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Flavonoids As DNA Topoisomerase I Poisons

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The therapeutic anticancer potential of flavonoids shown by recent research needs a greater understanding of these compounds. They are antioxidants and antimutagenic agents that can inhibit tumor promotion and transformation and can modify the activity of a large number of mammalian enzyme systems, such as human DNA-topoisomerases. Poisons of topoisomerases generate toxic DNA damage by stabilization of the covalent DNA-topoisomerase cleavage complex and some of them have therapeutic efficacy in human cancer. The present investigation has assayed ten flavonoids, isolated in our laboratory, as topoisomerase I poisons obtaining myricetin and myricetin-3-galactoside as two new topoisomerase I poisons. These two flavonoids, and the plant extract from which they were isolated, were assayed for cytotoxic activity against three human cancer cell lines using the SRB assay. Taking into account our previous research, structural requisites implicated in the topoisomerase poisoning are discussed.

Keywords: Topoisomerase poisons; SRB assay; Flavonols; Isoflavones; Myricetin

INTRODUCTION

The protection against some forms of cancer provided by many common foods containing non-nutritional components such as flavonoids, have been observed in different epidemiological studies. These reports have triggered much research activity which has shown that flavonoids are antioxidants and antimutagenic agents. They can also inhibit tumor promotion and transformation and can modify the activity of a large number of mammalian enzyme systems that are sometimes involved in important pathways that regulate cell division and proliferation.[1] Thus, flavopiridol (the first cyclin-dependent kinase inhibitor to be tested in Phase II clinical trials), genistein (whose antibody conjugates, B43-genistein and EGF-genistein, are currently in clinical development for the treatment of acute lymphoblastic leukemia and breast cancer, respectively) and catechin and its galates (major ingredients of green tea extract GTE-TP91, that has been conducted in adult patients with solid tumors as a Phase I study) are flavonoids emerging as prospective anticancer drug candidates.[2]

DNA topoisomerases (topos) are essential enzymes that govern DNA topology through transient DNA cleavage, strand passing and religation during fundamental nuclear metabolic processes, such as replication and transcription. Topo I acts by forming a transient single-strand break through which the other DNA strand passes to achieve relaxation. Poisons of topoisomerases allow the enzyme to cut and covalently bind to DNA, but prevent the subsequent rejoining of the molecule after relieving the torsional stress causing stabilization of the covalent topo-DNA cleavage complex. Stabilization of the cleavage complex on DNA may not be directly cytotoxic. It appears that there must be some secondary event to generate the toxic DNA lesion. One attractive model that has experimental support suggests that collision of DNA replication forks with cleavage complexes causes the complex to fall apart without rejoining DNA, thereby generating lethal double strands breaks.[3,4] Stabilization of cleavage complexes by topoisomerase poisons is thought to underlie their genotoxicity and efficacy as antineoplastic drugs.[4,5] Besides, tumoral cells have a higher topoisomerase level than normal cells.[6] Consequently, it seems of great interest to identify new topoisomerase poisons.

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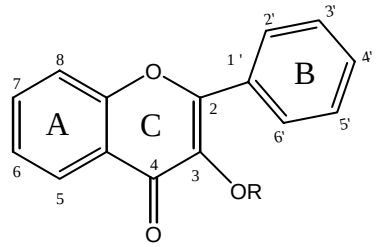
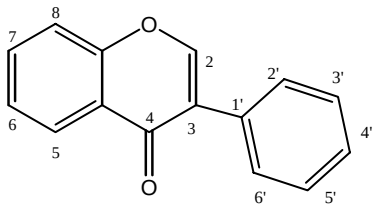


Flavonol



Isoflavone

Flavonols	3 (R)	5	7	3'	4'	5'	6'
myricetin (**1**),	H	OH	OH	OH	OH	OH	
myricetin-3-galactoside (**2**),	Gal	OH	OH	OH	OH	OH	
kaempferol (**3**),	H	OH	OH		OH		
kaempferol-3,7-dirhamnoside (**4**)	Rha	OH	ORha		OH		
kaempferol-3-glucoside-7-rhamnoside (**5**)	Glc	OH	ORha		OH		
rhamnazin (**a**)	H	OH	OMe	OMe	OH		
rhamnazin-3-glucose-arabinose (**b**)	Glc-Ara	OH	OMe	OMe	OH		
rhamnazin-3-glucose-apiose-arabinose (**c**)	Glc-Api-Ara	OH	OMe	OMe	OH		
Isoflavones							
genistein (**6**),		OH	OH		OH		
genistin (**7**),		OH	OGlc		OH		
daidzin (**8**),			OGlc		OH		
6'-methoxipseudobaptigenin (**9**)			OH	-OMeO-			OMe
6'-methoxipseudobaptigenin-7-glucoside (**10**)			OGlc	-OMeO-			OMe

FIGURE 1 Structures of the studied flavonoids (**1**–**10**). Flavonoids a–c are not assayed in the present work, but their structures are useful for discussing the structure–activity relationships.

With this purpose and as part of our continuing search of cytotoxic flavonoids that may interfere with DNA topoisomerase activity, we have evaluated ten flavonoids isolated in our laboratory as DNA topoisomerase poisons. These compounds were the flavonols myricetin (**1**), myricetin-3-galactoside (**2**), kaempherol (**3**), kaempherol-3,7-dirhamnoside (**4**) and kaempherol-3-glucoside-7-rhamnoside (**5**) and the isoflavones genistein (**6**), genistin (**7**), daidzin (8), 6′-methoxipseudobaptigenin (**9**) and 6′-methoxipseudobaptigenin-7-glucoside (**10**) (Fig. 1). Camptothecin was used as a positive control. The flavonoids myricetin (**1**) and myricetin-3-galactoside (**2**) were found to be topoisomerase I poisons. These two compounds and the plant extract from which they were isolated, have also been assessed for cytotoxicity against three human cancer cell lines using the SRB assay with etoposide as positive control.

MATERIALS AND METHODS

Enzymes Nucleic Acids and Chemicals

Purified enzyme, supercoiled DNA and the positive control campthotecin were purchased from Topo-Gen, Inc. (Columbus, OH, USA). Proteinase K and etoposide were from Sigma Chemical Co. The flavonoids myricetin (**1**), myricetin-3-galactoside (**2**) were isolated from an ethyl acetate extract from *Erica andevalensis*[7] kaempherol (**3**), kaempherol-3,7-dirhamnoside (**4**) and kaempherol-3-glucoside-7-rhamnoside from an aqueous extract from *Dorycnium rectum*[8] and the isoflavones genistein (**6**), genistin (**7**), daidzin (**8**), 6′-methoxipseudobaptigenin (**9**) and 6′-methoxipseudobaptigenin-7-glucoside (**10**) were isolated from different extracts from *Retama sphaerocarpa* Boissier[9] (additional detailed information on the preparation of the extracts and isolation of the compounds are available from the author). Stock solutions of these compounds were dissolved in dimethylsulfoxide (DMSO) at 40 mM and were diluted in water containing 2.5% DMSO before use.

Assay for Cytotoxic Activity on Human Cancer Cell Lines

The following three human cancer cell lines were used in these experiments: the human renal adenocarcinoma (TK-10), the human breast adenocarcinoma (MCF-7) and the human melanoma (UACC-62) cell lines. They were kindly provided by Dr. G. Cragg, Department of NCI, Maryland, USA. The human tumor cytotoxicities were determined following protocols established by the National Cancer Institute, National Institute of Health.[10]

Testing procedure and data processing: For the assay, cells were detached with 0.1% trypsin-EDTA (Sigma) to make single-cell suspensions, and viable cells were counted using a Coulter counter and diluted with medium to give final concentrations of 15×10^4, 5×10^4 and 100×10^4 cells/ml for TK-10, MCF-7 and UACC-62, respectively. 100 μl/well of these cell suspensions were seeded in 96-well microtiter plates and incubated to allow for cell attachment. After 24 h the cells were treated with the serial concentrations of compounds or extracts. These were initially dissolved in an amount of 100% DMSO (10 mM) and further diluted in medium to produce 5 concentrations. 100 μl/well of each concentration was added to the plates to obtain final concentrations of 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M for the compounds and 250, 25, 2.5, 0.25 and 0.025 μg/ml for the extract. The DMSO concentration for the tested dilutions was not greater than 0.25% (v/v), the same as in the solvent control wells. The final volume in each well was 200 μl. The plates were incubated for 48 h.

Sulphorhodamine B method: This colorimetric assay estimates cell number indirectly by staining total cellular protein with the dye SRB. After incubating for 48 h, adherent cell cultures were fixed *in situ* by adding 50 μl of cold 50% (w/v) trichloroacetic acid (TCA) and incubating for 60 min at 4°C. The supernatant was then discarded and the plates are washed five times with deionised water and dried. 100 μl of SRB solution (0.4%, w/v in 1% acetic acid) was added to each microtiter well and the culture was incubated for 30 min at room temperature. Unbound SRB was removed by washing five times with 1% acetic acid. The plates were then air-dried, bound stain solubilized with Tris buffer, and the optical densities read on an automated spectrophotometric plate reader at a single wavelength of 492 nm. IC_{50} values were calculated and at least three independent experiments were carried out for each compound or extract. Data are given as the mean ± SEM.

DNA Cleavage Reactions With Topoisomerase I

Cleavage topo I buffer contained 10 mM Tris–HCl pH 7.9, 1 mM EDTA, 0.15 M NaCl, 0.1 % BSA, 0.1 mM spermidine and 5 % glycerol. In the cleavage reaction (20 μl) contained water, cleavage buffer, compounds dissolved in 2μl dimethylsulfoxide/H_2O (2.5%), supercoiled DNA (0.25 μg in 1 μl of buffer), and 2.5 μl (5 units) of topoisomerase I storage buffer were mixed in this order in ice/water. Reactions were carry out by incubation at 37 °C for 30 min, terminated by the addition of 2 μl SDS 10% and 1 μl proteinase K 20 μg/ml and followed by an additional 30 min incubation at 37 °C. Subsequently, the samples were extracted with chloroform:isoamyl alcohol, and 2μl bromophenol blue. Samples were loaded on 1% agarose gels and electrophoresed at 3 V/cm for 6 h in Tris–acetate–EDTA buffer with ethidium bromide to a final concentration of 0.5 μg/ml. Gels were washed in a larger amount of water. For the quantitative determination of topo I activity, videoimpresion was densitometrically measured using PCBAS software. After integration of the bands, nicked open circle DNA (OC) form was expressed as percentage of total DNA.

RESULTS

The results depicted in Table I show the cytotoxic activity on the human cancer cell lines TK-10, MCF-7 and UACC-62 of the flavonoids myricetin (**1**), myricetin-3-galactoside (**2**) and the plant extract from which they were isolated. The antineoplastic agent etoposide was used as a positive control for comparison with the tested compounds. The ethyl acetate extract from *Erica andevalensis* was active on the three cancer cell lines and its activity on MCF-7 and UACC-62 cell lines is consider as a cytotoxic one by the NCI (USA) ($IC_{50} < 15$ μg/ml). The two flavonoids isolated from this extract showed cytotoxic activity on MCF-7 and UACC-62 cell lines at the recommended NCI (USA) doses although they were less active than etoposide. Their inactivity on TK-10 cell line and the different inactive compounds tested on the three cell lines in previous experiments in our laboratory,[11,12] could be regarded as genuine negative indicators, testifying the specificity of the designed bioassay systems.

Figure 3 shows the effects on topoisomerase I of the ten flavonoids used in the present study as well as that of the positive control camptothecin, at a final concentration of 100 μM. The gel presented in Fig. 2 shows the activity of five of the ten flavonoids tested and, as can be seen, myricetin

TABLE I Cytotoxic activity (expressed as IC_{50} ± SEM values) of flavonoids (**1**) and (**2**), the plant extract from which they were isolated and the positive control (etoposide) on the human cancer cell lines TK-10, MCF-7 and UACC-62. *n*: number of independent experiments

	n		TK-10	MCF-7	UACC-62
Ethyl acetate extract	3	IC_{50} (μg/ml)	51.3 ± 3.2	5.9 ± 0.3	14.1 ± 5.1
Myricetin (1)	3	IC_{50} (μM)	>100	19.9 ± 0.9	17.9 ± 0.6
Myricetin-3-galactoside (2)	3	IC_{50} (μM)	>100	21.2 ± 0.7	14.6 ± 2.1
Etoposide	1	IC_{50} (μM)	9.95 ± 0.08	0.87 ± 0.21	1.13 ± 0.21

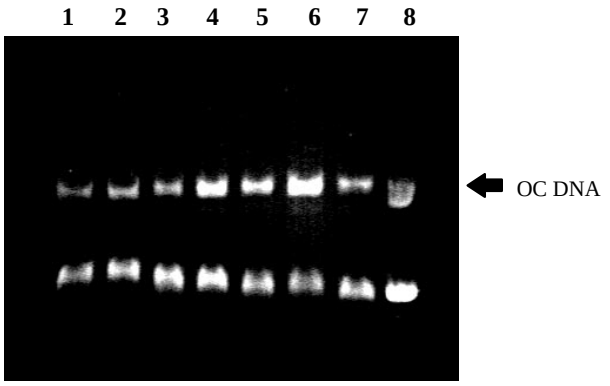


FIGURE 2 DNA topoisomerase I mediated DNA cleavage: **1**. Topo I + DNA (supercoiled) + 100 μM kaempferol-3,7-dirhamnoside, **2**. Topo I + DNA + 100 μM kaempferol-3-glucoside-7-rhamnoside, **3**. Topo I + DNA + 100 μM kaempferol, **4**. Topo I + DNA + 100 μM myricetin-3-galactoside, **5**. Topo I + DNA + 100 μM myricetin, **6**. Topo I + DNA + 100 μM camptothecin, **7**. Topo I + DNA, **8**. DNA.

(6.7%) and myricetin-3-galactoside (8.1%) induced the formation of OC DNA, acting as topoisomerase I poisons. (Camptothecin: 14.5%). The other five isoflavones (gel not presented because of their lack of activity) did not induce the stabilization of the cleavage complexes at the studied concentration and thus did not act as topoisomerase I poisons.

DISCUSSION

Taking into account these results and other works carried out in our laboratory we can discuss some structural requisites that can be implicated in topoisomerase poisoning. Initially, it can be observed that none of the five tested isoflavones acted as DNA topoisomerase I poison whereas the isoflavones **6**, **7**, **8** and **10** were active as DNA topoisomerase II poisons in a previous work carried out in our laboratory (isoflavones **7** and **8** were active at 250 and 500 μM and **10** at 500 μM. **6** was used as positive control at 100 μM).[13] This suggests that the isoflavone structure (B-ring at C-3 position) might produce selectivity against topo II poisoning. Another interesting structural feature is that most of the flavonoids found in the literature as possible anticancer agents are aglycones (flavonoids without sugar moieties) and although sugar moieties are not considered as an important element in the structure, our results show clearly that they can influence topoisomerase poisoning. Sometimes they increase topo I poisoning, as we can see if we compare flavonols **1** with **2** (Fig. 1 for structure). In the same way, we showed in another preceding work that two flavonol glycosides were topoisomerase I poisons whereas their aglycone was not (flavonols b, at 100 and 250 μM, and C, at 250 μM, were active whereas A was inactive at these two concentrations).[14] On the other hand, sugar moietes can reduce topo II poisoning, as occurs with the glycoside genistin (**7**) compared with its aglycone genistein (**6**).[13] Therefore, our results suggest that sugar moieties can increase the topo I activity of flavonols and reduce the topo II activity of isoflavones. Finally, we have observed that topoisomerase poisoning is not always dose-dependent (isoflavone **7** is more active at 250 μM than at 500 μM).[13]

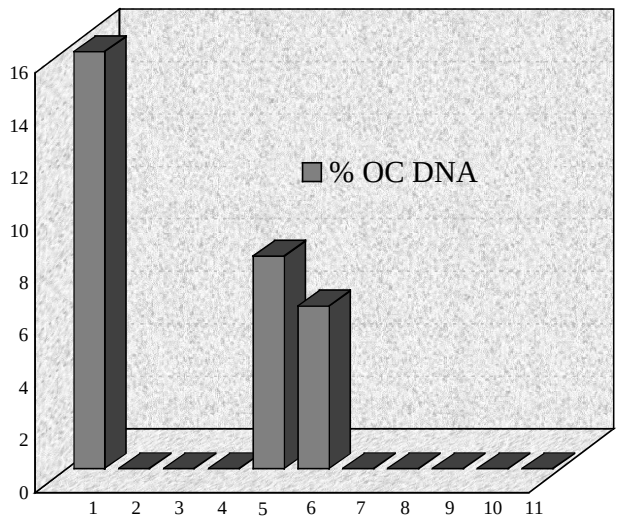


FIGURE 3 Topoisomerase I-mediated DNA cleavage, expressed as percentage of OC (open circular) DNA, induced by 1. campothecin, 2. kaempherol, 3. kaempherol-3,7-dirhamnoside, 4. kaempherol-3-glucoside-7-rhamnoside, 5. myricetin-3-galactoside, 6. myricetin, 7. genistein, 8. daidzin, 10. 6′-methoxipseudobaptigenin, 11. 6′-methoxipseudobaptigenin-7-glucoside. All the compounds were tested at a final concentration of 100 μM.

It is important to know that many flavonoids occur in our diet and that flavonoids are generally safe and without adverse effects. Flavones can be found in grains and herbs, flavonols and their glycosides in fruits and vegetables, flavanones in citrus juices or isoflavones in legumes.[15] In this way myricetin is presented in red wines, pineapple, orange, lime and berries among other foods[16–19] and although there are studies that show its antitumor activity,[20,21] this is the first report of myricetin acting as a topoisomerase I poison. Here we also present the first cytotoxic report of a galactoside of myricetin (**2**) that shows similar activity to myricetin on three tested human cancer cell lines its activity being slightly higher as a topoisomerase I poison.

Interest concerning topoisomerase I poisons has increased since 1997, when the camptothecin derivates topotecan and irinotecan were introduced in the clinic for the treatment of fluoropyridine-refractory ovarian and colorectal cancer, respectively, thus constituting topoisomerase I poisons as a novel family of antitumor agents.[22] Other reports show that the fungal toxin saintopin and the 7H-benzo-pyrido-indole intoplicine are able to stabilize the

catalytic intermediate of topoioserase I and II. Clinical trials showed that these substances may form a new class of antitumor drugs which are active on a variety of solid tumors and escape cross-resistance to drugs that target solely one type of topoisomerase.[23] Therefore, it is interesting to know that myricetin can act as a topoisomerase II poison[24] in a similar way, as we show here for topoisomemerase I. Another possibility for acting on the two topoisomerases at the same time would be by using a different poison for each enzyme. In this way Kancherla and co-workers have reported Phase I data on a topotecan and etoposide combination for patients with recurrent or refractory non-Hodgkin lymphoma.[25] Accordingly, and bearing in mind the potential anticancer effects of some dietary flavonoids acting as topoisomerase I poisons, such as quercetin[23] or myricetin, and taking into account their lack of adverse effects, it would be interesting to evaluate if the combination of flavonoids with the topoisomerase II poisons that nowadays are being used in clinical therapy, is able to reduce their toxic effects without decreasing their effectiveness.

Finally, in our opinion the increase in literature concerning flavonoids as potential anticancer agents is not only leading to new drug discoveries but could also influence our drinking and dietary habits.

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