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Anticancer activity of Noscapine, an opioid alkaloid in combination with Cisplatin in human non-small cell lung cancer

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ABSTRACT

The purpose of this study was to examine the efficacy of Noscapine (Nos) and Cisplatin (Cis) combination treatment in vitro in A549 and H460 lung cancer cells, in vivo in murine xenograft model and to investigate the underlying mechanism. The combination index values (<0.6) suggested synergistic effects of Nos + Cis and resulted in the highest increase in percentage of apoptotic NSCLC cells and increased expression of p53, p21, caspase 3, cleaved caspase 3, cleaved PARP, Bax, and decreased expression of Bcl_2 and surviving proteins compared with treatment with either agent. Nos + Cis treatment reduced tumor volume by 78.1 ± 7.5% compared with 38.2 ± 6.8% by Cis or 35.4 ± 6.9% by Nos alone in murine xenograft lung cancer model. Nos + Cis treatment decreased expression of pAkt, Akt, cyclin D1, survivin, PARP, Bcl_2, and increased expression of p53, p21, Bax, cleaved PARP, caspase 3, cleaved caspase 3, cleaved caspase 8, caspase 8, cleaved caspase 9 and caspase 9 compared to single-agent treated and control groups. Our results suggest that Nos enhanced the anticancer activity of Cis in an additive to synergistic manner by activating multiple signaling pathways including apoptosis. These findings suggest potential benefit for use of Nos and Cis combination in treatment of lung cancer.

1. Introduction

Lung cancer is one of the leading causes of cancer death and it is estimated that in the United States there will be 159,390 deaths in 2009 resulting from lung cancer accounting for 29% of all cancer deaths [1]. More than 85% of patients with lung cancer have non-small cell lung cancer (NSCLC). Chemotherapy with gemcitabine, taxanes or vinorelbine, together with a platinum drug is the first choice treatment in NSCLC [2,3]. Chemotherapy with Cisplatin (Cis) is associated with adverse side effects, such as anemia, neurotoxicity and nephrotoxicity [4]. Despite recent advances in chemotherapy, response rates in NSCLC remain <50% and a third of patients with stage IV disease have a 2-year survival rate of <20% [3,4]. To address this problem, attention has been focused on finding novel combination of anticancer agents with non-overlapping mechanisms of action to achieve enhanced efficacy with decreased side effects.

The effectiveness of microtubule-interfering agents in cancer therapy has been validated by the use of taxanes and vinca alkaloids for the treatment of variety of cancers [5]. However, the clinical success of taxanes + Cis combination treatment has been limited due to drug-resistance, need of i.v. infusion over a long period of time and associated severe toxicities [6,7]. Among antimicrotubule agents, the orally acting noscapinoids constitute an emerging class of compounds receiving considerable attention for treating cancers due to improved patient compliance and minimal side effects compared to taxanes [8–11]. Currently, phase I clinical trial of Noscapine (Nos) has been initiated for the treatment of relapsed or refractory multiple myeloma. Nos attenuates microtubule dynamics just enough to activate the mitotic checkpoints to stop cell cycle and do not alter the steady state monomer/polymer ratio of tubulin [12]. Nos was found to inhibit cell proliferation in wide variety of cancers [12–17] including many drug-resistant variants while evading normal [12–15]. Furthermore, Nos also showed little or no systemic toxicity to the body organs and did not inhibit primary humoral immune responses in mice [12,14,17]. Our previous studies demonstrated that oral administration of Nos showed significant reduction in tumor volume in NSCLC tumor xenograft in nude nice in a dose-dependent manner [18]. Landen et al. demonstrated that non-synergistic anticancer activity with Nos–Paclitaxel combination in murine B16LS9 melanoma model [12]. The use of Nos in combination with vincristine exhibits synergistic antitumor effects in leukemia cells in vitro [19]. However, anticancer potential of Nos in combination with approved anticancer agent in the treatment of lung cancer has not been explored yet. Nos in combination with anticancer agents may offer the possibility of effective management of cancer and thereby reduce dose and associated side effects. Therefore, there is a need for investigating the effect of Nos

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in combination with anticancer agents to realize its full chemotherapeutic potential in treatment of cancer.

The establishment of an optimal combination regimen with newer agents is an important step to improve the clinical outcome [20,21]. Several researchers have studied the combination of Cis and other agents and reported enhanced anticancer effects in treatment of lung cancer [22–28]. Both Nos and Cis have different mechanisms which may lead to potential additive/synergistic antitumor activity against lung cancer.

Therefore, we hypothesize that combination treatment of Nos with Cis may produce additive/synergistic antitumor activity in human NSCLC in vitro and in vivo possibly by enhancing multiple signaling pathway. This is the first report on the activity of the Nos in combination with Cis against lung cancer. The objectives of this study were to (a) examine the in vitro cytotoxicity and induction of apoptosis by Nos + Cis treatment against H460 and A549 cells and compare it to the Nos or Cis alone treatments and (b) evaluate the in vivo antitumor effect of Nos + Cis in murine H460 xenograft tumor model and investigate underlying mechanism.

2. Materials and methods

2.1. Materials

Noscapine and Cisplatin were purchased from Sigma Chemicals, St. Louis, MO, USA and Spectrum Chemicals, USA respectively. The human NSCLC cell lines H460 and A549 were obtained from American Type Culture Collection (Rockville, MD, USA). ApoTag Red *In Situ* Apoptosis detection kit® was purchased from Chemicon® International, CA, USA. DeadEnd™ Colorimetric Apoptosis Detection System was purchased from Promega (Madison, WI). Antibodies against p53, p21, pAkt, cyclin D1, survivin, PARP, cleaved PARP, Bcl_2, Bax, caspase 3, cleaved caspase 3, caspase 8 and caspase 9, were purchased from Cell Signaling Technology (Beverly, MA). Antibody to β-actin and secondary HRP were purchased from Santa Cruz Biotechnology. The cleaved caspase 3 (175) immunohistochemistry (IHC) kit was purchased from Cell Signaling (Beverly, MA). All other chemicals were either reagent or tissue culture grade.

2.2. Cell lines

H460 cells were grown in RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS). A549 cells were grown in F12K medium (Sigma, St. Louis, MO, USA) supplemented with 10% FBS. All tissue culture media contained antibiotic antimycotic solution of penicillin (5000 U/ml), streptomycin (0.1 mg/ml), and neomycin (0.2 mg/ml). The cells were maintained at 37 °C in the presence of 5% Co_2 in air.

2.3. Animals

Female Nu/Nu mice (6 weeks old form Harlan, Indianapolis, IN) were grouped and housed ($n = 8$ per cage) in sterile microisolator caging unit supplied with autoclaved Tek-Fresh bedding. The animals were kept under controlled conditions of 12:12 h light:dark cycle, 22 ± 2 °C and 50 ± 15% relative humidity. The mice were fed (irradiated rodent chow Harlan Teklad) and autoclaved water ad libitum. The animals were housed at Florida A and M University in accordance with the standards of *the Guide for the Care and Use of Laboratory Animals* and the Association for Assessment and Accreditation of Laboratory Animal Care.

2.4. In vitro cytotoxicity studies

The cancer cell lines (A549 and H460) were plated in 96-well microtiter plates, at a density of 1×10^4 cells/well and allowed to incubate overnight. The cells were treated with various dilutions of Cis in the presence or absence of Nos at 10–30 and 30–50 μM against H460 and A549 cells respectively. The plates were incubated for 72 h at 37 ± 0.2 °C in a 5% CO_2-jacketed incubator. Cell viability in each treatment group was determined by crystal violet dye assay.

2.5. Data analysis for the combination treatments

The interactions between Cis and Nos were evaluated by the isobolographic analysis, a dose-oriented geometric method of assessing drug interactions [29,30]. For 50% toxicity, the combination index (CI) values were calculated based on the equation stated below:

$$\mathrm{CI} = \frac{(D)_1}{(Dx)_1} + \frac{(D)_2}{(Dx)_2} + \alpha\frac{(D)_1(D)_2}{(Dx)_1(Dx)_2} \cdots$$

where $(Dx)_1$: dose of drug 1 to produce 50% cell kill alone; $(D)_1$: dose of drug 1 to produce 50% cell kill in combination with $(D)_2$; $(Dx)_2$: dose of drug 2 to produce 50% cell kill alone; $(D)_2$: dose of drug 2 to produce 50% cell kill in combination with $(D)_1$; $\alpha = 0$ for mutually exclusive or 1 for mutually non-exclusive modes of drug action. CI > 1.3 antagonism; CI 1.1–1.3 moderate antagonism; CI 0.9–1.1 additive effect; CI 0.8–0.9 slight synergism; CI 0.6–0.8 moderate synergism; CI 0.4–0.6 synergism; CI 0.2–0.4 strong synergism.

2.6. Induction of apoptosis in H460 and A549 cells

To detect apoptotic cells, the ApoTag Red *In Situ* Apoptosis detection kit® (Chemicon® International, CA, USA) was used. Cells were plated at a density of 1×10^6 cells/well in 6-well plates and incubated overnight. H460 cells were treated with Cis (0.8 μM), or Nos (30 μM), or combination and A549 cells were treated with Cis (2.5 μM), or Nos (40 μM), or combination. Untreated cells were used as control. After 72 h, cells were fixed in 4% paraformaldehyde and mounted onto slides using Cytospin® (Shandon). Equilibration buffer was added to slides and incubated for 10 min followed by incubation in working strength TdT enzyme at 37 °C for 1 h. The slides were incubated in stop/wash buffer for 10 min at room temperature. Working strength anti-digoxinenin conjugate (rhodamine) was added to each slide for 30-min incubation at room temperature. The images on the slides were visualized with an Olympus BX40 fluorescent microscope equipped with a computer-controlled digital camera (DP71, Olympus Center Valley, PA, USA). To quantify the apoptotic cells from terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay, 100 cells from 6 random microscopic fields were counted.

2.7. Western blot of NSCLC cells

Protein was extracted from untreated, Nos, Cis and Nos + Cis treated (72 h) cells in RIPA buffer (50 mM Tris–HCl, pH 8.0, with 150 mM sodium chloride, 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxychlorate, and 0.1% sodium dodecyl sulfate) with protease inhibitor (500 mM phenylmethylsulfonyl fluoride). Protein content was measured using BCA Protein Assay Reagent Kit (PIERCE, Rockford, IL). Equal amounts of supernatant protein (50 μg) from the control and different treatments were denatured by boiling for 5 min in SDS sample buffer, separated by 10% SDS-PAGE, transferred to nitrocellulose membranes for immunoblotting. Membranes were blocked with 5% skim milk in Tris–buffered saline with Tween 20 [10 mM Tris–HCl (pH 7.6), 150 mM Nacl, and 0.5% Tween 20] and probed with antibodies against p53 (1:500), p21 (1:500), caspase 3 (1:500), PARP (1:1000), cleaved PARP (1:1000), Bax (1:500), Bcl_2 (1:500), survivin (1:1000) and β-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Horseradish