

Inhibition of nasopharyngeal carcinoma cell proliferation and synergism of cisplatin with silvestrol and episilvestrol isolated from *Aglaia stellatopilosa*

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Abstract. Nasopharyngeal carcinoma (NPC) is a type of tumour that arises from the epithelial cells that line the surface of the nasopharynx. NPC is treated with radiotherapy and cytotoxic chemotherapeutic drugs such as cisplatin and 5-fluorouracil. However, current strategies are often associated with potential toxicities. This has prompted efforts to identify alternative methods of treatment. The present study aimed to investigate silvestrol and episilvestrol-mediated inhibition of cell proliferation in human NPC cells. The growth kinetics of NPC cells treated with silvestrol or episilvestrol were monitored dynamically using a real-time, impedance-based cell analyzer, and dose-response profiles were generated using a colorimetric cell viability assay. Furthermore, apoptosis was evaluated using flow cytometry and high content analysis. In addition, flow cytometry was performed to determine cell cycle distribution. Finally, the effects of combining silvestrol or episilvestrol with cisplatin on NPC cells was examined. Apoptosis was not observed in silvestrol and episilvestrol-treated NPC cells, although cell cycle perturbation was evident. Treatment with both compounds induced a significant increase in the percentage of cells in the G₂/M phase, as compared with the control. *In vitro* cultures combining silvestrol or episilvestrol with cisplatin showed synergistic effects against NPC cells. The results of the present study suggested that silvestrol and episilvestrol had an anti-tumour activity in NPC cells. Silvestrol and episilvestrol, particularly in combination with cisplatin, merit further investigation, so as to determine the

cellular mechanisms underlying their action(s) as anti-NPC agents.

Introduction

Nasopharyngeal carcinoma (NPC) is a type of cancer that affects the epithelial cells of the nasopharynx (1). Although NPC has a low prevalence among Caucasian populations, the disease has an exceptionally high incidence rate in the Eastern Malaysian state of Sarawak, particularly in the Bidayuh ethnic community (2). NPC is also prevalent among populations in Southeast Asia and Southern China, as well as Inuit populations in Alaska and certain ethnic groups in North Africa (1,3). Concurrent chemoradiation is the current standard therapy for NPC, although this method appears to be more effective in patients with early stage NPC, as compared with patients with advanced stage NPC and distant tumour metastasis (3,4). One of the treatment strategies being investigated for NPC involves the addition of another therapeutic agent to the combination of cisplatin and 5-fluorouracil, the standard chemotherapeutic drugs for the treatment of NPC (4). This approach requires a novel target agent that functions synergistically with the standard chemotherapy drugs to treat NPC.

The use of plant components as therapeutic agents has attracted much attention. Higher plants, specifically plants used in traditional medicine or as dietary supplements, are the source of a considerable number of natural product-derived drugs (5). *Aglaia* is a genus of plant belonging to the family Meliaceae, and can be found primarily in the forests in tropical Asia (6). Several species within the genus are known to be sources of cyclopenta[b]benzofuran flavaglines, a novel class of compound with a unique structure that has been shown to be antineoplastic (5). One member of this class of compounds, silvestrol and its 5'-epimer episilvestrol, are isolated from the twig, fruit, and bark of *Aglaia stellatopilosa*, a species endemic to Borneo (7). The mechanism underlying the anti-proliferative effects of the cyclopenta[b]-benzofurans occurs via inhibition of protein synthesis (8). Kinghorn *et al* (5) described novel plant bioactive agents with potential cancer chemotherapeutic properties, including silvestrol. Investigations into the

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phytochemical effects, synthetic methods, biological evaluation and mechanism of action of cyclopenta[b]-benzofurans are described in Pan *et al.* (9). Rocaglates, silvestrol and episilvestrol are translation initiation inhibitors (10). However, to the best of our knowledge, the role of silvestrol and episilvestrol in the treatment of NPC has yet to be evaluated.

The aim of the present study was to evaluate the capacity of silvestrol and episilvestrol to inhibit proliferation, induce apoptosis and perturb the cell cycle in NPC cells. The results demonstrated that both silvestrol and episilvestrol are effective at inhibiting the proliferation of NPC cells *in vitro* by blocking the G₂/M transition in the cell cycle. In addition, in combination with cisplatin, the two compounds exhibited a synergistic effect against NPC cells. These results suggested that silvestrol and episilvestrol may serve as NPC-targeting compounds in combination with existing chemoradiation treatment regimens.

Materials and methods

Chemicals. Silvestrol (Fig. 1) and episilvestrol (Fig. 2) were purchased from Cerylid Biosciences, Ltd. (Richmond, Australia).

Cell lines and culture. HK1, an Epstein-Barr virus (EBV)-negative NPC cell line (11), was provided by Professor George Tsao (Department of Anatomy, Faculty of Medicine, University of Hong Kong, Hong Kong, China). C666-1, an EBV-positive NPC cell line (12), was donated by Professor Kwok-Wai Lo (Department of Anatomical and Cellular Pathology, Faculty of Medicine, Chinese University of Hong Kong, Hong Kong, China). HK1 was maintained in the exponential growth phase in RPMI-1640 medium supplemented with 10% heat-inactivated foetal calf serum (FCS), 10 U/ml penicillin and 10 µg/ml streptomycin (all from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a humidified atmosphere containing 5% CO₂. C666.1 was maintained under similar conditions, although the FCS concentration was increased to 15%. Passage levels of the NPC cells were in the range of 10-30. The identity of HK1 and C666.1 cells were confirmed by DNA fingerprinting using an AmpFISTR Identifier® Polymerase Chain Reaction (PCR) Amplification kit (part no. 4322288; Applied Biosystems; Thermo Fisher Scientific, Inc.). The short tandem repeat profiles were consistent with published data (13). Detection of mycoplasma using an e-Mycotm Mycoplasma PCR Detection kit (cat. no. 25235; Intron Biotechnology, Inc., Seongnam, Korea) were conducted routinely and contamination-free cells were used throughout this study. Mycoplasma-free stocks were frozen in 10% v/v dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA), 40% v/v FCS and 50% v/v RPMI-1640, then stored in liquid nitrogen for subsequent re-culturing.

Sulforhodamine B (SRB) bioassay. SRB assays were conducted in order to ascertain the stability of silvestrol and episilvestrol activity against the NCI-H460 non-small cell lung cancer and MCF-7 breast cancer cell lines over a period of time. Both cell lines were obtained from American Type Culture Collection (Manassas, VA, USA), and were maintained in RPMI-1640 medium supplemented with 10%

heat-inactivated FCS at 37°C in 5% CO₂. For the SRB assay, 0.1% (w/v) gentamycin (Amresco, LLC, Solon, OH, USA) was added to the culture medium, after which 100 µl cells were plated in 96-well flat bottomed microtiter plates (Nalge Nunc International, Penfield, NY, USA) at 7,500 cells/well and 10,000 cells/well for NCI-H460 and MCF-7, respectively. The cells were incubated for 24 hours for recovery, after which 100 µl culture medium or culture medium containing silvestrol or episilvestrol (3819, 381.9, 38.19, 3.819 or 0.3819 nM) was added to the wells for 72 h. Subsequently, the cells were fixed with 10% (w/v) trichloroacetic acid (Scharlab, S.L., Barcelona, Spain) at 4°C for 1 h. After five washings with reversed osmosis water, the cells were stained with 0.4% (w/v) SRB dye (MP Biomedicals, Santa Ana, CA, USA) in 1% (v/v) acetic acid (Merck Millipore, Darmstadt, Germany) for 10 min at room temperature. Unbound stain was removed by washing three times with 1% acetic acid. The plates were then air-dried and the bound protein stain was solubilized with 100 µl of 10 mM Tris base (Avantor Performance Materials, Center Valley, PA, USA). The optical density was read at 515 nm using the Sunrise Basic Microplate Reader from Tecan Group Ltd. (Männedorf, Switzerland).

xCELLigence cell proliferation assay. HK1 cells were seeded at a density of 1x10⁴ cells/well into an E-Plate 16 (ACEA Biosciences, Inc., San Diego, CA, USA). For C666.1 cells, 3x10⁴ cells/well were seeded. At 24 h following seeding, the culture medium was aspirated and replaced with fresh medium containing 6.25 or 50 nM silvestrol or episilvestrol. The compounds were dissolved in DMSO with a final concentration of DMSO in the cell culture ≤1.0%. Vehicle control cultures received DMSO alone. Cells treated with 33.3 µM cisplatin served as the positive control. Cells were monitored dynamically for ~70 h using the impedance-based xCELLigence real-time cell analyzer (ACEA Biosciences, Inc.). The cell index, automatically calculated from the change in electrical impedance as the living cells interacted with electrodes in the E-plate wells, correlated with the number of cells, viability and/or cytotoxicity over time.

MTS cell viability assay. A total of 1x10⁴ HK1 cells/well or 3x10⁴ C666.1 cells/well were seeded into 96-multiwell microtiter plates using a Hamilton Microlab®STARlet robotic liquid handling workstation (Hamilton Robotics, Inc., Reno, NV, USA). At 24 h following seeding, the medium was aspirated and replaced with fresh medium containing various concentrations of silvestrol or episilvestrol. Vehicle control cultures received DMSO alone. The cells were then incubated for 24 h at 37°C in an atmosphere containing 5% CO₂. The number of viable cells at the end of the incubation period was measured using a CellTiter 96®AQ_{ueous} One Solution Cell Proliferation (MTS) assay (Promega Corporation, Madison, WI, USA). Absorbance at 490 nm was read using an EnVision multilabel plate reader (PerkinElmer, Inc., Waltham, MA, USA) and subtracted with non-specific absorbance measured at 630 nm. Wells containing medium without cells served as blanks. Cell viability was calculated as a percentage compared to the control cells, which were arbitrarily assigned 100% viability. The half maximal inhibitory concentration (IC₅₀) values, defined as the concentration that inhibited 50% cell

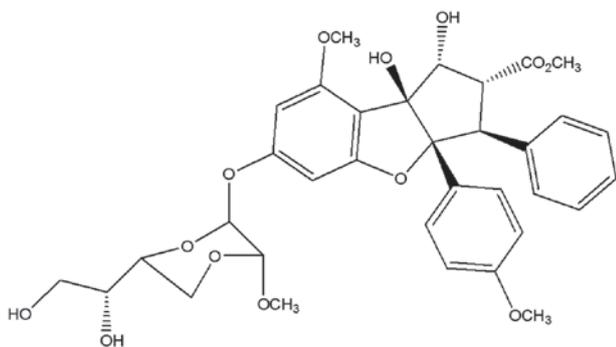


Figure 1. Chemical structure of silvestrol.

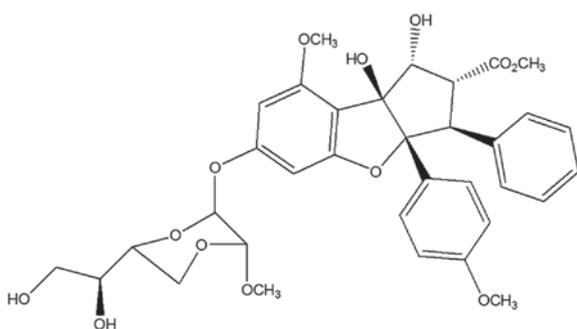


Figure 2. Chemical structure of episilvestrol.

growth relative to control cells, were graphically obtained from the dose-response curves.

Apoptosis assay. HK1 and C666.1 cells were seeded at a density of 1×10^6 cells/ml in culture dishes containing RPMI-1640 medium supplemented with 10 (HK1) or 15% (C666.1) heat-inactivated FCS, and allowed to adhere and reach ~80% confluence overnight at 37°C. Subsequently, the medium was aspirated and the cells were treated with 50 nM silvestrol or episilvestrol. Vehicle control cultures received DMSO alone, whereas cells treated with 100-175 μ M cisplatin served as the control. Apoptosis was evaluated at 24 h following treatment using a FACSCalibur flow cytometry system (model no. 342975; BD Biosciences, San Jose, CA, USA) using an Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit (cat. no. 556547; BD Pharmingen, San Diego, CA, USA). Data acquisition and analysis were performed using BD CellQuest Pro software, version 6.0 (BD Biosciences). A total of 1×10^4 events were collected for each sample. The lower right and upper right quadrants represented cells undergoing apoptosis. Annexin V-FITC-propidium iodide-stained cells were imaged using an IN Cell Analyzer 2000 (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) with a 20X objective. Hoechst 33342 (Cell Signaling Technology, Inc., Danvers, MA, USA) was used for nuclear staining. Briefly, 10 μ l Hoechst 33342 was added to the cell suspension to a final concentration of 1 μ g/ml, and then incubated in the dark for 15 min. The following filter combinations were used: Green (490/20 ex., 525/20 em.) for detection of Annexin V-FITC; red (579/34 ex., 624/40 em.) for detection of propidium iodide; and blue (350/50 ex., 455/50 em.) for detection of Hoechst 33342.

Cell cycle analysis assay. HK1 and C666.1 cells were seeded and grown in culture dishes, as described for the apoptosis assay. Cultured cells were pulsed with 10 μ M bromodeoxyuridine (BrdU) daily. Cell cycle progression was determined 24 and 48 h following treatment on a FACSCalibur flow cytometry system using an FITC BrdU Flow kit (cat. no. 559619; BD Pharmingen). ModFit LT™ software, version 3.3.11 from Verity Software House, Inc. (Topsham, ME, USA) was used to analyze the DNA patterns in the flow cytometry histograms.

Combined drug analysis. Cell seeding was performed as described above for the MTS cell viability assay using the Hamilton Microlab®STARlet robotic liquid handling workstation. To maintain similar experimental conditions, 96-multiwell microtiter plates were assigned simultaneously for single-drug and two-drug treatment (14). At 24 h following seeding, non-fixed ratio combinations of silvestrol-cisplatin or episilvestrol-cisplatin were evaluated (Table I). Following drug treatment, the microtiter plates were incubated for a further 24 h following which an MTS assay was conducted to determine cell viability. Drug interaction was determined using the previously described combination index (CI) method (14). CalcuSyn version 2.0 software (Premier Biosoft International, Palo Alto, CA, USA) was used to generate the dose-response curves, dose-effect analysis, and CI-effect plot. A CI <1 implied synergism, CI=1 was additive, and CI >1 implied antagonism. In addition, CI<0.1 implied very strong synergism, CI=0.1-0.3 implied strong synergism, CI=0.3-0.7 implied synergism, CI=0.7-0.85 was moderate synergism, CI=0.85-0.9 implied slight synergism (14).

Statistical analysis. Calculations were performed using IBM® SPSS® version 22.0 statistical software (IBM SPSS, Armonk, NY, USA). Differences between mean values were evaluated with a one-way analysis of variance and Tukey's post-hoc analysis. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Compound profiles. HPLC analysis of silvestrol and episilvestrol demonstrated that the compounds were pure (Figs. 3 and 4).

Stability of silvestrol and episilvestrol. Preliminary data (unpublished) from on-going experiments demonstrated that the silvestrol and episilvestrol compounds were stable over time. Silvestrol and episilvestrol reconstituted with 100% DMSO and stored at -20°C produced consistent GI_{50} results as determined by the SRB bioassay (72 h treatment period) on NCI-H460 non-small cell lung cancer and MCF-7 breast adenocarcinoma cell lines (Tables II and III).

Dynamic monitoring of cell proliferation. Based on screening experiments on NCI-H460 and MCF-7 cell lines (Tables II and III), the effective inhibition concentrations of silvestrol and episilvestrol were in the nano-molar range. Therefore, one low and one moderate dose were selected for use in the xCELLigence system; a real-time cell proliferation, viability and cytotoxicity analyzer. The cell index generated represents growth over time.

Table I. Description of CI values for each fraction of the cells and the corresponding DRI.

A, HK1 cell line						
Compound (nM)	Fraction affected	CI	Synergism	DRI ^a	Cisplatin (μ M)	Cisplatin DRI
Silvestrol						
5	0.211	0.04	Very strong	98.879	12.5	33.057
10	0.208	0.087	Very strong	47.213	25	15.128
50	0.347	0.016	Very strong	83.851	50	236.646
500	0.391	0.070	Very strong	15.067	100	298.111
1,000	0.336	0.294	Strong	3.603	150	62.114
5,000	0.394	0.645	Synergism	1.567	200	158.505
Episilvestrol						
10	0.172	0.209	Strong	778.01	25	4.811
50	0.270	0.025	Very strong	7.67x10 ⁶	50	40.295
500	0.324	0.014	Very strong	9.83x10 ⁷	100	71.468
1,000	0.261	0.093	Very strong	1.61x10 ⁵	150	10.720
5,000	0.440	0.003	Very strong	1.03x10 ¹¹	200	399.788
B, C666.1 cell line						
Compound (nM)	Fraction affected	CI	Synergism	DRI ^a	Cisplatin (μ M)	Cisplatin DRI
Silvestrol						
5	0.350	0.098	Very strong	83.104	33.3	11.638
10	0.467	0.048	Very strong	230.427	66.7	23.053
50	0.378	0.570	Synergism	12.633	266.7	2.037
500	0.445	0.644	Synergism	3.297	400	2.936
1,000	0.561	0.214	Strong	8.276	400	10.735
Episilvestrol						
5	0.461	0.033	Very strong	114.652	33.3	41.370
10	0.451	0.071	Very strong	52.574	66.7	19.103
50	0.647	0.038	Very strong	58.864	133.3	46.547
500	0.564	0.444	Synergism	2.786	266.7	11.700
1,000	0.670	0.325	Synergism	3.666	400	18.987

^aDRI values >1 are beneficial, and the greater the DRI values, the greater the dose reduction for a given therapeutic effect (14). CI, combination index; DRI, drug reduction index.

Proliferation of HK1 cells cultured in 6.25 nM silvestrol was not inhibited (Fig. 5A). However, 50 nM silvestrol exerted an immediate inhibitory effect and caused near-static cell index compared with the control cells. This observation suggests that a lower concentration of silvestrol (6.25 nM) enhanced proliferation more than the vehicle control-treated cells, whereas a higher concentration of silvestrol (50 nM) could inhibit cell proliferation. Similar observations were obtained with episilvestrol. A total of 50 nM silvestrol or episilvestrol were as effective as 33.33 μ M cisplatin in reducing C666.1 cell index (Fig. 5B). Following treatment, there was a rapid decline in cell index. However, C666.1 cell proliferation was not entirely inhibited by 6.25 nM silvestrol or episilvestrol, although the cell index generated was markedly lower compared with

that of the control, indicating that the hyper-proliferation effect observed in HK1 cells was cell-specific. The pattern of growth inhibition by 50 nM silvestrol and episilvestrol were comparable to that of cisplatin, a standard NPC chemotherapy drug. Since 50 nM silvestrol and episilvestrol were sufficient for inhibiting cell proliferation, all further experiments were conducted at this minimum concentration only.

Determination of viable cells. To further examine the anti-proliferative effects exhibited by the xCELLigence system, HK1 and C666.1 cells treated with various concentrations of silvestrol (Fig. 6A) and episilvestrol (Fig. 6B) were assessed for viability using an MTS assay. In HK1 cells, increasing the treatment concentrations from 50 nM to 10 and

Table II. GI₅₀ for silvestrol.

SRB bioassay performed	GI ₅₀ value (nM)	
	NCI-H460	MCF-7
First test	16.90	19.71
3 months	17.81	18.50
6 months	19.70	19.02
9 months	18.10	16.90
12 months	18.60	17.60

GI₅₀, concentration for 50% of maximal inhibition of cell proliferation; SRB, sulforhodamine B.

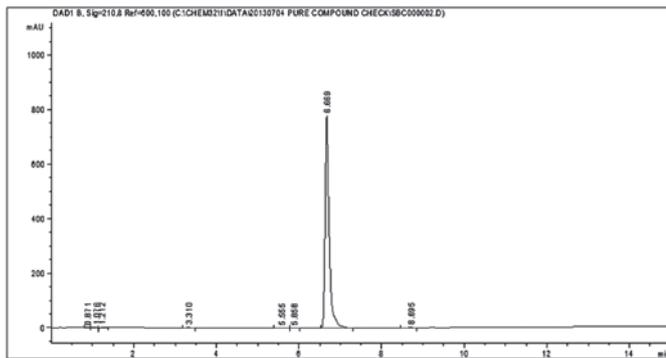


Figure 3. High-performance liquid chromatography profile of silvestrol.

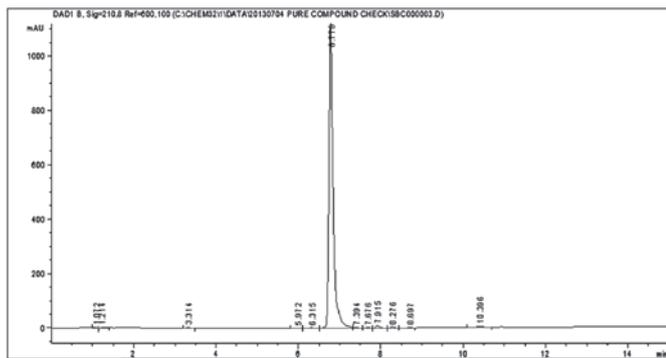


Figure 4. High-performance liquid chromatography profile of episilvestrol.

100-fold higher did not elicit further response, as evidenced by the near-plateau in cell viability ≥ 50 nM silvestrol and ≥ 50 nM episilvestrol. The trend in effect of silvestrol and episilvestrol against C666.1 cell proliferation was not as smooth, as compared with HK1. However, both compounds were potent against EBV-positive C666.1 NPC cells, with the effective concentrations required to inhibit IC₅₀ values attainable within 24 h compared with EBV-negative HK1 NPC cells (Table IV). These results are concordant with those obtained from xCELLigence dynamic monitoring of cell proliferation, whereby following treatment with 50 nM silvestrol or episilvestrol, the cell index of C666.1 cells continued to decline over time, whereas that of the HK1 cells remained static. The IC₅₀ value of episilvestrol in C666.1 cells was markedly lower

Table III. GI₅₀ for episilvestrol.

SRB bioassay performed	GI ₅₀ value (nM)	
	NCI-H460	MCF-7
First test	17.96	19.09
2 months	15.60	18.70

GI₅₀, concentration for 50% of maximal inhibition of cell proliferation; SRB, sulforhodamine B.

Table VI. IC₅₀ values for the anti-proliferative activity of silvestrol and episilvestrol against NPC cells.

Cell line	IC ₅₀ value at 24 h (nM)	
	Silvestrol	Episilvestrol
C666.1	484	259
HK1	>5,000	>5,000

IC₅₀, half maximal inhibitory concentration; NPC, Nasopharyngeal carcinoma.

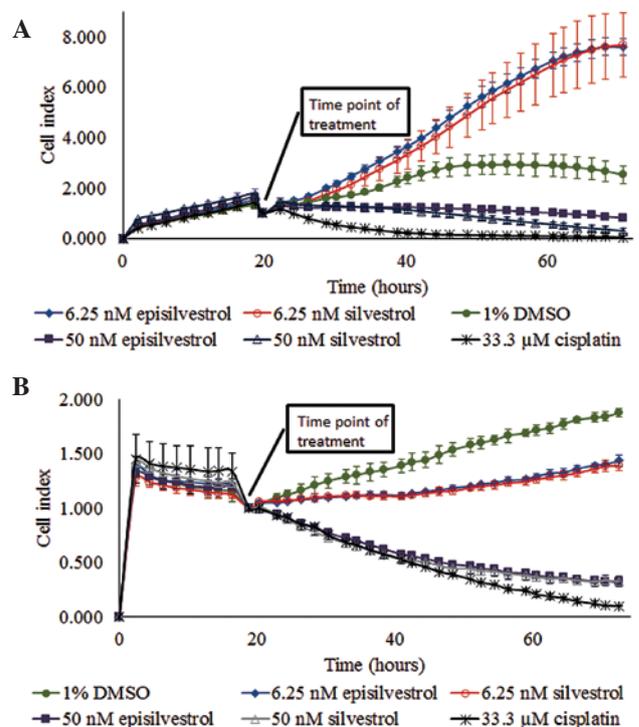


Figure 5. Real-time analysis of NPC cell proliferation with or without silvestrol or episilvestrol. The effect of various concentrations of silvestrol or episilvestrol on (A) HK1 and (B) C666.1 cell proliferation, monitored using the xCELLigence system. NPC, Nasopharyngeal carcinoma; DMSO, dimethyl sulfoxide.

compared with that of silvestrol, indicating increased efficacy and suggesting that stereoisomerism may be involved. DMSO showed no influence on cell viability (data not shown).

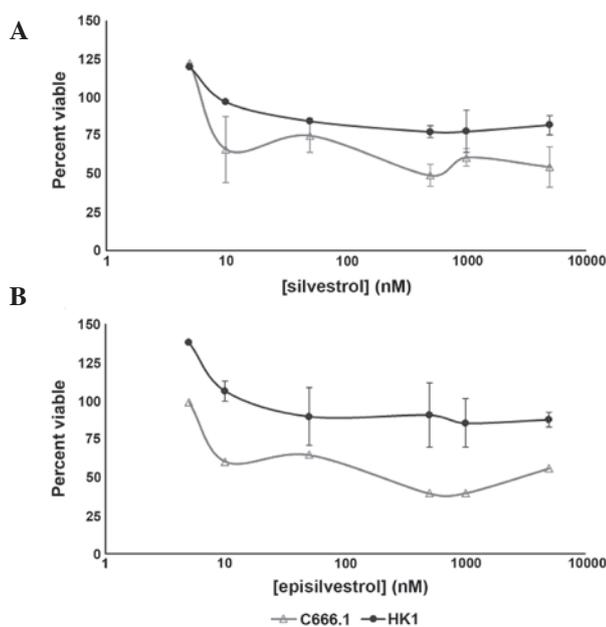


Figure 6. Viability curves of HK1 and C666.1 cells 24 h following culture in (A) silvestrol or (B) episilvestrol, as determined by the MTS assay. Two independent experiments were conducted. The results are presented as the mean \pm standard deviation.

Silvestrol and episilvestrol do not induce apoptosis. To determine if inhibited cell proliferation was associated with apoptosis induction, HK1 and C666.1 cells cultured in silvestrol or episilvestrol were subjected to Annexin V-FITC and propidium iodide assay, and apoptotic cells were identified by flow cytometry. Following 24 h exposure to 50 nM silvestrol or episilvestrol, there was no significant difference in the percentage of apoptotic cells between the treated cells and the control (Fig. 7A). Therefore, apoptosis did not account for the differences in cell proliferation observed in the present study. Cisplatin, an NPC chemotherapy drug, was used for instrument set-up and comparison of apoptosis induction (data not shown). Consistent with the flow cytometry results, no differences were detected in silvestrol or episilvestrol-treated cells compared with the control, as determined by the IN Cell 2000 high content cell analyzer. However, apoptosis was detected in cisplatin-treated HK1 and C666.1 cells, as shown in Fig. 7B and demonstrated by the greater quantity of green (Annexin V-FITC) and red (propidium iodide) stained cells. Cisplatin is used in cancer therapy due to its apoptosis-inducing activity. The amount of green and red fluorescence in silvestrol or episilvestrol-treated cells and vehicle control-treated cells were similar and were markedly lower compared with cisplatin-treated cells. These results were concordant with the flow cytometry results which demonstrated that silvestrol and episilvestrol did not induce apoptosis with the experimental dose and time.

Cell cycle analysis assay. DNA synthesis was monitored by BrdU-labelling and cell cycle progression by flow cytometry. When BrdU is added to cell cultures it is incorporated into newly synthesized DNA of cells entering and progressing through the S-phase (DNA synthesis phase) of the cell cycle. In HK1 cells, 24 h after cell culture in 50 nM silvestrol or episilvestrol, there

was no observable difference in BrdU-positive cells compared with the control (Fig. 8A). Silvestrol or episilvestrol did not affect cell distribution in the S-phase. However, there was a significant reduction of cells in the G₀/G₁ phase (silvestrol, $P=0.011$; episilvestrol, $P=0.002$) and increase in the G₂/M phase (silvestrol, $P=0.006$; episilvestrol, $P=0.016$) in treated cells compared with the control (Fig. 8B). This cell cycle inhibition observed in HK1 cells corresponds to the plateaued cell index observed following xCELLigence assay. The cells were neither proliferating nor dying. There was no significant observable effect on C666.1 cells within 24 h. However, after 48 h there was a significant reduction in BrdU-positive C666.1 cells (silvestrol, $P=0.019$; episilvestrol, $P=0.042$) compared with the control (Fig. 9A). This was accompanied by a significant increase in G₂/M phase cells (silvestrol, $P=0.000$; episilvestrol, $P=0.000$) compared with the control (Fig. 9B). The reduction of DNA synthesis in C666.1 cells may have caused the cell index (determined by the xCELLigence assay) to rapidly decrease. These differences in cellular effects and the time point at which the compounds exert their effects support the hypothesis that the activity of silvestrol and episilvestrol is cell-specific in HK1 and C666.1 cells.

Silvestrol and episilvestrol display synergistic effects in combination with cisplatin. As the results of the xCELLigence system and the MTS cell viability assay revealed that silvestrol or episilvestrol alone did not exert significant effects on HK1, both compounds were further investigated to determine whether they were able to sensitize NPC cells to the treatment and effect of cisplatin. Using CalcuSyn software, the CI was determined to ascertain the combinatorial effect of silvestrol or episilvestrol with cisplatin. The CI values are presented in Table I. The CI method (14), revealed a remarkable synergistic activity in HK1 cells treated simultaneously with silvestrol or episilvestrol and cisplatin (Fig. 10). Similarly, in C666.1 cells, silvestrol or episilvestrol synergized with cisplatin.

Discussion

Plants have been an important source of novel drugs of natural origin over the past decade (5). Our previous study demonstrated that quercetin, a polyphenolic flavonoid found in vegetables and fruits, and *trans*-cinnamaldehyde obtained from the stem bark of *Cinnamomum burmannii*, exhibited synergism with cisplatin in inhibiting the growth of NPC cells (15,16). Notably, the skeletal structures of the rocaglamide derivatives include a flavonoid unit and a cinnamic acid amide moiety (9). The synthesis of racemic rocaglamide from the benzofuran intermediate with *trans*-cinnamaldehyde was described by Pan *et al* (9).

The results of the present study showed that silvestrol and episilvestrol were able to inhibit the proliferation of EBV-positive C666.1 and EBV-negative HK1 NPC cells. NPC is often associated with the EBV. EBV-infected NPC cells exhibit type II latency and may express among others, latent membrane proteins 1 and 2 (LMP12A and B), of which LMP1 is oncogenic (3). According to Patton *et al* (17), silvestrol modulates direct anti-tumor activity against EBV-associated lymphomas while sparing innate and antigen-specific adaptive

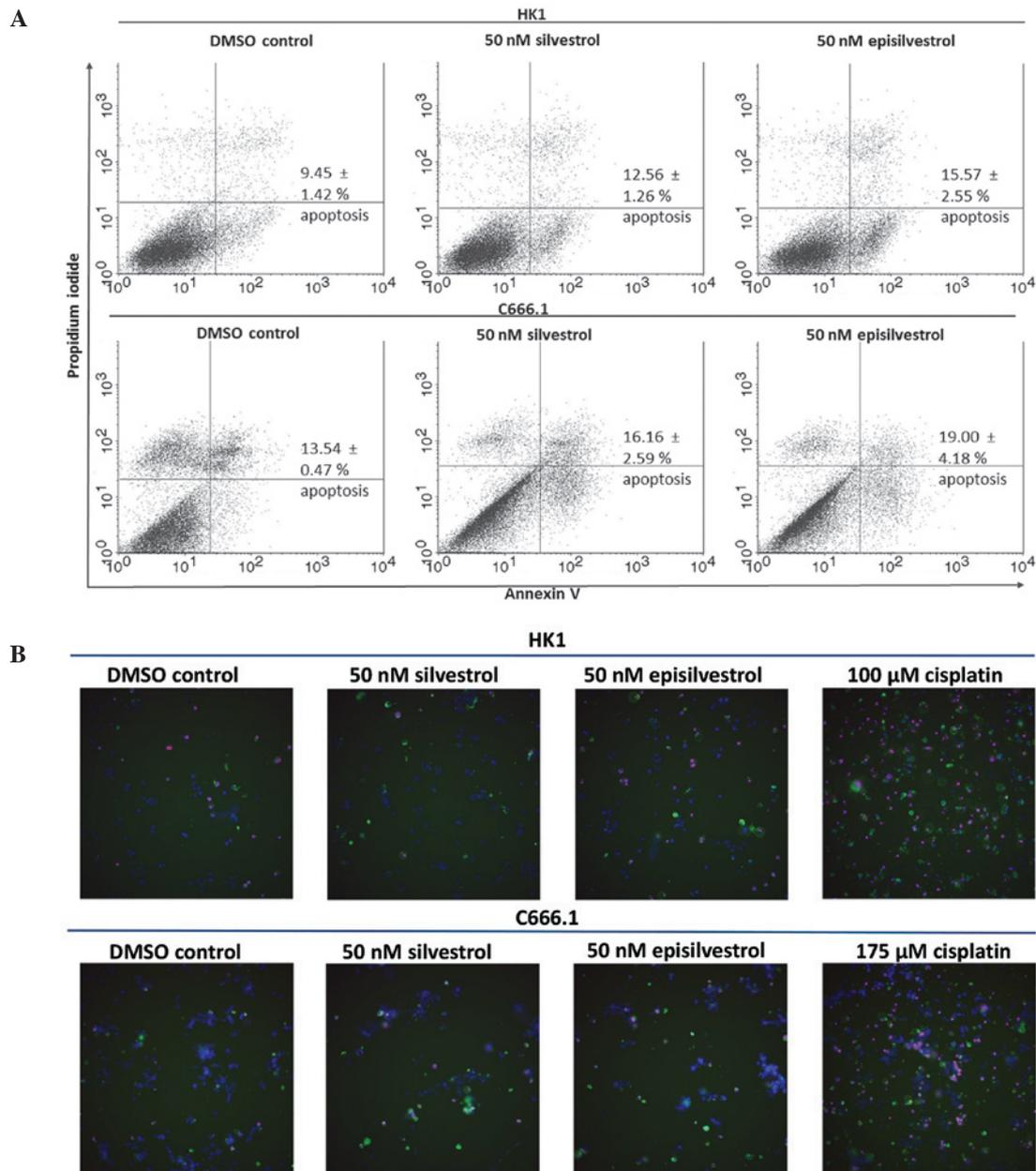


Figure 7. (A) Flow cytometry apoptosis assay with Annexin V-FITC and propidium iodide dual staining of HK1 and C666.1 cells exposed to 50 nM silvestrol or episilvestrol 24 h prior to analysis. The average percentage \pm standard deviation of total apoptotic cells is shown. (B) High content analysis for apoptosis. Representative fused Hoechst 33342, Annexin V-FITC and propidium iodide fluorescence images of cells 24 h post-treatment. A 20x objective was used in the IN Cell Analyzer 2000. FITC, fluorescein isothiocyanate; DMSO, dimethyl sulfoxide.

immunity. There is an urgent requirement for the development of anti-cancer agents that are effective against EBV-positive diseases. The study on EBV-transformed lymphoblastoid cell lines (EBV-LCLs) demonstrated that silvestrol (2-50 nM) was highly potent against anti-proliferation, with minimal cell death (18). These results were concordant with those of the present study that demonstrated that the inhibition of NPC cell proliferation was not accompanied by apoptosis induction at 50 nM. Notably, the study demonstrated that the anti-proliferative activity of silvestrol was associated with the loss of LMP1 expression (18). In addition, it has previously been reported that silvestrol treatment had indirect anti-proliferative effects on EBV-transformed lymphoblastoid cell lines by enhancing the innate immune function (17).

As a single agent, silvestrol was markedly effective, with IC_{50} values of 4-10 nM. Silvestrol exhibits nanomolar potency against multiple solid tumour cell lines (10,19,20). Similarly, the data of the present study obtained from the preliminary screening experiments conducted on NCI-H460 and MCF-7 showed a similar potency. The NPC results were in concordance with these findings. However, contrary to a previous study (10) which stated that silvestrol was approximately three times more potent than episilvestrol, the results herein suggested that episilvestrol had higher efficacy against NPC cells.

The present flow cytometry data indicated that silvestrol and episilvestrol inhibit the G_2/M transition in the NPC cell cycle. A previous study evaluating the cytotoxicity of silvestrol in LNCaP human prostate carcinoma cells indicated a similar

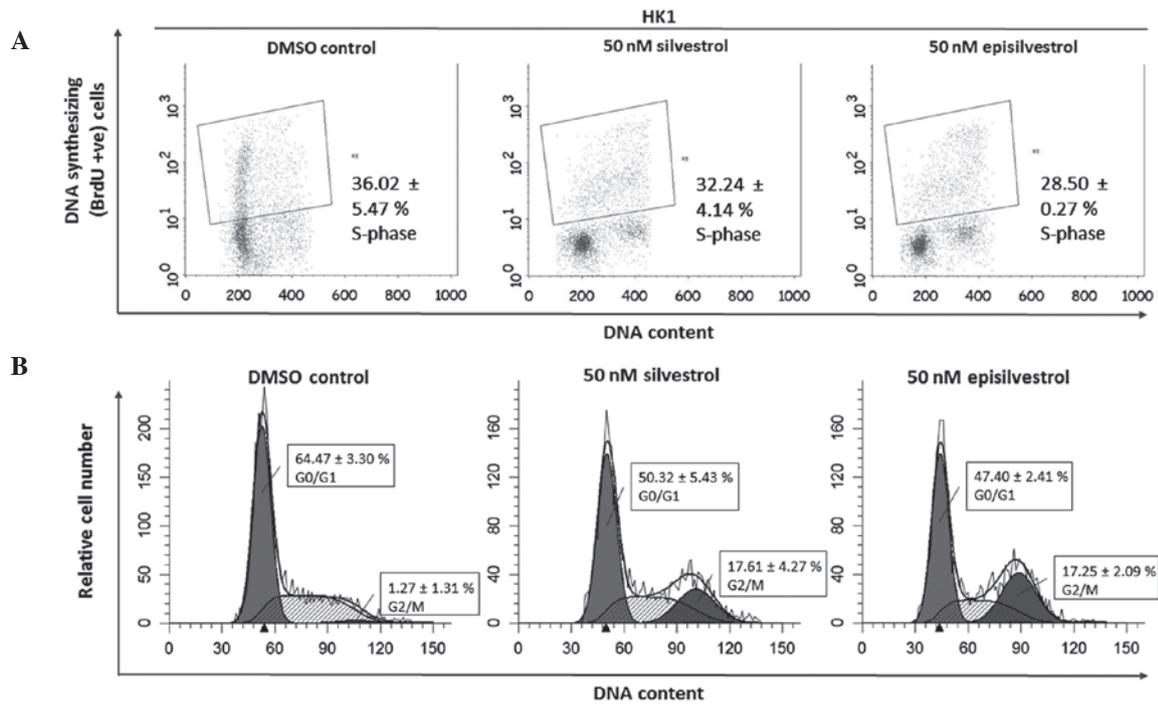


Figure 8. (A) DNA synthesis flow cytometry of HK1 cells that incorporated BrdU after 24 h (rectangle gates). (B) Cell cycle progression analysis by software modelling. The average percentage \pm standard deviation of total cells in the various phases are shown. DMSO, dimethyl sulfoxide; BrdU, bromodeoxyuridine.

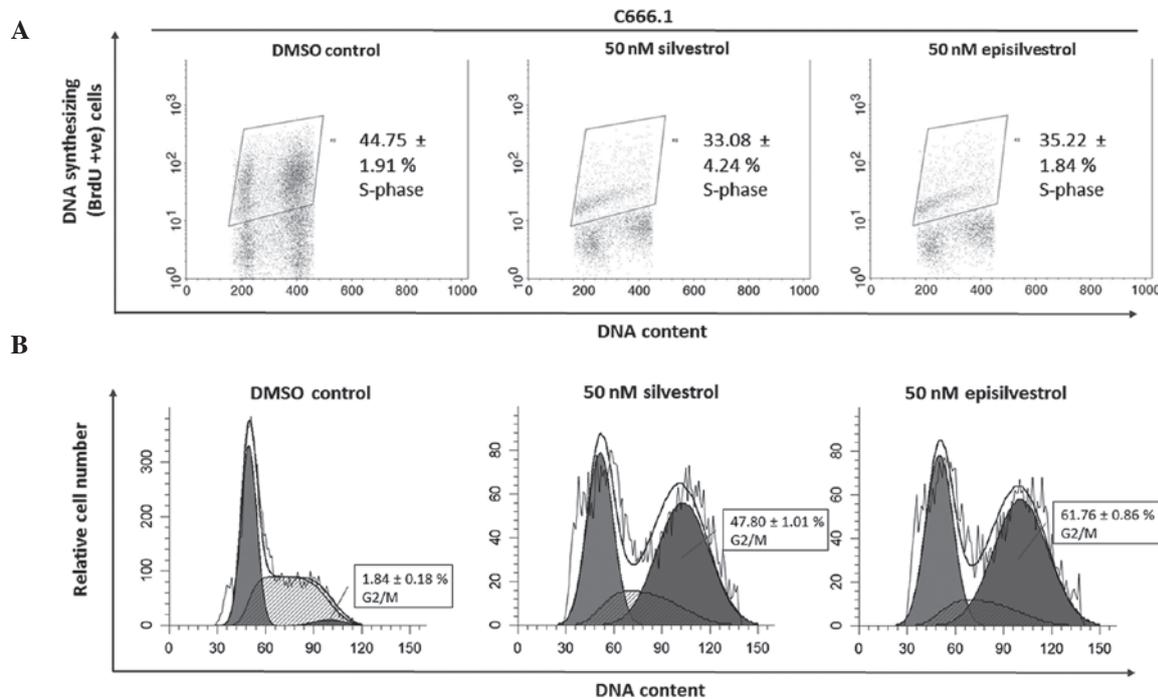


Figure 9. (A) DNA synthesis flow cytometry of C666.1 cells that incorporated BrdU after 48 h (rectangle gates). (B) Cell cycle progression analysis by software modelling. Mean percentage \pm standard deviation of total cells in the various phases are shown. DMSO, dimethyl sulfoxide; BrdU, bromodeoxyuridine.

mode of action (21). In addition, another study demonstrated that rocaglaol, which is a cyclopenta[b]benzofuran flavagline, inhibited G₂/M cell cycle progression (22). Tumours often have increased proliferation rates. Evaluation of the percentage of cells in S phase may be useful, as it may convey prognostic value. To evaluate the number of cells in S phase, a pulse-chase experiment was performed herein. In C666.1 cells, silvestrol

and episilvestrol were able to reduce DNA synthesis. However, this was not observed in HK1 cells under the present experimental conditions. Conversely, Kim *et al* (19) reported that while certain members of the plant-derived cyclopenta[b]benzofuran class were cytostatic against various human cancer cell lines, silvestrol exhibited a cytotoxic rather than cytostatic effect, as determined by a colony formation assay with LNCaP

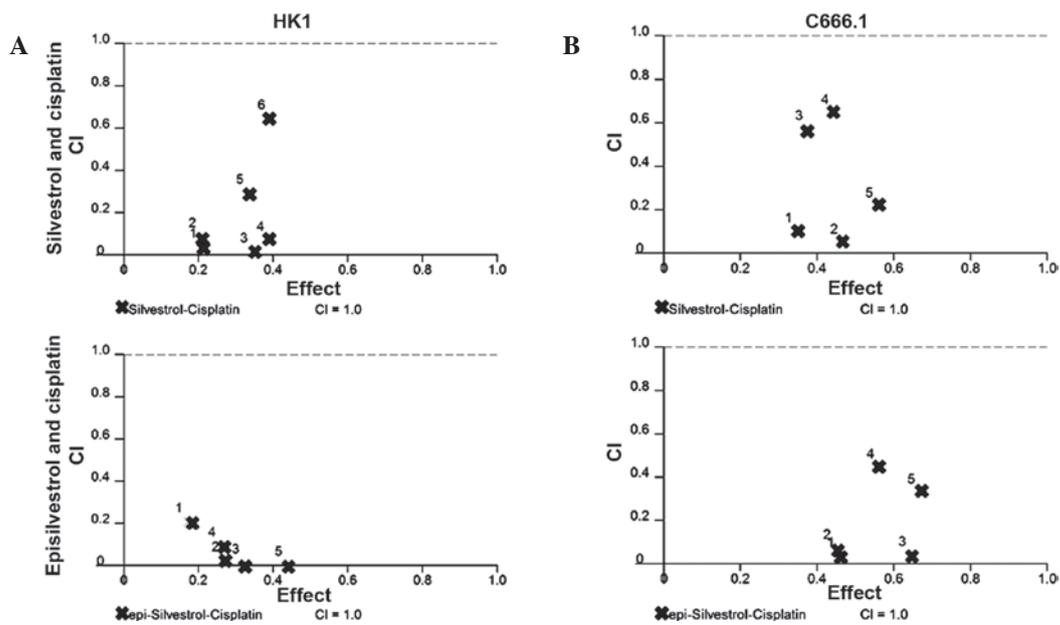


Figure 10. F_a -CI plots displaying the synergistic cytotoxic effect of 24 h simultaneous exposure to silvestrol or episilvestrol with cisplatin on (A) HK1 and (B) C666.1 cells. Values below the dashed line imply synergism. CI=1 is additive; CI>1 indicates antagonism. CI, combination index; F_a , fraction affected.

cells. Furthermore, silvestrol induced an apoptotic response. A previous study demonstrated that silvestrol induced cell growth arrest and apoptosis in AML cell lines and primary blasts (23). Another study reported the ability of silvestrol to inhibit eIF4F-dependent translation correlated with the ability to inhibit cell proliferation (24).

Concurrent chemotherapy and radiotherapy or chemoradiation is the standard treatment regimen for NPC. The method of treatment uses the chemotherapy drug cisplatin, 5-fluorouracil, or a combination of both in addition to radiotherapy to achieve local control of the disease (4). However, treatment of metastatic NPC remains a clinical challenge. One of the strategies currently under investigation involves the use of additional therapeutic agents in combination to the standard chemoradiation regimen to treat patients with metastatic NPC. The results of the present study demonstrated that silvestrol and episilvestrol, natural products from the *A. stellatopilosa* tree, exhibit *in vitro* synergism with cisplatin for the inhibition of NPC cell growth. Previous studies have shown that tumour cells can be sensitized by silvestrol to standard chemotherapeutic agents such as doxorubicin (8,25) or daunorubicin, etoposide or cytarabine-C (26), thereby producing improved therapeutic effects. One recent study demonstrated that the growth of hepatocellular cancer cell lines was inhibited by silvestrol, and that there was a synergistic effect of the compound with chemotherapy drugs sorafenib and rapamycin (27). The data obtained from the present investigation demonstrated that concomitant treatment of silvestrol or episilvestrol with cisplatin at various ratios showed marked synergistic growth inhibitory effects on NPC cells. The results also showed that the dose reduction index, which indicates how many fold the dose of each drug may be reduced in a synergistic combination compared with the dose of that drug alone, were >1. This is advantageous as a reduced dose leads to decreased toxicity in the host, while maintaining the same therapeutic efficacy (14). When administered as

a single agent to nude mice, *in vivo* anti-tumour effects of silvestrol were observed with no obvious toxicity (8). This allowed the use of silvestrol with established agents as a novel therapeutic strategy (9,26).

Silvestrol has been shown to exhibit *in vivo* anti-tumor activity in B-cell acute lymphoblastic leukemia and chronic lymphocytic leukemia (20). In a previous *in vivo* study, silvestrol administration suppressed the growth of MDA-MB-231 breast cancer and PC-3 human prostate cancer xenografts (8). Notably, vehicle and silvestrol-treated animals displayed similar blood cell profiles and silvestrol appeared to be well-tolerated in animals. However, the use of silvestrol in studies *in vivo* is hindered by poor absorption, distribution, metabolism and secretion and sensitivity to P-glycoprotein-mediated multidrug resistance (22). Gupta *et al* (28) described silvestrol as a substrate of phosphoglycolate phosphatase that was likely to result in the poor oral absorption of silvestrol observed in mice. Phosphoglycolate phosphatase efflux is a crucial aspect to overcome for the successful drug development of oral formulations (28). It was reported that intraperitoneal systemic availability was 100%; however, the oral bioavailability of silvestrol was markedly lower (29). A study conducting pharmacokinetics analysis of silvestrol via the development and validation of a sensitive LC-MS/MS method for accurate quantification of silvestrol in mouse plasma has previously been described (29). The data suggested an overall favorable pharmacokinetic profile of silvestrol in mice (29).

In conclusion, silvestrol and episilvestrol may function as adjunct therapeutic agents in the standard NPC treatment regimen of cisplatin and 5-fluorouracil. The synergism of these compounds with standard therapeutic agents may help in reducing drug toxicity in patients with NPC. Further investigation is required in order to understand the exact mechanism underlying the synergism between silvestrol or episilvestrol and cisplatin.

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