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WOODFRUTICOSIN (WOODFORDIN C), A NEW INHIBITOR OF DNA TOPOISOMERASE II

EXPERIMENTAL ANTITUMOR ACTIVITY

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Abstract—Woodfruticosin (woodfordin C) (WFC), a new inhibitor of DNA topoisomerase II (topo-II), was isolated from methanol extract of *Woodfordia fruticosa* Kurz (Lythraceae) and studied for *in vitro* and *in vivo* antitumor activities in comparison with Adriamycin® (ADR) and etoposide (ETP), well known inhibitors of topo-II. The inhibitory activity against DNA topo-II shown by WFC was much stronger than that shown by ETP or ADR. WFC inhibited strongly intracellular DNA synthesis but not RNA and protein synthesis. On the other hand, WFC had a weaker growth inhibitory activity against various human tumor cells than ETP or ADR, but it showed remarkable activity against PC-1 cells and moderate activity against MKN45 and KB cells. Furthermore, WFC had *in vivo* growth inhibitory activity against s.c. inoculated colon38. These results indicate that the mechanism by which WFC exhibits antitumor activity may be through inhibition of topo-II.

A large number of agents which interact with DNA show antitumor activities, but the mechanisms of action in the cell are still unknown. Recently, DNA topoisomerase II (topo-II‡), a novel biochemical target, has been identified as the action site for some of the most widely used antitumor drugs [1–8], such as etoposide (ETP) [1], Adriamycin® (ADR) [2] and actinomycin D (ACT-D) [3]. Most of the inhibitors, except ETP, have an intercalative moiety and bind to DNA and topo-II to stabilize a cleavable complex [9, 10]. However, despite the lack of the intercalative moiety, ETP also forms a cleavable complex. New topo-II inhibitors have been found recently, such as merbarone [11] and fosteriecin [12], which have no intercalative activity and do not cause DNA strand break via stabilization of a cleavable complex.

We have screened many materials extracted from microorganisms and plants with the purpose of finding an inhibitor of topo-II, which led to the isolation of woodfructicosin (woodfordin C) (WFC) (Fig. 1) from methanol extract of the leaves of *Woodfordia fruticosa* Kurz (Lythraceae) [13]. This compound, structurally unique in comparison with other inhibitors of topo-II, is a new dimeric hydrolysable tannin and has a large M_r of 1720.

In this study, we describe the inhibitory activity on topo-II and antitumor activity on cultured tumor cell lines of WFC in comparison with other topo-II inhibitors.

MATERIALS AND METHODS

Chemicals. Trizma base, ATP and dithiothreitol were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). P-11 phosphocellulose resin was purchased from Whatman Biosystems Ltd (Maidstone, U.K.). [^{3}H]Thymidine, [^{3}H]uridine and [^{3}H]leucine were obtained from Amersham Japan Co. (Tokyo, Japan). Other agents used were of analytical grade.

Topo-II assay. The topo-II assay was performed by the method of Miller *et al.* [14] with some modifications. The reaction for topo-II was carried out at 37° for 1 hr in a 10 μL reaction mixture containing 50 mM Tris–HCl (pH 7.4), 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM ATP, 1 mM dithiothreitol, 0.5 μg of kinetoplast DNA, 1 μL of sample solution and 1 μL of crude enzyme solution. After the reaction, the mixture was mixed with 2 μL of 75 mM EDTA, 75% glycerol and 0.075% bromophenol blue prior to the analysis of DNA products by 1.0% agarose gel electrophoresis. The gel was stained with 0.5 μg/mL ethidium bromide and phorographed under UV light (265 nm).

Preparation of crude enzyme. Rat ascites hepatoma, AH66F cells were maintained by intraperitoneal passage of ascites in Donryu rats and harvested from the peritoneal cavity 5 days after tumor inoculation. The cells were washed three times with Hanks' balanced salt solution and suspended in nuclear buffer (0.15 M NaCl, 5 mM $MgCl_2$, 1 mM KH_2PO_4, 1 mM EGTA, 0.1 mM dithiothreitol and 10% glycerol), and then homogenized with teflon homogenizer and centrifuged at 8000 *g*. The pellet was resuspended in nuclear buffer containing 1 M sucrose and 1 mM phenylmethylsulfonyl fluoride and further homogenized with tightly fixed teflon homogenizer. After centrifugation at 10,000 *g* for 10 min, the nuclear

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‡ Abbreviations: WFC, woodfruticosin; ADR, Adriamycin; ETP, etoposide; ACT-D, actinomycin D; topo-II, topoisomerase II.

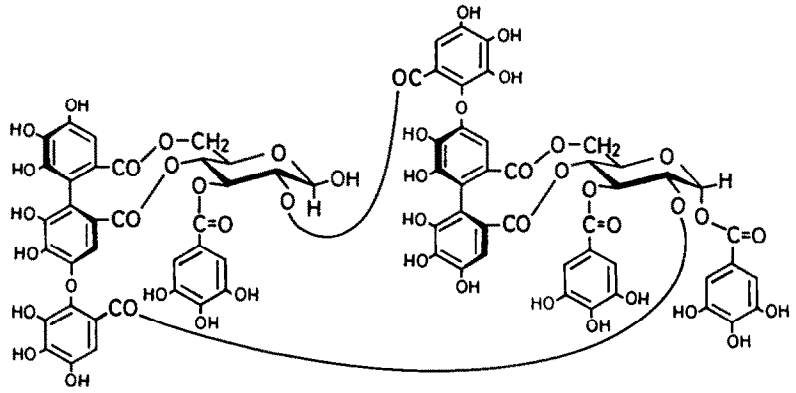

Fig. 1. Chemical structure of WFC.

Table 1. Growth inhibitory activities of WFC, ETP and ADR against various tumor cell lines

Cell	Origin	IC_{50} (μg/mL)* WFC	ETP	ADR
MKN45	Stomach cancer	1.73	0.67	0.018
SW1116	Colon adenocarcinoma	54.35	0.81	0.059
$HepG_2$	Hepatoma	13.21	0.25	0.041
Li-7	Hepatoma	21.29	0.22	0.020
PC-1	Lung carcinoma	0.07	0.12	0.015
MOLT-3	Leukemia	22.21	0.12	0.014
K562	Leukemia	27.20	0.23	0.011
KB	Nasopharyngeal carcinoma	5.58	0.17	0.0073
HeLa S_3	Cervix carcinoma	>200.00	0.46	0.026

* IC_{50} values were determined as described in Materials and Methods.

protein was extracted from the nuclear pellet by keeping the material for 20 min in ice-cold nuclear buffer containing 0.35 M NaCl and 1 mM phenylmethylsulfonyl fluoride and then centrifuged at 10,000 *g* for 10 min. The supernatant was collected and the extraction was repeated, resuspending the pellet in the same buffer. The supernatant obtained in the second extraction was combined with the supernatant obtained previously. The crude extract was fractionated through a P-11 Phosphocellulose column, which was eluted with a stepwise gradient of 0.2, 0.4, 0.6 and 0.8 M KCl at a flow rate of 0.25 mL/min. The active fractions were pooled and used as a crude topo-II.

Preparation of DNA substrate. Kinetoplast DNA was purified from *Crithidia fasciculata* (generous gift from Dr K. Tsutsui, Okayama University) by cesium chloride step gradient centrifugation [15].

Growth inhibitory activity against tumor cell lines. Table 1 shows the human tumor cell lines and culture conditions used in this study. The cells were incubated with WFC under 5% CO_2–95% air at 37°. After treatment with the drug, growth inhibition was observed. In K562 cell lines the growth inhibition was estimated by cell number counting assay using a Coulter counter, model ZBI, while in other cell lines it was estimated by dye staining assay [16].

Inhibitory activity on intracellular macromolecular synthesis. Mouse leukemia P388 cells were harvested from the peritoneal cavity of a DBA2 mouse and suspended in RPMI 1640 medium supplemented with 10% fetal bovine serum and 20 mM HEPES. They were seeded on a 96-well tissue culture plate and treated with WFC under 5% CO_2–95% air at 37° for 1 hr. After the treatment, 1 μCi of [^{3}H]-thymidine, [^{3}H]uridine or [^{3}H]leucine was added and incubated for another hour. Uptake of precursor to DNA and RNA were determined by counting the radioactivities collected on a glass filter using cell harvester (Skatoron, Conbi cell harvester). In the case of protein synthesis, the cells were lysed by addition of 5% trichloroacetic acid–1% sodium pyrophosphate. The lysate was collected on a Millititer HA filter plate with 0.45 μm mesh. The radioactivity on the filter was measured by a liquid scintillation counter.

Determination of cytocidal activity. Cytotoxicity assays of PC-1 and HeLa S_3 cells were carried out on exponentially growing cells treated with WFC for 1 or 24 hr and replated as single cell suspensions into Falcon 3002 6-cm dishes. Colonies made of more than 50 cells were stained with methylene blue after 12 days [17].

In vivo *antitumor activity against mouse C38*

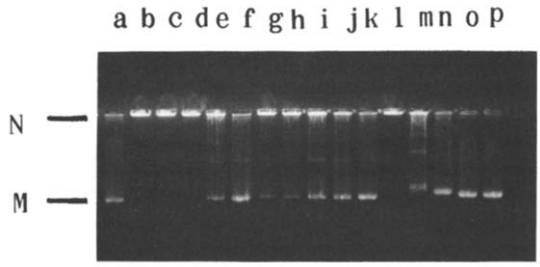

Fig. 2. Effect of WFC, ETP and ADR on the decatenation activity of DNA topo-II. Lane a, control; lane b, 20 μg/mL WFC; lane c, 10 μg/mL; lane d, 5 μg/mL; lane e, 2.5 μg/mL; lane f, 1.25 μg/mL; lane g, 20 μg/mL ETP; lane h, 10 μg/mL; lane i, 5 μg/mL; lane j, 2.5 μg/mL; lane k, 1.25 μg/mL, lane l, 20 μg/mL ADR; lane m, 10 μg/mL; lane n, 5 μg/mL; lane 0, 2.5 μg/mL; lane p, 1.25 μg/mL. The positions of kinetoplast network DNA and minicircle DNA are marked as N and M, respectively.

adenocarcinoma. A tumor fragment about 1.5 mm in size of colon38, mouse colon adenocarcinoma, was s.c. transplanted to the flank of a BDF_1 mouse. When the tumor volume reached about 50 mm^3, WFC was i.v. administered once daily for 5 consecutive days. Antitumor activity was estimated by tumor volume and T/C (%).

RESULTS

Inhibition of decatenation activity of topo-II

Figure 2 shows the result of the inhibitory effects of WFC on the decatenation activity of topo-II. ETP and ADR were included for comparison since they have been established previously as inhibitors of this enzyme [1, 2]. WFC completely inhibited the decatenation of kinetoplast network DNA at a concentration of 2.5 μg/mL. Similar results were obtained with ADR and ETP at 5.0 and 10 μg/mL, respectively. WFC was 10-fold more active than ETP and ADR when activity was compared at molar concentration.

Growth inhibition of WFC against various tumor cell lines

Inhibitory activity of WFC on the growth of various tumor cells is shown in Table 1. WFC remarkably inhibited the growth of PC-1 cells. The inhibitory activity against MKN45 and KB cells was moderate, but there was no effect against HeLa S_3 and SW1116. Thus, WFC showed a high inhibitory activity against some kinds of tumor cell line.

Inhibition of macromolecular synthesis by WFC

As shown in Fig. 3, WFC exhibited stronger inhibition of DNA synthesis than of RNA and protein synthesis. ETP, a non-intercalative inhibitor of topo-II, mainly inhibited DNA synthesis and ADR, an intercalative inhibitor of the enzyme, mainly inhibited RNA synthesis.

In vivo *antitumor activity against colon*38

The rate of inhibition of tumor growth by WFC on day 16 was 57.5 and 55% at 6 and 1.5 mg/kg/day, respectively. The body weight of the mice administered at 6 mg/kg/day decreased by only 8% compared to the body weight of mice before drug administration. Furthermore, when the mice were administered at 1.5 mg/kg/day, no significant change in body weight was observed. It is clear that WFC is effective on colon38 without decreasing the body weight. However, i.p. administered WFC did not show any effect on i.p. transplanted tumors, e.g. mouse leukemia P388 (data not shown).

Cytocidal activity of WFC against HeLa S_3 and PC-1 cells

As shown in Fig. 4, WFC dose- and time-dependently exhibited cytocidal effects on HeLa S_3 and PC-1 cells. Although the IC_{50} value of HeLa S_3 was higher than that of PC-1 (as shown in Table 1), the cytocidal activities of WFC against HeLa S_3 and PC-1 were similar.

DISCUSSION

This study demonstrates that WFC inhibited the catalytic activity of DNA topo-II and showed *in vitro* and *in vivo* antitumor activities. This compound with a large molecular mass has a unique structure when compared with well-known inhibitors [1–8], it is a dimeric hydrolysable tannin, and seems to have a novel character in its mechanism of action. WFC dose-dependently inhibited topo-II and the activity was much stronger than those of ETP and ADR (Fig. 2). However, WFC did not cause directly the DNA cleavage, DNA aggregation as observed in polycationic agents and DNA intercalation or inhibition of topoisomerase I (data not shown). When comparing the effects on DNA, RNA and protein synthesis, it was found that WFC as well as ETP inhibited DNA synthesis rather than RNA and protein synthesis. In contrast, intercalative inhibitors, such as ADR and ACT-D, inhibited RNA synthesis rather than DNA and protein synthesis. Therefore, WFC seems to exhibit an inhibitory mechanism different to those of ETP and ADR.

The inhibitory activity of WFC on the growth of various cultured cell lines was less effective than those of ADR and ETP except for PC-1 and MKN-45 (Table 1), whereas the inhibitory effect of WFC on topo-II *in vitro* was greater than those of ADR and ETP (Fig. 2). WFC may not be taken into cells based on the membrane characters of each cell because of its large molecular mass and a high anionic charge. The degree of uptake is under investigation now.

In the case of the cell killing kinetics of WFC, interesting results were obtained. Figure 4 shows the survival curve for the cytocidal activity of WFC against HeLa S_3 insensitive to WFC and PC-1 cells sensitive to WFC. The MLD_{90} values in HeLa S_3 cells treated with WFC for 1 and 24 hr were 40.2 and 5.47 μg/mL, respectively. The MLD_{90} values in PC-1 cells treated for 1 and 24 hr were 30.9 and 2.50 μg/mL, respectively. Thus, the cytocidal activity against both cell lines was almost equal, while the growth inhibitory activity was quite different. A possibility to be considered is that it might take several days

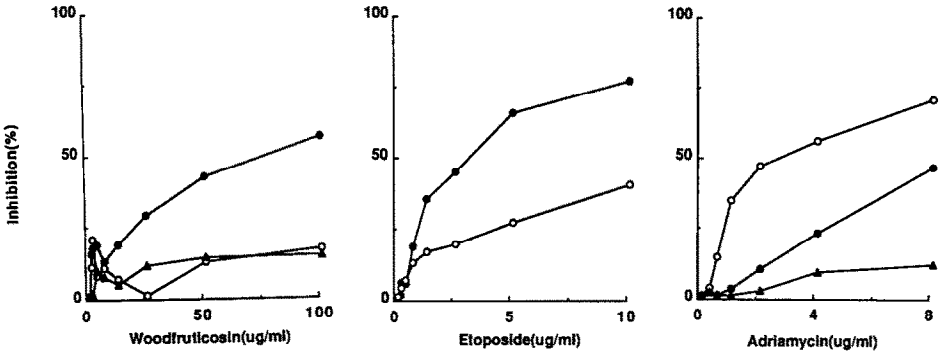

Fig. 3. Effects of WFC, ETP and ADR on intracellular macromolecular synthesis. Cells were incubated at 37° under 5% CO_2 for 2 hr and then treated with drugs for 1 hr. [^{3}H]Thymidine, [^{3}H]uridine or [^{3}H]leucine was added and incubation continued for another hour. The radioactivity incorporated by the cells was determined as described in Materials and Methods. Inhibition percentage on *y*-axis was calculated by the formula:

$$\text{Inhibition (\%)} = 100 - \frac{\text{Radioactivity taken into drug-treated cells}}{\text{Radioactivity taken into control cells}} \times 100.$$

Symbols express inhibition of the synthesis of DNA (●); RNA (○) and protein (▲).

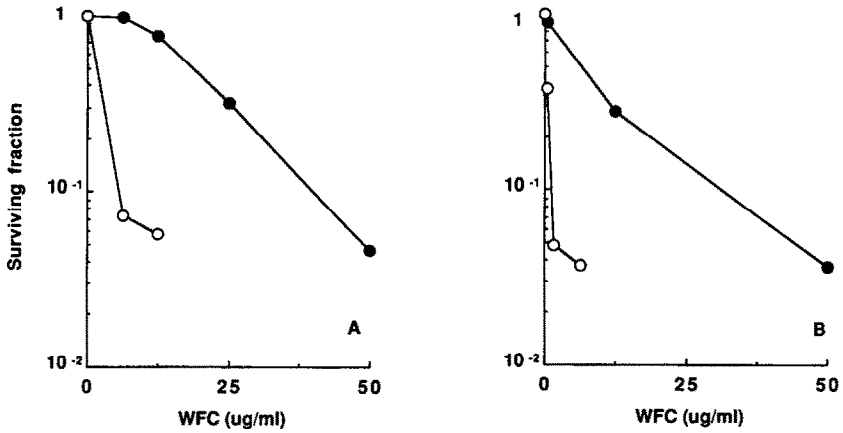

Fig. 4. Cytocidal effect of WFC against HeLa S_3 and PC-1. The exponentially growing cells, HeLa S_3 (A) and PC-1 (B), were treated with WFC for 1 (●) and 24 (○) hr. Colony formation was performed as described in Materials and Methods.

for lethal damage to HeLa S_3 cells caused by WFC to be expressed. The incubation time for colony formation after drug treatment was 12 days, while the incubation time for growth inhibition was 72 hr.

Recently, we investigated the effect of WFC on tumors s.c. inoculated in mice. WFC inhibited the tumor growth of colon38 adenocarcinoma. However, it showed no effects on i.p. transplanted tumors, e.g. mouse leukemia P388. Although WFC showed strong inhibition of topo-II, the effect against cultured tumor cells was moderate and there was no effect on rapidly growing tumors. The cytocidal effect and its effectiveness on solid tumor colon38 indicate that WFC affects the solid tumors in their slow phase of growth.

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