

Silvestrol inhibits nasopharyngeal carcinoma cells and synergizes with CX-5461: Insights from a proteomics study

MAELINDA DAKER^{1,2}, MUNIRAH AHMAD¹, ALAN SOO-BENG KHOO^{1,3,4}, LU PING TAN¹,
SIOK-FONG CHIN⁵, SIAW SAN HWANG² and PAUL M. NEILSEN⁶

¹Molecular Pathology Unit, Cancer Research Centre, Institute for Medical Research, National Institutes of Health, Ministry of Health, Shah Alam, Selangor 40170, Malaysia; ²School of Engineering and Science, Faculty of Engineering, Computing and Science, Swinburne University of Technology Sarawak Campus, Kuching, Sarawak 93350, Malaysia; ³School of Postgraduate Studies, International Medical University, Kuala Lumpur 57000, Malaysia; ⁴Department of Medical Oncology, Sidney Kimmel Medical College, Thomas Jefferson University, Philadelphia, PA 19107, USA; ⁵UKM Medical Molecular Biology Institute, National University of Malaysia, Cheras, Kuala Lumpur 56000, Malaysia; ⁶College of Clinical Sciences, School of Health, Medical and Applied Science, Central Queensland University, North Rockhampton, Queensland 4702, Australia

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Abstract. Nasopharyngeal carcinoma (NPC) is a cancer arising from the epithelial cells of the nasopharynx, which is rare in Western countries but extremely prevalent in Borneo and the Southern China region. Present-day hurdles in NPC treatment that lead to poor quality of life and poor survival include distant metastasis and resistance to chemoradiotherapy. Silvestrol and its 5"-epimer, episilvestrol, are compounds isolated from the plant *Aglaia stellatopilosa*, which is endemic to Borneo. Silvestrol, a protein synthesis inhibitor, preferentially inhibits the translation of cancer-associated mRNAs. CX-5461 is an inhibitor of RNA polymerase I that catalyzes rRNA synthesis. The present study sought to evaluate the potential of silvestrol as a new therapeutic for NPC with two authenticated NPC cell lines, C666-1 and HK1, and patient-derived xenografts. Concentration-response profiles were generated for silvestrol, episilvestrol, or CX-5461 as a single agent or in combination. Cell death and cell cycle progression studies were performed,

followed by proteomics profiling. The findings of the present study showed that silvestrol and episilvestrol, in the nM range, inhibited NPC cells and patient-derived xenografts. While CX-5461 alone induced S and G₂/M phase arrest in NPC cells, silvestrol or episilvestrol synergized with CX-5461 to arrest NPC cells at G₀/G₁ and increase NPC cell death. Differential protein expression profiling revealed that silvestrol-CX-5461 combination principally targeted processes critical to protein synthesis and downregulated proteins commonly upregulated in NPC, including eukaryotic initiation factor 5A (eIF5A), c-myc, and Cyclin D1. Combining a translation inhibitor with an rRNA synthesis inhibitor may synergize to prevent the synthesis of proteins that NPC cells depend on for growth and survival. Given that c-myc, Cyclin D1 and eIF5A are essential in cell cycle progression and cell proliferation, this finding puts forward a promising new synergistic treatment approach for NPC.

Introduction

In the Malaysian scenario, from 2012 to 2016, nasopharynx cancer was the fifth most common cancer amongst Malaysian men with an average age-standardized incidence rate (ASIR) of 5.2 per 100,000 (1). In Sarawak-a Malaysian region located in Borneo-there was a high incidence of nasopharynx cancer, particularly among males of the Bidayuh native ethnic group, with an ASIR of 24.6 per 100,000 (2). Other ethnic groups in Malaysia, such as Chinese, Malays, and other natives of Sarawak, for example, Iban and Melanau, are also at increased risk of nasopharynx cancer (2). From 2007 to 2011, the nasopharynx was the most common site of cancer among male residents of Sarawak, exceeding other major sites like colorectal and lung (2). Nasopharyngeal carcinoma (NPC) is a cancer of the nasopharynx's epithelial lining, located behind the back of the nose and above the oropharynx. NPC has a distinct geographical distribution pattern-highly prevalent in Southeast Asia, especially in Borneo, Southern China, North

Correspondence to: Dr Maelinda Daker, Molecular Pathology Unit, Cancer Research Centre, Institute for Medical Research, National Institutes of Health, Ministry of Health, 1 Jalan Setia Murni U13/52, Seksyen U13 Bandar Setia Alam, Shah Alam, Selangor 40170, Malaysia
E-mail: mae_cinnamon@yahoo.com

Abbreviations: ASIR, age-standardized incidence rate; CI, combination index; eIF5A, eukaryotic translation initiation factor 5A; IC₅₀, half maximal inhibitory concentration; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; NPC, nasopharyngeal carcinoma; PDX, patient-derived xenograft; UTR, untranslated region

Key words: c-myc, CX-5461, cyclin D1, eIF5A, episilvestrol, NPC, silvestrol, synergism, PDX

Africa, Greenland, and Alaska, but rare in other parts of the world (3,4). Among the multi-ethnic populations of Malaysia, the ASIR of NPC range extended from <1 to >30 per 100,000 (5). The etiology of NPC is multifactorial, including Epstein-Barr Virus (EBV) infection, genetic predisposition, diet, environmental exposure, and tobacco smoking (6). Radiotherapy is the primary treatment for NPC, whilst concurrent chemotherapy with radiotherapy is used for more advanced lesions (7). Yet, even today, most patients with late-stage NPC who have fully completed therapy, as per current standard practice, eventually succumb. Treatment resistance and metastasis remain clinical problems (8) and NPC may recur or progress when limited treatment options run out. In Malaysia, more than 60% of NPC cases were presented at late-stage, and the 5-year relative survival was 46% (1,9). Early detection would reduce late-stage cases. However, the strategies and cost-effectiveness of implementing screening exercises in regions with mixed high-risk and low-risk NPC populations await elucidation. Thus, it is imperative to discover new therapeutic strategies to improve treatment outcomes.

Silvestrol is isolated from *Aglaia stellatopilosa*, a species unique to Borneo, particularly in Sarawak, Sabah, and Kalimantan, but not Brunei. Apart from its timber for building, construction, and furniture, the genus *Aglaia* is known for compounds with insecticidal properties, anti-fungal, anti-viral, anti-bacterial, and anti-helminthic bioactivity (10). Silvestrol is a protein synthesis inhibitor, specifically targeting the eIF4A helicase to block the translation process (11). Silvestrol favors inhibition of 'weak' mRNAs [mRNAs with G+C rich, highly structured 5' untranslated regions (UTRs)] that encode short half-life proteins, including transcription factors, proto-oncoproteins and pro-survival factors including c-myc, Cyclin D1 and MCL1, without drastically influencing the translation of 'strong' mRNAs (mRNAs with short and unstructured 5'UTRs) that usually encode housekeeping genes like *ACTB* (β -actin) and *GAPDH* (12). Silvestrol is the first-in-class compound that can target the proto-oncogene c-myc. Cancer cells that depend on proteins with short half-lives may be susceptible to disruption of ongoing protein synthesis. *In vitro* evaluations identified silvestrol as a potent anti-proliferative agent, acting against hematological and solid tumor cells (13,14). Importantly, silvestrol with standard chemotherapeutic agents also showed *in vitro* synergistic effects in hematological and solid tumors (15,16). Silvestrol showed significant anti-neoplastic activity in lymphoma and solid tumor xenograft models (17,18). Toxicity studies mostly performed in murine models reported silvestrol to be well tolerated by test animals, with no observable adverse effects (19,20).

CX-5461 is a first-in-class small-molecule inhibitor of the synthesis of rRNA, the chief component of the ribosome. CX-5461 acts by suppressing RNA polymerase I (Pol I), the enzyme catalyzing rRNA synthesis (21). Just as with silvestrol, CX-5461 demonstrated *in vivo* antitumor activity in hematological and solid tumors (21-23). In a first-in-human, phase I dose-escalation study (24), CX-5461 was well tolerated in advanced hematologic malignancy patients, with no substantial hematologic toxicity presented by the patients. A total of 1 μ M CX-5461-treated normal bone marrow cells for two days exhibited minimal cell death.

Our previous study evaluated silvestrol and episilvestrol, isolated from *Aglaia stellatopilosa*-a plant genus found exclusively in Borneo- to treat NPC, a significant cancer in Borneo. The efficacy and synergistic effect of silvestrol and episilvestrol with cisplatin (a chemotherapeutic drug for NPC) in inhibiting NPC cell proliferation was revealed (25). However, the effects of silvestrol on its known and novel targets in NPC cells are yet to be known. In the present study, it was aimed to characterize the biological activities and protein expression changes exerted by silvestrol or episilvestrol in NPC as a single agent and with CX-5461. The present study is important to identify transcripts or proteins that could be inhibited by silvestrol or episilvestrol, which could then be sourced as a method to search for newer drug targets and to develop new drugs for the treatment of NPC.

Materials and methods

Cell lines and cell culture. C666-1 was acquired from Professor Kwok Wai Lo (Chinese University of Hong Kong, Hong Kong, China). HK1, NP69SV40T, NP460hTert and NPC43 cell lines were obtained from the deceased Professor Sai Wah Tsao (University of Hong Kong, Hong Kong, China). C666-1 was maintained in RPMI-1640 medium complete with 10% heat-inactivated fetal bovine serum (FBS), 10 U/ml penicillin, 10 μ g/ml streptomycin and 1X GlutaMAX™ Supplement (all from Gibco; Thermo Fisher Scientific, Inc.). HK1 was maintained in RPMI-1640 medium, supplemented with 10% FBS, 10 U/ml penicillin, and 10 μ g/ml streptomycin. NP69SV40T was cultured in keratinocyte serum-free medium (KSFM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 25 μ g/ml bovine pituitary extract and 0.2 ng/ml recombinant epidermal growth factor (Gibco; Thermo Fisher Scientific, Inc.). NP460hTert was sustained in a 1:1 ratio of defined KSFM supplemented with growth factor (Gibco; Thermo Fisher Scientific, Inc.) and EpiLife® Defined Growth Supplement (Cascade Biologics™; Thermo Fisher Scientific, Inc.). Cells were incubated in a 5% CO₂ humidified atmosphere at 37°C. The establishment and characterization of C666-1 (26), HK1 (27), NP69SV40T (28) and NP460hTert (29) were previously described. Cell line authentication was performed by DNA fingerprinting using AmpFLSTR Identifier PCR Amplification Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). Short tandem repeats of the cell line were consistent with published data (30). Detection of mycoplasma contamination was conducted routinely using e-myc Mycoplasma PCR Detection Kit (Intron Biotechnology, Inc.). Only mycoplasma contamination-free cells were used in the present study.

Chemicals. Silvestrol and episilvestrol (C₃₄H₃₈O₁₃; molecular weight, 654.66 Da) were isolated by Cerylid BioSciences Pty Ltd., and purified to 99% purity by Sarawak Biodiversity Centre (Kuching, Sarawak, Malaysia). Stock solutions (1,500 μ M) of silvestrol and episilvestrol were prepared in cell-culture-grade dimethyl sulfoxide (DMSO; MilliporeSigma) and kept in a non-auto-defrost -20°C freezer. CX-5461 was from Selleck Chemicals. CX-5461 was dissolved in 50 mM NaH₂PO₄ (pH=4.5; MilliporeSigma).

Assessment of concentration-dependent and time-dependent effects of silvestrol, episilvestrol and CX-5461 in nasopharyngeal epithelial cells and NPC cells by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. C666-1 (30,000 cells), HK1 (10,000), NP69SV40T (15,000) and NP460hTert (15,000) cells were seeded into 96-multi-well plates at 50 μ l/well and returned into the incubator at 37°C in a 5% CO₂ atmosphere for 2 h. Solutions of silvestrol or episilvestrol (99% purity; 0.8-200 nM) or CX-5461 (0.15-20 μ M) were prepared and dispensed into the 96-multi-well plates at 50 μ l/well. The multi-well plates were returned to the incubator for 1-3 days. One 96-multiwell plate was allocated for each assessment day. After 1-3 days of incubation, 20 μ l of CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS; Promega Corporation) reagent was added to each well, and the multi-well plates were returned to the incubator for 1-4 h, according to the optimal incubation time for MTS reagent. Absorbance was read at 490 nm and subtracted from background absorbance at 630 nm using an EnVision multilabel plate reader (PerkinElmer, Inc.), as previously described (31). DMSO at 0.1% final concentration (NaH₂PO₄ for CX-5461) functioned as vehicle control. Media without cells functioned as blanks. The percentage of MTS signal was calculated and compared with vehicle control. Concentration-response curves were plotted, and the concentration that inhibited 50% of the MTS signal (IC₅₀) was obtained using GraphPad Prism version 6.07 for Windows (GraphPad Software; Dotmatics).

Identification of synergism between CX-5461 with silvestrol or episilvestrol in NPC cells. To ensure experimental conditions were consistent throughout, 96-multiwell plates were allocated concurrently for single-drug and two-drug. C666-1 (30,000 cells) and HK1 (10,000) were seeded into 96-multiwell plates at 50 μ l/well and returned to the incubator for 2 h. Single-drug and two-drug solutions were prepared and diluted serially so that the mixtures remained at a constant ratio, for example, 2X IC₅₀, 1X IC₅₀, 0.5X IC₅₀, and 0.25X IC₅₀; thereby, each compound contributed equally in each combination (32). Solutions were dispensed at 50 μ l/well. The 96-multiwell plates were incubated for 3 days, and subsequently, the MTS assay, as aforementioned, was carried out. Concentration-response curves and combination index (CI) were generated using CalcuSyn Version 2.11 (Biosoft). Combination interaction was determined by the CI method (32) where CI >1 denotes antagonism, CI=1 is additive, and CI <1 is synergism.

Assessment of concentration-dependent effect of silvestrol and CX-5461 in patient-derived xenograft (PDX) by MTS assay. Four patient-derived xenografts (PDXs), namely Xeno-284, Xeno-B110, Xeno-B110-gfp-luc2, and Xeno-G517, were established in NOD SCID Gamma (NSG) immunodeficient mice. PDXs were harvested and washed twice using 1X Phosphate Buffered Saline (PBS; Takara Bio Inc.) supplemented with Antibiotic-Antimycotic (Gibco; Thermo Fisher scientific, Inc.). PDXs were dissociated to become PDX short-term *in vitro* cell culture, according to a previous study (33). Tissue dissociation enzymes were: 1 mg/ml Collagenase/Dispase®

(Roche Diagnostics) for Xeno-284; 2 mg/ml Collagenase from *Clostridium histolyticum* (MilliporeSigma) with 200 U/ml DNase I Solution (Thermo Scientific™; Thermo Fisher Scientific, Inc.) for Xeno-B110, Xeno-B110-gfp-luc2 and Xeno-G517. PDXs were minced using a Size-10 scalpel, followed by orbital shaking inside a 5% CO₂ incubator at 37°C for 2.5 h, after which enzymatic activity was neutralized with an equal volume of complete growth media. Formulation of complete growth media was achieved with: RPMI-1640 medium, 1X GlutaMAX™ Supplement, 1X Antibiotic-Antimycotic, 1X B-27 supplement, 1X Insulin-Transferrin-Selenium, 7.5% FBS, 10 ng/ml recombinant human fibroblast growth factor-basic, and 10 ng/ml recombinant human epidermal growth factor (all from GIBCO); 0.5 mg/ml Hydrocortisone and 10 μ M ROCK inhibitor (Y-27632) (all from Sigma-Aldrich; Merck KGaA). The consequential suspension was filtered through a 40 μ m cell strainer (Falcon®; Corning, Inc.). The filtrate was centrifuged at 132 x g for 5 min, then the supernatant was decanted. Up to 8 ml RBC Lysis Solution (Thermo Fisher Scientific, Inc.) was added, tubes were centrifuged, and the supernatant was decanted. RBC-free pellet was resuspended in PBS supplemented with Antibiotic-Antimycotic. This suspension was subjected to magnetic labelling and separation, following instructions from the Mouse Cell Depletion Kit (MACS; Miltenyi Biotec GmbH). Finally, the flow-through, enriched for PDX cells, was centrifuged, the supernatant decanted, and then the pellet resuspended in complete growth media. Xeno-284 (10,000 cells), Xeno-B110 (20,000), Xeno-B110-gfp-luc2 (15,000), and Xeno-G517 (10,000) were seeded into fibronectin-coated 96-multi-well plates at 50 μ l/well. Solutions of silvestrol (0.78-25 nM) and CX-5461 (0.23-7.5 μ M) were prepared and dispensed at 50 μ l/well. After 3 days of incubation, the aforementioned MTS assay was carried out. Concentration-response curves were plotted, and IC₅₀ was obtained using GraphPad Prism.

During the establishment of PDXs, NSG immunodeficient mice were housed in individually ventilated cages (IVCs) with paper chip bedding and a small amount of nesting material to reduce stress. Food and water were provided *ad libitum*, and all husbandry materials, including food and water, were sterile. To prevent contamination, acidified water was used. Each cage housed a minimum of one and a maximum of three mice. The animal room and IVC system were maintained at a temperature of 21-23°C, with relative humidity between 50-70%, and a 12/12-h light/dark cycle. For subcutaneous inoculation, analgesics were not administered because the procedure is minimally invasive and typically does not induce prolonged pain or distress. For subcutaneous inoculation, mice were sedated using isoflurane, which helps reduce stress by immobilizing the animals temporarily and preventing pain during the brief procedure. Isoflurane was administered via inhalation. In total, ~1 drop of isoflurane was applied to a piece of cotton placed inside a 15 ml tube, which served as a nose cone. Animal general health and behavior were monitored daily, with specific checks for food and water intake. Additionally, body scoring and tumor measurements were conducted three times per week. More than 15 mice were used. The establishment of PDXs lasted 21-30 days after subcutaneous tumor cell inoculation. Euthanasia was performed when tumors reached the required size for collection or on mice that did not show

tumor growth 150 days after subcutaneous tumor cell inoculation. Euthanasia via cervical dislocation (physical method) or CO₂ Chamber System was performed by trained personnel as per the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) recommendation and American Veterinary Medical Association (AVMA) guidelines. Deaths are rare but may occur due to fighting between mice. Death in mice was confirmed first with the absence of reflexes, such as a lack of response to toe pinch. Once unconsciousness was verified, cervical dislocation was performed as a secondary physical method to ensure death. This combination of reflex testing and physical confirmation is in line with accepted ethical and institutional guidelines to minimize animal suffering and ensure humane endpoints. Additionally, mice that showed any of the following signs would be euthanized: i) Weight loss of 15-20% or more; ii) severe signs of illness, such as dehydration, hunched posture, rough coat, trouble breathing, or not moving normally; iii) tumors that are too large (>1,500 mm³), ulcerated, or affecting movement; iv) not eating or drinking for 1-2 days; v) severe pain or distress (for example, self-injury, constant vocalizing); vi) no improvement after treatment within 2-3 days and vii) signs of severe disease, such as seizures, paralysis, or infection.

Identification of synergism in Xeno-G517 between CX-5461 with silvestrol. Xeno-G517 (10,000 cells) was used as a validation model for NPC PDX cells. The method for identification of synergism, as aforementioned, was repeated. Single-drug and two-drug solutions at 0.125-fold to 2-fold IC₅₀ value of each drug were assessed.

Flow cytometry analysis of cell viability and cell cycle progression. Hypotonic staining buffer was prepared from 500 ml autoclaved ddH₂O, 50 mg Propidium iodide (Merck KGaA), 10 mg Ribonuclease A (MP Biomedicals), 500 mg Sodium citrate (MilliporeSigma), and 1.5 ml Triton-X (MP Biomedicals, LLC). HK1 was seeded into 12-multiwell plates at 1.0x10⁵ cells/0.5 ml/well. Multiwell plates were returned to the incubator for 2 h. Solutions of silvestrol (5 nM), episilvestrol (10 nM), CX-5461 (1 μM), or combinations (1-fold IC₅₀ value of each drug) were prepared, then dispensed into the plates at 0.5 ml/well. After 2-3 days of incubation, cells (including any floating cells) were pelleted, resuspended in 500 μl of hypotonic staining buffer, stored on ice, and protected from light for 10 min before analysis on BD FACSCalibur (BD Biosciences). At least 10,000 events per sample were acquired with the CellQuest Pro version 6.0 (BD Biosciences) acquisition and analysis software, and the median fluorescence intensity was examined. The CellQuest Pro acquisition and analysis software gated out debris from cells active in different phases of the cell cycle, which were reported as the percentage of total events. DNA content pattern in the flow cytometry histograms was analyzed using ModFit LT™ software version 3.3.11 (Verity Software House).

FITC Annexin V/Propidium iodide (PI) apoptosis assay for flow cytometric analysis. HK1 was seeded into 12-multiwell plates at 1.0x10⁵ cells/0.5 ml/well. Plates were returned to the incubator for ~2 h. Solutions of silvestrol (5 nM), episilvestrol (10 nM), CX-5461 (1 μM), or combinations (1-fold IC₅₀ value

of each drug) were prepared, then dispensed into the plates at 0.5 ml/well. After 3 days of incubation, cells (including any floating cells) were harvested for assay using the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen; BD Biosciences). Cell pellets were stained according to the manufacturer's instructions, with slight modifications. Briefly, cell pellets were added with 100 μl 1X Annexin V Binding Buffer, then 5 μl FITC Annexin V, followed by 5 μl PI. Cells were gently vortexed and incubated for 15 min in the dark. A total of 400 μl of 1X Annexin V Binding Buffer was added, then the total volume was transferred into flow tubes and maintained cold on ice prior to analysis on the BD FACSCalibur. The software for data acquisition and analysis was BD CellQuest Pro version 6.0. In total 10,000 events were acquired per sample.

Preparation of cell pellet for protein analysis. C666-1 (3.0x10⁵ cells), HK1 (1.0x10⁵) and NP69SV40T (1.5x10⁵) were seeded into 12-multiwell plates at 0.5 ml/well, then returned to the incubator for about 2 h. Solutions of silvestrol (2.5-5 nM), CX-5461 (0.25-1 μM), or the corresponding combinations (2.5-5 nM silvestrol + 0.25-1 μM CX-5461) were prepared, then dispensed into the plates at 0.5 ml/well. After 40 h of incubation, cells (including any floating cells) were pelleted. Pellets were immediately stored at -80°C for mass spectrometry or western blot analysis.

Mass spectrometry analysis. Pellets were disrupted using an ultrasonicator (Misonix, Inc.) in cold 40 mM Tris-HCL, pH 7.4 buffer with 3% of DL-Dithiothreitol. Protein concentration was determined based on the Bradford method. For each sample, 20 μg protein was reduced, alkylated, and digested with trypsin. The obtained peptides were concentrated, desalted, and purified with Pierce® C18 Tips (Thermo fisher Scientific, Inc.) before vacuum drying using Concentrator plus™ (Eppendorf). Peptides were reconstituted in 0.1% formic acid in a sample vial, then subjected to analytical separation and analysis using an Eksigent ekspert™ nanoLC 415 system coupled to a TripleTOF® 5600+ System (SCIEX). Peptides were separated on a 75 μm x 15 cm ChromXP C18-CL 3 μm 120Å cHiPLC column in a 155-min gradient run (buffer A was 0.1% v/v formic acid, 2% v/v acetonitrile; buffer B was 0.1% v/v formic acid, 90% v/v acetonitrile) at 300 nl/min flow rate. Mass spectra were acquired in data-dependent mode (250 ms accumulation time per spectrum and mass range of 300-1,250 m/z) to obtain MS/MS spectra for the most abundant parent ions following each survey MS1 scan. For each mass spectrum, a maximum of 50 precursors with a charge state between 2 and 4 were selected for fragmentation. The tandem mass spectrum was acquired in high-sensitivity mode, in which signals were accumulated for a minimum of 50 msec per spectrum, and the dynamic exclusion time was set at 24 sec. Spectral data files were submitted to MaxQuant software version 1.6.0.1 (Max Planck Institute of Biochemistry) for simultaneous database matching for NPC and normal nasopharyngeal epithelial cells. The Human Uniprot fasta file was used for database searching. Trypsin digestion enzyme was selected as the cleaving agent, and a maximum of two missed cleavages were accepted. Variable modifications for the searches were acetyl (N-terminal) and oxidation (methionine), with a maximum of five modifications per peptide. Carbamidomethyl (C) was

Table I. IC₅₀ value of silvestrol, episilvestrol, and CX-5461 in NPC cell lines or nasopharyngeal epithelial cells, at 1-3 days post-treatment.

Cell line	Day(s) post-treatment	IC ₅₀ value		
		Silvestrol (nM)	Episilvestrol (nM)	CX-5461 (μM)
NPC	C666-1	1	68	56
		2	7	18
		3	5	10
	HK1	1	132	60
		2	7	13
		3	5	10
Nasopharyngeal epithelium	NP69SV40T	1	58	68
		2	8	19
		3	5	10
	NP460hTert	1	9	14
		2	9	20
		3	5	10

NPC, nasopharyngeal carcinoma; IC₅₀, half maximal inhibitory concentration; ND, not done.

Table II. IC₅₀ value of silvestrol and CX-5461 in PDXs, at 3 days post-treatment.

PDXs	IC ₅₀ value	
	Silvestrol (nM)	CX-5461 (μM)
Xeno-284	6	0.3
Xeno-B110	2	0.4
Xeno-B110-gfp-luc2	4	0.5
Xeno-G517	4	0.4

PDXs, patient-derived xenografts; IC₅₀, half maximal inhibitory concentration.

selected as a fixed modification. Initial search peptide tolerance was set at 0.07 Da, and fragment mass tolerance was at 0.006 Da. False discovery rates for peptide spectral matches and proteins were set to 0.01. Label-free quantification was set to a minimum ratio count of two, with normalization. Minimal peptide length was set to seven amino acids. A match between runs was selected for each experiment, and a match time window of 0.7 min was used. Under the identification tab, the minimum razor peptide was set to one. A minimum score of 40 was accepted for modified peptides.

A paired t-test was performed to find out if the fold change of each protein from the treated sample was significantly different from that of the untreated sample. Proteins were sorted and selected based on P<0.05, fold change <0.625 for downregulated proteins, or P<0.05, fold change >1.6 for upregulated proteins. Gene Ontology (GO) functional and integrative analyses were carried out using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) Knowledgebase v2023q4 (<<https://davidbioinformatics.nih.gov/>>)

(34,35), and overlapping protein IDs were identified using GeneVenn (<<http://genevenn.sourceforge.net/>>) (36).

Western blotting. Fresh 1X RIPA buffer was prepared daily from 1X RIPA (Cell Signaling Technology, Inc.), 1X protease/phosphatase inhibitor cocktail (Cell Signaling Technology, Inc.), 1 mM PMSF protease inhibitor (Thermo Fisher Scientific, Inc.), and 5 mM DTT (Bio-Rad Laboratories, Inc.). Pellets were vortexed vigorously in 1X RIPA buffer, followed by sonication, rocking on ice flakes in a 4°C chiller for 30 min, and cold centrifugation for 30 min. The supernatant was subjected to protein concentration assessment using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Inc.). Measurement at 595 nm was achieved on a SmartSpec Plus Spectrophotometer (Bio-Rad Laboratories, Inc.). Gel electrophoresis was performed on NuPAGE™ 4-12% Bis-Tris Protein Gels, 1.0 mm, 10-well with 1X NuPAGE™ MOPS SDS Running Buffer using the XCell SureLock™ Mini-Cell (all from Invitrogen; Thermo Fisher Scientific, Inc.). Gel lanes were loaded with samples of equal protein concentrations (23 μg) and 10 μl Amersham™ ECL™ Rainbow™ Marker-Full Range (Cytiva). After the electrophoresis run, dry electroblotting of proteins was performed using iBlot™ Dry Blotting System (Invitrogen; Thermo Fisher Scientific, Inc.) comprising of iBlot™ Gel Transfer Device and iBlot™ Gel Transfer Stacks (nitrocellulose, mini or regular size), as per the user guide. Membranes were blocked at room temperature on a rocker with Immobilon® Block-CH Chemiluminescent Blocker (Merck KGaA) for 1 h, rinsed in 1X Tris Buffered Saline with 0.1% Tween® 20 (TBST; Cell Signaling Technology, Inc.), incubated overnight at 4°C with primary antibodies, rinsed in 1X TBST, and then incubated for 1 h at room temperature with secondary antibodies. Primary antibodies were c-myc (1:10,000; cat. no. ab32072; Abcam), Cyclin D1 (1:10,000; cat. no. ab134175; Abcam), eIF5A (Cell Signaling Technology, Inc.; 1:1,000; cat. no. 20765) and β-actin (1:1,000; cat. no. 12620; Cell Signaling

Table III. Combination interaction description of each CI observed in C666-1 and HK1.

Cell line	Compound dilution at equipotency ratio ($\times$ IC ₅₀)		Combination index	Interaction
C666-1	Silvestrol	CX-5461		
	0.125	0.125	0.489	Synergism
	0.25	0.25	0.596	Synergism
	0.5	0.5	0.528	Synergism
	1	1	0.463	Synergism
	Episilvestrol	CX-5461		
	0.125	0.125	0.293	Strong synergism
	0.25	0.25	0.524	Synergism
	0.5	0.5	0.717	Moderate synergism
	1	1	0.993	Nearly additive
HK1	Silvestrol	CX-5461		
	0.125	0.125	0.526	Synergism
	0.25	0.25	0.440	Synergism
	0.5	0.5	0.393	Synergism
	1	1	0.189	Strong synergism
	Episilvestrol	CX-5461		
	0.25	0.25	0.658	Synergism
	0.5	0.5	0.487	Synergism
	1	1	0.346	Synergism

IC₅₀, half maximal inhibitory concentration.

Technology, Inc.). The secondary antibody was anti-rabbit IgG, HRP-linked (1:2,000; cat. no. 7074; Cell Signaling Technology, Inc.). Membranes were rinsed in 1X TBST. The protein bands were detected via chemiluminescence with Luminata™ Classico Western HRP Substrate (Millipore) on ImageQuant™ LAS 500 (Cytiva). Densitometric analysis of protein bands was performed using ImageQuant™ TL version 8.1 Image Analysis Software (Cytiva).

Statistical analysis. Differences between mean values were evaluated with one-way analysis of variance and multiple comparisons analysis, using IBM® SPSS® Statistics for Windows, Version 28 (IBM Corp.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Assessment of concentration-dependent and time-dependent effects of silvestrol, episilvestrol, and CX-5461 in nasopharyngeal epithelial cells, NPC cells and PDX. The efficacies of silvestrol (Fig. S1A) and its 5'''-epimer, episilvestrol (Fig. S1B), in inhibiting NPC cells and PDX were evaluated. Overall, IC₅₀ values of silvestrol and episilvestrol were in the lower nM concentrations, whereas IC₅₀ values of CX-5461 were between 0.4–6 μ M. Silvestrol was more potent than episilvestrol, except at 1-day post-treatment in NPC cell lines. Generally, silvestrol and episilvestrol had decreasing IC₅₀ values with increasing treatment time. Cells of nasopharyngeal epithelial lines representing normal controls

had similar IC₅₀ values relative to NPC cell lines (Table I). Likewise, silvestrol inhibited NPC PDX cells at low nM concentrations, while CX-5461 was consistently effective at 0.3–0.5 μ M (Table II).

Identification of synergism between CX-5461 with silvestrol or episilvestrol in NPC cells and PDX. Synergistic activity by silvestrol or episilvestrol with CX-5461 was examined in two NPC cell lines, C666-1 and HK1, and one NPC PDX model, Xeno-G517. Most of the dilutions tested yielded synergistic combination interaction in NPC cell lines, albeit to a different extent in NPC cell lines (Table III), but consistent strong synergism was observed in Xeno-G517 (Table IV).

Flow cytometric analysis of cell viability and cell cycle progression. Untreated cells and cells in vehicle controls had very similar sub-G₁ profiles (Fig. S2) and cell cycle distribution (Fig. S3). Compared with untreated control, combination treatments yielded a 4-fold increase in the percentage of cells at the sub-G₁ phase of the cell cycle (Fig. 1), which suggested that combination treatments increased cell death. In HK1, 1 μ M CX-5461 arrested cells at S-phase and G₂/M, while silvestrol-, episilvestrol-, and combination-treatment arrested cells at G₀/G₁ (Fig. 2).

FITC Annexin V/Propidium iodide (PI) apoptosis assay for flow cytometric analysis. To evaluate if apoptosis was induced, which could have contributed to inhibition by silvestrol, episilvestrol, or CX-5461, apoptotic cells were

Table IV. Combination interaction description of each CI observed in Xeno-G517.

Compound dilution at equipotency ratio (x IC ₅₀)		Combination index	Interaction
Silvestrol	CX-5461		
0.125	0.125	0.254	Strong synergism
0.25	0.25	0.295	Strong synergism
0.5	0.5	0.257	Strong synergism
1	1	0.157	Strong synergism
2	2	0.174	Strong synergism

IC₅₀, half maximal inhibitory concentration.

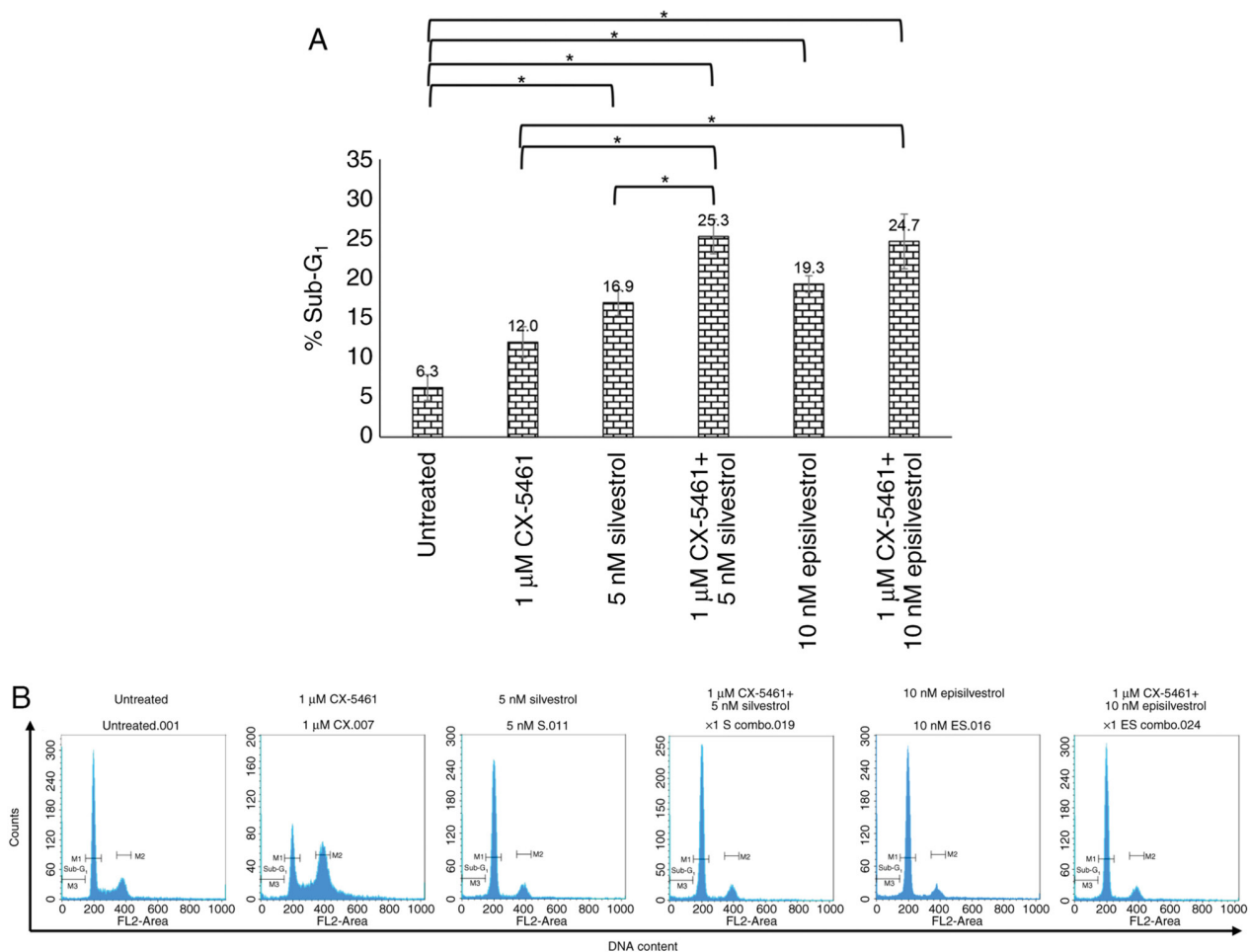


Figure 1. Flow cytometry analysis of HK1 cells treated with silvestrol, episilvestrol, CX-5461, or combinations, at 3 days post-treatment. (A) Percentage sub-G₁ in HK1. The data shows mean $\pm$ SEM. Values above each bar refer to the percentage sub-G₁. (B) Histograms of DNA content of HK1. Events below the G₀/G₁ peak were labelled as sub-G₁ and encompass dead cells. The x-axis annotated by FL2-Area represents DNA content. *P<0.05.

identified by flow cytometry. The percentage of apoptotic cells in the vehicle controls and untreated cells remained <10% (Fig. S4). The percentage total apoptosis in HK1 remained <20% after 3 days in 1 μ M CX-5461 (Fig. 3A), hinting that apoptosis is not the mode of action of CX-5461. The present results were in concordance with a previous study, which stated that apoptosis was not the key pathway through which CX-5461 influenced cellular viability in solid

tumor cell lines (23). HK1 cells cultured in combinations for 3 days produced a 6- to 7-fold higher percentage of total apoptosis compared with the untreated control and a 3-fold higher percentage of total apoptosis compared with CX-5461-treatment (Fig. 3A and B).

Identification of differentially expressed proteins as novel targets of combination therapy. As a single agent,

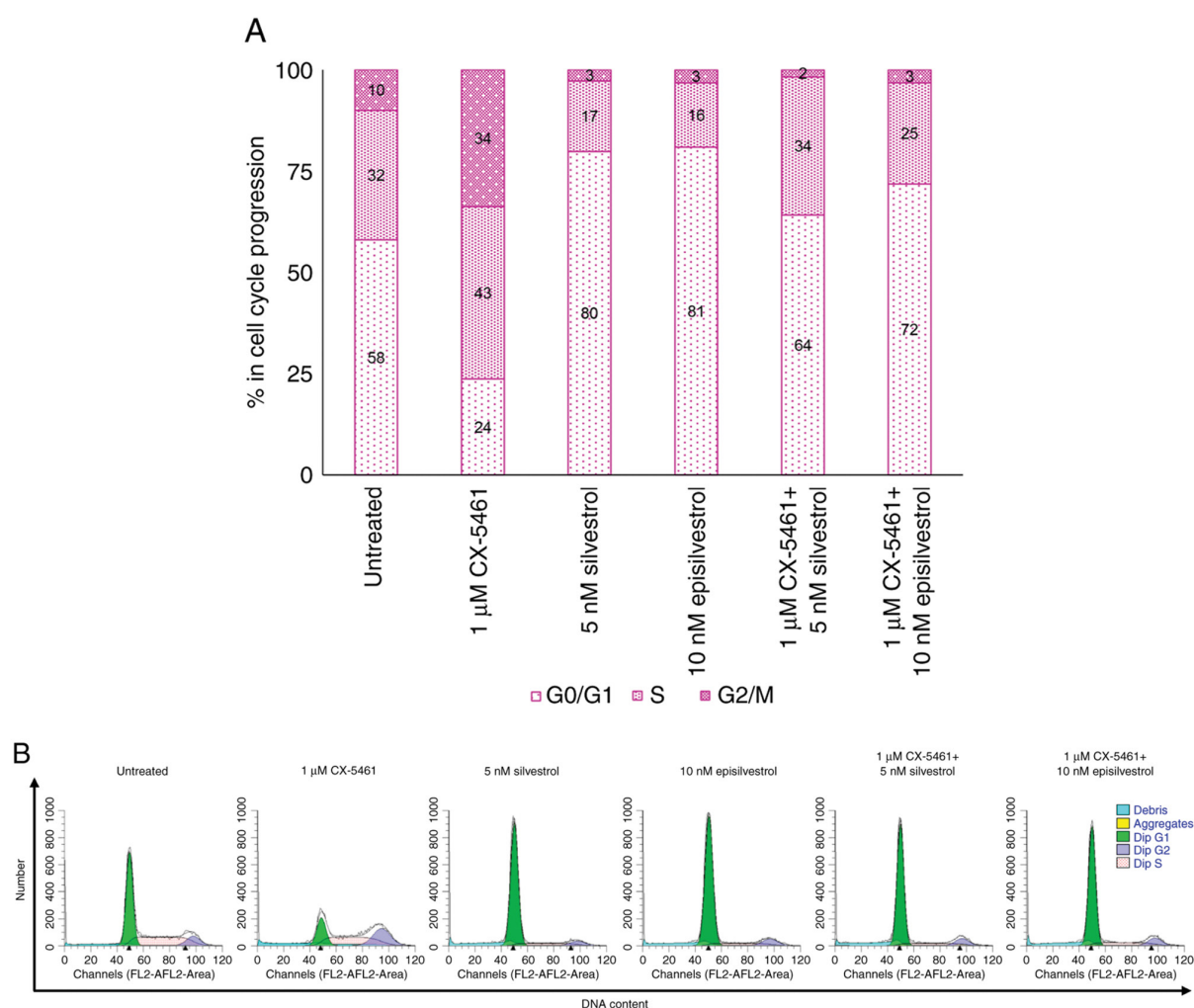


Figure 2. Flow cytometric analysis of cell cycle progression. (A) Percentage cell cycle progression of HK1 cells in the presence of CX-5461, silvestrol, episilvestrol, or combinations, at 2 days post-treatment. The data shown is the mean. Values rounded off to the nearest whole number within each bar refer to the percentage of cells in a specific cell cycle phase. (B) Representative histograms displaying the proportion of cells in G_1 , S and G_2 phases.

silvestrol, compared with episilvestrol, exhibited greater efficacy at inhibiting NPC cells (Table I). Henceforth, all subsequent experiments focused on silvestrol. Mass spectrometry-based quantitative proteomics was engaged in characterizing protein expression changes instigated by silvestrol-CX-5461 combination-treatment in C666-1, HK1 and NP69SV40T cells. Upon combination-treatment, no protein was consistently increased, while the abundance of seven proteins was consistently decreased in both NPC cell lines but not in normal nasopharyngeal epithelial cells (Fig. 4A, Table V). GO analysis of these seven proteins suggested their involvement in ribosome binding, RNA binding, mRNA transport and protein translation (Fig. 4B).

Western blot analysis showed that NPC cell lines had abundant levels of eIF5A compared with normal nasopharyngeal epithelial cells (Fig. 5A). Downregulation of eIF5A by combination-treatment was apparent in HK1 and less drastically observed in C666-1 (Fig. 5B). Additionally, it was found that silvestrol-treatment and combination-treatment consistently reduced c-myc and cyclin D1 levels in both NPC cell lines (Fig. 5C).

Discussion

NPC is a unique cancer with a heavy disease burden due to recurrence and metastasis, especially in Southeast Asia. To overcome resistance to targeted inhibitors, it is important to discover therapies that are specific and synergistic using cocktails of multiple inhibitors. The present study explored the efficacy, synergistic effect, apoptosis induction, cell cycle arrest, and protein expression changes exerted by silvestrol-CX-5461 combination-treatment in NPC. The approach of the present study is novel and unique because the production shutdown of transcription factors and oncoproteins has not been possible with other inhibitors.

Overall, silvestrol was more potent than episilvestrol; except at 1-day post-treatment in C666-1 and HK1, which was not uncommon, and was also observed elsewhere (25). At 3 days post-treatment, silvestrol had a markedly lower IC_{50} value relative to episilvestrol, suggesting stereoisomerism may have a role in efficacy. Concurrent treatment of silvestrol or episilvestrol with CX-5461 was synergistic and highly reproducible in NPC cells and NPC PDX. Synergism is important to cut down drug doses administered and eventually reduce toxic side effects

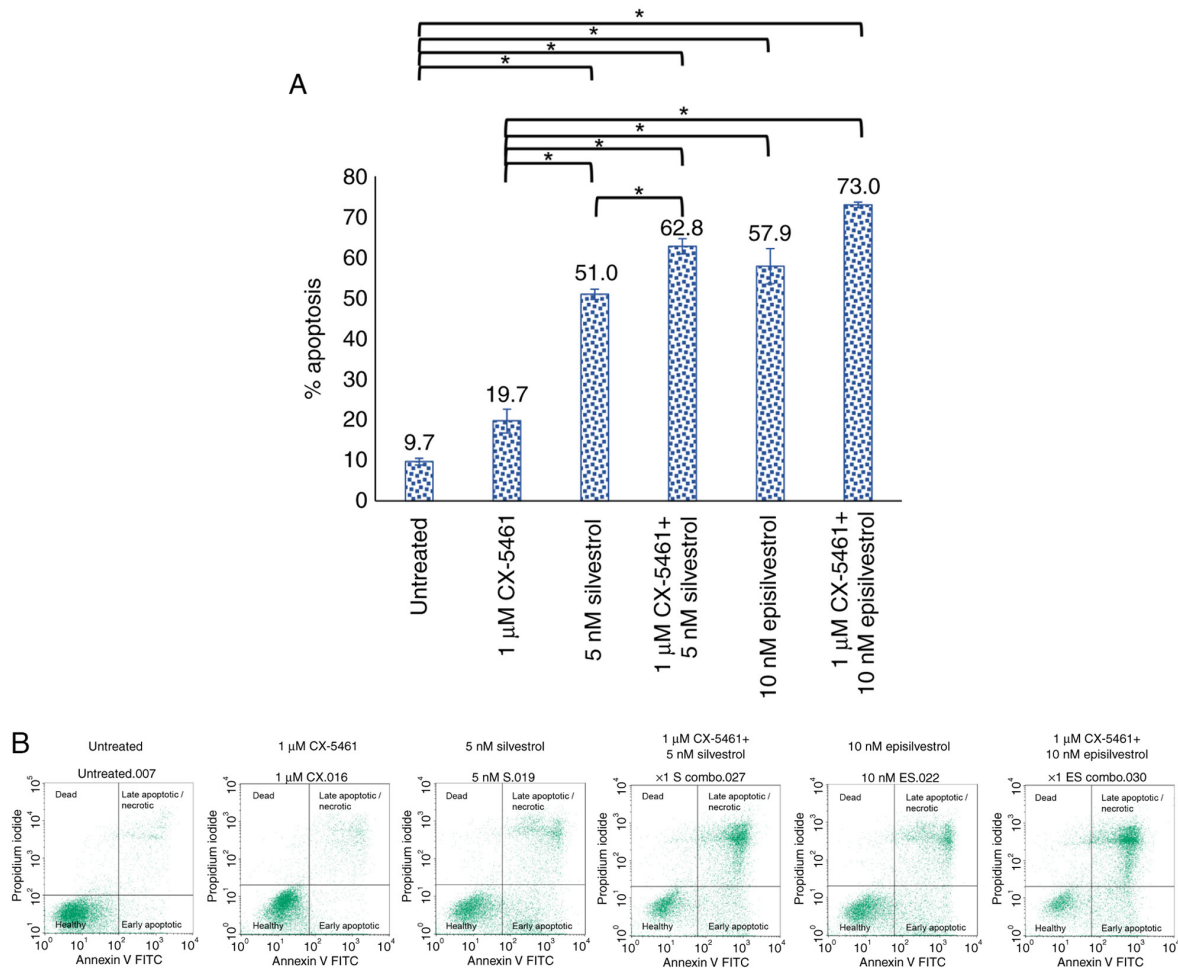


Figure 3. Flow cytometric apoptosis assay of HK1 cells treated with silvestrol, episilvestrol, CX-5461, or combinations, at 3 days post-treatment. (A) Percentage of apoptosis in HK1 measured using FITC annexin V and propidium iodide double staining. The data show mean $\pm$ SEM. Values above each bar refer to the percentage of total apoptosis. (B) Dot plots showing apoptotic, necrotic, dead and healthy events. * $P < 0.05$.

while simultaneously ensuring therapeutic efficacy is maintained. The use of multiple drugs with distinct modes of action may affect multiple targets and subpopulations concurrently, hence are more likely to increase therapeutic efficacy (32,37).

PDX is useful for performing experiments that would be unfeasible in humans, for example, pre-clinical cancer modelling for anticancer drug response assessment and drug development. To generate PDX, animals were implanted with human tumor tissue, not cell cultures that have been increasing indefinitely. To maintain PDX, tumor fragments were passed from animal to animal. PDX has gained interest because PDX closely resembles the original tumor in terms of morphology, histology and molecular profiling. Moreover, patient-derived cells grow serum-free *in vitro*, hence are highly relevant and representative of tumor growth in real-life. By contrast, established cell lines require growth media supplemented with serum; therefore, nutrients are abundantly available. Importantly, given the failure of treatment and because of metastasis, PDX allow the exploration of novel drugs or combinations for new and effective therapeutic approaches (38-40).

Studies of cell cycle distribution are essential to assess the cytostatic potential of candidate anticancer drugs. Furthermore, cell cycle investigation of cytotoxicity is important because drugs may block cell cycle progression, leading

to cell death. Proliferating cells progress through the cell cycle in the following sequence: G₀, G₁, S, G₂, and M phases. Progression through the phases of the cell cycle is facilitated by the cyclin-dependent kinase (Cdk) family of serine/threonine protein kinases and their regulatory subunits-the cyclins. Cdk4 and Cdk6 are activated by Cyclin D, and cells advance from G₀ into G₁. There are three types of Cyclin D: Cyclin D1, D2 and D3. Cyclin D1 facilitates the transition from G₁ to S-phase. The cyclin D1/Cdk4 or Cyclin D1/Cdk6 complexes are formed in early- or mid-G₁-phase. Cdk of the G₁-phase phosphorylates the retinoblastoma protein, pRb, a tumor suppressor and an anti-apoptotic factor, causing functional inactivation. The transition from one cell cycle phase to the next is maintained by checkpoints. pRb is important at the G₁ checkpoint and prevents progression into S-phase by binding to E2F transcriptional factors. Phosphorylation of pRb in early G₁ releases E2F, allowing transition into S-phase and DNA synthesis. Cyclin D1 overexpression results in continuous phosphorylation of pRb, giving rise to tumorigenesis (41,42).

The present study demonstrated the reduction of Cyclin D1 level in silvestrol-treated NPC cells and its apparent abolishment in combination-treated cells. This is a significant finding because NPC samples frequently demonstrated amplification of the Cyclin D1 gene, *CCND1* (43). In NPC,

Table V. Protein ID, gene symbol, and gene name of the seven key proteins significantly downregulated in NPC cells by silvestrol-CX-5461 combination-treatment.

UNIPROT accession	Gene symbol	Gene name
P55884	EIF3B	Eukaryotic translation initiation factor 3 subunit B
P63241	EIF5A	Eukaryotic translation initiation factor 5A
Q9GZV4	EIF5A2	Eukaryotic translation initiation factor 5A2
Q6IS14	EIF5AL1	Eukaryotic translation initiation factor 5A-like 1
P09429	HMGB1	High mobility group protein B1
P31942	HNRNPH3	Heterogeneous nuclear ribonucleoprotein H3
Q13813	SPTAN1	Spectrin alpha, non-erythrocytic 1

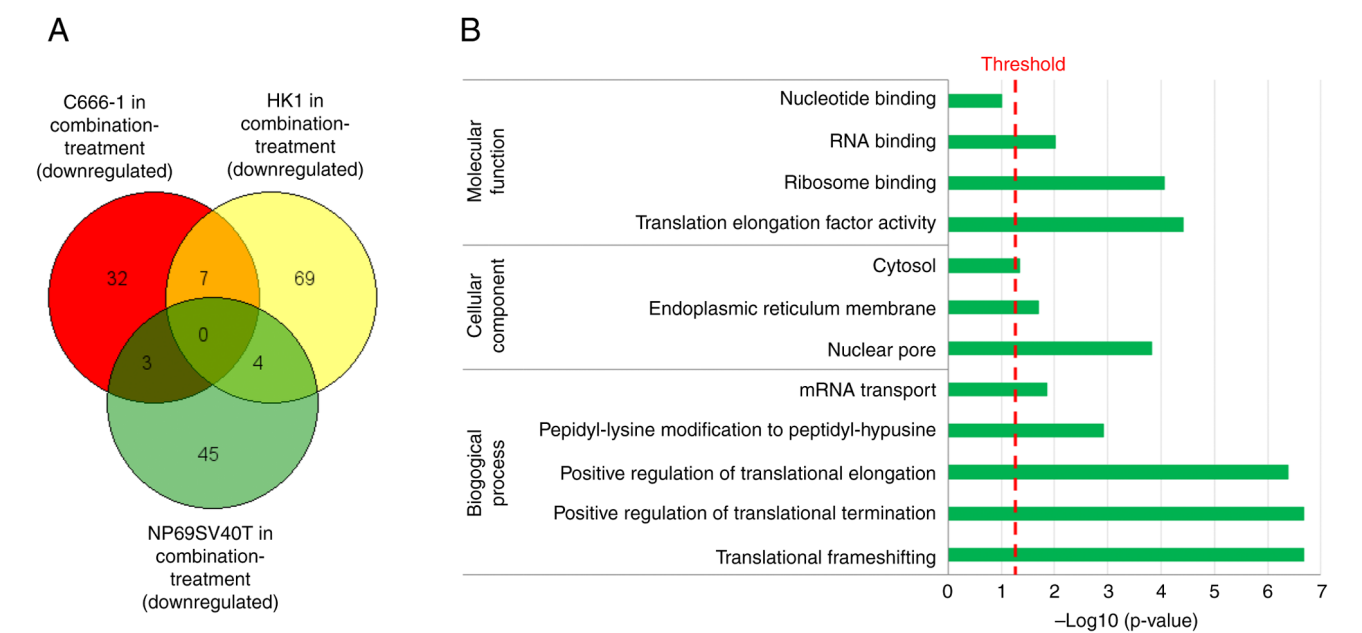


Figure 4. Identification of novel targets of silvestrol-CX-5461 combination-treatment by differential protein expression profiling. (A) Venn diagram depicting the number of proteins significantly downregulated following combination-treatment in individual cell lines and overlaps between cell lines, if any. Fold change <0.625; P<0.05. (B) Gene Ontology terms, according to biological process, cellular component, and molecular function, of the seven key proteins significantly downregulated in nasopharyngeal carcinoma cells by combination-treatment.

amplification of *CCND1* leads to Cdk4/6 dysregulation and Cyclin D1 overexpression (44). This overexpression is strongly linked to stable EBV infection and the pathogenesis of NPC, especially in the early stage (45). Depletion of Cyclin D1 reduced NPC cell proliferation extensively, suggesting the oncogenic role of *CCND1* (43). The current study suggested that silvestrol activated a mechanism that prevented the cell cycle from progressing beyond the S-phase. This proposition is anticipated given the association between Cyclin D1 and G₁ checkpoint regulation and the circumstance that silvestrol diminishes Cyclin D1 protein level in NPC cells.

myc participates in cell transformation by being involved in cell growth, cell cycle progression, cell proliferation and immortalization. c-myc has been associated with the transcriptional regulation of Cyclin D1, and Cyclin D/Cdk4 or Cyclin D/Cdk6 complexes were reported as targets of c-myc activity (46). myc, long considered to be undruggable, is an immensely appealing target for cancer therapy (47). In the present study, western blot analysis demonstrated that

silvestrol-treatment downregulated c-myc protein levels in NPC cells. This aligns with the observation that silvestrol decreased myc protein levels in colorectal cancer cells (48). In primary human fibroblasts, eIF5A is a target of myc that is directly linked to cell proliferation. myc induced eIF5A; hence, myc directly influences protein synthesis (49). Additionally, in cells experiencing DNA damage, activated p53 stimulates transcription of p21, a downstream gene of p53. Sequentially, p21 protein inhibits the Cyclin D1/Cdk4 complex and, as a result, prevents phosphorylation of pRb; the hypo-phosphorylated pRb remains bound to E2F, and cells are arrested at G₁-phase (41,50).

In summary, c-myc regulates *CCND1*, and myc induces eIF5A. c-myc is involved in cell cycle progression and survival, while cyclin D1 facilitates the transition from G₁ to S-phase. For this reason, exhausting short-lived pro-survival factors by interfering with their production may well be a therapeutic strategy (12). Our results showed that silvestrol could reduce c-myc, Cyclin D1, and eIF5A levels, but the level of β -actin

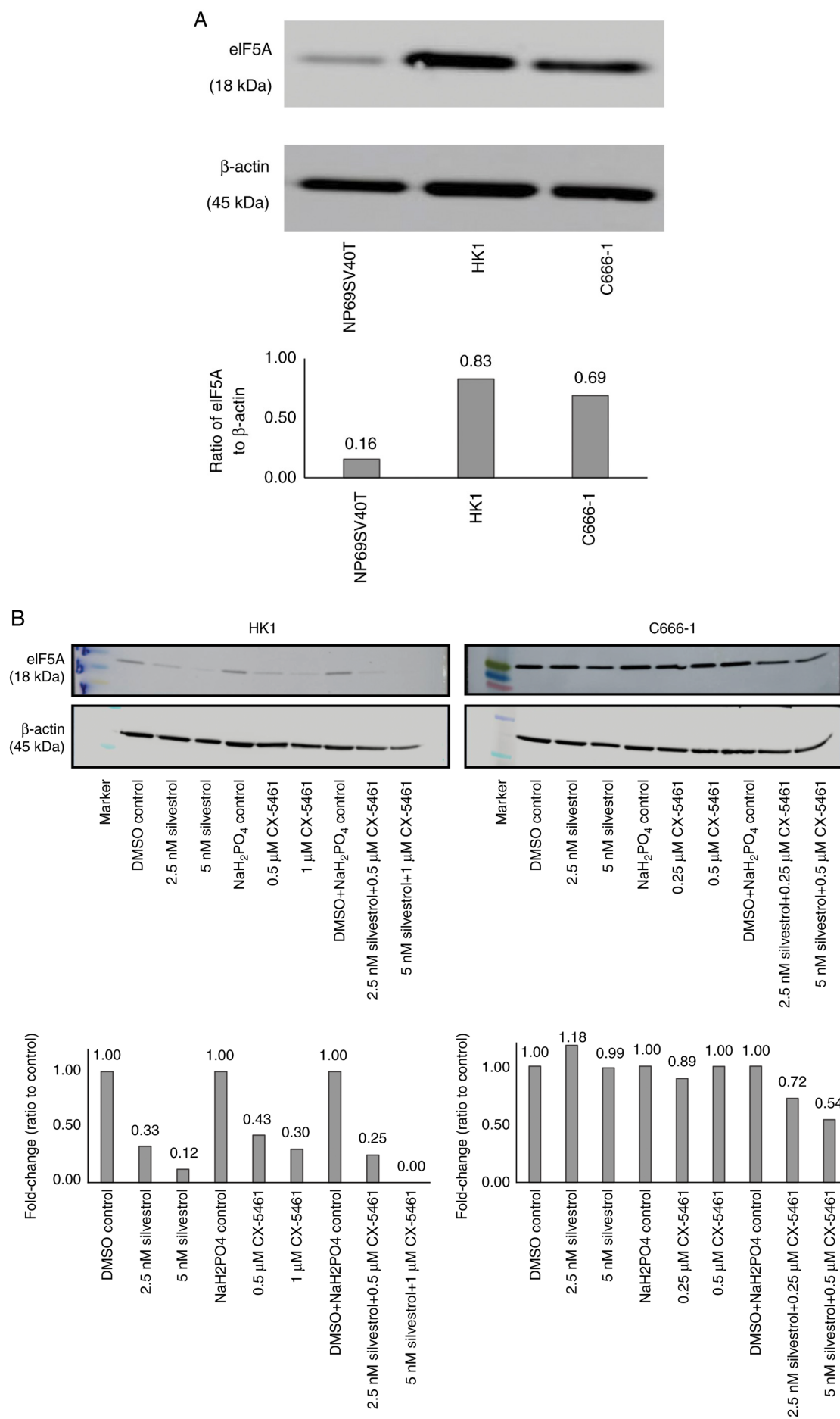


Figure 5. Continued.

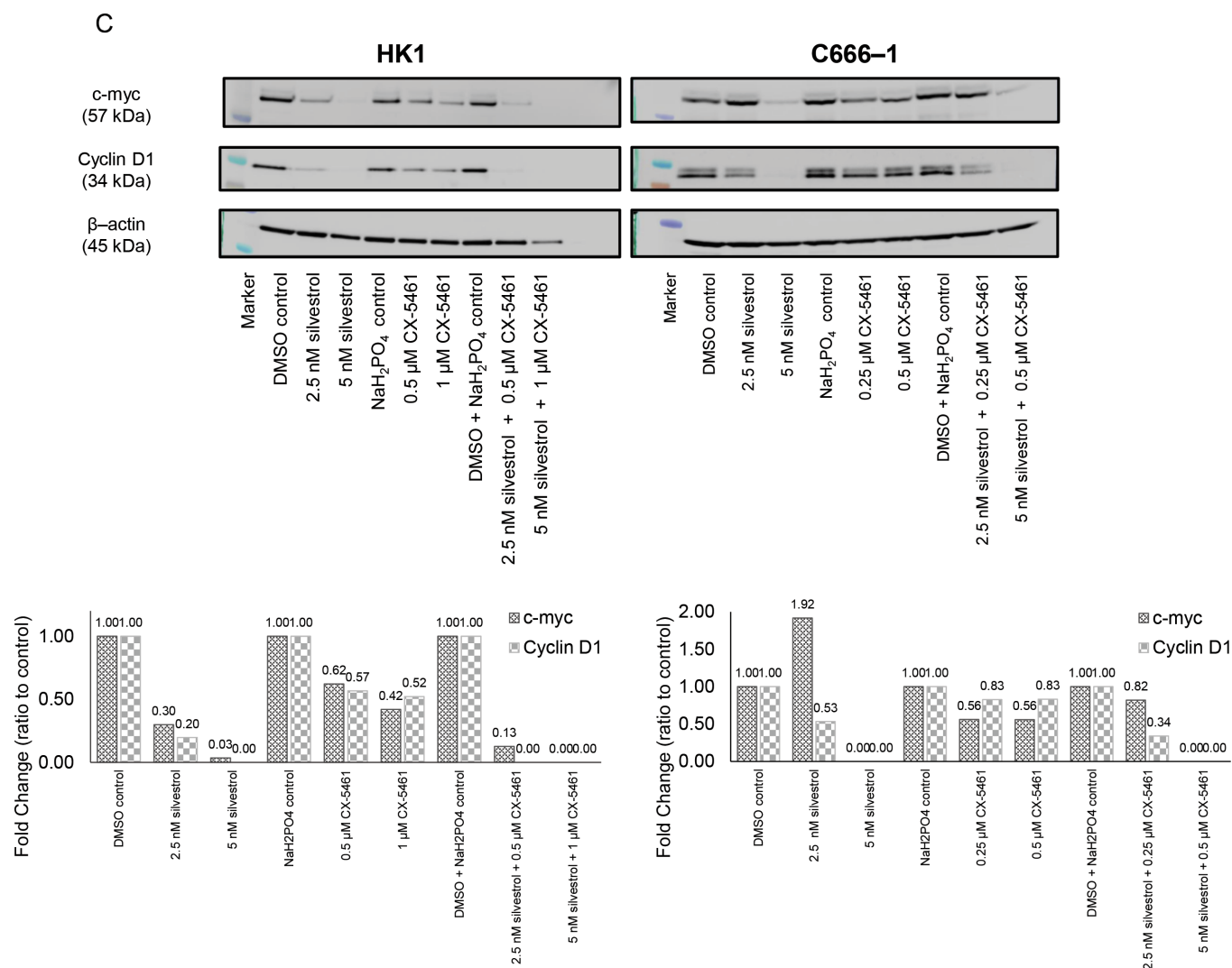


Figure 5. Validation of selected candidate protein from differential expression profiling. (A) Western blotting was used to detect eIF5A in nasopharyngeal carcinoma compared with nasopharyngeal epithelial cells. β -actin served as an internal control. The ratio of eIF5A expression to β -actin expression is indicated for each cell line. (B) Western blotting of eIF5A in HK1 and C666-1 treated with silvestrol, CX-5461, or combinations. β -actin served as an internal control. The fold change of eIF5A expression in HK1 and C666-1 relative to their respective control is indicated. (C) Western blotting of c-myc and cyclin D1 in HK1 and C666-1 treated with silvestrol, CX-5461, or combinations. β -actin served as an internal control. The fold change of c-myc and cyclin D1 expression in HK1 and C666-1 relative to their respective control is indicated. DMSO, dimethyl sulfoxide; eIF5A, eukaryotic translation initiation factor 5A; NaH₂PO₄, sodium dihydrogen phosphate.

remained unaffected, supporting that silvestrol selectively inhibits the synthesis of growth-related proteins. Insights into the link between Cyclin D1 and eIF5A, if any, remain to be discovered.

With the ability of CX-5461 to inhibit rRNA synthesis and the capacity of silvestrol to target translation, it is speculated that the coordinated specificity of each agent to act via impartial pathways was key to the synergistic interaction observed. The ultimate combined mechanism of action of silvestrol and CX-5461 in the central dogma of molecular biology would be the complete inhibition of protein synthesis. Briefly, combining a translation inhibitor with an rRNA synthesis inhibitor may synergize to prevent the synthesis of proteins that NPC cells depend on for growth and survival. Combination-treatment downregulated eIF5A in NPC cells, corroborated by proteomics analysis and western blotting. According to Open Targets Platform [<https://www.targetvalidation.org/>; (51)],

at present, no known drug has been approved or is in clinical trials for eIF5A. This void makes eIF5A an attractive target, and further research is required to explore if eIF5A inhibition by combination therapy could be a preclinical treatment strategy for NPC. eIF5A is a protein synthesis-promoting factor (52). Although initially identified as a translation initiation factor, eIF5A is an RNA-binding protein that associates with ribosomes and functions in translation elongation (52). eIF5A also plays a role in cell cycle progression, cell growth and proliferation (52,53). eIF5A is a stable protein with a long half-life (54). eIF5A promotes cell survival as well as cell death (53). Thus, the mechanisms of eIF5A should be taken as part of coordinated processes targeting proteins involved in cell growth or apoptosis (53). Depletion of eIF5A through manipulation of enzymatic reactions, which influence eIF5A post-translational modification, was associated with abrogation of cell cycle progression at the G₁/S transition in LAZ463

EBV-transformed human lymphoblastoid cells (55). Such findings implicated the role of eIF5A in cell cycle regulation. Similarly, in the present study, combination-treated NPC cells were arrested at G₀/G₁ and eIF5A-deprived.

In conclusion, silvestrol and its 5^{'''}-epimer, episilvestrol, in the low nM range, inhibited NPC cells and PDXs. CX-5461 synergized with silvestrol or episilvestrol in NPC cells and PDX and led to increased cell death. Silvestrol downregulated c-myc and Cyclin D1 protein levels in NPC cells. The effect was more distinct with silvestrol-CX-5461 combination-treatment. This finding was significant because NPC frequently overexpressed cyclin D1, promoting tumor development. Through comparative proteomics, eIF5A was identified to be markedly reduced by silvestrol-CX-5461 combination-treatment. The inhibitory effects of silvestrol and silvestrol-CX-5461 combination were associated with cell cycle perturbation at the G₁/S checkpoint and changes to protein synthesis regulation. This study is one of the earliest in NPC cells to associate silvestrol with a translation elongation factor in NPC via depletion of eIF5A, a protein with a long half-life. Additionally, the present study is the maiden investigation of CX-5461, showing efficacy in NPC cells and NPC PDX. It is also the first account of the combination and consequent synergism of CX-5461 and silvestrol or episilvestrol.

Among the limitations of this project are the lack of western blots of apoptosis-related proteins to verify results relating to promoting cell death. Additionally, there is a need for proliferation, colony formation, migration and invasion assays. To overcome limitations, functional characterization in NPC models, including animals, is required to elucidate and validate the mechanisms behind the cell death or cell cycle arrest caused by silvestrol, CX-5461, or silvestrol-CX-5461 combination. Efficacy of silvestrol, CX-5461, or silvestrol-CX-5461 combination ought to be researched in an *in vivo* model system. Such experiments would include observation of suppression of tumor growth via *in vivo* fluorescent or luminescent imaging or manual measurement, observation of changes in tumor weight, and tumor tissue histopathology analysis, including EBV *in situ* hybridization and Ki67 scoring.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

MD acquired and analyzed data, acquired funding and wrote the manuscript. LPT and SFC analyzed data and wrote the manuscript. MA, ASBK, SSH and PMN conceptualized, designed and supervised the study, and revised the manuscript. MD and MA confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was exempted from ethics approval by The Medical Research and Ethics Committee of Ministry of Health of Malaysia [reference no. KKM. NIHSEC. P18-969 (4)].

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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