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Cytotoxicity and cell cycle effects of the plant alkaloids cryptolepine and neocryptolepine: relation to drug-induced apoptosis

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Abstract

Cryptolepine and neocryptolepine are two indoloquinoline derivatives isolated from the roots of the african plant *Cryptolepis sanguinolenta*. These two alkaloids, which only differ by the respective orientation of their indole and quinoline rings, display potent cytotoxic activities against tumour cells and present antibacterial and antiparasitic properties. Our previous molecular studies indicated that these two natural products intercalate into DNA and interfere with the catalytic activity of human topoisomerase II. Here we have extended the study of their mechanism of action at the cellular level. Murine and human leukemia cells were used to evaluate the cytotoxicity of the drugs and their effects on the cell cycle were measured by flow cytometry. Cryptolepine, and to a lesser extent neocryptolepine, provoke a massive accumulation of P388 murine leukemia cells in the G2/M phase. With HL-60 human leukemia cells, the treatment with cryptolepine leads to the appearance of a hypo-diploid DNA content peak (sub-G1) characteristic of the apoptotic cell population. With both P388 and HL-60 cells, cryptolepine proved about four times more toxic than its isomer. But the use of the HL-60/MX2 cell line resistant to the anticancer drug mitoxantrone suggests that topoisomerase II may not represent the essential cellular target for the alkaloids, which are both only two times less toxic to the resistant HL-60/MX2 cells compared to the parental cells. The capacity of the drugs to induce apoptosis of HL-60 human leukemia cells was examined by complementary biochemical techniques. Western blotting analysis revealed that cryptolepine, but not neocryptolepine, induces cleavage of poly(ADP-ribose) polymerase but both alkaloids induce the release of cytochrome *c* from the mitochondria. The cleavage of poly(ADP-ribose) polymerase observed with cryptolepine correlates with the appearance of a marked sub-G1 peak in the cell cycle experiments. The proteolytic activity of Asp-Glu-Val-Asp- or Ile-Glu-Thr-Asp-caspases was found to be enhanced much more strongly with cryptolepine than with its isomer, as expected from their different cytotoxic potential. Despite the activation of the caspase cascade, we did not detect internucleosomal cleavage of DNA in the HL-60 cells treated with the alkaloids. Altogether, the results shed light on the mechanism of action of these two plant alkaloids.

Keywords: Cryptolepine; Neocryptolepine; Plant alkaloid; Topoisomerase II; Cytotoxicity; Apoptosis

1. Introduction

Cryptolepis sanguinolenta (Lindl.) Schlechter (Asclepiadaceae) is a climbing shrub traditionally used in Central and West Africa, Ghana and Nigeria essentially for the treatment of rheumatism, urinary tract infections and upper respiratory tract infections. In addition, decoctions of this plant are used for the treatment of malaria-related fevers and intestinal disorders (Boye and Oku-Ampofo, 1990). The roots of the plant proved to be a rich source of indoloquinoline alkaloids. More than 15 alkaloids have been isolated and the structures of about 10 of them were elucidated (Sharaf et al., 1996). The major alkaloid is cryptolepine (Fig. 1), which is highly cytotoxic to tumour cells (Bonjean et al., 1997) but has also revealed antibacterial and antiparasitic activities (Boakye-Yiadom and Heman-Ackah, 1979; Paulo et al., 1994a,b; Cimanga et al., 1996; Kirby et al., 1995; Grellier et al., 1996; Wright et al., 1996; Cimanga et al., 1997, 1998). For cryptolepine, the two nitrogen atoms of the indole ring and the *N*-methyl quinoline ring are situated on the opposite side of the

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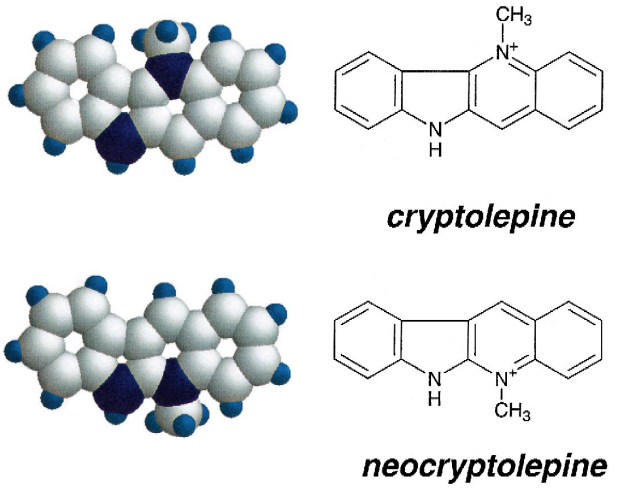

Fig. 1. Structures of cryptolepine and neocryptolepine. Energy minimized structures of the drugs are shown. The softwares HyperChem™ 5.01 and Alchemy 2000® were used to construct the structures.

planar molecule. In 1996, a new alkaloid for which the two nitrogens are located on the same side of the indoloquinoline chromophore was isolated from *C. sanguinolenta.* This isomeric form of cryptolepine was named neocryptolepine (Cimanga et al., 1996) to distinguish it from another known cryptolepine isomer, isocryptolepine (Pousset et al., 1995). Simultaneously, Sharaf et al. (1996) also identified 5-methyl-5*H*-indolo[2,3-b]quinoline from *C. sanguinolenta* which they christened cryptotackieine but the alkaloid is now commonly referred to as neocryptolepine (Alajarin et al., 1997; Molina et al., 1999).

To date, the molecular basis for the diverse biological effects of cryptolepine remains poorly understood. Only a few lines of the mechanism of action of the drug have been disclosed. We have previously shown that the planar alkaloid can intercalate into DNA, preferentially at GC-rich sequences, explaining how it can inhibit DNA synthesis in B16 melanoma cells (Bonjean et al., 1998). We have also demonstrated that cryptolepine is an inhibitor of the nuclear ubiquitous enzyme topoisomerase II which serves to regulate the topological states of DNA in cells. Like other well-known antitumour drugs such as etoposide, daunomycin or mitoxantrone, cryptolepine stabilizes topoisomerase II-DNA covalent complexes and stimulates the cutting of DNA by the enzyme. Both DNA and topoisomerase II are primary targets of cryptolepine (Dassonneville et al., 1999).

The mode of action of neocryptolepine remains largely unknown. Very recently, we reported that this cryptolepine isomer can also intercalate into DNA. Its affinity for DNA is slightly reduced compared to the parent alkaloid but neocryptolepine maintains a marked specificity for binding to GC-rich sequences. Our in vitro study also showed that the capacity of neocryptolepine to inhibit topoisomerase II is reduced compared to cryptolepine (Bailly et al., 2000). But these experiments were performed at the molecular level with purified DNA and topoisomerase II, in the test tube. Now we have extended our investigation at the cellular level. We compared the cytotoxicity and cell cycle effects of cryptolepine and neocryptolepine using the P388 murine leukemia cell line and two HL-60 human leukemia cell lines sensitive or resistant to the antitumour drug mitoxantrone.

2. Materials and methods

2.1. Alkaloids and chemicals

The procedures for the extraction and purification of cryptolepine and neocryptolepine from *C. sanguinolenta* have been reported previously (Cimanga et al., 1996). The drugs were dissolved in dimethylsulfoxide at 5 mM and then further diluted with water. The stock solutions of the drugs were kept at −20°C and freshly diluted to the desired concentration immediately prior to use. Etoposide was from Sigma. 3,3-Dihexyloxacarbocyanine iodide [$DiOC_6(3)$] was from Molecular Probes (Eugene, OR). All other chemicals were analytical grade reagents.

2.2. Cell cultures and survival assay

Human HL-60 and HL-60/MX2 promyelocytic leukemia cells were obtained from the American Tissue Culture Collection. The P388 murine leukemia cell line was kindly provided by Dr J.-F. Riou (Rhône-Poulenc Rorer, France). Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2 in RPMI 1640 medium, supplemented with 10% fetal bovine serum, glutamine (2 mM), penicillin (100 UI/ml) and streptomycin (100 μg/ml). The cytotoxicity of the alkaloids was assessed using a cell proliferation assay developped by Promega (CellTiter 96® AQ_{ueous} one solution cell proliferation assay). Briefly, 2×10^4 exponentially growing cells were seeded in 96-well microculture plates with various drug concentrations in a volume of 100 μl. After 72 h incubation at 37°C, 20 μl of the aqueous soluble tetrazolium dye (Cory et al., 1991) were added to each well and the samples were incubated for a further 2 h at 37°C. Plates were analyzed on a Labsystems Multiskan MS (type 352) reader at 492 nm. The LIVE/DEAD fluorometric assay was performed according to the supplier's recommended protocol (Molecular Probes). In this case, the flow cytometry analysis was performed at 530 nm for calcein-acetomethyl ester and 620 nm for the ethidium homodimer-1.

2.3. Cell cycle analysis

For flow cytometric analysis of DNA content, 10^6 HL-60 cells in exponential growth were treated with graded

concentrations of the test drug for 24 h and then washed three times with citrate buffer. The cell pellet was incubated with 250 μl of trypsin-containing citrate buffer for 10 min at room temperature and then with 200 μl of citrate buffer containing a trypsin inhibitor and RNase (10 min) prior to adding 200 μl of propidium iodide (PI) at 125 μg/ml. Samples were analyzed on a Becton Dickinson FACScan flow cytometer using the LYSYS II software, which is also used to determine the percentage of cells in the different phases of the cell cycle. Propidium iodide was excited at 488 nm, and fluorescence analyzed at 620 nm.

2.4. Mitochondrial energization

Mitochondrial energization was determined as the retention of the fluorescent dye $DiOC_6$. After the drug treatment, 10^6 cells in 2 ml of complete RPMI 1640 medium were loaded with the probe $DiOC_6$ (usually 25 nM unless otherwise stated) during 30 min at 37°C prior to the flow cytometric analysis. The same incubation time was applied to the controls and the drug-treated samples. Control experiments were performed by incubating cells with carbonyl cyanide *p*-chlorophenylhydrazone (50 μM, 10 min at 37°C), an uncoupling agent that abolishes $\Delta\Psi_{mt}$, and oligomycin (1.25 μg/ml, 10 min at 37°C), which is known to hyperpolarize the mitochondrial membranes. $DiOC_6$ was excited at 488 nm, and fluorescence analyzed at 525 nm after logarithmic amplification. Forward scattering and side scattering were analysed after linear amplification.

2.5. DEVD0-pNA and IETD-pNA cleavage

N-acetyl-Asp-Glu-Val-Asp-pNA (DEVD-pNA) and *N*-acetyl-Ile-Glu-Thr-Asp-pNA (IETD-pNA) cleavage activities were measured using the ApoAlert™ CPP32/caspase-3 and ApoAlert™ Caspase-8 assay kits (Clontech, Palo Alto) and the recommended protocol were followed. Briefly, 2×10^6 exponentially growing HL-60 cells in 2 ml of RPMI 1640 medium were treated with the test drug at the indicated concentration for 4 or 18 h at 37°C. Cells were pelleted by centrifugation and resuspended in 50 μl of the lysis buffer. The lysed cell mixture was then incubated on ice for 10 min prior to centrifugation (12,000 rpm, 3 min at 4°C). Fifty microliters of 2× reaction buffer supplemented with 10 mM dithiothreitol were then added to each tube incubated at 4°C. During this period, a control was prepared by adding 0.5 μl of 1 mM DEVD-fmk or *z*-IETD-fmk to a cell sample treated with 0.1 μM staurosporine (24 h at 37°C). The substrate DEVD-pNA or IETD-pNA was added to all tubes (5 μl, 50 μM) and the samples were incubated for 1 h at 37°C. The formation of *p*-nitroanilide was measured at 405 nm using a Labsystems Multiskan MS microtiter plate reader.

2.6. Poly(ADP-ribose) polymerase cleavage

Exponentially growing HL-60 cells (7×10^5) in a serum-free medium were treated with the alkaloids at the indicated concentration for 24 h at 37°C. Cells were pelleted by centrifugation, resuspended in 3 ml of lysis buffer containing 25 mM phosphate buffered saline, 0.1 mM phenylmethylsulfonyl fluoride, the protease inhibitors chymostatin, leupeptin, aprotinin and pepstatin A (5 μg/ml each). After centrifugation, the pellet is resuspended in the loading buffer containing 50 mM Tris–HCl pH 6.8, 15% sucrose, 2 mM EDTA, 3% SDS and 0.01% bromophenol blue. The mixture is sonicated for 30 s at 4°C and then boiled to 100°C for 3 min. For Western blotting, the cell lysates were fractionated on a 7.5% polyacrylamide gel containing 0.1% sodium dodecylsulphate (SDS), then transferred onto a Hybond-C nitrocellulose membranes (Amersham) for 40 min at 150 mA using a semi-dry transfer system. Membranes were blocked with 10% nonfat milk in PBST (25 mM phosphate bufferered saline pH 7.4, containing 0.1% Tween-20) for 30 min followed by incubation with anti-poly(ADP-ribose) polymerase monoclonal antibody (Clontech) (dilution 1:10,000 in PBST supplemented with 1% nonfat milk) for 30 min. The blots were washed three times (5 min each with PBST) and incubated with a goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Amersham Life Sciences, 1:10,000 dilution in PBST containing 1% nonfat milk) for 30 min. After three successives washes with PBST, the Western blot chemiluminescence reagent from NEN (Boston, MA) was used for the detection. Bands were vizualized by autoradiography.

2.7. Pro-caspase-3 processing

HL-60 cells (0.7×10^6 in 1 ml) were treated with the alkaloid at the indicated concentration for 24 h at 37°C. Cells were pelleted by centrifugation at 4°C, and washed twice with phosphate buffered saline (2×3 ml) at 4°C. After centrifugation, the pellet is resuspended in 25 μl of boiling buffer containing 10 mM Tris–HCl pH 7.4, 1 mM Na–vanadate, 1% SDS, 0.1 mM PMSF, and the protease inhibitors leupeptin (5 μg/ml), aprotinin (10 μg/ml) and pepstatin A (2.5 μg/ml). The mixture is incubated for 10 min at 4°C prior to adding 75 μl of the electrophoresis dye solution (15% sucrose, 50 mM Tris–HCl, 2 mM EDTA, 3% SDS and 0.01% bromophenol blue). Samples were passed through a 26-gauge needle to reduce the viscosity of the solutions and then boiled to 100°C for 3 min. For Western blotting, the cell lysates (containing about 30 μg of proteins) were fractionated on a 12.5% polyacrylamide gel containing 0.1% SDS, then transferred onto a Hybond-C nitrocellulose membranes (Amersham) for 40 min at 0.8 mA/cm^2 using a semi-dry transfer system. Membranes were blocked with 10% nonfat milk in PBST for 1 h at

room temperature (or overnight at 4°C) followed by incubation with a mouse monoclonal antibody directed against actin (1/1000, Oncogene Research Products) or a rabbit polyclonal antibody against pro-caspase-3 (1/1000, Pharmingen). Antibodies were diluted in PBST containing 2% nonfat milk and membranes were incubated for 4 h in the dark under gentle agitation. The blots were washed three times (15 min each with PBST) and incubated with a sheep anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (Amersham Life Sciences, 1:10,000 dilution in PBST containing 2% nonfat milk) for 1 h. After three successive washes (15 min each) with PBST, the Western blot chemiluminescence reagent from NEN was used for the detection.

2.8. Cytochrome c release

HL-60 cells (7×10^5 in 1 ml) were treated with the alkaloids at the indicated concentration for 24 h at 37°C. Cells were pelleted by centrifugation at 4°C, and washed twice with phosphate buffered saline (2×3 ml) at 4°C. After centrifugation, the pellet is resuspended in 25 μl of lysis buffer containing 10 mM Tris–HCl pH 7.4, 1 mM Na–vanadate, 1% SDS, 0.1 mM phenylmethylsulfonyl fluoride, and the protease inhibitors leupeptin (5 μg/ml), aprotinin (10 μg/ml) and pepstatin A (2.5 μg/ml). The mixture is incubated for 10 min at 4°C prior to adding 75 μl of the electrophoresis dye solution (15% sucrose, 50 mM Tris–HCl, 2 mM EDTA, 3% SDS and 0.01% bromophenol blue). Samples were passed through a 26-gauge needle to reduce the viscosity of the solutions prior to boiling at 100°C for 3 min. Cell lysates were then fractionated on a 12.5% polyacrylamide gel containing 0.1% SDS, then transferred onto a Hybond-C nitrocellulose membranes (Amersham) for 40 min at 0.8 mA/cm^2 using a semi-dry transfer system. Membranes were blocked with 5% nonfat milk in TBST (20 mM Tris–HCl pH 7.6, 137 mM NaCl, 0.05% Tween 20) for 30 min followed by incubation with anti-cytochrome *c* monoclonal antibody (Pharmingen) (dilution 1:1000 in TBST supplemented with 3% nonfat milk) for 1 h under gentle agitation. The blots were washed three times (15 min each with TBST) and incubated with a peroxidase-conjugated secondary antibody, as described above for the poly(ADP-ribose) polymerase experiments.

2.9. DNA fragmentation

HL-60 cells at a density of about 5×10^5 cells/ml were treated with various concentrations of the alkaloids for 24 h and then collected by centrifugation at $2500 \times g$ for 5 min. The resultant cell pellets were resuspended in PBS buffer containing 5 mM $MgCl_2$ and lysed in 500 μl of Tris–EDTA buffer containing 0.1% SDS and proteinase K (1.5 mg/ml) overnight at 37°C. After two successive extractions with phenol/chloroform, the aqueous layer was transferred to a new centrifuge tube. The DNA was precipitated with ethanol, resuspended in water (100 μl) and treated with RNase A (100 μg/ml) for 2 h at 37°C. Electrophoresis was performed in 1% agarose gel in Tris–borate buffer at about 12 V/cm for approximately 4 h. After electrophoresis, the gel was stained with ethidium bromide (1 mg/ml), washed and photographed under UV light.

3. Results

3.1. Cytotoxicity

Initially, the cytotoxicity of the two alkaloids was assessed by a cell growth inhibition assay using the P388 murine leukemia cell line. Under the experimental conditions used (3 days continuous exposure) cryptolepine exhibited a marked cytotoxic effect whereas neocryptolepine proved about four times less toxic to the P388 murine cells (Table 1). Similar results were obtained with a 24-h exposure time. To compare further the toxic potency of the alkaloids, we repeated the proliferation assay using two human leukemia cell lines sensitive (HL-60) or resistant (HL-60/MX2) to the antitumor drug mitoxantrone. Here again, we found that cryptolepine was significantly more cytotoxic than neocryptolepine. Interestingly, the IC_{50} value measured with cryptolepine is about fourfold lower than that obtained with neocryptolepine, as is the case with the murine cell line. HL-60/MX2 cells resistant to mitoxantrone showed reduced sensibility to two alkaloids. In both cases, the relative resistance index—RI defined by the ratio $[IC_{50}^{HL60/MX2}]/[IC_{50}^{HL60}]$—did not exceed 2.

Another procedure was used to compare the cytotoxic potential of the two alkaloids toward HL-60 cells. The cells were stained with two fluorescent markers: calcein and ethidium homodimer-1 (EthD-1), which stain simultaneously live and dead cells in green and red, respectively (Fig. 2). Cleavage of the ester group by intracellular esterases in live cells produces calcein which is intensely fluorescent whereas EthD-1 freely enters cells with damaged membranes, such as dead cells. Like ethidium bro-

Table 1
Cytotoxic properties of the alkaloids on murine (P388) and human (HL-60) leukaemia cells

	P388	IC_{50} (μM)[a]		RRI[b]
		HL-60	HL-60/MX2	
Cryptolepine	0.94 ± 0.05	3.2 ± 0.2	7.4 ± 0.6	2.3
Neocryptolepine	3.38 ± 0.7	12.7 ± 1.3	25.4 ± 1.8	2.0

[a] Drug concentration that inhibits cell growth by 50% after incubation in liquid medium for 72 h. Each drug concentration was tested in triplicate.

[b] The relative resistance index (RRI) is the ratio between the HL-60/MX2 IC_{50} value and the HL-60 IC_{50} value.

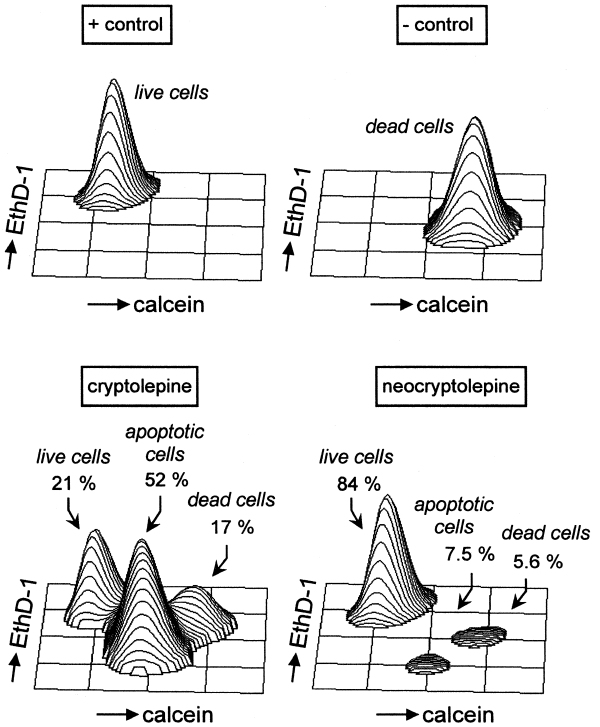

Fig. 2. Three-dimensional representation of the correlated distribution of calcein and EthD-1 fluorescence in HL-60 cells treated with 10 μM cryptolepine or neocryptolepine for 48 h. The positive control refers to untreated cells. Dead cells used as negative control were obtained by treatment with 2% methanol and 3% formaldehyde in PBS buffer for 30 min at 4°C.

mide, this dye becomes brightly fluorescent upon binding to DNA. Cells were treated with 10 μM cryptolepine or neocryptolepine for 48 h and then loaded with calcein and EthD-1 prior to analysis by flow cytometry. The populations of live (calcein$^+$) and dead (EthD-1$^+$) cells can be easily differentiated but in addition, a third population corresponding to cells stained both with calcein and EthD-1 can be detected. This doubly stained cell fraction represents 40% of the cells upon treatment with cryptolepine but only 3% with neocryptolepine. In a recent study (Kluza et al., 2000), we showed that these cells with an active metabolism (calcein$^+$) but which allow EthD-1 to penetrate and stain their nucleic acids correspond to apoptotic cells.

3.2. Cell cycle effects

In parallel to the cytotoxicity evaluation, we studied the variations of the cell cycle profile upon treatment of the different cell lines with the alkaloids. As shown in Fig. 3, treatment of the P388 cells with increasing concentrations of cryptolepine or neocryptolepine for 24 h led to profound changes of the cell cycle profiles. The flow cytometric analysis of propidium iodide-labelled cells indicates that the treatment with 5 μM cryptolepine induces a massive accumulation of cells in the G2/M phase. The G2 cell population increases from 16% in the control to 74%. With neocryptolepine, higher concentrations were required to observe a similar effect. A full blockage of the P388 cells in the G2/M phase was obtained using concentrations of neocryptolepine of 5–10 μM, i.e. about three times the concentrations required to detect a comparable efect with cryptolepine, exactly as expected from the cytotoxicity measurements.

No accumulation in the G2/M phase was observed with the HL-60 human cells, perhaps because these leukemia cells are p53 null (Wolf and Rotter, 1985; Collins, 1987) and prone to rapidly enter apoptosis. With neocryptolepine, we found relatively little perturbation of the cell cycle profile, be it with the sensitive (HL-60) or the resistant (HL-60/MX2) cell line (data not shown). Cryptolepine produced a more pronounced effect. Upon treatment with relatively high concentrations (≥ 5 μM), we found that the G1- and S-phase cell populations decrease considerably from 46% and 35% to 9% and 13%, respectively. In the mean time, the G2/M fraction almost completely disappeared in HL-60 cells treated with 5 μM cryptolepine. The same sort of effects were observed with the HL-60/MX2 mitoxantrone-resistant cells receiving concentrations of ≥ 10 μM. A hypo-diploid DNA content peak (sub-G1) representing 70% of the cell population can be easily seen when using 20 μM cryptolepine (data not

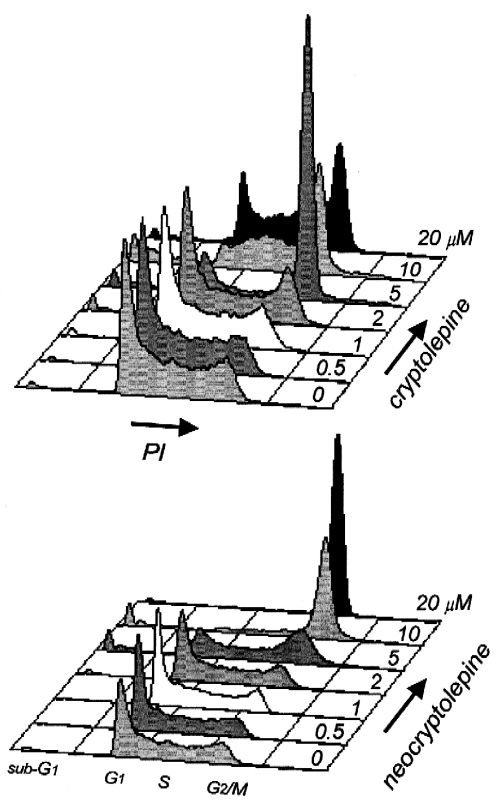

Fig. 3. Cell cycle analysis of P388 cells treated with graded concentrations of cryptolepine and neocryptolepine for 24 h. Cells were analyzed with the FACScan flow cytometer as described in Materials and methods.

shown). As a whole, the results of the cytometry data are consistent with the cytotoxicity measurements indicating a reduced anti-proliferative activity of neocryptolepine compared to its parent isomer. A marked hypo-diploid DNA content peak was detected with cryptolepine. The sub-G1 peak detected with cryptolepine refers to cells with DNA content less than G1, which are usually considered as apoptotic cells. Gross alterations in DNA content may result from the degradation of cellular DNA by activation of endogenous nucleases during apoptosis. This observation prompted us to investigate further the potential activation of the apoptotic machinery in the presence of the alkaloids.

3.3. Variations of the mitochondrial membrane potential

Mitochondria is essential for the propagation of apoptosis. We used the fluorochrome $DiOC_6$ to monitor the changes of the mitochondrial transmembrane potential, $\Delta\Psi_{mt}$ induced by the alkaloids. HL-60 cells were treated with 10 μM drug for 24 h and then analyzed by flow cytometry after $DiOC_6$ labelling. With both alkaloids we observed a slight increase in $DiOC_6$ fluorescence. Such a hyperpolarization effect has been previously detected with different antitumour drugs like etoposide and was correlated with the accumulation of the cells in the G2/M phase (Kluza et al., 2000). In addition with cryptolepine, we observed a second peak with a significant decrease of fluorescence intensity which reflects a marked reduction of the cellular uptake of the fluorochrome (data not shown). The collapse of $\Delta\Psi_{mt}$ is a signature for the opening of the mitochondrial permeability transition pores (Bernardi and Petronilli, 1996). The dissipation of $\Delta\Psi_{mt}$ observed upon treatment with cryptolepine is characteristic of apoptosis and has been commonly observed with a variety of anticancer drugs irrespective of the cell type. It generally defines early but already irreversible stage of apoptosis (Kroemer et al., 1998).

3.4. Caspase-3 activation

Programmed cell death is associated with activation of a number of aspartate-specific cysteine proteases, the caspases (Nuñez et al., 1998). In particular, caspase-3 is considered essential to the propagation of the apoptotic signal by several types of antitumour drugs (Kaufman, 1998). It was therefore of interest to determine whether this cysteine protease, which cleaves DEVD-type substrates, is also involved in the apoptosis induction by cryptolepine in HL-60 cells. We prepared lysates from cells treated for 4 h with various concentrations of the drug and then assayed for an activity capable of cleaving DEVD-pNA using a solution assay (Fig. 4A). Lysates were mixed with the pNA-tagged tetrapeptide and the absorbance of the released substrate was recorded at 405 nm using a 96-well plate reader. A marked activity was recorded in lysates from cells treated with 10 and 20 μM

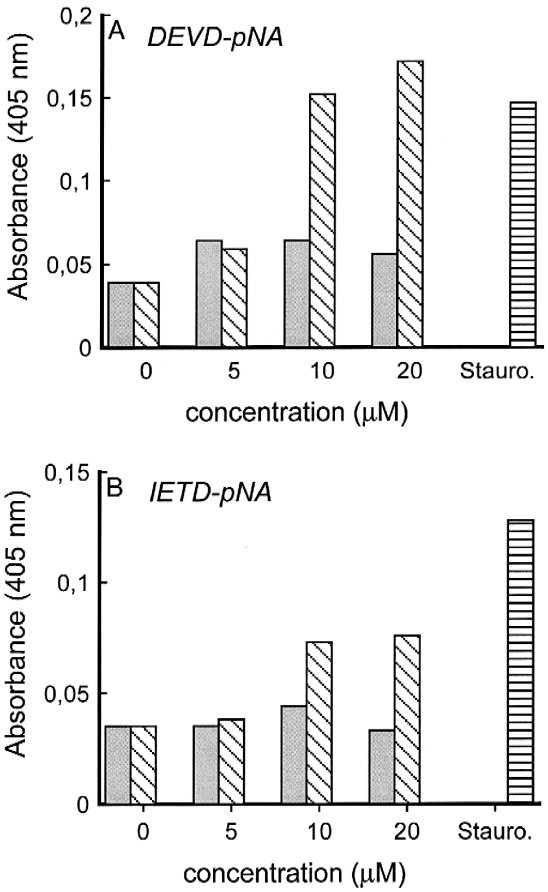

Fig. 4. (A) DEVD-pNA and (B) IETD-pNA cleaving activities. HL-60 cells were incubated with 5, 10 or 20 μM of (open bars) cryptolepine or (grey bars) neocryptolepine for 24 h prior to adding the caspase-3 (DEVD-pNA) or caspase-8 (IETD-pNA) substrate (50 μM each). Staurosporine, used as a positive control, was used at 0.1 μM. Assay mixtures were incubated for 1 h at 37°C prior to measurement of absorbance at 405 nm.

cryptolepine whereas neocryptolepine showed essentially no effect. Even when using 20 μM neocryptolepine, the absorbance at 405 nm is only marginally higher than that measured in the control (drug-free) lysates. In contrast, the effect of cryptolepine is very pronounced and comparable to that measured with positive control drugs such as etoposide, camptothecin as well as the protein kinase C inhibitor staurosporine which is known as a potent inducer of apoptosis (Fig. 4A). The caspase-3-mediated cleavage activity stimulated by cryptolepine was totally inhibited by the inhibitor *z*-DEVD-fmk (data not shown). The results suggest that cryptolepine activates caspase-3. However, DEVD is mainly cleaved by caspase-3 but may also be a substrate for a few other caspases, such as caspases-1 and -7 (Talanian et al., 1997). Moreover, a recent study of inhibitor specificity found that *z*-DEVD-fmk inhibits both caspases-3 and -7 (Garcia-Calvo et al., 1999). These considerations prompted us to use a second method for probing the involvement of caspase-3 in cryptolepine-induced apoptosis.

3.5. Cleavage of the poly(ADP-ribose) polymerase and processing of pro-caspase-3

Poly(ADP-ribose) polymerase is an enzyme involved in DNA repair, which catalyses the transfer of ADP ribose to a limited number of protein involved in chromatin architecture or in DNA metabolism (De Murcia and Ménissier-de Murcia, 1994). This ubiquitous enzyme represents a privileged substrate for caspase-3, one of the cysteine proteases at the heart of the apoptotic machinery (Kidd, 1998). Caspase-3 is involved in the apoptosis of HL-60 cells induced by topoisomerase inhibitors, such as camptothecin and etoposide (Shimizu and Pommier, 1997). The Western blot in Fig. 5 shows that the 116-kDa protein was cleaved into its characteristic 89-kDa fragment upon treatment of the cells with cryptolepine. In contrast, similar treatments with neocryptolepine showed no effect. A treatment for 24 h with 5 μM cryptolepine suffices to induce 50% cleavage of poly(ADP-ribose) polymerase in the leukemia cells and a quantitative cleavage was observed using 10 μM cryptolepine. The cleavage of poly(ADP-ribose) polymerase occurs essentially upon treatment of the cells with concentrations of ≥ 5 μM, i.e. the concentrations for which the hypodiploid peak (sub-G1) starts to appear in the cell cycle experiments. The lack of cleavage observed with neocryptolepine may be connected with the absence of a marked sub-G1 peak in the cell cycle experiments. The cleavage of poly(ADP-ribose) polymerase generally indicates that the cells engage into the irreversible apoptotic pathway. We also followed by immunoblot analysis the proteolytic activation of pro-caspase-3 induced by the alkaloids (Fig. 6). At 5 μM, cryptolepine induced a nearly quantitative cleavage of pro-caspase-3 and a similar effect was obtained with a fourfold higher concentrations of neocryptolepine, as expected from the cytotoxicity measurements.

Caspase-3 is an executioner protease that can be activated by at least two distincts mechanisms. Cytochrome *c*, which is often released from the mitochondria into the cytosol (Bossy-Wetzel et al., 1998), can induce ATP- or dATP-dependent formation of a complex of proteins that results in the proteolytic activation of pro-caspase-3 and the apoptotic destruction of the nuclei (Liu et al., 1996). Alternatively, distal caspases such as caspases-3, -6 and -7, can be directly activated by a proximal caspase such as caspase-8 (Muzio et al., 1997; Stennicke et al., 1998). The caspase-8 and cytochrome *c* pathways for caspase-3 activation are two independent pathways inhibited by IAP (inhibitor of apoptosis) proteins at distinct points (Deveraux et al., 1998). However, caspase-8 can also act through mitochondria to facilitate the efflux of cytochrome *c* (Kuwana et al., 1998). These considerations prompted us to determine the activity of caspase-8 and the variations of the level of cytochrome *c* in HL-60 cells treated with the two alkaloids.

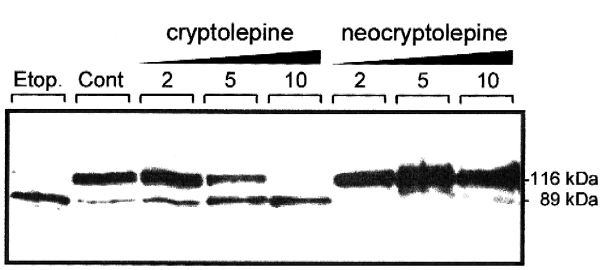

Fig. 5. Induction of poly(ADP-ribose) polymerase cleavage by cryptolepine. Western blot was used to detect cleavage of full length protein [116-kDa band] into the 89-kDa fragment in untreated cells (Cont) and cells treated with the alkaloids at the indicated concentration (μM) for 24 h. Whole cell lysates were subjected to SDS-PAGE followed by blotting with an anti-poly(ADP-ribose) polymerase monoclonal antibody. Etoposide (Etop.) was used at 10 μM.

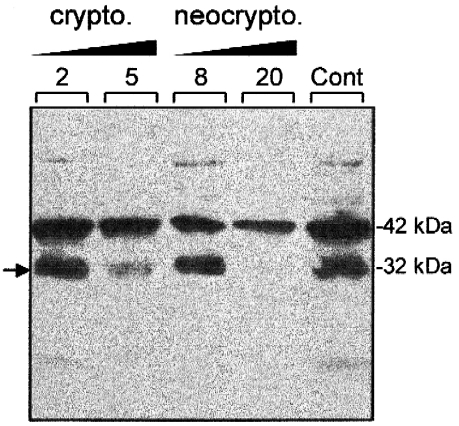

Fig. 6. Drug-induced processing of pro-caspase-3. Cells were treated with cryptolepine or neocryptolepine at the indicated concentration (μM) for 24 h. The control lane (Cont) refers to untreated cells. Whole cell lysates were subjected to SDS-PAGE followed by blotting with an anti-pro-caspase-3 antibody. The arrow points to the full-length protein [32-kDa band]. The 42-kDa band refers to actin that was detected with an anti-actin monoclonal antibody to evaluate the amount of protein in each lane.

3.6. Caspase-8 activation

Because the tetrapeptide IETD is a good substrate for caspase-8, we measured the rate of IETD-pNA hydrolysis in cells treated with graded concentrations of the alkaloids. A modest IETDase activity was recorded in lysates from cells treated with cryptolepine but not with its isomer (Fig. 4B). This moderate activity was abolished upon addition of the inhibitory peptide *z*-Ile-Glu-Thr-Asp-fluoromethylketone (*z*-IETD-fmk) but was inferior to that measured with drugs like staurosporine, camptothecin and etoposide. We concluded that the drugs have minimal, if any, effect on caspase-8.

3.7. Cytochrome c release

HL-60 cells were treated with 10 μM cryptolepine or neocryptolepine for 24 h and the release of cytotchrome *c*

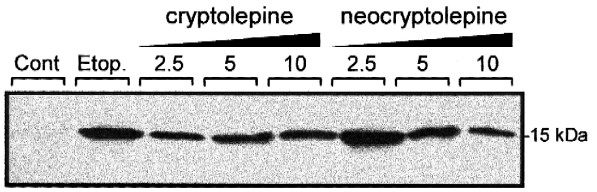

Fig. 7. Western blotting analysis for the release of cytochrome *c* induced by the alkaloids. Control (Cont) refers to untreated cells (Cont). HL-60 cells were treated with etoposide (Etop.) at 10 μM or with the alkaloids at the indicated concentration (μM) for 24 h. Whole cell lysates were subjected to a polyacrylamide gel electrophoresis followed by blotting with an anti-cytochrome *c* monoclonal antibody.

from mitochondria was detected by immunoblot analysis. Cytochrome *c* cannot be detected in drug-free cells whereas the appearance of free cytosolic cytochrome *c* was clearly evident in HL-60 cells treated with either cryptolepine or neocryptolepine (Fig. 7). The release of cytochrome *c* from the mitochondrial intermembranous space into the cytosol is usually considered as a necessary event for downstream caspase activation (Kluck et al., 1997; Yang et al., 1997). But our data suggest that this step may be necessary but not sufficient because neocryptolepine facilitates the leakage of cytochrome *c* from the mitochondria to the cytosol but does not activate caspases-3 and -8.

3.8. DNA fragmentation

The DNA of HL-60 cells treated with the alkaloids was extracted and analyzed by electrophoresis on agarose gels. The genomic DNA of the cells treated with neocryptolepine remained intact. When using concentrations of cryptolepine < 10 μM, the DNA was also unaffected but the treatment with a higher concentration (20 μM) resulted in DNA cleavage (data not shown). The genomic material became degraded but there was no internucleosomal cleavage, in contrast to the DNA profiles obtained uopn treatment with etoposide which is a well established topoisomerase II inhibitor and is known to efficiently promote the apoptotic cell death of HL-60 cells (Bertrand et al., 1993; Solary et al., 1993, 1994).

4. Discussion

Indoloquinoline alkaloids form a relatively rare group of natural products. In fact cryptolepine was first discovered in Nature more than twenty years after its total synthesis at the beginning of the XXth century. Cryptolepine has been isolated from diverse plants in Africa (*C. sanguinolenta* and *C. triangularis* N. E. Br), in Sri lanka (Sida sp., Malvaceae) and more recently from the Surinamese plant *Microphilis guyanensis* (A. DC) Pierre (Sapotaceae) (Yang et al., 1999). In contrast, neocryptolepine (cryptotackieine) was found only in the African plant *C. sanguinolenta* (Cimanga et al., 1996; Sharaf et al., 1996). The two alkaloids only differ by the orientation of the two nitrogen atoms, which are on the same (neocryptolepine) or on the opposite (cryptolepine) side of the indoloquinoline chromophore. The distinct chemical configuration has a significant impact on the cytotoxicity of the alkaloid. Cryptolepine is about three to four times more toxic to leukemia cells than neocryptolepine. However, they target the same molecules in cells, DNA and possibly topoisomerase II. The present study indicates that the action of the drugs on topoisomerase II is probably not as significant as expected from the molecular recognition experiments (Bailly et al., 2000). Both cryptolepine and neocryptolepine are only about two times less toxic to HL-60/MX2 cells resistant to the antitumor drug mitoxantrone. By comparison, the potent topoisomerase II poisons teniposide, amsacrine and mitoxantrone are respectively 24, 32 and 35 times resistant to the topoisomerase II deficient cell line compared to the parental cell line (Harker et al., 1991). The cross-resistance patterns observed with the two indoloquinoline alkaloids are comparable to those reported with anticancer drugs like actinomycin D and doxorubicin which are weaker inhibitors than the aforementioned drugs (Harker et al., 1991). For this reason, we are inclined to believe that topoisomerase II inhibition would play a relatively minor role in the cytotoxicity of the two plants alkaloids. HL-60/MX2 cells display altered topoisomerase II catalytic activity and reduced levels of topoisomerase IIα and IIβ but they also show an atypical multidrug resistance with the absence of P-glycoprotein overexpression (Harker et al., 1991, 1995). The slightly decreased sensitivity of the HL-60/MX2 cells to the two alkaloids may be attributed to their atypical MDR phenotype rather than to their reduced topoisomerase II functional activities. There are some specific efflux systems for mitoxantrones which may also play a role in resistance to the present compounds. However, the cytotoxicity evaluations are consistent with our previous experiments at the molecular level indicating that the two plants alkaloids are weak but noticeable topoisomerase II poisons (Dassonneville et al., 1999; Bailly et al., 2000). Topoisomerase II may not represent the essential cellular target for the alkaloids which likely interfere with several DNA-interacting proteins in addition to topoisomerase II. In other words, the mechanism of action is probably pleiotropic, as is the case with most anticancer agents.

The various biochemical and flow cytometry experiments reported here help to understand the mechanism of action of the test alkaloids. Both the cell cycle analysis and the measurements of caspase activities attest that cryptolepine induces apoptosis in chemosensitive HL-60 cells. The loss of the mitochondrial potential membrane ($\Delta\Psi_{mt}$) reveals that cryptolepine provokes marked changes of the mitochondrial functions. The collapse of $\Delta\Psi_{mt}$ is a signature of the opening of the mitochondrial pores responsible for an uncoupling of the respiratory chain and efflux of small molecules such as cytochrome *c* which is supposed to directly contribute to the stimulation of the proteolytic

activation of caspase-3 (Susin et al., 1997). Both alkaloids activate caspase-3 and induce the release of cytochrome *c* from the mitochondria to the cytosol. The mechanism of action of the two drugs is indentical; the different apoptotic effects recorded only reflect their different cytotoxic potential. Cryptolepine efficiently promotes the activation of caspase-3 but has a modest effect on caspase 8. This proximal caspase can act either upstream or downstream of mitochondria (Bossy-Wetzel et al., 1998). For example, caspase-8 activation occurs downstream of the $\Delta\Psi_{mt}$ dissipation in apoptosis induced by betulinic acid whereas caspase-8 cleavage occurs upstream of the $\Delta\Psi_{mt}$ collapse in doxorubicin-induced apoptosis (Fulda et al., 1998). The route leading to apoptosis induced by cryptolepine may be different from that seen with other anticancer drugs. This idea is supported by the DNA laddering experiments. In general, all topoisomerase II poisons, including etoposide and the indeno-quinoline derivative TAS-103 (Kluza et al., 2000), induce en masse breakage of DNA into oligonucleosome fragments. The degradation of DNA down to oligonucleosomal fragments is a late event of apoptosis (Compton, 1992). With cryptolepine, the apoptotic pathway does not reach this stage. This could explain, at least in part, the lower cytotoxic potential of cryptolepine compared to other topoisomerase II poisons.

In conclusion, the study reported here shows that the orientation of the indole moiety with respect to the quinoline moiety is important for the cytotoxicity of cryptolepine-type alkaloids. The reduced efficiency of neocryptolepine to intercalate into DNA and to inhibit topoisomerase II in vitro compared to its parent isomer (Bailly et al., 2000) accounts satisfactorily for its reduced cytotoxicity. This could explain also why neocryptolepine is a minor component of the alkaloids content in *C. sanguinolenta* whereas cryptolepine is much more abundant is the African climbing shrub and widely found in several plants not only in Africa but also in Asia (Sri Lanka) and South America (Suriname). In addition, the results provide direct evidences that cryptolepine induces apoptosis in HL-60 leukemia cells. Mitochondria and caspases play a central role in the activation of the executioner phase of cryptolepine-induced apoptosis. Inhibition of topoisomerase II may serve as an inducing signal triggering mitochondrial activation but we are inclined to believe that the alkaloid must produce cell death signals other than topoisomerase II-mediated DNA breakage. Further investigations are warranted to identify the initial signal(s) as well as the events downstream of DNA strand breaks and upstream of mitochondria, involved in activation of the apoptosis machinery. Identifications of these specific targets may have profound therapeutic implications.

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