By means of a loop equal amounts of the serial dilutions were placed onto the surface of the indicator plates (highest titer was 128x). The reciprocal of the highest dilution causing clear zones or lysing at least half of the bacteria at the corresponding site was regarded as one unit of megacine/ml (Holland and Roberts 1994, Ivánovics et al. 1959).

3.3. DNA thermal stability studies

For study the interaction of compounds with DNA, one of the most convenient is measuring the thermal stability of the DNA (Rad-niknam and Sharples 1990). Melting can be monitored by an increase in absorbance (hyperchromic effect) that results from the disruption of base stacking. The mid-point of the thermal denaturation curve is referred to as the "melting temperature, (Tm)" of the DNA. Highly polymerized type-1 calf thymus DNA sodium salt was purchased from Sigma. Stock solutions of DNA and the phenothiazines were prepared as follows: DNA (10 mg) was suspended in 10 ml 0.03 M Tris buffer containing 0.018 M NaCl adjusted to pH 7.0 and the solution was kept at 4°C for at least three days. Stock solution was diluted to the working conc. immediately before use. The concentrations the DNA solutions were determined spectrophotometrically in terms of nucleotide phosphate and calculated from an extinction coefficient at 260 nm of 6600/M/cm for calf thymus DNA (Angerer and Moudrianakis 1972, Wells et al. 1970) The phenothiazines and their metal complexes were dissolved in DMSO at a conc. of 10^{-3} M immediately before use.

Measurement was recorded on a Cary Varian Model IE spectrophotometer using a Cary temperature controller connected to a Cary 1/3 multicell block. The solutions were allowed to equilibrate for 20 minutes before increasing the temperature and the temperature then increased at a rate of 0.5 °C/minute. A cell containing DNA solution alone was always measured along side cells containing Drug/DNA mixtures to act as an internal standard. The blank cells in all measurements contained TRIS solution and all cells were stoppered with teflon caps. The mid point of the thermal denaturation profile of the solutions (T_m) was determined by calculating the average absorbance using the instruments thermal application software (Ebrahimi et al. 1992, 1995).

3.4. Inhibition of retrovirus replication

3.4.1. Cells

MT-2 and MT-4 cell line were applied as host of virus.

3.4.2. Viruses

MT-2 human T cell leukemia cell control and compounds tested and AZT treated cultures were infected with simian immuno-deficiency virus: SIV_{mac251} (a gift of R. C. Desrosiers, Southborough, MA) or with human immuno-deficiency virus: HIV producer H9 cells (isolate HTLV-III B, Popovic et al. 1984, a generous gift of R.C. Gallo) were used as a source for HIV in our studies.

3.4.3. Incubation - inhibition of virus replication in cell culture

The $2x10^6$ cells/ml infected and not infected and supplied by potential inhibitor drugs were cultured in vitro in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum in 5% CO_2 and 37 ^{0}C for 5 days and virus production was measured by follows:

3.4.4. Preparation of samples to reverse transcriptase assay

The virus was concentrated from clarified culture fluid by precipitation with polyethylene glycol (PEG). PEG-6000 (Fluka) 30% (w/v) in 0.7 M NaCl was added to the virus containing culture fluid to give a final concentration of 7.5% PEG. After overnight incubation at 4°C a virus containing pellet was obtained by centrifugation for 15 min at 3000 x *g* (Somogyi et al. 1990).

The SIV or HIV containing pellet was resuspended in a given volume of the reaction buffer (Somogyi et al. 1990) consisting of 50mM Tris-hydrochloride, pH 7.9, 5 mM dithiothreitol, 0.3 mM reduced glutathione, 5mM $MgCl_2$, 150 mM KCl, 0.5 mM cthylene glycol-bis (β-aminoethyl ether)-N,N,N',N', tetraacetic acid, 0.5 % Triton X-100, and the mixture was incubated at room temperature for 15-30 min.

3.5. Reverse transcriptase assay

In the case of direct inhibition of recombinant Moloney murine leukemia virus reverse transcriptase (M-MuLV RT: New England BioLabs) or HIV reverse transcriptase enzyme was added to the assay at the end of the protocol following below:

The assay is based on the poly(rA)$_n$-oligo(dT)$_{12-18}$ (New England BioLabs) directed incorporation of [^{3}H]dTTP (Amersham) into cDNA. The 10x reverse transcriptase buffer

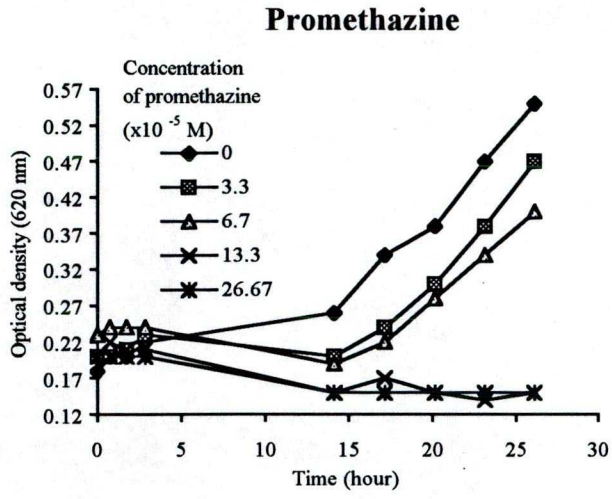
contained 500 mM Tris-HCl (pH 8.3), 80 mM $MgCl_2$, 300 mM KCl, and 100 mM DTT. In all experiments the final volume of reaction assay was 20 µl. This contained water, buffer, 20 µg/ml template-primer, 5 µM dTTP precursor (New England BioLabs), 7.4 kBq tritiated precursor, and compounds tested and then 2 U recombinant M-MuLV RT (or 10 µl of the virus suspension containing reverse transcriptase in case of inhibition of replication described above) initiating the reaction. This procedure was followed by an incubation for 40 min at 37 °C. 15 µl of the mixture was then transferred to a Whatman DE81 filter paper disc, washed by 5% dinatrium-hydrophosphate buffer (3x3 min), water and 96% ethanol and after drying and putting into 5 ml scintillation cocktail (OptiPhase 'HiSafe 3', Wallac) radioactivity measured by Packard Tri-Carb 2200 CE liquid scintillation counter (efficacy of measurement for ^3H was 35 %). The residual enzymatic activities were compared to the controls (no enzyme added<100 CPM, no drug added>8 000-20 000 CPM).

4. Results

4.1. Phenothiazine-metal complexes on plasmid and calf thymus DNA served as well adaptable model

On adding promethazine alone to the culture of *B. megaterium 216* no megacine production was observed (Figure 7/a). Promethazine pre-treatment was able to inhibit megacine production by inhibition of mitomycin induction (Figure 7/b). The Pz had concentration dependent antibacterial effect from 6.7 to 26.67×10^{-5}M (Figure 7/a.). The compounds had no effect on the production of megacine if the culture was treated by promethazine 10 minutes after the mitomycin-*C* induction. The quantity of megacine was followed by the growth-lysis curve (depending on the time of incubation). As a result of the bactericidal effect of megacine in the culture the cells lysed. Similar effect was found in the case of CPZ. Since the membrane effects of Pz and CPZ cannot be excluded on bacteria, some newly prepared metal complexes were tested to avoid the direct membrane injury caused by the phenothiazines. TFP one of the most potent phenothiazines had a concentration dependent inhibitory action on the megacine production (Figure 7/c). The most effective complex of TFP was the vanadium complex (Figure 7/d), while other metal-coordination complexes e.g. copper, nickel, palladium were less effective (Table 3, 4) showing that these three metals do not modify the biological action of TFP. The platinum complex of CPZ had similar effect to TFP (Table 3, 4). It was noted that the compounds do not neutralize the antibacterial action of preformed megacine (results not shown).

a/



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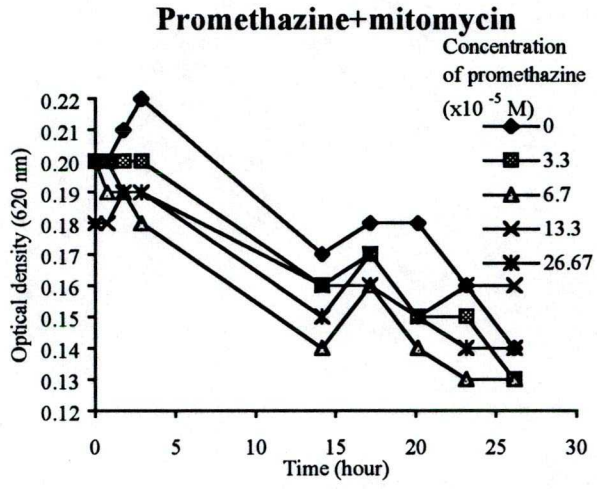

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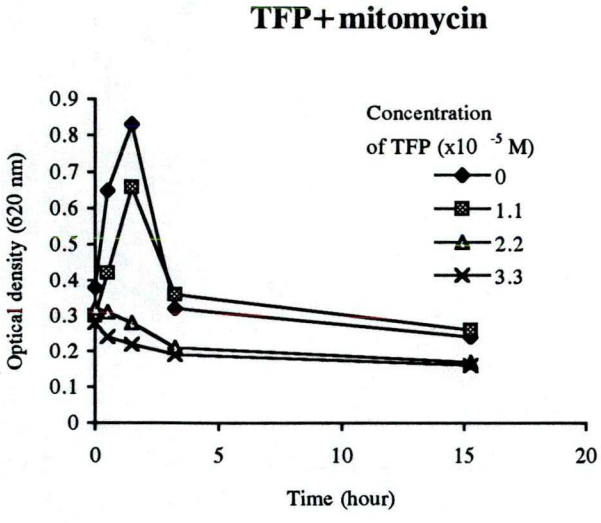

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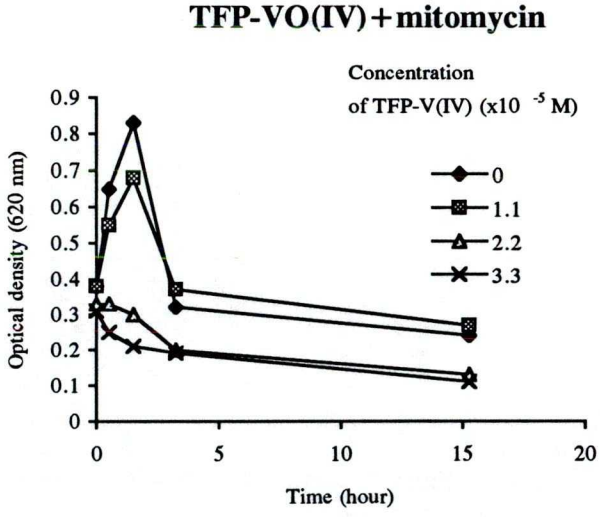

Figure 7.
Effect of pre-treatment of phenothiazines and their metal complexes on growth rate and induction of megacine by mitomycin-C in *B. megaterium 216*

Table 3. Effect of pre-treatment of metal complexes of phenothiazines on growth rate and induction of megacine in *B. megaterium 216*

		Concentration of compounds (10^{-5} M)			
		0	1,1	2,2	3,3
Compounds	**Time of incubation (hour)**	**Optical density of culture (620 nm)**			
TFP-Cu(II)	0	0,38	0,38	0,33	0,31
	0,5	0,65	0,55	0,36	0,24
	1,5	0,83	0,7	0,36	0,25
	3,25	0,32	0,25	0,2	0,25
	15,2	0,24	0,17	0,11	0,19
TFP-Pd(II)	0	0,38	0,36	0,35	0,25
	0,5	0,65	0,51	0,4	0,24
	1,5	0,83	0,64	0,36	0,23
	3,25	0,32	0,24	0,23	0,2
	15,2	0,24	0,17	0,17	0,17
TFP-Ni(II)	0	0,38	0,4	0,35	0,3
	0,5	0,65	0,58	0,48	0,3
	1,5	0,83	0,74	0,58	0,26
	3,25	0,32	0,32	0,24	0,22
	15,2	0,24	0,3	0,17	0,18
CPZ-Pt(II)	0	0,38	0,35	0,32	0,23
	0,5	0,65	0,41	0,3	0,18
	1,5	0,83	0,5	0,37	0,16
	3,25	0,32	0,26	0,27	0,14
	15,2	0,24	0,2	0,2	0,12

Table 4. Titer of megacine after the inhibition of mitomycin induction by phenothiazine and metal complexes

	Concentration of compounds (x10^{-5}M)			
	0	1.1	2.2	3.3
Compounds	**Titer of megacine**			
TFP	128x	128x	8x	0x
TFP-VO(IV)	128x	64x	4x	0x
TFP-Cu(II)	128x	128x	128x	1x
TFP-Ni(II)	128x	128x	128x	4x
TFP-Pd(II)	128x	128x	64x	0x
CPZ-Pt(II)	128x	128x	8x	8x

The measurements of thermal stability of DNA show some interaction between the phenothiazine metal complexes and DNA. The increased melting temperatures of DNA in the presence of metal complexes indicates an interaction with DNA and stabilization of the helix. CPZ and TFP can be seen to stabilize the DNA helix by intercalation causing a slight increase in the thermal denaturation temperature. A similar effect was seen with the TFP-Sn(IV), the CPZ-Sn(IV) and the TFP-Cu(II) coordination complexes indicating an interaction with the DNA helix whilst metal ions alone showed no significant stabilization of the helix. The chlorides of Pt, Pd and V degraded the DNA resulting in a linear thermal stability profile. The coordination complexes of these metals with CPZ and TFP however decreased the thermal stability of DNA. The phenothiazines appear to be exerting a protective effect, defending the DNA against total degradation caused by the metal ions alone (Table 5.).

Table 5. Change in Thermal Denaturation Temperature (T) of C T DNA in the presence of phenothiazines and metal complexes

Compounds	DNA: comp. = 10:1	ΔTm	DNA: comp. = 5:1	ΔTm	DNA: comp. = 2.5:1	ΔTm
CPZ		0.2		0.44		0.67
TFP		0.93		1.22		1.6
CPZ-Pt(II)				-2.7		
Pt(II)				*		
CPZ-Sn(IV)		1.2		1.4		1.34
Sn(IV)		0.2		0.1		0.23
TFP-Cu(II)		0.93		1.4		1.54
Cu(II)		0.70		-0.14		0.54
TFP-Ni(II)		2.38		0.93		2.3
Ni(II)		0,1		0.3		0.34
TFP-Pd(II)				-5.4		
Pd(II)				*		
TFP-VO(IV)		-2.3		-10.1		
VO(IV)				*		
TFP-Sn(IV)		2.0		1.0		1.4
Sn(IV)		0.2		0.1		0.23

* Resulted in degradation of the DNA

4.2. Phenothiazines and their metal complexes act on reverse transcription

Although sequence similarities among various DNA and RNA polymerases are weak, structural similarities are obvious. Therefore I could apply Moloney murine leukemia virus reverse transcriptase as a general model for inhibition of reverse transcription.

The effect of some phthalimido- and chloroethyl-phenothiazines were tested on reverse transcriptase. Although the ring substitution of these compounds may alter their interaction with plasmids, the reverse transcriptase enzyme inhibition of murine leukemia virus shows that the substituents introduced do not change significantly the antiretroviral effect of these compounds (Figure 8.).

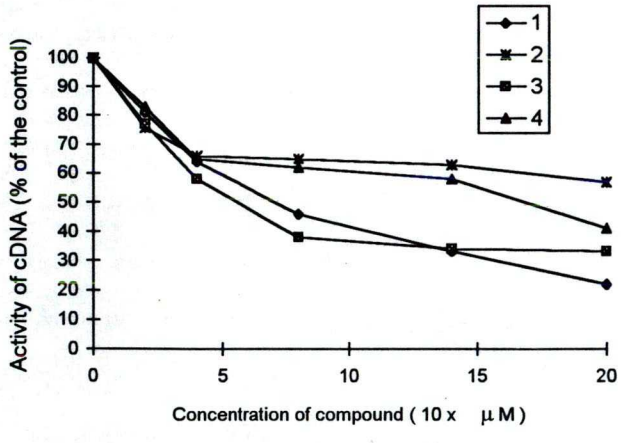

Figure 8.
Inhibition of Moloney murine leukemia virus reverse transcriptase by phenothiazine compounds. [1]: 10-(3-phthalimido)propyl-2-chloro-10H-phenothiazine,[2]:10-(4-phthalimido) butyl-2-chloro-10H-phenothiazine,[3]:1-(2-chloroethyl)-3-(2-chloro)-10H-phenothiazin-10-yl)butyl-1-urea,[4]:1-(2-chloroethyl)-3-(2-trifluoromethyl)-10H-phenothiazin-10-yl)propyl-1-urea.

The trifluoperazine inhibited the activity of Moloney leukemia virus RT in IC_{50} of 4.8 μM. The trifluoperazine-metal coordination complexes were more effective in our experiments than the parent trifluoperazine only. In comparison of IC_{50} values are following: TFP > TFP-Sn(IV) > TFP-Pd(II) > TFP-Ni(II) > TFP-Cu(II) ≥ TFP-VO(IV) (Figure 9.). By comparison of the effect of metal ions (IC_{50} values) the following activity order was found: VO(IV) > Ni(II) > Pd(II) > Cu(II) > Sn(IV). Calculating the additive effect of metal ions and TFP the IC_{50} values are in order: VO(IV) > Ni(II) > Pd(II) > Cu(II) > Sn(IV) (Figure 9).

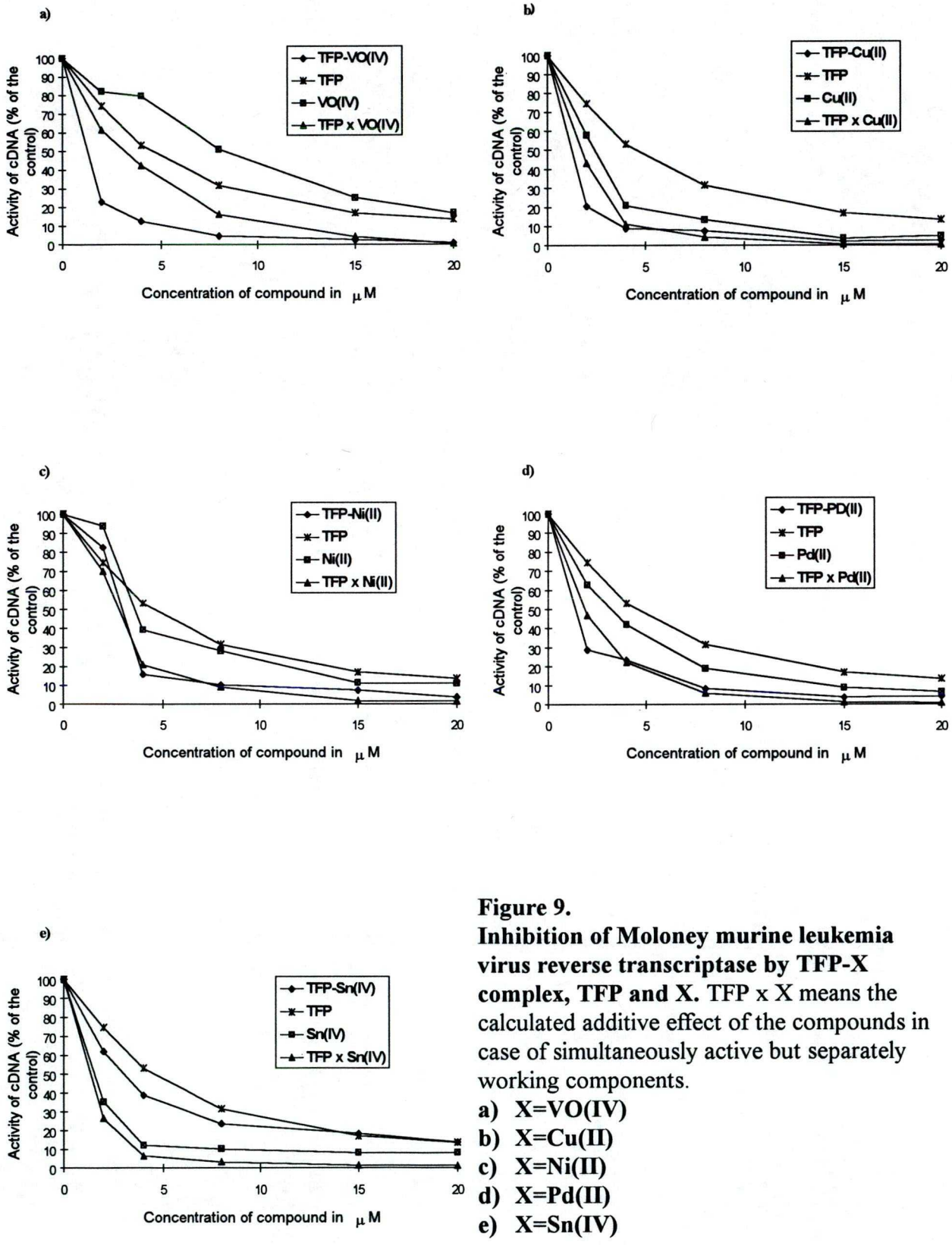

Figure 9.
Inhibition of Moloney murine leukemia virus reverse transcriptase by TFP-X complex, TFP and X. TFP x X means the calculated additive effect of the compounds in case of simultaneously active but separately working components.

a) X=VO(IV)
b) X=Cu(II)
c) X=Ni(II)
d) X=Pd(II)
e) X=Sn(IV)

This calculation was essential to avoid the effect of spontaneous complex formation or dissociation (decomposition) of complex forming components in the assay during the incubation period. Comparing the effects of complexes with the calculated values the following conclusion could be drawn out: 1) in case of the nickel(II), copper(II) and palladium(II) systems the differences can not be proved definitely. 2) The TFP-VO(IV) complex showed remarkable differences in all concentration range studied (Figure 9/a.). The vanadyl(IV) complex had much lower IC_{50} (1.2 μM) than it was expected from the additive effect (3.3 μM). The improved biological action was shown as a new feature of the metal coordination complexes. Comparing the corresponding curves for the inhibitory effects of organotin(IV)-TFP system the differences appeared remarkably as well. But the organotin(IV) complex showed definitively weaker action (IC_{50} = 3.1 μM) on the enzyme than the TFP (IC_{50} = 4.8 μM) and the metal ion (IC_{50} = 1.6 μM) together could do that (IC_{50} = 1.3 μM).

4.3. Ellipticines on reverse transcriptase

Few new ellipticine analogues were synthesized as a potential non-nucleoside inhibitors of reverse transcriptase and were tested on Moloney murine leukemia virus RT *in vitro*. Two of new nitrogen positional analogues of ellipticine (9a,b - Figure 6.) showed considerable inhibitor effect; ID_{50} was found to be in the range of 28 to 45 μM (Figure 10.). (Timari et al. 1996, Mathe et al. 1990, Acton et al. 1994, Kayitalire et al. 1992 and D'Incalci 1993).

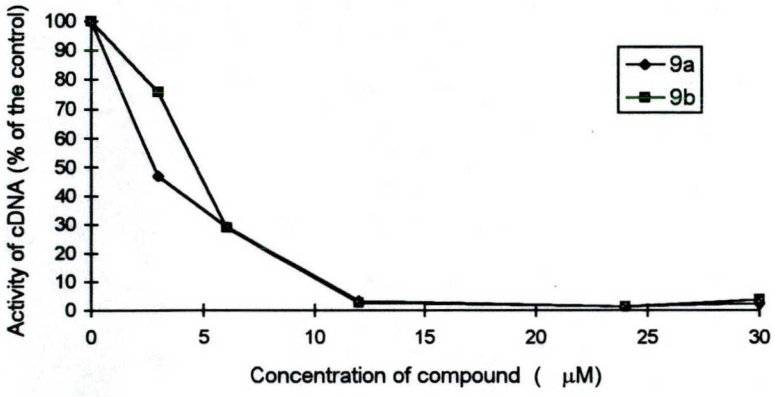

Figure 10.
Inhibition of Moloney murine leukemia virus reverse transcriptase by two ellipticine analogues

4.4. The nonderivatized fullerene inhibits the function of reverse transcriptase

Colloidal C_{60} and C_{60}-γCD supermolecule complex did not affect the viability of MT-2 cells (data not shown) when used in the concentration range (C_{60}: $1x10^{-7}$-$1x10^{-6}$ M, C_{60}/γCD: $1x10^{-7}/5x10^{-6}$-$1x10^{-5}/5x10^{-4}$ M) indicated in Table 6.

Production of SIV was significantly reduced in cultures treated with C_{60}-γCD or γCD alone. At each concentration ($1x10^{-5}$ M) applied, the C_{60}-γCD supermolecule complex resulted in a greater inhibition than the corresponding γCD control ($5x10^{-4}$ M).

In order to evaluate the effect of C_{60} itself, we treated SIV infected cells with colloidal C_{60}. As shown in Table 6., the addition of $1x10^{-6}$ M colloidal C_{60} also inhibited SIV replication.

In order to determine the potential target of nonderivatized C_{60}, we measured the effect of C_{60}-γCD supermolecule complex and colloidal C_{60} on the activity of M-MuLV reverse transcriptase. C_{60}-γCD and γCD itself inhibited the activity of this enzyme (Figure 11. a, b). At each concentration tested C_{60}-γCD ($0\text{-}18x10^{-6}/0\text{-}9x10^{-4}$ M) showed a greater inhibition than γCD at corresponding concentration ($0\text{-}9x10^{-4}$ M) alone. Colloidal C_{60} inhibited M-MuLV reverse transcriptase: $IC_{50} \approx 3$ μM (Figure 11. b). Comparing the activity of reverse transcriptase inhibited by C_{60}-γCD supermolecule complex (Figure 11.

a) to that of enzyme inhibited by γCD (Figure 11. c) as a control the division resulted a curve showing a concentration dependent inhibition (values of statistical analysis: $\beta = -0.85$; $F(1,4) = 10.2$; $p < 0.0004$) which is greater than the action of γCD or colloidal C_{60} only (Figure 11. d). For comparison, AZT-TP had an IC_{50} of 0,06 μM in our assay system.

Table 6. Activity of reverse transcriptase in SIV infected tissue culture inhibited by C_{60}-γCD, colloidal C_{60} and γCD

Compounds	Molar concentration of the compounds	Activity of reverse transcriptase (in % of control)*
	C_{60}:$1x10^{-5}$/γCD:$5x10^{-4}$	0,15
C_{60}-γCD	C_{60}:$1x10^{-6}$/γCD:$5x10^{-5}$	18
complex	C_{60}:$1x10^{-7}$/γCD:$5x10^{-6}$	31,6
	$1x10^{-6}$	37
Colloidal C_{60}	$5x10^{-7}$	95
	$1x10^{-7}$	87,5
γCD control	$5x10^{-4}$	6,6

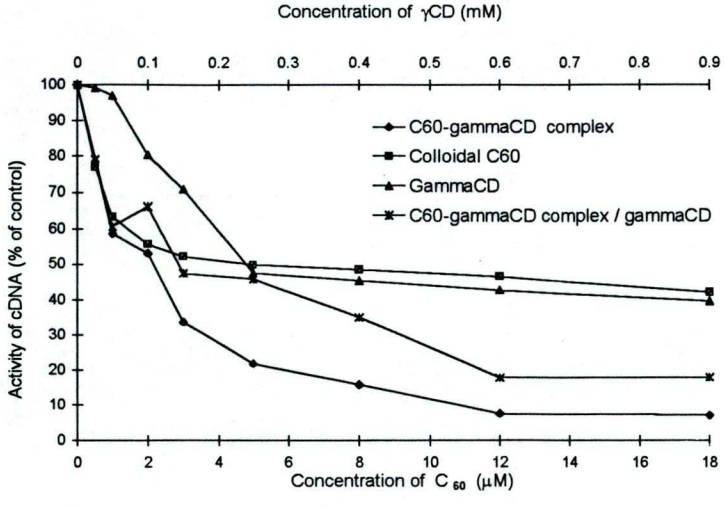

Figure 11.

Inhibition of Moloney murine leukemia virus reverse transcriptase by a) C_{60}-γCD supermolecule complex; b) colloidal C_{60} c) γCD and d) C_{60}-γCD supermolecule complex comparing to γCD as a control

5. Discussion

5.1. The most important findings

(i) The trifluoperazine is an effective inhibitor of reverse transcription in enzyme assay.

(ii) The inhibition of TFP-VO(IV) complex on reverse transcriptase remarkably exceeds that of TFP and this effect due to the new features of complex molecule.

(iii) Two newly synthesized ellipticine analogues show a significant inhibition on RT.

(iv) The nonderivatized C_{60} has an antiretroviral effect, especially arrests the function of reverse transcriptase.

5.2. Chlorpromazine, trifluoperazine and their metal complexes in stress induced gene-response mechanism

In order to test the DNA-phenothiazine interaction and the influence of phenothiazines on DNA-protein interaction in living system a plasmid coded bacterial stress gene protocol was chosen as a model experiment. Although a bacterial cell shows significant differences comparing to an eukaryotic cell, in the case of effect on DNA-protein interaction the potential mechanism of inhibition of reverse transcriptase can be suggested and explained.

The results of protein-DNA interaction model system indicate that phenothiazines and their metal co-ordination complexes are apparently able to reduce the SOS function in bacteria reducing the bacteriocin induction but do not imply its mechanism. At the same time it is known too that some stress gene encoded ABC transporter activities can be inhibited by these agents (Nacsa et al. 1998). Although the influence of phenothiazines on stress gene-regulator protein complex has not been clarified well so far it is recognized that the antitumor effect of chlorpromazine, trifluoperazine and other phenothiazines due to their interaction not only with some specific cell proteins such as calmodulin or p-glycoprotein but with the DNA as well (Lialiaris et al. 1992, Mizushima et al. 1993, Aas et al. 1994, Perez et al. 1992, Grief et al. 1989, Varga et al. 1996, Ganapathi et al. 1988, 1991). Some features of

phenothiazines clarified earlier supported that the inhibition of SOS function is due to the complex formation between the phenothiazines and the inducible SOS gene or some regulator proteins: the absorption spectra of calf thymus DNA shows two important changes upon binding to chlorpromazine, namely, the displacement of peaks to longer wavelength (ranging from 5-8 nm) and a decrease in the optical density. Chlorpromazine decreases the optical density at higher temperatures without affecting the Tm of DNA. In presence of chlorpromazine, the absorption spectra of purine deoxy-nucleosides (dA, dG) and of deoxy-nucleotides (dAMP, dGMP) are modified, i.e., the maxima are displaced to longer wavelength (ranging from 5-17 nm) and there is a general decrease in the optical density. No such effect is observed with pyrimidine deoxy-nucleosides (dC, dT) and deoxy-nucleotides (dCMP, dTMP) (Kantesaria and Marfey 1975). Chlorpromazine, 7,8-O_2-chlorpromazine, and methylene blue show an intercalation effect by fluorescence polarization and circular dichroism methods but at the same time other phenothiazines such as chlorpromazine sulphoxide and thiazinamium are not able to intercalate into Escherichia coli DNA, neither do amitriptyline and imipramine (Barabas et al. 1980). A combination of electrostatic binding of the amino group of phenothiazine sidechain with the negative phosphate groups of the DNA and a partial insertion of either of its two phenyl rings between the nucleotide base pairs of the DNA are plausible mode of binding of chlorpromazine and other phenothiazines to DNA. In spite of interaction with the DNA there appears to be no genotoxic activity associated with these drugs when tested under standard conditions. But UV irradiation of chlorpromazine and other chlorinated derivatives produces reactive free radicals which possess DNA damaging properties. Induction of gene mutation and chromosomal aberrations have been observed in appropriately designed photomutagenesis experiments. Enhancement but also reduction of UV induced skin tumour formation by chlorpromazine have been found (Gocke 1996).

In general the intercalation ability of phenothiazines is not necessarily related to their antiplasmid, antiviral or anticancer effect (Hahn 1979 and Barabas et al. 1980, Molnar et al. 1991) and their activity on plasmids can be due not only to the complex formation with the supercoiled form of plasmid DNA but also to the increased membrane permeability or inhibition of DNA gyrase which can lead to the cessation of plasmid

replication in the bacterial cells. (Molnar et al. 1992, 1995). However certain rules can be concluded on increased action of phenothiazine derivatives on plasmids: their activity seems to be enhanced by Cl- or CF3- substitution at 2 C atom and modified by the side chain length and charge at the L-region of the molecules, as well as by hydrophilicity (Motohashi et al. 1992).

Based on that working hypothesis namely the DNA binding properties of tested phenothiazines are responsible for the inhibition of SOS-gene regulator protein interaction the complex formation with native B form DNA was studied by measuring thermal stability of DNA.

The results suggest that the co-ordinating metals alter the fine chemical structure (superdelocalizibility of π-electrons) and biological action of phenothiazines.

Metal ions react with a variety of electron donor sites on polynucleotides. There are three main sites of interaction, the phosphate moieties of the ribose-phosphate backbone, the electron donor groups of the basis and the sugar part of the polynucleotides. The fourth but significant interaction could be occur by intercalation of metals or metal complexes and altering the hydrogen-bonding network. Interaction with ribose moieties under such an experimental conditions used is negligible. Reactions with the phosphate means stabilization of ordered structure or cleavage of phospodiester bonds at high temperature. Although the binding is not-specific, the result is to neutralize the array of negative charges on the double helix and thus to stabilize it. Such stabilization is accompanied by an increase in the melting temperature of DNA. Concerning the metal ions the preference for phosphate over the base association decreases in the order Mg(II) > Co(II) > In(II) > Mn(II) > Zn(II) > Cu(II) (Eichhorn and Shin 1968). Reaction with bases means destabilization of ordered structures, since metal ions or complexes can bind to the base stacking interactions that hold together the two strands of DNA. This interaction is accompanied by a decrease in the melting temperature.

It may be suggested from the thermal denaturation studies (Table 3) that the SOS inhibitory function and the reversal action on mdr of tumour cells is also due to the denaturing effect of Pd, Pt or V ions on the cellular DNA. Co-ordination of these ions with CPZ or TFP provides a means by which these ions may be selectively carried to the DNA helix, as the phenothiazines are known to interact with the DNA helix (Barabás and

Molnár 1980). At the helix the Pt, Pd and V ions act to destabilize the helix as evidenced by the observed decrease in the thermal stability of the DNA. A possible mechanism for this might be by the Pt, Pd or V ions interfering with hydrogen bonding between the nitrogenous base pairs, or the metal complexes interact with bases and destabilized the ordered structures.

The inhibitory effect on megacine induction can be improved by complex formation of phenothiazines with various metal ions. Therefore we can conclude, that the specific action of phenothiazine ring system can be basically modified by multivalent co-ordinating metal ions.

5.3. Influence of phenothiazines and their metal complexes in reverse transcription

The psychotherapeutic drugs phenothiazines and their derivatives have already been proven antiviral effects earlier (Lambrecht et al. 1991). Some of their actions on retroviral models were also observed: the 7,8-dioxo-*chlorpromazine* is able to block binding of fluorescein isothiocyanate-labeled anti-Leu3a and rgp120 to peripheral human blood T4 cells and blocks syncytia formation between gp120- and CD4-expressing cells. And it is also found that 7,8-dioxo-chlorpromazine blocks HIV infectivity of H9 cells and acts synergistically with zidovudine (Hewlett et al. 1997). The synergism with AZT can also be achieved by increased intracellular accumulation of the antiretroviral drug by trifluoperazine inhibiting the function of efflux pump and affects the sensitivity of HIV to AZT (Antonelli et al. 1992). The HIV induced apoptosis - which may be one of the major forms of T cell death in HIV-1 infection - can be also targeted by trifluoperazine, because calcium dependent apoptotic pathways being expected to involve calmodulin, one of the known target of TFP. (The role of the calmodulin activity in retrovirus infection was emerged earlier - Lewis et al. 1986.) The HIV-1 envelope glycoprotein, gp160, contains two known calmodulin-binding domains and the calmodulin antagonists trifluoperazine and tamoxifen are able to inhibit completely the pFN (a non-infectious HIV proviral clone which expressing wild-type gp160) enhancement of Fas-mediated apoptosis in Molt-4 cells (Pan et al. 1996).

In spite of these results the inhibitor effect of phenothiazines on the reverse transcriptase was not investigated. The action of several non-nucleoside inhibitors containing tricyclic condensed rings are already studied, mainly because of the therapeutic importance in the AIDS (Schafer et al. 1993 and Artico et al, 1996). It was found that nevirapine inhibited the HIV-1 RT in IC_{50} = 0.43 μM (Schafer et al. 1993). Testing some phthalimido- and chloroethyl-phenothiazines on reverse transcriptase based on structural similarities to other non nucleoside reverse transcriptase inhibitors their IC_{50} value was not less than 50 μM. Comparing their framework the ring substitution of these compounds did not alter their inhibition on RT significantly nevertheless they showed modified action on plasmids (Motohashi et al. 1997).

The corresponding value for TFP (Figure 3.) was found by us 4.8 μM (Figure 9.). A reasonable explanation for this inhibition seems to be the interaction with the NNI binding site because of structural similarities to other NNIs. Forasmuch the majority of the template/primer-RT interactions are thought to occur between the sugar-phosphate backbone of the DNA/RNA and p66, the TFP can block the primarily nascent DNA too. These interactions are presumably based on a partial insertion of either of its two phenyl rings between the nucleotide base pairs of the DNA in combination of electrostatic binding of the amino group of phenothiazine sidechain with the negative phosphate groups of the DNA.

The influence of some metals (Filler and Lever 1997) or metal complexes were also reported (Bruno et al. 1997) earlier. For example the 1-(2',6'-difluorophenyl)- 1H,3H-thiazolo [3,4-a]-benzimidazole(tri-n-propyl-phosphine) compound which is a highly potent non-nucleoside HIV-1 RT inhibitor lost its activity due to the complex formation with palladium(II), although the palladium itself showed a marked non-reversible inhibition of RT activity (Filler and Lever 1997). From our experimental data we conclude that the biological action of the newly synthesized vanadyl(IV) and organotin(IV) compounds is due to the complex formation of phenothiazine with the metals or transient metal salts. In case of vanadyl(IV) complex this enzyme inhibitory effect was improved. The effect of the organotin(IV) complex was reduced by comparing to the calculated additive effect of parent compounds. The modified effect of these compounds suggests some indirect and

direct action of complex forming metal ions. One possibility is that metal coordinating to the hetero atoms in the middle ring of phenothiazine stabilizes and/or rotates the sidechain and changes the folding angle between the two benzol ring producing new favorable or unbecoming structural features and binding properties to the NNI binding site on the palm of reverse transcriptase. On the other hand the catalytic residues in palm subdomain may bind metal ions forming a complex between the DNA primer (negative phosphate groups and/or nucleotide basepairs) - TFP (sidechain and/or benzol rings) - metal - metal binding site anchoring the nascent DNA to the enzyme, which would be an acceptable explanation of increased action of TFP-vanadyl(IV) compound comparing to calculated additive effect of TFP and metal ion.

The results show similarity to the effect of cationic substitution (Filler and Lever 1997), although the unsubstituted promazine and the substituted chlorpromazine were not tested and compared in this study. At any rate the stability of coordination complexes probably depends on the particular feature of other ring substituents e.g. H, Cl, Pd or CF_3 group at the second position on the phenothiazine framework.

Recent data show that the very first step of HIV replication the attachment interaction between gp120 and CD4 molecules can be also affected by phenothiazines and in addition this inhibitory effect can be improved in case of Pol coordination complex (Kidd et al. 1997). Other complexes e.g. gold and copper and the uncomplexed form of promazine and chlorpromazine were without effect on HIV replication.

5.4. Novel ellipticines

Most cytotoxic anticancer agents interact directly or indirectly with nuclear DNA, the ultimate target for this class of compounds. For a given type of drug both direct and indirect action at the DNA level usually causes various types of interference or damage. This multimodal mechanism of action is well illustrated by antitumor drugs in the ellipticine series, the planar polycyclic aromatic molecules, which may bind to DNA through intercalation, may undergo covalent binding, may generate oxidizing species, and may interfere with the catalytic activity of topoisomerase II (Figure 4.)(Auclair 1987,

Acton et al. 1994, Kayitalire et al. 1992 and D'Incalci 1993). Most experimental data clearly suggest that antitumor agents including DNA intercalative molecules (acridine derivatives, ellipticine and derivatives), or non intercalative ones (epipodophyllotoxines), exert their cytotoxic activity by stabilizing DNA-Topoisomerase II complexes (Vilarem et al. 1987). The cytotoxicity of most ellipticines is related to the presence of an hydroxy group at position 9 of the pyridocarbazole ring system and to their interaction with DNA topoisomerase II stabilizing the topoisomerase II-DNA covalent complex. This observation supports the idea that topoisomerase II is the primary target involved in the mechanisms of action of ellipticines (Fosse et al. 1990, 1994, Pognan et al. 1992)

Based on that recognition that the ellipticines represent effective inhibitors of function of DNA binding topoisomerase II stabilizing their complex formation they can be potent inhibitors also of reverse transcriptional and preintegrational steps (Auclair 1987, Vilarem et al. 1987, Fosse et al. 1990, 1994, Pognan F et al. 1992 and D'Incalci 1993). Therefore some newly synthesized ellipticine analogues were tested but only two of them represented remarkable action in RT assay *in vitro* with $28\mu M<IC_{50}<45\mu M$ value. Others also found ellipticine analogues showing antiviral activity (Jamison J et al., 1990) but some of them and their analogues were able to reduce the viremia drastically in mice infected with Friend virus as a retrovirus model for screening drugs which could be active on the HIV (Mathe et al. 1990, 1993, 1994). A series of cationic metalloporphyrin-ellipticine complexes were found to inhibit the cytopathicity of HIV-1 and simian immuno-deficiency virus in MT-4 cells at concentrations ranging from 1.4 to 17 micrograms/ml, i.e. at a concentration that was 2.5-30-fold below the cytotoxicity threshold. These compounds were also found to inhibit syncytium formation between persistently HIV-1-infected HUT-78 and uninfected Molt/4 cells, to interfere with HIV-1 binding to the cells, and to suppress HIV-1- associated reverse transcriptase activity (Ding et al. 1992).

The conformational rearrangement of the NNI binding site some 10 Å from the polymerase active site allows accommodation in regions of the surrounding protein interacting with chemically diverse, structurally unique compounds so with ellipticines too without changing the overall binding mode. Because of this structural plasticity the two

new nitrogen positional analogues of ellipticine founds further structure-action studies in reverse transcriptase inhibition (Timari et al. 1996, Mathe et al. 1990 and D'Incalci 1993)

5.5. Nonderivatized fullerene inhibits reverse transcription

Using a single agent to treat retroviral infection inevitably results in the emergence of drug resistant virus variants. The success of therapy is improved by the availability of new antiviral compounds and multiple combinations of drugs acting on different targets. One of the main difficulties should be worked out is the target site specific transportation of effector molecules. Fullerenes are considered as molecular skeletons which could be derivatized to carry functional groups inhibiting the activity of different enzymes, including those involved in the replicative cycle of retroviruses.

Some reports have described the anti-HIV activity of water soluble fullerene derivatives (Schinazi et al. 1993 and Friedman et al. 1993), but it was not clarified, however, whether the biological effects were due to the derivatized compound or to the C_{60} skeleton itself. The Buckminsterfullerene (C_{60}) is characterized by its insolubility in water and extreme hydrophobicity (Figure 5.)(Schinazi et al. 1995, Scrivens and Tour 1994). Therefore a water-soluble derivative of the "Buckyball", diamino diacid diphenyl fulleroid, was synthesized earlier to inhibit the HIV protease and reverse transcriptase, but the negatively charged functional groups were thought to be desirable to inhibit these enzymes (Sijbesma et al. 1993).

For further clarification we studied the action of nonderivatized C_{60} on the replication of simian retrovirus (SIV) and demonstrated its antiviral effect. C_{60} in C_{60}-γCD supermolecule complex and colloidal C_{60} itself could significantly reduce the activity of M-MuLV RT in our experiments. Shinazi et al. found that the 50% inhibitory concentration of a derivatized C_{60} is $\approx$ 5 μM when tested on HIV-1 RT (Schinazi et al. 1993). Our result using nonderivatized C_{60} was similar ($IC_{50} \approx 3$ μM) raising the possibility that the C_{60} skeleton is involved in RT inhibition in the case of functionalized C_{60} molecule as well. This observation will be important as additional information if one of the fullerene molecules acts as a carrier in drug delivery system. The stable Buckyball with its neutral

surface, without disorganization suggests hydrophobic interaction with plastic hydrophobic site(s) of the reverse transcriptase and its membrane affinity can produce antiretroviral action primarily *in vitro* too. In conclusion the antiretroviral effect of nonderivatized C_{60} modulates its perspectives as a drug carrier molecule.

5.6. Potential role of new findings in non-nucleoside reverse transcriptase inhibition of retroviruses

Since the importance of multiple drug combination of anti-HIV therapy has been recognized, the discovery of new antiretroviral compounds has been definitely promoted world-wide. Respecting expenses of the drug production and high adaptability of the evolutionary immature virus the chemotherapeutic intervention is probably not the ultimate solution of the epidemic. However biological ways (e.g. immunization or gene therapy) raise other complicated questions and to answer may need longer term. Therefore during the following years chemotherapeutic agents will probably play main role in the struggle against AIDS and the influence of the pharmaceutical companies - motivated by also complicated economical considerations - remains determinate factor. Among these scientific and social conditions two main angles come to the front in reference to the new drugs against HIV: effectiveness and specificity. These considerations led attention to the registered psychotropic medicines as chlorpromazine or trifluoperazine or to the drug carrier candidate fullerenes. The complex formation with metals can yield totally new features for the phenothiazines so that they keep their tolerability and get higher or different specificity. The new findings related to phenothiazines and new ellipticine analogues mean extension of spectrum of antiretroviral candidates. As the fullerenes are becoming increasingly more interesting effector carrier can be manipulated by drug designers, the additional information on their influence on retrovirus replication are getting important.

6. Summary

The scientific and technical development is accompanied by abuses which create new environment for living beings. This process subserves relatively newly recognized challenging agents such as retroviruses but at the same time provides new biological knowledge and tools such as retrovirus vectors for gene therapy. The human immuno-deficiency virus (HIV), the causative agent of the acquired immuno-deficiency syndrome (AIDS) is the mostly targeted representative of this group because of its world-wide extending and fatal nature. Its replicative cycle needs essential enzymes and other elements and their simultaneous inhibition by multiple drug combination means the most effective therapy currently. Besides the nucleoside analogue reverse transcriptase (RT) and protease inhibitors applied in patients, one of the major groups of drugs investigated also is non nucleoside reverse transcriptase inhibitors. I aimed to study the antiretroviral action of some modified or newly synthesized molecule of such groups which had shown direct or indirect interaction with DNA or more complex effects on some intracellular proteins earlier. Few of the newly prepared phenothiazines and some phenothiazine-metal complexes produced remarkably more expressive anti-RT activity than the parent phenothiazine showed. Their molecular interaction with DNA suggests some possible explanations which are implicit in direct inhibition of enzyme-substrate complex, but the allosteric action cannot be excluded neither. Testing several novel ellipticine analogues, which are members of a molecule group, known inhibitors of other DNA binding enzyme (topoisomerase), two of them showed notable inhibition in RT assay. These results reveal the possibility of significantly increased specificity and effectivity of these compounds modified by further structure-action studies. Synthesis of the fullerene molecules represents a novel approach to be used as drug carriers. Respecting their relative neutrality in biological systems the functional groups of derivatized fullerenes were thought to have inhibitor effect on HIV enzymes. Results of this study clarify that the C_{60} skeleton itself has antiretroviral impact including the RT inhibition on its hydrophobic site. Application of results described above may contribute to the design and development as well as practical application of future against HIV and other retroviruses.

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