Inhibitory effects of asiatic acid and CPT-11 on growth of HT-29 cells

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Abstract: Asiatic acid is a pentacyclic triterpene contained in medicinal plants. The cytotoxic effect of this compound and its augmentative effect on the anticancer drug irinotecan hydrochloride (CPT-11) were investigated in the human colon adenocarcinoma cell line HT-29. Asiatic acid dose-dependently showed cytotoxicity in HT-29 cells. DNA fragmentation, annexin-positive apoptotic cells, and caspase-3 activation were observed in a dose-dependent manner. A caspase-3 inhibitor suppressed the DNA ladder formation in a concentration-dependent manner. Bcl-2 and Bcl-xL proteins were decreased by asiatic acid treatment. These results indicate that asiatic acid induced apoptosis in HT-29 cells via caspase-3 activation. Cytotoxic effects of combined treatment with CPT-11 and asiatic acid on HT-29 cells were further examined. Simultaneous treatment or sequential exposure first to asiatic acid and then to CPT-11 showed an additive effect. Synergism was observed when cells were first exposed to CPT-11 and then to asiatic acid. These results suggest that asiatic acid can be used as an agent for increasing sensitivity of colon cancer cells to treatment with CPT-11 or as an agent for reducing adverse effects of CPT-11. J. Med. Invest. 52: 65-73, February, 2005

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INTRODUCTION

Asiatic acid is an active principle in Centella asiatica Linn., a medicinal plant. C. asiatica has been shown to inhibit the proliferation of transformed cell lines and to retard the development of solid and ascites tumors (1). We have reported that C. asiatica extract inhibited the formation of azoxymethane (AOM)-induced aberrant crypt foci (ACF) and AOM-induced tumorigenesis in the rat colon (2). Asiatic acid has a common structure of pentacyclic triterpenes and belongs to the amyrin ursolic acid group. Ursolic acid is widely distributed in medicinal herbs and edible plants (3, 4) and has been shown to exhibit growth inhibi-

hition properties against many human cancer cell lines (5-8). Lee et al. (9) reported that asiatic acid induces apoptosis in HepG2 human hepatoma cells. However, its activity for inducing apoptosis in various cancer cell lines has not been examined.

Apoptosis has recently become a subject of much interest in cancer chemotherapy. Bcl-2 family proteins are involved in the regulation of apoptosis either as death antagonists or death agonists (10-12). These antiapoptotic proteins act at upstream processes of activation of apoptotic proteases such as caspase-3 by preventing apoptotic signaling in cells (12, 13). While many tumor cells overexpress antiapoptotic proteins Bcl-2 and Bcl-xL to become resistant to chemotherapy and radiotherapy (14, 15), some triterpenes have been reported to affect the level of expression of bcl-2 (16, 17).

Irinotecan hydrochloride (CPT-11), a water-soluble derivative of camptothecin (18), presents a wide spectrum of antitumor activity through inhibition of DNA
topoisomerase I. CPT-11 prevents DNA-religation reaction, resulting in DNA double-strand breaks and eventually leading to apoptosis (19). It has shown cytotoxic activity in several malignant tumors, including cervical, breast, lung, ovarian, pancreatic, renal, colon and oesophageal cancers, leukemia and lymphoma (20-30). However, leukopenia and diarrhea are two major side effects in patients receiving CPT-11, often accompanied by cramping, flushing, and sweating. Grade 3-4 leukopenia and diarrhea were noted in several studies (26, 27, 31). Weekly chemotherapy regimens incorporating multiple drugs into one regimen have been developed to obtain the maximum antitumor effect with reduced adverse effects of the drugs (32). Recently, the antitumor effect of combinational therapy using an anticancer drug and a phytochemical has been studied (33-35). We previously reported that betulonic acid, a pentacyclic triterpene isolated from medicinal plants, augments the cytotoxic effect of vincristine on B16F10 melanoma cells (36). Since asiatic acid has been reported to induce apoptosis in hepatoma cells (9) and has a common structure of triterpenic acid, we expected that combinational treatment with CPT-11 and asiatic acid would show additive or synergistic cytotoxicity for human colon tumor cells.

In this study, we found that asiatic acid induced apoptotic cell death via caspase-3 activation. Furthermore, we evaluated the effectiveness of combinational treatment with CPT-11 and asiatic acid for HT-29 human colon cancer cells by isobologram analysis (37, 38).

MATERIALS AND METHODS

Chemicals

Asiatic acid (Fig.1A) was purchased from Funakoshi Co., Ltd. Tokyo, Japan. CPT-11 (irinotecan hydrochloride trihydrate) was purchased from Toronto Research Chemicals Inc., North York, ON, Canada. Other reagents were reagent grade or higher and obtained from Wako Pure Chemical Industries (Osaka, Japan).

Cell lines and culture conditions

Cells of the human colonic adenocarcinoma cell line HT-29(American Type Culture Collection, Rockville, USA) were grown at 37°C in a fully humidified atmosphere containing 5% CO₂. HT-29 cells were cultured in McCoy’s 5A medium (ICN Biomedicals, Ohio, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO-BRL, Grand Island, NY), 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate. The number of viable cells was determined using a hemocytometer based upon their exclusion of 0.2% trypan blue dye.

Cytotoxicity

Cells were plated at 1×10⁵ cells/well in 96-well culture plates, cultured for 24 h, and then treated with asiatic acid and/or CPT-11. Asiatic acid at final concentrations ranging from 10 to 60 μg/ml and CPT-11, from 10 to 200 μM, were added to the cultures in triplicate in a final volume of 100 μl. For combination studies, three different schedules of exposure were tested: asiatic acid and CPT-11 simultaneously for 24 h, CPT-11 for 24 h and then asiatic acid for 24 h, and asiatic acid for 24 h and then CPT-11 for 24 h. After drug exposure, the media in the control and drug-containing wells were removed and 100 μl of fresh media were added. Then 20 μl of 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 H-tetrazolium, inner salt (MTS) (Promega, Corp., Madison, WI, USA) solution was added to each well according to the manufacturer’s
instructions. The solutions were incubated for a certain
time, and the absorbance was then measured at 490
and 630 nm. The results are calculated as follows: relative
viability (%) = (experimental absorbance — background
absorbance) / (absorbance of untreated controls — background absorbance) × 100.

DNA fragmentation analysis

DNA fragmentation in cells treated with asiatic acid
was analyzed by the procedure of Ohyama (39). Briefly,
1×10⁶ cells were lysed in 100 μl of chilled lysis buffer
containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl,
2.5 mM EDTA, and 1% Triton X-100. The samples
were held on ice for 15 min, and then 2 μl of RNase A (1 mg/
ml, Wako Pure Chemical Industries, Ltd., Osaka, Japan)
and 2 μl of RNase T1 (3,340 units/ml, Wako) were added and
the mixtures were incubated at 37°C for 1h. The lysed
samples were treated with 2 μl of proteinase K (Wako)
at 50°C for 30 min. DNA was precipitated with isopropyl
alcohol and dried by a speed vacuum concentrator
(Tomy Seiko, Tokyo, Japan). The pellet was dissolved in
Tris-EDTA buffer (pH 7.4). DNA (25 μg/lane) was elec
trophoresed in 2% agarose gel using Tris-borate EDTA
buffer (pH 7.4) with a voltage of 100 V for 45 min, and
DNA bands were stained with 0.5 μg/ml ethidium
bromide and visualized under a UV transilluminator.
If necessary, a caspase-3 inhibitor, Z-Asp-Glu-Val-Asp-
FMK (Z : benzoyloxycarbonyl, FMK : fluoromethylketone ; MBL Medical & Biological Laboratories Co., Ltd., Nagoya, Japan), was added at a concentration of 0.1-
30 μM.

Assessment of cell death using flow cytometry

Cells(1×10⁶) were seeded onto 6-well plates. After
treatment with asiatic acid, cells were harvested and
resuspended in 100 μl of a reaction mixture containing
annexin V-FITC and propidium iodide (PI) according to
the instructions of the manufacturer (R & D Systems,
Minneapolis, MN). Cells binding annexin V but exclud
PI were judged to be early apoptotic cells, whereas
cells binding annexin V and accumulating PI were judged
to be late apoptotic cells. In all experiments, fluores
cence was determined from the combined collection of
floating and attached cells by a flow cytometer (Coul
ter Epics XL-MCL, Beckman Coulter, Tokyo, Japan).

Western blot analysis

Whole cell lysates were prepared as described previou
(40). Briefly, cells were harvested by centrifugation
at 1,000 g for 5 min at 4°C. The cell pellets (5×10⁶ cells)
were washed once with ice-cold PBS and resuspended
with 100 μl of the chilled lysis buffer containing 20 μg/
ml leupeptin, 20 μg/ml aprotinin and 0.2 mM phenyl-
methylsulfonylfluoride (PMSF). Cells were disrupted
by passing 10 times through a G 27 needle. After sequen
tial centrifugation at 750 g for 5 min and at 15,000 g for
15 min at 4°C, the supernatants were divided into aliq
ots and stored at −20°C. Protein concentration
was determined using a Coomassie protein assay kit
(Pierce, Rockford, USA) according to the manufac
turer’s instructions. Samples were subjected to 12%
SDS-polyacrylamide gel (Wako) electrophoresis with
200 V for 35 min and transferred to polyvinylidene
difluoride (PVDF) membranes (Bio-Rad, Hercules,
CA, USA) with 20 V for 40 min. The membranes were
blocked in TBS (TWEEN 20-Tris-buffered saline) con
taining 2% bovine serum albumin (BSA) for 1 h and
probed overnight with a primary antibody mouse anti
Bcl-2 and mouse anti-Bckβ [1 : 1,000 ; BD Transduction,
Japan] or with mouse anti-β-actin [1 : 10,000 ; Sigma
Chemical Co., St. Louis, MO]) at 4°C. Primary anti
body binding was detected with a goat anti-mouse IgG
conjugated with alkaline phosphatase (1 : 2,000; Sigma)
and visualized by an enhanced chemiluminescence
method using disodium 3-(4-methoxyspiro (1,2-dioxetane,
3,2’-(5’-chloro) tricyclo[3.3.1.1²⁶] decan-4-yl)phenyl
phosphate (CSPD) (Boehringer, Manheim Germany).

Assay of caspase-3 activity

The activity of caspase-3 in HT-29 cells was measured
using apopain substrate (Sigma) according to the user’s
manual (BD Biosciences Clontech Co., Palo Alto, CA).
Cells(1×10⁶) were plated for 24 h and exposed to asi
atic acid for a further 12 h. Cells were collected by cen
trifugation, resuspended in 50 μl of the chilled lysis
buffer as described above, and held on ice for 15 min.
Fifty μl of the reaction buffer containing 1 μl of 1 M
DTT and 5 μl of 1 mM acetyl-Asp-Glu-Val-Asp-7-amido-
4-methylcoumarin (Ac-DEVD-AMC) was added to the
cell lysate, and the mixture was incubated at 37°C
for 1h. The fluorescence emitted at 450 nm (λex=365
nm) was measured with a microplate reader spec
trofluorometer (MTP-32 microplate reader, Corona
Electric, Japan).

Drug interaction analysis

Additive or synergistic interaction between asiatic
acid and CPT-11 was determined by using isobologram
analysis as described in detail previously (37, 38).
The type of interaction between asiatic acid and CPT-11
was evaluated by comparing the cytotoxic effects ob
tained after simultaneous or sequential exposures to
the drugs with the ones observed after exposure to
asiatic acid or CPT-11 alone. Interaction indices were
calculated by the following equation: interaction index = asiatic acid \( c \)/asiatic acid \( e \) + CPT-11 \( c \)/CPT-11 \( e \), where asiatic acid \( e \) and CPT-11 \( e \) are concentrations of asiatic acid and CPT-11 that inhibit 50, 60, 70% of proliferation when used alone, and asiatic acid \( c \) and CPT-11 \( c \) are concentrations of asiatic acid and CPT-11 that produce the same effect when used in combination. According to this method, an interaction index of less than 1.0 indicates synergistic interaction between two drugs, an interaction index of more than 1.0 indicates antagonism, and an index of 1.0 indicates additive interaction.

RESULTS
Cytotoxicity of asiatic acid

The viability of HT-29 cells exposed to asiatic acid for 24 h decreased in a dose-dependent manner to 4.8% of the control level at a dose of 60 \( \mu \)g/ml (Fig.1B). The concentration of 50% inhibition was 37.0±1.32 \( \mu \)g/ml. This result indicates that asiatic acid is cytotoxic to HT-29 cells.

DNA fragmentation

DNA ladder formation was observed in HT-29 cells after 24 h of incubation with asiatic acid at concentrations of 30, 40, 50 and 60 \( \mu \)g/ml in a dose-dependent manner (Fig.2A). It was observed in parallel with growth inhibition. DNA ladder formation caused by treatment with 50 \( \mu \)g/ml of asiatic acid for 24 h was suppressed by addition of the caspase-3 inhibitor Z-Asp-Glu-Val-Asp-FMK dose-dependently (Fig.2B).

Detection of early and late apoptotic cells by flow cytometric analysis

Early apoptotic cells appeared in a dose-dependent manner after treatment with 12.5 and 25 \( \mu \)g/ml of asiatic acid for 24 h (10.3 and 33.0%, respectively) (Fig.3). Late apoptotic cells accounted for 8.6 and 95.4% of total cells after treatment with 25 and 50 \( \mu \)g/ml of asiatic acid for 24 h, respectively.

Bcl-2 and Bcl-xL expression in HT-29 cells

To elucidate involvement of Bcl2 and BclxL proteins in the asiatic acid-induced apoptosis in HT-29 cells, the levels of these proteins were analyzed by Western blotting (Fig.4). When HT-29 cells were exposed to 50 \( \mu \)g/ml of

![Fig 2](image_url) DNA fragmentation in HT-29 cells treated with asiatic acid. (A) Cells \((1 \times 10^6)\) were cultured in the absence or presence of 10-60 \( \mu \)g/ml of asiatic acid for 24 h. (B) HT-29 cells were treated with 0.1-30 \( \mu \)M Z-Asp-Glu-Val-Asp-FMK before adding 50 \( \mu \)g/ml of asiatic acid. DNA was then extracted from the cells and analyzed by 2% agarose gel electrophoresis. M, DNA size markers (100-bp ladders).

![Fig 3](image_url) Flow cytometric analysis of apoptosis in HT-29 cells exposed to asiatic acid. Cells were treated with various concentrations of asiatic acid for 24 h and then stained with annexin V-FITC and PI. Values in the quadrants indicate percentages in the total cells.
asiatic acid for 6, 12 and 24 h, the levels of Bcl-2 and Bcl-xL proteins decreased time-dependently. After 24 h, the levels of Bcl-2 and Bcl-xL proteins standardized with the level of β-actin reached 0% and 26.3% compared with those at 0 h. These results suggest that down-regulation of the anti-apoptotic proteins Bcl-2 and Bcl-xL may correlate with apoptosis in HT-29 cells.

Fig 4. Western blot analysis. The expression levels of Bcl-2 (A) and Bcl-xL(B) proteins in HT-29 cells after treatment with 50 µg/ml of asiatic acid were determined by Western blot analysis. Twenty µg of protein was loaded in each lane in 12% SDS-polyacrylamide gel electrophoresis. The protein recognized by each antibody is indicated on the side. β-actin was detected as an internal standard.

Fig 5. Activation of caspase-3 in HT-29 cells treated with asiatic acid. HT-29 cells (1×10⁶) were incubated with 25, 50 and 100 µg/ml of asiatic acid for 12 h. Values are means±SD. Bars with * are significantly different from the 0 µg/ml treatment group at p<0.0001.

Fig 6. Effect of combinational treatment of asiatic acid and CPT-11 on the growth of HT-29 cells. (A) The cells were treated with asiatic acid and CPT-11 simultaneously. (B) The cells were treated with asiatic acid for 24 h and then with CPT-11 for a further 24 h. (C) The cells were treated with CPT-11 for 24 h and then with asiatic acid for a further 24 h. Concentrations of CPT-11 were 0 µM (open box), 20 µM (hatched box), 40 µM (crossed box), 80 µM (latticed box), 100 µM (dotted box) and 150 µM (closed box). Cytotoxicity was evaluated using the MTS assay. Data are expressed as means±SD of three independent experiments.
Activation of caspase-3 in HT-29 cells

To investigate the activation of caspase-3 during apoptosis, activity of caspase-3 was measured by fluorometric analysis using Ac-DEVD-AMC, a caspase-3-specific synthetic substrate. Fig.5 shows significant activation of caspase-3 in a dose-dependent manner in HT-29 cells (p<0.0001). Caspase-3 activity had increased to 6.3 fold and 7.1 fold of the solvent control (P<0.0001) at 12 h after asaiatic acid treatment at concentrations of 50 and 100 µg/ml, respectively.

Cytotoxicity of the asiatic acid/CPT-11 combination

CPT-11 alone inhibited the growth of HT-29 cells in a dose-dependent manner (Fig.6). Asiatic acid alone also inhibited the growth of HT-29 cells in a dose-dependent manner (Fig.6). Since asiatic acid and CPT-11 showed cytotoxicity after 24 h at the concentrations of 10 to 50 µg/ml (Fig.1) and 20 to 150 µM (Fig.6), respectively, combinational treatment was done at these concentrations for 24 h. When the cells were treated simultaneously with asiatic acid and CPT-11, interaction indices at 50, 60 and 70% inhibition of proliferation were 0.98±0.17, 0.92±0.08 and 0.93±0.09, respectively, indicating an additive effect (Fig.6A). When cells were first exposed to asiatic acid and then treated with CPT-11, interaction indices were 0.99±0.10, 0.93±0.15 and 0.88±0.17, respectively, indicating an additive effect (Fig.6B). When cells were sequentially exposed to CPT-11 and then asiatic acid, interaction indices at 50, 60 and 70% inhibition of proliferation were 0.87±0.08, 0.81±0.05 and 0.76±0.03 (Fig.6C). A weak synergism was observed in this treatment.

These results indicate that asiatic acid induced apoptosis through activation of caspase-3, which cleaves DNA fragmentation factor 45 (DFF45) in the DFF45/DFF 40 complex (42) and produces active DFF40 to trigger chromosomal DNA fragmentation.

Bcl-2 is an antiapoptotic protein, predominantly present in the outer mitochondrial membrane, the endoplasmic reticulum membrane and the nuclear membrane (13). Bcl-2 has been shown to inhibit cytochrome c release from mitochondria into the cytosol by inhibiting insertion of the proapoptotic protein Bax in the mitochondria or by directly or indirectly inhibiting Bax-channel activity (13). Bcl-xL is also an antiapoptotic protein present in the cytosol, and it works to close the channel (13, 43). Many tumor cells overexpress these antiapoptotic proteins and become resistant to chemotherapy and radiotherapy (14,15). Ohmori et al. (44) reported that bcl-2 can modulate the cytotoxicity of some anti-cancer agents such as CPT-11 and mitomycin C by inhibiting the process of apoptosis. In the present study, the levels of Bcl-2 and Bcl-xL were decreased by treatment with asiatic acid, suggesting that down-regulation of Bcl-2 and Bcl-xL in response to asiatic acid may cause apoptosis. Since HT-29 cells carry high levels of non-functional p53(45), asiatic acid-induced apoptosis in these cells may not be mediated by activation of p53 but triggered by decrease in levels of Bcl-2 and Bcl-xL after asiatic acid treatment.

Systemic chemotherapy of colorectal cancer using new agents that target specific molecular processes of cell proliferation, vascularization, metastasis and apoptosis inhibition have been developed (32). CPT-11 is a topoisomerase I inhibitor (18) and has been clinically applied for treatment of patients with colorectal cancer that is refractory to treatment with fluorouracil (26, 27). Sensitivity of tumor cells to topoisomerase inhibitor depends on topoisomerase I activity (19), tumor-associated deficiency of p53(46), and the easiness of apoptosis induction (47, 48). Combination with apoptosis-inducing agents would enhance the chemotherapeutic response of colorectal cancer treated with topoisomerase I inhibitors. In this study, we examined effects of combinational treatment with CPT-11 and asiatic acid on cytotoxicity for HT-29 cells according to three kinds of protocol: 1) simultaneous treatment, 2) first exposed to asiatic acid and then treated with CPT-11, 3) first exposed to CPT-11 and then treated with asiatic acid. Simultaneous treatment and sequential treatment in which cells were first treated with asiatic acid and then with CPT-11 showed an additive cytotoxic effect. Since asiatic acid is a derivative of ursolic acid, which has been reported to block cell cycle progression in the

DISCUSSION

Asiatic acid is an active principle in C. asiatica. Crude extracts of this medicinal plant have shown chemopreventive effects in in vivo tumor models (1, 2). In an in vitro experiment, asiatic acid induced apoptosis in HepG2 human hepatoma cells (9). In this study, we examined asiatic acid-induced apoptosis in human colon tumor-derived cells, HT-29 cells. Asiatic acid dose-dependently showed cytotoxicity in HT-29 cells (Fig. 1B). After asiatic acid treatment, DNA ladder formation was observed (Fig.2A) and flow cytometric analysis showed that annexin-positive cells increased dose-dependently (Fig.3). Caspase-3, one of the effector proteases in an apoptosis process (41), was activated by asiatic acid (Fig.5). In the presence of the caspase-3 inhibitor Z-Asp-Glu-Val-Asp-FMK, DNA fragmentation triggered by asiatic acid was inhibited (Fig.2B).

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G1 phase (49), it is possible that a part of the HT-29 cell population treated with asiatic acid is blocked in G1 phase and can no longer respond to CPT-11. The remaining part of the cell population might enter S phase and respond to CPT-11. On the other hand, synergism was observed when cells were first exposed to CPT-11 and then treated with asiatic acid. In CPT-11-pretreated cells, asiatic acid-mediated decrease in the antiapoptotic proteins Bcl-2 and Bcl-x, might enhance the apoptotic process, resulting in synergistic cytotoxicity. Hayward et al. (48) reported that antisense-mediated Bcl-x, knockdown enhances the response to topoisomerase I inhibition in the colorectal cancer cell line HCT 116. Although cytotoxic intensity of the combinational treatment was dependent on the order of the treatment, asiatic acid could possibly be used to enhance the tumor cell-killing effect of the anticancer drug CPT-11.

Combinational treatment with CPT-11 and asiatic acid revealed additive or synergistic cytotoxicity in HT-29 cells. The total triterpenic fraction of *C. asiatica*, in which asiatic acid is one of main constituents, has been used for treatment of venous hypertension in clinical studies at a dose of 30 or 60 mg/day for 10 weeks or 4 months (50, 51). Taken together, the results of this study suggest that asiatic acid enhances the sensitivity of a tumor to anticancer drugs and reduces the adverse effects of chemotherapy. The mechanism of asiatic acid-induced apoptosis in colon cancer cells should be clarified for introducing this compound to clinical use.

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