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INHIBITION OF HIV-1 INTEGRASE BY FLAVONES, CAFFEIC ACID PHENETHYL ESTER (CAPE) AND RELATED COMPOUNDS

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Abstract—The inhibition of HIV-1 integrase by flavones and related compounds was investigated biochemically and by means of structure–activity relationships. Purified enzyme and synthetic oligonucleotides were used to assay for three reactions catalysed by integrase: (1) processing of 3′ termini by cleavage of the terminal dinucleotide; (2) strand transfer, which models the integration step; and (3) "disintegration," which models the reversal of the strand transfer reaction. Inhibitions of all three reactions by flavones generally occurred in parallel, but caffeic acid phenethyl ester (CAPE) appeared to inhibit reaction 2 selectively. CAPE, however, inhibited reactions 1 and 3 effectively when preincubated with the enzyme, suggesting that this compound differs from the flavones primarily in requiring more time to block the enzyme. The core integrase fragment consisting of amino acids 50–212 retained the ability to catalyse reaction 3, and flavones and CAPE retained the ability to inhibit. Hence, the putative zinc-finger region that is deleted in this fragment is probably not the target of inhibition. Inhibition by flavones usually required the presence of at least one ortho pair of phenolic hydroxyl groups and at least one or two additional hydroxyl groups. Potency was enhanced by the presence of additional hydroxyl groups, especially when present in ortho pairs or in adjacent groups of three. Inhibitory activity was reduced or eliminated by methoxy or glycosidic substitutions or by saturation of the 2,3 double bond. These structure–activity findings for flavones were generally concordant with those previously reported for reverse transcriptase and topoisomerase II. These findings are discussed in the context of a review of the effects of flavones on various enzymes, the possible mechanisms of inhibition, and the potential for building upon a general pharmacophore to generate target specificity.

Key words: HIV, AIDS, integrase, flavone, caffeic acid esters

HIV-1‡ infection remains a worldwide health concern with, presently, only a limited armamentarium of clinically active antiretroviral agents [1, 2]. After viral RNA is processed by reverse transcriptase, the life cycle of the virus proceeds with the integration of the double-stranded DNA transcript into the host genome, a process that is mediated by retroviral integrase [3, 4]. Integrase presents an attractive possibility as an antiviral target because host cells do not make or require such enzymes. Although some HIV gene products may be expressed in the absence of integrase [5], and the possibility of HIV replication in some cell types in the absence of integrase has not been eliminated, recent evidence shows that HIV replication in T-lymphoid cells requires integrase function [6, 7]. The ability to block integrase function by means of drugs, therefore, could be a useful addition to a therapeutic armamentarium.

Our previous investigation of potential integrase inhibitors focused on DNA binders and topoisomerase blocking drugs [8]. Many DNA binders were found to inhibit HIV integrase, perhaps due to a non-specific block of DNA function. Topoisomerase inhibitors, such as camptothecin and epipodophyllotoxins, which do not bind DNA, did not inhibit HIV integrase. However, some other compounds that do not bind DNA were found to inhibit HIV integrase, e.g. the flavone quercetin and a natural product made by bees, CAPE.

Integrase acts in two steps. The first step processes the linear viral DNA by removing two nucleotides from each 3′ end, leaving recessed 3′-OH termini. The second step, strand transfer, is a transesterification of phosphodiester bonds in which a host DNA strand is cut, and the 5′ end of the cut is joined to a processed viral 3′ terminus. These two steps were assayed together in a model reaction using labeled oligonucleotides of viral terminal sequence in a cell-free system devised by Craigie *et al.* [9].

In the current work, we have added assay variations to measure the strand transfer reaction directly and to measure the reverse reaction which has come to be called "disintegration" [10]. We have carried out a structure–activity study of flavones and

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‡ Abbreviations: HIV-1, human immunodeficiency virus type 1; CAPE, caffeic acid phenethyl ester; MOPS, 4-morpholinepropanesulfonic acid; TBE, Tris-borate and EDTA.

related compounds. In addition, we report kinetic studies on the relative inhibition of the processing and strand transfer steps, and a study of the effects of inhibitors on a truncated functional enzyme.

MATERIALS AND METHODS

Materials

HIV-1 integrase protein (2 pmol/reaction), produced via an *Escherichia coli* expression vector as described [11], was obtained through the generosity of Dr R. Craigie and stored at −70° in protein storage buffer [1 M NaCl, 20 mM HEPES, pH 7.6, 1 mM EDTA, 1 mM dithiothreitol, and 20% glycerol (w/v)]. CAPE was supplied by Dr Dezider Grunberger, Columbia University, New York City, NY. All flavones not available through the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, NCI, were obtained through the Indofine Chemical Co., Somerville, NJ.

Oligonucleotide substrates

Oligonucleotides were obtained from the Midland Certified Reagent Co., Midland, TX, and were purified by HPLC before use. The following complementary oligonucleotides were used as substrates:

AE118: 5′-GTGTGGAAAATCTCTAGCAGT
AE117: 5′-ACTGCTAGAGATTTTCCACAC
AE119: 5′-GTGTGGAAAATCTCTAGCA
AE146: 5′-GGACGCCATAGCCCCGGCGCG-GTCGCTTTC
AE156: 5′-GTGTGGAAAATCTCTAGCAGG-GGCTATGGCGTCC
AE157: 5′-GAAAGCGACCGCGCC

The blunt-end oligonucleotide was 5′ end-labeled with ^{32}P by reacting AE118 (1 μL of 0.1 mg/mL) with [^{32}P]ATP and polynucleotide kinase at 37° for 45 min followed by 15 min at 85° to inactivate the kinase. It was subsequently slowly annealed by mixing with 1 μL of 0.4 mg/mL of AE117. Unincorporated nucleotides were separated from labeled oligonucleotide by passage through a G-25 quick-spin column (Boehringer Mannheim). The precleaved oligonucleotide was created in a similar manner by substituting oligonucleotide AE119 for oligonucleotide AE118. The "Y" oligonucleotide was made by labeling AE157 with polynucleotide kinase as described for the blunt-end and precleaved oligonucleotides. After heat inactivation of the kinase, this was annealed with equimolar amounts of oligonucleotides AE146, AE117 and AE156 as described (see Fig. 1).

HIV integrase reactions

The stock enzyme (0.44 mg/mL) was first diluted 1:3 in protein storage buffer. Subsequent enzyme dilution was performed 1:20 into reaction buffer to give 50 mM NaCl, 1 mM HEPES, 50 μM EDTA, 50 μM dithiothreitol, 10% glycerol, 7.5 mM $MnCl_2$, 0.1 mg/mL BSA, 10 mM β-mercaptoethanol and 25 mM MOPS, pH 7.2. Reaction volume was 16 μL. All reactions were done in 10% DMSO to enhance the reaction efficiency and as a universal drug solvent. Reactions were for 1 hr with ^{32}P-labeled double-stranded oligonucleotide (0.2 pmol). Reactions were stopped by the addition of an equal volume of Maxam–Gilbert loading buffer to each sample. Subsequently, 4 μL of each sample was run on a 20% denaturing polyacrylamide gel in 1 × TBE buffer. Quantitation of radioactivity on dried gels was accomplished by means of a Betascope 603 blot analyser (Betagen Corp., Waltham, MA). The displayed images were obtained by autoradiography on Kodak XAR-2 film.

Preincubation reactions

HIV-1 integrase (in protein storage buffer) was preincubated with drugs or DMSO for the indicated times at a concentration of 0.044 mg/mL in 15 mM $MnCl_2$, 200 μg/mL BSA, 20 mM β-mercaptoethanol before final dilution into reaction buffer by addition of DNA substrate and MOPS to give the 16 μL reaction buffer.

Quantitation

Dried gels were analysed using a Betascope 603 blot analyser. Radioactivity was counted in the 19-mer cleavage band, the larger integration bands (to avoid interference from the 21-mer origin band), and the lane total. Percent inhibition was calculated as:

$$100 \times [1 - (D - C)/(N - C)]$$

where C, N and D are fractions of 21-mer converted to 19-mer or integration products for DNA alone, DNA + integrase, and DNA + integrase + drug, respectively. The IC_{50} values were calculated from a sigmoid model using the formula:

$$y = [100\, x^n]/[(IC_{50})^n + x^n]$$

where x is the concentration of the compound tested, y is the percent inhibition, and n is the Hill coefficient that was set at 1.2, a mean [12]. The fraction disintegrated and the fraction of labeled 15-mer ligated to produce a 30-mer (see Fig. 1) were also calculated.

RESULTS

HIV integrase catalytic assays

The previously employed assay for integrase inhibitors, devised by Craigie *et al.* [9], included the 3′-processing step and the subsequent strand transfer step within the same reaction tube [8]. To obtain a direct assay of the strand transfer step, we utilized an oligonucleotide duplex lacking the 3′-dinucleotide that is ordinarily removed by the processing step [13]. This construct served as direct substrate for strand transfer. In addition, we assayed for inhibition of reverse strand transfer ("disintegration") [10, 14].

The three assays employed are diagrammed in Fig. 1. In the first (left panels), a 21-mer blunt-end oligonucleotide corresponding to the U-5 end of the HIV-1 proviral DNA, 5′ end-labeled with ^{32}P, is reacted with purified HIV-1 integrase. The initial enzyme step processes the 3′ end of the 21-mer by removing a terminal dinucleotide, yielding a labeled 19-mer product. The second or strand transfer step joins the recessed 3′ end to the 5′ end of a strand break in the target DNA. The strand break is generated by the enzyme as part of the strand

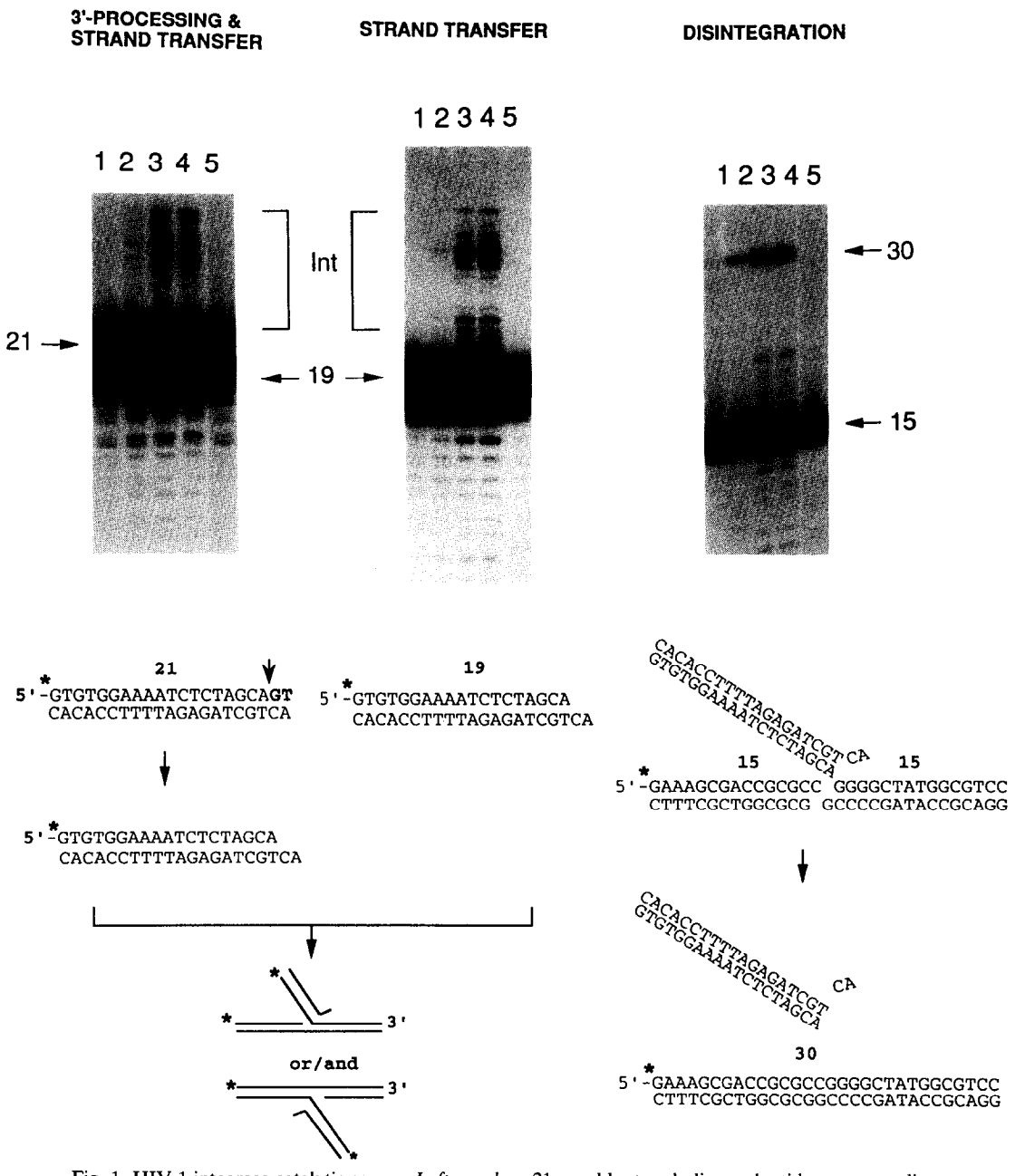

Fig. 1. HIV-1 integrase catalytic assays. *Left panels:* a 21-mer blunt-end oligonucleotide corresponding to the U-5 end of the HIV-1 proviral DNA, 5′ end-labeled with ^{32}P, is reacted with purified HIV-1 integrase. The initial enzyme step involves nucleolytic cleavage of two bases from the 3′ end, resulting in a 19-mer oligonucleotide. The second or strand transfer step involves joining these recessed 3′ ends to the 5′ end of an integrase-induced break in another identical oligonucleotide, which serves as the target DNA. Electrophoresis in a 20% denaturing acrylamide gel allows separation of the reaction products into integration products (Int) larger than 21 nucleotides, the original 21-mer oligo, and the 19-mer cleavage product. Hence, this assay (Fig. 1, left panel) is able to analyse both the 3′-processing (cleavage) and the strand transfer steps. *Middle panels:* a 5′ end-labeled oligonucleotide, where the GT dinucleotide product of the initial nucleolytic cleavage step has been removed, is used to selectively assay the strand transfer step of the enzyme. The target is again another identical oligonucleotide. Electrophoresis visualizes the integration products (Int) and the original oligonucleotide (19-mer). *Right panels:* the starting oligonucleotide mimics the product of a strand transfer step, a Y oligonucleotide containing a 15-mer oligonucleotide 5′ end-labeled with ^{32}P. HIV-1 integrase mediates the strand transfer to a second 15-mer when "disintegration" occurs. Thus, on a denaturing gel, the initial substrate appears as a 15-mer, while the disintegration product appears as a 30-mer. In all three assays, results were quantitated using a β-emissions detector and were expressed as fraction processed and percent inhibition. An asterisk (*) indicates the position of the ^{32}P label. Quercetagetin was tested at 10, 1 and 0.1 μM (lanes 1, 2 and 3). Lane 4 in each gel represents the control lane (solvent DMSO), while lane 5 in each gel represents DNA in the absence of enzyme.

Table 1. Structure–activity of flavones against HIV-1 integrase
(A) Active compounds*

Flavones	IC_{50} Cleavage (μM)	IC_{50} Integration (μM)	$\frac{IC_{50}\ CL}{IC_{50}\ INT}$	Substitutions								
				3	5	6	7	8	2′	3′	4′	5′
Quercetagetin	0.8 ± 0.3	0.1 ± 0.1	6.9	OH	OH	OH	OH			OH	OH	
Baicalein	1.2	4.3			OH	OH	OH					
Robinetin	5.9 ± 1.9	1.6 ± 0.7	3.7	OH			OH			OH	OH	OH
Myricetin	7.6 ± 0.6	2.5 ± 1.0	3.0	OH	OH		OH			OH	OH	OH
Quercetin	23.6 ± 6.6	13.6 ± 3.4	1.7	OH	OH		OH			OH	OH	
Fisetin	28.4 ± 6.5	8.5 ± 3.6	3.3	OH			OH			OH	OH	
Luteolin	32.9 ± 9.4	25.0 ± 8.6	1.3		OH		OH			OH	OH	
Myricetrin	39.6 ± 14.1	10.3 ± 5.0	3.8	RH	OH		OH			OH	OH	OH
NSC 339192	51.7 ± 10.5	22.6 ± 6.2	2.3		OH		†				OH	
Quercetrin	60.0 ± 11.7	38.5 ± 4.4	1.6	RH	OH		OH			OH	OH	
Rhamnetin	61.6 ± 8.2	28.7 ± 5.5	2.1	OH	OH		MeO			OH	OH	
Avicularin	66.3 ± 29.5	25.1 ± 14.5	2.6	A	OH		OH			OH	OH	
Gossypin	69.7 ± 17.9	22.5 ± 4.8	3.1	OH	OH		OH	GL		OH	OH	
Morin	76.5 ± 19.6	31.7 ± 3.1	2.4	OH	OH		OH		OH		OH	
6-MeO Luteolin	94.3 ± 17.0	39.1 ± 4.8	2.4		OH	MeO	OH			OH	OH	
Kaempferol	97.8 ± 9.2	64.7 ± 18.1	1.5	OH	OH		OH				OH	

Abbreviations: A = arabinose; GL = glucose; and RH = rhamnose.

* The IC_{50} ± SD values ($N \geq 3$) for cleavage and integration reactions and the ratio of IC_{50} cleavage to IC_{50} integration are listed. Ring substitutions are on the flavone structure, as shown in Fig. 2. MeO indicates methoxy substitution.

† See Fig. 2.

transfer reaction in which a phosphodiester bond in the target strand is cleaved while a new phosphodiester bond is created, joining target DNA with integrating DNA. This occurs through a single displacement reaction at phosphorus, as evidenced by inversion of chiral configuration in a model thiophosphate structure [13].

Electrophoresis in a 20% denaturing acrylamide gel allows separation of the reaction products into strand transfer products larger than the original 21-mer, and 3′-processing products that would be of 19-mer size. Hence, this assay (Fig. 1, left panel) detects the 3′-processing and strand transfer steps simultaneously in a single reaction system.

The second assay (Fig. 1, middle panels) employs a staggered duplex oligonucleotide in which the 5′ end-labeled strand lacks the 3′ terminal GT and serves as direct substrate for strand transfer. In this reaction, as in the previous combined assay, the same oligonucleotide serves as target and as integrating species. Electrophoresis visualizes only the integration products and the original oligonucleotide.

In the third assay (Fig. 1, right panels), the starting oligonucleotide models the strand transfer product. HIV-1 integrase is reacted with a Y-shaped oligonucleotide containing a 15-mer oligonucleotide 5′ end-labeled with ^{32}P. In exact reversal of the integration reaction, the enzyme cleaves one branch of the "Y" and joins the labeled 15-mer to the stem of the "Y," thereby creating a 30-mer labeled product. Thus, on a denaturing gel, the initial substrate appears as a 15-mer, while the disintegration product appears as a 30-mer.

Structure–activity relationship

A variety of flavonols (3-hydroxyflavones) and related compounds were tested for their ability to inhibit 3′-processing and strand transfer by HIV integrase. The compounds examined in this survey were available from commercial sources or from the Developmental Therapeutics Program of the National Cancer Institute. The compounds are listed in Tables 1 and 2, and their chemical structures are indicated in Fig. 2. Table 1A includes compounds that inhibited 3′-processing or strand transfer with an IC_{50} of less than 100 μM. Table 1B lists compounds that produced weak or undetectable inhibitory activity at 100 μM.

(The following compounds produced detectable activity, but less than 50% inhibition at 100 μM: apigenen, orientin, demefline, isoquercetrin, kaempferol-3,4′-methyl ether, and taxifolin. The other compounds listed in Table 1B produced no detectable inhibition at 100 μM.)

The most potent compound, quercetagetin, a hexahydroxyl flavone, had an IC_{50} below 1 μM both for 3′-processing and for strand transfer. Quercetagetin has hydroxyl substitutions at three adjoining carbons at positions 5, 6 and 7 on the benzopyranone ring and a pair of ortho hydroxyl substitutions at positions 3′ and 4′ on the phenyl ring. Next in potency were three compounds: robinetin, myricetin and baicalein. What these compounds have in common is three hydroxyl groups on adjoining carbons of an aromatic ring. The adjacent hydroxyls are either on the phenyl ring (robinetin and myricetin) or on the benzopyranone ring (baicalein). The only other compound tested that had three adjacent hydroxyls was myricetrin, which is like myricetin with a rhamnose substitution at position 3. The rhamnose substituent diminished the potency by a factor of 4–5.

The third most potent group of compounds

Table 1—continued.
(B) Inactive compounds (IC_{50} > 100 μM)‡

Flavones	Substitutions							
	3	5	6	7	8	3′	4′	5′
Apigenin		OH		OH			OH	
Orientin		OH		OH	GL	OH	OH	
Demefline	Me			MeO	CH_2NMe_2			
Isoquercetrin	GL	OH		OH		OH	OH	
Kaempferol-3,4′-Me ether	MeO	OH		OH			MeO	
Rutin	RU	OH		OH		OH	OH	
Sinensetin		MeO	MeO	MeO		MeO	MeO	
Pratol				OH			MeO	
Myricetin trimethyl ether	OH	OH		OH		MeO	MeO	MeO
3-OH flavone	OH							
7-OH flavone				OH				
Dimefline	Me			MeO	CH_2NMe_2			
Flavone acetic acid					CH_2COOH			
Quercetin tetramethyl ether	MeO	OH		MeO		MeO	MeO	
5-OH Flavone		OH						
Peltatoside	AG	OH		OH		OH	OH	
7-OH-3,4′-MeO flavone	MeO			OH			MeO	
4′,5,6,7-MeO flavone		MeO	MeO	MeO			MeO	
3-OH-3′,4′,5,7-MeO flavone	OH	MeO		MeO		MeO	MeO	
5,7-OH-3′,4′,5′-MeO flavone		OH		OH		MeO	MeO	MeO
3′-Benzyloxy-5,7-OH-3,4′-MeO flavone	MeO	OH		OH		OCH_2Ph	MeO	
Acacetin		OH		OH			MeO	
Chrysin		OH		OH				
Gardenin		OH	MeO	MeO	MeO	MeO	MeO	MeO
Genkwanin		OH		MeO			OH	
Luteolin tetramethyl ether		MeO		MeO		MeO	MeO	
Tangeritin		MeO	MeO	MeO	MeO		MeO	
6,4′-OH flavone			OH				OH	
3′,4′-Dihydroxy flavone						OH	OH	
6,7-OH flavone			OH	OH				
Genistein (isoflavone)		OH		OH				OH
Taxifolin (flavanone)	OH	OH		OH		OH	OH	

Abbreviations: AG = arabinoglucose; GL = glucose; and R = rutinose.

‡ Taxifolin is a flavanone and lacks the double bond between 2 and 3 (see Fig. 2). Genistein is an isoflavone in which the phenyl ring attaches at carbon 3 not 2 as in the flavones. Ring substitutions are on the flavone structure shown in Fig. 2.

Table 2. Effect of divalent cation cofactor on CAPE and quercetagetin inhibition of HIV-1 integrase full size (IN^{1-288}) and truncated (IN^{50-212}) enzymes

	Concn (μM)	Fraction disintegrated		
		IN^{1-288}		IN^{50-212}
		Mn^{2+}	Mg^{2+}	Mn^{2+}
DMSO (control)	0	0.098	0.014	0.018
Quercetagetin	0.1	0.107	0.010	0.013
	1	0.029	0.009	0.009
	5	0.003	0.006	0.002
	10	0.002	0.001	0.001
CAPE	25	0.087	0.014	0.016
	50	0.092	0.011	0.014
	100	0.018	0.010	0.008

The truncated protein was unable to disintegrate when magnesium was used as a cofactor. Results are from a typical experiment. In all samples, enzyme was preincubated with drug or DMSO for 20 min.

included quercetin, fisetin and luteolin, all of which have a pair of adjacent hydroxyls on the phenyl ring at positions 3′ and 4′. The other compounds with these ortho hydroxyls had various kinds of substitutions that were found to reduce activity.

In this regard, several variations of the quercetin structure were tested, and all diminished or abolished activity. Substitutions at position 3 with rhamnose (in the case of quercetrin) or arabinose (in the case of avicularin) diminished the potencies by factors of approximately 2–3. Substitutions at position 3 with glucose (isoquercetrin) or rutinose (rutin) almost completely inactivated the compounds (IC_{50} > 100 μM). Substitution at position 3 with arabinoglucose (peltatoside) gave a compound with no detectable activity. Substitution of a methoxy group at position 7 (rhamnetin) or addition of glucose to position 8 (gossypin) diminished the potencies by factors of approximately 2–3. Saturation of the 2,3 double bond gives the flavanone, taxifolin, which was nearly inactive.

Some similar variations on luteolin diminished or abolished activity. The addition of a methoxy group

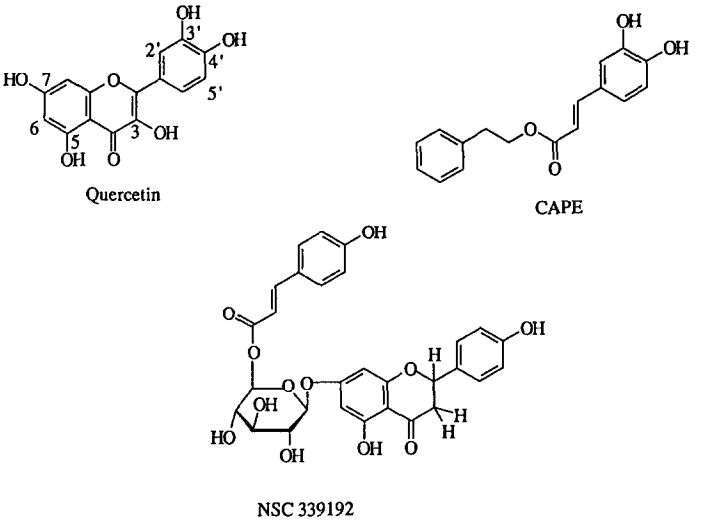

Fig. 2. Structures of quercetin, CAPE, and NSC 339192. The numbering convention for flavones is shown on the quercetin structure.

at position 6 reduced the potency by a factor of approximately 1.5 to 3. Addition of glucose to position 8 (orientin) made the compound nearly inactive ($IC_{50} > 100\ \mu M$).

Although a pair of ortho hydroxyls appears to be a minimum requirement for high potency, it was not an absolute criterion for activity. The presence of a pair of ortho hydroxyls without any additional hydroxyl groups gave inactive compounds (3′,4′-dihydroxyflavone and 6,7-dihydroxyflavone). Hence, it seems that at least three hydroxyls are needed for activity. On the other hand, two tetrahydroxy compounds (morin and kaempferol) had detectable, albeit weak, activities despite the absence of an ortho pair.

The isoflavone genistein, a topoisomerase II inhibitor [15, 16], was inactive.

Variations in the potency ratios for inhibition of 3′-processing relative to inhibition of strand transfer by active flavones were observed (Table 1), but the available data do not suggest a structural correlate.

A moderately potent compound that did not seem to fit the above structure–activity principles was NSC 339192 (submitted in 1980 by W. Rahman, Aligarh, India, for testing in the NCI anticancer screen). This compound consists of a flavanone moiety joined via a sugar constituent to a CAPE-like moiety (Fig. 2). The molecule has only two hydroxyls and these are not in ortho configuration, but rather are on different ring systems. Computer modeling by K. Raghavan in our laboratory suggested that the molecule may fold so as to stack the two phenolic groups approximately above each other.

Relative inhibition of 3′-processing and strand transfer

Response curves for the inhibition of 3′-processing and strand transfer by selected flavones of different potencies are shown in Fig. 3. The flavones showed no marked deviations between inhibition of the two reactions.

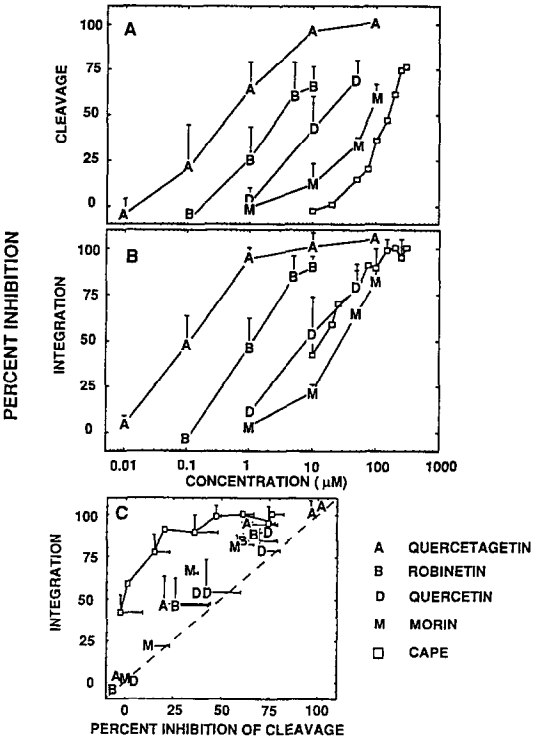

Fig. 3. Concentration–response curve for inhibition of HIV integrase cleavage (panel A) and integration (panel B) by quercetagetin (A), robinetin (B), quercetin (D), morin (M) and CAPE (□). In panel C, percent inhibition of cleavage vs percent inhibition of integration is shown. Reactions were for 1 hr at 30°. Enzyme was not preincubated with drugs. Values are means ± SEM, N ≥ 3.

Representative lanes from electrophoresis gels were analysed for integration patterns. It can be seen in Fig. 1 (lanes 2 and 3, left and center panels) that different integration sites were inhibited equally.

As previously reported [8], CAPE was unusual in that it was 10-fold more potent as an inhibitor of strand transfer ($IC_{50} = 19\ \mu M$) than as an inhibitor of 3′-processing ($220\ \mu M$) (Fig. 3C). In these experiments, inhibitor was added at the start of the two-step reaction. For further investigation, therefore, CAPE was included as well as the most potent of the flavones tested, quercetagetin.

Precleaved oligonucleotide

The previous assays of strand transfer were indirect, because the 3′-processing and strand transfer steps were combined. To obtain a direct assay of integration, the precleaved 19-mer oligonucleotide was used as substrate (Fig. 1). This direct assay showed that CAPE and quercetagetin inhibit the strand transfer step (Fig. 1). The combined evidence from the two assays demonstrated that CAPE and quercetagetin inhibit both 3′-processing and strand transfer.

Disintegration

In disintegration assays employing the "Y" oligonucleotide, CAPE was inactive at concentrations up to $100\ \mu M$. This was unexpected because disintegration should be merely a reversal of the integration step. In contrast, quercetagetin was a potent inhibitor of disintegration (Fig. 1).

Altering the relative enzyme concentration over a 10-fold concentration range had no detectable effect on the level of inhibition of 3′-processing, strand transfer or disintegration by CAPE or quercetagetin.

Effects of preincubation

The origin of these diverse results was explored in experiments in which the enzyme was preincubated with drug prior to the addition of oligonucleotide substrate. Preincubation was for 20 min in the presence of Mn^{2+} and β-mercaptoethanol. Control preincubations included DMSO solvent without drug. Preincubation of CAPE with HIV integrase greatly enhanced the inhibition of 3′-processing, but only modestly enhanced the inhibition of integration (Fig. 4). Under preincubation conditions, therefore, the apparent selectivity of CAPE for inhibition of strand transfer was greatly diminished. Moreover, the disintegration reaction, which was not detectably inhibited by CAPE in the previous assay, was clearly inhibited when enzyme and drug were preincubated together (Fig. 5).

In the case of quercetagetin, the inhibition of 3′-processing and strand transfer were nearly unaffected by preincubation (Fig. 4), whereas the inhibition of disintegration was diminished (Fig. 5).

Preincubation of HIV-1 integrase in the absence of drug consistently produced a small increase in the efficiency of the disintegration reaction (Fig. 5), but the magnitude of this effect was small compared with the effects on drug-induced inhibition.

The time-dependence of the preincubation effect was evaluated. In these experiments, CAPE was added to the reaction at times between 10 min before to 5 min after the addition of the 21-mer double-stranded oligonucleotide (Fig. 6). Inhibition of 3′-processing required CAPE to be added at or before the addition of oligonucleotide. At $25\ \mu M$ CAPE, the magnitude of inhibition increased with duration of preincubation up to 10 min.

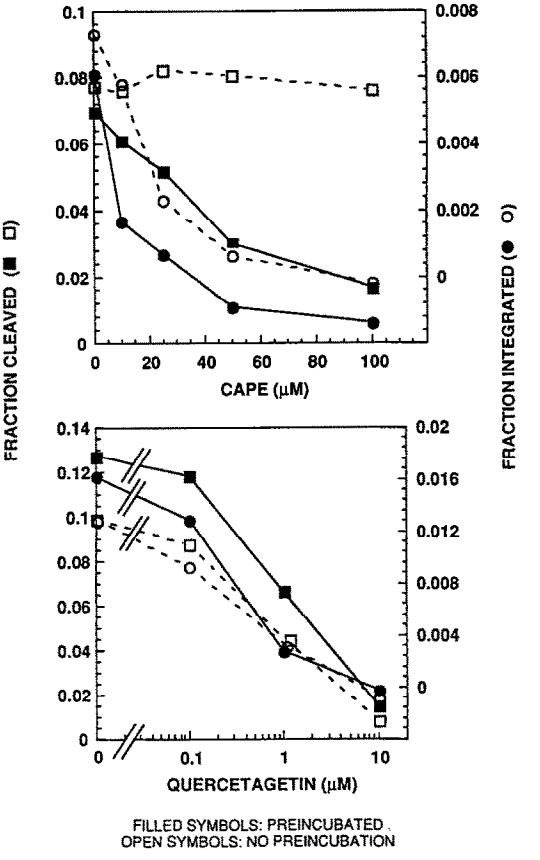

Fig. 4. Effect of preincubation on inhibition of HIV integrase cleavage and integration by CAPE (top) and quercetagetin (bottom). Squares represent fraction of total DNA cleaved to 19-mer. Circles represent fraction of total DNA converted to integration products. Either DNA, enzyme and drug were added simultaneously (open symbols), or enzyme was preincubated with drug for 20 min before adding DNA (filled symbols). Control points (DMSO solvent without drug) are means of at least three independent determinations, and these fell within a range of ±15% of the indicated value.

Kinetic studies of the HIV integrase reactions in the absence of drug (10% DMSO only) revealed that (under these conditions of excess enzyme) the 3′-processing and disintegration reactions began immediately, while there was a 4–6 min lag before integration products began to appear (Fig. 7).

These results suggest that the apparent selectivity of CAPE for the inhibition of the integration step, when the inhibitor was added at the start of a sequential cleavage–integration process, may have been an indirect effect. The action of CAPE on the

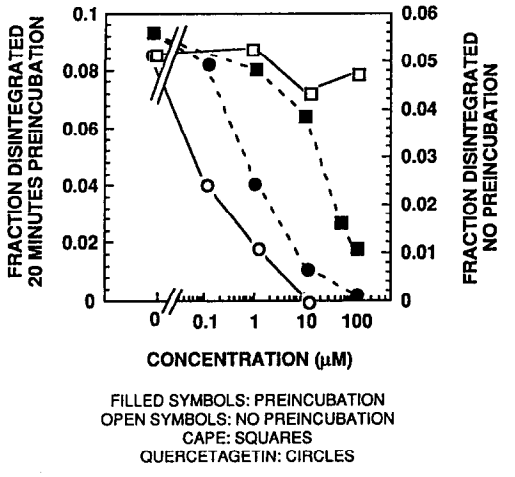

Fig. 5. Effect of preincubation on inhibition of HIV integrase disintegration by CAPE and quercetagetin. The enzyme was preincubated with drug or DMSO for 20 min. A representative experiment is shown.

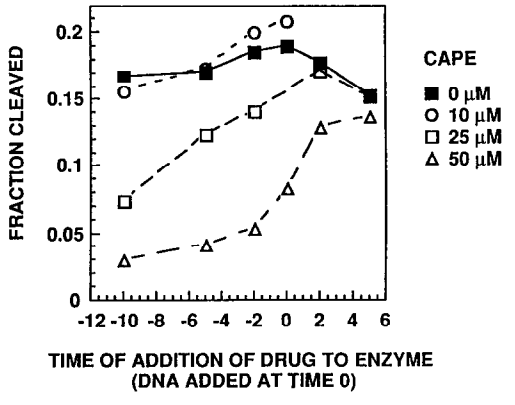

Fig. 6. Effect of time of addition of CAPE on the cleavage reaction. CAPE was added to HIV-1 integrase at the time (given in minutes) indicated on the abscissa. The 21-mer double-stranded oligonucleotide was added at time zero. Reactions were continued for 20 min in the presence of enzyme, inhibitor and oligonucleotide. The fraction of 21-mer cleaved to 19-mer is plotted on the ordinate. The control reactions (0 μM CAPE) contained DMSO solvent.

enzyme may take a few minutes to become effective. Thus, 3′-processing and disintegration reaction, which begin without delay, may be well under way by the time the inhibitory action of CAPE becomes effective. The integration reaction, on the other hand, is delayed 4–6 min due to the requirement for prior cleavage, and therefore may commence after CAPE has had time to exert its effect on the enzyme.

Effect of divalent cation

The HIV-1 integrase functions with either manganese or magnesium as its required divalent cation. Reaction efficiency, however, is better with manganese [17]. Since manganese may be better able to coordinate with phenolic hydroxy groups of the inhibitors, we tested whether the inhibition of the integrase by CAPE or quercetagetin depends upon the type of divalent cation. This was most easily tested in disintegration reactions where either manganese or magnesium can serve as the divalent metal cofactor, and where the low background allows accurate quantitation. The efficiency of the reaction was about eight-fold less with magnesium than with manganese. Disintegration reactions were performed after 20 min of preincubation to optimize the inhibitory effects of CAPE and quercetagetin. Both CAPE and quercetagetin remained active inhibitors of disintegration when magnesium was used as a cofactor (Table 2). The IC_{50} for inhibition of disintegration for CAPE using magnesium as a cofactor was 155 μM, whereas for manganese it was 65 μM. The corresponding values for quercetagetin were 0.8 μM with magnesium and 0.4 μM with manganese (data not shown). Thus, in addition to the integrase being more efficient when manganese rather than magnesium was used as a cofactor, CAPE and quercetagetin were more efficient as inhibitors (by a factor of 2) when used with manganese than with magnesium.

Truncated integrase

Because of the previous suggestion that some inhibitors may interact with the zinc-finger domain of the enzyme, we tested the effects of inhibitors on an active enzyme fragment that lacks this domain. The truncated enzyme consists of amino acids 50–212, which includes the DD-35-E region, but lacks the C-terminal zinc-finger region, as well as part of the N-terminal region [18]. Although the truncated enzyme has lost 3′-processing and strand transfer activities, it retains disintegration activity [18]. Quercetagetin and CAPE were tested for their ability to inhibit disintegration with or without preincubation. Manganese was used as divalent metal cofactor, since the truncated enzyme was inactive with magnesium as cofactor. CAPE inhibited the truncated enzyme, but, as in the case of the intact enzyme, only when preincubated (Fig. 8). Quercetagetin inhibited the truncated enzymes with or without preincubation, as was the case with the intact enzyme. Inhibition of disintegration by quercetagetin and CAPE, therefore, does not require the zinc-finger domain. Also tested was dihydroxynaphthoquinone, which had been found to inhibit HIV integrase and whose structure suggested possible chelation to zinc [8]; this compound, however, also was an effective inhibitor of the zinc-finger-less truncated enzyme (data not shown).

DISCUSSION

Structure–activity relationships

Naturally occurring bioflavonoids, including flavones such as quercetin and its relatives, have been reported to exert a remarkable range of biochemical effects. A number of structure–activity studies in various systems have been reported, but there seems

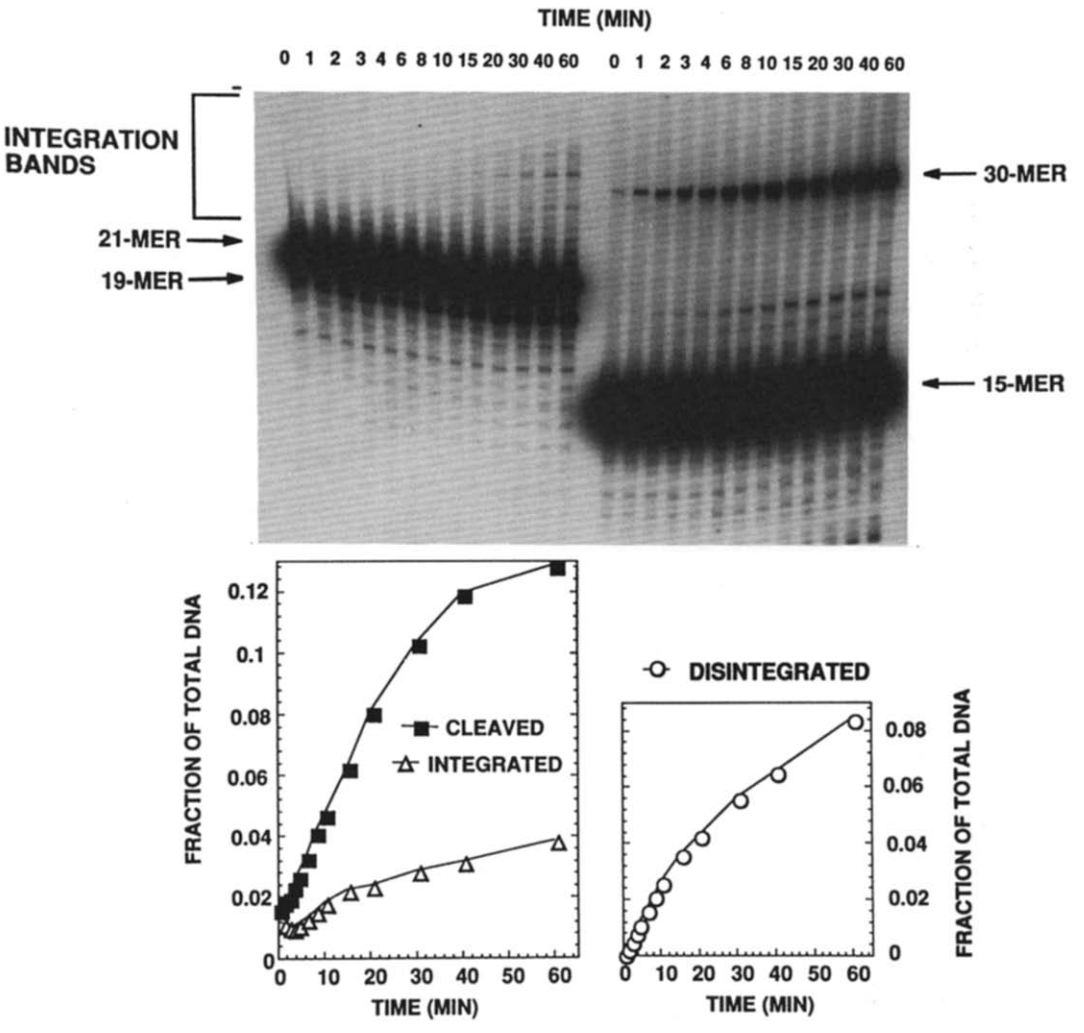

Fig. 7. Kinetics of HIV-1 integrase-mediated DNA cleavage and integration (left panels) and disintegration (right panels) reactions. Enzyme was added at time 0. Aliquots were removed at various times and reactions stopped by the addition of Maxam–Gilbert "blue" solution. Lower panels plot the fraction of DNA cleaved, integrated or ligated (disintegrated).

to have been no recent attempt to integrate these findings. The structure–activity findings for flavones in the current study of the inhibition of HIV integrase can be summarized as follows: (1) activity required the presence of at least three hydroxyl groups; (2) potency was enhanced in proportion to the number of ortho hydroxyl pairs, and especially when three adjacent hydroxyls were present in the same aromatic ring; and (3) activity was reduced or eliminated by the presence of glycosyl or methoxy substituents. This applies to both steps of the integrase reaction: 3′-processing and strand transfer. It should be noted, however, that not all possible positions of substitution were covered in the present study. The importance of ortho hydroxyls on an aromatic ring is consistent also with the activity of CAPE which, in addition, shares with the flavones an analogously located keto group.

The flavones consist of a planar system of three aromatic rings with polar groups appended at various positions. The planarity, aromaticity and polarity may allow these compounds to bind by stacking with adenine or guanine, or to compete with purine moieties for binding to enzyme sites. Many of the molecules also have oxidation–reduction and metal chelation capacities. A considerable literature has accumulated concerning various actions of flavonoids, and these accumulated data allow some suggestive interpretations in relation to our integrase inhibition data.

Stacking interactions are at the heart of DNA intercalation. Intercalative interactions were demonstrated by means of helix twist assays for quercetin, quercetagetin, myricetin and baicalein [15], and these were among the most potent integrase inhibitors listed in Table 1. Extensive untwisting was observed at 50 μM but not at 5 μM concentrations of these flavones, indicating low binding affinity, as

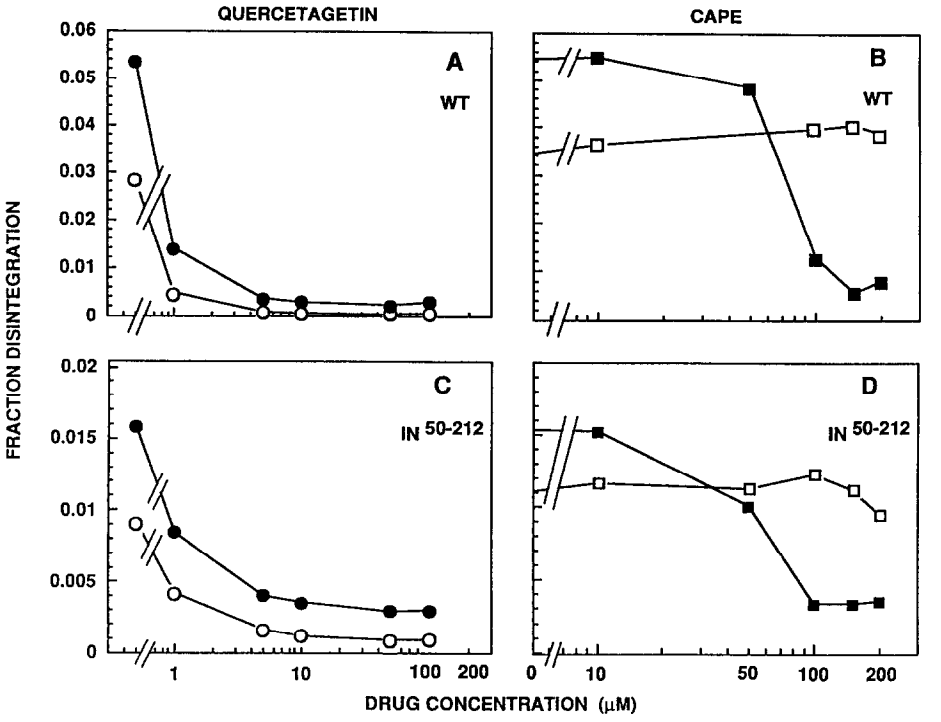

Fig. 8. Effects of CAPE and quercetagetin on disintegration activity of wild-type (WT) (IN^{1-288}) and (IN^{50-212}) mutant HIV-1 integrase under standard and preincubation conditions. CAPE (squares, panels B and D) and quercetagetin (circles, panels A and C) were tested without (open symbols) or with 20 min of preincubation (closed symbols) for the ability to inhibit either the wild-type (panels A and B) or truncated (panels C and D) integrase protein.

expected for small uncharged polycyclic DNA binders. Stacking with DNA bases at the active site could play a role in the mechanism by which these compounds inhibit integrase. This would be analogous to a proposed mechanism by which specific drugs block topoisomerases by stabilizing cleavable complexes [19–22]. The proposed mechanism involves stacking of drug with DNA bases and interaction between drug and enzyme. An analogous proposal could apply to the interaction of flavones with integrase, even though some details of enzyme mechanisms differ significantly, particularly in that the integrase does not form covalent DNA–enzyme intermediates.

Austin *et al.* [15] reported the enhancement of topoisomerase II DNA cleavable complexes by several flavonoids. Their structure–activity findings are, in most respects, coherent with our findings for HIV integrase. Quercetin, quercetagetin, myricetin, baicalein and luteolin were effective in blocking topoisomerase II, and these are high on our list of potent integrase inhibitors (Table 1). Chrysin, which has only 2 OH groups, had no detectable effect on either enzyme, nor did gardenin or rutin, which bear methoxy or glycosyl substituents. Quercetrin, a glycosylated derivative of quercetin, has reduced activity against topoisomerase II [15, 23], as well as against HIV integrase (Table 1). The only discrepancy between the studies of the two enzymes concerned ortho hydroxyls, a configuration indicated by our data to be essential for potent inhibition of integrase. Although there was agreement for morin, which bears five hydroxyls without an ortho pair and had reduced activity against both enzymes, the results for apigenin and possibly also kaempferol were discrepant. Apigenin, with only three hydroxyls and no ortho pair, exhibited little inhibition of HIV integrase at 100 μM (Table 1B), yet was reported to be an active topoisomerase II blocker. Kaempferol, with four hydroxyls and no ortho pair, exhibited low potency against integrase (Table 1A), but was listed as active against topoisomerase II [15]. Despite these perhaps minor discrepancies, the available data are consistent with a similarity of structure–activity dependence for the inhibition by flavones of HIV integrase and topoisomerase II.

This similarity of structure–activity dependence among flavones, however, does not extend to isoflavones, since the isoflavone genistein blocks topoisomerase II, producing cleavable complexes [16, 24] (Pommier *et al.*, unpublished data), but in the current study genistein showed no activity against HIV integrase. Moreover, deviations between activity against the two enzymes were found with other types of chemical agents, notably the topoisomerase II inhibitors etoposide, teniposide and amsacrine, which showed no significant inhibition of HIV integrase [8].

Another enzyme for which the structure–activity dependence for inhibition by flavones appears to be similar to that reported here for HIV integrase is reverse transcriptase. Ono *et al.* [25] studied reverse transcriptase from Rausher murine leukemia virus and HIV-1, and reported that the most potent group of flavones (50% inhibition at <0.5 μg/mL) included myricetin > quercetin > quercetagetin > baicalein, in that order. Less potent (about 50% inhibition at 2–5 μg/mL) were luteolin and quercetrin. Slight activity was noted for morin, rutin and 6,7-dihydroxyflavone. No detectable inhibition was found for apigenin, kaempferol, chrysin, 3-hydroxyflavone or 7-hydroxyflavone. Except for the difference in potency order of the compounds in the most potent group, these results are coherent with our findings with HIV integrase.

More quantitative results were reported by Chu *et al.* [26] for reverse transcriptase from Moloney murine leukemia virus. Although there was general agreement for the relative potencies of most of the compounds tested with respect to our integrase data, the LD_{50} values were usually nearly two orders of magnitude lower. The reverse transcriptase assay, however, was conducted at pH 8.3 and 3 mM Mg^{2+}, whereas our integrase assays were at pH 7.2 and 7.5 mM Mn^{2+}. Since the flavones differ in basicity and metal chelation ability, more work is needed to compare the magnitudes of the effects on the different enzymes under constant conditions.

Results with DNA polymerases have deviated from the pattern of general consistency described above [27]. Differences in structure–activity dependence were observed among the DNA polymerases, but this might have been due, in part, to differences in pH and divalent metal ion conditions, which were optimized for each enzyme. Such differences in assay conditions might also have contributed to the minor discrepancies observed in the structure–activity dependencies between HIV integrase, topoisomerase II and reverse transcriptase.

An inverted order of potency among flavones was reported for the NADPH-dependent monooxygenase activities of two dealkylases in rat liver microsomes [28]. Potency of inhibition of these enzyme activities *decreased* with increasing number of hydroxyl groups. We do not have a ready explanation for this inversion of the more usual structure–activity pattern.

The inhibitions of eukaryotic DNA polymerases α, β and γ and of *E. coli* RNA polymerase by various flavones have been found to be competitive with respect to polynucleotide template and non-competitive with respect to nucleoside triphosphates [27]. Similarly, the inhibition of reverse transcriptase from Rous murine leukemia virus was competitive for polynucleotide template, but not for nucleoside triphosphates; this was demonstrated for quercetin, baicalein, quercetagetin and myricetin [25]. For HIV reverse transcriptase, however, the inhibition was not purely competitive with respect to template-primer, suggesting that the exact structure of the active site of an enzyme may influence the details of mechanism.

Quercetin was found to inhibit the kinase $pp60^{src}$ competitively with respect to ATP or GTP, but non-competitively with respect to protein substrate [29]. The kinetic K_i constants for inhibition (assayed at pH 7.1, 10 mM Mg^{2+}) were about 10-fold higher than for reverse transcriptase. Competitive inhibition with respect to ATP by quercetin has also been described for phosphatidylinositol-3-kinase [30, 31] and for Ca^{2+}-ATPase [32]. Competitive inhibition with respect to cAMP in cAMP phosphodiesterase has been reported for quercetin and fisetin [33]. In addition, flavones may inhibit DT-diaphorase competitively for NADH [12, 34].

Another class of ATP-dependent enzymes inhibited by flavones are ion-transporting ATPases, including the plasma membrane Ca^{2+} pump [35]. The order of activity for inhibition of the latter was myricetin > luteolin > robinetin, while quercetin and several other flavonols were inactive. The limited data suggested that ortho hydroxyls favor inhibitory activity. Interestingly, the inhibition of erythrocyte Ca^{2+}-pumping ATPase by myricetin was enhanced markedly by preincubating the plasma membranes with the flavone [36].

This broad range of enzyme inhibitions by flavones thus seems to include different mechanisms, sometimes based on competition for binding of nucleotide and sometimes for binding of nucleic acid. Their ability to intercalate in DNA suggests that, in addition to binding to enzyme sites, the flavones may be able to interact directly with nucleic acid substrate, and possibly with nucleotides. In the case of integrase, there is no nucleotide cofactor participant in the reaction; hence, the flavones presumably interact with DNA binding sites on the enzyme and/or with the DNA itself. The interference could be with the donor or with the acceptor nucleotides, or both. Kinetic studies may help to resolve this question.

The chemical interaction of flavones may involve oxidation–reduction reactions and divalent metal chelation. This is especially true for compounds having ortho hydroxyl pairs (ortho quinones), a configuration that we found necessary for potent inhibition of integrase. The rates of auto-oxidation of flavones have been found to be highly pH dependent; this dependence was much stronger for myricetin than for quercetin and was enhanced markedly by FeEDTA [37]. This underscores the importance of pH and divalent metal ion when comparisons of structure–activity relations of flavones are made against different targets.

Our structure–activity data for HIV integrase are consistent with the ortho hydroxyl configuration engaging in an oxidation–reduction reaction at the active site of enzymes. The ortho configuration in flavone polyhydroxy compounds was found to favor the inhibition of mitochondrial NADH-oxidase (NADH-coenzyme Q oxidoreductase) [38] and to be essential for protection against cytotoxicity induced by hydrogen peroxide [39]. Inhibition of glutathione reductase (an NADPH-requiring enzyme assayed at pH 7.6 without added divalent metal ions) by flavones and related natural products seemed to be favored by the presence of ortho hydroxyls, although quercetagetin was anomalous in being nearly as low in potency as kaempferol [40]. Lipid peroxidation (enzymatic or non-enzymatic) is

inhibited by flavones, but did not seem to require ortho hydroxyls, although these were usually present in the most potent compounds [41, 42]. A final example of an oxidase-like enzyme inhibited by flavones is prostaglandin endoperoxide synthase [43]. For inhibition by flavones, this enzyme (assayed at pH 8.1) appeared to require either an ortho hydroxyl configuration or hydroxyls at positions 3 and 5, both of which, being adjacent to the keto group, would favor chelation. In the case of quercetin, the inhibition was competitive with respect to arachidonate, suggesting that the interaction is with the enzyme site that adds an oxygen atom to a double bond. These types of enzymes may oxidize ortho hydroxyls on flavones to form semiquinone radicals which conceivably could react with enzyme either directly or by way of superoxide or hydroxyl radicals. An oxidation–reduction or free-radical reaction could inactivate an enzyme irreversibly. It remains to be determined whether integrase enzyme activity would recover after separating the enzyme from an inhibitor flavone, and whether inhibition requires the presence of molecular oxygen.

A flavanone derivative that violated the ortho hydroxyl rule was NSC 339192 (Fig. 2), which we found to be a moderately potent integrase inhibitor (Table 1A). This interesting structure may bring together two hydroxyls from different phenyl rings in a stacked configuration.* Chelation, but not oxidation, would be possible in this configuration, which presents an interesting starting point for further development.

The remarkable versatility of flavones as enzyme inhibitors is underscored by their competitive inhibition of hyaluronidase, which has nothing obvious in common with the enzymes considered above [44]. In this case, ortho hydroxyls were not required for high potency. Quercetin has also been found to inhibit the transcriptional induction of heat shock proteins, probably by interacting directly with heat shock factor and blocking its binding to heat shock elements on DNA [45, 46].

Taking all of these data together, a plausible view of the inhibition of integrase by flavones would be that these compounds bind to enzyme sites that normally interact with DNA bases. Flavones would furthermore be capable of chelating a divalent metal ion bound to the enzyme and of reacting chemically, perhaps by way of a free radical mechanism, with essential groups at the site of enzyme activity. Alternatively, flavones may bind by stacking with DNA bases at the enzyme site and then reacting chemically or by chelation with the enzyme.

From the wide variety of enzymes inhibited by flavones, one might reasonably question their potential usefulness as specific inhibitors. On the other hand, the compounds generally have very low toxicity, and it seems possible that selectivity could be built in by judicious manipulation of chemical structure guided in part by structure–activity data. The structure–activity data define a simple pharmacophore that presumably can access active sites present in a variety of enzymes. Since these sites are unlikely to be identical in the different target enzymes, it seems likely that specificity could be induced by the addition of steric or functional groups to the essential pharmacophore structure.

Mechanism of action

Previous assays of integrase inhibitors combined the 3′-processing and strand transfer reactions [8]. The current work included assays that clearly separate these two steps, and also an assay for the reversal of the integration reaction ("disintegration" reaction). These assays showed that flavones and CAPE can inhibit all three of these reactions. In the case of CAPE, however, it was necessary to preincubate with the enzyme in order to demonstrate inhibition of 3′-processing or of disintegration (Figs. 4 and 5). It appears, therefore, that inhibition of integrase by CAPE occurs slowly over a period of a few minutes. Thus, the 3′-processing and disintegration reactions, which kinetic studies show to begin without delay (Fig. 7), are inhibited by CAPE only if assayed with preincubation. The flavones act rapidly, and the inhibition is, therefore, not much affected by preincubation. Strand transfer, on the other hand, is a delayed reaction (Fig. 7), and is inhibited by CAPE even without preincubation. These kinetic differences may explain why CAPE showed disproportionately strong inhibition of strand transfer relative to 3′-processing when the two steps were combined in a single assay system [8].

The prominence of ortho hydroxyl groups in the structure–activity relationships discussed above suggested oxidation–reduction and divalent metal chelation as important factors in the mechanism of inhibition of HIV integrase, as well as several other enzymes. An oxidation–reduction or free radical mechanism, however, would probably have to be local and protected within the micro-environment of the enzyme, because the inhibitions were not sensitive to the presence or absence of 10 mM 2-mercaptoethanol (data not shown), nor to superoxide dismutase or catalase (Mazumder A and Pommier Y, unpublished data). The tendency of CAPE to oxidize would be expected to be favored by the double bond conjugated to the phenyl ring; an analog structure synthesized without this double bond, however, retained the ability to inhibit the integrase (Burke T, Fesen M, Mazumder A, Kohn KW and Pommier Y, unpublished data).

A role of chelation is suggested by our finding that the inhibition of integrase by quercetagetin or CAPE was more effective when the assay included Mn^{2+} rather than Mg^{2+}. The chelation of Zn^{2+} in a putative zinc-finger region of HIV integrase is not necessary for inhibition, in view of our finding that the activity of a truncated integrase, which lacked the zinc-finger region, retained the ability to be inhibited by quercetagetin and CAPE (Fig. 8).

Previous studies describing the central core region of retroviral integrases have found several highly conserved amino acids whose mutation abolishes all activity, thus suggesting that these residues are critical to the function of the active catalytic site of the enzyme [18, 47–49]. These include and correspond to Asp 64, Asp 116 and Glu 152 of the HIV-1 integrase. These authors have noted the

* Computer modeling by K. Raghavan, personal communication. Cited with permission.

similarity between these amino acids of the integrase and the 3′–5′ exonuclease activity of the DNA polymerase I (Klenow fragment) which share similar conserved amino acids (Asp 355, Asp 501, Glu 357, and Tyr 497) and are also involved in phosphoryl transfer reactions employing divalent cations. Based on the crystal structure of the Klenow fragment, Beese *et al.* [50] have described a two-metal-ion phosphoryl transfer model of this reaction. A similar mechanism for the conserved acidic residues of the integrase has been suggested [47, 48]. The difference in inhibitory effect of CAPE or quercetagetin when Mn^{2+} rather than Mg^{2+} was used as a cofactor is consistent with this possibility, as is the retained inhibitory action of flavones and CAPE on the truncated integrase containing only amino acids 50–212 (Fig. 8). A further observation that fits with this site of action is the inhibition by quercetagetin of the 3′–5′ exonuclease activity of DNA polymerase I (Klenow fragment) (data not shown).

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