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DNA topoisomerase inhibitors: biflavonoids from *Ouratea* species

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Abstract

Topoisomerase inhibitors are agents with anticancer activity. 7"-O-Methyl-agathisflavone (I) and amentoflavone (II) are biflavonoids and were isolated from the Brazilian plants *Ouratea hexasperma* and *O. semiserrata*, respectively. These biflavonoids and the acetyl derivative of II (IIa) are inhibitors of human DNA topoisomerases I at 200 µM, as demonstrated by the relaxation assay of supercoiled DNA, and only agathisflavone (I) at 200 µM also inhibited DNA topoisomerases II-α, as observed by decatenation and relaxation assays. The biflavonoids showed concentration-dependent growth inhibitory activities on Ehrlich carcinoma cells in 45-h culture, assayed by a tetrazolium method, with IC_{50} = 24 ± 1.4 µM for I, 26 ± 1.1 µM for II and 10 ± 0.7 µM for IIa. These biflavonoids were assayed against human K562 leukemia cells in 45-h culture, but only I showed 42% growth inhibitory activity at 90 µM. Our results suggest that biflavonoids are targets for DNA topoisomerases and their cytotoxicity is dependent on tumor cell type.

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- Human K562 leukemia

DNA supercoiling is a precisely regulated process that influences DNA replication, transcription and packaging. DNA topoisomerases are enzymes that modulate the topological state of DNA. There are two classes of topoisomerases: type I acts by transiently nicking one of the two DNA strands and type II nicks both DNA strands and is ATP dependent (1). Interest in these enzymes has increased in the last few years because they are targets for many drugs effective in cancer treatment. Interestingly, flavonoids ubiquitously occurring in green plants have inhibitory activity on a variety of enzymes including topoisomerases (2) and are useful for the treatment of various diseases. For example, *Ginkgo biloba* containing flavonoids and terpenoids is used to prevent coronary disease and cancer (2), and several Leguminosae plants containing isoflavonoids have estrogenic activities.

The interest of our group in studying the chemical constituents of Brazilian plants led us to isolate biflavonoids from the Ochnaceae family. This family is pantropical and has the highest density of genera and species in the tropical zones of South America. The *Ouratea* genus has been characterized as a good source of biflavonoids (3,4).

In the present study, we describe the effect of the biflavonoids 7"-O-methyl-agathisflavone (biflavonoid I), isolated from

Ouratea hexasperma Bail (St. Hill) collected in Amapá, Brazil, and amentoflavone (biflavonoid II) obtained from *O. semiserrata* Mart (Engl.) collected in Ouro Preto, MG, Brazil, and the acetyl derivative of II (biflavonoid IIa) on Ehrlich ascitic carcinoma cells, human K562 leukemia cells (5) and action on the human DNA topoisomerases I and II-α.

Biflavonoids I and II, 7''-O-methyl-agathisflavone [1,4',5,7-trihydroxyflavone-(6→8'')-4''',5''-dihydroxy-7''-methoxyflavone] and amentoflavone [2,4',5,7-trihydroxyflavone-(3→8'')-4''',5'',7''-trihydroxyflavone], respectively, were isolated from a methanolic extract of the leaves using chromatographic techniques, and II was also acetylated with acetic anhydride in the presence of pyridine to yield IIa. The structures of the natural products (biflavonoids I and II) were established on the basis of 1-D and 2-D NMR, MS, IR and UV spectral data, including results obtained for the acetyl derivative IIa (3,4).

The antiproliferative activities of biflavonoids were assayed *in vitro* against murine Ehrlich carcinoma (1 x 10^5 cells) and human K562 leukemia (1 x 10^4 cells) in RPMI complete medium (supplemented with 5% fetal calf serum, 100 µg/ml streptomycin and 100 IU/ml penicillin), and were seeded onto 96-well microplates. The compounds in 0.3% (v/v) DMSO were prepared at concentrations of 200, 100, 50 and 25 µM, and were added to cells and incubated for 48 h at 37ºC in the presence of 5% CO_2 (6). The same volume of DMSO was used as control. Cell viability was assayed in the absence or presence of I, II, and IIa, quercetin and etoposide (the last two as positive control), using the Mossman assay (7). After 45 h of cell culture, MTT [3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide] was added to the samples and absorbance was measured at 570 nm after 3 h at 37ºC. The IC_{50} values (µM) were reported as means ± SD of three experiments.

The biflavonoids presented concentration-dependent growth inhibiting activities on cultured Ehrlich carcinoma cells. The IC_{50} values were 24 ± 1.4, 26 ± 1.1 and 10 ± 0.7 µM for I, II and IIa, respectively, compared with 38 ± 1.4 µM for etoposide, a powerful anticancer agent. Furthermore, the additional inhibitory effect 10 ± 0.7 *vs* 26 ± 1.1 µM of the biflavonoid acetylated on the hydroxy groups (IIa) may be related to the presence of a more lipophilic moiety (acetyl ester *vs* hydroxyl group) than II. This lipophilic compound could probably cross the cell membrane, providing better inhibition of Ehrlich carcinoma cells. During 45 h of exposure of K562 leukemia cells to all biflavonoids assayed, only marginal activity was obtained for I with 42% of growth inhibition at 90 µM. No cytotoxicity was observed with etoposide under these conditions.

The inhibitory effects of biflavonoids on DNA topoisomerases were studied by a relaxation assay for topoisomerases I and II, and by a decatenation assay for topoisomerase II-α. Topoisomerase I was assayed by relaxation of supercoiled plasmid DNA as suggested by TopoGen, Columbus, OH, USA. Briefly, 0.125 µg of supercoiled pBR322 was incubated with 2 units of human topoisomerase I in the presence or absence of different drug concentrations for 30 min at 37ºC in reaction buffer (10 mM Tris-HCl, pH 7.9, 1 mM EDTA, 0.15 M NaCl, 0.1 mM spermidine, 4% glycerol, and 0.1% BSA) and the reaction was stopped with 0.1 volume of stopping solution (5% SDS, 0.025% bromophenol blue and 40% glycerol). The relaxation products were analyzed in TAE buffer (40 mM Tris-acetate, pH 8.5, and 10 mM Na_2EDTA) by electrophoresis on 1% agarose gels at 45 V. Gels were stained with ethidium bromide and photographed under UV light. Topoisomerase II was assayed by decatenation of kDNA as described by TopoGen. Reactions contained 0.125 µg, 50 mM Tris-HCl, pH 8, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol,

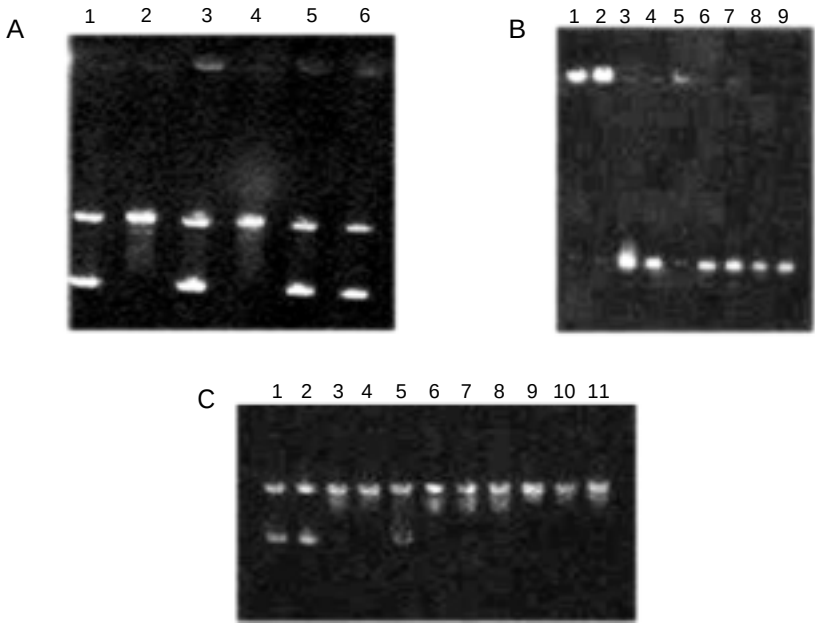

Figure 1. Agarose gel electrophoresis showing the effect of drugs on the relaxation of supercoiled DNA by topoisomerase I, and on decatenation of kDNA and relaxation of supercoiled DNA by topoisomerase II-α. *A*, 0.125 µg pBR322 DNA alone (lane 1) was incubated with topoisomerase I (4 U) (lane 2) in the presence of 200 µM biflavonoid I (lane 3), 200 µM rutin (lane 4), 200 µM biflavonoid II (lane 5), and 50 µM campothecin (lane 6). *B*, kDNA alone (lane 1), kDNA + topoisomerase II-α (2 U) + 100 µM etoposide (lane 2), and kDNA + topoisomerase II-α (2 U) (lane 3), in the presence of 50 µM biflavonoid I (lane 4), 200 µM biflavonoid I (lane 5), 50 µM biflavonoid II (lane 6) and 200 µM biflavonoid II (lane 7), 50 µM biflavonoid IIa (lane 8) and 200 µM biflavonoid IIa (lane 9). *C*, pBR322 DNA alone (lane 1) was incubated with topoisomerase II-α (4 U) (lane 3) in the presence of 100 µM etoposide (lane 2), 50 µM biflavonoid I (lane 4) and 200 µM biflavonoid I (lane 5), 10, 50 and 200 µM biflavonoid II (lanes 6, 7 and 8, respectively), and 10, 50 and 200 µM biflavonoid IIa (lanes 9, 10 and 11, respectively).

30 µg/ml BSA, and 2 units of human topoisomerase II in the presence or absence of varying amounts of drugs. The reaction mixtures were incubated for 30 min at 37ºC and stopped with 0.1 volume of stopping solution. For the topoisomerase II-α relaxation assay, the same buffer was incubated for 30 min and 1 µg/ml proteinase K and 0.5% SDS were added and incubated for an additional 30 min at 37ºC under the same conditions as described above. DNA topoisomers were separated by electrophoresis.

The inhibitory effects of 200 µM biflavonoids I, II and IIa on topoisomerase I are shown in Figure 1A. In the presence of I, II and IIa only the band corresponding to the supercoiled DNA was observed. The same effect was obtained with campothecin (50 µM), used as a positive control. However, the glycosylated flavonoid rutin, used as negative control, did not inhibit the topoisomerase I activity.

The inhibition of human topoisomerase II-α by biflavonoids demonstrated by decatenation assay was observed only at a concentration of 200 µM, with a result similar to 100 µM etoposide (Figure 1B). Figure 1C shows the inhibitory effects of biflavonoids determined by the relaxation assay for topoisomerase II-α with an inhibitory pattern similar to that shown above. Despite small differences in chemical structure of the biflavonoids tested here, they have a similar effect on DNA topoisomerase I and a differential effect on topoisomerase II-α. Biflavonoid I (agathisflavone) affects the catalytic activity of human DNA topoisomerase and is a dual inhibitor. Interestingly, it also showed a borderline inhibitory effect on K562 leukemia cells.

Our results suggest that biflavonoids I and II are cytotoxic against Ehrlich tumor and that DNA topoisomerases are involved in their cytotoxicity, with cytotoxic potency being dependent on the type of tumor. Further studies of flavonoids and their derivatives may lead to the elucidation of the role of DNA topoisomerase inhibitors, thus contributing to the search for better natural chemotherapeutic and cancer preventing agents.

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