Isostrychnopentamine, an Indolomonoterpenic Alkaloid from *Strychnos usambarensis*, Induces Cell Cycle Arrest and Apoptosis in Human Colon Cancer Cells

MICHEL FRÉDÉRIC, MOHAMED BENTIRES-ALJ, MONIQUE TITS, LUC ANGENOT, ROLAND GREIMERS, JACQUES GIELEN, VINCENT BOURS, and MARIE-PAULE MERVILLE

Natural and Synthetic Drug Research Center, Laboratory of Pharmacognosy and Structural Chemistry (M.F., M.T., L.A.); Center for Molecular and Cellular Therapy, Laboratory of Medical Chemistry and Medical Oncology (M.B.-A., J.G., V.B., M.-P.M.); and Laboratory of Cell Pathology, Institute of Pathology (P.G.), University of Liège, Liège, Belgium

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ABSTRACT

Isostrychnopentamine (ISP) is an indolomonoterpenic alkaloid that is present in the leaves of *Strychnos usambarensis*, a well known African shrub or little tree. The roots contain quaternary alkaloids, which are used to make a curare-like arrow poison. However, tertiary alkaloids isolated from the same plant possess cytotoxic activities against mammalian cells and protozoa. The effect of ISP has been investigated on the growth and viability of HCT-116 colon cancer cells during their exponentially growing phase. ISP induced apoptotic cell death as shown by the translocation of phosphatidylserine from the inner layer to the outer layer of the plasma membrane, chromatin condensation, DNA fragmentation, and caspase-3 and -9 activation. ISP provoked also cell cycle arrest in the G$_2$-M phase. We also showed that the expression of p53 was not modified in ISP-treated cells, but that p21 was induced in a p53-independent manner. Finally, we demonstrated that ISP did not affect the catalytic activity of human topoisomerases I and II. In conclusion, ISP, which promotes cell death by a p53-independent apoptotic pathway, could be an interesting lead for cancer chemotherapy.

Although interest in natural products as a source of innovation in drug discovery has decreased in the last few decades, therapeutic agents from microbial or plant origin account for more than 30% of the current worldwide human drug sales. For anticancer and anti-infective treatments, even 60% of the approved drugs and of the new drug candidates (excluding biologics) are of natural origin for the period 1989 to 1995 (Cragg et al., 1997).

Isostrychnopentamine (ISP) (Fig. 1) is an asymmetrical monoterpenoid bisindole alkaloid possessing an atypical structure with five nitrogen atoms. This alkaloid was isolated for the first time in our laboratory more than 20 years ago from the leaves of the plant *Strychnos usambarensis* Gilg (Angenot et al., 1978). *S. usambarensis* is a little tree or shrub distributed in almost all tropical Africa, from the Ivory Coast to South Africa. ISP is extracted [yield 0.2% (w/w)] from the leaves of the plant, which makes the supply of the raw material relatively easily obtained, if need be. This *Strychnos* was mainly known for its toxicity, due to quaternary curarizing alkaloids (Angenot, 1971; Angenot et al., 1975). Nevertheless, it is known that it contains also tertiary alkaloids, which possess cytotoxic activities against mammalian cells (Bassleer et al., 1982; Tits et al., 1984) and antiprotozoal activities (Wright et al., 1991; Frédéric et al., 1999).

Our interest in isostrychnopentamine stemmed from the previous studies conducted on its stereoisomer strychnopentamine. We have showed that strychnopentamine inhibits RNA synthesis and induces cytological modifications, such as lamellar bodies, vacuoles, and blebs in the cytoplasm of B16 mouse melanoma cells (Bonjean et al., 1996). Strychnopentamine, along with isostrychnopentamine and usambarensine (a closely related alkaloid from the roots of *S. usambarensis*) were then submitted to the National Cancer Institute for an in vitro screen of 60 cell lines (V. L. Narayanan, National Cancer Institute, unpublished data). Because ISP displayed an interesting activity pattern, we decided to investigate how ISP induced cell death in HCT-116 colon cancer cells.

**ABBREVIATIONS:** ISP, isostrychnopentamine; WST-1, 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium; DAPI, 4′,6-diamidino-2-phenylindole; PI, propidium iodide; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorting; PAGE, polyacrylamide gel electrophoresis; PARP, poly(ADP-ribose) polymerase; FLUOS, fluorescein.
In the present study, we report that ISP treatment of colon cancer cells induces G2 cell cycle arrest and apoptosis through the intrinsic pathway. These findings provide for the first instance a role of ISP in cancer cell-induced apoptosis.

Materials and Methods

Drugs, Chemicals, and Plant Material. The leaves of *S. usambarensis* were collected by Professor F. Sandberg (University of Uppsala, Uppsala, Sweden) in Tanzania. Voucher specimens of the plant were deposited in the herbarium of the National Botanical Garden of Belgium (Meise, Belgium) and in the herbarium of the Pharmaceutical Institute (Liége, Belgium) (voucher no. Leeuvenberg 10826). ISP was isolated from *S. usambarensis* as described previously (Tavernier et al., 1987; Frédérich et al., 1999). Stock solutions (1 mM) were prepared with 5% dimethyl sulfoxide. Daunomycin-hydrochloride (Rhône-Poulenc-Rorer, Paris, France) and camptothecin and etoposide (Sigma Chemical, Bornem, Belgium) were stored in stock solutions, protected from light.

Cell Culture. Cell lines were obtained from American Type Culture Collection (Manassas, VA) and have been described previously (Hellin et al., 1998, 2000). HCT-116 and HCT-15 human colon carcinoma cells were grown in McCoy’s 5A and RPMI 1640 medium supplemented with 1% l-glutamine (200 mM), 100 IU/ml penicillin, 50 μg/ml streptomycin, and 10% fetal bovine serum (Invitrogen, Carlsbad, CA).

In Vitro Cytotoxicity Assay. This assay was performed as described previously (Bentires-Alj et al., 1999). Briefly, cells were seeded at the concentration of 7 × 10^5 cells/well on 96-well flat-bottom microplates in 0.2 ml of medium. After 48 h of incubation with the drug, cell viability was measured by a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 (Roche Diagnostics). The formation of a florigenic product was measured at 460 nm (excitation wavelength, 355 nm) using SoftMAX Pro software and a SpectraMAX spectrofluorometer from Molecular Devices Corp. (Sunnyvale, CA).

Annexin V-HEPES solution (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 5 mM CaCl2) and incubated on ice for 30 min in the dark.

Cell Cycle Analysis. DNA content was measured as described by the manufacturer (Cycle Test Plus DNA reagent kit; BD Biosciences, San Jose, CA).

FACS Analysis. Cells (for Annexin V test and cell cycle analysis) were analyzed with a FACStar Plus (BD Biosciences) with a 100-mW air-cooled argon laser (Spinnaker 1161; Spectra Physics, Mountain View, CA) and the CellQuest software (Macintosh, Facstation; BD Biosciences).

Protein Extraction and Western Blotting. To obtain whole cell protein extract, control or ISP-treated cells were treated as described previously (Hellin et al., 1998). Then, 10 μg of total proteins was resolved on a 10% SDS-PAGE and then transferred to a polyvinylidene difluoride membrane (Roche Diagnostics). The membrane was blocked overnight with Tris-buffered saline-Tween buffer plus 5% dry milk, incubated 1 h with the primary antibody, washed with Tris-buffered saline-Tween buffer plus 5% dry milk, incubated 1 h with the primary antibody, washed, and then incubated with secondary antibody conjugated with peroxidase (1:10,000; DAKO, Glostrup, Denmark). The signal was detected using a chemiluminescent detection system (ECL kit; Amersham Biosciences AB, Uppsala, Sweden). The used antibodies were as follows: anti-PARP 85/116-kDa antibody (1:200; BD PharMingen, San Diego, CA); anti-p53 antibody (1:4000; Oncogene Research, San Diego, CA); anti-p21WAF1 antibody (1:100, Oncogene Research), anti-cytochrome-c antibody (1:500; BD PharMingen); anti-β-actin antibody (1:500; Sigma Biosciences, Bornem, Belgium); anti-caspase-9 antibody (1:2000, BD PharMingen); and anti-caspase-8 antibody (1:600; BD PharMingen).

Cytoplasmic Nonmitochondrial Extracts. HCT-116 cells were harvested as described previously (Yang et al., 1997). Briefly, cells were centrifuged at 600 g for 10 min at 4°C. The cell pellets were washed once with ice-cold PBS and resuspended in 5 volumes of buffer A (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1 mM MgCl2, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride) containing 250 mM sucrose. The cells were homogenized with 12 strokes of a Teflon homogenizer, and the homogenates were centrifuged twice at 750 g for 10 min at 4°C. The supernatants were centrifuged at 100,000 g for 1 h at 4°C, and the resulting supernatants were divided into samples (eventually frozen at −80°C) and used for cytochrome c detection by Western blotting.

Caspase-3 Activity Assay. Caspase-3 activity was measured using the specific Ac-Asp-Glu-Val-Asp-amino-4-methylcoumarine (Ac-DEVD) caspase-3 substrate from Calbiochem (San Diego, CA). Exponentially growing HCT-116 cells (2 × 10^6) were incubated with ISP for various lengths of time. Extracts were prepared as described by the manufacturer. Proteins (50 μg) from this extract were then incubated with 150 μl of lysis buffer (Tris pH 8, 0.2 mM NaCl, 37 mM; 10% glycerol, and 1% Nonidet P-40) with 20 mM caspase-3 substrate for 4 h at 37°C. The formation of a florogenic product was then measured at 460 nm (excitation wavelength, 355 nm) using SoftMAX Pro software and a SpectraMAX spectrophotometer from Molecular Devices Corp. (Sunnyvale, CA).

DNA Laddering Assay. After treatment with ISP, HCT-116 cells were washed once in PBS, trypsinized, pooled with cells recovered from the supernatant, and centrifuged at 5000 g for 2 min. Cells were washed again in PBS and treated with two cycles of lysis buffer (1% Nonidet P-40 in 20 mM EDTA, 50 mM Tris-HCl, pH 7.5, 10 μl/10^6 cells, minimum 50 μl). After centrifugation for 5 min at 1600 g, the supernatant was collected, treated with RNase A for 120 min at 56°C, brought to 1% (w/v) SDS, and finally digested with proteinase K (final concentration, 2.5 μg/ml) for at least 120 min at 37°C. DNA was then precipitated and analyzed by gel electrophoresis on 1% agarose gel.

Topoisomerase I and II DNA Cleavage Reaction. Native supercoiled pKMp27 DNA (0.5 μg) (lane DNA) was incubated for 30 min at 37°C with 4 units of human topoisomerase in the absence or presence of ISP at various concentrations (micromolar). Reaction
was stopped with sodium dodecylsulfate and treatment with proteinase K. DNA samples were separated by electrophoresis on agarose gels containing ethidium bromide. After electrophoresis, the gels were washed with water and photographed under UV light. Etoside and camptothecine were used as positive controls for topoisomerase I and II assays, respectively.

**Results**

**Evaluation of ISP Cytotoxicity on Cancer Cells.** Compared with other structurally related alkaloids such as strychnophenamine and usambarensine, ISP possessed a higher toxicity against colon and breast cancer cell lines (National Cancer Institute’s in vitro 60-cell lines antitumor screen, unpublished data). We investigated then the effect of ISP on colon cancer HCT-116 and HCT-15 cells (human colon carcinoma cells containing a mutated p53 gene) by the WST-1 assay (Fig. 2). ISP promoted cell death in a dose-dependent manner in the two cell lines (IC₅₀ = 7 and 15 μM for HCT-116 and HCT-15 cell lines, respectively).

**ISP Induces Apoptosis in HCT-116 Cells.** Cells exposed to 10 μM ISP for 12 to 24 h shrank, rounded up, and detached from the dishes (data not shown), suggesting that they underwent apoptosis. To confirm this hypothesis, we used different methodological approaches. We analyzed the fluorescence of the nuclei of cells stained with the DNA-specific dye DAPI after 24 h of treatment. In untreated cells, we observed normal nuclei staining (Fig. 3B). In contrast, ISP-treated cells (10 μM) displayed typical condensed chromatin and fragmented nuclei (blebbing) (Fig. 3A). DNA fragmentation was further examined by the classical DNA laddering on agarose gel. Formation of internucleosomal fragments was observed after 24-h exposition of HCT-116 cells to 5 and 10 μM ISP (Fig. 4).

Annexin V is a calcium-dependent phospholipid-binding protein with high affinity for phosphatidylserine. It is used as a sensitive probe to evidence the translocation of the phospholipid from the inner to the outer layer of an apoptotic cell. The marking of cells by PI indicates a modification of cell permeability and is a sign of cell necrosis. Living cells, exposed to Annexin V-fluorescein and PI staining after 14 or 28 h of ISP expositions (15 μM), or without exposition to ISP (control) were analyzed by FACS. As shown in Fig. 5A, in the control cultures, 92% of the cells were normal living cells with low Annexin and low PI staining. In contrast, ISP-induced apoptosis in 94% of treated cells as shown by their high Annexin and low PI staining (Fig. 5, B and C). Among these treated cells, very few (12% in cells exposed 28 h to 15 μM ISP) were necrotic as shown by dual Annexin and PI staining.

Two complementary experimental approaches were then used to determine whether caspase-3 participated in ISP-induced apoptosis in the HCT-116 cell line. The enzyme activity was first measured using the Ac-DEVD fluorimetry assay as described in the methods. Figure 6A illustrates that ISP, at a concentration of 15 μM, activated caspase-3 after 14 h of exposition; this activation increased up to 4.5-fold after 30 h of exposition. Likewise, we examined the cleavage of PARP, an enzyme involved in DNA repair and a substrate for caspase-3. By Western blotting (Fig. 6B), we showed that the native 116-kDa PARP protein was cleaved into its characteristic 89-kDa fragment upon treatment of the cells with the alkaloid. The 24-kDa fragment was not detected because the anti-PARP antibody recognized only the COOH terminus of the protein. The activation of caspase-3 shown by the Western blotting experiment fully agreed with the fluorometric assay. Those events are hallmarks of apoptosis and confirmed that ISP cytotoxicity is mainly induced by apoptosis.

**Detection of Caspase-8, Caspase-9, and Cytochrome c.** To further characterize the apoptotic pathway activated by ISP, extracts from HCT-116 cells were examined by Western blotting using caspase-8- and caspase-9-specific antibodies (Fig. 7). No change of the caspase-8 content was detected in this cell line. In contrast, the amount of caspase-9 protein (48 kDa) decreased after 6 h of exposition to 15 μM ISP, whereas the cleaved active (37-kDa) form of caspase-9 increased significantly. We then examined whether a cytoplasmic release of cytochrome c occurred. Figure 7 showed that cytochrome c was detected in the cytosol after 6- to 12-h exposition to 15 μM ISP. Cytochrome c release was maximum at 12 h and decreased strongly 24 h after ISP treatment. Altogether, the data strongly supported the conclusion that the mitochondrial pathway is used to trigger apoptosis after an ISP exposure.

**Changes in Cell Cycle after Exposition to ISP.** HCT-116 cell cycle was examined 3 and 12 h after addition of 15 μM ISP to the culture medium (Fig. 8). Without treatment, most cells (83%) were in G₁ and S phases, due to the high

![Fig. 2. Effect of ISP on HCT-116 and HCT-15 cell growth and viability. One day after seeding, cells were treated with increasing concentrations of ISP for 48 h. The number of cells was determined by WST-1 assay. Each value represents the mean of three measures ± S.D.](image-url)
proliferative state of this cell line. After 12 h of exposition to 15 μM ISP, we observed a marked increase in the percentage of cells in the G2-M phase of the cell cycle. This increase was accompanied by a decrease in the percentage of cells in G1 phase (from 49 to 32%), which indicated a cell cycle arrest in G2-M phase.

Role of p53 and p21 Proteins in Cell Cycle Arrest and Apoptosis. Extracts from HCT-116 cells after ISP treatment at various concentrations were examined by Western blotting using p53- and p21-specific antibodies. p53 expression was unchanged in the variously ISP-treated HCT-116 cells (Fig. 9A). ISP until 50 μM did not induce any p53 recruitment (data not shown). In contrast, an increase in the p21 protein content occurred after treatment with 10 or 15 μM ISP (Fig. 9A). To confirm that ISP can induce p21 in a p53-independent manner, we examined p21 protein content in HCT-15 cells after treatment by ISP. HCT-15 cells are human colon carcinoma cells where p53 gene is deleted. Figure 9B shows that p21 protein content was raised in HCT-15 cells after treatment with 10 to 15 μM ISP for 12 to 24 h. Apoptosis was also confirmed in these HCT-15 cells by observation of PARP cleavage after treatment with 10 to 15 μM ISP during 24 h (data not shown).

ISP Treatment Does Not Inhibit Topoisomerases I and II. The effect of ISP on the catalytic activity of human topoisomerases I and II was investigated using a conventional plasmid DNA relaxation assay (Bailly, 2001). ISP did not affect the electrophoretic mobility of the DNA in the presence of either topoisomerase I or II, even at a high concentration of 100 μM. The antitumor drugs camptothecin and etoposide, used as positive controls in the same experiment, strongly promoted permanent single- and double-stranded DNA cleavages due to topoisomerases I and II inhibition (data not shown). ISP is therefore not a topoisomerase poison.
Many naturally occurring indolomonoterpenic alkaloids (camptothecin, ellipticine, vincristine) are or were used as anticancer agents. Their chemotherapeutic effect is mediated by different biochemical modes of action, such as inhibition of topoisomerase I and II and inhibition of microtubules formation. Herein, we investigated the mechanisms responsible for the cell death-promoting activity of isostrychnopentamine, a Strychnos alkaloid whose derivatives usambarensine and strychnopentamine were known for their cytotoxicity (Bonjean et al., 1996). Our results clearly showed for the first time that ISP (15 μM) induced cell cycle arrest and subsequent apoptotic death in the human colon cancer cell line HCT-116. Necrotic or lytic effects were excluded by the lack of plasma Fig. 6. Caspase 3 activation. A, fluorometric assay. HCT-116 cells were incubated without or with 15 μM ISP for 6, 14, or 30 h before the addition of the caspase-3 substrate (Ac-DEVD), as described under Materials and Methods. The cell lysate was incubated for 4 h at 37°C and fluorescence was measured at 460 nm (excitation wavelength, 355 nm). B, Western blot was used to detect cleavage of full-length PARP (116-kDa band) into the 89-kDa fragment in untreated cells (control) and cells treated with ISP for 6, 12, or 24 h at 15 μM. Whole cell lysates where subjected to SDS-PAGE (as described under Materials and Methods) followed by blotting with an anti-PARP antibody (BD PharMingen). Fig. 7. Western blot analysis of caspase-8, caspase-9, and cytochrome c proteins in cytoplasmic nonmitochondrial or total extracts from untreated cells (control) and cells treated with 15 μM ISP for 6 to 24 h. The protocol was described under Materials and Methods. Actin blotting is shown to assess that equivalent amounts of proteins were loaded in each line.

Discussion

Many naturally occurring indolomonoterpenic alkaloids (camptothecin, ellipticine, vincreistine) are or were used as anticancer agents. Their chemotherapeutic effect is mediated by different biochemical modes of action, such as inhibition of topoisomerase I and II and inhibition of microtubules formation. Herein, we investigated the mechanisms responsible for the cell death-promoting activity of isostrychnopentamine, a Strychnos alkaloid whose derivatives usambarensine and strychnopentamine were known for their cytotoxicity (Bonjean et al., 1996). Our results clearly showed for the first time that ISP (15 μM) induced cell cycle arrest and subsequent apoptotic death in the human colon cancer cell line HCT-116. Necrotic or lytic effects were excluded by the lack of plasma.
membrane permeability as assessed by the absence of PI uptake.

We clearly observed many of the typical structural and ultrastructural modifications that happen during the apoptotic process, including translocation of phosphatidylserine to the outer layer of the plasma membrane, condensation of

Fig. 8. Effect of ISP on HCT-116 cell cycle. One day after seeding, cells were untreated (A) or treated with 15 μM ISP for 3 h (B) or 15 μM ISP for 12 h (C). Distribution of the cells in G₀-G₁, S, and G₂-M phases was analyzed by flow cytometry as described under Materials and Methods.
nuclear chromatin, internucleosomal DNA fragmentation, caspase-3 activation, and PARP cleavage. Further investigations of the apoptotic process showed that the effector caspases were activated by the mitochondrial pathway, caspase-9 activation, and cytochrome c release from mitochondria.

The weak difference of ISP cytotoxicity on HCT-15 and HCT-116 cells could be linked to the mutation of p53 in HCT-15 cells. However, we showed that triggering of apoptosis in HCT-116 cells did not require p53. Multidrug resistance in HCT-15 cells (M. Bentires-Alj, unpublished data) could be an alternate explanation. Further experiment should be done to address whether the multidrug resistance expression reduces ISP cytotoxic effect.

Camptothecin (and its hemisynthetic analogs topotecan and irinotecan), ellipticine, vincristine, and vinblastine are antitumor agents that are ISP biosynthetically related alkaloids. However, in contrast to the indolomonoterpenic alkaloids camptothecin and ellipticine, ISP inhibited neither topoisomerases I or II. On the other hand, disruption of microtubule structure by vincristine or vinblastine results in induction of tumor suppressor gene p53 and in phosphorylation of Bcl-2, leading to apoptosis (Wang et al., 1999). Here, we clearly showed that ISP-induced HCT-116 cell death was not associated with a change in p53 expression. ISP cytotoxicity is thus probably mediated by a different biochemical mechanism.

The protein p21 is an inducer of cell cycle arrest by inhibition of cyclin-dependent kinases, which regulate transitions between different phases of the cell cycle. The expression of p21 is increased in response to a variety of stressfull stimuli, including DNA-damaging drugs (such as daunomycin), ionizing radiation, and agents affecting mitosis (such as paclitaxel or vincristine). The response to these factors are mediated primarily through transcriptional activation of the p21-WAF1 gene by p53 (for review, see Roninson, 2002). Nevertheless, p21 gene expression can uncommonly be regulated by p53-independent mechanisms in response to ultraviolet c (Haapajarvi et al., 1999), reactive oxygen species (Qiu et al., 1996), growth factors (Michieli et al., 1994; Datto et al., 1995), and alkylphospholipids (Patel et al., 2002), or by some natural drugs such as the isoflavone genistein (Choi et al., 2001) or indole-3-carbinol (Chinni et al., 2001). p53-independent p21 induction is probably regulated in part at the level of transcription, through various cis-regulatory sites in the p21 promoter, and in part at the level of mRNA and protein stability (Gartel and Tyner, 1999). Here, we have demonstrated that the ISP induction of p21 was independent from p53, both in wild-type p53 HCT-116 and in p53-deleted HCT-15 colon cancer cells. ISP might be used as a tool to help to understand the mechanisms regulating p53-independent inductions of p21.

The resistance of cancer cells to classical chemotherapy may be due in part to the high frequency of mutation in p53 that impairs p53-dependent apoptosis. Most of the common anticancer agents such as doxorubicin, etoposide, paclitaxel, vincristine, or 5-fluorouracil induce apoptosis via a p53-dependent pathway (Lowe et al., 1993; Mesner et al., 1997). The requirement of p53 tumor suppressor gene for activation of apoptosis by these agents provides an attractive explanation for their poor efficacy on p53 mutant tumors. Thus, chemotherapeutic agents such as ISP, which induce p21-dependent cell cycle arrest and apoptosis independently from the p53 apoptotic pathway are of a major interest. It is now important to investigate the molecular targets and effectors, which are affected by ISP to trigger programmed cell death.

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