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Original Article

Novel combination of Vincristine and COX-2 Inhibitor Nimesulide provides synergistic anti-proliferative and pro-apoptotic effects in KOSC-2 oral squamous carcinoma cells

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Abstract: Oral cancer is one of the most common malignant tumors in head and neck cancer. Vincristine (VCR) is an antimitotic agent widely used for cancer treatment. Nimesulide (NIM) is a selective cyclooxygenase-2 (COX-2) inhibitor which exhibits antitumor effects in several human tumor types. The present study examined the interaction between VCR and NIM in a human KOSC-2 oral squamous carcinoma cell line. Our data showed that each inhibitor alone reduced cell growth and the combination of VCR with NIM synergistically inhibited cell growth and invasion and increased apoptosis. To better understand the molecular mechanisms underlying the synergistic anti-tumor activity of the combination, we investigated the expression profile of the combination-treated KOSC-2 cell line using western blot analysis. Combination treatment significantly altered expression levels of several proteins in KOSC-2 cell line. Protein functionally involved in pro-apoptosis and anti-invasion were predominantly up-regulated, while proteins implicated in anti-apoptosis, invasion, proliferation and multidrug resistance were mainly down-regulated upon treatment. In conclusion, our in vitro findings indicate that the COX-2 selective inhibitor NIM enhances the drug toxicity of VCR to KOSC-2 cells, which correlates with P-gp expression down-regulation and P-gp function inhibition in KOSC-2 cells.

Keywords: Human oral squamous carcinoma cells, KOSC-2 cells, nimesulide, vincristine, multidrug resistance

Introduction

Oral cancer is the sixth most common malignancy worldwide. Although the clinical diagnosis and management of early-stage oral squamous carcinoma has improved significantly, oral squamous carcinoma prognosis is still extremely poor [1]. Furthermore, advanced oral squamous carcinoma is a highly aggressive tumor with a low or no response to common therapies which results in chemotherapy failure [2]. Multidrug resistance (MDR) was the main reason which causes the cells to be resistant to chemotherapeutic drugs and results in chemotherapy failure [3]. Drug resistance might be developed by the over expression of mdr-1 gene-encoded P-glycoprotein (P-gp) [3-5]. However, the drug resistance of tumor cells can be effectively reversed by suppressing P-gp expression and function [6]. Therefore, new effective and well-tolerated therapy strategies are urgently needed.

Cyclooxygenase-2 (COX-2) is an enzyme in the pathway leading to production of prostaglandin E2 (PGE2) [7]. This pathway is known to play a role in inflammation, cell division cycle and tumor growth, invasiveness and metastasis, inhibition of apoptosis and angiogenesis. Inhibition of COX-2 has been shown to be a promising antitumor and antiangiogenic strategy in several tumor types including human oral carcinoma cells [8-12]. Besides, COX-2 selective inhibitors also enhance the toxicity of anticancer drugs to carcinoma cells [13-15].

In preclinical models, COX-2 inhibition has been shown to exhibit activity as a single agent as well as in combination with immunotherapy and chemotherapy [16-18]. Recent findings have indicated that COX-2 expression is positively correlated with P-gp expression in tumor tissues [12]. Relevant studies have found that COX-2 inhibitors increase the sensitivity of cancer cells to chemotherapeutics by regulating the activity of membrane transport enzymes affiliated with the ABC superfamily [19, 20]. VCR, a vinca alkaloid isolated from the leaves of the periwinkle plant Catharanthus roseus, is an antimitotic anticancer agent. Intensive chemotherapy including VCR and other anti-tumor drugs is often combinational, and is used for many types of cancers [21]. Whether or not combination of VCR and NIM inhibit oral squamous carcinoma growth or reverses MDR by suppressing P-gp expression is not yet clear.

Here we investigated the effects of combination of VCR and NIM on invasion, proliferation and apoptosis of human KOSC-2 oral squamous carcinoma cells which were VCR-resistant and over-expressing P-gp. Furthermore, we assessed the sensitivity of KOSC-2 cells to VCR by suppressing the expression of P-gp with NIM. We found that NIM inhibited invasion and proliferation of KOSC-2 cells, promoted cell apoptosis, and enhanced cell sensitivity to VCR.

Materials and methods

Materials

RPMI-1640 medium, Click-iT Edu imaging kit and fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA). Penicillin/streptomycin and paraformaldehyde were from Genom (Hangzhou, China). Triton X-100 was from Bio Basic (Markham Ontario, Canada). Bovine serum albumin was from Whiga (Shanghai, China). Mouse anti-GPADH antibody was obtained from ISGB-Bio (Houston, USA). Rabbit polyclonal anti-caspase-3, MMP-2 and TIMP-2 antibody was from Epitomics (California, USA). Rabbit polyclonal anti-Bcl-2, Bax, Survivin and P-gp antibody was from Abcam (Cambridge, UK). Goat anti-Rabbit and Mouse IgG IR Dye 800cw were from Odyssey (Licor, USA). The Cycle Test plus DNA Reagent kit and Annexin V-FITC Apoptosis Detection were from BD (New Jersey, USA).

Cell cultures

The human oral squamous carcinoma cell line, KOSC-2, was purchased from Nanjing KeyGen Biotech Co, Ltd (Nanjing, China). The KOSC-2 cell line with CD44+SSEA-4+ surface markers is more resistant to chemotherapy drug than the other subpopulations. The KOSC-2 cells were cultured in RPMI-1640 culture medium containing 10% fetal calf serum at 37°C with 5% CO_2. When the cultures reached about 80% confluence, the cells were treated with NIM/VCR for the indicated time period.

MTT assay

KOSC-2 cells at the logarithmic phase were collected, incubated in a 96-well plate at a concentration of 5×10^3 cells and cultured for 24 h. Following the cell attachment to the wall, RPMI-1640 medium containing different concentrations of VCR or NIM were supplemented at a final volume of 200 μL/well, the control group was replaced with new medium without treatment. Following culture for 12, 24 and 48 h, the 20 μL (1 g/L) MTT solution was added and the cells were continuously cultured for 4 h. The supernatant was then removed and 150 μL DMSO was added to each well, and afterwards cells were slightly shaken for 15 min. The optical density value was detected by Microplate Reader (BioTek, ELx800) at a wavelength of 490 nm. MTT assays were performed in six duplicates on three independent experiments.

In vitro invasion analysis

An *in vitro* invasion assay was carried out to examine the invasion of KOSC-2 cells as previously described [22]. Briefly, 24-well transwell units with 8 μm polycarbonate Nucleopore filters (Corning, NY) were coated with 0.1 mL 0.8 mg/mL Englebreth Holm-Swarm sarcoma tumor extract (EHS Matrigel) at room temperature for 1 h to form a genuinely reconstituted basement membrane. Human KOSC-2 cells (5×10^4 cells) were placed in the upper compartment, and 500 μL RPMI-1640 culture medium containing 10% fetal calf serum was added to the lower compartment. The transwell plates were incubated at 37°C for 36 h in a humidified atmosphere with 5% CO_2 and stained with 10% crystal violet. Invading cells were defined as cells that had degraded the Matrigel and moved into the lower surface of the membrane, and

the non-invading cells which were retained on the upper surface of the membrane were removed by a cotton swab.

Click-iT EdU Test

Half of medium for KOSC-2 cells were replaced with fresh medium containing 20 μM EdU which is a nucleoside analog of thymidine and incorporated into DNA during active DNA synthesis. After incubation for 24 h, the cells were fixed by 1 mL 3.7% formaldehyde in PBS for 15 min at room temperature. The permeabilization was carried out by 1 mL 0.5% Triton X-100 in PBS. After washing with 1 mL 3% BSA in PBS, 0.5 mL Click-iT reaction cocktail containing Alexa Fluor 594 was added to each well. Detection is based on a click reaction, in which a copper-catalyzed covalent reaction occurred between an azide of Alexa Fluor 594 and an alkyne of EDU. The samples were kept from light for 30 min at room temperature. After washing with 1 mL 3% BSA in PBS, the cells were mixed with 1 mL 1× Hoechst 33342 solution for 30 min, and then were proceeded to imaging and analysis. Results were expressed as the percentage of proliferating KOSC-2 cells. Values were expressed as means ± SD of three separate experiments, each performed in triplicate.

The cell cycle analysis by flow cytometry

The cells were collected by centrifugation at 400 *g* for 5 min after treatment with VCR or NIM for 24 h, and then cells were resuspended with 250 μL trypsin buffer. After 10 min, 200 μL trypsin inhibitor and RNase buffer were added and mixed with 200 μL cold propidium iodide stain solution. The samples were gently mixed and incubate for 10 min on ice in the dark. The flow cytometry analysis will be carried out within 3 h.

Apoptosis assays by flow cytometry

Phosphatidylserine (PS) exposure was measured using the binding of fluorescein-isothiocyanate-labelled (FITC) Annexin-V to PS according to the manufacturer's protocol. Briefly, cells (2×10^6/mL) were washed with PBS and suspended in 500 μL binding buffer. Annexin V FITC (5 μL) and propidium iodide (5 μL) were added to the cells for 15 min in the dark at room temperature and analyzed with flow cytometer (FACSCalibur; Becton Dickinson, Mountain View, CA, USA) equipped with argon laser (488 nm) and filtered at 530 and 585 nm for FITC and phycoerythrin respectively. Low fluorescence debris and necrotic cells, permeable to propidium iodide, were gated out before analysis and 10^4 events were collected. Data were analyzed using Cell Quest software (Becton Dickinson). This assay was also used to measure spontaneous apoptosis in freshly isolated KOSC-2 cells.

Western blot

KOSC-2 cells were harvested and homogenized in a SDS sample buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% SDS, 1 mM each of PMSF, NaF, $NaVO_3$, 1 μg/mL each of leupeptin, pepstatin, aprotinin). The concentration of proteins was determined by the Bradford method with BSA as a standard [23]. The 40 μg of protein loaded per lane was separated by 10% polyacrylamide SDS gel electrophoresis. Antibodies such as Caspase-3, Bcl-2, Bax, P-gp and GAPDH were first incubated with membranes for 2 h at room temperature respectively. Goat anti-rabbit and mouse IgG IR Dye 800cw (1:15,000), respectively, was added to incubate for 1 h at room temperature. The blots were then imaged by Odyssey analysis system. The pictures were quantified using the Gel-Pro analyzer (Alpha Innotech Corp. CA), and protein levels were expressed as the relative values of the controls.

Detection of intracellular P-gp function by flow cytometry

KOSC-2 cells at the logarithmic phase were incubated in a 6-well plate at a density of 1×10^6/mL per well. After cell adhesion to the wall, VCR (1.0 μmol/L), NIM (100 μmol/L) VCR plus NIM (1 μmol/L+100 μmol/L, respectively) were added to the wells. The cells were then further cultured in medium supplemented with Rho123 (5 μg/mL) for 30 min and washed with PBS twice after centrifugation. The cells were re-suspended and incubated at 37°C with 5% CO_2 for 60 min. The fluorescence intensity of intracellular Rho123 after the Rho123 effect was detected by flow cytometry (BD, FACSCalibur). The mean of the data was considered as the final value after three repetitions.

Statistical analysis

Data are expressed as means EM of the indicated number of experiments performed in

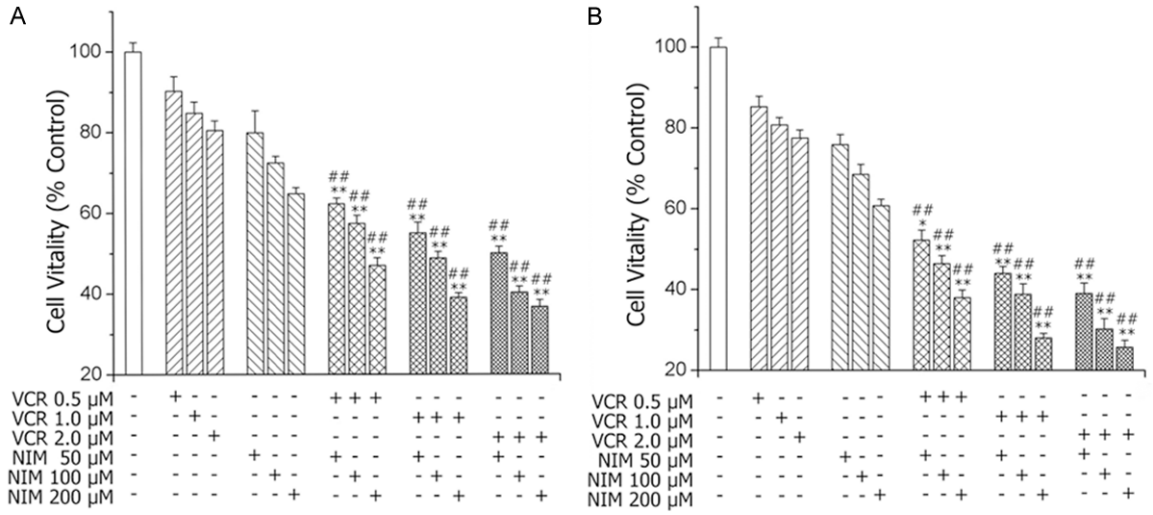

Figure 1. Effect of Vincristine and Nimesulide individually and in combination on viability of KOSC-2 cells. Cell vitality was assessed by the MTT assay. A. KOSC-2 cells were treated for 24 h with the indicated concentrations of VCR and NIM either alone or in combination. B. KOSC-2 cells were treated for 36 h with the indicated concentrations of VCR and NIM either alone or in combination. Data are expressed as the percentage of control cells and are the means ± SD of three separate experiments, each of which was performed in triplicate. $^{**}P < 0.01$ versus VCR alone, $^{\#\#}P < 0.01$ versus NIM alone.

Table 1. The combined treatment analysis of KOSC-2 cells

	Cell vitality (% Control) after 24 h			Cell vitality (% Control) after 36 h		
	$VCR_{0.5}$+NIM_{50}	VCR_1+NIM_{100}	VCR_2+NIM_{200}	$VCR_{0.5}$+NIM_{50}	VCR_1+NIM_{100}	VCR_2+NIM_{200}
VCR	90.29 ± 3.59	84.79 ± 0.85	80.52 ± 2.41	85.29 ± 2.59	80.79 ± 1.85	77.52 ± 2.01
NIM	79.91 ± 5.53[c]	72.50 ± 1.58[c]	64.79 ± 1.58[c]	75.91 ± 2.53[c]	68.50 ± 2.58[c]	60.79 ± 2.50[c]
VCR+NIM	62.27 ± 0.48[a,b]	48.81 ± 1.62[a,b]	36.77 ± 1.68[a,b]	52.37 ± 2.48[a,b]	38.81 ± 2.62[a,b]	25.77 ± 1.68[a,b]
F	41.303	514.774	397.136	56.236	218.968	306.172
p	0.000	0.000	0.000	0.000	0.000	0.000
q	1.266	1.199	1.156	1.228	1.207	1.203
Interaction	Synergism	Synergism	Synergism	Synergism	Synergism	Synergism

$VCR_{0.5}$: 0.5 μM VCR treatment; NIM_{50}: 50 μM NIM treatment; VCR_1: 1 μM VCR treatment; NIM_{100}: 100 μM NIM treatment; VCR_2: 2 μM VCR treatment; NIM_{200}: 200 μM NIM treatment; [a]VCR+NIM vs VCR, $P < 0.01$; [b]VCR+NIM vs NIM, $P < 0.01$; [c]VCR vs NIM, $P < 0.05$; [c]VCR vs NIM, $P < 0.01$.

independent cultures. Significance of differences was evaluated by ANOVA followed by the Student-Newman-Keuls post-hoc test when more than two conditions were evaluated. Isobologram analysis was performed to analyze the interactive effect between NIM and VCR using the IsoBoloGram software packages. Significant changes are indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$.

Results

Combination of VCR and nim synergistically reduces cell viability and proliferation in KOSC-2 cells

Using the MTT assay we first assessed the effects of Vincristine and NIM on the viability of KOSC-2 cell line. As shown in **Figure 1**, the combined treatment of VCR and NIM significantly enhanced the inhibitory effect of VCR on KOSC-2 cell growth. Following 24 h and 36 h treatment, the growth inhibition rate in cells treated with VCR plus NIM was significantly lower than that in cells treated with VCR or NIM alone ($P < 0.01$; **Figure 1**). All of the q values were greater than 1.15 after NIM was combined with VCR treatment for 24 h and 36 h, suggesting that combined treatment results in a synergistic effect (**Table 1**). The growth inhibition rate of KOSC-2 cells was about 50% under the combined treatment of 1.0 μmol/L VCR and 100 μmol/L NIM for 24 h. To better reflect its pharmacological effects, the 1.0 μmol/L VCR and 100 μmol/L NIM doses were selected for next studies.

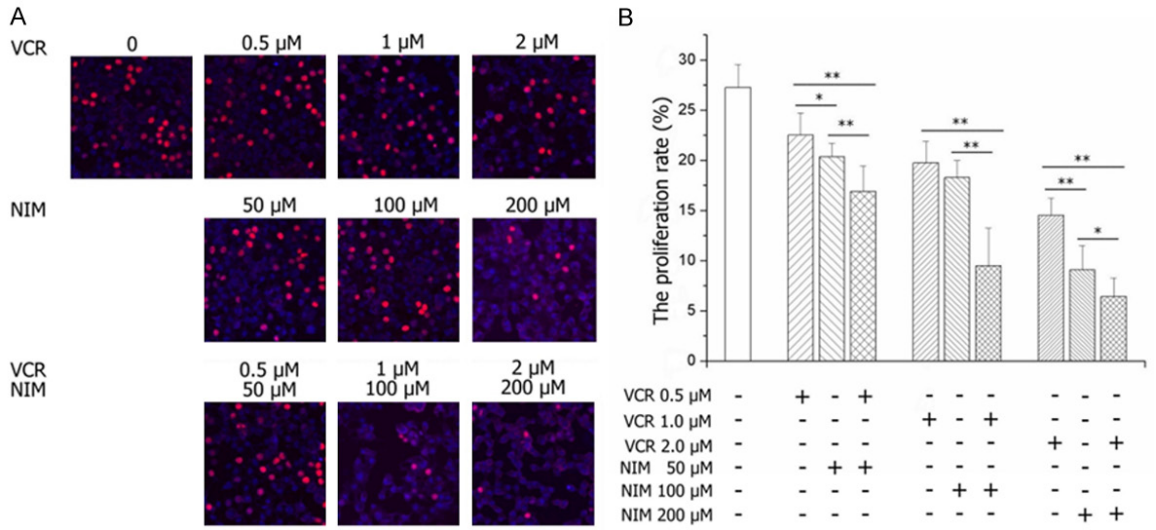

Figure 2. Effect of Vincristine and Nimesulide individually and in combination on proliferation of KOSC-2 cells. A. Click-iT EdU Test was performed to measure KOSC-2 cells proliferation. KOSC-2 cells are incubated with EdU (10 μM) and the signal is subsequently amplified to reveal proliferating DNA that showed red and other cells which were stained with Hoechst 33342 show blue. B. Quantification of KOSC-2 cells cells proliferation ratio inhibited with VCR or NIM. The data are expressed as the means ± SEM of n=3 experiments. $^{**}P < 0.01$.

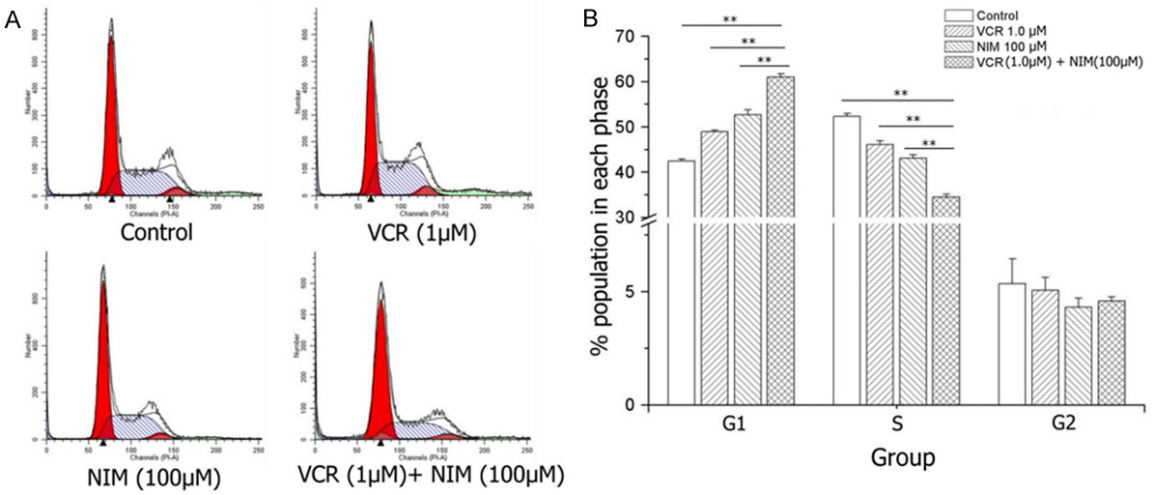

Figure 3. Effect of Vincristine and Nimesulide individually and in combination on cells cycle of KOSC-2 cells. A. Analysis of cell cycle. Cells were plated overnight and exposed to VCR and NIM alone or in combination at the indicated concentrations for 24 h. After treatment cells were harvested and used to analysis of cell cycle, by which we found VCR or NIM increased the population in the G1 phase with a corresponding decrease in the S phase. B. Quantification of cell population changes when treatment with VCR and NIM individually and in combination. The data are expressed as the means ± SEM of n=3 experiments. $^{**}P < 0.01$.

We next investigated the effects of VCR and NIM combination on proliferation and cells cycle in KOSC-2 cell line using Click-iT EdU Test and flow cytometry. The proliferation rate of KOSC-2 cells significantly decreased after combined treatment and was significantly lower than that in other groups (**Figure 2A** and **2B**). To explore the mechanism of VCR and NIM inhibiting KOSC-2 cells proliferation, flow cytometry was implemented, the results showed that VCR and NIM individually and in combination increased proportion of the cells population in the G1 phase. But a corresponding decrease in the S phase was found. The combined treatment of VCR and NIM significantly enhanced proportion of the cells population in the G1 phase and decreased proportion of the cells population in the S phase compared with VCR or NIM alone (**Figure 3A** and **3B**). These results corroborate the potent inhibitory

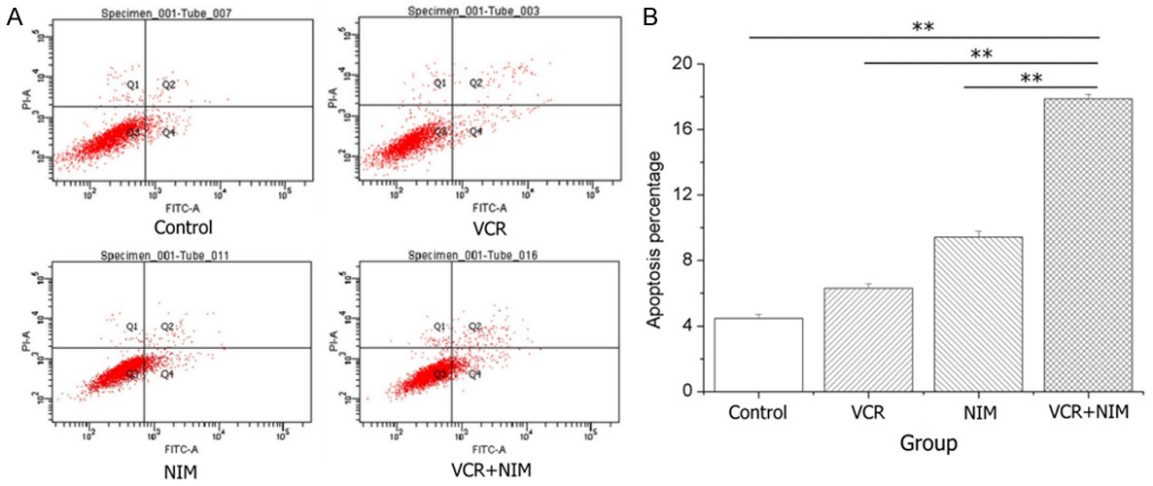

Figure 4. Vincristine and Nimesulide induced apoptosis of KOSC-2 cells. A. KOSC-2 cells were treated with VCR and NIM individually and in combination at indicated doses for 24 h before assay of apoptosis by FITC Annexin-V and propidium iodide (PI) method using flow cytometry. B. The apoptosis proportion of KOSC-2 cells in the combination treatment group is higher than the control group and VCR or NIM group alone. The data are expressed as the means ± SEM of n=3 experiments. **P < 0.01.

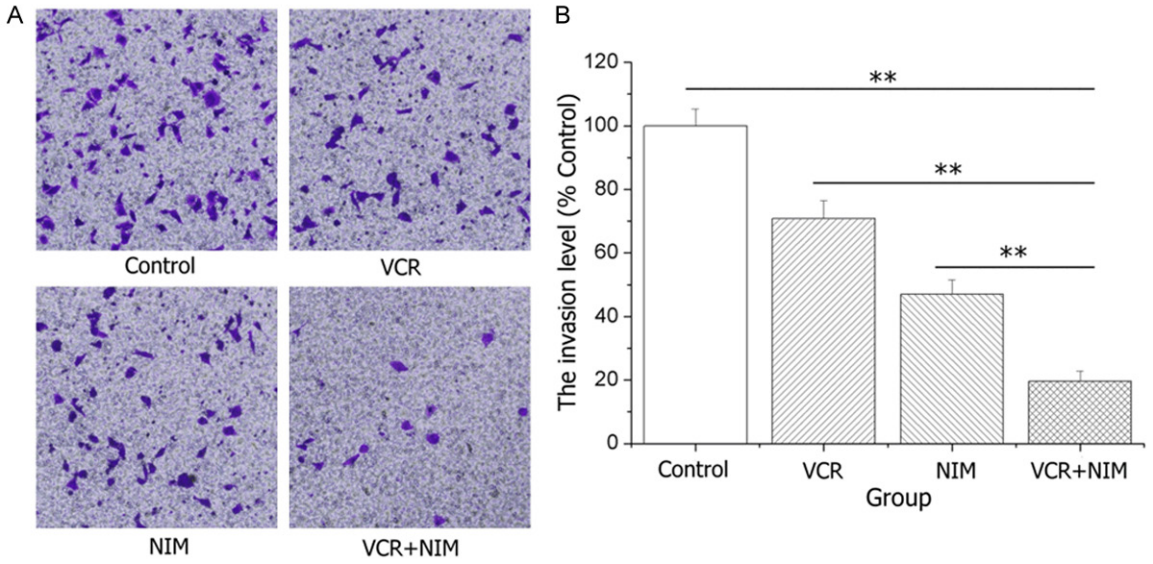

Figure 5. In vitro invasion assay of KOSC-2 cells by a transwell assay. A. Effect of Vincristine and Nimesulide individually and in combination on invasion of KOSC-2 cells. Migrated cells on the membranes were stained with crystal violet for 10 min. B. Quantification of migration of KOSC-2 cells inhibited by VCR or NIM. Each bar represents the mean ± SEM of three independent observations. **P < 0.01.

effect of VCR and NIM on DNA synthesis which was assessed by the EdU assay.

Effect of the combination on apoptosis of KOSC-2 cells

The apoptosis proportion in combination treated KOSC-2 cell line significantly increased compared with VCR or NIM alone. NIM significantly promoted and enhanced inducing effect of VCR on the apoptosis in KOSC-2 cell line. Additionally, the apoptosis rate of KOSC-2 cells in VCR and NIM individually and in combination were 6.30%, 9.43% and 17.87%, respectively, which were significantly higher than that of the control (4.47%, P < 0.05; **Figure 4A** and **4B**).

The inhibitory effect of combination on invasion of KOSC-2 cells

The invasion of KOSC-2 cells was significantly inhibited by VCR plus NIM, compared to VCR or

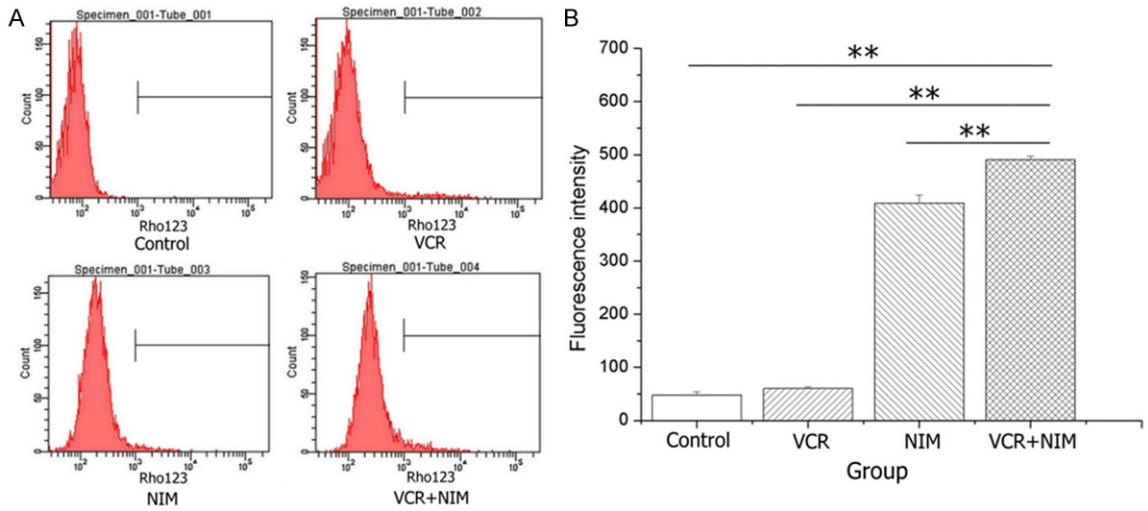

Figure 6. The effect of Nimesulide on P-gp pump function in KOSC-2 cells. A. The amount of Rho123 accumulated in KOSC-2 cells was detected by flow cytometry to reflect P-gp function. B. The fluorescence intensity of Rho123 in the four treatment groups was 47.33 ± 6.66, 60.00 ± 3.61, 408.33 ± 16.07 and 490.33 ± 7.09, respectively. The fluorescence intensity in NIM plus VCR-treated cells was significantly higher than those of the other three treatment groups. **P < 0.01.

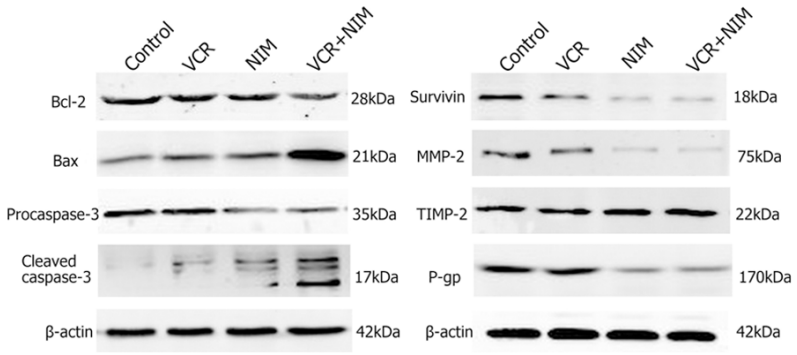

Figure 7. Effect of Vincristine and Nimesulide individually and in combination on expression levels of Survivin, Bax, Bcl-2, Caspase-3, MMP-2, TIMP-2 and P-gp proteins. Cells were treated for 24 h with the indicated concentrations of VCR and NIM and their combinations. After treatment cells were harvested and lysed and equal amounts of extracted protein were analyzed for Bax, Bcl-2, Caspase-3, Survivin, MMP-2, TIMP-2 and P-gp expression by Western blotting. The data represent two independent experiments with comparable outcomes.

NIM alone. VCR, NIM and VCR+NIM decreased the number of migrated cells to 72.04%, 47.70% and 18.42%, respectively, compared to control which considered as 100%, quantitative data derived from three independent experiments supported VCR plus NIM effectively inhibited the invasion of KOSC-2 cells (**Figure 5A** and **5B**).

The weakening effect of NIM on pump function of P-gp in KOSC-2 cells

Rho123 is a fluorescence substrate that is applied to investigate P-gp functional activity. When functional activity of P-gp declines, the accumulation of the Rho-123 substrate within cells increases and vice versa. Our results indicated that the amount of Rho123 accumulation in cells treated with NIM alone and cells treated with NIM plus VCR was significantly higher than that in cells treated with VCR alone and control cells (**Figure 6A** and **6B**), suggesting that NIM increases intracellular accumulation of Rho123 by inhibiting P-gp pump function in in human KOSC-2 cells.

Effect of VCR and NIM individually and in combination on expression levels of Survivin, Bax, Bcl-2, Caspase-3, MMP-2, TIMP-2 and P-gp proteins

Survivin, which overexpressed in essentially all human cancer cells is linked to interactions with other proteins and the formation of multi-protein complexes that control proliferation and cell death [24]. In this study, our results indicated that the combined treatment with VCR+NIM and NIM alone significantly lower Survivin expression in KOSC-2 cells (all P < 0.01). In addition, the relative gray-scale value in cells treated with VCR plus NIM was

Table 2. The combined treatment analysis of KOSC-2 cells

	Fold change (% Control)				
	VCR	NIM	VCR+NIM	F	P
Bcl-2	93.21 ± 4.63	93.73 ± 4.13	34.29 ± 3.90^{a,b}	212.772	0.000
Bax	99.39 ± 5.61	115.08 ± 15.20^{C}	531.5728 ± 72.46^{a,B}	99.222	0.000
Procaspase-3	95.02 ± 1.69	31.68 ± 1.52^{c}	18.70 ± 2.15^{a,b}	2178.674	0.000
Cleaved caspase-3	105.66 ± 9.55	190.99 ± 29.11^{c}	599.40 ± 58.17^{a,b}	156.270	0.000
Survivin	34.32 ± 5.23	10.77 ± 1.26^{c}	4.40 ± 1.27^{a,b}	749.410	0.000
MMP-2	40.05 ± 4.52	10.65 ± 0.97^{c}	6.40 ± 0.86^{a,b}	1015.165	0.000
TIMP-2	114. 16 ± 3.95	114.43 ± 0.23	141.58 ± 8.18^{a,B}	43.878	0.000
P-gp	98.20 ± 7.03	24.43 ± 5.17^{c}	14.55 ± 0.87^{a,b}	332.371	0.000

VCR: 1 μM VCR treatment; NIM: 100 μM NIM treatment; ^{A}VCR+NIM vs VCR, $P < 0.05$; ^{a}VCR+NIM vs VCR, $P < 0.01$; ^{B}VCR+NIM vs NIM, $P < 0.01$; ^{b}VCR+NIM vs NIM, $P < 0.01$; ^{C}VCR vs NIM, $P < 0.05$; ^{c}VCR vs NIM, $P < 0.01$.

significantly lower than that in cells treated with NIM alone ($P < 0.01$, **Figure 7** and **Table 2**).

Given the profound roles of the Bcl-2 and caspase family in triggering apoptosis [25-29], we next examined if anti-apoptotic protein Bcl-2, pro-apoptotic protein Bax and caspase-3 were involved in combination treatment-induced apoptosis. The results in **Figure 7** indicated that neither VCR nor NIM alone had any detectable effect on the expression of Bcl-2 and Bax, however, the combination treatment of VCR with NIM led to noticeable down-regulation of Bcl-2 and up-regulation of Bax in KOSC-2 cells. Our results showed that the combined treatment with VCR plus NIM and NIM alone significantly lower Procaspase-3 expression in KOSC-2 cells and increase expression of Cleaved-caspase-3 (all $P < 0.01$, **Figure 7** and **Table 2**). Besides, the relative gray-scale value of Procaspase-3 and Cleaved caspase-3 in cells treated with VCR plus NIM was significantly lower and more respectively than that in cells treated with NIM alone ($P < 0.01$). No statistical significance was noted between cells treated with VCR and control cells in terms of the relative gray-scale value ($P > 0.05$; **Figure 7** and **Table 2**). Those data showed that VCR and NIM-induced apoptosis of KOSC-2 cells is probably mediated by the mitochondrial pathway.

TIMP-2 and MMP-2 play a role in the process of several tumor invasion and metastasis [38, 39]. In our screening, we observed decreased expression of MMP-2 in KOSC-2 cells upon VCR or NIM treatment which was further potentiated following combination treatment. Similarly, the combination treatment increased TIMP-2 protein expression more than each agent used alone (**Figure 7** and **Table 2**).

The reversal of P-gp-mediated MDR can be achieved either by decreasing P-gp expression or by inhibiting its drug-transport activity [12, 30]. In this paper, our results showed that the relative gray-scale values in cells treated with VCR plus NIM and the NIM alone were significantly lower than those in cells treated with VCR and the control cells (all $P < 0.01$). The relative levels of P-gp in the KOSC-2 cells treated with VCR plus NIM were significantly lower than those treated with NIM alone, although VCR alone did not decrease P-gp expression. No statistical significance was noted between cells treated with VCR and control cells in terms of the relative gray-scale value ($P > 0.05$; **Figure 7** and **Table 2**).

Discussion

Human oral squamous carcinoma is a complex disease which needs interacting approaches for effective therapy. A multi-targeting-based approach is of particular relevance in the treatment of oral squamous carcinoma, thus combination therapy would be more appropriate and may increase therapeutic efficacy. Given that VCR is the standard of care in the first-line setting for advanced oral squamous carcinoma patients, the new agents and new drug combinations must be compared head-to-head with VCR. However, to our knowledge, there are few data examining in detail the effects of VCR in combination with other anti-cancer drugs in oral squamous carcinoma. Therefore, to inhibit multiple signaling pathways involved in oral squamous carcinoma, in the present study we investigated whether in KOSC-2 lines, the combinations of VCR+NIM have more potent antitumor effects than VCR alone. In addition, we also examined the changes in the transcription-

al profiles for KOSC-2 cell line upon combined VCR+NIM treatment.

In this study, we found showed that each inhibitor alone can reduce cell growth; however the VCR+NIM combination displayed a synergistic effect in terms of cell growth inhibition and apoptosis induction. Of particular significance is our observation on Survinin protein expression after combination treatment. Survivin is overexpressed in essentially all human cancer cells and expression has not only been associated with the acquisition of several of the so-called tumor cell traits [31, 32], but also with maintenance of tumor cell viability in vitro and in vivo. The ability of Survivin to do so is often linked to interactions with other proteins and the formation of multi-protein complexes that control proliferation and cell death [24]. More recently, Survivin expression was also shown to enhance the metastatic potential of cancer cells by promoting, together with XIAP, NF-kB-dependent transcription and secretion of fibronectin [33].

Previous studies have indicated NIM could induce apoptosis in a wide variety of tumor cells [8, 9]. In this study, we observed NIM displayed the effect on apoptosis induction of KOSC-2 cells and the effect on apoptosis induction of VCR was further potentiated following NIM treatment. The Bcl-2 family consists of anti- and pro-apoptotic members, such as Bcl-2 protein and Bax protein. The ratio of anti- and pro-apoptotic members within the Bcl-2 family plays an important role to determine the cell fate [34, 35]. An increase or over expression of anti-apoptotic factor Bcl-2 protein would promote cell survival, and endow cells with drug resistance [25-27]. However, the over-expression of pro-apoptotic factor Bax, which could trigger Caspase-mediated apoptotic cell death, would increase the sensitivity of malignant cancer cells to chemicals [28, 29]. Another factor, such as Caspase-3, is a key factor in apoptosis induction. Both the mitochondria-initiated intrinsic pathway and the death receptor-triggered extrinsic pathway can lead to Caspase-3 activation. Afterwards, it further initiates downstream signals of pathway to induce apoptosis. Activation of Caspase-3, and its cleavage by proteases, is considered as a hallmark of the apoptotic process [25]. In this study, we examined the expression of Bcl-2, Bax, Caspase-3 and cleaved Capasee-3 in KOSC-2 cells after combined treatment, our results provided the evidence that the VCR+NIM combination induced apoptosis was mediated by Bcl-2 family, which was also called as the mitochondrial pathway.

Oral cancer, especially squamous carcinoma is a malignant tumor that is pathologically characterized by metastasis and marked local invasiveness, such as tumor invading into the adjoining maxilla and mandible [36, 37]. Therefore, exploring the effect of VCR and NIM on invasion of KOSC-2 cells has extremely vital significance. Previous studies have shown NIM inhibited and blocked invasion in a wide variety of tumor cells, such as RCC [10]. In this study, we found that combined treatment significantly inhibited invasion of KOSC-2 cells by increasing TIMP-2 and decreasing MMP-2 which play a role in the process of colorectal cancer invasion and metastasis [38, 39], our results showed that the combinations of VCR+NIM maybe as an option to inhibit the oral tumor metastasis.

MDR is a common clinical problem for the treatment of cancers in chemotherapy. The escape of cancer cells from chemotherapy through MDR is a major reason for cancer treatment failure [40, 41]. Previous studies indicated the effectiveness of many drugs is attenuated due to the chemotherapeutic drugs are pumped outside of cells by P-gp [42]. Therefore, the cytotoxicity of drugs to tumor cells is reduced and the drug resistance of carcinoma to chemotherapy developed. The drug resistance of tumor cells can be effectively reversed by suppressing P-gp expression and function [43]. Numerous reports indicated that COX-2 selective inhibitors, including NIM, might significantly contribute to MDR overcoming and enhance the sensitivity of cancer cells [12, 19]. These reports coincide with our results that NIM can reduce the P-gp expression and enhance the sensitivity of KOSC-2 cells to VCR.

In conclusion, combined VCR+NIM treatment displayed strong synergistic cytotoxic effects in KOSC-2 cells. These analyses, as well as consecutive validation studies based on protein expression levels, identified several new protein targets of individual drugs and of the VCR+NIM combination. Finally, our findings suggest the possible application of combined VCR+NIM therapy in oral squamous carcinoma patients.

Disclosure of conflict of interest

None.

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