Bull Yamaguchi Med Sch 37(3-4) : 169-180, 1990

Development of Anti-human Immunodeficiency Virus (HIV) Agents

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(Received August 8, revised August 16, 1990)

Key Words: Human immunodeficiency virus, Anti-HIV agents, 2',3'-Dideoxynucleosides, Sulfated polysaccharides

Introduction

The unprecedented speed with which the etiologic agent of AIDS, human immunodeficiency virus (HIV), was isolated and characterized, and then effective drugs were discovered is truly remarkable. Discovery of some anti-HIV compounds suggested that antiviral chemotherapy of AIDS was fesible and opened the search for more potent and more selective anti-HIV agents.

HIV belongs to the Retroviridae, a virus family of enveloped RNA viruses which have been known to cause a variety of naturally occuring disorders in several animal species. Retroviruses have been classified according to their type of transmission, with respect to their host range, according to their morphology and budding characteristics, and according to their biological or pathogenic properties[1]. However, all retroviruses possess a unique enzyme, reverse transcriptase (RT, RNA-dependent DNA polymerase), encoded by one of the three typical retroviral genes, the *pol* gene (other two genes are *gag* and *env*).

A few years after the isolation of HIV[2-4], novel retroviruses have been isolated which are genomically and antigenically related each other, but clearly distinct from the prototype HIV[5,6]. Thus, the virus initially known as HIV has been named HIV-1. This form of the virus is primary responsible for the global AIDS epidemic. A later isolate, named HIV-2, is geographically restricted to areas in West-Africa.

This review will focus on the *in vitro* cell-culture systems which are used in our laboratory for the evaluation of anti-HIV substances. The anti-HIV compounds that were investigated by our methods are also described.

Strategy for development of anti-HIV drugs

When developing anti-viral agents for the chemotherapy of AIDS, several events in the replicative cycle of HIV could be considered as targets for chemotherapeutic attack, i.e. virus attachment to the target cells, fusion of the viral envelope with cellular membrane, penetration of the viral capsid into the cells, uncoating of the viral capsid, reverse transcription of HIV-RNA genome to proviral DNA, circulation and integration of the proviral DNA into the cellular genome, transcription of the proviral DNA to mRNA, translation of mRNA to viral precursor proteins, which are then modified by myristylation, proteolytic cleavage and glycosylation, assembly and, finally, budding of the virus particles from the infected cells[7].

Most of these events require the action of the specific proteins encoded by the viral

genome. Virus adsorption requires a specific interaction between HIV glycoprotein gp120 and the cellular CD4 receptor. The reverse transcription of viral RNA to DNA is catalyzed by the virion-associated reverse transcriptase (RT), and the remaining RNA template is degraded by ribonuclease H. The integration of proviral DNA into the cellular genome is carried by another viral enzyme, named integrase or endonuclease. Proteolytic cleavage of the viral precursor proteins is effected by the viral proteinase, *gag* and *gag-pol* gene products are translated as polyproteis, which are subsequently processed by HIV specific proteinase to yield structural proteins of the virus core (p17, p24, p9 and p7), together with essential viral enzymes including the proteinase itself. Furthermore, virus replication is under control of a set of regulatory genes which either stimulate expression of viral proteins (*tat*), increase the infectivity of the virus particle (*vif*) or supress expression of viral proteins (*nef* and *vpu*). Another regulatory protein (*rev*) positively regulates expression of virion proteins but negatively regulates expression of regulatory genes (including the *rev* gene itself)[7] (Fig. 1).

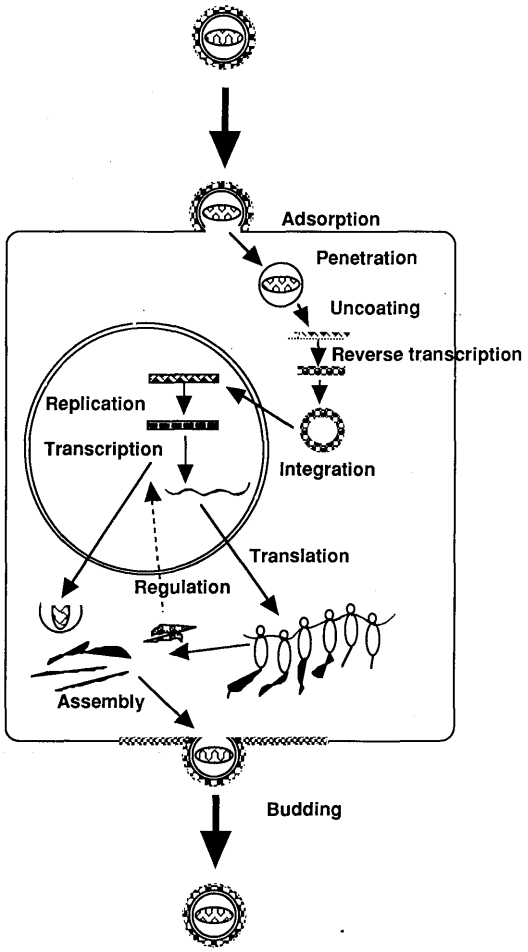

Fig. 1 Replicative cycle of HIV.

In vitro evaluation methods

Virus-cell culture systems, which allow the monitorings of anti-HIV activity of compounds *in vitro*, play a pivotal role in the strategy for intervention against HIV infection. Several factors should be considered in the development of a rational approach; (1) the large numbers of compounds that need to be evaluated; (2) the requirement that potential anti-AIDS drugs inhibit HIV replication, irrespective of the type of the host cell; (3) the necessity that anti-HIV activity be seen at concentrations that do not impair the normal cellular functions; (4) the need to assess all steps of the replicative cycle; and (5) the requirement that *in vitro* assay systems relate as much as possible to the *in vivo* situation.

Various assay methods have been developed for the evaluation of antiviral compounds against HIV, using continuous human CD4-positive T cell lines (e.g. ATH8, H9, CEM, MOLT-4 and MT-4 cells). These target cells differ in their susceptibility to HIV-induced cytopathogenicity as well as the amount of CD4 that they express at the cell surface. CD4 glycoprotein is an essential component of the receptor for HIV[9-11]. Among of these cell lines, we have reported that an HTLV-I transformed cell line, MT-4 cell, has proven to be the most sensitive to HIV infection[12,13]. The cytophathic effects (CPE) induced by HIV infection resulted in complete cell death within 4 to 5 days. HIV replication can be measured by a variety of techniques. For example, the viral antigen expression in the host cell and production of viral particles or proteins that are associated with intact disrupted virions. HIV replica-

tion results in a variety of effects on the host cell. The most apparent of these, are HIV induced CPE. They can be observed microscopically and result from either cell death or gp120-CD4-mediated cell fusion.

If inactive in these primary assays, the compounds were not further evaluated. So far, no compounds have been found active in other cell culture systems when those compounds have no activity in the MT-4 cell system. If selective anti-HIV activity was found in the MT-4 cell system, the compounds were further examined in other T-cell systems (MOLT-4, CEM, freshly isolated peripheral blood lymphocytes) and monocytes/macrophages in order to study their activity in cellular systems which may differ in drug metabolism or sensitivity towards HIV-infection. In these systems antiviral activity was determined by inhibition of HIV antigen expression or by using other parameters, such as HIV antigen (p24) capture ELISA assay[14].

Parameters of HIV replication

1) CPE (Primary screening)

The evaluation parameter for antiviral efficacy in the primary *in vitro* assay was HIV-induced cytopathicity. The protection of virus-infected cells against the HIV-induced CPE on MT-4 cells was initially determined by viability staining techniques such as the trypan blue dye exclusion method. This method is based on the principle that only dead cells become permeable to the polar stain. As the trypan blue dye exclusion method is rather cumbersome when extensive numbers of compounds need to be examined, a more practical assay for the evaluation of anti-HIV agents in MT-4 cells has been developed. The protective effects against the HIV-induced CPE as well as the host cell cytotoxicity of the compounds were monitored by the viability of both HIV- and mock-infected cells which were assessed spectrophotometrically *via* the *in situ* reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2 H-tetrazolium bromide (MTT)[15]. The procedure was optimized by marking use of multichannel pipettes, microprocessor-controlled dispensing and optical density reading.

2) HIV-specific antigens expression (Secondary screening)

In all of the HIV-sensitive cell line, including MT-4 cells, viral antigens are expressed some time after HIV infection. These HIV specific antigens were revealed by an indirect immunofluorescence staining procedure using polyclonal or murine monoclonal antibodies as probes. The antigens were visualized by an immunofluorescence microscope. This assessment of HIV antigen expression was later replaced by laser flow cytometry[16].

3) Plaque assay

We have established a quantitative assay system, a plaque assay, using chemically adhered MT-4 cells[12,17]. This plaque assay is considered to be a highly accurate, sensitive method for the evaluation of anti-HIV compounds.

4) HIV antigen capture ELISA

Permissive cells exposed to HIV release virus particles into the cell culture medium. These virus particles can be either defective or infectious. HIV core protein production (p24), which reflects release of both infectious and defective but mature virus particles, could be quantified by an antigen capture sandwich ELISA. In this assay, microtiter plates coated with HIV antibodies are used. After incubation with the cell culture supernatants containing the HIV antigens, the antigen-antibody complex is revealed by a sandwich technique using a scond antibody-enzyme conjugate. The detection is photometric, using a standard ELISA reader[14].

5) $CD4^+$ HeLa focal immunoassay

Recently, a focal immunoassay using $CD4^+$ HeLa cells has proven suitable for monitoring HIV infection[18] and represents a highly accurate and sensitive system for the evaluation of potent anti-HIV compounds[19].

In our anti-HIV compound evaluation unit, the latter three methods, 3), 4) and 5), are used for confirmatory studies. In addition, the susceptibility of different HIV-1 and HIV-2 strains was determened (Fig. 2).

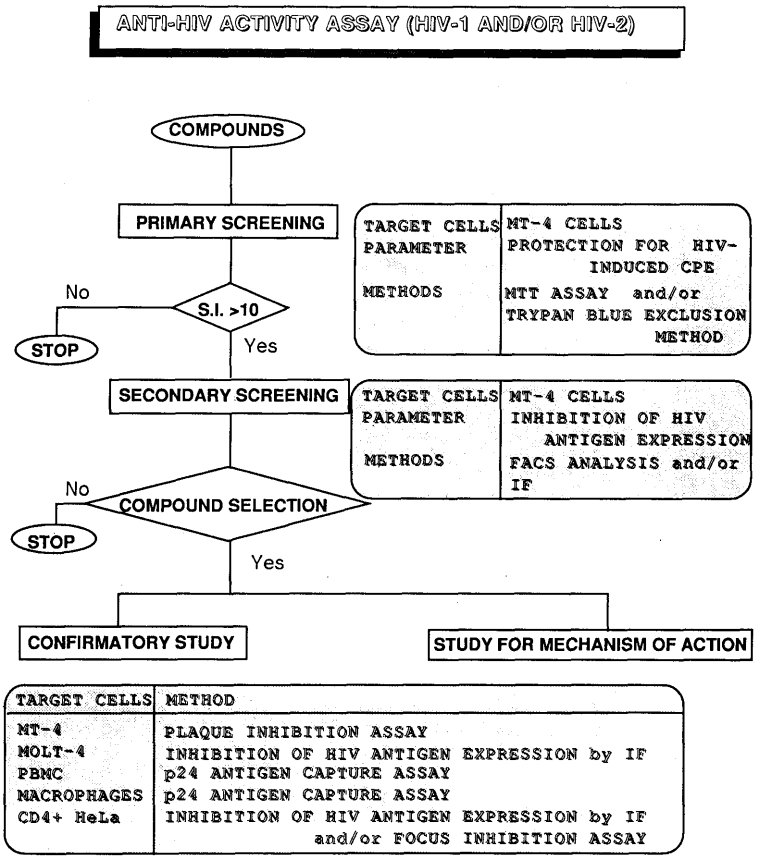

Fig. 2 Schematic flow-diagram of the *in vitro* anti-HIV activity analysing systems.

Mechanism of action for anti-HIV agaents

1) Binding of virion to $CD4^+$ cells

If compounds had anti-HIV activity, studies of the mechanism were carried out in order to identify the stage at which antiviral compounds may interact with the replication cycle of HIV (Fig. 3). The first step in the HIV replicative cycle involves interaction of the HIV envelop glycoprotein (gp120) with the CD4 receptor. We reported a virus binding assay that was based on the radioimmunoassay using anti-HIV serum and ^{125}I-labeled anti-human IgG[20]. This method was devised whereby virion binding to the cell membrane was visualized by an indirect immunofluorescence assay using anti-HIV serum, rabbit anti-human-IgG-F $(ab')_2$-fluorescein isothiocyanate and laser flow cytometry[21].

2) Syncytium (multinuclear giant cell) formation

HIV is tropic and cytopathic for $CD4^+$ cells and can infect these cells either directly following binding of cell-free virus to cells or indirectly by a cell-to-cell fusion process by which multinuclear giant cells are formed. The cell fusion of the HIV-infected cells and uninfected cells depends on a specific interaction between the viral gp120 of the HIV-infected cells and the CD4 receptor of the uninfected cells. This process can be inhibited by polyclonal neutralizing antibody against HIV or anti-CD4 monoclonal antibody.

We have reported that the co-culture of

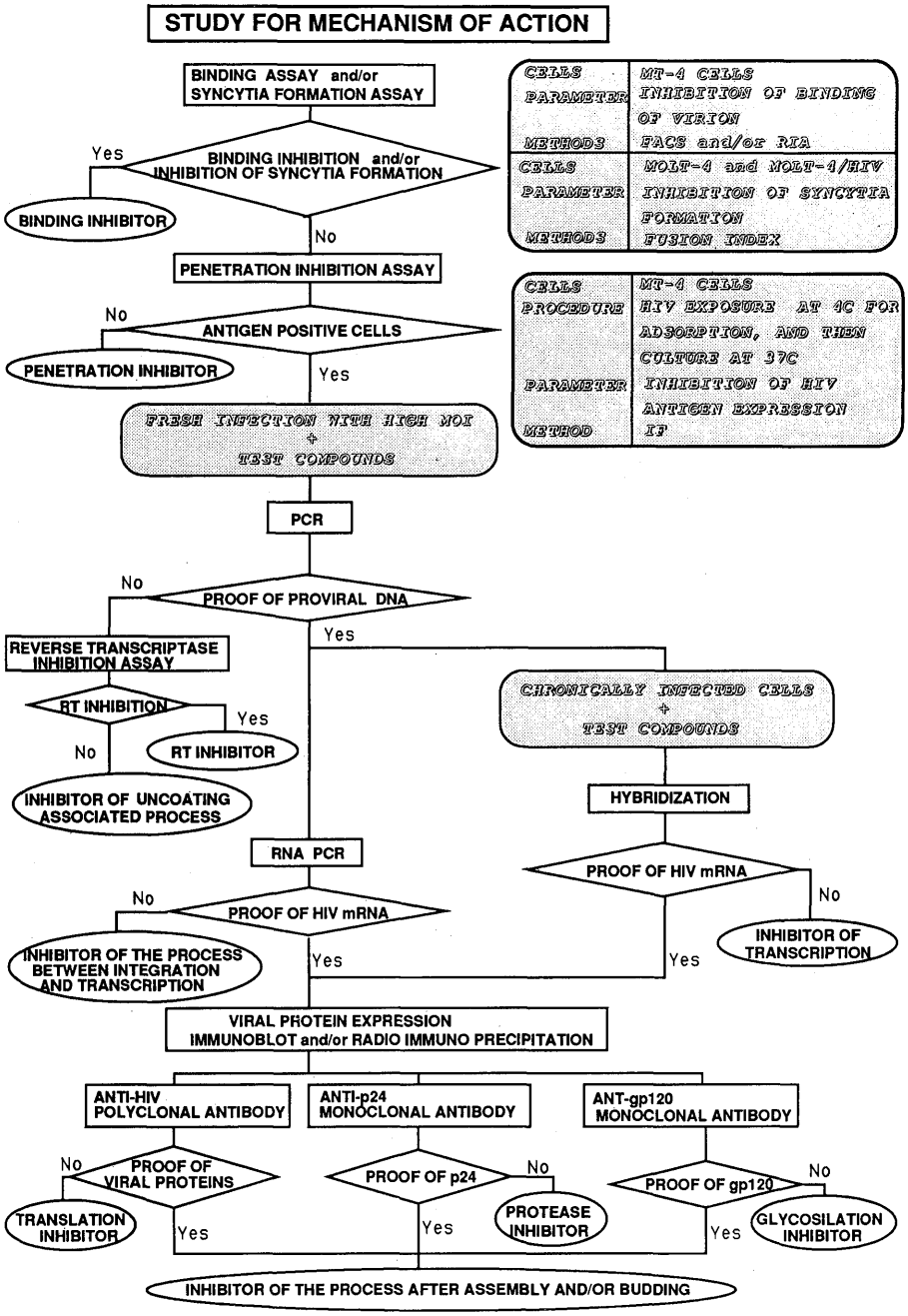

Fig. 3 Schematic flow-diagram of the study fro mechanism of action of anti -HIV compounds.

persistently HIV-infected MOLT-4 cell (MOLT-4/HIV) with uninfected MOLT-4 cells led to cell fusion and formed multinuclear giant cell very effectively[22]. It is considered that this multinuclear giant cell formation occurs *in vivo* and contributes to the dramatic decrease in $CD4^+$ cells observed in AIDS patients. Multinuclear giant cell formation could be readily observed microscopically. In addition, the value of giant cell formation was estimated by fusion index[23] or cell multisizer profile[24], quantitatevely. Compounds which interfere with the virus-cell binding also inhibit this multinuclear giant cell formation.

3) Reverse transcriptase assay

The reverse transcriptase (RT) is a viral encoded polymerase that catalyzes the conversion of the viral RNA into DNA. This enzyme which is present inside the HIV particles, can be adequately detected in cell lysate as well as in the culture medium.

Inhibition of RT activity has been studied with the enzyme derived from partially purified and detergent-treated viral particles as well as RT obtained by recombinant techniques and purified RT from avian myeloblastosis virus. The inhibitory effects of the compounds on RT were performed with exogenous poly(rA) template and oligo $(dT)_{12-18}$ as primer as described elsewhere[25,26].

4) Post-reverse transcription steps of HIV-replicative cycle

After reverse transcription, the proviral DNA is integrated into the cellular genome, and is replicated concomitantly with the replication of cellular DNA. Thereafter, the proviral DNA is transcribed to mRNA, translated to precursor proteins, which are modified by myristylaiton, proteolytic cleavage and glycosylation. The proviral DNA and/or mRNA in HIV infected cells, which were cultured with anti-viral compounds, might be detected by recently established polymerase chain reaction (PCR). In fact, PCR technique is widely used for detection of HIV proviral DNA from peripheral blood lymphocytes of patients with HIV infection.

Immunoblot and radio immunoprecipitation assays are also useful in detecting whether each structural proteins are present. If HIV proteinase activity and/or glycosilation were inhibited by antiviral compounds, the maturation of HIV core proteis, including p24 or envelope glycoproteins, such as gp120 would be interrupted. Thus, the infectivity of progeny virus particles should be reduced.

Promising anti-HIV compounds

1) Dideoxynucleoside analogues

Azidothymidine (3'-azido-2',3'-dideoxythymidine, AZT) was the first among the 2',3'-dideoxynucleoside (ddN) analogues shown to be a potent and selective inhibitor to HIV replication *in vitro*[27,28]. Following AZT, several other ddN analogues, i.e. 2',3'-dideoxycytidine (DDC), 2',3'-dideoxyadenosine (DDA) and 2',3'-dideoxyinosine (DDI), were reported to inhibit HIV[29]. Further search for new ddN analogues has produced a wealth of active compounds, i.e. the 2',3'-didehydro derivatives of dideoxycytidine and dideoxythymidine, named 2',3'-didehydro-2',3'-dideoxycytidine (D4C) and 2',3'-didehydro-2',3'-dideoxythymidine (D4T), respectively[30,31]. All ddN analogues may be assumed to act in a similar fashion as AZT, which means that they must be phosphorylated intracellularly to their 5'-triphosphate derivatives before they can interact with their target enzyme, the HIV-associated reverse transcriptase. As a rule, the ddN triphospates (ddNTPs) have a greater affinity for the HIV RT than for the cellular DNA polymerase alpha, and for HIV RT they also have affinities some 50-fold greater than those of the corresponding 2'-deoxynucleoside 5'-triphosphate (dNTPs)[32]. At the RT level, the ddNTPs may act as either competitive, i.e. prevent the incorporation of the natural substrates (dNTPs), or alternate substrates, and thus be incorporated (as ddNMP) in the growing DNA chain. This incorporation must lead to termination of the DNA chain, since the ddNMPs do not offer the 3'-hydroxyl groups that are necessary for chain elongation.

AZT has so far remained the only drug that is officially licenced for the treatment of AIDS. It is also effective in children with

symptomatic HIV infection, especially those with encephalopathy or dementia[33]. A notorious side effect of AZT is its toxicity for the bone marrow[34]. This is reflected by the development of anemia and other peripheral cytopenias during AZT administration *in vivo*. Following AZT, DDC has also reached clinicla trials, and, while it may be limited as a single agent by the painful peripheral neuropathy that it causes, an alternating DDC-AZT regimen may reduce the toxic side effects of both drugs. In addition to AZT and DDC, clinical studies have also been undertaken with DDA and DDI, and will be initiated with D4T.

2) Sulfated polysaccharides

While we were searching new anti-viral substances, we found that an extract from the marine red alga, *Schizymenia pacifica*, has an inhibitory effect on the RT activity[25]. Following the purification of the active substance form the sea alga, we noticed that several sulfated polysaccharides, i.e. carrageenans and dextran sulfate showed not only RT inhibition but also potent anti-HIV activity, probably due to virus adsorption inhibition[26]. This observation prompted us to design new antiviral compounds against HIV by sulfation of polysaccharides and other substances that per se have no such activity[35].

The anti-HIV activity of the sulfated polysaccharides is critically dependent on the sulfate content: two sulfate groups per monosaccharide are required to accomplish full anti-HIV activity. In addition to the degree of sulfation (or sulfate content), the molecular weight also plays an important role in the anti-HIV activity of sulfated polysaccharides: for dextran sulfate, anti-HIV activity increases with the molecular weight increasing from 1kd to 10 kd and then levels off. With heparin fragments, no anti-HIV activity is noted if the molecular weight of the fragment falls below the 5-kd threshold[36].

The anti-HIV activity of the sulfated polysaccharides resides primarily, if not solely, in the inhibition of virus adsorption to the outer cell membrane. This has been domonstrated by several techniques based on either cell-associated radioactivity following exposure of the cells to radiolabeled HIV virions[36.37], flow cytometric measurements of cell-associated immunofluorescence[21] or radioimmunoassay of cell-bound virus[20]. Additional assets of this class of compounds include inhibitory effects on a wide variety of enveloped virus, including herpes simplex virus type 1 (HSV-1), type 2 (HSV-2) and cytomegalovirus (CMV)[38], and that they also block syncytium (multinuclear giant cell) formation between HIV infected and uninfected cells[35.39]. It is considered that the syncytium formation leads to a specific destruction of the uninfected $CD4^+$ cells, which means that one infected cell can wipe out hundreds if not thousands of innocent bystander cells. As this phenomenon may play an important role in the pathogenesis of AIDS, by the depletion of $CD4^+$ T-lymphocytes, any compound that blocks syncytium formation may be therapeutically advantageous.

3) Acyclouridine derivatives

A 6-substituted acyclouridine derivative, 1-[(2-hydroxy-ethoxy)methyl]-6-phenylthiothymine (HEPT), is unique among the anti-HIV agents in that it is specifically inhibitory to HIV-1: it does not inhibit HIV-2 or any other retrovirus[40]. Although HEPT is an acyclic thymidine (5-methyluridine) analogue, it does not compete with the phosphorylation of [metyhyl-^{3}H]thymidine by MT-4 cell extracts. In fact, HEPT does not need to be converted to its triphosphate to inhibit HIV replication. This suggests that HEPT is not a substrate for thymidine kinase. HEPT does not interfere with an early event (i.e. adsorption, penetration or uncoating) of the virus replication cycle.

Among of many acyclouridine derivatives, some HEPT analogues also proved to be potent and selective inhibitors of HIV-1 replication. From a structure-function relationship point of view, substitution of the phenylthio goup at C-6 of HEPT by a cyclohexylthio goup does not markedly affect anti-HIV-1 activity. However, substitution of the phenylthio group by hydrogen, halogens, or linear carbon chains completely abolishes the anti-HIV-1 activity of HEPT.

It is assumed, therefore, that the three-dimensional conformation of this part of the molecule is an important determinant for anti-HIV-1 activity. When the oxygen at C-2 position is replaced by a sulfur, the compound becomes more active but also more cytotoxic (Baba et al.: in preparation). Based on their unique ability to discriminate between HIV-1 and HIV-2, HEPT and its analogues could be advocated as specific markers to distinguish HIV-1 from HIV-2 in clinical isolates. The reason for this discriminative capacity, as well as the target for the mode of action for the HEPT congeners, remain to be elucidated.

4) Tetrahydro-imidazo[4,5,1-jk][1,4]-benzodiazepin-2(1H)-one and thione (TIBO) derivatives

A novel series tetrahydro-imidazo[4,5,1-jk][1,4]-benzodiazepin-2(1H)-one and thione derivatives (i.e. TIBO derivatives), which are structurally unrelated to any other antiviral agents, such as ddNs, are highly specific and potent inhibitors of HIV-1 replication, but not of HIV-2 or any other DNA or RNA viruses. Replication of HIV-1 was inhibited in different cell culture systems at nanomolar concentrations that were 10,000 to 100,000-fold lower than those that were cytotoxic[41]. This unprecedented specificity may be due to a unique stereospecific interaction with a reverse transcriptase-associated process.

5) Proteinase inhibitors

During the replicative cycle of HIV, *gag* and *gag-pol* gene products are translated as polyproteins. They are subsequently processed by a virally-encoded proteinase to yield structural proteins of the virus core (p17, p24, p9 and p7), together with essential viral enzymes including the proteinase itself. HIV proteinase, which has been classified as an aspartic proteinase, was considered as a potential target for AIDS therapy. Recently, it was reported by several institutes that a series of peptide derivatives based on the transition-state mimetic concept has been designed that inhibit the proteinase from HIV[42-44]. The more active compounds inhibit both HIV-1 and HIV-2 proteinases in the nanomolar range with little effect at 10 micromolar against the structurally related human aspartic proteinases. Proteolytic cleavage of the HIV-1 *gag* polyprotein (p55) to the viral structural protein p24 was inhibited in chronically infected CEM cells. Antiviral activity was also observed in the nanomolar range in different cell-culture systems, as measured by p24 antigen capture and syncytium formation.

6) Soluble CD4 and other HIV binding inhibitors

The interaction between HIV envelope glycoprotein, gp120, and the cellular receptor CD4 is a critical determinant in both cell free virus infection and cell-to-cell syncytium formation. This interaction of the attachment of gp120 with CD4 provides an attractive approach to therapeutic intervention in AIDS. An antibody directed against gp120 could block the site that binds to CD4. Soluble forms of CD4 protein also block the virus binding[45-49]. An anti-idiotypic antibody to gp120 is made by taking a monoclonal antibody against CD4 and forming an antibody to it. Chimeric molecules, which occupy the sites on CD4 and splice them onto the constant region of a human immunoglobulin (antibody) molecule, could be more stable than soluble CD4. It will be considered that certain parts of the so-called heavy chain of the immunoglobulin morecule may be able to activate other parts of the immune system into destroying the virus.

Conclusion

An effective chemotherapy for AIDS is a major task requiring the combined forceds of several disciplines including virology, chemistry, biochemistry, molecular biology, pharmacology, toxicology, clinical medicine, patent law, business, economics and drug regulation. However, the qualities of the ideal anti-HIV agents (i.e. *i* low toxicity, *ii* high specificity for HIV and HIV infected cells, *iii* protects uninfected cells, *iv* penetrates CSF, *v* orally absorbed, *vi* long half-life, *vii* produced at low cost) are difficult to satisfy. In addition several processes are involved in the development of effective anti-HIV drugs: 1) chemi-

cal synthesis of the compounds, 2) evaluation of its *in vitro* activity against HIV, 3) evaluation of its *in vivo* activity against retrovirus infections in animal models, 4) study for mechanism of action, 5) study for its pharmacokinetics (absorption, distribution, metalbolism and clearance), 6) toxicological examination, 7) clinical trials in HIV infection patients.

While AZT is the only anti-AIDS drug that has been licenced for clinical use, several new compounds have been identified which are potent and selective inhibitors of HIV. Among these candidate drugs for the chemotherapy of AIDS, sulfated polysaccharides, dideoxynucleoside analogues, soluble CD4 and its fragments or derivatives are most promising. In addition, proteinase inhibitor and glycosylation inhibitor are also being considered because of their inhibitory activity against HIV. Acyclouridine derivatives and TIBO derivatives are unique compounds because of their specificity against HIV-1, not HIV-2 nor other retroviruses. Additional investigations are required to assess their efficacy. Moreover, we reported the anti-HIV activity of glycyrrhizin[50] and soybean saponins[51]. Glycyrrhizin has been clinically used for allergic disorders and hepatitis. Soybean products have long been a very important source of protein in the diet of oriental people. The *in vivo* bioavailability of those compounds, their absorption, excretion upon systemic administration and side effect/toxicity to individuals require further study.

Testing and considerable compounds against HIV replication are shown in Fig. 4. The true therapeutic potential of these compounds can only be assessed after *in vivo* efficacy and their pharmacokinetic and toxicologic profiles have been established.

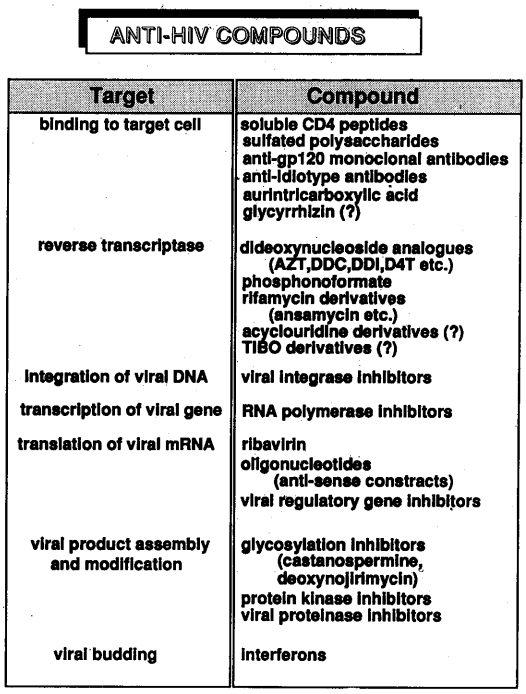

Fig. 4 Anti-HIV compounds. All of the compounds on the list have shown some activity against HIV in the test tube. Many of them are now in various stages of clinical trials.

Acknowledgements

I wish to gratefully acknowledge valuable discussion and revision provided by Professor N. Yamamoto. I also wish to thank Miss N. Fukuda for her editorial assistance.

Originally presented at the Kiso Igaku Club, Yamaguchi University School of Medicine, 27 July 1990.

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