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Short communication

Transcriptional suppression of the HIV promoter by natural compounds

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Abstract

Tannins and lignins are natural compounds contained in plants such as tea leaves. Previously, we demonstrated that tannic acid represses 12-*o*-tetra-decanoyl phorbol-13-acetate (TPA)-induced human immunodeficiency virus (HIV) promoter activity. Furthermore, we demonstrated that a 30-bp element located just downstream of the NF-κB element in the HIV promoter responds negatively to tannic acid. However, the kinds of molecules responsible for this suppressive effect have remained unknown, because tannic acid is a mixture of various galloylglucoses. Here, we examined structure-defined natural compounds for HIV promoter-suppressive effects. We found that ellagitannins suppress TPA-induced HIV promoter activity to the same extent as tannic acid. 3-Phenylcoumarins, isoflavone and chalcones have more suppressive effects than ellagitannins. On the other hand, other flavonoids and acetogenins have no suppressive effect. 3-Phenylcoumarins and chalcones showed no suppressive effect on the cytomegalovirus (CMV) promoter, suggesting that they act specifically on the HIV promoter. These results suggest that 3-phenylcoumarin or chalcone compounds could be used to develop novel anti-HIV drugs with an action targeted at HIV promoter activity.

Keywords: TPA; HIV promoter; Cytomegalovirus; Ellagitannin

1. Introduction

Nuclear events such as DNA replication, repair, and transcription are thought to be regulated by the poly(ADP-ribosyl)ation of chromosomal proteins (Althaus and Richter, 1987; Miwa and Sugimura, 1990). We have investigated the physiological significance of poly(ADP-ribose) metabolism in gene expression (Tanuma, 1989; Uchida et al., 1993). The synthesis of poly(ADP-ribose) is known to be catalyzed by poly(ADP-ribose) polymerase (PARP) (de Murcia and Menissier de Murcia, 1994; Lindahl et al., 1995), while the degradation of poly(ADP-ribose) is mainly regulated by poly(ADP-ribose) glycohydrolase (PARG) (Maruta et al., 1991; Tanuma et al., 1986; Braun et al., 1994). Previously, we found that tannins and related compounds (Tsai et al., 1992; Aoki et al., 1993) and lignins (Tanuma et al., 1989) potently inhibit the PARG activity. We also showed that tannic acid suppresses glucocorticoid-induced mouse mammary tumor virus (MMTV) gene expression (Tsai et al., 1992). Furthermore, we reported that a 50-bp element in the MMTV promoter is responsible for the transcriptionally negative effect of tannic acid (Uchiumi et al., 1998). Moreover, we identified a core sequence, ACTG, in this element as essential for the negative effect (Uchiumi et al., 1998). We also observed that tannic acid represses 12-*o*-tetra-decanoyl phorbol-13-acetate (TPA)-induced human immunodeficiency virus (HIV) promoter activity, and found a putative tannic acid-responsive element between positions −133 and −104 of the HIV promoter (Uchiumi et al., 1996). Interestingly, the 30-bp region possesses an ACTG motif. This supports the repressive effect of the core ACTG motif.

Although we have demonstrated that tannic acid, a mixture of galloylglucoses can suppress TPA-induced HIV

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promoter activity in T-cells (Uchiumi et al., 1996), we did not know the chemical structures of the natural compounds that are responsible for that suppressive effect. So, we proceeded to test structure-defined natural compounds related to tannins and lignins for HIV promoter suppression effects. Here, we show that 3-phenylcoumarins and chalcones suppress TPA-induced HIV promoter activity more effectively than tannic acid. These compounds or derivatives thereof may be used as anti-HIV drugs interfering with the viral gene expression regulatory mechanism.

2. Materials and methods

2.1. Polyphenols

Polyphenols tested in this study were those isolated from the following plants: ellagitannins from *Reaumuria hirtella* (Yoshida et al., 2000) and *Quercus coccifera* (Ito et al., 2002) (Fig. 2 for structures); glycyrrhisoflavone, glycycoumarin, licopyranocoumarin, tetrahydroxymethoxychalcone, licocoumarone, licochalcones A and B (Fig. 3), liquiritigenin, liquiritin (Fig. 4) from licorice (Hatano and Yoshida, 1998); torachrysone gentiobioside, rubrofusarin gentiobioside from *Cassia tora* (Hatano et al., 1999a); cassiaoccidentalins A and C from *Cassia occidentalis* (Hatano et al., 1999b); aspidins AB and BB from *Dryopteris fragrans* (Ito et al., 2000b); chromene glucoside from *Eucalyptus cypellocarpa* (Ito et al., 2000a). Anthraquinones were obtained from a commercial rhubarb (Fig. 4).

2.2. Cell culture

Jurkat is a human acute lymphocytic lymphoma cell line (Uchiumi et al., 1992). The cell line was cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and antibiotics (RPMI growth medium).

2.3. Plasmid constructs

A luciferase (Luc) reporter plasmid carrying the HIV promoter region was constructed as follows. The LTR regions of HIV (from −452 to +80) was obtained from pHIV-CAT (pUR3R-III; Rosen et al., 1985) digested with *Bgl*II, and ligated into the *Bgl*II site of a pGL-2 control vector (Promega). The Luc expression plasmids (Uchiumi et al., 1996) obtained were used for the transient transfection experiments.

2.4. Transfection assays

Plasmid DNAs were transfected into Jurkat cells by the DEAE-dextran method (Uchiumi et al., 1992). Cells (2×10^6) were suspended in 0.4 ml TBS (25 mM Tris (pH 7.4), 137 mM NaCl, 5 mM KCl, 0.6 mM Na_2HPO_4, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$) containing 2.5 μg of the reporter plasmid and 500 μg of DEAE-dextran per milliliter for 30 min at room temperature. The cells were then washed with TBS to remove the unadsorbed DNA and cultivated for another 48 h in RPMI growth medium. Next, TPA (Sigma) and tannic acid (Sigma), or the lignin-containing fractions, or various natural compounds were added to the culture medium. After 15 h incubation, the cells were collected and used for the preparation of Luc samples. Luciferase assays were performed using the Luciferase assay system (Promega) as described in the product manual. Briefly, the collected cells were lysed with 150 μl of 1 × cell culture Lysis Reagent (25 mM Tris-phosphate (pH 7.8), 2 mM DTT, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% glycerol, 1% Triton X-100), mixed, and centrifuged (12,000 × *g* for 5 s). The supernatant was transferred to a new tube and stored at –80 °C before using for the Luc assay. Luc assays were performed according to the instruction manual. Luc assay reagent (100 μl) was added to 20 μg of the protein sample and briefly mixed. Immediately, the chemiluminescence was measured for 7.5 s with a Luminometer. The light intensity was referred to directly as a Luc activity.

3. Results

3.1. Establishment of the HIV promoter-Luc expression assay system

We have already analyzed HIV promoter activity by the HIV-CAT expression vector transfection assay. To analyze suppressive effects on the TPA-induced HIV promoter in a more sensitive way, we constructed a Luc reporter plasmid that expresses the Luc gene under the control of the HIV promoter. As shown in Fig. 1, tannic acid (100 μg/ml) suppressed the HIV promoter activity to 70% of the activity in TPA-treated cells (columns 2 and 4). A suppressive effect was not observed for TPA non-treated cells (columns 1 and 3). Although the Luc activity of pGL-2 transfected cells was elevated by TPA treatment (compare columns 5 with 6, or 7 with 8), tannic acid did not directly affect the Luc activity (compare columns 5 with 7, or 6 with 8). These results are essentially similar to previous data using the CAT reporter plasmid system (Uchiumi et al., 1996). Moreover, the assay was shown to be sensitive and reproducible. Thus, we decided to use this assay system in further experiments in the search for natural compounds that suppress TPA-induced HIV promoter activity.

3.2. Effect of various natural compounds on TPA-induced HIV promoter activity

The previous report (Uchiumi et al., 1996) suggest that certain compounds responsible for the suppression of HIV promoter activity are included in extracts such as the

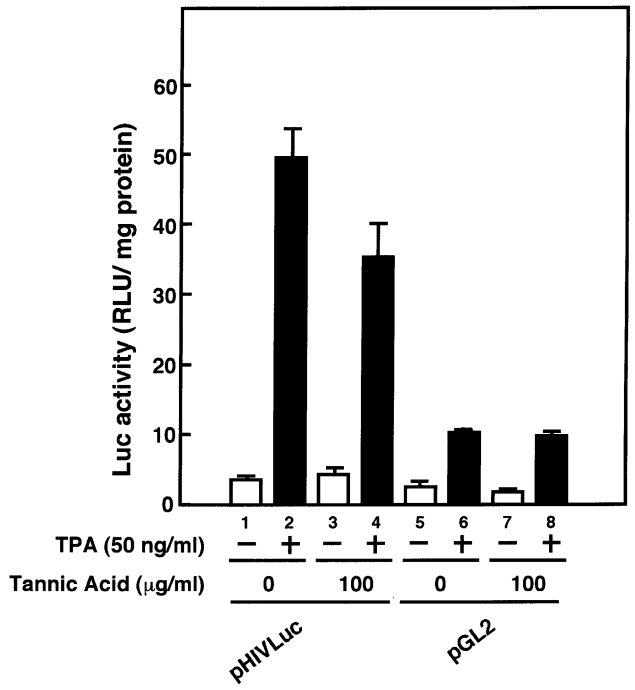


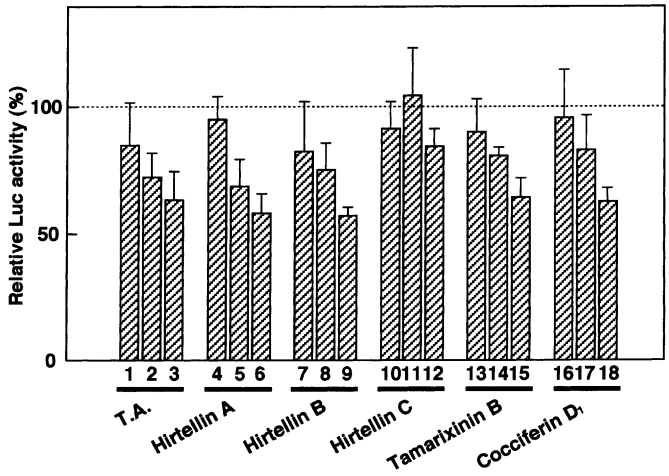
Fig. 1. Establishment of an assay system for HIV promoter activity by Luciferase expression. The pHIVLuc plasmid was transfected into Jurkat cells. After 48 h of transfection, the cells were treated with (closed columns) or without (open columns) 50 ng/ml of TPA in the presence (columns 3, 4, 7, and 8) or absence (columns 1, 2, 5, and 6) of tannic acid (100 μg/ml). The Luc activities were measured by a Luminometer. Results are shown as averages and S.E.M. of the absolute chemiluminescence of four independent experiments.

tannin-containing fractions obtained from plants. Hypothetically, it is possible that some other natural compounds in those extracts have effects on the HIV promoter. To address this hypothesis, we examined various natural compounds whose chemical structures have already been defined. Since we have found that tannic acid, although it is a mixture of galloylglucoses, has an inhibitory effect on the HIV promoter, we tested tannins and related compounds in the HIV promoter-Luc expression HIV promoter-Luc expression assay system (Fig. 2). Hirtellin A, hirtellin B, hirtellin C, tamarixinin B, and cocciferin D1, which are classified as ellagitannin dimers, showed suppressive profiles similar to that of tannic acid (Fig. 2). Although, aspidin AB, BB, and chromene glucoside have hexagonal structures, they showed no suppressive effect, possibly because they have no circular chain composed of phenols and saccharides (Fig. 4). Since these circular structures, which are characteristic of ellagitannins, have been suggested to be responsible for the inhibition of PARG (Aoki et al., 1995), the suppressive effects on the HIV promoter by these compounds suggest that poly(ADP-ribose)*n* degradation by PARG may be involved in the onset of HIV promoter activity.

Next, we examined the effect of coumarins and chalcones, which are also contained in plants. As shown in Fig. 3, glycyrrhisoflavone, glycycoumarin, and licopyranocoumarin, which include isoflavone and 3-phenylcoumarin structures, suppress the HIV promoter more than ellagitannins. Tetrahydroxymethoxychalcone, licochalcones A and B, which carry the chalcone structure, potently reduce the HIV promoter activity, as well. Since our experimental system uses TPA (50 ng/ml), cells are further damaged by the high concentration (100 μg/ml) of 3-phenylcoumarin or chalcones. To avoid the cytotoxic effect of these compounds, we performed the same experiment using low doses (10 μg/ml) of the compounds (Fig. 3, columns 4, 7, 10, 13, 16, 19, and 22). Although the response of the HIV promoter was differentially affected by the compounds, the results suggest that natural compounds carrying 3-phenylcoumarin and chalcone structures can suppress HIV transcription. Interestingly, licocoumarone was found to have a similar suppressive effect, although it has a different molecular structure (Fig. 3). Although liquiritigenin, liquiritin, cassiaoccidentalin A, and cassiaoccidentalin C have molecular structures related to chalcones, they have no obvious suppressive effect on the HIV promoter (Fig. 4). It is noteworthy that these flavonoid compounds have a phenolic residue at the *ortho*-position, unlike coumarins, which have it at the *meta*-position. Torosachrysone gentibioside, rubrofusarin gentiobioside, chrysophanol, emodin, and aloe-emodin, which have a naphthalene or anthraquinone structure classified as acetogenins, show no suppressive effect (Fig. 4); instead, they somewhat induce TPA-induced HIV promoter activity. The above results together suggest that natural compounds carrying 3-phenylcoumarin or chalcone structures have a negative effect on HIV transcription, possibly because they bind to some specific protein factors.

3.3. Coumarins and chalcones specifically suppress the TPA-induced HIV promoter

As shown in Fig. 1, the pGL-2 control vector itself expresses Luciferase that responds to TPA treatment, suggesting the involvement of coumarin/chalcone-responsive element(s). In order to confirm that the inhibitory effect of coumarin/chalcone is not a non-specific effect, we investigated this effect in pGL-2, which is a control vector for pHIVLuc, and pCMVLuc reporter plasmid transfected cells (Fig. 5A and B). All the 3-phenylcoumarins and chalcones tested suppressed the TPA-induced HIV promoter, whereas they did not cause an apparent reduction in the Luc activity in pCMVLuc transfected cells. Instead, these compounds increased the Luc activity (Fig. 5A columns 4–6, 13–15, 22–24, 31–33, and Fig. 5B columns 21 and 22). Because similar responses to these compounds were observed in pGL-2 vector-transfected cells, it is possible that the positive response may result from the pGL-2 vector sequence itself. These results suggest that there is no coumarin/chalcone-responsive suppressor sequence within the pGL-2 or pCMVLuc vectors.



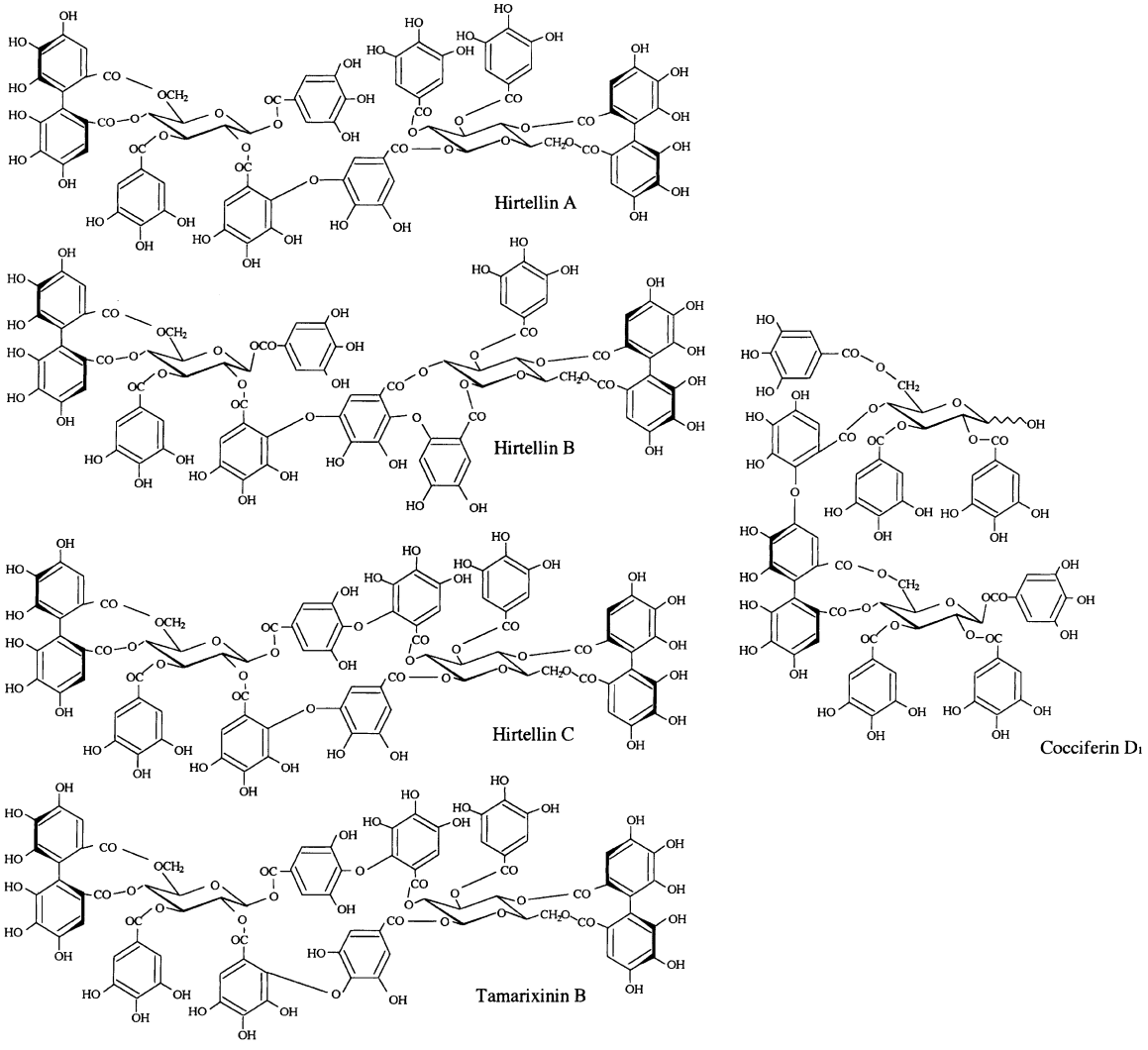
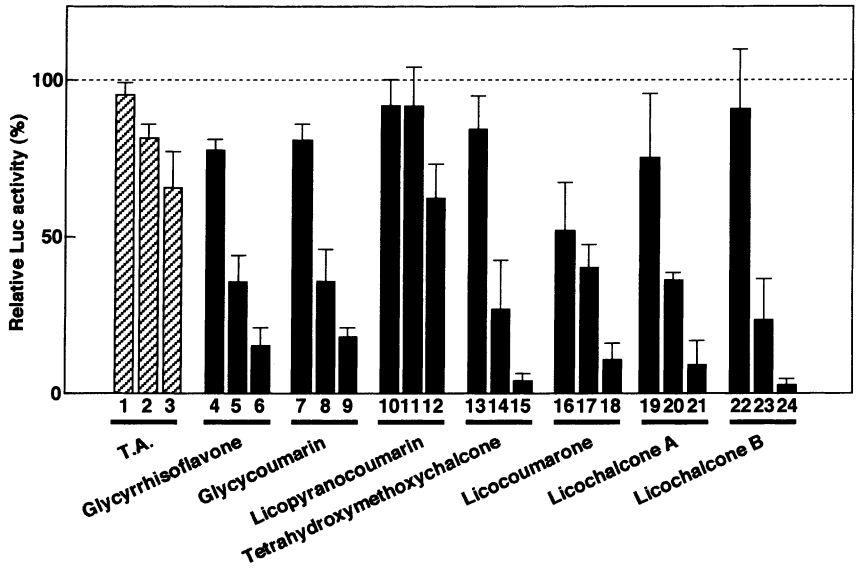


Fig. 2. Ellagitannins suppress TPA-induced HIV promoter activity. Similar experiments as described in the legend to Fig. 3 were performed using various natural compounds instead of tannic acid. pHIV-Luc transfected Jurkat cells were treated with TPA (50 ng/ml) in the presence of tannic acid (columns 1–3), hirtellin A (columns 4–6), hirtellin B (columns 7–9), hirtellin C (columns 10–12), tamarixinin B (columns 13–15), or cocciferin D1 (columns 16–18). The concentration of each compound was 25 (1, 4, 7, 10, 13, and 16), 50 (2, 5, 8, 11, 14, and 17), or 100 μg/ml (3, 6, 9, 12, 15, and 18). Luc activities were normalized to that of TPA-induced pHIV transfected Jurkat cells and shown as relative Luc activities. Histograms show the averages with S.D. values from at least three independent experiments. The structure of each compound is shown.



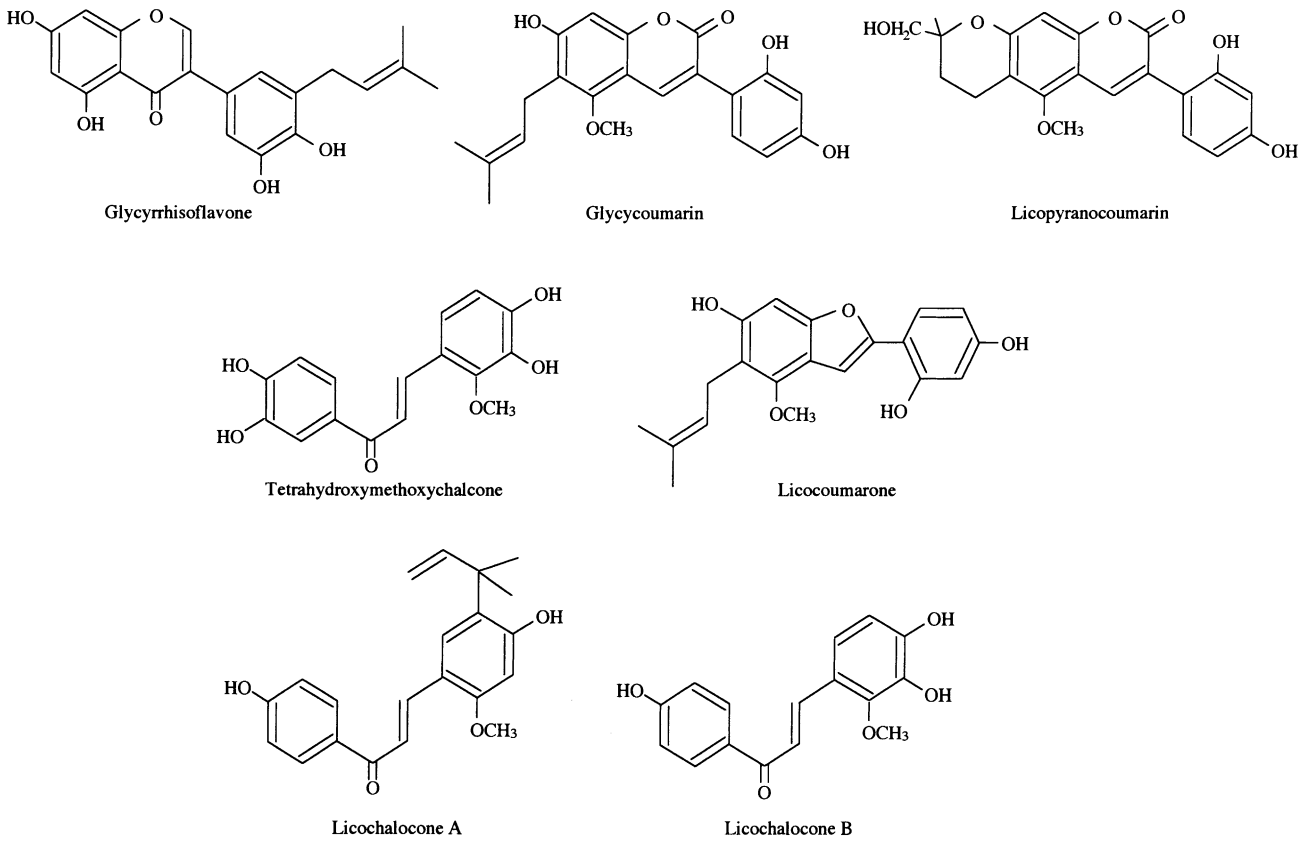


Fig. 3. Coumarins and chalcones suppress TPA-induced HIV promoter activity. pHIV-Luc transfected Jurkat cells were treated with TPA (50 ng/ml) in the presence of tannic acid (columns 1–3), glycyrrhisoflavone (columns 4–6), glycycoumarin (columns 7–9), licopyranocoumarin (columns 10–12), tetrahydroxymethoxychalcone (columns 13–15), licocoumarone (columns 16–18), licochalcone A (columns 20–22), or licochalcone B (columns 23–25). The concentration of each compound was 10 (4, 7, 10, 13, 16, 19, and 22), 25 (1, 5, 8, 11, 14, 17, 20, and 23), 50 (2, 6, 9, 12, 15, 18, 21, and 24), or 100 μg/ml (column 3). Luc activities were normalized to that of TPA-induced pHIV transfected Jurkat cells and are shown as relative Luc activities. Histograms show the averages with S.D. values from at least three independent experiments.

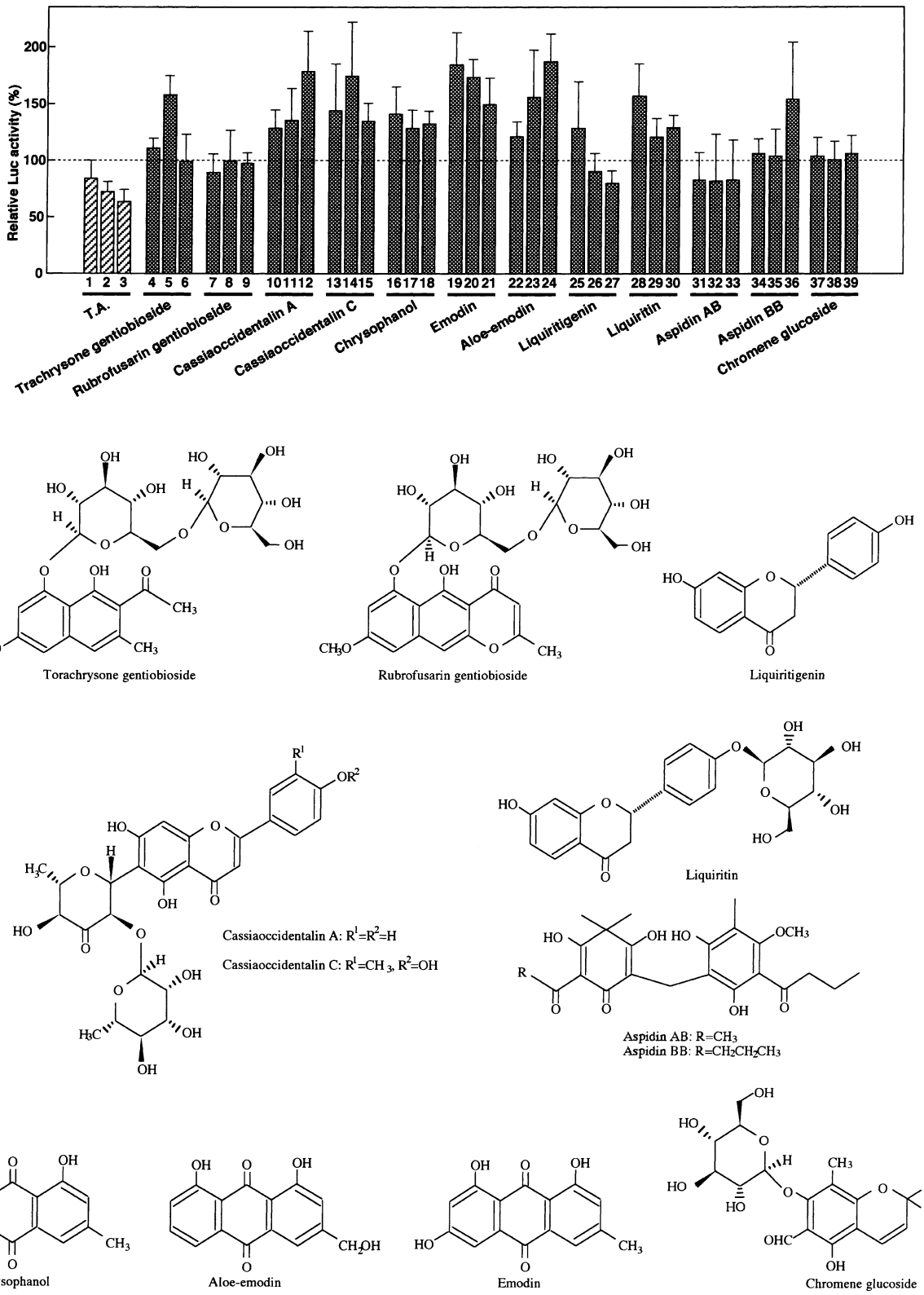


Fig. 4. Flavones, naphthalenes, and anthraquinones do not affect TPA-induced HIV promoter activity. pHIV-Luc transfected Jurkat cells were treated with TPA (50 ng/ml) in the presence of tannic acid (columns 1–3), torachrysone gentiobioside (columns 4–6), and rubrofusarin gentiobioside (columns 7–9), cassiaoccidentalin A (columns 10–12), cassiaoccidentalin C (columns 13–15), chrysophanol (columns 16–18), emodin (columns 19–21), aloe-emodin (columns 22–24), liquiritigenin (columns 25–27), liquiritin (columns 28–30), aspidin AB (columns 31–33), aspidin BB (columns 34–36), or chromene glucoside (columns, 37–39). The concentration of each compound was 25 (1, 4, 7, 10, 13, 16, 19, 22, 25, 28, 31, 34, and 37), 50 (2, 5, 8, 11, 14, 17, 20, 23, 26, 29, 32, 35, and 38), or 100 μg/ml (3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, and 39). Luc activities were normalized to that of TPA-induced pHIV transfected Jurkat cells and shown as relative Luc activities. Histograms show the averages with S.D. values from three independent experiments.

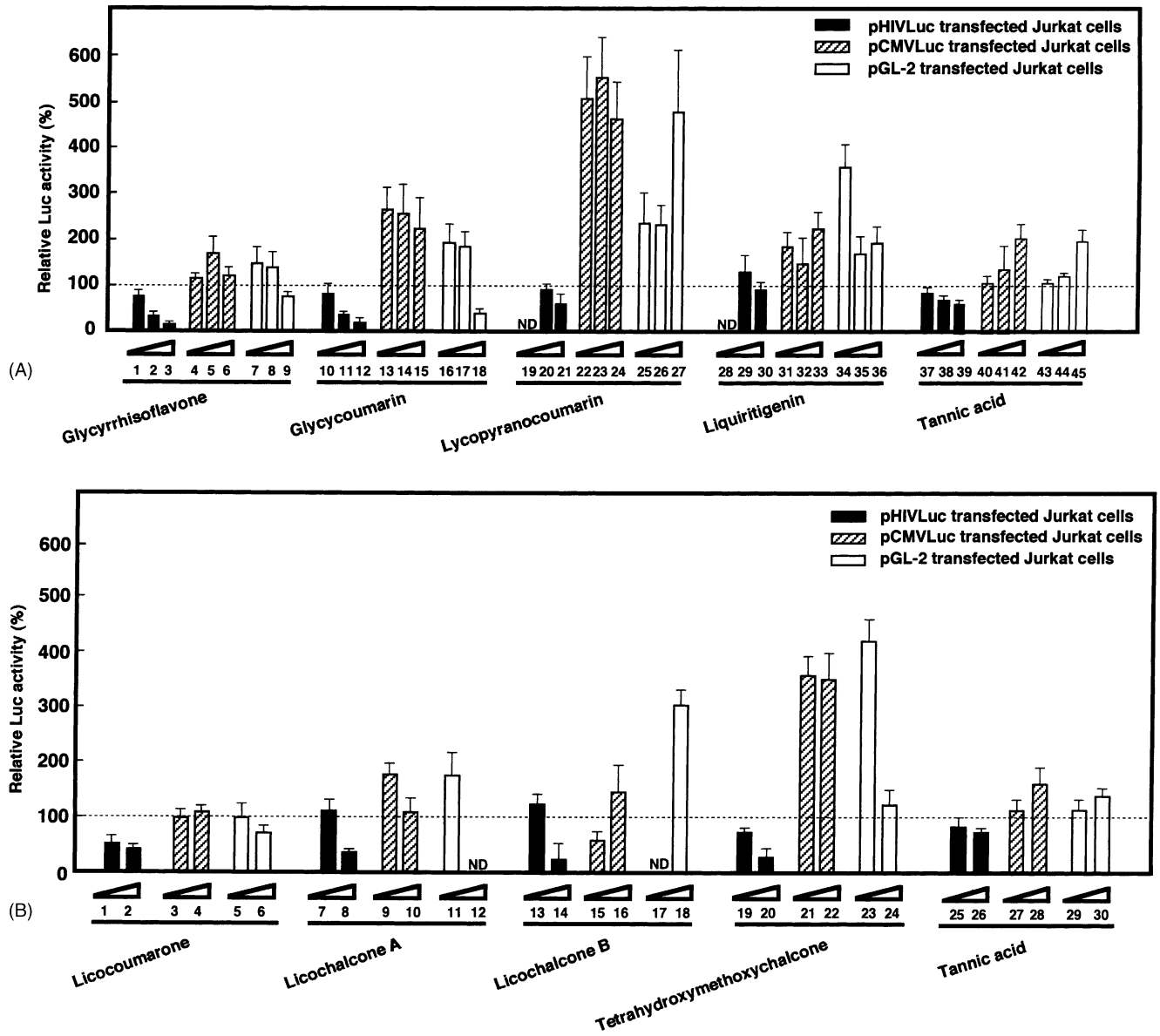


Fig. 5. Coumarins and chalcones specifically suppress TPA-induced HIV promoter activity. Experiments similar to those described in the legends to Figs. 3 and 4 were done using pCMVLuc and pGL-2 control vector as the reporter plasmid. (A) pHIVLuc (columns 1, 2, 3, 10, 11, 12, 19, 20, 21, 28, 29, 30, 37, 38, and 39), pCMVLuc (4, 5, 6, 13, 14, 15, 22, 23, 24, 31, 32, 33, 40, 41, and 42), and pGL-2 (7, 8, 9, 16, 17, 18, 25, 26, 27, 34, 35, 36, 43, 44, and 45) transfected Jurkat cells were treated with 12.5 μg/ml (1, 4, 7, 10, 13, 16, 19, 22, 25, 28, 31, and 34), 25 μg/ml (2, 5, 8, 11, 14, 17, 20, 23, 26, 29, 32, 35, 37, 40, and 43), 50 μg/ml (3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 38, 41 and 44) or 100 μg/ml (39, 42, and 45) of glycyrrhisoflavone (columns 1–9), glycycoumarin (10–18), lycopyranocoumarin (19–27), liquiritigenin (28–36) or tannic acid (37–45). (B) pHIVLuc (columns 1, 2, 7, 8, 13, 14, 19, 20, 25, and 26), pCMVLuc (3, 4, 9, 10, 15, 16, 21, 22, 27, and 28), pGL-2 (5, 6, 11, 12, 17, 18, 23, 24, 29, and 30) transfected Jurkat cells were treated with 12.5 μg/ml (1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, and 23), 25 μg/ml (2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 25, 27, and 29) or 50 μg/ml (26, 28, and 30) of licocoumarone (columns 1–6), licochalcone A (7–12), licochalcone B (13–18), tetrahydroxymethoxychalcone (19–24), or tannic acid (25–30). Histograms show Luc activities relative to that of TPA-treated cells transfected with pHIVLuc plasmid or TPA-non-treated pCMVLuc or pGL-2 transfected cells. Histograms show the averages with S.D. values from three independent experiments. ND: not determined.

4. Discussion

In this study, we established a sensitive system for screening HIV promoter-suppressor compounds using the pHIVLuc reporter plasmid. The negative effect of coumarin and chalcone structure-containing natural products was revealed to be greater than that of ellagitannins. On the other hand, naphthalene- or anthraquinone-related compounds did not suppress TPA-induced HIV promoter activity. Previously, tannins and lignins were reported to be potent inhibitors of HIV expression (Mizuno et al., 1992) and adsorption to host cells (Nakashima et al., 1992). These

antiviral effects are thought to result from the multiple biological activities of these compounds, including the inhibition of PARG (Tanuma et al., 1989) and superoxide radical-scavenging effects (Hatano et al., 1989; Satoh et al., 1996; De Bruyne et al., 1999). Since our experimental system is based on the transient transfection of reporter constructs carrying the HIV promoter sequence, the results presented here suggest that coumarins and chalcones directly or indirectly influence transcription factors associated with the 30-bp element. By performing similar experiments, we have identified a tannic acid-responsive 50-bp element in the MMTV promoter (Uchiumi et al., 1998). We are now investigating protein factors that bind to these tannic acid-responsive elements. These factors may be poly(ADP-ribosyl)ated; alternatively, they may be affected by other poly(ADP-ribosyl)ated protein factors.

So far, we have observed reproducible inhibitory effects of tannins and ellagitannins on TPA-induced HIV promoter activity (Uchiumi et al., 1996). As shown in Fig. 2, coumarins and chalcones have greater effects on the HIV promoter than tannins. The biological effects of coumarin derivatives have been studied. Those compounds have been shown to have antiallergic effects (Saraf et al., 1993), to improve brain injury induced by transient cerebral ischemia (Calapai et al., 1995), and to accumulate in leukocytes (Squadrito et al., 1993). Chalcones are known to be cytotoxic, anticancer chemopreventative compounds (Dimmock et al., 1993). Recently, chalcone derivatives have been shown to have anti-HIV and cytotoxic activities (Mishra et al., 2001). The cell surface expression of the ICAM protein (Tanaka et al., 2001) and iNOS expression (Herencia et al., 2001) levels have been reported to be lowered by chalcone derivatives, suggesting that the negative effect may come from the transcriptional suppression of those genes. Interestingly, a recent study has suggested that chalcone derivatives disrupt the MDM2/p53 protein complex to cause increased p53 levels (Stoll et al., 2001). However, the molecular mechanism(s) for these biological actions require further investigation.

As shown in Fig. 5, the negative responsive sequence is suggested to be located within 0.5-kbp of the HIV promoter region. Although the responsible element has not yet been determined, we suspect that it might be located in the 30-bp tannic acid-responsive region. The mechanism(s) for the HIV promoter-suppressive effects of these natural compounds have not yet been reported, but they might involve a specific binding protein factor(s), because liquiritin or liquiritigenin, which have structures similar to but are different from coumarins, do not show any suppressive effects. Thus, the position of the phenolic residue may play an important role in transcriptional regulation. In other words, the 3-phenylcoumarin structure, but not that of flavonoids, may prefer to bind to certain cellular signal transduction factor(s) or transcription factor(s). On the contrary, naphthalene- and anthraquinone-related compounds rather stimulated HIV promoter activity. These compounds may bind to (an)other cellular protein(s) that regulates HIV gene expression positively. The results suggest that some ligand-receptor system might be involved in the HIV transcription regulatory system.

At present, numerous drugs for AIDS patients have been developed (Johnston and Hoth, 1993). For example, reverse transcriptase inhibitors such as azidothymidine (Fauci, 1993), and protease inhibitors (McQuade et al., 1990; Ashorn et al., 1990) are known to be effective in the treatment of AIDS. Although these drugs are biologically effective, there are some problems associated with their use, namely, the emergence of resistant strains and serious side effects. Recently, chemokines were found to prevent infection by HIV (Cocchi et al., 1995; Feng et al., 1996; Bates, 1996). The use of chemokines has also been reported to be effective in inhibiting viral uptake and replication, suggesting that chemokine treatment can be applied to clinical therapy (Simmons et al., 1997; Chen et al., 1997). In short, multiple drugs, interacting at different steps in the life cycle of HIV, are available at present (Richman, 2001). Recently, p53 was reported to regulate HIV transcription (Gualberto et al., 1995). Benzothiophene derivatives, which have been shown to have both anti-inflammatory and anti-HIV effects (Boschelli et al., 1995), inhibit calucineurin activity and transactivation of the HIV promoter by p53 (Gualberto et al., 1998). Moreover, p300, which is known as a histone acetyltransferase, has been shown to play a role in the regulation of HIV transcription (Hottiger et al., 1998).

Also polyphenolic compounds or coumarin/chalcone derivatives suppress HIV gene expression at the transcriptional level. Therefore, such compounds will add a new method for suppressing HIV gene expression. Moreover, we believe that utilizing by-products of foods made from cacao, such as chocolate, for AIDS treatment may have an advantage in terms of cost. Thus, it will be necessary to purify and synthesize the main compounds responsible for the suppression of HIV gene expression as well as to initiate the molecular characterization of the factors with which these compounds interact.

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