

Cytotoxic Effect of the Pentacyclic Oxindole Alkaloid Mitraphylline Isolated from *Uncaria tomentosa* Bark on Human Ewing's Sarcoma and Breast Cancer Cell Lines

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Key words

- *Uncaria tomentosa*
- Rubiaceae
- cat's claw
- oxindole alkaloid
- mitraphylline
- cytotoxic activity

Abstract

Preparations from *Uncaria tomentosa*, a South American Rubiaceae, have been used in the Peruvian traditional medicine for the treatment of infective, inflammatory and tumoral processes. In this study, the pentacyclic oxindole alkaloid mitraphylline was isolated from the dried inner bark of this plant species, and its structure elucidated by analysis of NMR spectroscopic data. Mitraphylline was differentially identified from its stereoisomeric pair isomitraphylline by ¹⁵N-NMR. Its antiproliferative and cytotoxic effects have been tested on human Ewing's sarcoma MHH-ES-1 and breast cancer MT-3 cell lines, using cyclophosphamide and vincristine as reference controls. A Coulter counter was used to determine viable cell numbers, followed by the application of the tetrazolium compound MTS [3-(4,5-dime-

thylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium] an inner salt. A colorimetric method was employed to evaluate cell viability in this cytotoxic assay. Micromolar concentrations of mitraphylline (5 μM to 40 μM) inhibited the growth of both cell lines in a dose-dependent manner. The IC₅₀ ± SE values were 17.15 ± 0.82 μM for MHH-ES-1 and 11.80 ± 1.03 μM for MT-3 for 30 hours, smaller than those obtained for the reference compounds. This action suggests that the pentacyclic oxindole alkaloid mitraphylline might be a new promising agent in the treatment of both human sarcoma and breast cancer.

Supporting information available online at <http://www.thieme-connect.de/ejournals/toc/plantamedica>

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Introduction

Uncaria tomentosa (Willdenow ex Roemer & Schultes) DC. (Rubiaceae) is a Peruvian thorny liana, which is commonly known as “cat's claw” or “uña de gato”. Peruvians have used it as a bark infusion for the treatment of a wide range of health problems such as cancer, gastric ulcers, viral infections, recovery from child birth, arthritis and other inflammatory processes [1,2].

Many different chemical constituents have been isolated and identified in the extracts of cat's claw, including quinovic acid glycosides, sterols, tannins, procyanidins, flavonoids, ursane polyhydroxylated triterpenes and at least 17 oxindole alkaloids [3–9]. These compounds, acting in synergy, may be responsible for many pharmacological activities described for this plant [10]. The most prominent compounds are alkaloids. The chemical structure identification of these compounds from the *Uncaria* genus has been an ob-

ject of study for decades [11–13]. It was found that two botanic chemotypes of *Uncaria tomentosa* with different alkaloid patterns occur in nature. Most of the pharmacological activity has been attributed to the one which contains more pentacyclic (rather than tetracyclic) oxindole alkaloids [14,15].

Numerous investigations concerning the anti-inflammatory properties of several extracts containing different quantities of pentacyclic oxindole alkaloids have been reported [16–18]. Moreover, it has been shown that cat's claw possesses immunostimulant properties as it induced human endothelial cells to release a lymphocyte-proliferation regulating factor [19] and prolonged lymphocyte survival [20].

Furthermore, one of the most interesting properties of cat's claw extract is inhibiting cell proliferation. Thus, a commercially available water extract of the plant has demonstrated an antiproliferative effect in human leukemia and lymphoma

cell lines [21]. In order to investigate the bioactive constituents responsible for its antiproliferative activity, Riva et al. [22] tested some chromatographic fractions of cat's claw extract on the MCF7 breast cancer cell line. More recently, Pilarski et al. [23] investigated the antiproliferative potency of several preparations with different quantitative and qualitative alkaloid contents on HL-60 acute promyelocytic human cells. In previous investigations, our group demonstrated a potent antiproliferative effect of the oxindole alkaloid mitraphylline on human glioma (GAMG) and neuroblastoma (SKN-BE) cell lines [24]. In the present study, we report the isolation and identification of this pentacyclic oxindole alkaloid from the inner bark extract of *Uncaria tomentosa* and further investigate the cytotoxic capacity of mitraphylline upon two other human tumoral cell lines: Ewing's sarcoma and breast cancer.

Materials and Methods



Bark extract

The bark material from the Peruvian forest was supplied by the practitioner Dr. Carlos S. González and was identified in the Botanic Department of the San Lorenzo Chemical Sciences School from the Asuncion University in Paraguay. The bark extract was produced as follows: 500 g of *Uncaria tomentosa* dried inner bark were treated with ammonium hydroxide and dichloromethane. After filtration, the obtained solution was concentrated *in vacuo* to afford a residue, which was dissolved in a hydrochloric acid solution (3%). Ammonium hydroxide and dichloromethane were added again. After concentration *in vacuo*, the purified alkaloid fraction was obtained as a brown residue and the yield was 0.15%.

Gas-chromatography/mass-spectrometry (GC/MS) analysis of the alkaloid fraction

Gas-chromatography/mass-spectrometry analysis were realized, using a CARLO ERBA/KRATOS MS 80 RFA apparatus. Helium (99.99%) was the carrier gas (1 mL/min). One μL of the sample dissolved into dichloromethane was injected into the gas chromatograph. The injector and detector temperatures for the gas chromatograph were 275 °C and 325 °C, respectively. The column oven temperature was increased linearly from 230 °C to 300 °C (4 °C/min). The ionization mode was electron impact (EI). NBSL 1 B2 library was used to recognize all derivatives found in the fraction.

Isolation and identification

The dried residue of the alkaloid fraction (0.5 g) was subjected to a silica gel column chromatography, compacted with silicagel 0.063–0.2 mm (0.8 × 25 cm), followed by a gradient elution with various mixtures of *n*-hexane, CH_2Cl_2 and MeOH. Twenty-five fractions of about 15 mL each were collected. In the fractions 14 to 16, eluted with $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (9.5 : 0.5), an isolated compound was obtained by the method of TLC (SiO_2 , $\text{CH}_2\text{Cl}_2/\text{MeCO}$ (5 : 4) Rf: 0.83; ethyl ether/EtOAc (1 : 1) Rf: 0.73; $\text{CH}_2\text{Cl}_2/\text{EtOH}$ (95 : 5) Rf: 0.68) and visualized as a brown and orange spot with the reagents sulphuric acid/ $\text{CH}_3\text{COOH}/\text{H}_2\text{O}$ (1 : 20 : 4). This was followed by heating at 120 °C and the use of Dragendorff's reagent. ^1H and ^{13}C NMR experiments were performed for the compound identification using an AVANCE 500 spectrophotometer. The following 2D NMR experiments COSY-DQF, (^1H - ^{13}C)-HSQC, (^1H - ^{13}C)-HMBC and NOESY correlation were used to elucidate its struc-

ture. Furthermore, the 15 N chemical shifts of the isomeric oxindole alkaloids, using (^1H - ^{15}N)-HMBC experiments, were utilized to facilitate their characterization. The solvent used for NMR spectra was CDCl_3 .

Cell cultures

We used the human cell lines Ewing's sarcoma MHH-ES-1 and breast cancer MT-3 from DMSZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen. Cell lines were seeded in 75 cm^2 tissue culture flasks (Falcon). These cell lines were maintained in RPMI 1640 (Invitrogen-Gibco) supplemented with 10% heat-inactivated fetal bovine serum according to the culture conditions suggested by the manufacturer. The cell lines were subcultured in 75 cm^2 tissue culture flasks. The medium was renewed every two days and the cell cultures were incubated at 37 °C in a humidified atmosphere at 95% air and 5% CO_2 .

Drug treatments

To determine the 50 inhibition concentration (IC_{50}), different concentrations (5, 10, 20, 30 and 40 μM) of mitraphylline dissolved in ethanol were evaluated in the MHH-ES-1 and MT-3 cell lines. Cyclophosphamide (Merck Generics) and vincristine (Ciclum Farma) were used as reference controls at the same doses.

Proliferation assay

Cell proliferation was evaluated using the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), according to the manufacturer's instructions (CellTiter 96 Aqueous One-Solution Cell Proliferation Assay; Promega Corp.) and as described by Muñoz et al. [25]. Both cell lines were cultured for 4–5 days to let them grow as monolayer for MHH-ES-1 and in colonies (not necessarily as a monolayer) for MT-3. Cells were harvested by trypsinization and Trypan blue exclusion to determine cell viability. They were quantified using a Coulter counter. The experiments were made in 96-well plates, each well containing 10^4 cells in a total volume of 100 μL . The plates were inoculated with drugs and incubated for a period (first doubling time) of 30 h (MT-3) or 28 h (MHH-ES-1), respectively. After that, 20 μL of MTS reagent was added to each well. 90 min later, the samples were read at 492 nm in a multiscanner microplate reader (TECAN Spectra classic). The quantity of product, as measured by optical density, was directly proportional to the number of living cells. Each experimental condition was assayed in duplicate and all experiments were performed at least three times. The IC_{50} was calculated with a curve fitting parameter.

Statistical analysis

Results are expressed as mean \pm SE. All measurements were evaluated statistically using Student's t-test, taking $p < 0.05$, $p < 0.01$ and $p < 0.001$ as the levels of significance.

Supporting information

Gas-chromatogram of the alkaloid fraction and a table with comparison data of the different signs for mitraphylline and isomitraphylline obtained from the literature are provided as Supporting Information.

Position	δ_{1H} (ppm)	Multiplicity	J (Hz)	δ_{13C} (ppm)	δ_{15N} (ppm)
1	7.70	s	–	–	132.6
2	–	–	–	180.9	–
3	2.37	m	–	74.6	–
4	–	–	–	–	63.6
5	3.36; 2.47	m; m	–	54.3	–
6	2.47; 2.02	m; m	–	35.2	–
7	–	–	–	55.6	–
8	–	–	–	133.4	–
9	7.17	d	7.8	123.0	–
10	7.01	td	7.7; 1.1	122.6	–
11	7.16	td	7.6; 1.2	128.0	–
12	6.83	d	7.6	109.5	–
13	–	–	–	140.7	–
14	2.37; 1.17	m; dd	–; 11.8, 10.7	28.4	–
15	2.09	m	–	30.5	–
16	–	–	–	107.0	–
17	7.40	d	1.35	154.1	–
19	4.35	qd	6.6; 3.1	73.9	–
20	2.08	m	–	40.5	–
21	3.19; 1.82	dd; t	10.4, 2.5; 10.4	54.3	–
22	–	–	–	167.1	–
23	3.57	s	–	50.7	–
24	1.09	d	6.6	14.9	–

Table 1 Signs, multiplicity and coupling constants (J) from the NMR spectra of the isolated compound (δ values, in $CDCl_3$).

s: singlet, d: doublet, t: triplet, dd: double doublet, td: triple doublet, cd: quadruple doublet, m: multiple

Results and Discussion

One of the most recognized alkaloids in *Uncaria* is the oxindole alkaloid mitraphylline which has been identified in 20 of the 34 species [2]. Alkaloids have been mainly separated and identified by HPLC [13], but we used GC/MS to recognize the alkaloid profile of a purified alkaloid fraction as previously described by Phillipson and Hemingway [11]. In the gas-chromatogram (Fig. 1S, Supporting Information) a main peak could be observed, at a retention time of 24.22 min. This compound represented 87.3% of the total alkaloid fraction. Its mass spectrum EI-MS data were: m/z 368 (M^+ , 63), 223 (100), 208 (35), 180 (2), 146 (6), 145 (6), 144 (7), 130 (13), and 69 (30). Its molecular formula was determined to be $C_{21}H_{24}N_2O_4$.

Approximately 200 mg of white crystal were obtained from 500 mg of the dried residue of the alkaloid fraction by silica gel column chromatography. Data from the NRM experiments performed with the isolated compound are listed in Table 1. These signals were in agreement with those reported in the literature [6]. Data from the (1H - ^{15}N)-HMBC spectrum allowed us to confirm the differential structural characteristics of mitraphylline compared to its stereoisomeric pair isomitraphylline (Table 1S, Supporting Information). (Copies of the original spectra are obtainable from the author of correspondence.) These have already been used by other authors to distinguish between stereoisomeric pairs of oxindole alkaloids [6, 26, 27]. The structure of the alkaloid is illustrated in Fig. 1. These chemical pentacyclic types of alkaloids are considered to be biochemical marker of cat's claw and are responsible, in part, for its immunomodulatory and cytostatic activities.

From our experiments, growth inhibition of the MHH-ES-1 sarcoma and MT-3 breast cancer cell lines was observed after the addition of increasing concentrations of mitraphylline at the first doubling time of incubation. Moreover, the treatment of both cell lines with the alkaloid resulted in a concentration-dependent cytotoxicity. The calculated mitraphylline concentration required

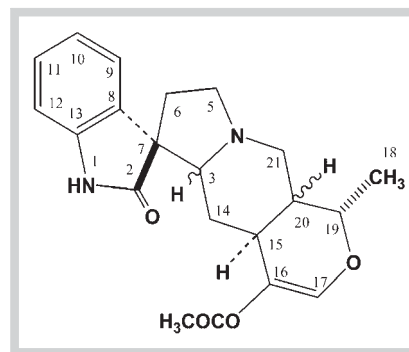


Fig. 1 Chemical structures of the oxindole alkaloid mitraphylline.

for a 50% reduction in MHH-ES-1 sarcoma proliferation (IC_{50}) was $17.15 \pm 0.82 \mu M$ for 30 hours. This dose was smaller than those calculated for cyclophosphamide and vincristine: $67.60 \pm 2.30 \mu M$ and $39.16 \pm 1.21 \mu M$, respectively. For MT-3 human breast cancer cells, the alkaloid IC_{50} was $11.80 \pm 1.03 \mu M$ for 30 hours. Also in this case, the dose is smaller than those for cyclophosphamide and vincristine, $38.01 \pm 2.21 \mu M$ and $44.66 \pm 2.72 \mu M$, respectively. Maximum inhibition was observed when mitraphylline was present at a concentration of $40 \mu M$ during the culture period ($p < 0.001$ against the control untreated cells) for both tumoral cell lines. At the first doubling time, a strong decrease in the cell number of the two lines studied was found at intermediate concentrations ($p < 0.05$ at $10 \mu M$; $p < 0.01$ at $20 \mu M$ and $p < 0.001$ at $30 \mu M$) and almost no remaining living cells were observed at the maximum concentrations.

Other authors have demonstrated a cytotoxic potency of different pentacyclic alkaloids. One of these studies shows uncarine F as the most antiproliferative alkaloid towards leukemic cells [28]. In another study, Muhammad et al. [6] isolated and identified five stereoisomer oxindole alkaloids and reported uncarine D, with IC_{50} values in a range of 30–35 $\mu g/mL$, as the most cytotoxic from them, against three tumoral human cell lines. On the other

hand, Bacher et al. [29] found a strong apoptotic effect of pteropodine and uncarine F on acute leukemic lymphoblasts, but mitraphylline was not as effective as them against this cell line. As it can be observed, there is no full agreement on the cytotoxicity potential of these alkaloids, which show differential sensitivity towards different tumoral cell lines.

On a previous work, we had already observed that mitraphylline had stronger antiproliferative effect than vincristine and cyclophosphamide on two human brain tumoral cell lines [24] using the same colorimetric assay. Here, we report for the first time on the cytotoxic effect of the highly purified mitraphylline alkaloid isolated from the bark of *Uncaria tomentosa* on a sarcoma (MHH-ES-1) and a breast cancer (MT-3) cell line. This action suggests that mitraphylline might be a new promising agent in the treatment of both human sarcoma and breast cancer, besides brain cancer.

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