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INDUCTION OF MAMMALIAN TOPOISOMERASE II DEPENDENT DNA CLEAVAGE BY NONINTERCALATIVE FLAVONOIDS, GENISTEIN AND OROBOL

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Abstract—Two isoflavones, genistein (4′,5,7-trihydroxyisoflavone) (**1**) and orobol (5,7,3′,4′-tetrahydroxyisoflavone) (**2**) induced mammalian topoisomerase II dependent DNA cleavage *in vitro*. The cleavage activities of **1** and **2** were comparable to those of known antitumor agents with topoisomerase II dependent DNA cleavage activity such as 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA) and demethylepipodophyllotoxin ethylidene-β-D-glucoside (VP-16). Two flavones, fisetin (3,7,3′,4′-tetrahydroxyflavone) (**3**) and quercetin (3,5,7,3′,4′-pentahydroxyflavone) (**4**) showed topoisomerase II dependent DNA cleavage activity with similar potentials to that of Adriamycin®. Addition of salt (0.5 M NaCl) to the reaction mixture containing genistein and topoisomerase II resulted in a great reduction of DNA cleavage, suggesting that the mechanism of the topoisomerase II dependent DNA cleavage induced by flavonoids is through the cleavable complex formation as seen with *m*-AMSA and VP-16. DNA unwinding assay using mammalian topoisomerase I showed that both **1** and **2** did not intercalate into DNA but both **3** and **4** intercalated like *m*-AMSA. Other structurally related flavonoids could not induce topoisomerase II dependent DNA cleavage, indicating that the restricted structures of flavonoids were required for the cleavage activity.

DNA topoisomerases I and II are enzymes that catalyze the concerted breaking and rejoining of DNA strands, thereby controlling the topological states of DNA (reviewed in Ref. 1). Much interest has focused on the functional role of these two topoisomerases. Recent experiments suggest that they are involved in many processes of DNA metabolism, including replication, transcription, recombination and chromosome segregation at mitosis (reviewed in Ref. 1). In addition to the study of the intracellular function of topoisomerase, one of the most significant findings has been the identification of topoisomerase II as the primary cellular target for a number of clinically important antitumor agents which include intercalating agents (e.g. *m*-AMSA†, Adriamycin® and ellipticine) as well as nonintercalating epipodophyllotoxins (VP-16 and VM-26) [2–5]. Several lines of evidence indicate that these antitumor drugs have the common property of stabilizing the DNA–topoisomerase II complex ("cleavable complex") which upon exposure to denaturing agents results in the induction of DNA cleavage. Structure–activity studies of a large number of acridine derivatives and epipodophyllotoxin congeners have shown a strong correlation between cytotoxicity and the ability to induce cleavable complexes [6, 7]. It has been suggested that the cleavable complex formation by antitumor drugs produces bulky DNA adducts that can lead to cell death.

According to this attractive model, a specific new inducer of DNA–topoisomerase II cleavable complex could be an antitumor drug as well as a tool for further understanding the physiological role of topoisomerase II. We have screened antitumor antibiotics and cultures of actinomycetes and fungi for their abilities to induce topoisomerase II dependent DNA cleavage (TDC) *in vitro*, and have now found that isoflavones such as genistein (**1**) and orobol (**2**) show a potent TDC activity *in vitro*. These flavonoids are the first examples of new inducers of the "cleavable complex" which have been found by the mechanistically oriented screening method using mammalian topoisomerase II. In this report, we describe the TDC activity of flavonoids and discuss their biological activities related to cytotoxicity and antineoplastic activity.

MATERIALS AND METHODS

Enzymes, nucleic acids and chemicals. DNA topoisomerase II was isolated from calf thymus as described by Halligan *et al.* [8] and partially purified with Bio-Rex70, Hydroxylapatite and P-11 phosphocellulose column chromatography. DNA topoisomerase I was isolated from nuclear fraction of the human tumor cell line Kato III as described by Drake *et al.* [9] and partially purified with Bio-Rex70 and Hydroxylapatite column chromatography. Proteinase K was from the Sigma Chemical Co. Supercoiled pBR322 DNA was purified from *Escherichia coli* as described [10]. VP-16 was obtained from the National Cancer Institute and *m*-AMSA

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† Abbreviations used: *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; VP-16, demethylepipodophyllotoxin ethylidene-β-D-glucoside; SDS, sodium dodecyl sulfate; CCC-DNA, covalently closed circular DNA; OC-DNA, open circular DNA; VP-26, 4′-demethylepipodophyllotoxin thenylidene-β-D-glucoside; and TDC, topoisomerase II dependent DNA cleavage.

(NSC249992) was a gift from the Warner-Lambert Co. Orobol and tectrigenin were synthesized by the Pharmaceutical Research Laboratories of the Kyowa Hakko Kogyo Co. Other flavonoids were obtained from commercial sources. All of the drugs and flavonoids were dissolved in dimethyl sulfoxide at 50 mM as stock solutions and diluted in methanol containing 20% dimethyl sulfoxide to the desired concentration before use.

Agarose gel assay for topoisomerase II dependent DNA cleavage (TDC assay). Reactions (20 μL) containing 50 mM Tris–HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 1 mM ATP, 0.5 mM dithiothreitol, 0.5 mM EDTA, 30 μg/mL of bovine serum albumin, 0.4 μg of pBR322 DNA, calf thymus DNA topoisomerase II and drugs were incubated at 37°. After 60 min, reactions were terminated by the addition of 2 μL of a solution containing 5% SDS and 2.5 mg/mL proteinase K. Following an additional 60-min incubation at 37°, the samples were electrophoresed through a 1.2% agarose gel in 89 mM Tris–borate (pH 8.3), 2 mM EDTA buffer containing 0.1% SDS. After electrophoresis, gels were stained with ethidium bromide and photographed under UV illumination, and negative film was scanned by a Shimazu microdensitometer. The peak area of the Gaussian peak was measured and the percent of linear DNA was calculated. None of the drugs and flavonoids modified DNA migration.

DNA unwinding measurements. DNA unwinding effects of intercalators were assayed according to the method described by Chen *et al.* [3] Briefly, relaxed pBR322 DNA was prepared by treatment of plasmid pBR322 DNA with excess DNA topoisomerase I in the same buffer described above for TDC assay except that ATP and $MgCl_2$ were not included. After phenol extraction and ethanol precipitation, relaxed pBR322 DNA resuspended in Tris–EDTA buffer was used in further experiments as substrate. For the unwinding assay, each reaction mixture (20 μL each in the same reaction mixture as described above) contained 0.4 μg of relaxed pBR322 DNA, excess DNA topoisomerase I and drugs. The concentration of topoisomerase I used in the unwinding experiments was at least ten times higher than that required to generate full relaxation of any substrate we have used. After 60 min at 37°, reactions were stopped by addition of 3.5 μL of prewarmed stopping solution (5% SDS, 15% Ficoll and 0.25% bromophenol blue). Electrophoresis was done as described for the TDC assay.

RESULTS

Topoisomerase II dependent DNA cleavage by flavonoids. In the course of screening for new compounds with TDC activity, we found that genistein was the active component of a culture broth of *Penicillium* sp. We have conducted studies on TDC activity of flavonoids using purified calf thymus DNA topoisomerase II and supercoiled plasmid pBR322. Figure 1 shows a photograph of agarose gels comparing TDC activity in the presence of various amounts of flavonoids such as isoflavones (genistein, orobol, and daidzein) and flavones (quercetin, fisetin and chrysin). As the concentrations of genistein and orobol were increased (from 12.5 μM in lanes d and g to 250 μM in lanes f and i), the linear form DNA progressively appeared. For comparison, the antitumor drugs *m*-AMSA and VP-16, which have been shown to promote TDC, were included (Fig. 1 A and B, lanes m–o, respectively). The TDC activities of genistein and orobol were comparable to those of *m*-AMSA and VP-16, but much stronger than that of the known antitumor drug Adriamycin® (data not shown). The flavones quercetin and fisetin also showed TDC activity in a potential similar to that of Adriamycin®. On the other hand, treatment with daidzein and chrysin in the same concentration range had no effect (Fig. 1, A and B, lane j–l, respectively).

Stabilization of the cleavable complex by flavonoids. Several lines of evidence have indicated that many antitumor drugs such as *m*-AMSA and VP-16 stabilize a "cleavable complex" formed between mammalian DNA topoisomerase II and DNA [2–5]. Inhibition of the strand-passing activity of topoisomerase II is accompanied by the stabilization of the cleavable complex [3]. Genistein and orobol inhibited the relaxation of pBR322 DNA by calf thymus topoisomerase II in a potential similar to that of VP-16, whereas daidzein, which has no TDC activity, did not (data not shown). DNA strand breaks are the result of protein denaturing agent treatment of this cleavable complex, and the DNA cleavage is suppressed by increasing the salt concentration in a reaction mixture [11]. To see whether genistein also has an effect similar to those of *m*-AMSA and VP-16, in stabilizing the cleavable complex, we tested the salt reversibility of TDC *in vitro*. As shown in Fig. 2, when NaCl was added to a preincubated reaction mixture (0.5 M NaCl final), the protein-linked DNA breaks (linear form) were suppressed after a short preincubation time, consistent with the cleavable complex hypothesis. In the case of *m*-AMSA and VP-16, the salt reversal was not complete even after 30 min. These results suggest that the mechanism of the TDC induced by flavonoids is through the cleavable complex formation as seen with *m*-AMSA and VP-16, but the stability of their complex may be much weaker than that of *m*-AMSA and VP-16.

Effects of genistein and orobol on the intercalation of DNA. Most of the antitumor drugs which have TDC activities are intercalative drugs, such as *m*-AMSA, Adriamycin® and ellipticine, and so we examined whether flavonoids can intercalate plasmid DNA, using a DNA unwinding assay [3, 12]. As shown in Fig. 3, genistein and orobol did not show the unwinding activity even at a concentration as high as 250 μM. In contrast, the unwinding effects of quercetin and fisetin were clearly detected, although their potencies were lower than that of *m*-AMSA (Fig. 3). These results indicated that flavonoids with TDC activities are classified into two groups according to their intercalative activities: genistein and orobol are nonintercalative like VP-16, and quercetin and fisetin are intercalative like *m*-AMSA, Adriamycin® and ellipticine.

A slight upward shift of the topoisomer bands was observed at 250 μM (Fig. 3, lanes d and g), but no more changes in the elctrophoretic pattern of DNA were detected up to 2.5 mM genistein and orobol.

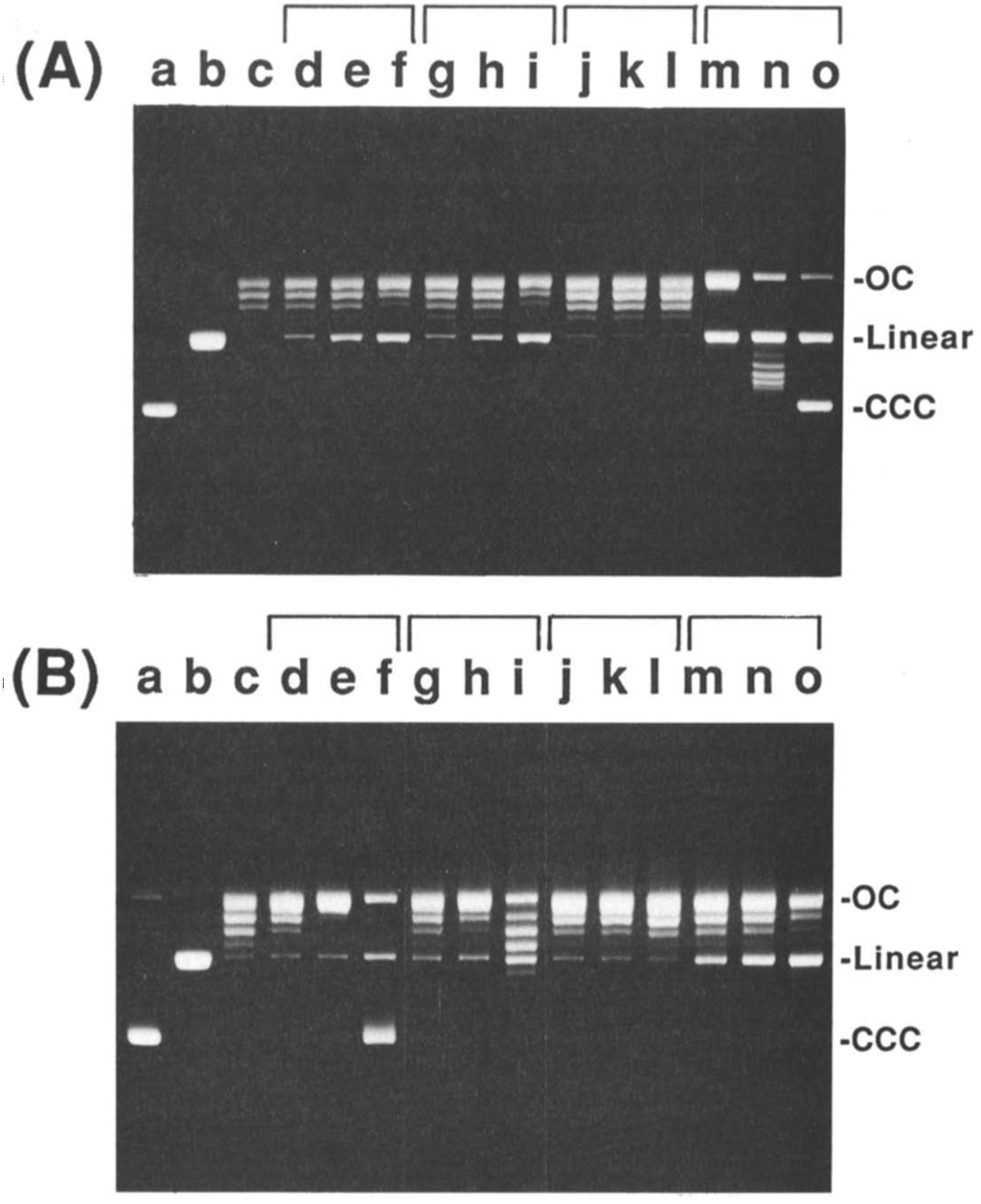


Fig. 1. Agarose gel assay for topoisomerase II dependent DNA cleavage effects of flavone compounds and antitumor drugs. Cleavage of DNA was analyzed by the agarose gel assay described in Materials and Methods. (A) (Lane a) CCC-DNA control (no enzyme, no drug); (lane b) linear DNA control; (lane c) no drug; (lanes d–f) genistein; (lanes g–i) orobol; (lanes j–l) daidzein; (lane m–o) *m*-AMSA. Drug concentrations were 12.5 μM (lanes d, g, j and m), 50 μM (lanes e, h, k and n) and 250 μM (lanes f, i, l and o). (B) Lanes a, b and c were the same as (A) respectively. (Lanes d–f) quercetin; (lanes g–i) fisetin; (lanes j–l) chrysin; (lanes m–o) VP-16. Drug concentrations were 12.5 μM (lanes d, g, j and m), 50 μM (lanes e, h, k and n) and 250 μM (lanes f, i, l and o).

The possibility that the lack of further unwinding at higher drug concentrations was due to the inhibitory effect of the drug on topoisomerase I was excluded by the following experiments. First, using the DNA relaxation assay under the condition that contains topoisomerase I with an activity equivalent to that used in the unwinding experiments, the catalytic activity of topoisomerase I was not inhibited by high concentrations of genistein and orobol. Second, using the unwinding assays with relaxed and supercoiled DNA, similar amounts of relaxed DNA were formed as final products. Thus, the activity of topoisomerase I used in the unwinding assay was not inhibited by genistein and orobol. Furthermore,

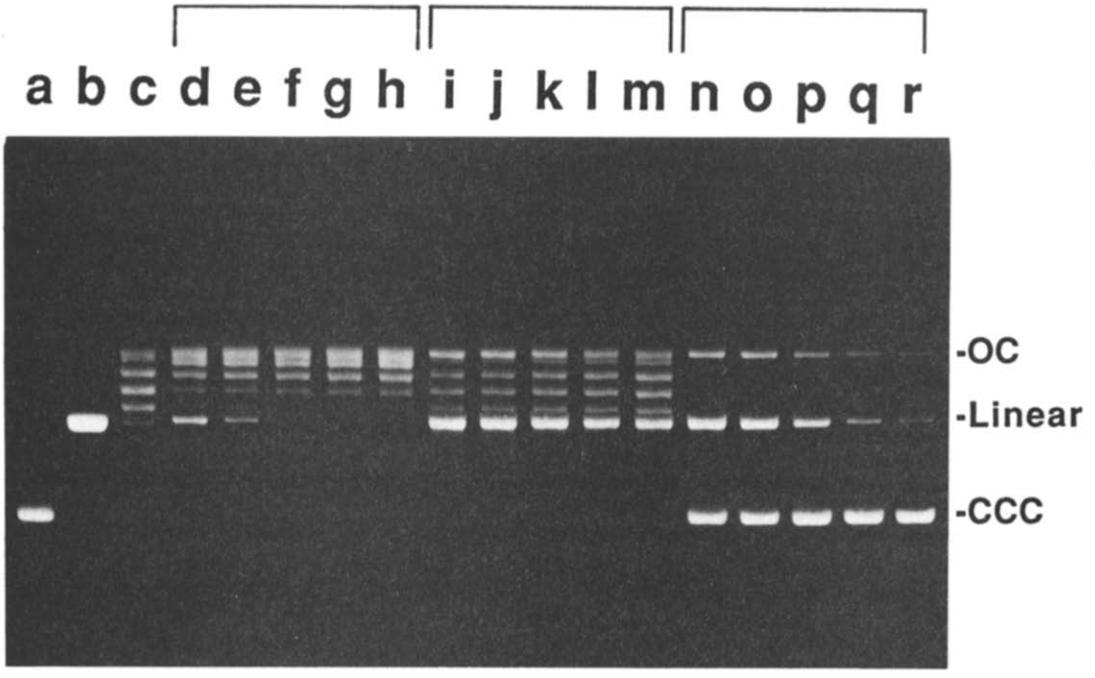


Fig. 2. Salt reversal of genistein and drug-induced DNA cleavage. DNA cleavage assays were done as described under Materials and Methods. Large reaction mixture (120 μL) containing either genistein, VP-16 or *m*-AMSA (250 μM for each compound) were incubated at 37° for 15 min. Then 1/9 vol. of 5 M NaCl was added to each mixture (0.5 M NaCl final) and the reactions were continued at 37°. Aliquots (20 μL) were withdrawn at various times after the second incubation. SDS and proteinase K treatment were done as described under Materials and Methods. (Lane a) CCC-DNA control (no enzyme, no drug); (lane b) linear DNA control; (lane c) control (no drug); (lanes d–h) samples containing genistein withdrawn at 0.5, 1, 5, 15, 30 min after the second incubation; (lanes i–m) same as lanes d–h, respectively, except that VP-16 was used; (lanes n–r) same as lanes d–h, respectively, except that *m*-AMSA was used.

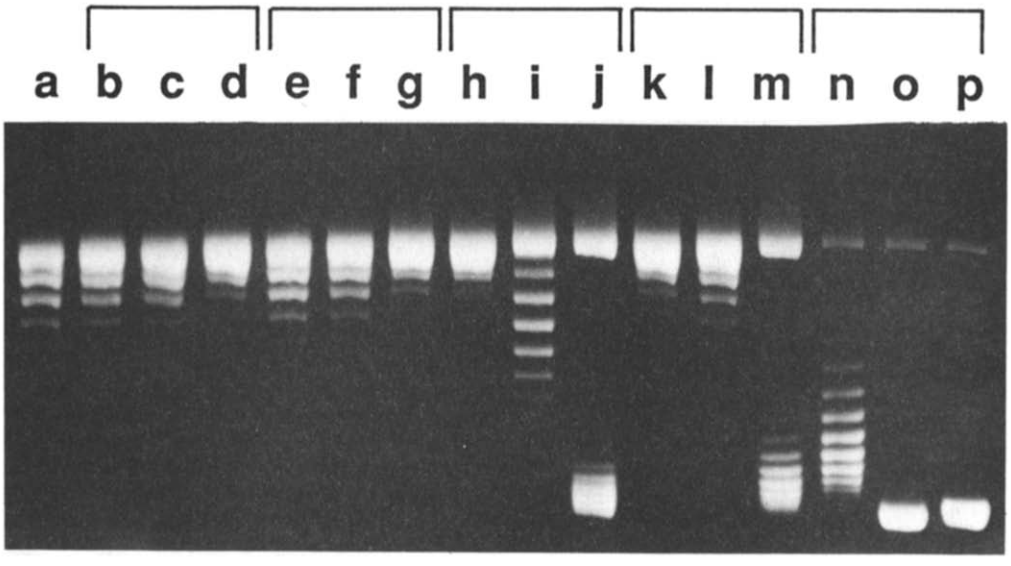


Fig. 3. Effects of flavone compounds on DNA unwinding assay. Unwinding measurements were done as described under Materials and Methods. (Lane a) DNA control (no drug); (lanes b–d) genistein; (lanes e–g) orobol; (lanes h–j) quercetin; (lanes k–m) fisetin; (lanes n–p) *m*-AMSA. Drug concentrations were 12.5 μM (lanes b, e, h, k and n), 50 μM (lanes c, f, i, l and o) and 250 μM (lanes d, g, j, m and p).

unlike quinolone compounds such as norfloxacin and nalidixic acid which have been reported to cause DNA unwinding in the presence of Mg^{2+} [13], genistein and orobol showed no DNA unwinding activity under the condition with Mg^{2+} (data not shown).

From these results, we concluded that genistein and orobol are nonintercalator like VP-16. But it is possible that genistein and orobol interact with DNA in a manner other than the typical intercalation. Further study is required to determine the detailed mechanism in which these flavonoids stabilize DNA–topoisomerase II complex and induce TDC activity *in vitro*.

Structure–activity relationship of flavonoids. We have screened more than thirty flavonoids for their abilities to induce TDC and intercalation activities. As shown in Table 1, which summarizes a part of these results, genistein and orobol were the most potent inducers of TDC activity and, in addition to the flavonoids described above, myricetin, morin and prunetin also showed TDC activities. As a result of this experiment, some information on the structure–activity relationship of flavonoids can be pointed out as follows.

First, since flavanones which differ from flavones by a saturated C2–C3 bond did not show either TDC or intercalation activity (for example, compare the effect of quercetin to that of dihydroquercetin in Table 1), flavone and isoflavone structures may be required for interaction with DNA and topoisomerase II leading to cleavable complex formation.

Second, the position of OH substitution on the flavonoid is important for TDC activity. In contrast to genistein, for example, another structurally related isoflavone, daidzein, which has no OH substitution at position 5 (R2 in Table 1), did not induce TDC activity (Fig. 1A and Table 1). Similar results indicating that slight differences in the position of a substitution have a great influence on cleavable complex formation are well known in the case of *m*-AMSA and its inactive isomer *o*-AMSA [2].

Third, the existence of a bulky substituent, such as a methoxy or sugar, suppresses TDC activity as seen from a comparison in activities of genistein with prunetin and also quercetin with rutin in Table 1.

DISCUSSION

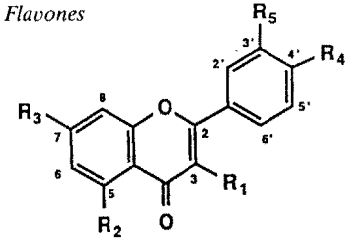
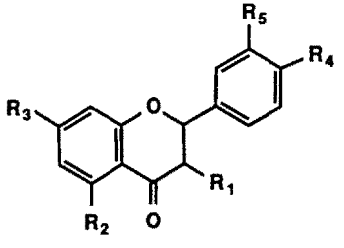
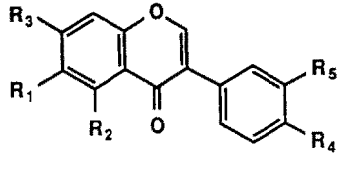
Over the past several years, evidence has accumulated that DNA topoisomerase II is an important cellular target for a number of antineoplastic DNA intercalators, as well as the nonintercalating drugs etoposide (VP-16) and teniposide (VM-26) [2–5, 14, 15]. Although the derivatives of these topoisomerase inhibitors have been used to establish a quantitative relationship between drug-induced cleavable complex formation and cytotoxicity [6, 7], other new inhibitors that induce TDC activity have not been reported yet. In this study, we found that flavonoids showed TDC activities *in vitro* like the antitumor drugs described above. The isoflavones, genistein and orobol were the most potent flavonoids among thirty flavonoids screened for their abilities to induced TDC *in vitro*. In addition to TDC activities, intercalation activities like those of *m*-AMSA and Adriamycin® were detected for some flavone compounds such as quercetin and fisetin. Because most of the previously reported topoisomerase II targetting drugs were intercalative compounds, except for the epipodophyllotoxins VP-16 and VM-26, it is very interesting that TDC activities of flavonoids were detected for both intercalative and nonintercalative compounds. The structures of genistein and orobol are more simple than those of known topoisomerase II active compounds and so the structure–activity relationship described in this report should provide a valuable research tool to understand the molecular properties of "cleavable complex" formation.

Since flavonoids occur widely in plants, they have been considered to be non-toxic (early reports suggested an essential vitamin-like role of dietary flavonoids [16]. Because of their potent TDC activities *in vitro*, we are now interested in the possible functions of flavonoids to regulate the growth of plants and in the reevalutation of antitumor activities of flavonoids, in particular, isoflavones such as genistein and orobol. Genistein and structurally related flavonoids were reported to have no significant antineoplastic activity in the screening program of the National Cancer Institute [17]. Akiyama *et al.* showed that genistein is a highly specific inhibitor for tyrosine-specific protein kinases ($ID_{50} = 0.7$ to 8.0 μg/mL) and inhibits epidermal growth factor-stimulated phosphorylation in cultured cells *in vitro* [18]. In a previous paper, however, they reported that genistein does not show any toxic effect in mice at 500 mg/kg [19]. Recently, Okura *et al.* reported that genistein inhibits both topoisomerase I and II and suppresses selectively the growth of *ras*-transformed NIH 3T3 cells in prolonged culture [20].

In this paper we reported that some flavonoids, such as quercetin and fisetin, show not only TDC activity but also intercalation activity *in vitro*. Quercetin is one of the most well-known flavonoids studied for its biological effect [21]. Quercetin has been reported to be mutagenic (Ames test) [22], to inhibit many cellular enzymes such as Na^+, K^+- [23] and Ca^{2+}, Mg^{2+}-ATPase [24] and some protein kinases including protein kinase C [25] and tyrosine-specific protein kinase [26], and to inhibit the growth of tumor cells (L1210 leukemia cells, P388 leukemia and Ehrlich ascites) [27]. Cunningham *et al.* have reported recently that 3′,4′-dihydroxyflavone and quercetin inhibit the growth of Abelson-transformed NIH3T3 cells which express the Abelson tyrosine-protein kinase, with IC_{50} values of 8 and 91 μM respectively [28]. Although many researchers have speculated that the inhibitory effects of quercetin on several of the enzymes noted above are probably involved in the mechanism of its cytotoxicity, our results show another possibility. TDC and the intercalation activity of quercetin may be important for its cytotoxic effect *in vitro*.

Why are the potent TDC activities of flavonoids not correlated to their cytotoxicity and, in particular, their antitumor activity *in vivo* in the murine tumor model? One possible reason is that flavonoids are metabolized to inactive compounds in mammalian cells, such that the active flavonoids cannot reach their target, the nucleus. Several metabolic pathways of flavonoids, such as the hydrolysis of its glycoside,

Table 1. Formulas and TDC and intercalation activity of flavonoid compounds (listed by the chemical structure)

	Compound	Substitutions OH at,	TDC	Intercalation
Flavones 	Flavone	—	+/−	+/−
	Flavonol	R1	—	—
	Chrysin	R2,R3	—	—
	Apigenin	R2,R3,R4	+/−	—
	Fisetin	R1,R3,R4,R5	+	+
	Quercetin	R1,R2,R3,R4,R5	+	+
	Myricetin	R1,R2,R3,R4,R5, 5′	+	−
	Rutin	R2,R3,R4,R5, R1 = Rutinose	−	−
	Morin	R1,R2,R3,R4, 2′	+	+/−
Flavanones 	Naringenin	R2,R3,R4	—	—
	Dihydro-quercetin	R1,R2,R3,R4,R5	—	—
	Dihydro-myricetin	R1,R2,R3,R4,R5, 5′	—	—
Isoflavones 	Daidzein	R3,R4	—	—
	Genistein	R2,R3,R4	++	—
	Prunetin	R2,R4,R3 = OMe	+	—
	Puerarin	R3,R4, 8 = Glucose	−	−
	4′,6,7 OH-isoflavone	R1,R3,R4	−	−
	Orobol	R2,R3,R4,R5	++	−
	Tectorigenin	R2,R3,R4,R1 = OMe	+/−	−

TDC activity was compared by the amount of a linear DNA generated in the presence of 250 μM flavonoid. As a positive control (not shown), the TDC activities of both *m*-AMSA and VP-16 were indicated by (+ + −) which means that linear DNA generated was in excess of 30% of total DNA under the same experimental conditions. Intercalation activity was compared by the degree of the unwinding which was detected as a conversion of plasmid DNA from the relaxed form to the supercoiled form in the DNA unwinding assay. In the TDC column, the symbols used indicate: (+ +) linear DNA generated was 20–30% of total DNA; (+) linear DNA was 10–20%; (±) linear DNA was less than 10%; (−) same as no drug control. In the intercalation column: (+) almost complete unwinding; (±) partial unwinding; (−) no effect.

oxidation, methylation and conjugation with glucuronic acid, have been reported in several species including human [29], but further study will be required to discuss the therapeutic application of flavonoids.

The data described in this paper should be useful for further studies in the following aspects: (i) At present, little is known about how topoisomerase active drugs form a cleavable complex between enzyme and DNA. Apparently, nonintercalative drugs have been preferred for studies on this point; however, only VP-16 and VM-26 have been known as the nonintercalative topoisomease poison. In the present paper, we showed that both nonintercalative and intercalative flavonoids, such as genistein and quercetin, can induce cleavable complex formation; therefore, these flavonoids will provide useful tools for future experimental approaches to understanding the mechanism itself. (ii) Topoisomerase active drugs may potentially act synergistically with other antineoplastic drugs having different mechanisms of action. Schnipper *et al.* showed that the topoisomerase II inhibitor, novobiocin, potentiated *cis*-platinum cytotoxicity in mammalian cells.* Since flavonoids are considered to be non-toxic, topoisomerase active flavonoids may be valuable in combination chemotherapy. (iii) With regard to antitumor drug design, the studies on the structure–activity relationship of flavonoids are described in this paper can provide an important point of departure for further analogue synthesis.

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