Two New Flavonol Glycosides as DNA Topoisomerase I Poisons

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Flavonoids are secondary plant metabolites whose anticancer properties are actually being studied from an epidemiological and pharmacological point of view. They are believed to be implicated in the lower risk of some forms of cancer observed in Asian countries, due to their capacity to control cell proliferation, to act on certain regulatory enzymes as protein kinases or topoisomerases. Based on these precedents, three flavonols isolated from a cytotoxic butanol extract from Retama sphaerocarpa Boissier have been assessed to study their topoisomerase I and II activity. Two new rhamnazin glycosides were found to have the ability to stabilize the cleavage complex human DNA topoisomerase I at concentrations in the 100–250 μM range, acting as topoisomerase I poisons.

Introduction

DNA topoisomerases (topos) are essential enzymes that control 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 and topo II is able to do so with the two strands that make up duplex DNA, creating a DNA-linked protein gate through which another intact duplex passes (Wang, 1985).

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 a secondary event to generate the toxic DNA lesion. One attractive model that has experimental support claims that collision of DNA replication forks with cleavage complexes causes the complex to fall apart without rejoining DNA, thereby generating lethal double strand breaks (Hsiang et al., 1989; Kaufmann, 1998).

Stabilization of cleavage complexes by topoisomerase poisons is thought to underlie their genotoxicity and efficacy as antineoplastic drugs (Pommier, 1993; Kaufmann, 1998). Besides, tumor cells have higher topoisomerase level than normal cells (Cardellini and Durban, 1993). So, it seems to be interesting to identify new topoisomerase poisons.

With this objective and as part of our continuing search for cytotoxic flavonoids from Retama sphaerocarpa Boissier (López-Lázaro et al., 1998, 1999, 2000; Martín-Cordero et al., 1999a, 1999b, 2000), we have studied three flavonols, rhamnazin (1), rhamnazin-3-O-β-D-glucopyranosyl-(1→5)-α-L-arabinofuranoside (2) and rhamnazin-3-O-β-D-glucopyranosyl-(1→5)-[β-D-apiofuranosyl-(1→2)]-α-L-arabinofuranoside (3), isolated from a cytotoxic BuOH extract from Retama sphaerocarpa Boissier, as topoisomerase I and II poisons and we have found that the two new flavonols glycosides (2) and (3) are topoisomerase I poisons.

Materials and Methods

Plant material

The aerial parts of R. sphaerocarpa were collected at Zahara de la Sierra (Cádiz, Spain) in May 1996, during the flowering period. The identity was kindly verified by Dr A. Aparicio (Laboratory of Botany of the Faculty of Pharmacy, University of Sevilla) and a voucher specimen was deposited in the herbarium of this Faculty (SEV-F).
Enzymes, nucleic acids and chemicals

Purified enzymes, supercoiled DNA and the positive controls camptothecin (for topo I) and etoposide (for topo II) were purchased from TopoGen, Inc (Columbus, OH, USA). Proteinase K was from Sigma Chemical Co. Stock solutions of these drugs were dissolved in dimethylsulfoxide at 40 mM and were diluted in water containing 2.5% dimethylsulfoxide before use.

The flavonols used in the present study, rhamnazin (1), rhamnazin-3-O-β-D-glucopyranosyl-(1→5)-α-L-arabinofuranoside (2) and rhamnazin-3-O-β-D-glucopyranosyl-(1→5)-[β-D-apiofuranosyl-(1→2)]-α-L-arabinofuranoside (3) were isolated from a cytotoxic BuOH extract (López-Lázaro et al., 2000) from Retama sphaerocarpa according to the method of López-Lázaro et al. (1998).

The selected method was the Netien and Lebreton (1964) technique, slightly modified by López-Lázaro et al. (1998): air-dried, powdered aerial parts (500 g) of R. sphaerocarpa were extracted by soxhlet successively for 24 h with Et2O and for 48 h with MeOH. The MeOH extract was evaporated to dryness and suspended in 50 ml H2O, then it was extracted successively with CHCl3, EtOAc and n-BuOH (yielding 54 g dry material). The dry residue was fractionated by column chromatography on silicagel 60 (Merck) and Sephadex LH-20 (Pharmacia), using different proportions of ethyl acetate/methanol/water and dichloromethane/methanol as solvent systems.

DNA cleavage reactions with topoisomerase I and II

Cleavage topo I buffer contained 10 mM tris-HCl pH 7.9, 1 mM EDTA, 0.15 mM NaCl, 0.1% BSA, 0.1 mM spermidine and 5% glycerol. The cleavage reaction (20 μl) contained water, cleavage buffer, flavonols dissolved in 2 μl dimethylsulfoxide/H2O (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 carried out by incubation at 37 °C for 30 min, terminated by the addition of 2 μl SDS (sodium dodecyl sulfate) 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:isooamyl alcohol, and 2 μl bromophenol blue. Samples were loaded on 1% agarose gels and electrophoresed at 3V/cm for 6 h in Tris (tris[hydroxymethyl]aminomethane)-acetate-EDTA buffer (with ethidium bromide to a final concentration of 0.5 μg/ml) and gels were washed in a bigger amount of water.
For topo II assay, the cleavage buffer contained 30 mM Tris-HCl, pH 7.6, 60 mM NaCl, 15 mM mercaptoethanol, 8 mM MgCl₂, 3 mM ATP. The DNA used was pRYG DNA (0.25 μg in 1 μl of buffer) and we used 2 μl (4 units) of topoisomerase II. The second incubation were carried out at 37 °C for 15 min. The gels were electrophoresed at 6V/cm for 2.5h without ethidium bromide, stained with ethidium bromide and washed in water.

For the quantitative determination of topo I and II activity, the bands were densitometrically measured using a PCBAS software. After integration of the bands, linear DNA (topo II assay) and nicked open circle (OC) DNA forms were expressed as percentage of total DNA.

Results and Discussion

The gel presented in Figure 1 shows the three flavonols studied at concentrations of 100 and 250 μM and the positive control camptothecin, at a concentration of 100 μM, in the topo I assay. Compound (3) at a concentration of 250 μM and (2) at the two tested concentrations induce formation of OC DNA, but at a concentration of 100 μM (3) and the aglycone rhamnazin at the two tested concentrations were found to be inactive.

In the topoisomerase II assay, using the positive control etoposide, none of the three flavonoids tested, at concentrations of 100 and 250 μM, induced OC and linear plasmid DNA.

These results show two new compounds as topoisomerase I poisons, and this topoisomerase-mediated DNA damage seems to be a possible mechanism, by which (2) and (3) may exert their cytotoxic activity.

Bearing in mind the structures of the three tested flavonoids and the percentage of OC induced (Figure 2), we can see that the two glycosylated flavonoids are active as topoisomerase I poisons but the aglycone rhamnazin is not. Apparently, the presence of some sugar chain in the aglycone is necessary to act as a topoisomerase I poisons. However, these results seem to disagree with the higher cytotoxicity shown by the aglycone rhamnazin, in relation to the two glycosides, on three human cancer cell lines in a previous work carried out in our laboratory (López-Lázaro et al., 2000). We assumed that the hydrophylic nature of sugars, or the increased molecular size of glycosides could interfere with their transport through cellular membranes and/or that the cytotoxic mechanism of action of rhamnazin must be another one.

Recent research has confirmed that often common food contains non-nutritive components, such as flavonoids, that may provide protection against...
chronic diseases including some forms of cancer (Peterson, 1995; Barnes, 1995, 1997; Fotsis et al., 1997; Pollard and Luckert, 1997; Santibáñez et al., 1997). Epidemiological investigations support this hypothesis (Stavric, 1994; Adlercreutz et al., 1995, 1997; Wiseman, 1996; Wu et al., 1996), because the high level of these compounds are found in countries or regions with low cancer incidence. These epidemiological studies consistently show the cancer-protective effect of fruit and vegetable consumption, but show little understanding of which phytochemicals account for this observation. Several plant derived flavonoids have been previously reported to inhibit certain regulatory enzymes including protein kinase C or DNA topoisomerase I and II (Akiyama et al., 1987; Yamashita et al., 1995; Constantiou et al., 1995).

The present study shows two new flavonols glycosides as topoisomerase I poisons and although they are not potent ones, higher concentrations of these compounds with little toxicity are present in human diet (Herman, 1988; Peterson and Dwyer, 1998; Goda et al., 1999). So, our results are useful to increase the number of known phytochemicals with possible anticancer activity and justify the protective anticancer properties of flavonoids from a pharmacological point of view.

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