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Effect of Steviol on SaOs2 Cells as a Potential Anticancer Agent: A Molecular and Cellular Study

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KEYWORDS

Steviol; osteosarcoma; U2OS cells; apoptosis; cell cycle arrest; mitochondrial pathway; chemotherapeutic candidate; Bax/Bcl-2; p53; steviol glycosides

Abstract

Introduction: Steviol, a primary colonic metabolite of steviol glycosides, exhibits low systemic availability and a higher half-maximal inhibitory concentration (IC_{50}) relative to standard chemotherapeutics such as 5-fluorouracil (5-FU) and doxorubicin. Despite these pharmacokinetic characteristics, emerging evidence suggests potential anticancer activity, warranting further investigation.

Objectives: To evaluate the anticancer effects of steviol on the human osteosarcoma U2OS cell line and elucidate the underlying mechanisms regulating cell proliferation, cell cycle progression, and apoptosis.

Methods: U2OS cells were treated with varying concentrations of steviol, doxorubicin, or 5-FU. Cell proliferation assays assessed dose- and time-dependent responses. Cell cycle distribution was analyzed, and colony-forming assays evaluated long-term proliferative capacity. Mechanistic studies examined mitochondrial apoptotic markers, including the Bax/Bcl-2 ratio, and the activation of cyclin-dependent kinase inhibitor 1 (p21), tumor protein 53 (p53), and cyclin-dependent kinase. Caspase-3 and survivin levels were assessed to determine pathway involvement.

Results: Steviol significantly inhibited U2OS cell proliferation in a dose- and time-dependent manner, achieving antitumor efficacy comparable to doxorubicin and 5-FU. Treatment induced G1 phase cell cycle arrest and reduced colony formation. Mechanistically, steviol activated a mitochondrial apoptotic pathway, evidenced by increased Bax/Bcl-2 ratio and upregulation of p21, p53, and cyclin-dependent kinase. The anticancer activity occurred independently of caspase-3 activation and survivin suppression.

Conclusions: Steviol demonstrates notable anticancer effects against U2OS osteosarcoma cells, inducing G1 arrest and mitochondrial apoptosis through a caspase-3- and survivin-independent mechanism. Its minimal systemic presence during metabolism and higher median lethal dose relative to 5-FU highlight its potential as a promising, potentially safer chemotherapeutic candidate.

1. Introduction

Steviol glycosides, a family of popular natural non-nutritive sweeteners from leaves of Stevia rebaudiana bertoni. At present, a few studies have been reported about the cytotoxicity of steviol on human resource cells.Steviol exhibits a kaurene diterpenoid structure, similar to that of gibberellin[1] . Its rearrangement product isosteviol and steviol itself have been used as starting reagents for synthetic medicines [2]. The acceptable daily intake of steviol is 4 mg/kg body weight/day [3] and its median lethal dose (LD50) value is 15 g/kg body weight in rats and mice, irrespective of the gender [1]. During the metabolism of steviol or steviol glycosides, steviol is not detectable in blood, and half maximal inhibitory concentration (IC50) value of steviol is much higher than that of current chemotherapy agents such as 5-fluorouracil (5-FU) and doxorubicin (6). Therefore, if steviol could efficiently inhibit cancer cells with a clear mechanism, it could be expected as a chemotherapy agent applied in large doses.

High-dose chemotherapy and chemoresistance are the typical features of osteosarcoma treatment, which is a primary malignant bone cancer with high morbidity [4,5]. Patients with metastasis exhibit a 5-year survival rate of only 20% [6,7]. Efficient treatment of osteosarcomas requires systemic chemotherapy prior and subsequent to surgery[8]. The majority of chemotherapy regimens applied in OS are based on the following drugs: High-dose methotrexate with leucovorin

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rescue [5], doxorubicin (Adriamycin®, ADM), cisplatin, and ifosfamide[9] . These regimens are associated with marked short- and long-term collateral toxic effects [10], including acute alopecia, myelosuppression, mucositis and nausea[11] .

In addition, rare ADM-regimen cases of toxic mortalities caused by early or late cardiac failure have been identified, which were due to ADM toxicity and sepsis following febrile neutropenia [12]. Therefore, many efforts have been made to develop novel drugs to increase the number of options for chemotherapy in OS, such as: Rapamycin ; ampelopsin ; JQ1 (a BET protein inhibitor) in combination with rapamycin; and few other small molecules [13]. However, only a small number of studies have been conducted on the use of natural medicines such as evodiamine [14], riccardin D [15] and piperine [4]. The anticancer activity of steviol has not been well examined. Boonkaewwan and Burodom [16] suggested that unpurified steviol did not present cytotoxicity on Caco-2 cells at 0.1-100 μmol/l, but it suppressed lipopolysaccharide

(LPS)-mediated tumor necrosis factor-α, interleukin (IL)-1β and IL-6 release, and attenuated the production of LPS-induced pro-inflammatory cytokines. However, higher steviol dosage (200-800 μmol/l) decreased cell viability of T84, Caco-2 and HT29 cells [15]. Steviol also inhibited renal cyst growth in a mouse model of polycystic kidney disease [17]. A two-stage carcinogenesis model on mouse skin demonstrated that steviol markedly inhibited the promotion and initiation stages of lymphoblastoid cells [17,18]. These results suggest that steviol may be a potential chemotherapy agent for cancer treatment.

At present, with the exception of some aforementioned studies investigating the inhibition of the proliferation of cancer cells by steviol, including lymphoblastoid cells [18], none have explored its possible molecular mechanisms. Our preliminary study indicated an anti-cancer activity of steviol on human osteosarcoma SaOs2 cells (data not shown). Therefore, the present study focused on the in vitro anti-proliferative effects of steviol on human osteosarcomaSaOs2 cells and the potential molecular mechanisms involved.

2. Methods

Materials and Chemicals:

Steviol was obtained from Sigma-Aldrich, while doxorubicin (ADM) and 5-fluorouracil (5-FU) were biological-grade reagents. Additional reagents included dimethyl sulfoxide (DMSO), Na_2CO_3, $NaHCO_3$, NaCl, KCl, $Na_2HPO_4 \cdot 12H_2O$, $NaH_2PO_4 \cdot 2H_2O$, EDTA disodium, SDS, glycocoll, bromoxylenol blue, ammonium persulphate, tris(hydroxymethyl)aminomethane, Ponceau, tetramethylethylenediamine (TEMED), xylene brilliant cyanin G (G250), and phenylmethylsulfonyl fluoride (PMSF), all of biological grade. Trypsin-EDTA, propidium iodide (PI), Triton X-100, RNase A, MTT, penicillin-streptomycin solution (100X), BeyoECL Plus, polyvinylidene fluoride membranes, RIPA lysis buffer, and JC-1 dye were purchased from Beyotime Institute of Biotechnology. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were also used. All other reagents were analytical grade and used as provided unless otherwise noted.

Cell Culture:

The SaOs2 human osteosarcoma cell line was purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science. Cells were cultured in DMEM supplemented with 10% FBS, 1% glutamine (200 mmol/l), 100 IU/ml penicillin, and 100 mg/l streptomycin in a 5% CO_2 humidified incubator at 37°C.

MTT Assay on Cell Proliferation:

The antiproliferative effect of steviol on SaOs2 cells was assessed using the MTT assay. Logarithmic-phase cells were digested with 0.25% trypsin and seeded at 5,000 cells/well in 96-well plates with DMEM. After 24 hours of incubation, cells were treated with varying concentrations of steviol and incubated for 48 hours at 37°C. MTT reagent (0.5 mg/ml) was added, followed by a 4-hour incubation. Formazan crystals were dissolved in DMSO, and absorbance was measured at 570 nm to determine cell growth inhibition. Doxorubicin and 5-FU were used as positive controls.

Colony Formation Assay:

SaOs2 cells were seeded in 6-well plates at 1,000 cells/well and treated with 0, 5, 10, or 20 μg/ml of steviol. After 14 days, cells were methanol-fixed and stained with crystal violet, and colonies were counted under a microscope.

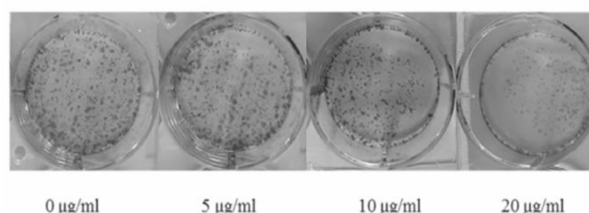

Figure 2. Steviol inhibits colony formation in SaOs2 cells. Cells treated with steviol for 14 days.

Cell Cycle Analysis:

SaOs2 cells (2×10 cells/well) were treated with steviol (0, 50, 100, and 200 μg/ml) for 48 hours, harvested, and fixed with 70% ethanol. Fixed cells were stained with PI and RNase and analyzed by flow cytometry to assess the distribution of cells across the cell cycle.

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Mitochondrial Membrane Potential Detection and Hoechst 33342 Staining:

Following steviol treatment, mitochondrial membrane potential was measured using a JC-1 assay. Cells were incubated with JC-1 dye and analyzed under a fluorescence microscope. For nuclear staining, SaOs2 cells were incubated with steviol, fixed with formalin, and stained with Hoechst 33342. Fluorescent images were captured. Apoptotic cells were quantified using Annexin V-FITC/PI staining. SaOs2 cells were treated with steviol, stained with Annexin V-FITC and PI, and analyzed by flow cytometry.

Statistical Analysis:

Data were expressed as mean ± standard deviation from triplicate experiments. Group comparisons were performed using one-way ANOVA with post hoc Student-Newman-Keuls tests. Statistical significance was considered at $P<0.05$. Analyses were conducted using SPSS software.

3. Results

Inhibition of steviol on the viability of SaOs2 cells.

To compare steviol's effectiveness, two commonly used chemotherapy agents, doxorubicin (ADM, LD50 570 mg/kg, oral, mouse) and 5-fluorouracil (5-FU, LD50 115 mg/kg, oral, mouse), were included as positive controls. As shown in Fig. 1, steviol exhibited a similar inhibition rate to 5-FU but was less potent than ADM, with all three agents inhibiting SaOs2 cell viability in a time- and dose-dependent manner. The IC50 values after 24 hours of treatment were 200 µg/ml for steviol, 1.2 µg/ml for ADM, and 250 µg/ml for 5-FU. Additionally, Fig. 2 revealed a dose-dependent reduction in colony formation with increasing concentrations of steviol. Microscopic observations suggested signs of cell apoptosis, which was further investigated in subsequent experiments.

Table I. Regulation of steviol on cell cycle progression of SaSo2 cells.

	Cell cycle stage		
Dosage (µg/ml)	G1(%)	S(%)	G2(%)
Control	48.07	31.31	17.93
Steviol - 50	60.82	26.76	10.92
Steviol - 100	63.17	17.83	19.71
Steviol - 200	68.54	18.42	13.81

Steviol causes G1 phase arrest and apoptosis in U2OS cells.

Flow cytometry was used to assess cell cycle distribution and detect cell death in SaOs2 cells treated with steviol. Steviol exposure significantly increased the percentage of SaOs2 cells in the G1 phase, while reducing the S and G2 phase populations (Fig. 3; Table I), indicating that steviol induces G1 cell cycle arrest in SaOs2 cells. The underlying mechanism is discussed later. Apoptosis was further confirmed using Hoechst staining (Fig. 4A), JC-1 staining (Fig. 4B), and Annexin V-FITC/PI flow cytometry (Fig. 4C and D). As steviol concentration increased, the intensity of green fluorescence (JC-1) also increased (Fig. 4B), demonstrating a concentration-dependent effect on apoptosis. The total apoptosis rate rose from 0.04% to 24.10% after 48 hours of treatment with steviol at concentrations up to 200 µg/ml (Fig. 4C). These findings suggest that steviol inhibits SaOs2 cell viability by inducing G1 phase arrest and promoting apoptosis.

Figure 4. Concentration-dependent effect of steviol on mitochondrial membrane potential of SaOs2 cells. As steviol concentration increased, the green fluorescence intensity increased.

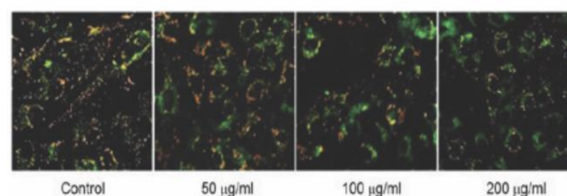


Figure 4. Steviol induced apoptosis of SaOs2 cells Photomicrographs of Hoechst 33342 staining.

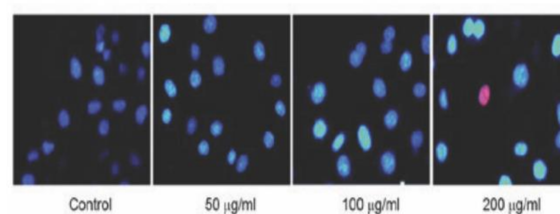
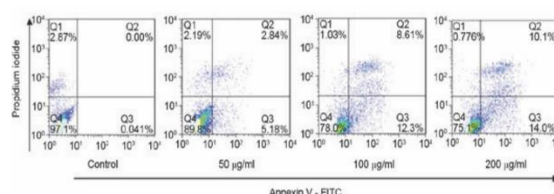


Figure 4 . SaOs2 cells were treated with steviol for 48 h and stained with Annexin V-propidium iodide.



4. Discussion

Steviol, despite its widespread use as a natural sweetener, has not been extensively studied for its anticancer potential or its

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mechanism of action in cancer cells. The findings from Boonkaewwan and Burodom (2008) [16] indicated that steviol reduces cell viability at concentrations ranging from 200 to 800 µmol/l (approximately 63.7–254.8 µg/ml) in colorectal cancer cell lines such as T84, Caco-2, and HT29. The IC50 values observed were between 400 and 800 µmol/l, aligning closely with the results obtained in the present study. In both studies, steviol was 90% pure, derived from the oxidation of stevioside. This consistency reinforces steviol's potential as an anticancer agent across different cell lines, though its precise mode of action remains underexplored.

A similar study by Cappadone et al. [19] highlighted that NSC743420, an indole derivative, exerted a cytostatic and differentiating effect on SaOs2 osteosarcoma cells by inducing G0/G1 cell cycle arrest and increasing alkaline phosphatase activity. While NSC743420 targets similar pathways, the present study delved deeper into the cell cycle arrest induced by steviol and its role in regulating G1 phase-associated proteins, such as Cyclin D1, p21, p53, and CDK2 (as shown in Figs. 3-6). These proteins play a crucial role in controlling the progression through the G1 phase, and their modulation by steviol suggests that it disrupts the normal cell cycle, leading to a halt in proliferation.

Moreover, steviol's pro-apoptotic effect was demonstrated by the upregulation of the Bax/Bcl-2 ratio and activation of apoptosis-related pathways independent of Survivin and Caspase 3. This suggests a mitochondrial-driven mechanism of apoptosis, consistent with intrinsic apoptotic pathways [20]. Steviol's unique ability to induce apoptosis without relying on Survivin or Caspase 3 distinguishes it from many traditional chemotherapeutic agents.

Given these findings, steviol demonstrates significant anticancer activity, not only by inhibiting cell proliferation through G1 cell cycle arrest but also by triggering apoptosis via mitochondrial pathways. However, further research is needed to validate these in vitro results in vivo, and to determine whether steviol can be developed as a novel, natural anticancer agent, potentially offering a safer alternative to traditional chemotherapy drugs like 5-fluorouracil and doxorubicin.

5. Conclusion

This study demonstrates that steviol exerts anticancer effects on human osteosarcoma U2OS cells, comparable to established chemotherapeutic agents such as 5-fluorouracil and doxorubicin. Steviol induces G1 phase cell cycle arrest by suppressing Cyclin D1 and upregulating p21, p53, Cyclin E, and CDK2. It also triggers mitochondrial apoptosis, as evidenced by an increased Bax/Bcl-2 ratio, via a mechanism independent of Survivin and Caspase 3. These findings suggest that steviol, a natural sweetener metabolite, holds potential as an anticancer agent. However, further in vivo studies are necessary to confirm its therapeutic efficacy in osteosarcoma.

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