"Cytotoxic and antitumor potentialities of aporphinoid alkaloids"

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Abstract

Aporphinoids form an important group of plant secondary metabolites. Some of these compounds are used for a long time in traditional medicine for the treatment of various diseases, from benign syndromes to more severe illnesses. More than 500 aporphine alkaloids have been isolated from various plant families and many of these compounds display potent cytotoxic activities which may be exploited for the design of anticancer agents. Here we review the origin, biosynthesis, structure and cytotoxic properties of the prominent members of this class of compounds. Simple aporphinoids (boldine, dicentrine) as well as oxo-, pro- and dehydro-aporphines, and dimeric forms such as thalicarpine, are discussed here. Their mechanisms of action are not well known but DNA-manipulating enzymes such as polymerases and topoisomerases are among the most frequently cited targets for these benzylisoquinoline compounds. This review presents an updated view of the cytotoxic properties of the aporphinoids and ...

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Induction of Apoptosis in Human Promyelocytic Leukemia Cells by a Natural Trachylobane Diterpene

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Abstract. Background: Trachylobane diterpenes are secondary metabolites, quite rare in nature, and their bioactivities are poorly understood. Recently, we have described the cytotoxic activity of ent-trachyloban-3'-ol isolated from the leaves of Croton zambesicus, a plant used in African folk medicine. Materials and Methods: Cell viability on several cell lines, cell morphology, DNA laddering, annexin V and caspase-3 activation experiments were undertaken in order to analyse the cytotoxicity of trachylobane diterpene and to determine if this compound is able to induce apoptosis. Results: ent-Trachyloban-3'-ol exerts a dose-dependent cytotoxic effect, which varies between cell lines. Induction of apoptosis in HL-60 cells could be detected at a concentration of 50 μM after 24-h treatment. Conclusion: We show here, for the first time, that a trachylobane diterpene is able to induce apoptosis in human promyelocytic leukemia cells via caspase-3 activation in a concentration-dependent manner.

For centuries, traditional and folk medicine has exploited the properties of plants for therapeutic purposes and many drugs on the market today are of plant origin, particularly in the field of cancer. Camptothecin derivatives, Cantharanthus alkaloids, teniposide and etoposide or paclitaxel are all anticancer compounds derived from plants. In view of the effectiveness of some of these cytotoxic and antiproliferative drugs, we have been interested in finding new active compounds from natural sources and studying their biological effects. ent-Trachyloban-3β-ol (Figure 1) is a new secondary metabolite isolated by a bio-guided fractionation from a cytotoxic extract of the leaves of Croton zambesicus (1), a plant widespread in tropical Africa (2).

The first trachylobanes were found in Copal, the resin of Trachylobium verrucosum (Leguminosae), by a group led by Ourisson (3, 4). This type of diterpenes is characterized by a pentacyclic carbon skeleton with a tricyclo 3,2,1,0 octane system for the rings C, D and E and all the trachylobanes isolated so far from natural sources belong to the enantiomeric series. Since their first identification, they have been isolated from a wide range of families and genus of plants, but mainly from Asteraceae (Helianthus), Labiateae (Sideritis), Annonaceae (Xylopia) and Euphorbiaceae (Croton) (5). The bioactivities of most trachylobanes have not been explored, although some trachylobane diterpenes have been shown to possess antimicrobial, antifeedant and antifungal properties (6-11).

Recently, we described the cytotoxic activity of ent-trachyloban-3β-ol isolated from the leaves of Croton zambesicus on HeLa cells (1). However, the molecular mechanism of cytotoxic activity has not yet been clarified. The aim of this study was to determine whether ent-trachyloban-3β-ol induces apoptosis in HL-60 cells.

Materials and Methods

Source and preparation of ent-trachyloban-3β-ol. ent-Trachyloban-3β-ol was isolated from a dichloromethane extract of the leaves of Croton zambesicus and characterised by spectroscopic methods such as IR, EIMS, 1H- and 13C- NMR, as recently described (1). The purity of the compound was determined as 98% by GC/MS analysis. A stock solution (10 mg/ml) of ent-trachyloban-3β-ol was prepared in DMSO (Sigma-Aldrich, St Louis, MO, USA) and stored at 4°C.
Cell culture. HL-60 (Human Promyelocytic Leukemia cells) were routinely grown in suspension in RPMI 1640 medium (Gibco BRL, Paisley, Scotland, UK) supplemented with L-glutamine (0.33%), non essential amino acids (1%), sodium pyruvate (1%), antibiotics (100 IU penicillin/ml, 100 µg streptomycin/ml) and heat-inactivated fetal calf serum (10%, Gibco BRL), in a 5% CO2 humidified atmosphere at 37°C. WI-38 (Human Lung Fibroblast, non-cancer cells) were grown in DMEM (Gibco BRL) containing antibiotics (100 IU penicillin/ml, 100 µg streptomycin/ml) and supplemented with fetal calf serum (10%, Gibco BRL).

Cytotoxicity assays. The cytotoxicity of ent-trachyloban-3'-ol was evaluated using tetrazolium salt WST-1 (Boehringer-Mannheim, Indianapolis, IN, USA) for HL-60 and MTT (Sigma-Aldrich) for WI-38. These colorimetric assays are based on the reduction of the tetrazolium salt in formazan by dehydrogenases in viable cells (12). The assays were performed following a previously described method (13). Cells were treated for 24 h or 72 h and negative control cells received fresh medium with analogous concentrations of DMSO. The relative optical density was expressed as percent of the control cells set at 100%. Camptothecin (Sigma-Aldrich) was used as an apoptosis inducer reference compound. All experiments were performed at least in triplicate. The concentrations of drugs giving 50% cell viability (IC50) were calculated from linear regression. A Student’s t-test was performed (statistical significance was set at p<0.01).

Cell morphology. 5x10⁵ HL-60 cells were inoculated into each well of a 96-well tissue culture plate in 100 µl of RPMI 1640 medium. Cells were treated with 100 µl of the different dilutions of ent-trachyloban-3'-ol stock solution. Control cells received fresh medium with the same concentration of DMSO and camptothecin (1µM) was used as positive control. After 24-h treatment, cytocentrifuge preparations were made from the cell suspension and, following air drying, the cells were fixed with methanol, stained with Giemsa and analysed by light microscopy.

DNA fragmentation assay. 5x10⁵ HL-60 cells in 1 ml medium were inoculated into 24-well tissue culture plate. One ml of the given concentration of drugs was added to each well. After 24-h treatment, the cells were collected into tubes and washed with PBS. The cells were lysed overnight at 56°C in 400 µl of lysis buffer (100 mM Tris-HCl, pH 8.5, 10 mM EDTA, 0.5% SDS) containing 0.5 mg/ml proteinase K. DNA was extracted from cell lysates with one volume of phenol followed by one volume of phenol/chloroform/isoamyl alcohol (25:24:1) and finally with one volume of chloroform/isoamyl alcohol (24:1). In order to precipitate the DNA, 0.1 volume of sodium acetate and 2 volumes of ethanol were added to the final aqueous phase and the latter was centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was discarded and the precipitate was treated for 10 min at room temperature with 80 µl RNase (5 µg/ml). An aliquot (8-16 µl) of this solution was transferred to a 1.8% agarose gel containing ethidium bromide (0.5 µg/ml) and electrophoresis was carried out at 100 V for 90 min in TAE (0.8 mM Tris-Acetate, 0.02 mM EDTA) as running buffer. DNA in the gel was visualised under UV light.

Annexin V - 7-AAD. This test was performed using the Annexin V-FITC/ 7-AAD kit (Beckman Coulter, Fullerton, CA, USA). In brief, 1 ml of cell suspension containing 1x10⁶ HL-60 cells was treated with drugs for 24 h. The cells were obtained by centrifugation and washed 3 times with PBS. The cell pellets were resuspended in 100 µl of "Binding Buffer" and 10 µl of Annexin V-FITC and 20 µl of 7-AAD were added. The sample preparations were analysed on a Becton Dickinson FACScan flow cytometer, using Cell Quest software, treated with Fit mode on WinMDI 2.8. Ten thousand cells were counted.

Caspase-3-like protease activity. This assay is an adaptation of the method described by Nicholson et al. (14). 1x10⁷ cells in 10 ml of RPMI-1640 medium were treated with the test drug at the indicated concentration for 24 h at 37°C. The cells were pelleted by centrifugation, washed twice with PBS and then lysed in 200 µl of lysis buffer [HEPES (10 mM, pH 7.4), CHAPS (0.1% w/v), DTT (5 mM), EDTA (2 mM), PMSF (1 mM), aprotinin (10 µg/ml), pepstatin A (10 µg/ml), leupeptin (20 µg/ml)]. Ten µl of the lysate were combined with 10 µl of acetyl-Asp-Glu-Val-Asp-
pNitroaniline (2 mM) as substrate. The volume was brought to 100 μl with assay buffer [HEPES (50 mM, pH 7.4), NaCl (100 mM), CHAPS (0.1%, w:v), DTT (10 mM), EDTA (1 mM), glycerol (10%, v:v)]. The specificity of the assay was tested by the addition of 10 μl of acetyl-Asp-Glu-Val-Asp-aldehyde (1 μM). Absorbance was monitored at room temperature with a microplate reader over 120 min at 405 nm. The results were expressed as pmol of pNA/min.μg of protein. In parallel, the protein content of the cell lysate was determined using the 2-D quan Kit (Amersham Biosciences, Piscataway, NJ, USA).

Western blotting. 1x10^6 HL-60 cells were treated with 50, 70 or 90 μM of ent-trachyloban-3'-ol for 24 h. The cells were washed twice with PBS and lysed with 100 μl lysis buffer [HEPES (10 mM, pH 7.4), CHAPS (0.1% w:v), DTT (5 mM), EDTA (2 mM), PMSF (1 mM), aprotinin (10 μg/ml), pepstatin A (10 μg/ml), leupeptin (20 μg/ml)]. The cell lysates were centrifuged and equivalent amounts of protein (7.5 μg) were separated on 14% Tris-Glycine gel (Novex, Invitrogen, Carlsbad, CA, USA). After electrophoresis, the proteins were transferred to nitrocellulose (Hybond-C, Amersham Biosciences). The membranes were blocked with BSA (5%) and then immunoblotted with primary antibodies (mouse monoclonal...
antibody to human caspase-3 at a dilution of 1/1000 (Oncogene research products, La Jolla, CA, USA), mouse monoclonal antibodies to β-actin (Sigma-Aldrich). This was followed by the addition of a goat anti-mouse peroxidase-conjugated secondary IgG antibody (Transduction laboratories, San Diego, CA, USA) and the bands were visualised by electrochemiluminescence Western Blotting Detection Reagents (Pierce, Rockford, IL, USA).

Statistical analysis. The results are reported as means with the standard error (SE). Statistical analysis was performed using the Student’s t-test (statistical significance was set at *p*≤0.01). The Mann-Whitney U-test was used when heteroscedasticity was suspected.

Results

Cytotoxic effect of ent-trachylobane-3'-ol. The cytotoxicity of ent-trachylobane-3'-ol on Human Cervix Carcinoma cells (HeLa) has already been determined (IC_{50}=25.3 μM after 72-h treatment) (1). In order to compare the effect of this compound in cancer and non-cancer cell lines, cytotoxicity assays were made on HL-60 (IC_{50}=40 μM) and WI-38 (IC_{50}=54.2 μM) (Figure 2). Slight differences in sensitivity could be observed between each cell line, with the cancer cell lines (mainly HeLa cells) being slightly more sensitive to ent-trachylobane-3'-ol than the non-cancer cell line (WI-38). A clear dose-dependent effect was observed and only slight differences were observed between HL-60 cells treated for 24 h (IC_{50}=53 μM) and 72 h (IC_{50}=40 μM), suggesting that the cytotoxic effect of this compound occurred mainly during the first 24 h of treatment. Thus we decided to detect apoptosis only after 24-h treatment. In each case, camptothecin was used as the positive control (IC_{50} on HL-60=1.2 μM after 24 h, 0.05 μM after 72 h) (15).

Effect of ent-trachylobane-3'-ol on cell morphology. In order to determine the type of cell death involved by treatment with ent-trachylobane-3'-ol, the morphology of HL-60 cells was examined. After treatment for 24 h with 50, 70 and 90 μM of ent-trachylobane-3'-ol, we could observe, by light microscopy, all the features of apoptotic cell death, namely cell shrinkage, vacuoles, plasma and nuclear membrane blebbing, chromatin condensation and apoptotic bodies (16).

Effect of ent-trachylobane-3'-ol on DNA fragmentation. The degradation of nuclear DNA into nucleosomal units is one of the main biochemical features of apoptotic cell death (17, 18). This DNA fragmentation is characterised by the formation of a ladder after gel electrophoresis analysis. Our results (Figure 3) showed an increase of the DNA fragmentation in HL-60 cells after treatment with 50-90 μM ent-trachylobane-3'-ol for 24 h.

Effect of ent-trachylobane-3'-ol on translocation of phosphatidylserine. An early marker of apoptosis is the externalisation of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane (19). This feature can be detected by annexin V staining. Annexin V is a Ca^{2+}-dependent protein with a high affinity for PS (20). As shown in Figure 4, ent-trachylobane-3'-ol treatment led to the exposure of PS on the outside of the plasma membrane. As compared to the untreated cells, the cells treated with ent-trachylobane-3'-ol 70 μM and 90 μM showed a high proportion of annexin V-positive cells. For cells treated with 90 μM, 7-AAD staining showed that a small proportion (4.7±0.9%) of the cells was in the early stage of apoptosis, the majority being in the late stage of apoptosis or in necrosis (36.2±2.4%). The same phenomenon was also observed with the positive control (camptothecin at 1 μM).

Effect of ent-trachylobane-3'-ol on caspase-3 activation. Studies with anticancer drugs have shown that programmed cell death is associated with the activation of a number of aspartate-specific cysteine proteases (caspases) (21, 22). In particular, caspase-3 (CPP32) is considered essential for the propagation of the apoptotic signal by several types of
antitumour drugs (23). For example, caspase-3, which cleaves DEVD-type substrates, is involved in camptothecin-induced apoptosis in HL-60 cells (24). It was therefore of interest to determine whether this cysteine protease is also involved in apoptosis induction by ent-trachyloban-3β-ol. To determine the activation of this protease, we used a colorimetric assay based on the cleavage of DEVD-pNA specifically by activated caspase-3. The results clearly showed that caspase-3 was already activated after 24 h of treatment by 70 μM of ent-trachyloban-3β-ol (Figure 5). The activation of caspase-3 was concentration-dependent. The results on caspase-3 activation were confirmed by immunoblotting with mouse monoclonal antibodies to human caspase-3 of the protein content of treated cells. As a band corresponding to activated caspase-3 appeared in cells treated with 50 μM of ent-trachyloban-3β-ol, it seems that Western blot is a more sensitive technique for determining caspase-3 activation than the enzymatic colorimetric assay.

**Discussion**

Many natural diterpenes have been shown to possess interesting antitumour properties, among them the well known macrocyclic diterpene paclitaxel. Diterpenes, as well as phorbol esters from the genus Croton are well known for their toxicities, as the tumor promoters phorbol esters present in several species of Croton. Other diterpenes have also shown promising activity on several tumour cell lines. Salvicine, a diterpene quinone derived from the Chinese herb Salvia prionitis, is a potent apoptosis inducer (25). Another example is triptolide, a triepoxide diterpene isolated from another Chinese herb (Tripterygium wilfordii) used in Chinese folk medicine to treat auto-immune disease. This compound has cytotoxic and immunosuppressive properties and induces apoptosis (26-28). Trachylobanes have a very uncommon chemical structure and their cytotoxicity or anticancer properties have been little studied. The only report on cytotoxic activity concerns ent-trachyloban-3β-ol (1).

In this study, we have shown that ent-trachyloban-3β-ol was able to induce apoptosis in human promyelocytic leukaemia cells in a dose-dependent manner. The initial signs of apoptosis induction in HL-60 cells could be observed at 50 μM after 24-h treatment and were associated with caspase-3 activation. Even if the active concentrations are relatively high, it would be interesting to test derivatives which may be more effective. A study of the pathway involved in the apoptotic process would also be worthwhile.

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