Anticancer Effect of a New Benzophenanthridine Isolated from Zanthoxylum madagascariense (Rutaceline)

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Abstract. Fractionation of the cyclohexane extract from the stem bark powder of Zanthoxylum madagascariense led to the isolation of a new benzophenanthridine-type alkaloid, hydrochloride of 2,3-methylendioxy-8-hydroxy-7-methoxy-benzo[C]phenanthridine (Rutaceline), characterized on the basis of its spectral data. Rutaceline was evaluated for its antiproliferative capacity on the human colorectal adenocarcinoma (Caco-2) and the African green monkey kidney (Vero) cell lines. The 50% inhibition of cell growth (IC₅₀) obtained after 24 h incubation was similar for both cell lines (110-115 µg/ml, i.e. 269-281 µM), but at 48 h the IC₅₀ value for the Caco-2 cells was lower than for the Vero cells (20 µg/ml, i.e. 49 µM versus 90 µg/ml, i.e. 220 µM) indicating a higher cell growth inhibitory effect on the colon adenocarcinoma cells. At the respective IC₅₀ concentrations, Rutaceline did not significantly induce apoptosis but induced cell cycle arrest in the G0/G1 phase, as well as a decrease of cells in S phase. Rutaceline also induced DNA fragmentation in both cell lines, as revealed by agarose gel electrophoresis, and a dose-dependent clastogenic effect in both cell lines as revealed by the Comet assay.

Cancer is one of the leading causes of death in the world that claims more than seven million lives per year (1). Every year, thousands of children and teenagers under twenty years of age are diagnosed with cancer in the United States. With the introduction of new therapeutic strategies during the past thirty years, survival for many diagnostic groups has increased tremendously. The surveillance, epidemiology and end results program estimate that the overall 5-year survival rate in 1998 was 80% for the group of cancer patients under twenty years old. Nevertheless, cancer remains the leading medical cause of death among children between one and nineteen in the United States (2).

Natural products have been the main anticancer prodrugs for centuries and represent 50% of drugs used clinically in developed countries, 25% of which are derived from higher plants. The plant kingdom represents an enormous potential of molecules to be discovered, since it was estimated one decade ago that more than 90% of plant species have not yet been exhaustively studied (3).

Recent investigations have been focused on the activity of the components found in Citrus species, including flavonoids, carotenoids and limonoids, especially in terms of their anticarcinogenic effects. The antioxidant micronutrients, carotenoids, vitamin C, vitamin E and polyphenols, especially flavonoids, play an important role in the prevention of cancer (4). Although whole organic foods should be the first line of defense against cancer, certain supplements have been shown to help in the prevention of certain types of cancer. For example, folic acid (B vitamin family member), found
in many vegetables, beans, fruits, whole grains, and fortified breakfast cereals, may hold promise as an anticarcinogen in colon cancer. Deficient intake of folic acid, which plays an important role in DNA synthesis and repair, has been linked to an increased risk of colon cancer amongst others. Animal studies have shown that diets lacking in folic acid are associated with DNA strands breaks in the organs most susceptible to cancer, leading to the development of the disease (5).

The search for novel natural drugs has resulted in the identification of several benzophenanthridine alkaloids present in plants as promising anticancer agents. For example, there is evidence that sanguinarine, which derives from the root of Sanguinaria canadensis, induces apoptosis in the A431 skin carcinoma cell line and is a potential antiproliferative agent that could be developed for chemotherapy of skin cancer (6). This root was classically used in traditional medicine for the treatment of gingivitis and dermatitis (7). Sanguinarine and other quaternary benzo[c]phenanthridine alkaloids (QBA) have a number of interesting pharmacological activities including antimutural (8), antimicrobial (9), antifungal (10) and antioxidant (11) properties.

Other benzophenanthridine alkaloids have not been studied as much as sanguinarine. Among them, fagaronine and its synthetic derivative ethoxidine inhibit DNA topoisomerase (12). This inhibition is also observed for chelerythrine, a benzophenanthridine which inhibits protein kinase C (13) and causes mitotic disruption by interaction with tubulin (14).

An extract of Zanthoxylum americanum (Rutaceae) has shown cytotoxicity (15) and recently a new benzophenanthridine called Rutaceline has been identified in Zanthoxylum madagascariense (Rutaceline). In the present work its antiproliferative effect, capacity to alter the cell cycle, induce apoptosis, DNA fragmentation and genotoxic damage were examined in a human intestinal cancer cell line Caco-2 and Vero cells from monkey kidney.

Materials and Methods

**Materials.** Dubelcco’s modified Eagle’s medium (DMEM), antibiotics, penicillin, streptomycin, Dulbecco’s phosphate-buffered saline (PBS) and agarose low melting point (LMP) were provided by Gibco-BRL (Egggenstein, Germany). Fetal bovine serum (FBS) was purchased from Invitrogen (Carlsbad, CA, USA). Trypsin EDTA solution C (0.05% trypsin – 0.02% EDTA) was obtained from Biological Industries (Kibbutz Beit Haemet, Israel). Tris (hydroxymethyl) aminomethane was purchased from Aldrich-Chemie (Steinheim, Germany), DNAase free RNase from Roche Diagnostic (Mannheim, Germany) and Annexin V/FITC Kit from Bender System MedSystem. RPMI, 1% sodium N-laurylsarcosinate (w/v), Proteinase K, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide) and other reagents were provided by Sigma Chemical Co (St. Louis, MO, USA).

**Rutaceline extraction.** The stem bark of Zanthoxylum madagascariense was collected from Fianarantsoa and was authentically identified at the herbarium of Botanical and Zoological Park of Tsimbazaza, Antananarivo, Madagascar. The dried stem bark powder (600 g) was soaked extracted with cyclohexane over seven hours to give a dark viscous extract (70 g). The powder defatted was extracted with 80% ethanol over one hour. The ethanolic extract was filtered and concentrated under vacuum giving a honey like viscous residue (20 g). This was treated with HCl aqueous 1 N and extracted with CHCl₃. The aqueous layer was made basic to pH 8 with NH₄OH. The solution was extracted with CHCl₃, the CHCl₃ dried with Na₂SO₄ was filtered and evaporated to yield 4 g of residue. This was chromatographed on 160 g Al₂O₃ (activity III) using a gradient of hexane and AcOEt to give 120 fractions. The eluant hexane AcOEt 60/40 yielded 50 mg of rutaceline, a yellow powder. The concerted use of one and two dimensional NMR methods 1H, 13C, (COSY, HMBC, HMQC, NOESY) and mass spectrum allowed the identification of rutaceline as a hydrochloride of 2,3-methylenedioxy-8-hydroxy-7-methoxy-benz[o]phenanthridine (MM=409).

**Cell culture.** The human colorectal adenocarcinoma cell line, Caco-2, and African green monkey kidney, Vero cells, were purchased from ATCC. The Caco-2 cell line was cultured in DMEM supplemented with 10% heat inactivated FBS and 0.1% antibiotic; and the Vero cell line was cultured in RPMI supplemented with 10% heat inactivated FBS and 1% antibiotic and 1% glutamine. Both cell lines were cultured as a monolayer at 37°C in a humidified incubator with 5% CO₂.

**MTT assay.** The assay was performed using the method described by Mossman (16) slightly modified. Samples containing 200 µl cell suspension (2x10⁴ cells/ml) were plated in 96-well flat-bottomed microtiter plates. After adherence of the cells within 24 h of incubation at 37°C, Rutaceline at concentrations ranging from 1 µg/ml to 100 µg/ml was added (3-4 wells per concentration). After additional incubation time (24 and 48 h) at 37°C in a humidified incubator with 5% CO₂, 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dissolved in PBS and sterile filtered was added to all the wells at a final concentration of 1 mg/ml. Following 1 h of incubation, the generated formazan was dissolved with 100 ml dimethylsulphoxide (DMSO) per well. The optical density was measured using an enzyme-linked immunosorbent assay (ELISA) plate reader (Merek Whitehouse Station NJ USA, ELISA system MIOS version 3.2.) at 550 nm. The Rutaceline concentration that caused 50% inhibition of cell growth (IC₅₀) was calculated for each cell line.

**Cell cycle analysis.** The cell cycle was assessed through flow cytometry by using a fluorescence-activated cell sorter (FACS). The cells were cultured in 6-well flat bottomed microtiter plates containing 2 ml of cell suspension. The number of cells was determined by calculation according to the number of cells/well in the 96-well plates (3,500). After 24 h of incubation at 37°C with 5% CO₂, Rutaceline was added at the respective IC₅₀ concentration for each cell type. Following 24 and 48 h of incubation, cells were harvested by mild trypsination, collected by centrifugation and stained in Tris-buffered saline (TBS) for 1 h at 4°C. FACS analysis was carried out at 488 nm in an Epics XL flow cytometry (Coulter Corporation, Hialeah, FL, USA). Data from
12,000 cells were collected and analysed using a Multicycle program (Phoenix Flow Systems, San Diego, CA, USA). All experiments were performed in triplicate.

Assessment of apoptosis. Apoptosis was assessed using an Annexin V-FITC kit binding assay and analyzed by FACS. Cell culture, treatment with Rutaceline and cell collection, were carried out as described in the cell cycle analysis section. Thereafter, cells were resuspended in binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl2). The Annexin V-FITC was added according to the product insert and incubated for 30 min at room temperature in the dark. One min before FACS analysis, propidium iodide (PI) was added at a concentration of 20 µg/ml. Approximately 500,000 viable cells were counted to assess apoptosis. Experiments were performed in triplicate.

DNA fragmentation assays. Cells were cultured (5x10⁵ cell/ml) in 25 cm² flasks, total vol. 10 ml/flask at 37°C. Rutaceline was added at concentrations corresponding to the IC₅₀ concentration for each cell type or omitted for the controls. After 24 h incubation, the cells were washed with PBS (5 ml) and the DNA was extracted as follows: cells were harvested in 2 ml of 20 mM Tris-HCl, pH 8.0, containing 5 mM EDTA and 1% sodium N-lauryl-sarcosinate (w/v). After 5 min, proteinase K (final concentration 200 µg/ml) was added and the mixture incubated overnight at 37°C. Subsequently, 1 vol. of 7.5 M ammonium acetate and 1 vol. of chloroform/phenol (1:2) were added. The mixture was shaken for 20 min, and centrifuged at 2,200 xg for 5 min at 20°C. The upper phase was collected and combined with one vol. of SEVAG (chloroform/isoamyl alcohol, 34:1, v/v) to eliminate all trace of phenol. Then, the mixture was added to 2,200 xg for 5 min at 20°C. The aqueous phase was collected and the RNA was digested with DNase free RNase A (final concentration 9.8 µg/ml) at 37°C for 30 min. Then, 1 vol. of SEVAG was added followed by centrifugation at 2,200 xg for 5 min at 20°C. The upper phase was collected and the DNA was precipitated with two vol. of ice-cold absolute ethanol for 2 h at −20°C. After centrifugation at 2,500 xg for 45 min at 4°C, the pellet was washed with a solution of ice-cold 70% ethanol, the pellet was air dried and the DNA was redissolved in 1 ml of 20 mM Tris-HCl, pH 8.0, containing 5 mM EDTA for quantification by UV-spectrophotometry at 254 nm, the purity of DNA was controlled by the ratio of absorbance at 260 and 280 nm, respectively. The acceptable ratio was set at 1.8.

Under such conditions, 10-20 µg of DNA was loaded onto a 1% agarose gel for electrophoresis (70 V, 30 mA for 1 h approximately). The gel was stained with ethidium bromide and photographed under UV irradiation.

Single cell gel electrophoresis (SCGE). Cells were cultured in 6-well flat-bottomed microtiter plates containing 2 ml of cell suspension (200,000 cells approximately). After 24 h of incubation at 37°C with 5% CO₂, Rutaceline was added at its respective IC₂₀ concentration for each cell type. Methionine methanosulfonate (MMS) at 300 µM was used as positive control. Following 24 h of incubation, Caco-2 and Vero cells were detached from the well with 0.05% trypsin solution and collected by centrifugation. Cells were embedded in 0.6% LMP agarose prepared in MilliQ water (18 MQ) and layered on commercial pre-coated slides (Trevenig®, Gaithersburg MD, Maryland, USA). The slides were placed in lysing buffer (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris pH 10, N-lauryl-sarcosine 1% (w/v)) with 1% Triton X-100 for 1 h at 4°C. The DNA of the nuclei in the agarose gels was unwound for 40 min in electrophoresis buffer (1 mM Na₂EDTA and 300 mM NaOH, pH >13). The SCGE slides were then electrophoresed for 30 min at 25V and 300 mA at 4°C. After neutralization with 400 mM Tris buffer (pH 7.5), the slides were dried at room temperature. For image analysis the slides were hydrated and stained with 10 µl 4,6-diamidino-2-phenylindole (DAPI).

Statistical analysis. Results are expressed as Tail Moment (migrated DNA × tail length). The data are presented as mean±SEM and analyzed using the Mann-Whitney U-test and Student’s t-test. A limit of p<0.05 was accepted for significant differences.

Results

Antiproliferative effect. The IC₅₀ concentrations were obtained by the MTT test. Rutaceline concentrations were plotted against the percentage of cell proliferation after 24 and 48 h of incubation. The IC₅₀ values obtained in the Caco-2 cells were: 110±4 µg/ml (269 µM) for 24 h and 20±2 µg/ml (49 µM) for 48 h (Figure 1A). The IC₅₀ values obtained in the Vero cells were: 115±2 µg/ml (281 µM) for 24 h and 90±2 µg/ml (220 µM) for 48 h (Figure 1B).

Cell cycle analysis and apoptosis. Cell cycle analysis of the Caco-2 and Vero cells was performed after 24 and 48 h of incubation with Rutaceline at the respective IC₅₀ concentrations. Compared to the untreated cells, Rutaceline induced cell cycle arrest in the G0/G1 phase as well as a decrease of cells undergoing the S phase, after 24 and 48 h on both cells lines (Figure 2). The student’s t-test was used to analyze the differences observed between the controls and the Rutaceline treated samples.

The assessment of apoptosis in Caco-2 and Vero cells was performed after 24 and 48 h of incubation with Rutaceline, at the same concentrations mentioned above for the analysis of the cell cycle. FACS analysis was used to differentiate viable cells (Annexin V⁻ and PI⁻), early apoptotic cells (Annexin V⁺ and PI⁻), late apoptotic/ necrotic cells (Annexin V⁺ and PI⁺), late apoptotic/necrotic cells (Annexin V⁺ and PI⁺) and necrotic cells (Annexin V⁺ and PI⁻). The results showed that Rutaceline did not induce apoptosis in either the Caco-2 cells or the Vero cells at IC₅₀ concentrations following 24 or 48 h of incubation (Figure 3).

DNA damage. The results indicated that Rutaceline induced DNA fragmentation in the Caco-2 cells and in the Vero cells as shown by a diffuse pattern characteristic of cellular death by necrosis (Figure 4 A and B).

The results of the comet assay (expressed as Tail Moment) are shown in Figure 5 for each cell line, for all the different treatments with or without Rutaceline, (p<0.05).

The comet test results showed a concentration-dependent clastogenic effect of Rutaceline in both cell lines and at
IC$_{20}$ was slightly higher than that produced by MMS. In our experiment, the Vero cells showed more sensitivity to the genotoxic compound than the Caco-2 cells.

The percentage of apoptotic cells (identified as nuclei showing a characteristic "hedgehog" image) was lower than 3% in both the Caco-2 and Vero Rutaceline treated cells at IC$_{10}$ and IC$_{20}$ and these were not significantly different from that of untreated cells.

**Discussion**

The present experiments were designed to study the antiproliferative effect of Rutaceline, a new benzophenanthridine isolated from *Zanthoxylum madagascariense*. The data clearly demonstrate that Rutaceline inhibited cell proliferation in the Caco-2 and Vero cell lines with IC$_{50}$ in the µM range (48 µM - 220 µM). Preliminary studies on the Caco-2 cell line showed that Rutaceline inhibited the synthesis of DNA (data not shown). The present data corroborated this fact since a disruption in G0/G1 phase of the cell cycle of this cell line was observed preventing cells from entering into the S phase of the cell cycle, and preventing cell proliferation. This indicates that Rutaceline has similar effects as Sanguinarine and Chelerythrine regarding cytotoxic capacity (10, 11).

The fact that the IC$_{50}$ concentrations decreased from the incubation time of 24 h to 48 h may be explained by a biotransformation of Rutaceline into a more toxic derivative in both cell lines or by its accumulation inside the cells. Both hypothesis need to be further investigated.

The antitumor effect of a drug can be due to cytostatic and/or cytotoxic effects. A substance is cytostatic if it prevents cell growth and/or produces a disruption in any one of the three phases of the cell cycle. On the other hand, a cytotoxic substance causes cellular death by apoptosis or

**Figure 1. Effect of Rutaceline on cell proliferation of Caco-2 cells (A) and Vero cells (B), after 24 and 48 h of incubation. The relative percentage of cell proliferation was calculated considering that untreated cells at 24 and 48 h show 100% cell proliferation. Each point represents the mean of triplicate experiments.**

**Figure 2. Distribution of percentage in different phases of the cell cycle of untreated and treated Caco-2 cells (A), and Vero cells (B), after 24 and 48 h incubation with Rutaceline, at respective IC$_{50}$ concentrations. Data shown are mean value of three independent experiments. * p<0.05. CT: control (untreated).**
Figure 3. Percentage of early and late apoptotic and necrotic cells assessed by flow cytometry analysis of Annexin V-FITC staining and PI accumulation after exposure of Caco-2 cells (A) and Vero cells (B) to Rutaceline at respective IC\textsubscript{50} concentrations after 24 and 48 h of incubation. Values are expressed as means±SEM of three independent experiments. CT: control.

Figure 4. Agarose gel electrophoresis analysis of DNA from Caco-2 cells (A) and Vero cells (B). Cells were incubated in the absence of Rutaceline (line 1) treated with Rutaceline at IC\textsubscript{50} concentrations for 24 h (line 2). M: DNA size marker.

Figure 5. Results of comet assay on (A) Caco-2 cells (CCT: Control Caco-2; C10: Caco-2 IC\textsubscript{10}; C20: Caco-2 IC\textsubscript{20}; MMSC: Caco-2 with Methionine Methanousulfonate (MMS) positive control) and (B) Vero cells (VCT: Control Vero; V10: Vero IC\textsubscript{10}; V20: Vero IC\textsubscript{20}; MMSV: Vero positive control MMS.
necrosis. Rutaceline inhibits cell proliferation and induces cell cycle disruption in the G0/G1 phase accompanied by a slight increment of apoptotic cells after 48 h of treatment and DNA fragmentation at IC50 concentrations in both cell lines. This apoptotic cell death may be increased with increasing Rutaceline concentrations in the culture medium.

In general, the Vero cells showed more sensitivity to the genotoxic compound than Caco-2 cells. From the percentage of "hedgehog" comets observed, the increase in tail moment values could not be attributed to apoptosis (17, 18).

Caco-2 cells have been shown by Berger et al. (19) to bear the multidrug resistance protein-2, (MRP-2), which confers on them some resistance to several antitumor drugs. Since they are very sensitive to Rutaceline and since the IC50 even decreases after 48 h incubation indicating a higher sensitivity to this drug, it may be hypothesized that Rutaceline is not refluxed from Caco-2 cells by MRP-2. This is an additional interesting indication for further applications.

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References


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