Antiviral Research, 12 (1989) 99–110
Elsevier

AVR 00368

Inhibition of reverse transcriptases by flavonoids

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(Received 16 December 1988; accepted 9 May 1989)

Summary

Selected naturally occurring flavonoids were shown to inhibit three reverse transcriptases (RT): avian myeloblastosis (AMV) RT, Rous-associated virus-2 (RAV-2) RT and Maloney murine leukemia virus (MMLV) RT when poly (rA)oligo$(dT)_{12-18}$ or rabbit globin mRNA were used as template. Amentoflavone, scutellarein and quercetin were the most active compounds and their effect was concentration-dependent. The enzymes exhibited differential sensitivity to the inhibitory effects of the flavonoids. The compounds also inhibited rabbit globin mRNA-directed, MMLV RT-catalyzed DNA synthesis. Amentoflavone and scutellarein inhibited ongoing new DNA synthesis catalyzed by RAV-2 RT. Kinetic studies were performed in an attempt to elucidate the mechanism of action of amentoflavone and scutellarein. A model is proposed for the mechanism of action of the flavonoids on RT activity.

Reverse transcriptase; Flavonoid; Kinetics; Quercetin; Amentoflavone; Scutellarein

Introduction

The group of low molecular weight plant secondary metabolites known as flavonoids (phenylbenzo-γ-pyrones) possess antiviral activity, both antiinfective and antireplicative (Kaul et al., 1985; Ishitsuka et al., 1982; Van Hoof et al., 1984; Vrijsen et al., 1987; Veckenstedt et al., 1987; Musci, 1984; Musci and Pragai, 1985), but no systematic study of their potential as anti-RT agents has been published. We wondered whether certain flavonoids might possess anti-RT activity and herein

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we report that several naturally occurring flavonoids are inhibitors of two avian RTs and one mammalian RT.

Materials

The flavonoids used in this investigation were purchased from several sources. Amentoflavone, eriodictyol, fisetin, formononetin, galangin, hinokiflavone, isorhamnetin, kaempferol, luteolin, quercetin, rhamnetin, scutellarein and silybin were purchased from Sarsynthese, Merignac, France. Taxifolin was obtained from Extrasynthese, Genay, France, and (+)-catechin and morin from Sigma, St. Louis, MO. Myricetin was purchased from Aldrich Chemical, Milwaukee, WI. Nobeletin was a generous gift from Dr. James Tatum, Department of Agriculture, Lakeland, FL.

The avian myeloblastosis virus reverse transcriptase (AMV RT) was purchased from Boehringer Mannheim Biochemicals (BMB), Indianapolis, IN, and the Bethesda Research Laboratories Life Technologies (BRL), Gaithersburg, MD. The Maloney murine leukemia virus reverse transcriptase (MMLV RT) and the Rous-associated virus-2 reverse transcriptase (RAV-2 RT) were purchased from BRL and Amersham, Arlington Heights, IL, respectively. Nuclease-free bovine serum albumin (BSA) was from BRL and methyl-[^{3}H]thymidine 5′-triphosphate (^{3}H-dTTP) was purchased from Amersham and rabbit globin mRNA from BRL. The template primers, poly(rA)oligo(dT)$_{12-18}$ and oligo (dT)$_{12-18}$(5′-phosphate) were purchased from Pharmacia, Inc., Piscataway, N.J. and the nucleotides, 2′-deoxyadenosine 5′-triphosphate (dATP), 2′-deoxycytidine 5′-triphosphate (dCTP), 2′-deoxy-guanosine 5′-triphosphate (dGTP) and dTTP from BMB. All other reagents and solvents used were of analytical grade.

Methods

Stock solutions of 1.0 or 1.25 mM flavonoids in absolute ethanol were prepared. The addition of flavonoids to experimental tubes was accomplished by adding the required amount of the various flavonoids in ethanol and then the solvent was evaporated off either in a dessiccator or under a stream of nitrogen gas.

The enzyme preparations were diluted in appropriate enzyme storage buffers comprising 200 mM potassium phosphate buffer, pH 7.2, 2 mM dithiothreitol (DTT), 0.2% NP40 and 50% (v/v) glycerol for AMV RT and RAV-2RT and 20 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.01% NP40, 0.1 mM EDTA, 100 mM NaCl and 50% (v/v) glycerol for MMLV-RT.

Effect of flavonoids on AMV RT

Each reaction mixture comprised 0.5 mM dTTP, 1 μg of the synthetic template primer poly(rA)oligo(dT)$_{12-18}$, 10 μg of BSA, 2 μl of 0.5 mM ^{3}H-dTTP and buffer,

pH 8.3 (50 mM Tris-HCl, 40 mM KCl, 1 mM DTT and 6 mM $MgCl_2$) with or without added flavonoid. The total reaction volume was 100 µl. The reaction mixture was preincubated at 37°C for 3 min. The reaction was then started by addition of 2 U of AMV RT enzyme. After 10 min at 37°C, the reaction was terminated with 1 ml of cold stop solution (10% TCA, 0.1 M sodium pyrophosphate, 1 mM disodium EDTA and 10 mM Tris-HCl, pH 7.5). The reaction tubes were vortexed and immersed in ice for about 20 min. The contents were filtered through Whatman glass fiber filters (presoaked in cold 5% TCA) with a vacuum pump. The filters were washed three times with 3 ml of 5% TCA and once with 3 ml of absolute alcohol. The filters were then air dried and the radioactivity of the TCA-precipitable material incorporated in the glass fibre filters was determined by liquid scintillation counting.

Effect of flavonoids on RAV-2 RT

The assay to determine the effect of flavonoid on RAV-2 RT activity was carried out as described above except that 0.25 U of enzyme was added and the incubation was for 5 min at 37°C.

Effect of flavonoids on MMLV RT

The reaction mixture for the studies comprised 1.5 mM dTTP, 1 µg of poly(rA)oligo$(dT)_{12-18}$, 10 µg of BSA, 2 µl of 0.5 mM ^{3}H-dTTP and made up to the reaction volume of 50 µl with buffer, pH 8.2 (50 mM Tris-HCl, 75 mM KCl, 10 mM DTT and 3 mM $MgCl_2$). The rest of the assay was carried out as described in the previous section except that 1 U of MMLV RT was used to start the reaction and the incubation period was 5 min.

To determine MMLV RT activity – using a natural message, rabbit globin mRNA, as template – the reaction mixture comprised 1 mM each of dATP, dCTP, dGTP, 0.5 mM dTTP, 10 µg of rabbit globin mRNA, 0.5 µg of oligo$(dT)_{12-18}$ as primer, 10 µg of BSA, 5 µl of 0.5 mM ^{3}H-dTTP with or without flavonoids and made up to 100 µl with buffer, pH 8.2 (100 mM Tris HCl, 10 mM $MgCl_2$ and 10 mM DTT). After preincubation for 3 min at 37°C, the reaction was started with 100 U of MMLV RT. After various time intervals at 37°C, the reaction was terminated by removing 15 µl aliquots of the reaction mixture into 1.0 ml of cold stop solution. The samples obtained were processed for liquid scintillation counting as described in the previous section for AMV RT.

Time course of flavonoid inhibition of RAV-2 RT

To study the effect of flavonoids on RAV-2 RT activity over time, the reaction mixture was similar to that described in the previous section for AMV RT. The reaction mixture (100 µl volume) without flavonoid was preincubated at 37°C for 3 min. Two units of RAV-2 RT were added to begin the reaction which proceeded at 37°C. After 3 min flavonoid was introduced. To the control tubes an equal vol-

ume of absolute ethanol was added. The final concentration of ethanol in the reaction mixture was 2% (v/v). At various time intervals, the reaction was terminated by removing 10 µl aliquots of the assay mixture and adding them to 1.0 ml of cold stop solution. The samples were processed for liquid scintillation counting as described above under 'Effect of flavonoids on AMV RT'.

All results are presented as mean ± SEM of at least three identical experiments.

Results

The effect of various flavonoids on AMV RT

In order to ascertain whether some flavonoids might possess anti-RT activity we examined the effect of 18 compounds representing seven different chemical classes (see Fig. 1). In addition, a known RT inhibitor, aurintricarboxylic acid (Givens and Manley, 1976), was included. All compounds were studied at 50 µM concentration. As can be seen in Fig. 2, aurintricarboxylic acid was an effective RT inhibitor (83%). Two flavonoids, amentoflavone and scutellarein exhibited moderate activity, inhibiting by 51 and 37%, respectively. The commonly studied flavonoid, quercetin, inhibited AMV RT by 23%. The inhibitory activity of amentoflavone, scutellarein and quercetin was concentration-dependent as shown in Fig.

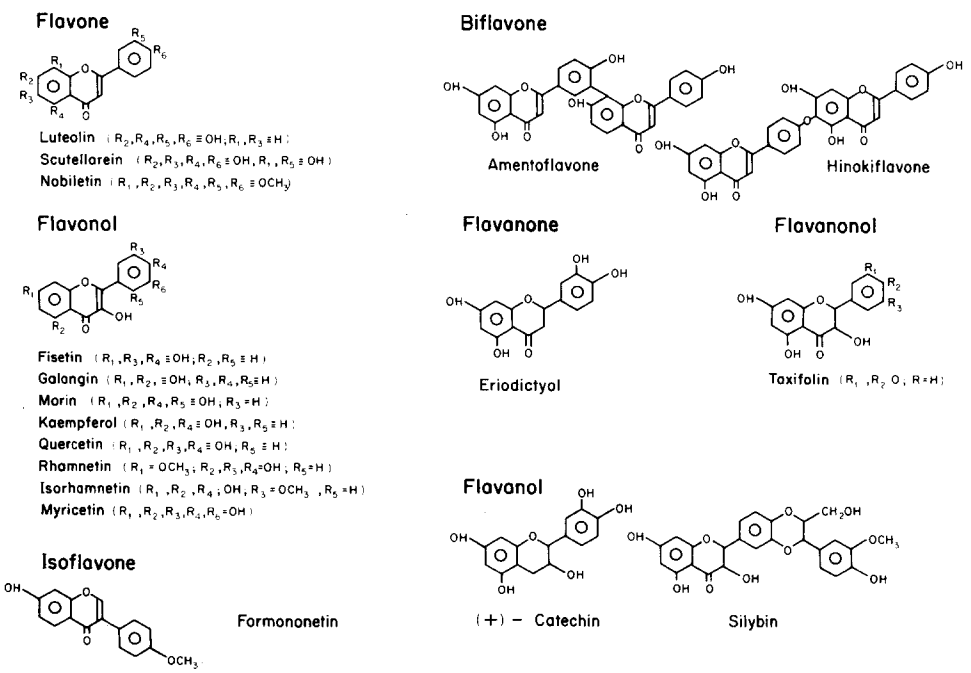

Fig. 1. Structural formulae of flavonoids studied.

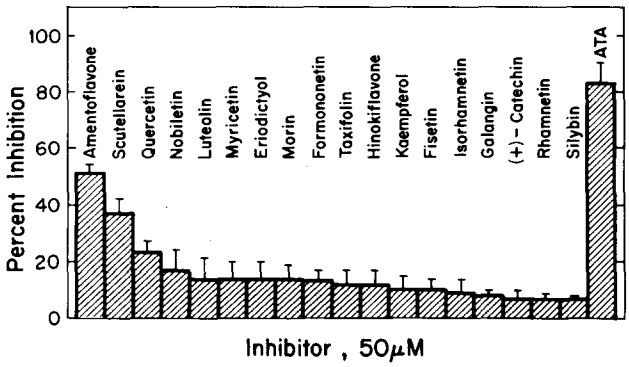

Fig. 2. Inhibition of AMV-RT by flavonoids and aurintricarboxylic acid (ATA; a known RT inhibitor). The control (0% inhibition) contained 3305±445 cpm.

3A. The approximate IC_{50} values for amentoflavone and scutellarein were 50 and 70 μM, respectively.

Effect of amentoflavone, scutellarein and quercetin on RAV-2 RT

Since amentoflavone and scutellarein were shown to possess some inhibitory activity against AMV RT we examined the effect of these flavonoids and quercetin on another avian RT, RAV-2 RT. As shown in Fig. 3B, each compound caused a concentration-dependent inhibition of the RAV-2 RT. Moreover, scutellarein was considerably more active at 10 μM and 20 μM concentrations against RAV-2 RT as compared to its effect on AMV RT. Approximate IC_{50} values for amentoflavone and scutellarein were 70 μM and 30 μM, respectively. Also, quercetin appeared slightly more inhibitory against RAV-2 RT as compared to AMV RT.

Effect of amentoflavone, scutellarein and quercetin on MMLV RT

The findings with these three flavonoids using AMV and RAV-2 RTs suggested that these compounds might also affect a viral RT in a mammalian system. To test this possibility we examined their effect on the RT obtained from MMLV. As seen in Fig. 3C, each flavonoid produced a concentration-dependent inhibition of the enzyme. Interestingly, the effect of the compounds on MMLV RT was greater than their effect on the avian RTs. For each flavonoid, the IC_{50} was approximately 10 μM.

We wondered whether MMLV RT could utilize mammalian mRNA as template with oligo$(dT)_{12-18}$ as primer for the synthesis of new DNA in the presence of the four required nucleotides. The results shown in Fig. 4 indicate very clearly that this system is capable of synthesizing new DNA. Moreover, amentoflavone and scutellarein produced marked inhibition of the MMLV RT in this reaction system.

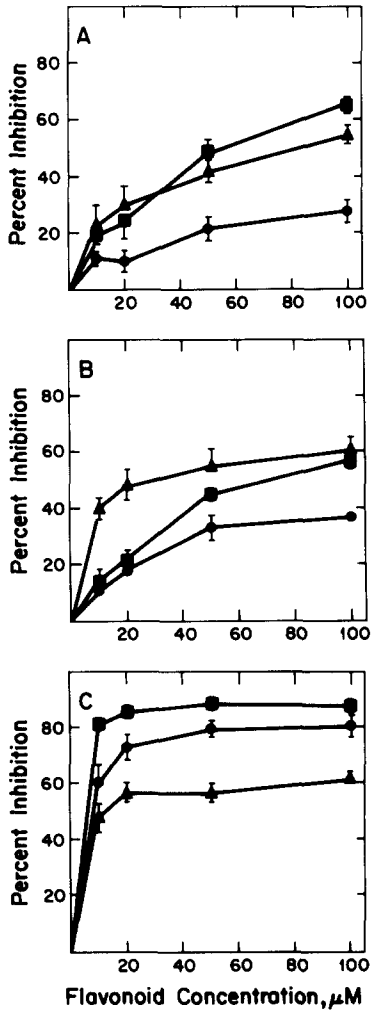

Fig. 3. Inhibition of (A) AMV RT, (B) RAV-2 RT and (C) MMLV RT by amentoflavone (■), scutellarein (▲) and quercetin (●). The controls (0% inhibition) contained (A) 3320±745 (SD) cpm, (B) 3527±445 cpm and (C) 417±95 cpm.

Inhibition of ongoing RT activity

To study the mechanism of inhibition of RAV-2 RT by amentoflavone and scutellarein, we studied the effect of adding the flavonoids after the RT reaction had been initiated. The conditions were as described above for RAV-2 RT. As seen in Fig. 5, the addition of amentoflavone at 3 min after reaction initiation led to a gradual decrease over the next 5 min of the rate of ^{3}H-dTMP incorporation into the TCA insoluble material. After 8 min no further label was incorporated. Fig. 5 also shows the effect of adding scutellarein at 3 min into the ongoing reaction.

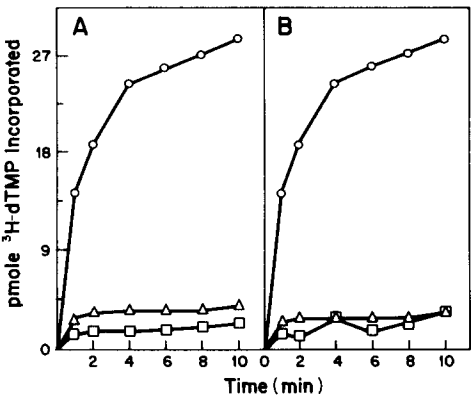

Fig. 4. Effect of 0 (○), 50 (△) and 100 μM (□) of scutellarein (A) and amentoflavone (B) on MMLV RT activity in the presence of rabbit globin mRNA and oligo(dT)$_{12-18}$ as primer instead of poly(rA)oligo(dT)$_{12-18}$ template-primer. Specific activity of ^{3}H-dTTP was 222 dpm/pmole.

It can be seen that scutellarein was less effective than amentoflavone in decreasing the rate of ^{3}H-dTMP incorporation and that the scutellarein effect did not plateau up to 14 min.

Studies of the mechanism of inhibition by amentoflavone and scutellarein

Kinetic studies were performed varying the concentration of dTTP and the poly(rA)oligo(dT)$_{12-18}$ template-primer in the presence (50 μM) and absence of

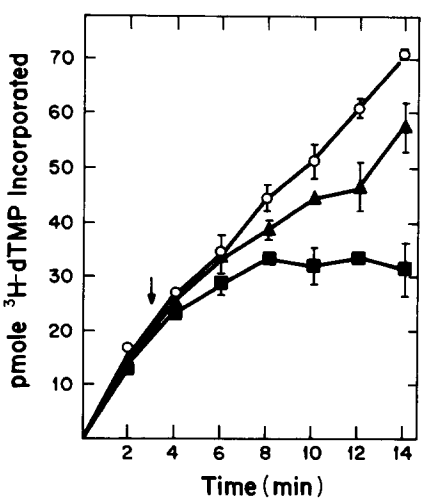

Fig. 5. Time course of RAV-2 RT inhibition by 50 μM amentoflavone (■) and scutellarein (▲). The arrow indicates time of addition of the flavonoids or ethanol to the control (○). Results presented as mean ± SEM. Specific activity of ^{3}H-dTTP was 178 dpm/pmole.

flavonoids. These assays were done at substrate concentrations with little or no deviation from linearity. The amount of ^{3}H-dTMP incorporated was proportional to incubation time and amount of enzyme. AMV RT and RAV 2 RT were used for these studies with varying concentrations of dTTP; RAV-2 was employed for experiments utilizing varying concentrations of template primer. The nature of the inhibition and the kinetic constants were determined from Lineweaver-Burke and Hanes-Woolf plots (Segel, 1975).

As seen in Fig. 6 the data for AMV with dTTP as substrate suggests a noncompetitive type of inhibition with scutellarein and an uncompetitive type of inhibition with amentoflavone. The Hanes-Woolf plot seen in the insert in Fig. 6 supports this interpretation of the inhibition mechanism. The Km value obtained for AMV RT was 0.2 mM with respect to dTTP as substrate and the apparent Km values with amentoflavone and scutellarein were 0.08 mM and 0.20 mM, respectively.

By comparison, using RAV-2 RT, we found the inhibition pattern caused by amentoflavone and scutellarein suggestive of an uncompetitive inhibition mechanism. The Km values were 0.25 mM for the enzyme and the apparent Km value with amentoflavone was 0.07 mM and with scutellarein, 0.10 mM (data not shown).

In other experiments using RAV-2 RT we analyzed the mechanism of inhibition with varying concentrations of poly(rA)oligo(dT)$_{12-18}$ template-primer. As seen in Fig. 7 amentoflavone at a concentration of 50 μM appeared to produce a mixed type inhibition. With 50 μM scutellarein the plot did not permit a conclusion regarding the mechanism of inhibition.

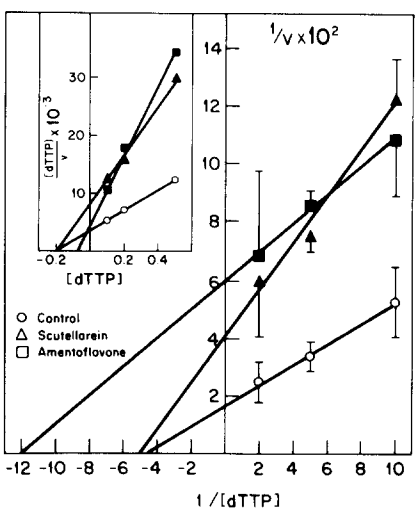

Fig. 6. Lineweaver-Burke plot of AMV RT (○) and the effects of 50 μM amentoflavone (□) and scutellarein (△) on the enzyme. Insert shows the Hanes-Woolf plots. Assay conditions were as described under 'Effect of flavonoids on AMV RT' except that 2.0 U of AMV RT enzyme was used. The velocity (v) and [dTTP] were expressed as pmole ^{3}H-dTMP incorporated/nmol and mmol, respectively. Specific activity of ^{3}H-dTTP was 88 dpm/pmole.

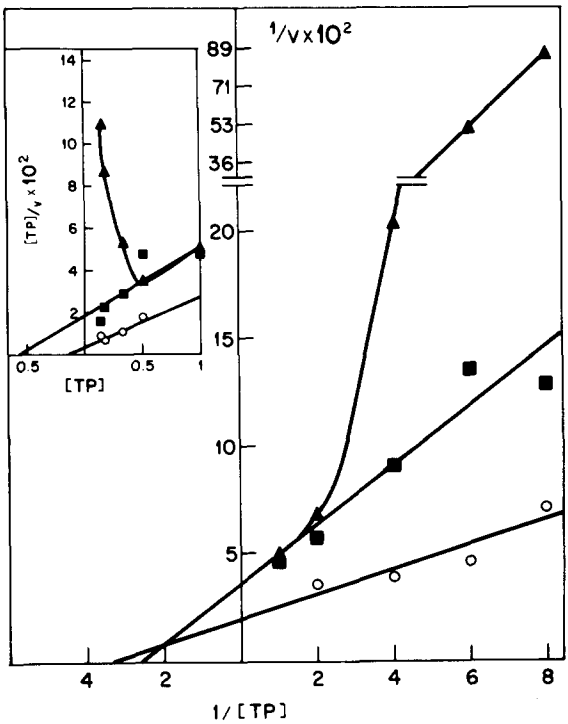

Fig. 7. Lineweaver-Burke plot of RAV-2 RT (○) and the effects of 50 μM amentoflavone (■) and scutellarein (▲) on the enzyme. Insert shows the Hanes-Woolf plots. Assay conditions were as described under 'Effect of flavonoids on RAV-2 RT' except that 1.0 U of RAV-2 RT and 0.2 mM dTTP were used. The velocity (v) and concentration of template-primer [TP] were expressed as pmole ^{3}H-dTMP incorporated per min and μg/assay, respectively. Specific activity of ^{3}H-dTTP was 178 dpm/pmole.

Discussion

The present experiments unequivocally demonstrate that certain naturally occurring flavonoids possess anti-reverse transcriptase (RT) activity. This is a potentially very important observation since RT is the enzyme critically involved in retrovirus replication within infected cells. The present observations demonstrate that a biflavone, amentoflavone (a dimer of apigenin), scutellarein, a tetrahydroxy flavone, and quercetin, a pentahydroxy flavone (see Fig. 1) possess antireverse transcriptase activity.

In our initial experiments, 18 flavonoids were examined for their anti-RT activity using AMV RT, an enzyme commonly employed to screen inhibitors of RT. Amentoflavone and scutellarein were found to possess moderate inhibitory activity at a concentration of 50 μM followed by somewhat less activity with quercetin. Therefore, these three flavonoids were further studied with the RAV-2 and MMLV RTs.

Three different reverse transcriptase preparations, i.e., AMV, RAV-2 and

mMLV RTs were found to be inhibitable by several flavonoids in a concentration-dependent manner. The most active compounds were amentoflavone, scutellarein and quercetin. The flavonoids were somewhat less active than the known reverse transcriptase inhibitor, aurintricarboxylic acid, in the experiments with AMV RT. Of interest is the fact that the three different reverse transcriptase enzymes exhibited differing degrees of sensitivity to the flavonoids. AMV RT was least sensitive, RAV-2 of intermediate sensitivity, and mMLV RT was the most sensitive. In view of the apparently increased sensitivity of the RAV-2 and MMLV RTs to amentoflavone, scutellarein and quercetin (Fig. 3), further screening with additional flavonoids is clearly called for not only with the RTs we have studied but also with human retroviral RTs. As each of these RT preparations utilizes the same reactants (enzyme, template-primer poly(rA)oligo$(dT)_{12-18}$ and dTTP) the reason for the differential sensitivity of the enzymes to flavonoid inhibition is not clear.

The finding that catalysis of the synthesis of new DNA by the mammalian MMLV RT in the presence of rabbit globin mRNA, primer [oligo$(dT)_{12-18}$] and the necessary nucleotides, could be inhibited by the flavonoids, amentoflavone and scutellarein was of interest. Indirectly, this finding suggests that other mammalian retroviral RTs might be inhibited by selected flavonoids.

From the present experiments no clearcut structure-activity relationships emerged. For example, while amentoflavone, a biflavone which is a C-C dimer, was an active inhibitor of AMV RT (51% at 50 μM), hinokiflavone, which is a C-O-C or ether-linked dimer, possessed only weak activity (12% at 50 μM). Similarly, scutellarein was the only one of three flavones to exhibit activity (37%) as compared to luteolin (14%) and nobiletin (17%). Of the seven flavonols tested, inhibition of AMV RT by 7–23% was observed. The other flavonoids representing different chemical classes (Fig. 1) inhibited by 14% or less. It should be noted that preliminary observations were reported in abstract form in 1975 (Fischer et al., 1975; Apple et al., 1975) suggesting that flavonoids bearing certain structural features could inhibit oncornavirus RT. No specific flavonoids were described, however.

We attempted to ascertain the mechanism of action of amentoflavone and scutellarein inhibition of AMV and RAV-2 RTs and found that amentoflavone inhibition was uncompetitive and that scutellarein caused a noncompetitive inhibition of AMV RT with respect to dTTP (Fig. 6).

The Lineweaver-Burke plot of RAV-2 RT activity with respect to varying concentrations of poly(rA)oligo$(dT)_{12-18}$ template-primer showed that amentoflavone was a mixed type inhibitor (Fig. 7). This finding was supported by the Hanes-Woolf plot (insert, Fig. 7). On the other hand, for scutellarein we could not identify the type of inhibition from the Lineweaver-Burke and Hanes-Woolf plots. The Lineweaver-Burke plot did, however, suggest that high concentrations of poly(rA)oligo$(dT)_{12-18}$ were associated with decreased inhibition by scutellarein. Although atypical, this inhibition could be construed as being competitive. In comparable experiments of Eriksson et al. (1982), inhibition of AMV RT by imidodiphosphonate exhibited similar, nonlinear, double-reciprocal plots also suggesting competitive-like inhibition.

We also examined the effect of adding amentoflavone or scutellarein to an ongoing reaction catalyzed by RAV-2 RT. Amentoflavone inhibited the further incorporation of ^{3}H-dTMP 5 min after addition to the reaction mixture. Likewise, scutellarein also inhibited the incorporation of label into the ongoing reaction; however, at no time up to 14 min did the effect of scutellarein plateau as in the case of amentoflavone (Fig. 5). This also suggests that the mechanism of action of the two flavonoids may differ.

We have attempted to develop a model based on the assumption that reverse transcriptases are processive rather than distributive enzymes (Gregerson et al., 1980). The processive hypothesis proposes that the template-primer and enzyme are continuously associated throughout the addition of nucleotides to the growing new DNA daughter strand.

Based on the arguments of Eriksson et al. (1982), our data suggest that scutellarein might inhibit RAV-2 RT competitively with respect to template-primer (Fig.7). Scutellarein, however, was less potent as an inhibitor of an ongoing reaction (Fig. 5) compared to its potent inhibitory activity when present at the beginning of an RT-catalyzed reaction (Fig. 3B). This observation could be accounted for if template-primer and enzyme become associated in a spatial manner which reduces the possibility of the flavonoid to block the template-primer-enzyme interaction. We postulate, therefore, that the site of action of scutellarein on RT is at the binding site of the enzyme for template-primer. The ability of scutellarein to uncompetitively inhibit the enzyme with respect to dTTP could result from its interaction at the template-primer binding site.

We found amentoflavone to cause noncompetitive inhibition of RAV-2 RT with respect to dTTP and mixed type inhibition with respect to template-primer. Therefore, the interaction of amentoflavone with the enzyme indirectly affects both the dTTP and template-primer binding sites. Perhaps this accounts for the ability of amentoflavone to effectively turn off an ongoing DNA synthetic reaction (Fig. 5).

Because of the potential implications of these findings with respect to human diseases caused by retroviruses we wished to report our early results on the effect of flavonoids on three different nonhuman reverse transcriptases. It is obviously essential that the selectivity of the flavonoid effects on reverse transcriptases be determined. Thus we plan to study their possible activity on cellular DNA polymerases and ultimately on their ability to inhibit retroviral infection of selected cells in tissue culture. Finally, it will be necessary to examine their properties as potential inhibitors of retroviral infection in animal models.

In summary, it is clear that certain naturally occurring flavonoids can inhibit reverse transcriptases of different origins. The mechanism of action of different flavonoids differs depending on the source of the enzyme. It is likely that other naturally occurring and synthetic flavonoids will be found which will possess anti-RT activity. The present observations suggest that flavonoids should be further studied for their potential as antiretroviral agents.

Acknowledgements

This work was supported in part by grants from the Margaret Duffy and William Cameron Troup Fund of the Buffalo General Hospital and from Tsumura Juntendo, Inc.

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