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Inhibition of HIV infection by flavanoids

Naheed Mahmood[a], Cosimo Pizza[b], Rita Aquino[b], Nunziatina De Tommasi[b], Sonia Piacente[b], Susan Colman[a], Andrew Burke[a] and Alan J. Hay[c]

[a]*MRC Collaborative Centre, 1-3 Burtonhole Lane, Mill Hill, London, UK,* [b]*Universita degli Studi di Napoli, Dipartmento di Chimice delle Sostanze Naturali, Via Domenico Montesano, Napoli, Italy and* [c]*National Institute for Medical Research, The Ridgeway, Mill Hill, London, UK*

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Summary

Of a variety of flavanoids, the flavans were generally more effective than flavones and flavanones in selective inhibition of HIV-1, HIV-2 or SIV infection. Studies of their effects on the binding of sCD4 and antibody to gp120 indicated that the effective compounds interact irreversibly with gp120 to inactivate virus infectivity and block infection.

Flavanoids; HIV; gp120-CD4 interaction

Introduction

Two classes of flavanoids can specifically inhibit the replication of picornaviruses by distinct mechanisms. On the one hand, various 4′-hydroxy-3-methoxyflavones such as 3-methyl quercetin block the replication of poliovirus, apparently by selectively inhibiting the synthesis of genomic RNA (De Meyer et al., 1991; González et al., 1990). On the other hand, certain flavan derivatives, e.g., 4′,6-dichloroflavan, are members of a diverse class of inhibitors of the replication of human rhinoviruses which interact specifically with the VP1 capsid protein to prevent virus uncoating (McKinlay and Rossmann, 1989; Ninomiya et al., 1985).

Various flavones have been shown to inhibit in vitro the reverse

Correspondence to: N. Mahmood, MRC Collaborative Centre, 1-3 Burtonhole Lane, Mill Hill, London NW7 1AD, UK.

transcriptases (RT) of certain retroviruses including human immunodeficiency virus (HIV) as well as cellular DNA polymerases (Ono et al., 1989, 1990). Whereas baicalen specifically inhibited HIV RT in vitro at concentrations which did not affect DNA polymerases α and β, myricetin inhibited DNA polymerase α and *E. coli* DNA polymerase I at comparable concentrations.

Here we report studies of the antiviral activities of a number of flavones, flavans and flavanones which showed that two flavones, including myricetin, and five flavan derivatives selectively inhibited HIV replication. Like a number of polyanionic compounds including sulphated polysaccharides, polyhydroxycarboxylates and various tannins (Baba et al., 1988; Mitsuya et al., 1988; Schols et al., 1990, 1991; Weiler et al., 1990; Nonaka et al., 1990; Weaver et al., 1992; Mahmood et al., 1993), these flavanoids interact with the surface glycoprotein gp120 to prevent binding of virus to the CD4 receptor.

Materials and Methods

Compounds

Flavones. **1**, Chrysin (5,7-dihydroxyflavone); **2**, baicalein (5,6,7-trihydroxyflavone); **3**, apigenin (4′,5,7-trihydroxyflavone); **4**, kaempferol (3,4′,5,7-tetrahydroxyflavone); **5**, morin (2′,3,4′,5,7-pentahydroxyflavone); **6**, quercetin (3,3′,4′,5,7-pentahyroxyflavone); and **7**, myricetin (3,3′,4′,5,5′,7-hexahydroxyflavone) were obtained from Sigma. **8**, Myricetin-3-*O*-rhamnoside; **9**, quercetin-3-*O*-rhamnoside; **10**, quercetin-3-*O*-arabinoside; and **11**, kaempferol-3-*O*-glucoside, were isolated from the methanol extract of *Befaria cinnamomea* and are reported for the first time. **12**, Quercetin-3-*O*-glucoside; **13**, quercetin-3-*O*-galactoside; and **14**, isorhamnetin (3,4′,5,7-tetrahydroxy-3′-methoxy-flavone) were isolated from the methanol extract of the leaves of *Myntostachis setosa* and are reported for the first time. **15**, Isorhamnetin-3-*O*-rutinoside; **16**, isorhamnetin-3-*O*-rutinoside-4′-*O*-glucoside; and **17**, isorhamnetin-3-*O*-rutinoside-7-*O*-glucoside were isolated from *M. annua* (Aquino et al., 1987).

Flavans. **18**, (+)Gallocatechin; and **19**, (−)epigallocatechin (3,3′,4′,5,5′,7-hexahydroxyflavan) were previously isolated from *Croton draconoides* (Aquino et al., 1991a). **20**, (−)epicatechin (3,3′,4′,5,7-pentahydroxyflavan; **21**, (+)catechin; **22**, (−)epicatechin-3-*O*-gallate and **23**, (+)catechin-7-*O*-gallate were isolated from *Detarium microcarpum* (Aquino et al., 1991b).

Flavanones. **24**, Hesperitin (3′,5,7-trihydroxy-4′-methoxy-flavanone); and **25**, naringenin (3,4′,5,7-tetrahydroxyflavanone) were obtained from Sigma. **26**, Narirutin (naringenin-7-*O*-rutinoside); and **27**, (2′,5,5′,7-tetrahydroxy-flavanone-7-*O*-rutinoside) were isolated from *Hamelia patens* (Aquino et al., 1990). **28**, 4′-methoxy-5,7-dihydroxyflavanone-7-*O*-rutinoside was isolated from *Myntostachis setosa* and is reported for the first time.

Plants

Myntostachys setosa (Labiatae) and *Befaria cinnamomea* (Ericaceae) were collected at Cordillera del Condor (Ayabaca Province), Perù, in August 1990 and were identified by Professor Ramon Ferreyra, Museo de Historia Natural 'J. Prado' de la Universidad Nacional Mayor de San Marcos, Lima. Voucher samples are deposited at the Herbarium of this Institute.

General procedures

The following instruments were used: HPLC, Waters 6000 A equipped with a refractive index detector; NMR Bruker 500; AMX-32 spectrometer; FAB-MS in negative ions mode, Kratos MS 902 mass spectrometer equipped with Kratos FAB source. The NMR spectra were recorded in CD_3OD.

Extraction and isolation

Air-dried leaves (0.5 kg), defatted in Soxhlet with light petroleum and $CHCl_3$, were extracted with MeOH at room temperature. The crude extracts were fractionated by a standard procedure for flavonoids as reported previously by Aquino et al. (1987; 1990). Pure compounds were obtained by semipreparative HPLC separation on a C-18 u-Bondapak column (30 cm × 7.8 mm). The purity (100%) and identity of compounds **8, 9, 10, 11, 12, 13, 14** and **28** were established by NMR and FAB-MS spectra by comparison with published data.

Antiviral assays

The anti-HIV and anti-SIV activities and toxicities of compounds were assessed in C8166 cells infected with HIV-1_{III-B}, HIV-2_{Rod} or SIV_{Mac}. Cells were grown in RPMI 1640 with 10% foetal calf serum. 4×10^4 cells per microtiter plate well were mixed with 5-fold dilutions of compound prior to addition of 10 $CCID_{50}$ (50% cell culture infectious dose) units of virus and incubated for 5–7 days. Formation of syncytia was examined from 2 days post-infection. Gp120 antigen produced after 5–7 days was measured by ELISA, using the lectin GNA (from *Galanthus nivalis*) to capture the glycoprotein and human anti-HIV serum for detection, as described by Mahmood and Hay (1992). The EC_{50} is the concentration of compound in μg/ml which reduced the production of gp120 by 50%. Cell viability of virus-infected and uninfected control cells was measured by the MTT-Formazan method as described by Pauwels et al. (1988). The TC_{50} is the concentration of compound which reduced the viability of uninfected cells by 50%.

Antiviral activity against herpes simplex virus type 1 (HSV-1) (strain 17-I) was determined by measuring viral antigen produced in infected Vero cells as described previously (Mahmood et al., 1993). 5-Fold dilutions of compounds were added to duplicate wells just before adding virus at a multiplicity of infection of 0.01 plaque-forming units per cell. After incubating 16–18 h at 37°C the cells were fixed with 3% formalin for 1–2 h and antigen detected by ELISA using rabbit anti-HSV-1 antibodies (Dakopatts, Denmark).

Infectivity assay

Virus was titrated in microtiter plates of C8166 cells using doubling dilutions of freshly collected supernatants from infected C8166 cells. The end point was determined by examining syncytium formation and by the MTT-Formazan assay and the virus titre ($CCID_{50}$) is expressed as the reciprocal of the dilution which gave a 50% end point. To measure the effects of compound on virus infectivity, HIV-1_{IIIB} (10^5 – 10^6 $TCID_{50}$) was incubated with compound at room temperature for 1 h, the virus was serially diluted and the infectivity end-point determined. In all cases compound was diluted to well below the EC_{50} such that residual compound did not interfere with the virus titration.

Gp120-sCD4 and gp120-antibody-binding assays

The ELISA assays were done as previously described (Mahmood and Hay, 1992; Mahmood et al., 1993). Briefly, the lectin GNA (Vector Laboratories) was bound to microtitre plate wells to capture gp120 from extracts of HIV-1 infected C8166 cells. Various dilutions of compound were added and after incubating for 60 min at 37°C, sCD4 was added either in the presence of compound or after its removal. The binding of sCD4 was monitored using the anti-CD4 monoclonal antibody OKT4 and anti-mouse Ig conjugated to alkaline phosphatase. Binding of anti-gp120 monoclonal antibodies ADP323, ADP358, ADP360 and ADP388 (supplied by the MRC AIDS reagent project, NIBSC, Potters Bar, Herts) was assayed in a similar manner, in the presence of various concentrations of compound.

Results and Discussion

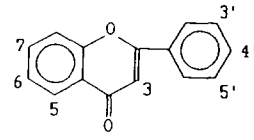
Antiviral activity

As a group the flavans exhibited the greatest selective anti-HIV-1 activity (Table 1), only one of the six tested proving to be inactive. There was a good correlation between the reduction in gp120 production, measured by ELISA, and the degree of protection against virus-induced cell death, determined using the MTT-formazan assay (Pauwels et al., 1988) (Fig. 1). The galloyl derivative, (−)epicatechin-3-*O*-gallate **22**, consistently exhibited the greatest activity, EC_{50} of 1 μg/ml and selectivity index >100, and was somewhat more active than (−)epicatechin **20**. Differences between isomers were noted in the lower activity of (+) catechin **21** and particularly dramatically in the absence of any selective activity of (−)epigallocatechin **19** in contrast to (+)gallocatechin **18**. Substitution of the hydroxyl group at position 7 of (+)catechin **21** by a gallate moiety in **23** caused a reduction in antiviral activity and increase in cytotoxicity (Fig. 1, Table 1).

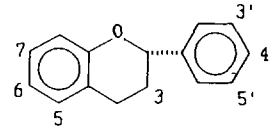
Of seventeen flavones (unsaturated pyrone ring) tested, only two – **7** and **11** – caused significant inhibition of HIV-1 infection at non-toxic concentrations. The selective activity of myricetin **7**, selectivity index = 20, contrasted with the inactivity of quercetin **6**, which differed only in the absence of a 5′ hydroxyl,

TABLE 1

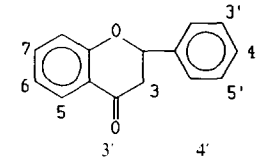
Anti-HIV-1 activities of flavanoids



Flavone	3	5	6	7	3'	4'	5'	EC_{50}	TC_{50}
1	H	OH	H	OH	H	H	H	20	50
2	H	OH	OH	OH	H	H	H	inactive	50
3	H	OH	H	OH	H	OH	H	inactive	2
4	OH	OH	H	OH	H	OH	H	inactive	10
5	OH	OH	H	OH	2'OH	OH	H	inactive	100
6	OH	OH	H	OH	OH	OH	H	inactive	10
7	OH	OH	H	OH	OH	OH	OH	2	40
8	ORha	OH	H	OH	OH	OH	OH	100	>200
9	ORha	OH	H	OH	OH	OH	H	50	>100
10	OAra	OH	H	OH	OH	OH	H	inactive	>100
11	OGlc	OH	H	OH	H	OH	H	10	100
12	OGlc	OH	H	OH	OH	OH	H	inactive	>100
13	OGal	OH	H	OH	OH	OH	H	inactive	>100
14	OH	OH	H	OH	OCH_3	OH	H	inactive	>100
15	OGl-Rha	OH	H	OH	OCH_3	OH	H	inactive	>200
16	OGl-Rha	OH	H	OH	OCH_3	OGlc	H	inactive	>100
17	OGl-Rha	OH	H	OGlc	OCH_3	OH	H	inactive	100



Flavan	3	5	6	7	3'	4'	5'	EC_{50}	TC_{50}
18	(+)OH	OH	H	OH	OH	OH	OH	5	>80
19	(−)OH	OH	H	OH	OH	OH	OH	inactive	>100
20	(−)OH	OH	H	OH	OH	OH	H	2	>100
21	(+)OH	OH	H	OH	OH	OH	H	4	>100
22	(−)Ogallate	OH	H	OH	OH	OH	H	1	>100
23	(+)OH	OH	H	Ogallate	OH	OH	H	10	>100



Flavanone	3	5	6	7	3'	4'	5'	EC_{50}	TC_{50}
24	H	OH	H	OH	OH	OCH_3	H	inactive	15
25	H	OH	H	OH	H	OH	H	inactive	10
26	H	OH	H	OGlc-Rha	H	OH	H	inactive	16
27	H	OH	H	OGlc-Rha	2'OH	OH	H	inactive	>100
28	H	OH	H	OGlc-Rha	H	OCH_3	H	inactive	40

Ara, arabinose; Gal, galactose; Glc, Glucose; Rha, rhamnose.EC_{50}, concentration (μg/ml) which reduces by 50% the production of gp120 in infected C8166 cells. TC_{50}, concentration (μg/ml) which causes 50% cytotoxicity to uninfected C8166 cells. 'Inactive' indicates that EC_{50} and TC_{50} values were indistinguishable.

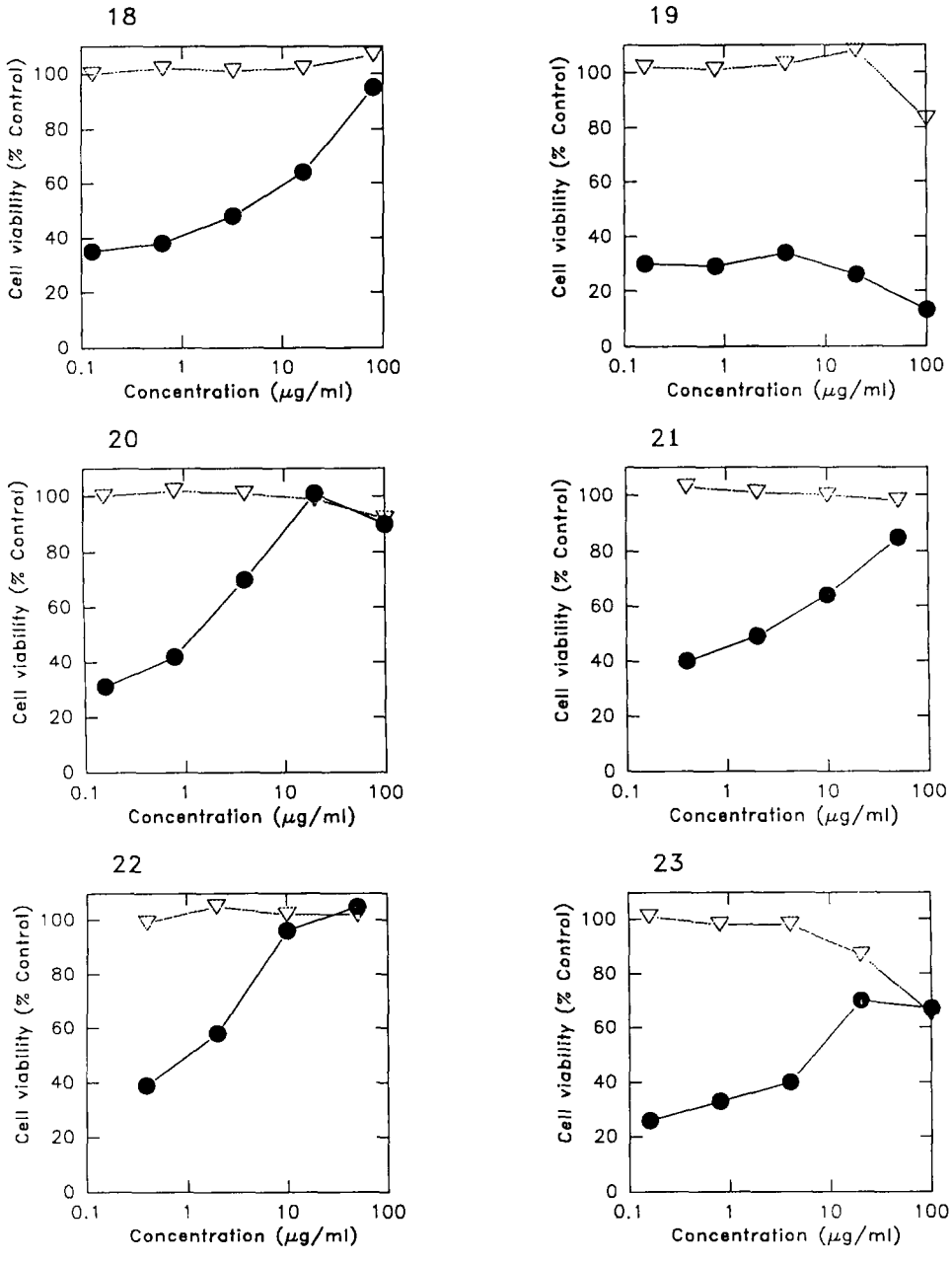


Fig. 1. Effect of flavans 18–23 on the viability of HIV-1-infected and uninfected C8166 cells. The viability of the cells was determined by the MTT -formazan assay 5 days after infection. (●) infected; (▽) uninfected.

indicating that all three hydroxyl groups at 3′, 4′ and 5′ positions of ring B are required for activity. The 3-*O*-rhamnosides of myricetin **8** and quercetin **9**

TABLE 2

Antiviral activities against HIV-2, SIV and HSV-1

Compound	HIV-2	SIV	HSV-1	Vero cells
	EC_{50}	EC_{50}	EC_{50}	TC_{50}
1	40	ND	10	>50
6	>50	>50	20	>50
7	2	2	2	>100
18	8	10	32	>80
19	>100	>100	>100	>100
20	2	2	10	>50
21	5	5	20	>50
22	1	1	1	>100
23	ND	ND	10	>100

C8166 cells were infected with HIV-2_{ROD} or SIV_{MAC} and Vero cells were infected with HSV-1. Values are in μg/ml.

exhibited only very slight selective antiviral activity. In contrast, glucosidation of position 3 of the pyrone ring of kaempferol **4**, which lacks a further 3′ hydroxyl on ring B, elicited selective anti-HIV activity in compound **11**, selectivity index = 10.

None of the flavanones (carbonyl at position 4 of the saturated pyrone ring) selectively inhibited HIV-1 replication. These compounds were generally more cytotoxic than the flavan and flavone derivatives studied.

The active compounds elicited a comparable effectiveness against HIV-2 and SIV infections of C8166 cells (Table 2) and compound **22** had the same EC_{50} against HIV-1 infected peripheral blood lymphocytes. Although somewhat less effective in general against herpes simplex virus infection of Vero cells (Table 2), it is apparent that the anti-viral activities of the flavanoids are not limited to lentivirus infections.

TABLE 3

Effect of the time of addition of compound

Compound	Concentration (μg/ml)	Cell viability (% of control)			gp120 production (% of control)		
		0 h	1 h	4 h	0 h	1 h	4 h
22	20	100	71	35	0.5	10	34
	4	64	49	26	20	32	52
DS_{500}	10	83	57	33	4	10	35
	2	39	31	20	35	45	83

DS_{500}, dextran sulphate molecular weight 500 000. Compounds were added to C8166 cells at the times indicated, relative to addition of HIV-1.

TABLE 4

Effect of compounds on virus infectivity

Compound	Concentration (μg/ml)	Virus titre ($TCID_{50} \times 10^{-3}$)
1	100	80
7	100	3
18	80	5
19	200	20
20	100	5
21	100	10
22	100	0.3
23	100	5
DS_{500}	100	160
Heparin	25	160
Control	0	160

Mechanism of action

Since (−)epicatechin-3-*O*-gallate **22** possessed the greatest anti-HIV activity it was selected for more detailed analysis of its antiviral action. Like dextran sulphate, compound **22** was much more effective when added prior to or at the time of virus infection (Table 3) indicating that they act at an early stage of infection. Pretreatment of cells with compound and removal prior to virus infection had little effect. This may account for its reported lack of selective activity when added to MT-4 cells after infection (Nakashima et al., 1992). Brief treatment of chronically infected H9 cells with 10 μg/ml of compound 22 reduced by greater than 90% syncytium formation with uninfected H9 cells, whereas similar treatment of the uninfected target cells prior to mixing had no effect. Unlike the action of dextran sulphate which was readily reversible on removal of drug, the flavanoids irreversibly inactivate virus infectivity. For example, incubation of virus with a 100 μg/ml of compound **22** for 60 min at room temperature reduced virus infectivity by as much as 500-fold, whereas

TABLE 5

Inhibition of gp120/sCD4-binding

Compound	Concentration μg/ml	% Inhibition	
		Washed*	Unwashed
22	20	89	97
	4	45	53
	0.8	35	38
DS_{500}	10	20	81
	2	8	76
	0.4	4	42

*Compound removed before addition of sCD4 to immobilised gp120.

TABLE 6
Inhibition of antibody-binding to gp120

Compound	Concentration (μg/ml)	% Inhibition of antibody binding			
		323	358	360	380
1	10	0	0	0	16
6	10	0	7	0	40
	1		0		48
7	10	0	90	0	98
	1		50		97
19	10	0	0	0	0
20	50	0	96	0	91
	5		44		87
	0.5		17		36
21	50	0	59	0	96
	5		24		24
22	10	0	98	0	99
	2		78		85
	0.4		11		37
DS_{500}	10	0	92	0	93
	2		26		0
	0.4		0		0

dextran sulphate (MW 500 000) and heparin had no effect (Table 4).

As shown for **22** in Table 5, treatment of immobilized gp120 with the compounds also irreversibly blocked the binding of sCD4. This differed from the reversible effect of dextran sulphate (MW 500 000), which to be effective had to be present during the binding reaction. Some degree of specificity in the interaction of the various flavanoids with gp120 was apparent from the selective inhibition of antibody binding. Thus, for example, whereas they blocked (in a dose-dependent manner) the interaction of monoclonal antibodies 358 and 380 with the V3 loop and CD4-binding regions of HIV-1 gp120, respectively (Cordell et al., 1991), the compounds had no effect on the binding of the monoclonal antibodies 360 and 323 to the N- and C-termini, respectively of the molecule. There was a correlation between the degree of inhibition of antibody and sCD4-binding by various flavanoids and their relative effectiveness in inhibiting virus infection.

Thus, although some compounds (e.g., quercetin) can inhibit HIV reverse transcriptase in vitro, it is apparent that this inhibitory action is non-specific (Moore and Pizza, 1992) and that inhibition of virus infection is principally due to a more selective interaction with gp120. In this respect the anti-HIV action of these flavanoids is similar to the actions of various tannins (Weaver et al.,

1992; Mahmood et al., 1993) and polyanionic compounds (Schols et al., 1991), which interact irreversibly with gp120 to block virus infection.

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