SCREENING OF NATURAL PRODUCTS AS HIV-1 AND HIV-2 REVERSE TRANSCRIPTASE (RT) INHIBITORS

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INTRODUCTION

The human immunodeficiency virus type-1 (HIV-1) is the etiologic agent for acquired immunodeficiency syndrome (AIDS)[1], which has reached pandemic proportions in recent years. A second immunodeficiency virus (designated HIV-2), which is serologically distinct from HIV-1, was later isolated from West African patients with AIDS[2,3]. Research efforts have intensified in an attempt to discover new therapeutic modalities for the treatment of this fatal disease. The complex replication process of the HIV in its host cell offers many potential target points for therapeutic intervention[4,5]. Examples include viral attachment and uncoating, reverse transcription of the viral genome, proviral DNA integration into the host cell DNA and subsequent transcription and translation of this genetic material into viral proteins. As these viral proteins in turn undergo processing, e.g., protein glycosylation, which then leads to the assembly and release of progeny virus particles, additional target points are obvious.

Emphasis will be placed on the HIV reverse transcriptase (RT) enzyme since it is specific and essential for the replication and infectivity of retroviruses including the HIV. The viral RT has no counterpart in the normal cell, and is hence unique to the virus. Since viral replication is essential for the progression of AIDS, HIV RT represents one of the most important targets for the development of selective anti-HIV compounds. Chemotherapeutic agents designed to inhibit the function of retroviral RT currently comprise the only entities used for the treatment of AIDS[5].

Pitfalls of current antiviral chemotherapy include the toxicity of agents like azidothymidine (AZT) and other nucleoside analogs, in addition to the emergence of viral resistance to AZT[5]. Most reverse transcriptase inhibitors also inhibit cellular DNA polymerases and the non-discriminatory nature of such agents accounts for their toxicity[6,7]. Hence, the discovery and characterization of agents capable of specifically inhibiting the HIV RT without mediating a toxic response remain a high priority. In addition, novel inhibitors of this enzyme may function by unique mechanisms of action.

Natural products represent a rich and largely untapped source of structurally novel chemicals which are worth investigating as specific inhibitors of HIV RT. As yet, very few natural products have been shown to inhibit HIV RT specifically. This chapter summarizes

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progress in the development of a bioassay and the screening of natural products for potent inhibitors of HIV-1 and HIV-2 RT. This includes the establishment of a protocol and definitions of concentration limits for activity, solvent selection, application of the bioassay to a bioactivity-guided fractionation procedure, and the development of strategies involved in the general screening of plant extracts. The sensitivity and selectivity of this bioassay procedure will also be described. In addition, various structural classes of HIV-1 and HIV-2 RT inhibitors will be presented, with a brief discussion on their possible mechanisms of action.

HIV-1 AND HIV-2: A COMPARISON

HIV-2 is similar to HIV-1 in morphology, ultrastructure and genomic organization, with both possessing homologous structural and replicative proteins including the RT. Biological properties are also conserved in these cytopathic viruses in that they selectively infect and kill OKT4$^+$ helper cells resulting in immune suppression. However, genetic comparison of HIV-1 and HIV-2 has revealed significant sequence divergence at both nucleotide and amino acid levels[3]. The RTs of HIV-1 and HIV-2 also show significant sequence divergence, having approximately 60% of their amino acids in common. Nevertheless, the DNA polymerase activities of both enzymes share similar biochemical properties such as ion and template-primer preferences[8,9]. Genetic analyses also suggest that the model previously proposed for HIV-1 RT[10] may not strictly apply to HIV-2 RT[11].

Given the limited amount of sequence homology, it is important to identify and compare the biological properties of these two enzymes. Preliminary studies have shown that despite differences at the molecular level, the HIV RTs are functionally similar[11]. It is critical from the viewpoint of AIDS therapy to determine if this functional similarity extends to the susceptibility of these enzymes to inhibitors. The total conservation of biological and functional properties would ultimately imply that strategies for HIV-1 antiviral therapy may be of value in treating diseases associated with HIV-2. Additionally, such a comparison could provide important information on the structure of both enzymes. In order to probe the structure and activity of these enzymes, it is appropriate to evaluate the effect of a diverse group of compounds. We have recently performed such a study with a variety of natural products. It was found that the two HIV-associated RTs do show small but significant differences in their sensitivities to certain natural product inhibitors[12].

STRUCTURE AND FUNCTION OF HIV-1 AND HIV-2 RT

RT converts the RNA genome of the virus into a duplex DNA intermediate that can be integrated into the genome of the host cell. This proviral DNA is then transcribed leading to the production of viral proteins. HIV RTs are multifunctional, as are other retroviral RTs. A single protein possesses DNA polymerase activity that can transcribe utilizing either RNA or DNA templates. In contrast, the ribonuclease H (RNase H) activity is capable of degrading RNA only when it is part of an RNA - DNA heteroduplex[13]. All these domain-mediated functional activities of the enzymes provide reasonable targets for the action of anti-HIV drugs.

Structural analysis has identified proteins of M_r 66 000 (p66) and 51 000 (p51) as the HIV-1 RT[14]. HIV-2 RT is also a heterodimer, consisting of M_r 68 000 (p68) and 55 000 (p55) proteins. Amino acid sequence analysis has shown that the p51 subunit of HIV-1 RT is generated by the carboxy-terminal processing of p66; the two subunits have a common sequence at the amino terminus[15]. By analogy, the p55 subunit of the HIV-2 RT is proposed to be a product of processive cleavage of the p68 subunit. Sequence homology studies also revealed that all retroviral RTs (including the HIV-1 RT) have the DNA polymerase domain as the amino-terminal portion of the molecule and the RNase H as the carboxy-terminal portion. The same was found with HIV-2 RT[11].

MECHANISMS OF ACTION OF RT INHIBITORS

RT inhibitors may function by several mechanisms of action. Substrate or template-primer analogs of RTs act by mimicking the natural nucleotides and nucleic acids with which the enzyme interacts. AZT and the 2',3'-dideoxynucleosides[16] such as ddI are substrate analogs of HIV RT, while template-primer analogs that inhibit RT activity include 5-mercapto-polycytidylic acid[17]. Phosphonoformate and other pyrophosphate[18] derivatives constitute product analogs of RTs. Template-primer binding agents such as the benzophenanthridine alkaloids[19] are thought to intercalate with nucleic acids and, hence, interfere with their template properties. Agents which bind metal ions, such as the thiosemicarbazones, either deplete the system of divalent cations necessary for enzyme activity, or they may interact with the metal ion which is complexed with the enzyme[20].

Compounds which bind selectively to the active site of the enzyme should yield useful responses. Unfortunately, such agents are not frequently encountered. Compounds that bind to proteins in a relatively nonspecific fashion such as polyphenolic compounds[21] are observed to have more generalized effects on a variety of enzyme systems. This property often contributes significantly to the toxicity of such compounds.

Similarly, compounds that exert their inhibitory action by complexing with synthetic template-primers are typically not very specific for the RT, and may be cytotoxic. The RNA genome of the virus (70S RNA), though a novel feature of retroviruses, does not appear to offer any uniqueness from a physical and chemical standpoint that would distinguish it from cellular nucleic acids. However, approaches based on specificity (such as antisense oligonucleotides) offer promise[22].

SCREENING OF NATURAL PRODUCTS FOR HIV-1 AND HIV-2 RT INHIBITORY ACTIVITY

The HIV RT Assay

The assay developed for the detection of RT activity in virions[23], involving polyadenylic acid [poly (rA)], oligodeoxythymidylic acid [oligo (dT)], and radiolabeled thymidine triphosphate ([^{3}H]TTP), has been adopted in our laboratory as a simple method for screening the HIV-1 and HIV-2 RT inhibitory potential of natural products[12,24]. Although the optimal conditions for the assay of HIV-2 RT have not been systematically determined per se, identical assay conditions for both enzymes yielded good rates of substrate incorporation. This is expected because of the conservation of biological properties in both enzymes.

HIV-1 And HIV-2 RT

The efficient expression of enzymatically active HIV-1[25] and HIV-2 RT[26] in bacterial systems has made available large quantities of the enzymes for biochemical, genetic, immunological and proteolytic analyses. The 66 kDa polypeptide of HIV-1 RT, when expressed in *Escherichia coli*, has biochemical properties that closely resemble the dimeric p66/p51 form of the enzyme purified from virions[25]. Furthermore, the p66 enzyme was shown to be active in RT assays and exhibit inhibitory properties with several known antiretroviral agents (e.g., AZT and suramin) that are indistinguishable from the viral enzyme[27]. The purified recombinant enzyme was sufficiently similar to the viral enzyme that it can be substituted for the latter in drug screening assays[28]. Hence, the intercomparison of data obtained with monomeric and dimeric forms of HIV-1 and HIV-2 RT is not expected to commonly cause any discrepancies.

Solvent Selection

Adaptation of the RT assay for the evaluation of natural products necessitates the

selection of appropriate solvents. Various solvents have been tested and found to exert different degrees of inhibitory effect on the polymerization reaction. In general, the monomeric forms of the RTs were found to be more susceptible to solvent inhibition than the dimeric forms, possibly due to the greater stability of the latter. This, however, does not influence the inhibitory potential of compounds and extracts determined with the system since the solvent effect is monitored as the negative control. DMSO at a final concentration of 10% (v/v) is well-tolerated in systems incorporating either structural form of HIV-RT. At this concentration, only slight inhibition of enzyme activity was noted. Satisfactory dissolution of test compounds and extracts may be expected, except for those which are very nonpolar in nature. The aqueous nature of the enzyme reaction mixture also precludes the use of nonpolar organic solvents and hence the testing of nonpolar compounds. Certain compounds which appear to dissolve completely in DMSO may precipitate when added to the final reaction mixture. A lower concentration of compound may be tested in these cases. Methanol and ethanol at final concentrations of 10% (v/v) are poorly tolerated resulting in approximately 55% and 100% inhibition of monomeric HIV-1 RT activity, respectively[24]. Much lower concentrations of these solvents [e.g., 2% (v/v)] may be used when DMSO is not suitable.

Evaluation of Experimental Parameters

The most fundamental and yet crucial part of the development of the HIV-RT assay involves the evaluation of experimental parameters. Attempts should be made to optimize all assay conditions with respect to the ratios and concentrations of template-primers, monovalent and divalent ion concentrations, and pH of the reaction buffer. The concentration of reagents used (especially that of the enzyme, template-primer and substrate) will determine the catalytic efficiency of the enzyme, and ultimately its susceptibility to inhibition by agents with varied mechanisms of action. For example, the use of saturating enzyme, template-primer or substrate concentrations may attenuate the inhibitory effect of compounds acting competitively with respect to the enzyme, template-primer or substrate, respectively. On the other hand, when higher template-primer concentrations are used, greater inhibition values will be obtained for a compound that acts by an uncompetitive mode of action with respect to the template-primer. Hence, it is clear that the inhibitory potency of a particular agent depends not only on its concentration, structure and mode of action, but also on the assay conditions.

In addition, the concentration of RT, template-primer and substrate must be suitable from a kinetic viewpoint. Typical kinetic information to be examined includes the time-course profile and the saturation curves for the template-primer, substrate and enzyme[24]. Substrate incorporation should increase linearly with time for the duration of the assay and, in general, due to the aforementioned reasons, subsaturation conditions should be employed preferably. Thus, the standardization of experimental conditions is especially important when the inhibitory potential of a single agent is to be compared in systems incorporating two different enzymes. Wherever possible, the effects of inhibitors should be evaluated at the same region on the saturation curves of the two enzymes in an attempt to standardize the polymerization activity of each system and hence their susceptibility to the inhibitors.

A question frequently raised concerning the *in vitro* evaluation of compounds as potential antiviral agents is the relevance of the template-primer employed in the enzyme assay. As a prerequisite, the template-primer used should be sensitive in order to achieve good substrate incorporation and hence, a system perceptive towards the presence of inhibitory compounds. However, inhibitors of nucleic acid-polymerizing enzymes frequently demonstrate template specificity. Such is the case with fagaronine chloride[29] and *O*-methylpsychotrine sulfate heptahydrate[30], which have a preference for A:T (adenine:thymine) rich templates. Ideally, the RNA genome of the virus in question should be used for assessing the RT inhibitory potential of prospective antiviral agents. This is often not practical. However, since the genomes of the HIV-1[31] and HIV-2[3] are A:T rich, the use of $(rA)_n \cdot (dT)_n$ as a sensitive template-primer in the

screen may be considered appropriate and of physiological relevance. Other template-primers such as $(rC)_n \bullet (dG)_n$ may be examined during the mechanistic analysis of active inhibitors.

Plant constituents which bind nonspecifically to proteins (e.g., polyphenolic compounds) may be frequently encountered during a screening procedure. The inclusion of bovine serum albumin (BSA) as a protective agent in an enzyme assay is optional. Its presence, however, will decrease the inhibitory activity of these compounds toward the enzyme of interest, and thus, serves as an effective means of screening out interfering substances. It is also worthwhile to optimize the concentration of BSA in the assay from the viewpoint of enzyme activity.

Since HIV-1 RT demonstrates a preference for Mg^{2+} over Mn^{2+} ions for most template-primers[23], the former is normally used in an assay of this enzyme activity. However, on occasion the type of divalent ion used in the RT assay has been found to have significant effects on the inhibitory activity demonstrated by certain compounds[32].

Assay Protocol and Strategies for Screening

A strategic and economical method for conducting the assay that results in highest efficiency involves the prescreen of only one concentration of pure compound or plant extract at a concentration of 200 μg/ml[24]. A dose-response is then obtained for active compounds and extracts by testing at least five concentrations in duplicate. The IC_{50} values (concentration giving 50% inhibition of enzyme activity) may be calculated from linearly regressed dose-response plots of percent control activity versus concentration or log concentration of compound or extract. Any RT inhibitor [e.g., fagaronine chloride, IC_{50} = 5 μg/ml (13 μM)[33]] may be used as the positive control substance. Negative control assays are performed without the compounds or extracts, but an equivalent amount of solvent is added.

Assay Sensitivity

The HIV-1 RT assay developed was found to be equally sensitive in detecting selected positive control inhibitors (e.g., fagaronine chloride) in the absence and presence of up to 200 μg/ml of an arbitrarily chosen plant extract, which had previously been determined to be inactive in the HIV-1 RT system[24]. The lower limit of detection was found to be 0.01% w/w fagaronine chloride in the plant extract.

Concentration Limits for Activity

Inhibition data generated from different laboratories are difficult to compare critically due to variations in assay conditions and test protocols used. Therefore, a certain compound may appear significantly active in one assay while relatively inactive in another. Such inconsistencies necessitate the standardization of an enzyme assay when it is applied to natural product screening. Similarly, activity cut-offs should be individually tailored for each enzyme assay protocol. Thresholds for activity can usually be determined after a preliminary round of screening involving a few hundred natural product entities. Consequently, for the HIV-1 RT screen, the following thresholds were established. After the screening of approximately 200 pure compounds it was deduced that a pure natural product may be classified as active (IC_{50} < 50 μg/ml), moderately active (50 μg/ml < IC_{50} < 150 μg/ml) or weakly active (150 μg/ml < IC_{50} < 200 μg/ml)[24]. During the prescreen, however, compounds demonstrating 50-100% inhibition at 200 μg/ml were selected for further activity evaluation.

Screening of Plant Extracts

The screening of crude extracts may result in a significant proportion of actives (>50% inhibition at 200 μg/ml). A response of this magnitude is indicative of the presence of high

concentrations of potent inhibitory compounds in plant extracts. Frequently, this may be ascribed to polyphenolic compounds which are abundant in the plant kingdom, and which have been reported to inhibit RTs from RNA tumor viruses[34]. While certain tannins have HIV RT inhibitory activity, in our laboratory non-tannin inhibitory compounds are of primary interest. Tannin-precipitating reagents offer a quick and convenient way to verify the presence of tannins. A dark blue to brown solution with or without a precipitate results with the use of ferric chloride, whereas a gelatin/sodium chloride mixture gives a white precipitate in the presence of tannins. Relative to tannins, most non-polyphenolic inhibitory compounds are present in much lower concentrations in plant extracts. It was observed that when potent HIV RT inhibitors such as fagaronine chloride or nitidine chloride were tested at concentrations between 0.002 and 0.4 μg/ml in the presence of an inert extract (simulating a 0.001 - 0.2% composition in a 200 μg/ml plant extract), only 15 to 20% inhibition was obtained[24]. Thus, in the absence of tannins, responses of this magnitude must be considered significant. However, because it is generally not known from the outset whether the inhibition mediated by a plant extract is due to tannins or other substances, chromatographic fractionation of active extracts is required, and inhibition obtained with concentrated fractions will serve as a better indication of the presence of non-tannin inhibitory compounds. Alternatively, tannins can be removed prior to testing of the extract in the preliminary screen.

Evaluation of Methods for Tannin Removal

Due to the interfering nature of polyphenolics, it becomes mandatory to find quick and simple procedures for their removal that do not significantly affect the efficiency of the screening process. Methods that have been evaluated included the use of precipitating agents such as gelatin/sodium chloride solution, caffeine solution, soluble and insoluble polyvinylpyrrolidone (PVP), in addition to polyamide column chromatography[24].

The interaction leading to the removal of tannins is the formation of hydrogen bonds between the tannin phenolic hydroxy groups and the amide link (CONH) of the precipitating agent which results in the formation of insoluble complexes. Therefore, these procedures also remove non-tannin inhibitory compounds with phenolic hydroxy groups (e.g., flavonoids), except those that have catechol groups which are internally hydrogen bonded. Due to this structural characteristic, hydrolyzable tannins have been reported to elude such tannin removal procedures. Furthermore, quinones are irreversibly bound to these agents due to a covalent interaction[35].

It was previously reported that chromatography on polyamide is effective for the selective removal of polyphenolics[36]. With slight modification, this procedure may be accomplished quickly and conveniently with only a few milligrams of plant extract. This procedure is advantageous in that bound non-tannin inhibitory compounds with two or three phenolic hydroxy groups can be eluted by flushing with methanol.

The use of PVP, on the hand, may result in the removal of all compounds with phenolic hydroxy groups. The insoluble form of PVP facilitates its removal by centrifugation. However, soluble PVP has been found to be equally efficient, even though an excess will result in an increase in viscosity of the enzyme reaction mixture. A slight stimulation of enzyme activity was observed in the presence of soluble PVP. Nonetheless, at this stimulatory concentration, it did not interfere with the inhibitory effect of test compounds as demonstrated by the identical inhibition values observed in its presence and absence[24].

All other procedures examined were found to be unsuitable for tannin removal. Difficulty was encountered in using such procedures quantitatively because the reagents themselves were added as aqueous solutions, and there was an uncertainty as to the amount to add to the plant extract for the complete precipitation of tannins. The viscosity of these reagents and excess amounts in solution also interfered with the enzyme assay.

Thus, no single quantitative procedure involving the addition of a precipitating reagent was found to be suitable for tannin removal from plant extracts in preparation for the HIV RT assay, and no generalizations could be made regarding the concentrations or type of reagents that should be adopted. Different plant species contain different types of polyphenolics at highly variable concentrations. In general, since tannins are frequently present in high concentrations in plant extracts, all precipitating agents should have a high capacity for adsorbing these polyphenolic compounds. The methods of choice are obviously polyamide column chromatography and precipitation with insoluble PVP, procedures which do not introduce any extraneous material to the plant extract to be tested.

Bioactivity-Guided Fractionation Procedure

The utility of a bioassay in the process of drug discovery from natural sources relies on its capacity to detect minute quantities of active constituents in plant extracts derived from various stages of a fractionation procedure, and hence guiding this often lengthy process to ultimately yield an active compound(s). When the HIV-1 RT assay was applied at various stages of the fractionation scheme of the bark of *Plumeria rubra* L. (Apocynaceae), known to contain the active iridoid fulvoplumierin, it proved capable of directing the isolation process to yield the active compound from the petroleum ether extract after silica gel column chromatography[24]. The ability of the assay to detect active constituents in plant extracts depends, in part, on the concentration of extract tested, in addition to the concentration and inhibitory potency of the active constituent(s) present. Therefore, in a bioactivity-guided fractionation scheme, *relative* % inhibition values should be of concern rather than the *actual* % inhibition. For example, in the case of *P. rubra*, a petroleum ether fraction demonstrated only 35% inhibition at 200 μg/ml, from which fulvoplumierin, a moderately active HIV-1 RT inhibitor, was later isolated. This % inhibition value was not outstanding in itself, but it warranted attention when compared with the aqueous and chloroform fractions which were almost devoid of detectable inhibitory activity[24].

Data Interpretation

A variety of interactions occurring in an *in vitro* enzyme system can modulate the catalytic rate of DNA synthesis and the effect of potential inhibitors. An awareness of such factors will facilitate the correct interpretation of enzyme inhibition data. Some examples of such interactions include the effect of the type of divalent cations (Mn^{2+} or Mg^{2+}) on DNA synthesis obtained with different template-primers, the role of chelating agents or cation binders, thiol reducing agents and BSA in the reaction buffer, and the effect of using different concentrations of enzyme, template-primer and substrate. Thiols may react with certain potential inhibitors and alter their inhibitory activity while the detergent effect on the activity of rifamycins is well documented[37]. In addition, certain natural products such as saponins may have surfactant properties, and their enzyme inhibitory effect may well be due to an alteration of the physicochemical properties of the reaction medium. Indeed, it was observed with the HIV-1 RT assay currently used in our laboratory[24] that the inhibitory effect of these compounds is quite irreproducible (unpublished observations). The interaction between certain compounds and nucleic acids results in the immediate precipitation of the complex. This artifact, if overlooked, may be misconstrued as a legitimate inhibition of enzyme activity. The effect of compounds which bind nonspecifically to proteins is worth reiterating. Thus, it is imperative to evaluate potential inhibitors of HIV RT under carefully controlled conditions.

NATURAL PRODUCT INHIBITORS OF HIV-1 RT

Benzophenanthridine Alkaloids

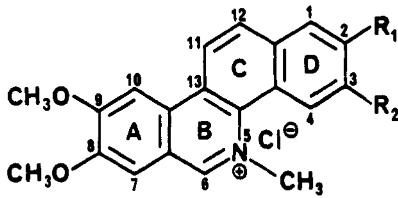
Benzophenanthridine alkaloids (Figure 1) are a class of isoquinoline alkaloids found in

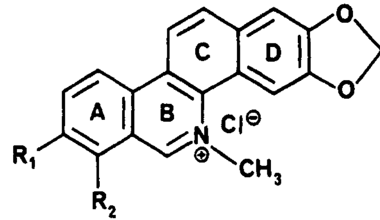
the plant families Rutaceae and Papaveraceae that possess interesting biological and pharmacological properties[38]. Fagaronine chloride (1), isolated from *Fagara xanthoxyloides* Lam. (Rutaceae), demonstrated potent antileukemic (P-388 and L1210 lymphocytic leukemias)[33] activity but was considered inactive in a few solid tumor models investigated[39]. Nitidine chloride (2), isolated from *Fagara macrophylla* (Oliv.) Engl. (Rutaceae), was found to inhibit Lewis lung carcinoma and murine L1210 and P388 leukemias[40]. Sanguinarine chloride (3) and chelerythrine chloride (4) have been shown to possess antimicrobial activity[41,42]. Enzyme systems affected by the benzophenanthridine alkaloids include catechol *O*-methyltransferase[43] and transfer RNA methyltransferase[43,44] of rat liver, NaK-ATPase of guinea pig brain[45,46] and protein kinase C of rat brain[47]. Benzophenanthridine alkaloidal salts such as fagaronine chloride, *O*-methylfagaronine fluorosulfonate, nitidine chloride, allonitidine methylsulfate (5) and 6-methoxy-5,6-dihydronitidine bromoacetate (6) were reported to be potent inhibitors of avian myeloblastosis virus (AMV) RT and Rauscher murine leukemia virus (RMLV) RT demonstrating IC_{50} values between 6-18 µg/ml for fagaronine chloride and *O*-methylfagaronine fluorosulfonate, and 40-60 µg/ml for the latter three alkaloids[19,48]. HIV-1 RT was later shown to be similarly affected, with fagaronine chloride (IC_{50} = 10 µg/ml) and nitidine chloride (IC_{50} = 49 µg/ml) being the most potent compounds tested[24].

The inhibitory effects of these alkaloids on several mammalian polymerase enzymes have also been described[49]. While reverse transcriptase and DNA polymerase α activities were strongly inhibited by these alkaloids, RNA polymerases I and II were only moderately affected. Polyadenylic acid (poly A) polymerase activity was only weakly inhibited. Fagaronine chloride and nitidine chloride gave IC_{50} values in the range of 20-45 µg/ml with both viral and mammalian DNA polymerases. Additionally, fagaronine chloride inhibited protein synthesis in cell-free systems, and caused irreversible inhibition of DNA, RNA and protein syntheses in KB cells[50]. An interaction with the ribosomal system was proposed.

A comparison of the effect of 15 structurally-related benzophenanthridine alkaloids revealed that fagaronine chloride possesses optimum structural features for AMV RT inhibitory activity[51]. Decreased activity was observed when the phenolic hydroxy group at position 2 was methylated or when the methoxy group at position 9 was replaced by a phenolic hydroxy group. Also, nitidine chloride and allonitidine methylsulfate which have a methylene dioxy group at positions 2 and 3 or 8 and 9, respectively, showed less inhibition of AMV RT. Saturation of ring B (e.g., 6-methoxy-5,6-dihydronitidine bromoacetate) or rings B and C [e.g., chelidonine (7)] with the concomitant loss of the charge on the nitrogen atom, was observed to produce a significant loss of activity. Likewise, demethylation of fagaronine chloride resulted in the loss of its antileukemic, bactericidal, cytotoxic and RT inhibitory effects[39,24]. Furthermore, substituents at positions 7 and 8 (e.g., chelerythrine chloride) or at any other positions [e.g., chelirubine chloride (8)] on the molecule reduced the RT inhibitory effect. These data indicate that the quaternary nitrogen and the methoxy groups at positions 8 and 9 are essential for optimum activity. These general considerations have also been shown to apply to the HIV-1 RT[24].

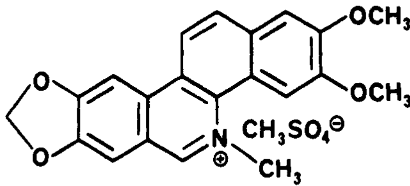
It has also been suggested that the RT inhibitory activity of these alkaloidal salts is in accordance with the postulated active site (iminium ion $-C=N^{+}-CH_3$) for antitumor and other biological activities[51]. In aqueous solution at suitable pH, benzophenanthridine alkaloids covalently take up hydroxide ions to form a pseudobase or alkanolamine adduct. The alkaloids have been shown to attain different iminium ion concentrations in solution, the equilibria of which is highly dependent on the pK_a of the molecule and the effect of substituent groups[52]. A dichotomy of biological activies was observed. While a high iminium ion concentration correlated with potent antitumor activity (fagaronine chloride and nitidine chloride), a low concentration of the ion was favorable for antimicrobial activity and NaK-ATPase inhibition (sanguinarine chloride and chelerythrine chloride). The carbon of the iminium function



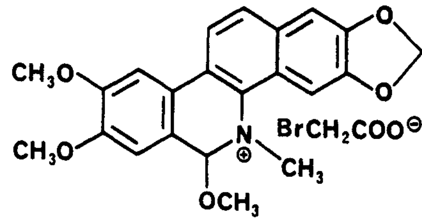


1 Fagaronine chloride (R_1 = OH; R_2 = OCH_3)
2 Nitidine chloride (R_1, R_2 = OCH_2O)

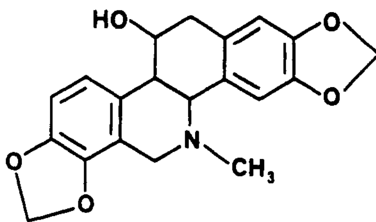
3 Sanguinarine chloride (R_1, R_2 = OCH_2O)
4 Chelerythrine chloride (R_1, R_2 = OCH_3)



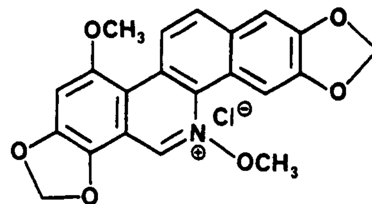
5 Allonitidine methylsulfate



6 6-Methoxy-5,6-dihydronitidine bromoacetate



7 Chelidonine



8 Chelirubine chloride

FIGURE 1. BENZOPHENANTHRIDINE ALKALOIDS

represents an effective alkylation site. The presence of substituents at positions 7 and 8 such as in sanguinarine chloride and chelerythrine chloride could sterically block nucleophilic attack at this iminium carbon, and may thus account for the absence of antitumor and antileukemic effects in these molecules[53]. A substituent at position 7 could also interact sterically with the peri-H of ring B and destabilize the iminium ion, shifting the equilibrium in favor of the alkanolamine ion[52]. From the perspective of structure-activity relationship (SAR), it would seem that the iminium ion is also necessary for RT inhibitory activity. The iminium-alkanolamine equilibrium, nevertheless, is very much dependent on the pH of the reaction medium. Sanguinarine chloride and chelerythrine chloride have lower pK_a values than the other members of the benzophenanthridine alkaloids. Consequently, there are significant amounts of pseudobase forms present at physiological pH[54].

It has also been reported that many nonalkylating antileukemic alkaloids possess two oxygen atoms and a nitrogen atom that define a triangle of certain optimum interatomic distances[55]. Any deviations from these specified interatomic distances is postulated to result in decreased antileukemic activity. This observation was found to apply to the benzophenanthridine alkaloids as well[53].

Marked template-primer preference was observed for the benzophenanthridine alkaloids. A significant degree of RT inhibition was obtained for activated DNA, 70S viral RNA and template-primers rich in A:T (adenine:thymine) or dA:T (deoxyadenine:thymine) base pairs compared to those containing G:C (guanine:cytosine) base pairs. Therefore, these alkaloids were suggested to bind to template-primers, interacting specifically with A:T base pairs of nucleic acids[19,29]. Circular dichroism measurements[56] and ultraviolet spectrophotometric titrations[57] furnished direct proof of interaction of these alkaloids with nucleic acids. Only those alkaloids which are potent RT inhibitors showed induced CD bands at longer wavelengths when admixed with $r(A)_n \cdot (dT)_{12\text{-}18}$. However, contrary to the base specificity proposed, ultraviolet spectral perturbations suggestive of an intercalation process were obtained when fagaronine chloride was admixed with calf thymus DNA, $(dA\text{-}dT)_n$, $(dG)_n \cdot (dC)_n$ or with other double-stranded nucleic acids. Hence, the binding of fagaronine chloride is not limited to polynucleotides containing only A and T. In contrast, DNase I footprinting studies have revealed that sanguinarine chloride and chelerythrine chloride failed to interact with $(AT)_n$ sequences on DNA due to the unique structure of the DNA helix in such a region[54]. Although the mode of action of fagaronine chloride has not been unequivocally characterized, interaction with the template-primer appears to be a major mechanism. Consistent with this suggestion, the kinetics of inhibition of RTs by the benzophenanthridine alkaloids was competitive with respect to the template-primer[56]. Interaction of fagaronine chloride with the enzyme protein itself has not been established. When these alkaloids were added to the enzyme reaction mixture after the initiation of the polymerization reaction, enzyme activity was abruptly decreased, further supporting the notion that the alkaloids interacted with the template-primer, thereby, affecting polynucleotide elongation instantly[19,49]. In summary, the quaternary nitrogen atom appears to serve as an anchor through interaction with the negatively charged phosphate backbone of nucleic acids enabling the fused coplanar aromatic ring system to intercalate[58,59]. This proposition is advocated by the fact that *N*-demethylfagaronine failed to interact effectively with double stranded nucleic acids[57]. Interactions of this type could also explain why poly A polymerase, which requires a single stranded RNA primer with a free 3' terminus as the template for activity, is the only polymerase enzyme relatively unaffected by these alkaloids[49]. Noteworthy is the fact that it is the iminium ion rather than a quaternary N atom, in general, that mediates the interaction with nucleic acids and, hence, RT inhibitory activity. Quaternary alkaloids of other structural classes, e.g., echitamine chloride (an indole alkaloid) and magnoflorine chloride (an aporphine), were virtually inactive in the HIV-1 RT system[24].

A good correlation was observed between the RT inhibitory activities of the benzophenanthridine alkaloids and their antileukemic effects, implying common molecular

features responsible for activity. The role that RT inhibition plays in the antineoplastic activity of these alkaloids is uncertain at this point. In addition, the role of cellular metabolic activation cannot be overlooked where the *in vivo* effects of the benzophenanthridine alkaloids are concerned. For example, rat liver preparations have been shown to substantially enhance the bactericidal activity of fagaronine[57]. Unfortunately, the usefulness of these alkaloids as potential antiviral or anticancer agents is limited by their toxicity[53,57] and potent anti-HIV activity in cell culture remains to be demonstrated.

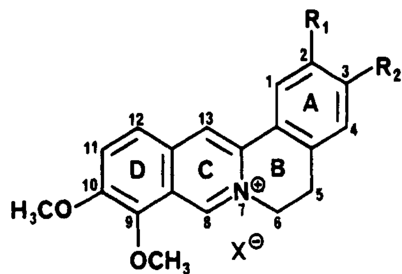
Protoberberine Alkaloids

Protoberberine alkaloids (Figure 2) which are widely distributed in plants of the Berberidaceae, Menispermaceae, Ranunculaceae and Rutaceae, are structurally related to the benzophenanthridine alkaloids. Berberine chloride (9), a typical representative of these alkaloids, has been extensively studied with respect to its chemical and pharmacological properties[60]. Much of the biological activities of the benzophenanthridine and protoberberine alkaloids are similar. Both berberine chloride and palmatine (10) were also effective against experimental tumors but lacked antileukemic activity against P-388 murine lymphocyte leukemia[61]. However, the synthetic dehydroprotoberberine coralyne chloride (11) exhibits inhibitory properties against both leukemias L1210 and P388 in mice[62,63]. Furthermore, protoberberine alkaloids were reported to inhibit a number of enzymes including horse liver[64] and yeast[65] alcohol dehydrogenase, tyrosine decarboxylase[66], tryptophanase[67] and catechol *O*-methyl transferase[62] by distinct mechanisms. The potent RT inhibitory effects of the benzophenanthridine alkaloids prompted an investigation on the related protoberberine alkaloids[68]. The IC_{50} values of palmatine and berberine chloride were in the range of 30-35 μg/ml and 100-105 μg/ml, respectively, for RTs from AMV, RMLV, and simian sarcoma virus type 1 (SSV-1). Similar IC_{50} values were observed for the HIV-1 RT[24].

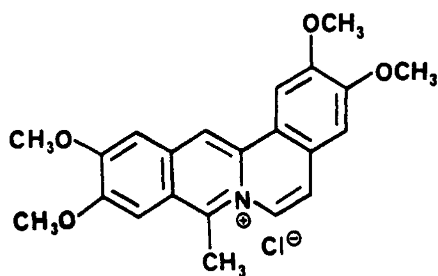
The RT inhibitory properties of these alkaloids are identical to those of the benzophenanthridines. The use of template-primers containing only G:C base pairs resulted in weak inhibition of enzymatic activity. Based on this indirect evidence, a preference for A:T base pairs of nucleic acids was suggested[69]. The inhibition mediated by these alkaloids exhibited competitive type kinetics with respect to the template-primer but interaction with the template-primer binding site of the enzyme has not been conclusively demonstrated. A previous report related the antileukemic activity of the protoberberine alkaloids with molecular conformation and DNA binding properties of the molecules[61]. It became apparent that in berberine chloride and palmatine, the plane of ring A is twisted slightly out of that of the CD rings by the completely saturated ring B, thus lowering their ability to intercalate relative to the completely planar coralyne. A change in the absorption spectrum of coralyne acetosulfate when added to a calf thymus DNA solution has also been reported[70]. Other studies have concurred that the biological activities of berberine and coralyne are due to their ability to bind DNA probably by intercalation[71,72,73]. The concept of the triangular N-O-O pharmacophore[54] which was found among a number of antileukemic compounds may also be applied to coralyne and the protoberberine alkaloids.

SAR studies reveal that the presence of methoxyl groups at positions 2 and 3 of ring A and positions 9 and 10 or 10 and 11 of ring D was essential for optimum RT inhibitory activity[68]. Saturation of ring C as in tetrahydropalmatine (12) also yielded an inactive compound. Even though SARs are not as well defined in this series of alkaloids, they are observed to be weaker inhibitors of RT than the benzophenanthridines possibly due to differences in intercalating ability imposed by structural differences. While the benzophenanthridine molecule constitutes a fused planar aromatic ring system, that of the protoberberine does not[61].

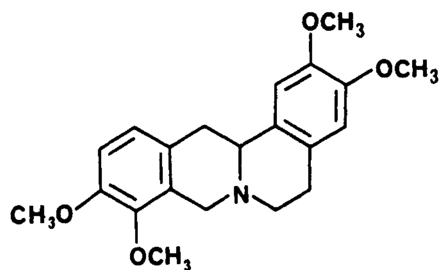
Earlier studies indicated that the RT inhibitory property of benzophenanthridine alkaloids corresponded well with their antileukemic activity but this did



9 Berberine chloride (R_1, R_2 = OCH_2O ; X = Cl)
10 Palmatine (R_1, R_2 = OCH_3; X = OH)



11 Coralyne chloride



12 Tetrahydropalmatine

FIGURE 2. PROTOBERBERINE ALKALOIDS

not seem to be the case for the protoberberine alkaloids in general[74]. The mechanism of RT inhibition may well follow similar arguments, although additional mechanisms cannot be ruled out. Analogous to the benzophenanthridine alkaloids, the potential clinical utility of these compounds as antivirals or chemotherapeutic agents is hampered by their toxicity[75]. The anti-HIV activity of this group of compounds has yet to be determined.

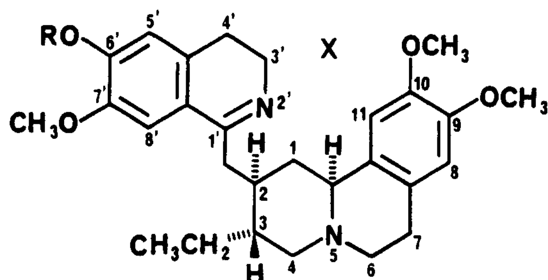
Ipecac Alkaloids

Previous efforts in the screening of diverse structural classes of natural products[24] revealed two novel inhibitors from the ipecac class of alkaloids (Figure 3), *O*-methylpsychotrine sulfate heptahydrate (MP) (13) and psychotrine dihydrogen oxalate (PDO) (14). Relative to approximately 200 natural product drugs that were evaluated for inhibitory activity, MP and PDO were two of the most potent inhibitors uncovered. MP and PDO demonstrated IC_{50} values in the HIV-1 RT system of 10 μg/ml (14 μM) and 6 μg/ml (9μM), respectively, relative to fagaronine chloride, which demonstrated an IC_{50} value of 5 μg/ml (13 μM). As a result, additional members of the ipecac alkaloids were tested for HIV-1 RT inhibitory activity with the aim of defining relevant structure-activity relationships[30].

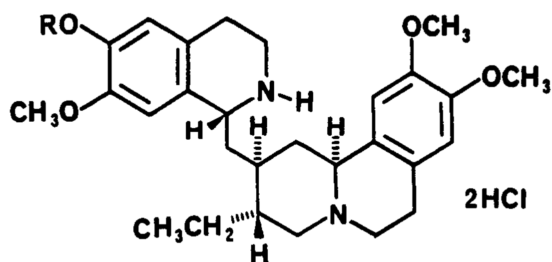
MP is a minor alkaloid of ipecac, the dried rhizome and root of *Cephaelis ipecacuanha* (Brotero) A. Richard (Rubiaceae)[76]. Like the benzophenanthridine and protoberberine alkaloids, ipecac alkaloids are biosynthesized from isoquinoline intermediates. Ipecac yields more than 2% (w/w) of ether-soluble alkaloids, with the three principal ones being emetine, cephaeline and psychotrine[77]. These structurally-related alkaloids are prepared as their inorganic acid salts for the improvement of solubility and stability. The therapeutic properties of the ipecac alkaloids as emetics, expectorants and amebicides have long been recognized. The pharmacological effects of the two major alkaloids, emetine and cephaeline, include antiamebic[78], antitumor[79], and antiviral activities[80], in addition to the irreversible inhibition of DNA synthesis in cell culture[81,82]. They did not, however, affect cellular thymidine kinase or the incorporation of TTP into DNA with *in vitro* assay systems[81]. The synthesis of cellular RNA is unaffected by emetine but viral RNA synthesis in polio virus infected HeLa cells was strongly inhibited[81]. However, the toxicity of the drug, manifested primarily as cardiotoxicity when used for extended periods, precludes its therapeutic use as an antiviral.

Emetine and cephaeline are also potent inhibitors of protein synthesis in mammalian, yeast and plant cells, while bacterial cells are resistant to the effects of these alkaloids[81]. All biological effects of emetine and cephaeline, both therapeutic and toxic, are consistent with the inhibition of protein synthesis as the primary mode of action. Protein synthesis is thought to be inhibited at the stage of translocation[83,84], possibly by a direct effect of these compounds on an elongation factor. In contrast, *O*-methylpsychotrine and isoemetine were reported to have very weak effects on protein synthesis (<1% of the activity of emetine)[81].

An attempt has been made to characterize the mechanism of inhibition mediated by MP[30]. The inhibition was noncompetitive with respect to TTP and uncompetitive with respect to $(rA)_n$ and $(dT)_{12\text{-}18}$ (4:1) at low template-primer concentrations but competitive at high concentrations (> 200 μM). Identical non-Michaelis-type kinetics were observed when activated DNA was used as the template. The biphasic nature of the double-reciprocal plots and Hill coefficients of less than one indicated that MP functions as an allosteric inhibitor of the enzyme that appears to possess multiple active sites which interact in a cooperative (negative) fashion in the presence of the inhibitor. MP was selective for recombinant HIV-1 RT (p66) utilizing $(rA)_n$ and $(dT)_{12\text{-}18}$ (4:1) as template-primer. Greater inhibition was observed with this template-primer as compared to other natural and synthetic template-primers tested. Even though A:T or dA:T systems were strongly inhibited, spectroscopic studies did not reveal changes in the absorption spectra of MP in the presence of various concentrations of A or T rich homopolymers or activated DNA. Hence, it appears that MP does not interact with nucleic



13 *O*-Methylpsychotrine sulfate heptahydrate (R = CH_3; X = H_2SO_4)
14 Psychotrine dihydrogen oxalate [R = H; X = $2(COOH)_2$]



15 Emetine hydrochloride (R=CH_3)
16 Cephaeline hydrochloride (R=H)

FIGURE 3. IPECAC ALKALOIDS

acids and, therefore, does not interfere with their template properties. Since the p66 monomeric enzyme was used for the assay it can be concluded that a putative binding site(s) for MP resides on this catalytic subunit of the HIV-1 RT even though the recombinant p66/p51 heterodimer was equally susceptible to inhibition. Data further supported the conclusion that MP binds to a site other than the substrate or template-primer binding site of the enzyme possibly through hydrophobic or hydrogen bonding interactions.

MP had significantly less effect on AMV RT as well as mammalian or bacterial DNA and RNA polymerases. Other members of the ipecac class of alkaloids, e.g., emetine dihydrochloride (15) and cephaeline dihydrochloride (16), were inactive against all of these enzymes, including HIV-1 RT. Conversely, MP did not inhibit *in vitro* protein synthesis, a property manifested by all the other ipecac alkaloids tested. The inhibition of protein synthesis was observed to bear an apparent inverse correlation with RT inhibition. The imine functionality at positions 1' and 2' of MP and PDO appeared to be the key structural requirement for activity. It is also of necessity that these compounds be in the salt form in order to demonstrate HIV-1 RT inhibitory activity. MP and PDO demonstrated similar potency in the HIV-1 RT system suggesting that the presence of the methoxy group at position 6' of MP had no consequence on the binding of the compound to its putative binding site on the enzyme. The salt form of the active molecules may impart a positive charge on the N atoms in solution and hence facilitate interaction with the enzyme in some unknown way. It is uncertain at this point if the nature of the compounds (salt or free base) will influence the molecular conformation adopted in solution.

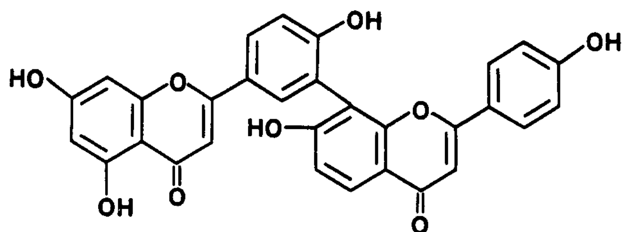
The fact that they do not inhibit protein synthesis implies that psychotrine, MP and PDO may be devoid of some toxic effects mediated by other biologically active ipecac alkaloids. However, while the inhibition of protein synthesis is structurally specific, the emetic action of these compounds does not correlate with their ability to inhibit protein synthesis[81]. *O*-Methylpsychotrine is very bitter and induces nausea when ingested. If compounds of this type are to find therapeutic use it will be necessary to explore chemical modifications that may reduce bitter and emetic properties while retaining or even improving HIV-1 RT inhibitory properties.

Flavonoids

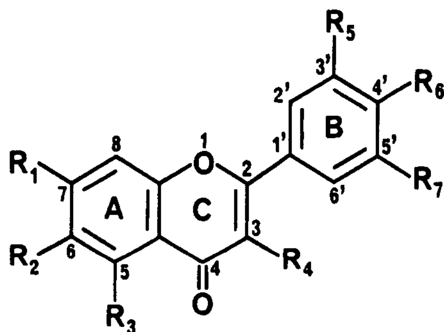
Flavonoids (Figure 4), 2-phenyl benzo-γ-pyrone derivatives, are of wide occurrence in vascular plants including fruits, vegetables and grains. Flavonoids are classified according to ring substitution patterns and the degree of benzopyrone ring saturation as flavones, flavanones, isoflavones, isoflavanones, anthocyanins, chalcones and aurones, to name a few. These groups in turn show varying degrees of hydroxylation, methoxylation and glycosylation[85].

This large group of low molecular weight compounds has a wide spectrum of pharmacological properties[86], the mechanisms of which remain to a large extent unknown. Hence, flavonoids exert diverse effects on mammalian enzyme systems which contribute to their potential use in the treatment of a wide variety of human ailments[85]. These natural products are potent inhibitors of enzymes such as iodothyronine deiodinase[87], cyclic AMP phosphodiesterase[88], protein kinase C[89] and catechol-*O*-methyl-transferase[90]. The enzyme inhibitory properties of flavonoids were observed to show stringent conformational requirements. No unifying mechanism explains the effect of this rather homogenous group of molecules on such a perplexing number and variety of enzymes[91]. Flavonoids structurally resemble nucleosides, isoalloxazine and folic acid, and this similarity may be the basis of many of the current hypotheses of their physiological action[86].

Progress in the realms of molecular modelling and conformational analysis has permitted the visualization of the crystal and molecular conformation of flavonoids[92,93]. This facilitates the study of SAR involved in the host of biological activities mediated by



17 Amentoflavone



18 Scutellarein (R_1, R_2, R_3, R_6 = OH; R_4, R_5, R_7 = H)
19 Quercetin (R_1 ,R_3, R_4, R_5, R_6 = OH; R_2, R_7 = H)
20 Baicalein (R_1, R_2, R_3 = OH; R_4, R_5, R_6, R_7 = H)
21 Myricetin (R_1, R_3, R_4, R_5, R_6, R_7 = OH; R_2 = H)
22 Quercetagetin (R_1, R_2, R_3, R_4, R_5, R_6 = OH; R_7 = H)
23 Kaempferol (R_1, R_3, R_6 = OH; R_2, R_5, R_7 = H)

FIGURE 4. FLAVONOIDS

these molecules. Conformational analyses have indicated that the basic ring system of flavones, aurones and chalcones are planar. In contrast, saturation of the C_2-C_3 bond causes the pyran ring of flavanones, anthocyanins and isoflavanones to be puckered, thereby significantly affecting the conformation of these molecules. However, when a double bond is present, an extended conjugated resonance system is created which may play a role in their interaction with enzymes.

Numerous flavonoids have demonstrated promising antiviral activity[94-96] via mechanisms of action ranging from binding to virus capsid proteins (i.e., antiinfective)[97] to interference with viral RNA or protein synthesis (i.e., antireplicative)[98]. Preliminary results were published on the oncornavirus RT inhibitory activity of flavonoids[99]. It was later confirmed that these compounds are potent *in vitro* RT inhibitors. A systematic study of the potential RT inhibitory effects of 18 flavonoids representing seven different chemical classes has been conducted[100]. Amentoflavone (17), scutellarein (18) and quercetin (19) were found to inhibit AMV RT, Rous-associated virus-2 (RAV-2) RT and Moloney murine leukemia virus (MMLV) RT in a concentration-dependent manner. For each of these flavonoids, the IC_{50} value was approximately 10 μM. The effect of these compounds on MMLV RT was observed to be much greater than that on the avian RTs. In another study, baicalain (5,6,7-trihydroxyflavone) (20) inhibited the activity of RTs from RMLV, MMLV and HIV-1 at concentrations as low as 2 μg/ml, while the activities of mammalian DNA polymerase α and β were not affected in this concentration range[32]. Moreover, the degree of inhibition was observed to be greater when Mn^{2+} was used in the assay mixture instead of Mg^{2+}. Quercetin, baicalein, myricetin (21) and quercetagetin (22) were also found to inhibit the RTs from RMLV and HIV, demonstrating IC_{50} values of less than 0.5 μg/ml[101]. All three flavonoids demonstrated differential effects on DNA polymerases α, β, and γ purified from KB-III cells, terminal deoxynucleotidal transferase obtained from calf thymus, and DNA and RNA polymerases from *Escherichia coli*. The activity of flavonoids on nucleotide polymerizing enzymes[102,103] may account for their cytotoxicity[104]. In addition, quercetin was observed to inhibit the activity of murine RNA polymerase II[105]. During a screen aimed at identifying natural product HIV-1 RT inhibitors specifically[24], flavonoids such as quercetin and kaempferol (23) emerged as weak inhibitors of the enzyme (IC_{50} = 150-200 μg/ml).

When inhibition kinetics were analyzed, flavonoids exhibited varied modes of action. With few exceptions, the inhibition of RT was competitive or mixed with respect to the template-primer $[r(A)_n \cdot (dT)_{12\text{-}18}]$ used and noncompetitive with respect to the TTP substrate. In the case of RMLV RT, for example, quercetin, baicalein, myricetin and quercetagetin competed with the polynucleotide template-primer but not with the triphosphate substrate[101,103]. However, when HIV RT was used, the mode of inhibition was of the mixed type with respect to the template-primer. These results indicate that flavonoids act by binding to the template-primer binding site of the enzyme or to the template-primer itself. The molecular planarity of flavones such as quercetin and myricetin is thought to facilitate the intercalation of these molecules with the template-primer. It has been suggested that polar flavonoids have this intercalative ability[106]. The planarity of flavonoids has also been correlated with their ability to produce open circular DNA from a covalently closed cyclic plasmid, an observation which may be used as a measure of intercalating ability[102].

A comparative study of numerous flavonoid molecules was conducted to define SARs involved in RMLV and HIV RT inhibition[101]. It was concluded that the presence of both the unsaturated double bond between positions 2 and 3 of the flavonoid pyrone ring, and the hydroxy groups at positions 5, 6 and 7 (i.e., baicalein) were prerequisites for RT inhibition. Neither flavone nor various mono- and dihydroxyflavones (except 6,7-dihydroxyflavone) showed appreciable inhibitory activity up to 10 μg/ml. The fact that phenolic hydroxy groups contribute to the activity of flavonoids was clearly demonstrated by glycosidation of the hydroxyl groups at position 7 of baicalein and position 3 of quercetin which greatly reduced the inhibitory effects of their respective parent compounds. No clear-cut SARs have emerged from the

present state of knowledge on the RT inhibitory activity of flavonoids. It can be postulated, however, that in addition to the requirement of structural planarity, the phenolic hydroxy groups on these molecules, which are often present in numbers greater than two, have a profound effect on the RT inhibitory potential of flavonoids.

Several flavonoids including quercetin have been reported to show *in vivo* antiviral effects[107,108]. Controversy still shrouds the effect of flavonoids on the common cold[109]. Preliminary results have shown some of these flavonoids to be toxic to cultured cells at concentrations that did not inhibit HIV-1 induced cytopathogenicity[101]. Nevertheless, the toxicity of flavonoids is typically low in humans and it is possible that pharmacologically significant concentrations could be reached in tissues and influence the outcome of viral infections[86]. Structural modifications to minimize toxicity and enhance activity may further resolve these problems.

Miscellaneous Compounds

Numerous other natural products have been shown to inhibit the RT of various retroviruses. Selected examples of these compounds are shown in Figure 5.

Even though the inhibitory effect of polyanionic substances on the replication of enveloped viruses were reported more than two decades ago, it did not generate much interest until dextran sulfate and heparin were found to be highly inhibitory to the replication of HIV-1 *in vitro*[110]. Until now, this antiviral effect was considered to be largely nonspecific. Pentosan polysulfate[111] also emerged as a potent anti-HIV compound which was associated with low toxicity and anticoagulant activity[112]. Consistent with previous findings[113], anionic polysaccharides have been shown to inhibit virus adsorption to cells. Pentosan polysulfate also acts as a competitive inhibitor of HIV-1 RT with respect to the template-primer, but whether this compound is actually taken up by cells to enable RT inhibition to play a role in its anti-HIV effects, remains subject for further study. However, it was noted that the concentrations of sulfated polysaccharides required to inhibit HIV-1 RT *in vitro* were considerably higher than those required for inhibition of HIV-1 replication in cell culture[112]. The IC_{50}s for HIV-1 RT inhibition for pentosan polysulfate, fucoidan, dextran sulfate and heparin were 19.1, 29.5, 32.9, and 410 µg/ml, respectively[112]. HIV-1 and AMV RT inhibitory carrageenans have also been isolated from sea algae[114].

All the polysaccharides that inhibit RT activity have sulfate residues. Those which do not (e.g., dextran and lentinan), were essentially devoid of RT inhibitory activity. Thus, sulfate residues play a key role in RT inhibition. Indeed, the addition of sulfate groups to glycyrrhizin and other nonsulfated polysaccharides resulted in the endowment of RT inhibitory activity to the substances involved[115]. It was also reported that the sulfated side chains of polyvinyl sulfate competed with the template-primer for the active site of RT[116]. It was observed that the HIV-1 RT inhibitory effect of these compounds was negated if bovine serum albumin was added to the reaction mixture, indicating nonspecific binding to protein[112].

Avarol (29) is a sesquiterpenoid hydroquinone present in the marine sponge *Dysidea avara*. Both avarol and its quinone derivative avarone (24) exhibit a dose-dependent inhibition of HIV-1 replication in human H9 cells *in vitro*[117]. Avarol and avarone, however, were devoid of any significant inhibitory effect on HIV-1 RT as well as mammalian DNA polymerases[118]. Subsequently, several novel secondary metabolite derivatives of avarol and avarone, namely avarone A (25), B (26) and E (28), and avarol F (31) were found to inhibit all the three catalytic activities associated with the HIV-1 RT (DNA-dependent and RNA-dependent DNA polymerase and ribonuclease H activities)[119]. Kinetic experiments performed with avarone E revealed that the compound binds RT molecules at sites different from the binding sites of the template-primer or the substrate TTP. Avarol C (30) and avarone D (27) which have 6'-acetyl substituents were devoid of any inhibitory activity against the HIV-1 RT. Taken together, these observations indicate that the hydroxyl group at the *ortho* position to the carbonyl group of the

quinone ring (as in the case of derivatives A, B, E and F) is required for potent HIV-1 RT inhibitory activity.

Analogous to avarone, various quinones have also been reported to inhibit retroviral RTs including the HIV-1 RT[120-123]. Quinone compounds interfere with the binding of the template-primer to the enzyme, most probably by interaction with the enzyme. Structure-activity studies of a diverse group of quinone molecules revealed a common feature. Hydroxy groups critically situated play a pivotal role in the activity of these compounds. Most often activity is attributed to hydroxy groups situated *ortho* to the quinone carbonyl. Studies have also shown that quinones function as electron acceptors and, therefore, may oxidize sulfydryl groups in the active site of the enzyme[124]. Furthermore, it was demonstrated that hypericin (32), an anthraquinone dimer, binds to proteins in a nonspecific manner[123]. This property may be partially responsible for its HIV-1 RT inhibitory activity.

In an extensive survey of plant extracts for HIV-1 RT inhibitory activity, four phloroglucinol derivatives, mallotojaponin, mallotochromene (33), mallotophenone and mallotolerin isolated from the pericarps of *Mallotus japonicus* Muell. Arg. (Euphorbiaceae), were found to be active[125]. Mallotojaponin and mallotochromene were particularly active with IC_{50} values of less than 10 µg/ml. Differences in activity were attributed to the nature of the side chains present. Mallotojaponin was also found to interfere with the binding of the template-primer to the enzyme.

Taspine (34) is a dilactone tertiary alkaloidal base isolated from *Croton lechleri* M. Arg. (Euphorbiaceae). Taspine hydrochloride inhibited RMLV, SSV-1 and AMV RT with IC_{50} values falling in the range of 70-98 µg/ml[126]. The alkaloid was more specific for RT than for cellular DNA polymerases or *E. coli* DNA or RNA polymerases. Preliminary experiments involving UV spectrophotometric titrations also indicated that the alkaloid interacted with calf thymus DNA.

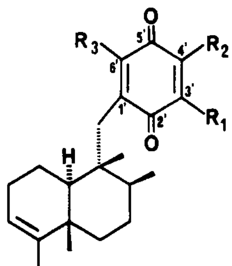
Pretazettine (35), an alkaloid isolated from the bulbs of *Narcissus tazetta* L. "Sacred lily" was claimed to be the first RT inhibitor discovered that binds to the AMV RT and affects steps subsequent to the formation of the enzyme-template-primer complex[127]. The immediate cessation of polymerization upon the addition of the alkaloid to an ongoing reaction suggested that the inhibitor interfered with initiation or elongation of the polymerization process, rather than with the initial binding of the template to the enzyme.

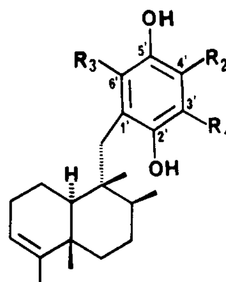
Many antibiotics such as the antitumor anthracyclines adriamycin and daunomycin are known inhibitors of retroviral RTs[128]. The predominant mode of action is via an interaction with the template-primer, hence, these compounds have received relatively little attention as antiviral agents due to their toxicity. In contrast, quinoline quinone antibiotics such as streptonigrin (36)[129] and the ansamysin antibiotic rifamycin SV (37)[130] have been shown to interact with the enzyme molecule itself. Where quinone compounds such as streptonigrin are concerned, a specific site of interaction on the RT molecule, referred to as the "quinone pocket", has been proposed[131]. A comparative study of the inhibitory properties of antibiotics on HIV RT, AMV RT and cellular DNA polymerases has also been published[132].

As already mentioned, the application of the HIV-1 RT assay recently developed to the bioactivity guided fractionation scheme of the bark of *Plumeria rubra*, yielded the active iridoid fulvoplumierin (38) (IC_{50} = 45 µg/ml in the HIV-1 RT system)[24]. Additional iridoids evaluated such as plumericin and plumieride were inactive.

CONCLUSION

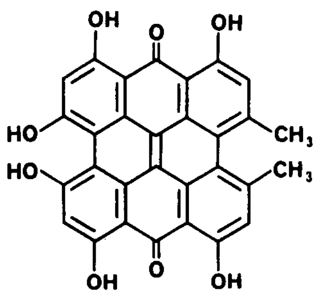
Therefore, the standard reverse transcriptase assay is a specific, sensitive, simple and



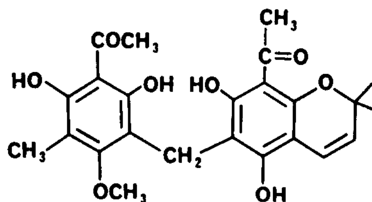


24 Avarone (R_1, R_2, R_3 = H)
25 Avarone A (R_1 = OH; R_2, R_3 = H)
26 Avarone B (R_1 = H; R_2, R_3 = OH)
27 Avarone D (R_1, R_2 = H; R_3 = CH_3COO)
28 Avarone E (R_1 = H; R_2 = OCH_3; R_3 = OH)

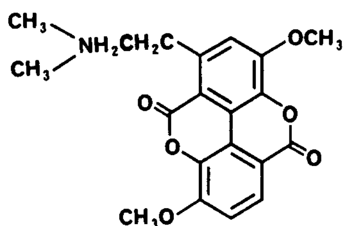
29 Avarol (R_1, R_2, R_3 = H)
30 Avarol C (R_1, R_2 = H; R_3 = CH_3COO)
31 Avarol F (R_1, R_2 = H; R_3 = OH)



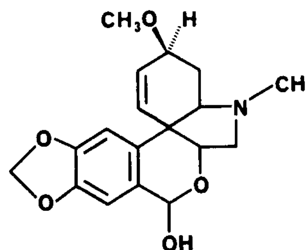
32 Hypericin



33 Mallotochromene

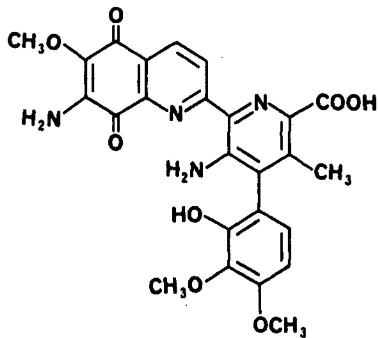


34 Taspine

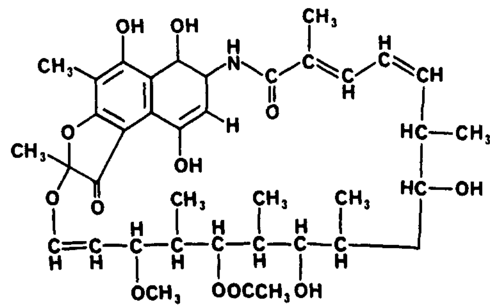


35 Pretazettine

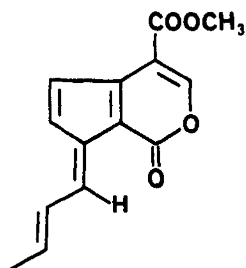
FIGURE 5. MISCELLANEOUS COMPOUNDS



36 Streptonigrin



37 Rifamycin SV



38 Fulvoplumierin

FIGURE 5. MISCELLANEOUS COMPOUNDS, CONT.

reliable method for screening the HIV-1 and HIV-2 RT inhibitory potential of natural products. Results are reproducible, and the procedure is applicable to both pure natural products and plant extracts. Moreover, the method has proven to be useful in a bioactivity-guided fractionation procedure. However, tannins are active inhibitors of reverse transcriptase and, in general, only non-tannin inhibitory compounds are of interest. Since no clear-cut threshold for activity could be established for plant extracts containing tannins, these substances are preferably removed before the assay is performed.

Since retroviruses are thought to play an important role in carcinogenesis, a variety of inhibitors of the retroviral RT has been uncovered from the viewpoint of understanding the mechanism of malignant transformation of virus-infected cells. Interest in these RT inhibitors rekindled with the discovery of AIDS. The clinical utilization of AZT has established the potential value of RT inhibitors in the management of AIDS. In spite of our knowledge of a great variety of RT inhibitors, however, an absolutely specific HIV-1 or HIV-2 RT inhibitor is not yet available. The potential of higher plants to provide novel prototype drugs is considerable. The present review clearly demonstrates that a diverse group of natural products possesses RT inhibitory activity. The empirical screening of structurally diverse materials may be the most efficient way to identify novel HIV-1 and HIV-2 RT inhibitors, until such time as the structure of the enzymes is completely understood, and alternative methods are defined.

Once active leads are identified, further screening of structural analogs is generally warranted. Mechanistic studies are in order to determine the selectivity of the mode of action manifested. One very important approach to demonstrate selectivity of compounds is to compare their inhibitory effects against various cellular DNA polymerases. Finally, it will be necessary to examine their properties as potential inhibitors of HIV infection and replication in cell culture followed by more advanced testing in animal models.

Although complete HIV eradication and a cure of AIDS cannot be achieved with agents that disrupt the replication of the viral genome, they are considered extremely important for improving the quality of life and survival of the host, and they are of value in combination regimens of chemotherapy. Chemotherapeutic intervention of the viral replicative cycle is also a prerequisite for immunotherapy aimed at reconstruction of the immune system. In addition, substances found to function as HIV RT inhibitors may serve as inhibitors of other types of retroviruses.

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