in vivo *23*: 1-6 (2009)

Review

Drug Resistance of Human Immunodeficiency Virus and Overcoming it by Natural Products

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Abstract. *Acquired immune deficiency syndrome (AIDS) caused by infection with the human immunodeficiency virus (HIV) represents a major health problem worldwide, especially in developing countries. Combinational anti-HIV therapy has largely improved patients' lives, but is not widely available in developing countries. Moreover, drug resistance is a main problem during HIV treatment. Therefore, there is a continuous need for new drugs effective against otherwise drug-resistant HIV strains. Chemical compounds from natural sources provide a large variety of potential anti-HIV compounds. The most promising natural products are discussed in this review.*

Even though the number of new human immunodeficiency virus (HIV) infections is decreasing, acquired immune deficiency syndrome (AIDS) is still one of the leading causes of deaths worldwide. In 2007, an estimated 33.2 million people globally were infected with AIDS. The virus represents a major threat in developing countries, where over 95% of all infected people worldwide are living (1). In Africa, AIDS remains the main cause of death. Sub-Saharan Africa is most rigorously affected, with over two-thirds of the population infected with HIV. Even though there is still no cure for AIDS, advances in HIV treatment have improved patients' quality of life and prolonged their lives (2). However, the high cost of treatment is a major problem in developing countries. Additionally, there are several limitations to standard HIV therapy such as drug resistance and dose-limiting side-effects. Therefore, medicinal plants and their chemical constituents with activity against HIV have come into the focus of virus research.

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Key Words: Antiviral agents, chemotherapy, drug resistance, human immunodeficiency virus, natural products, pharmacognosy, review.

HIV and Standard Anti-HIV Therapy

HIV is a lentivirus belonging to the *Retroviridae* family. Its genome contains three major genes: *gag*, *pol* and *env*. The *gag* gene encodes for a Gag precursor protein, which is cleaved by the viral protease into matrix, capsid and nucleocapsid proteins. The *pol* gene encodes viral enzymes, which are initially synthesized as GagPol precursor proteins. The precursor proteins are cleaved by the viral protease resulting in formation of the viral enzymes polymerase, integrase and protease. The envelope glycoprotein (Env) is also first expressed as a precursor polyprotein, which is then cleaved by cellular proteases into the surface protein gp120 and the transmembrane protein gp41 (3).

HIV infects $CD4^+$ T-lymphocytes, resulting in their depletion and in subsequent immunodeficiency. The CD4 molecule was the first and principal retroviral receptor identified (4). Binding of the virus to the CD4 receptor *via* gp120 represents the initial step of host cell infection. However, for membrane fusion and virus entry, additional binding of gp120 to a co-receptor is necessary. M-tropic viruses generally use the CCR5 chemokine co-receptor ("R5" virus), while T-tropic viruses usually bind to the chemokine CXCR4 receptor ("X4" virus). Receptor binding induces a conformational change of the gp120 protein and subsequently a change in the gp41 protein, the latter being responsible for fusion of the virus with the host cell membrane (5). Following membrane fusion, the viral core is released into the host cytoplasm. The virus mRNA is then reverse transcribed into cDNA by the viral enzyme reverse transcriptase (RT). This enzyme also synthesizes the complementary DNA strand and exerts RNAse activity. The double-stranded DNA is translocated into the nucleus, where its integration into the host genome is catalyzed by the viral enzyme integrase (IN) (6). Inside the host genome, the so-called provirus can remain latent for a long period of time. Once transcribed, both spliced and

unspliced RNAs are generated and transported into the cell cytoplasm. After synthesis of viral proteins, virus particles assemble at the host cell membrane. This process is mainly regulated by the Gag precursor proteins. Eventually, the virus particle is released by budding of the cell (7). In order to be infectious, additional maturation of the virus is necessary. During this process, which takes place during or shortly after release of the virus (3), the Gag and GagPol precursor proteins are cleaved into the mature Gag and Pol proteins by the viral protease.

Anti-HIV drugs target the different steps of the virus life cycle. Currently 24 drugs for HIV treatment are approved by the Food and Drug Administration (FDA) of the U.S.A. They can be classified into four different groups: fusion or entry inhibitors (FI), reverse transcriptase inhibitors, integrase inhibitors (IN), and protease inhibitors (PI) (Table I). Current anti-HIV protocols are fairly individualized, based on the patient's medical history, $CD4^+$ T-cell count, viral load and resistance assays (8). During standard HIV therapy, which is also referred to as HAART (highly active antiretroviral therapy), a combination of three or more drugs is administered. This combinational therapy reduces the emergence of resistant virus particles, which represents a major problem in anti-HIV therapy.

The group of nucleoside analogue reverse transcriptase (RT) inhibitors (NRTIs) is composed of various nucleoside analogs. Inside the host cell, these prodrugs need to be metabolically activated by phosphorylation. They inhibit the activity of RT by termination of chain elongation. Non-nucleoside reverse transcriptase inhibitors (NNRTIs) do not need metabolic activation. They inhibit RT in a non-competitive manner by binding to the enzyme. Most PIs are administered in combination with ritonavir, which boosts the effect of other PIs by inhibiting the cytochrome P450 (CYP) 3A4 isoenzyme (8). As CYP 3A4 metabolizes PIs, co-administration of ritonavir allows reduced dosing of these PIs.

The FIs either prevent formation of the entry pore by binding to gp41 (enfuvirtide) (9) or block the CCR5 co-receptor (maraviroc) (10). The latter drug is only active against R5 viruses as X4 viruses bind to the CXCR4 co-receptor. The first IN inhibitor, raltegravir, was only recently approved by the FDA in October 2007 (11).

Table I. *Anti-HIV drugs currently approved by the Food and Drug Administration (FDA) of the U.S.A. Anti-HIV drugs can be grouped into four different inhibitor classes. The largest classes are reverse transcriptase inhibitors and protease inhibitors, while only two entry inhibitors and one integrase inhibitor are currently FDA-approved.*

Reverse transcriptase		Protease	Entry	Integrase
NRTI	NNRTI			
Abacavir	Delavirdine	Amprenavir	Enfuvirtide	Raltegravir
Didanosine	Efavirenz	Atazanavir	Maraviroc	
Emtricitabine	Etravirine	Darunavir		
Lamivudine	Nevirapine	Fosamprenavir		
Stavudine		Indinavir		
Tenofovir		Lopinavir		
Zidovudine		Nelvinavir		
		Ritonavir*		
		Saquinavir[1]		
		Tipranavir		

NRTI: Nucleoside analogue reverse transcriptase inhibitors; NNRTI: non-nucleoside reverse transcriptase inhibitors. *Used in combination with other protease inhibitors to boost their effect; [1]no longer marketed.

Drug Resistance

Drug-resistant mutations arise and accumulate frequently in the HIV genome. This is mainly due to two reasons: (a) the lack of proof-reading activity of the RT, resulting in a high mutation rate during virus replication (about 3.4×10^{-5} mutations per bp per replication cycle) (12) and (b) the high replication rate of HIV (13). All current anti-HIV treatments eventually lead to drug-resistant mutants, which is one of the main reasons for therapy failure (14). As drug resistance is a major problem in anti-HIV therapy, mutations leading to resistance have been widely investigated and mutation profiles for the currently approved drugs have been developed. For a comprehensive review of mutation profiles, the reader is referred to Johnson *et al*. (15). Moreover, cross resistance to drugs of the same class is a major problem, as illustrated by thymidine analogue-associated mutations (TAMs). TAMs are selected by thymidine anaolgues such as zidovudine and stavudine and lead to cross-resistance to all currently approved NRTIs (16, 17). In NNRTI-associated mutations, cross-resistance to drugs of the same class frequently emerges by the simple occurrence of only one mutation at codon 103 or 166, respectively.

Three or more mutations are often required for PI-associated resistance depending on the drug used. Resistance to FIs is caused mainly by mutations in the *env* gene. Depending on the drug target, these mutations are primarily but not exclusively found in the *gp41* gene (enfuvirtide) or the *gp120* gene (maraviroc).

Mutations in genes of two pathways have been identified as mediating resistance towards the IN inhibitor, raltegravir. Gene mutations of each pathway comprise at least one mutation in the integrase gene.

Considering the significant problem of drug resistance during anti-HIV therapy, there is a continuing interest in finding new agents which are not only well tolerated, convenient and less expensive, but which are also active against resistant virus strains and do not show cross resistance to other drugs.

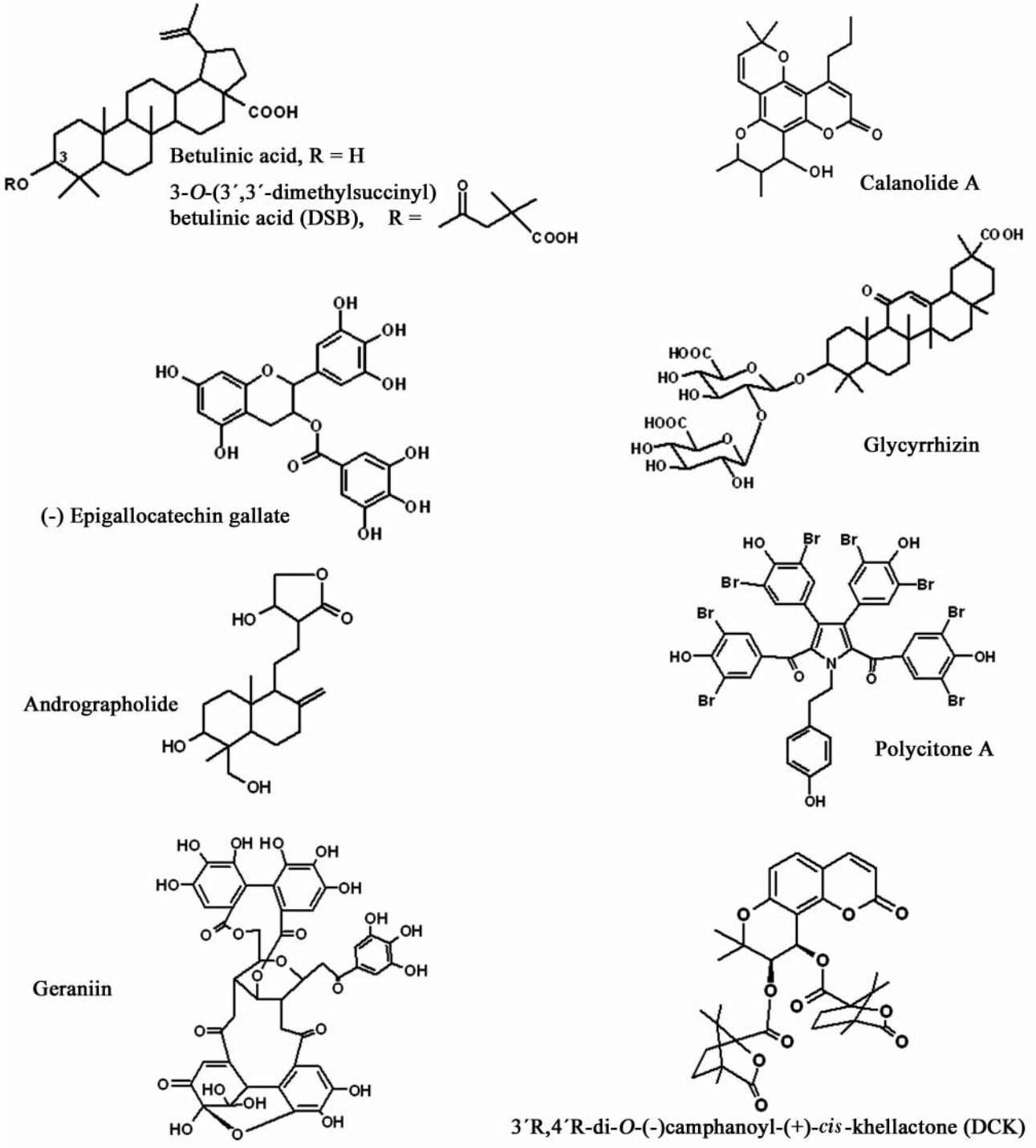

Figure 1. *Chemical structures of anti-HIV natural compounds.*

Natural Products

A vast number of natural products from medicinal plants such as terpenoids, coumarins, alkaloids, polyphenols, tannins and flavonoids, have been shown to possess anti-HIV activity. Some of these natural products have already entered clinical trials. In this review, we focused on the most promising small molecules (Figure 1); macromolecules, such as proteins and polysaccharides, were not considered. Criteria for inclusion of natural products or their derivates were: (a) an effective concentration $(EC)_{50}$ value below 1 μM and known

mechanism of action and/or (b) promising *in vivo* activity or clinical trial data. For further details, the reader is referred to several recent reviews (18-21).

Betulinic acid (**1**) is a triterpene isolated from *Syzygium claviflorum* and many other plants. Betulinic acid was shown to inhibit HIV replication *in vitro* with an EC_{50} of 1.4 μM (22). Modifications of betulinic acid lead to even more potent derivatives. Structural modifications at the C3 position of betulinic acid showed that an ester group at this position is important for anti-HIV activity (21). The most promising of these derivates, 3-*O*-(3'3'-dimethylsuccinyl)betulinic acid (DSB, bevirimat, PA-457) exhibits remarkable anti-HIV activity with an EC_{50} value of 0.35 nM (21). Bevirimat also showed promising reduction of viral load and a promising safety profile in several clinical studies (23). A double-blind, randomized phase IIa study showed significant reduction of the viral load in HIV-infected patients treated with bevirimat compared to a placebo group when administered orally (in solution) and daily for ten days at a dose of 100 or 200 mg. Viral load decreased by about 90% . Preliminary results of a currently ongoing phase IIb study also showed a significant overall viral load reduction in drug-resistant patients failing standard HIV therapy. However, reduction of viral load was less than expected. A reduction of virus load was observed in bevirimat-treated patients by application of a daily tablet dose of 400 mg compared to placebo. The bevirimat plasma concentrations were also lower than expected, which may be due to galenic properties of the tablets used (23).

Anti-HIV activity of bevirimat is mediated through maturation inhibition. Maturation of the virus involves cleavage of the Gag and GagPol precursor proteins into the mature Gag proteins and the viral enzymes. Bevirimat was shown to block cleavage of the capsid protein precursor (p25) into the mature capsid protein (p24). This is a unique mechanism of action compared to any other approved HIV drug so far. This might explain why bevirimat is also active against strains which are resistant to other currently approved drugs (23, 24).

Various calanolides, isolated from *Calophyllum langerium* reveal anti-HIV-1 activity (25). The most promising compound, calanolide A (**2**), is an NNRTI with EC_{50} values between 0.02 and 0.5 μM. In contrast to currently approved NNRTIs, which inhibit RT non-competitively, calanolide A's mechanism of RT inhibition is at least partly competitive (26). Calanolide A interacts with RT at two sites: the active site and presumably the pyrophosphate-binding site of the enzyme (27). The compound is active against a broad range of HIV-1 strains, including otherwise drug-resistant variants (28). Phase Ia clinical trials with calanolide A have been performed and have shown the compound to be safe and well tolerated at oral doses between 200 and 800 mg (28). A phase Ib study in HIV-infected, therapy-naive patients showed a significant mean viral load reduction with oral dosage of 600 mg calanolide A *bid* (twice daily) (29). Phase II studies are ongoing (21). Making it an even more interesting candidate for therapeutic use, calanolide A also showed synergistic effects with other anti-HIV drugs (30).

(–)Epigallocatechin gallate (EGCG, **3**) is a flavonoid isolated from the green tea plant *Camellia sinensis*. EGCG inhibits several steps in the HIV life cycle such as post-adsorption entry and protease kinetics (31). Most remarkable is its IC_{50} value of 0.01-0.02 μg/ml for inhibition of the viral RT (32). Moreover, EGCG binds with high affinity to $CD4^+$ T-cells and inhibits binding of gp120 to the $CD4^+$ cells at a physiologically relevant concentration of 0.2 μM (33). Besides antiviral activities, EGCG also has antitumor promoting, antiinflammatory and antioxidative activities (31).

Glycyrrhizin (GL, **4**), a terpenoid from the roots of *Glycyrrhiza glabra*, improved the $CD4^+$ T-cell count in AIDS patients (34). The $CD4^+$ T-cell count was significantly higher (243.6/l) in a group treated with glycyrrhizin and HAART compared to a group of patients receiving HAART only (170.8), *In vitro*, GL had an IC_{50} of 0.15 mM (18). Its action seems to be mediated through inhibition of virus binding to the host cell and inhibition of protein kinase C (PKC) (35). Additionally, GL has affinity to HIV surface proteins (36).

Andrographolide (**5**) is a diterpenoid lactone isolated from *Andrographis paniculata*. In a phase I clinical trial, andrographolide caused a significant rise in $CD4^+$ T-cells from 405 cells/ml to 501 cells/ml in HIV-positive patients at a dosage of 10 mg/kg (7). However, the viral load did not decline significantly in patients and the trial was discontinued due to severe side-effects. Side-effects such as headache, fatigue and altered sense of taste were mild to moderate, but one HIV-positive individual developed an anaphylactic reaction during the clinical trial. Andrographolide probably acts through inhibition of the dysregulation of the cell cycle in HIV infected cells.

Polycitone A (**6**), an aromatic alkaloid isolated from marine *Polycitor* sp., inhibits RT of wild-type and some resistant HIV-1 strains as well as of HIV-2 strains. Polycitone A has a low IC_{50} of 245 nM for RNA-directed DNA polymerase function of RT and an IC_{50} of 470 nM for DNA-directed DNA-polymerase function. The mechanism for inhibition of DNA-polymerase activity of HIV-1 RT has been investigated (38). These studies proposed polycitone A to be an allosteric RT inhibitor which interferes with DNA primer extension and also inhibits formation of the RT-DNA complex. Notably, polycitone A has five hydroxyl groups, which seem to be important for its inhibitory activity. Inhibition of the RT-DNA complex formation is an anti-HIV mechanism not used by any currently approved drug. Therefore, polycitone A is an interesting model compound for drug development.

Geraniin (**7**) was isolated from *Phyllanthus amarus*. Geraniin inhibits HIV-1 replication in MT-4 cells (EC_{50}=0.22 μM). This natural product acts through two

different mechanisms. First, it acts as a competitive RT inhibitor. Second, it inhibits viral uptake. This makes geraniin interesting for therapeutic use, since inhibiting the viral life cycle at two different sites presumably slows down the emergence of resistant mutants. Additionally, geraniin has also been reported to inhibit several viral strains resistant to currently approved NRTIs and NNRTIs (39).

3'R,4'R-di-*O*-(–)camphanoyl-(+)-*cis*-khellactone (DCK) (**8**), a derivative of suksdorfin, a pyranocoumarin isolated from *Lomatium suksdorfii,* demonstrated promising anti-HIV activity *in vitro* (EC_{50} of 0.049 μM) (40). Several modifications of DCK were performed to further increase potency and bioavailability (40). *In silico* studies, investigating the binding mode of DCK analogs, suggested a mechanism of action that is similar to other RT inhibitors (41). Previous studies, however, did not show inhibition of viral enzymes or RT and proposed that DCK inhibits HIV at a point after virus entry and before integration into the host DNA (40).

Conclusion

The swift emergence of resistant mutants, an enormous problem during HIV treatment, limits the use of currently approved drugs and continuously calls for new anti-HIV drugs. Access to conventional drugs is rather limited in developing countries, even though a vast majority of HIV-infected people live there. Natural products and their derivatives provide an excellent source for new anti-HIV drugs. Since many natural products are present in medicinal plants, which can be abundantly found in developing countries, a straightforward idea might be to propose administration of natural anti-HIV products in forms of tea or by direct consumption of the plant by patients in these countries. However, there are several limitations to this kind of application. First, concentration of the compound may not be high enough for its anti-HIV activity in a tea extract or even in the plant itself. Second, bioavailability may not be sufficient after oral uptake and serum concentrations may be too low for anti-viral effects. Remarkably, suboptimal concentrations can facilitate the emergence of drug-resistant virus strains, which should be avoided under any circumstances. Moreover, some of the most promising compounds are derivatives of natural products. Hence chemical modifications are sometimes necessary for improved antiviral activity.

Out of the innumerable compounds reported in the literature as revealing anti-HIV activity, this review points out the most promising natural products, some of which show unique ways of action. A few of them have already been investigated in clinical trials. The vast majority of natural compounds have, however, only been analyzed *in vitro* leaving the treasure of potential novel HIV drugs from natural origin to be unearthed in the future.

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Received September 12, 2008
Revised December 4, 2008
Accepted December 8, 2008