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Inhibition of Protein Tyrosine Kinase Activity and Induction of Apoptosis in Epithelial Cells by Oxindole Alkaloids of *Uncaria tomentosa* (Willd.) D. C

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Root bark of *Uncaria tomentosa* (Willd.) D. C. is used for a variety of therapeutic purposes, including reported efficacy as both an immunostimulant and anticancer therapy. The oxindole alkaloids (OAs) of *U. tomentosa* were investigated for cytotoxicity toward epithelial cells, specifically focusing on lines derived from breast (MB-MDA-231, MB-MDA-468, BT-20 and SKBR-3) and skin (A431) carcinomas, as well as a non-cancer line (Cos). Alkaloid extracts were shown to be minimally cytotoxic at concentrations as low as 27μM, with an IC_{50} in the range of 0.9 to 1.7 mM, depending on the cell line tested. Microscopic evaluation of cells treated with marginally cytotoxic levels of OAs, and stained with fluorescent indicators, propidium iodide and Hoechst 33342, specifically demonstrate the induction of apoptosis, rather than necrosis. Evaluation of protein tyrosine kinase (PTK) activity in treated cells further demonstrated that minimally cytotoxic concentrations of the OAs inhibited tyrosine phosphorylation of endogenous proteins in MB-MDA-231 cells, when compared to controls. Given the recognized role of protein tyrosine kinases in the coordination of cell proliferation, in this research, propose that inhibition of PTK activity by the OAs contributes to the apoptotic cell-death, and perhaps other biological activities associated with these compounds.

Key words: *Uncaria tomentosa*, oxindole alkaloid, protein tyrosine kinase, cytotoxic

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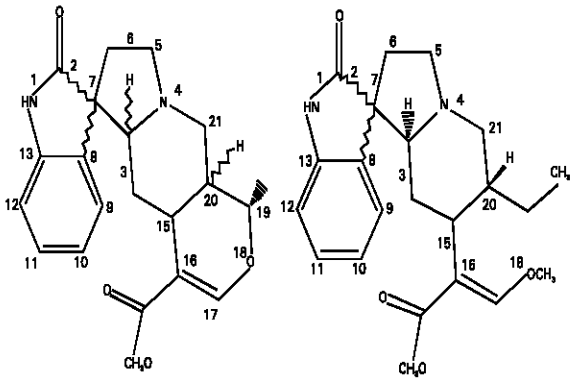
Introduction

The South American liana, *Uncaria tomentosa* (Willd.) D. C. (Rubiaceae), represents an important component of the traditional pharmacopoeia of the Peruvian Amazon (Kepplinger *et al.*, 1999; Reinhard, 1999). Additionally, herbal preparations from root bark of this plant are marketed extensively, under the name "Uña de Gato," throughout Latin America for treatment of a variety of ailments. More recently, Uña de Gato, or "Cat's Claw," has been exported worldwide as a part of the emerging herbal market. Prescribed uses include treatment of arthritis, gastritis and ulcers, as well as other applications associated with a reported immunostimulatory activity (Wagner *et al.*, 1985), and purported efficacy against cancer.

An isomeric series (Fig. 1) of six pentacyclic, as well as two tetracyclic, oxindole alkaloids (OAs) have been isolated by Philipson and Hemingway (1975) Montenegro *et al.* (1976) from *U. tomentosa*, and largely linked to the efficacy of the herbal preparation (Wagner *et al.*, 1985; Stuppner *et al.*, 1992). Specifically, it has been suggested (Kepplinger *et al.*, 1999; Reinhard, 1999) that mixtures of the pentacyclic oxindole congeners are most effective, though studies on the tetracyclic OAs from *U. rhyncophylla*, an Asian relative, have demonstrated various pharmacological activities as well. Biological activity, including antiviral and anti-inflammatory activity (Aquino *et al.*, 1989, 1991), has also been demonstrated for unrelated quinovic acid glycosides found in *U. tomentosa*, and these constituents likely play a role in the chemotherapeutic potency of this herbal preparation.



	C3	C7	C20	C7	
Pteropodine	S	R	S	Rhynchophylline	R
Isopteropodine	S	S	S	Isorhynchophylline	S
Mitraphylline	S	R	R		
Isomitraphylline	*S*	*S*	*R*		
Uncarine F	R	R	S		
Speciophylline	R	S	S		

Fig.1: Structure of oxindole alkaloids isolated from *Uncaria tomentosa* (Willd.) D. C

Much of the investigation of bioactivity has focused on the purported immunostimulant activity of the OAs from this herbal preparation. Wagner *et al.* (1999), for instance, isolated pteropodine, isopteropodine, mitraphylline, isomitraphylline, rhynchophylline and isorhynchophilline from collections of *U. tomentosa*, and showed that four of these six alkaloids (with the specific exception of mitraphylline and rhynchophylline) stimulated phagocytosis by macro phages *in vivo* and *in vitro*. More recently, it has been found (Wurm *et al.*, 1998) that an isomeric mixture of the OAs of *U. tomentosa* stimulated the proliferation of lymphocytes by an unidentified cue secreted from endothelial cells. More recently, Lemaire *et al.* (1999) found that OAs induce production of interleukin-1 and -6 by macro phages. Other studies have demonstrated that pentacyclic OAs of *U. tomentosa* are cytotoxic, specifically inhibiting the proliferation of leukemic cells *in vitro* (Stuppner *et al.*, 1993). To further elucidate the biological activity of the OAs from *U. tomentosa* investigated the cytotoxicity against several epithelial cell-lines.

Though relatively little data exists for the biological activity of the OAs of *U. tomentosa*, a fair amount of research has elucidated biological activities of synthetic, oxindole-based compounds. In particular, a growing number of independent reports (Shirashi *et al.*, 1987; Graziani *et al.*, 1993; Buzzetti *et al.*, 1993; Mohammadi *et al.* 1997) have documented inhibition of the protein tyrosine kinase (PTK) activity of oncoproteins by synthetic, indolinone analogs. Mohammadi *et al.* (1997) demonstrated that the oxindole ring-system of these compounds "mimics" the adenosine moiety of ATP required for phosphorylation, competing for the ATP binding site of the kinase domain. Based on this, and recognizing the role of PTK activity in cell proliferation, explored the hypothesis that OAs from *U. tomentosa* may likewise inhibit PTK activity.

Materials and Methods

Plant material and alkaloid isolation: Alkaloids were isolated from finely ground bark of the root of *U. tomentosa* (Willd.) D.C., provided by Oscar Schuler of Tracker, S. A. (Lima, Peru) and Norma Pestano of Nutrivida™ as samples from a commercial batch (Batch #NV0001) of the gelatin-encapsulated, herbal preparation. Herbal preparations contains only ground bark of *U. tomentosa* harvested regularly from cultivated plots near Pucallpa, Peru; "voucher samples" of the batch are deposited at Cornell University, and are available upon request. Typically, 20-50 g of dried material removed from gelatin capsules was prepared for extraction.

Alkaloids of *U. tomentosa* were extracted by the method of Wagner and Bladt (1996). Powdered plant material was ground with NH_4OH (1 ml g^{-1} of dried material) for 2 minutes. The ground material was then mixed with basic aluminum-oxide (approximately 7 g/2 g of dried plant material), which was subsequently packed into a glass column (2 cm diameter) from which a crude extract, enriched for alkaloids, was eluted with chloroform. The OAs were subsequently isolated to apparent purity (as determined by GC/MS analysis) by partitioning the chloroform extract with 1.2 M HCl in 50% MeOH to remove the alkaloids as their hydrochlorides, and subsequently re-extracting the "free bases" following basification 2 M NaOH. Aliquots of extracted OAs were dried under N_2, and redissolved to an appropriate concentration (100 mg ml^{-1}) in dimethylsulfoxide (DMSO), or in MeOH for cytotoxicity assays and analytical chromatography, respectively.

Analytical chromatography of oxindole alkaloids: Oxindole alkaloids composition of extracts were analyzed by high-performance liquid chromatography (HPLC) using a method modified from that of Stuppner *et al.* (1992). For HPLC analysis, alkaloid extracts were dried under N_2, re-dissolved in MeOH and syringe-filtered. Alkaloids were separated by reverse-phase HPLC (Waters™ 600 Pump/Controller and Millenium® Chromatography Manager software) on a Waters™ C-18 column (3.9 x 150 mm) with a decreasing gradient (over 30 min) of 60-30% phosphate buffer (10 mM, pH 6.6) in MeOH/acetonitrile (1:1), as per Stuppner *et al.* (1992). Alkaloids were detected by UV_{245} absorbance (Waters™ 996 Photodiode Array Detector). Oxindole alkaloids were evaluated additionally by TLC on silica gel, EtOAc/isopropanol/NH_4OH (100:2:1) with detection by "short wave" UV absorbance (254 nm), and dragendorff reagent, as described by Wagner and Bladt (1996). Purity of the extracted alkaloids was additionally determined by Gas Chromatography/Mass Spectrometry (GC/MS) using a Hewlett Packard® HP 6890 Series GC and Mass Selective Detector.

Determination of cytotoxicity: Epithelial lines, including those from human breast cancers (MB-MDA-468, MB-MDA-231, BT-20 and SKBR-3), human skin cancer (A431) and Green Monkey kidney (Cos) cells, were cultured as per standard protocols in Dulbecco's Modified Eagle's Medium (Sigma® Chemical Co., St. Louis, MO),

supplemented with 10% heat-inactivated Fetal Bovine Serum and antibiotics (Antibiotic Antimycotic Solution, Sigma® Chemical Co., St. Louis, MO) at 37 °C with 5% CO_2. Cells were plated and grown to 80 % confluency (1.5 x 10^5 cells/ml) in either 60 mm dishes or 96-well plates for assays.

To determine IC_{50} values of OAs, dilution series (1 to 0.001 mg ml^{-1}) of the extracted OAs in DMSO (along with negative controls of DMSO-only) were added to confluent cultures in 60 mm Petri dishes. Percent inhibition (after 48 hours), relative to an untreated control, was measured colorimetrically based on the relative reduction of the oxidation-reduction indicator, Alamar Blue™ (Trek™ Diagnostic Systems Inc. Westlake, OH), added to the culture medium (at approximately 10% v/v), as per the method recommended by Trek™ Diagnostic Systems Inc. IC_{50} values were calculated based on regression analysis of concentration and percent inhibition, and by determining the concentration estimated accordingly for 50% inhibition. Minimum inhibitory concentrations (MICs) were additionally determined for a "microdilution series" of the tested against the same cell lines cultured (as above) in 96-well titer plates, and likewise using the color change of Alamar Blue™ (10% v/v) to determine "end points" of growth inhibition qualitatively. Cytotoxicity was confirmed additionally by microscopic evaluation of cells in culture, compared to untreated controls, by fixing cells in 4% paraformaldehyde (pH 7.4), and staining with Crystal Violet (Sigma™ Chemical Co., St. Louis, MO).

Fluorescence microscopy for apoptosis and necrosis: Cytological detection of apoptosis and necrosis was accomplished by fluorescence microscopy of treated and untreated MB-MDA-231 cells, "double-stained" (Mitovic *et al.*, 1998) with propidium iodide and Hoechst 33342 (Molecular Probes., Inc., Eugene, OR). Specifically, cells were seeded onto flame-sterilized, glass coverslips, and cultured in 6-well plates, as described previously, for 24 h. Cells were then treated with 0.1 mg ml^{-1} of OAs extract for 72 h. Aliquots of medium, and coverslips with attached cells, were stained with 50 μl of propidium iodide (20 μg ml^{-1}) and Hoechst 33342 (140 μg ml^{-1}) in PBS (pH 7.40) at 37 °C for 15 minutes. Coverslips were mounted on flame-sterilized, glass slides with 1 drop of Aqueous Mounting Medium with Anti-Fading Agents (Biomeda Corp., Foster City, CA), and stained cells were evaluated by fluorescence microscopy (Carl Zeiss, Inc., Thornwood, NY).

Protein tyrosine kinase (PTK) assays: Detection of tyrosine-phosphorylated proteins in cell lysates was used to investigate inhibition of protein tyrosine kinase (PTK) activity. MB-MDA-231 cells cultured (as above) to approximately 80% confluency in 60 mm dishes, were treated with minimally inhibitory concentrations (0.1 mg ml^{-1}) of the extracted alkaloids (along with solvent controls of DMSO alone, and negative controls of untreated cells). After 24 h, cells were washed twice with ice-cold TRIS-buffered saline plus 0.6 mM sodium orthovanadate, and lysed with ice-cold lysis-buffer (1 mM orthovanadate, 23 μM leupeptin and 5-10 x 10^{-3} TIU ml^{-1} aprotinin in Triton X-100). Protein concentrations for lysates were determined spectrophotometrically by the Bio-Rad method (Bio-Rad Laboratories®, Inc., Hercules, CA), and equivalent amounts of protein lysate separated by SDS-polyacrylamide gel-electrophoresis (10% polyacrylamide). The presence of tyrosine-phosphorylated proteins was detected by "Western-blotting" with PY99 anti-phosphotyrosine antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), followed by detection with horseradish peroxidase-labeled, polyclonal, anti-mouse secondary-antibody (ECL™, Amersham Pharamacia Biotech, Inc., Piscataway, NJ).

Results

Extraction of alkaloids over basic alumina, followed by "acid-base extraction," yielded an apparently pure, enantiomeric mixture of pentacyclic OAs (approximately 0.3% w/w of the dry weight), as

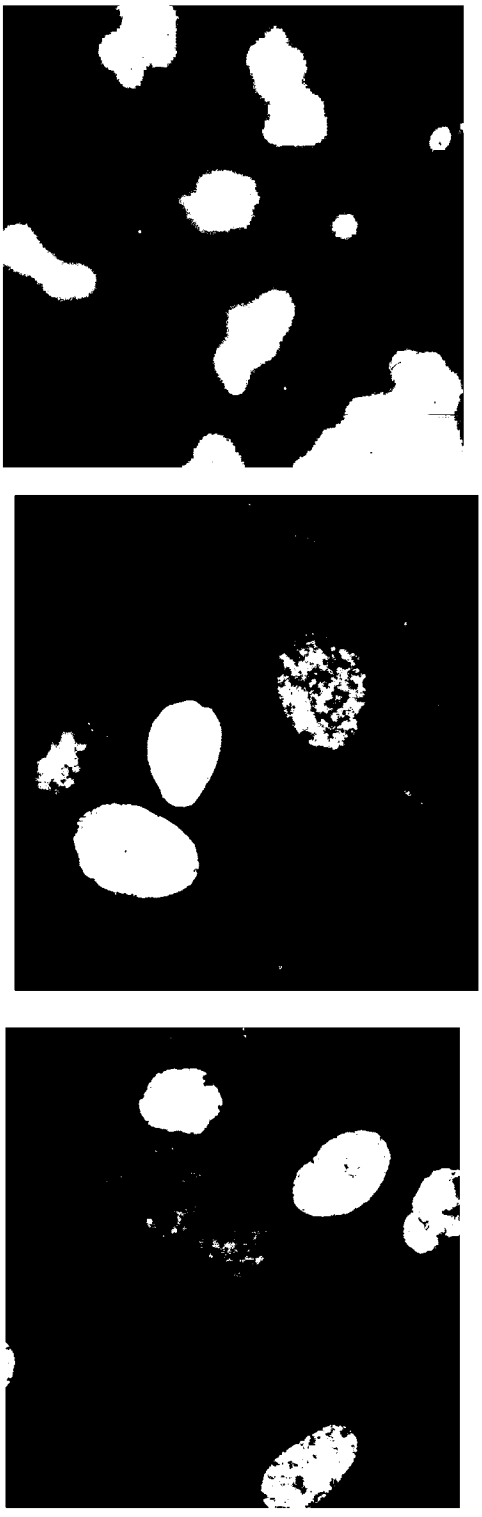


Fig. 2: Fluorescence micrograph of MB-MDA-231 cells (1000x total magnification) "double-stained" with propidium iodide and Hoechst 33342, showing apoptotic cells in cultures treated with OAs (C), but not in untreated (A) or solvent-treated (B) controls.

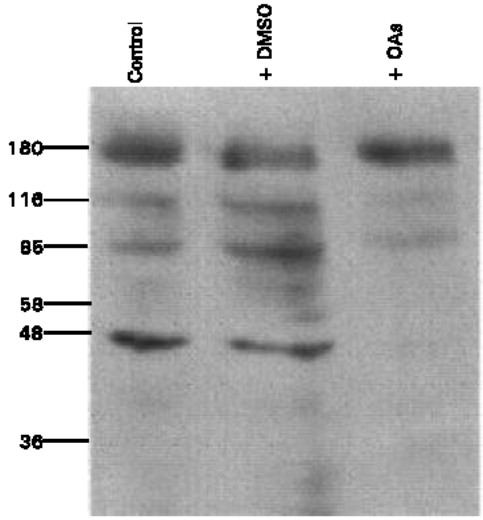


Fig. 3: Western blot" of lysates from untreated (Control), solvent-treated (+DMSO) and OA-treated (+OA) cultures of MB-MDA-231 cells, showing inhibition of the protein tyrosine kinase (PTK) activity by OAs
DMSO = Dimethyl sulph oxide, OAs = oxindole alkaloids

determined by GC/MS, which resolved into 2-component peaks with mass spectra characteristic of the pentacyclic alkaloids. Specifically, the major OAs isolated were pteropodine, isopteropodine, mitraphylline and isomitraphylline, with approximately 55 and 45% of the isomeric pairs of these C-20 enantiomers, as is typical for *U. tomentosa* (Stuppner *et al*., 1992). In this study the individual enantiomers were not purified.

Cytotoxicity was observed for all cell-lines tested. Calculated IC_{50} values varied over a range of 900 μM to 1.7 mM for different cell lines (Table 1). Minimum inhibitory concentrations (MICs) were as low as 27 μM (0.01 mg mL^{-1}) for breast cancer lines, BT-20 and SKBR-3, and as high as 2.7 mM (1 mg mL^{-1}) for breast and skin cancer lines, MB-MDA-468 and A431, respectively.

Cytotoxicity was further investigated by "double-staining" with propidium iodide and Hoechst 33342 to differentiate apopototic versus necrotic cell-death, specifically, for alkaloid-treated MB-MDA-231 cells. Cytotoxicity of the OAs was found to be exerted by induction of apoptosis in these cells, with almost all of the alkaloid-treated cells showing the indications of condensed, fragmented chromatin, loss of nuclear envelope and membrane "blebbing" that is characteristic of apoptotic cells (Fig. 2). Both untreated and DMSO-treated cells were normal in appearance. No necrotic cells (which appear swollen, because of damage to the cell membrane, and which fluoresce red to pink with this staining procedure) were observed for alkaloid-treated, DMSO-treated or control cells.

Treatment of MB-MDA-231 cells at minimally inhibitory concentrations (0.27 mM in DMSO) of extracted OAs inhibited the phosphorylation of several, endogenous proteins, as detected by immunoblotting with a phosphotyrosine-specific antibody, when compared to both untreated and DMSO-treated cells (Fig. 3). However, phosphorylation of all protein was not apparently inhibited, as evidenced by identical bands for tyrosine-phosphorylated proteins in both treated and untreated cells (Fig. 3)

Discussion

Protein phosphorylation is a fundamental mechanism of the signaling pathway which, coordinates cell proliferation and differentiation. Alterations in protein kinase activity have been linked to various forms of cancer (Bishop, 1987; Aaronson, 1991). Phosphorylation of key enzymes, including the p53 and *myc* gene alkaloids of *U. tomentosa* reported here is the first known example of PTK inhibition by plant-derived OAs. Though PTK inhibition was not observed for all endogenous protein substrates in immunoblots (Fig. 3), investigations of synthetic oxindoles (Mohammadi *et al*., 1997) have, likewise, found that inhibition of PTK activity by synthetic, oxindole compounds also varies with protein substrate, as well as cell-line. Preliminary experiments with various other cell-lines treated with oxindole alkaloids (data not shown) likewise suggests that cytotoxicity and PTK inhibition by these compounds may also vary with cell-line, presumably reflective of both kinases and protein substrates in these cells. The degree to which structural differences in the OAs determines relative inhibition of PTK and cytotoxicity remains to be determined, however, as individual OAs are known to spontaneously isomerize in solution (Laus *et al*., 1996), and were therefore not purified for these experiments.

PTKs have a widely recognized role in the regulation of numerous, cellular processes particularly including coordination of cell division and differentiation, as well as programmed cell death. Given this, it is suggested that the observed PTK inhibition by OAs is responsible for the induction of apoptosis observed for OAs here. Additionally, tyrosine phosphorylation is well documented to be involved in cell signaling pathways related to cytokine release and related responses associated with lymphocyte proliferation and differentiation (Baird *et al*., 1999). It is, therefore, proposed that PTK inhibition may also be involved in other reported biological activities, including immunostimulatory activities, and particularly the observed effects on lymphocyte development (Stuppner *et al*., 1993; Wurm *et al*., 1998; Lemaire *et al*., 1999).

Further investigation of structure-function relationship will continue to clarify the potential of these widely used phytotherapeutics.

Table 1: Cytotoxicity of oxindole alkaloids from *U. tomentosa* root bark against breast cancer skin cancer and Green-monkey kidney (Cos) cells

Cell type	Breast cancer				Skin Cancer	Green monkey kidney
Cell Line:	BT-20	MB-MDA-468	MB-MDA-231	SKBR-3	A431	Cos
IC_{50} (mM)	0.9 (±1.0)	1.5 (±0.7)	1.2(±2.9)	1.2(±0.4)	1.7(±1.7)	1.1(±0.9)
MIC(mM)	0.03	2.70	0.27	0.03	2.70	0.27

Confidence interval (95%) given in parentheses

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