The influence dose of nicotine exposure on H520 smoking-related Non-Small-Cell Lung carcinoma cell growth and toxicity

Ching-Yi Peng[2], Jia-Ping Wu[1]

[1]Research Center for Healthcare Industry Innovation, National Taipei University of Nursing and Health Sciences, Taipei City 11219, Taiwan, R.O.C.
[2]Department of Gerontological Health Care, College of Nursing, National Taipei University of Nursing and Health Sciences, Taipei City 11219, Taiwan, R.O.C.

Correspondence author: Jia-Ping Wu[1]
[1]Research Center for Healthcare Industry Innovation, National Taipei University of Nursing and Health Sciences. No. 365, Mingde Rd., Beitou Dist., Taipei City 11219, Taiwan, R.O.C.
Phone:886-2-2822-7101 Ext. 4216
Fax: 886-2-2820-6729
EMAIL: affymax0823@yahoo.com.tw

Running title: Dose-dependent effects of nicotine exposure on growth.

Abbreviations:
NSCLC, Non-Small-Cell Lung carcinoma cell
α7nAchR, α7nicotinic-acetylcholine receptors
EGFR, epidermal growth factor receptor
BSA, Bovine serum albumin
CYP2A6, cytochrome c P450 2A6
CYP2A13, cytochrome c P450 2A13
CYP1B1, cytochrome c P450 1B1
NOS2, Nitric oxide synthase 2

Abstract

Background and Objectives: Nicotine exposure may affect NSCLC is associated with lung cancer in humans. Whether nicotine exhibits carcinogenesis promoted activities in tumor growth still unknown. Nicotine is known to have dichotomous effects on cancer biology, acting like a pro- or anti-

carcinogenesis agent. There are different functions between adenocarcinoma and squamous NSCLC cancer cells. Excess generation of nicotine may inhibit mitochondrial metabolism, protein modification, and DNA cleavage. **Materials and Methods:** We used the H520 NSCLC line obtained from human lung epithelial cells to detected nicotine growth and toxicity using MTT assay and western blotting. The concentration of nicotine stimulated cell growth to correspond to low concentration, while high concentration was cytotoxic. **Results:** According to MTT assay results, at 1.0 µM nicotine has significantly enhanced the H520 cell viability (%). Nicotine induced lung cancer carcinogenesis through mechanisms of α7nAchR, EGFR, HDAC2/4/5, Cyclin D/Cyclin E, Bcl-2, p-Akt, and inflammatory proteins of NF-KappaB and COX2 increases at 1.0 µM. Apoptosis proteins were decreases at 1.0 µM nicotine by p21, p27, c-jun, and p38α using western blotting. Nicotine stimulates tumor growth is mediated through α7nicotinic-acetylcholine receptors (α7nAChR), possibly involving inflammation. On the other hand, at high nicotine concentrations (> 1.0 µM) with consistent cytotoxic effects and appeared to be due to direct cell kill. Nicotine can prevent apoptosis induced by NSCLC. **Conclusion:** Therefore, the effects on chemotherapeutics by NSCLC malignant cell lines, nicotine in concentrations as low as 1.0 µM decreased. These mechanisms are responsible for the genotoxic effects caused by nicotine. This leads to downstream effects on decreased apoptosis, increased cell proliferation and transformation. The malignant NSCLC cells respond to the treatment with nicotine in lung cancer, the nicotine-mediated induction of growth may provide one of its links to α7nAchR or EGFR.

Keywords: Nicotine, carcinogenesis, growth, cytotoxicity, carcinoma, Non-Small-Cell Lung.

Introduction

Cigarette smoking has been considered one of risk factors for lung cancer cells. Nicotine constitutes the dry weight of cigarette. Nicotine exposure is an important component in cigarette but is a non-carcinogen [1]. Nicotine exerts its biologic function mainly through nicotinic acetylcholine receptors (nAChR) and/or EGF receptors (EGFR) in the lung cancer cells. Among various subtypes of nicotinic receptors, α7nAChR, can bind nicotine with highest affinity and mediate multiple effects of nicotine in lung cancer. α7nAChR and EGFR expressed on lung carcinoma form a part of a proliferative network facilitating the growth of NSCLC cells [2]. Nicotine could induce the proliferation of a variety of lung carcinoma cell clines, but there is no evidence that nicotine itself provokes cancer. Nicotine alone is generally accepted as a tumor promoter, but not a tumor initiator in carcinogenesis [3]. Effects of nicotine on cancer growth have been demonstrated on NSCLC cells. Nicotine promoted NSCLC lung cancer in all patients, the role of nicotine underlying mechanisms through α7nAChR nicotine receptor signaling in lung cancer [4]. Nicotine increases migration and invasion of lung cancer cells through activation of the α7nAChR. Nicotine binds to α7nicotinic-acetylcholine receptors (α7nAchR) and EGF receptor, leading to activation of the HDAC2/4/5 and cell cycle signaling pathway [5]. Nicotine directly regulated COX2 expression in a α7nAchR dependent manner. Its activation resulted in regression of tumor cell growth and inactivation of cellular apoptosis via cell cycle arrest phase in lung cancer cells [6, 7]. Cellular cytotoxicity was associated with inhibition of DNA synthesis, not stimulation of DNA synthesis [8]. The role of the NF-kappa B activation in protecting the cells is involved in cell survival, proliferation, and apoptosis by

activating anti-apoptotic mechanisms [9]. Nitric oxide (NO) from L-arginine by NO synthase (NOS2) contributed in inflammatory response [10]. The activities of c-jun and p38 MAP kinases show to be involved in apoptosis [11]. The specific development of cancer growth roles of individual HDACs is closely linked to regulation of cell proliferation, apoptosis, and cell cycle. Therefore, nicotine alone is a generally accepted as a tumor promoter, but not a tumor initiator in carcinogenesis.

Materials and Methods

Cell culture and nicotine treatment

H520 NSCLC lung cancer lines were obtained from human lung epithelial cells. Lung cancer cells grown in RPMI-1640 medium with 10% fetal bovine serum containing 100 units/mL penicillin, and 100 μg/mL streptomycin, in a 5% CO_2 atmosphere at 37 °C. Modified to contain Earle's Balanced Salt Solution, non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate. This reduced level of sodium bicarbonate (NaHCO3, 1.5 g/L) is intended for use in 5% CO2 in air. Additional sodium bicarbonate may be required for use in incubators containing higher percentages of CO2. All lung cancer cells were purchased from Bioresource Collection and Research Center (BCRC 60124) in Taiwan. For nicotine reagent nicotine (N3876; Sigma-Aldrich Co., St. Louis, MO) treatment, H520 lung cancer cells were received 0, 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 uM nicotine for 36 h for designed doses.

Western blot analysis

H520 NSCLC cells proteins were separated on 12% SDS-PAGE gel, and blotted onto PVDF membranes. After incubated with primary antibodies

including α7nAchR (ab216485)(abcam, Cambridge, MA, USA), EGFR (sc-373746), HDAC2 (sc-9959), HDAC4 (sc-46672), HDAC5 (sc-133225), Cyclin D (sc-8396), Cyclin E (sc-377100), Bcl-2 (sc-7382), p-Akt (sc-514032), p21 (sc-6246), p27 (sc-1641), COX2 (sc-376861), c-jun (sc-74543), p38α (sc-81621) (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) and ß-actine (ab179467) (abcam, Inc., Cambridge, MA, USA). And incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (abcam, Inc., Cambridge, MA, USA). After wash, immunoreactive bands were detected with enhanced chemiluminescence (ECL) reagent.

Immunofluorescence staining

H520 NSCLC cells were grown on 6 cm dish poly-Llysine-coated cover slips at 37°C. After next day, the cells were treated with 0, 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 uM concentration of nicotine for 36 h for the treatment doses. After 36 h treatment, the cells were fixed in 4% paraformaldehyde in PBS for 10 min. Permeabilised with 0.1% Triton X-100 for 20 min, and blocked with 10% BSA in PBS for 30 min. Followed processing lung cancer cells incubated with HDAC2 primary antibody at 4°C overnight and FITC-conjugated secondary antibody (1:500, Invitrogen), and nuclei were labeled with DAPI. Extensive washing with PBS will be performed between each step and examined by confocal microscope (OLYMPUS FV500/BX Laser Scanning Confocal Microscope).

Statistical analysis

All data are presented as the mean ± SEM. Three independent repeats were conducted in MTT assay. Error bars represent these repeats. Statistical analyses were performed using the Student t test. $P < 0.05$, $P < 0.01$ was

accepted as statistically significant. All statistical analyses were performed with the GraphPad Prism 6 software package.

Results

During nicotine in concentrations as low as 1.0 µM, nicotine activates cell migration, proliferation, survival, and anti-apoptotic effects exerted and modulation chemotherapeutics on several different malignant cell lines. On the other hand, at high nicotine concentration (> 1.0 µM) with consistent cytotoxic effects and appeared to be due to direct cell kill. The concentrations of nicotine promote cell proliferation correspond to the low concentrations, while high concentrations are cytotoxic (3.0 µM, $p<0.0001$). As is well known, lung cancer remains one of the most common types of fatal malignancies. That is because we found at 1.0 µM nicotine concentration has the highest cancer growth cell visability (%) increases (Figure 1). We obtained a dose-response curve at 0, 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 µM during 24~72 h. The concentration of 0.5 µM nicotine could find at 12, 24 and 72 h have significant difference increases ($p<0.05$). The concentration of 1.0 µM nicotine found at 72 h has significant difference ($p<0.05$) (Figure 2). Too high nicotine concentration is toxicity. Even though human body cell or cancer cell. Especially, exposure nicotine to long time high dose. Thus, we found lower 1.0 µM nicotine induced lung cancer growth. This result we could find on the concentration of 0.5 and 1.0 µM nicotine exposure at 36 h increased cell viability (%) (Figure 3A). In contrast, above 3.0 µM nicotine had a significant decrease ($p<0.0001$). These effects of nicotine occur at the concentrations of 1.0 µM or less lead to disruption of the critical balance between cell death and growth. Smoking level increases nicotine receptor binding in dose-dependent manner. We detected HDAC2 signaling

pathways involved in nicotine regulation of growth of human non-small-cell lung carcinoma cell (NSCLC) by immunofluorescence staining. Figure 3B showed HDAC2 at 1.0 μM nicotine has higher than other dose. This express was HDAC2 signaling related nicotine in the cytosol and nuclear. Result showed α7 nicotinic-acetylcholine receptor (α7nAChR) was with nicotine increasing doses. Although nicotine has been implicated as a potential factor in the pathogenesis of human NSCLC lung cancer, because nicotine contribute to carcinogenesis by activating α7nAChR or EGFR on lung tumors and epithelial cells, its mechanism of action in the development of lung cancer remains largely unknown, we detected whether differential expression of α7nAChR or EGFR expression in H520 NSCLC cells after nicotine treatment could explain the different association with smoking. We detected EGFR and HDAC2/4/5 expression in H520 NSCLC cells, EGFR was pivotal in inducing tumor promotion and anti-apoptosis in cancer cells by HDAC2/4/5 inducing COX2 and NFκB. Results showed α7nAChR, EGFR, HDAC2, HDAC4 and HDAC5 protein expression levels at 1.0 μM nicotine concentration H520 NSCLC cancer cell have the highest dose ($p<0.01$) (Figure 3C and 3D). Inflammation factors, COX2 and NF-KB, protein expression levels at 1.0 μM nicotine concentration H520 NSCLC cancer cell have the highest dose ($p<0.01$ and $p<0.05$, respectively) (Figure 3E). Inflammation induces lung cancer cell growth after nicotine treatment. May through Bcl2/p-Akt and cell cycle critical pathways activation associated with tumorigenesis and tumor growth. From Figure 4, Bcl2/p-Akt and cell cycle check proteins, Cyclin D1 and Cyclin E, have significant increased dose at 1.0 μM nicotine concentration ($p<0.01$) (Figure 4A and 4B), but suppression genes, p21 and p27, have significant difference decreases at 1.0 μM nicotine concentration ($p<0.01$ and $p<0.05$, respectively)

(Figure 4C). As dose increased above 1.0 µM concentration, p21 and p27 protein were increases by nicotine induced cell apoptosis. The same results also observed in pro-inflammation of c-jun and p38α decreased at 1.0 µM nicotine concentration in H520 squamous cancer cell (Figure 4D). Higher than 1.0 ~ 3.0 µM concentration, p21, p27, c-jun and p38α were induced H520 NSCLC cancer cell apoptosis (p<0.01). Higher nicotine concentration will induce cell cytotoxicity. CYP 2A6 and CYP 2A13 have higher expression at above 1.0 µM nicotine concentration in H520 squamous cancer cell (Figure 5B). The production of NO was assessed by NOS2. As low as the concentration of 0.1 µM nicotine, it increased the pro-inflammatory cytokines, NO production, NOS2 (p<0.01) (Figure 5A) and CYP1B1 (p<0.01) (Figure 5B). In contrast, above 0.1 µM nicotine concentration, NOS2 and CYP1B1 have significant difference decreases (p<0.01). That may suggest nicotine induced cytotoxicity increases lead to cell apoptosis, not cancer therapy effects.

Discussion

Develop cigarette smoking strategies for the interplay lung cancer with nicotine as well as potentially cell growth in NSCLC. The underlying mechanisms responsible of the nicotine dose-experimental works has described for the carcinogenic potential of HDACs content in apoptosis and cell cycle regulation of H520 NSCLC cells [12]. Low concentrations of nicotine are reported to promote inflammation led to tumor growth, whereas high nicotine influx acts as a potent tumor repressor, leading to cytotoxicity and apoptosis [13, 14]. Like anything that enters the body, nicotine can be metabolized in lung and kidney via several enzymes such as cytochrome P450 2A6 (CYP2A6), cytochrome P450 2A13 (CYP2A13), cytochrome P450 1B1 (CYP1B1) [15]. The nicotine

concentration in blood plasma of smokers varies. The average human plasma concentration of nicotine in heavy smokers is 0.6 µM. The half-life of nicotine in human plasma is around 2 h [16, 17]. The concentration of nicotine stimulated cell growth to correspond to low concentration was needed [18, 19]. Exposure to environmental nicotine have been shown to cause nephrotoxicity [20]. Nicotine also stimulates tumor growth mediated through α7nAChR and EGFR, possibly involving inflammation production of COX2 and NF-KB [21, 22]. Our data showed the modulation of cell growth by exposure to nicotine is mediated by cell cycle (Cyclin D/Cyclin E) and growth inhibition (p27/p21). Therefore, our data revealed the expression of Cyclin D/Cyclin E and p27/p21 was dose-response curve increases and decreases at 1.0 µM concentration of nicotine. On the other hand, NSCLC cells included adenocarcinoma and squamous lung cancer cells [23]. HDACs inactivation resulted in regression of tumor cell growth and activation of cellular apoptosis via c-jun and p38α activation and Bcl2/p-Akt suppression [24, 25]. Our data showed anti-apoptotic proteins Bcl-2 and p-Akt increased and pro-apoptotic proteins decreased with exposure nicotine. The nicotine concentration in the tumor microenvironment plays a key role in the promotion of tumor growth, angiogenesis, and metastasis [26]. Therefore, we suggest that the molecular basis of NSCLC cell growth as well as the level of growth signaling pathway proteins were the dose- and duration-dependent curve effects of concentration of nicotine exposure.

Conclutions

Standardizing cigarette smoking levels is also likely to be important. Nicotine increases binding the receptors in a dose- and duration-dependent manner. Nicotine is unusual in comparison to most drugs, as its concentration profile

changes from induced tumor growth from inflammation to cytotoxic with doses.

There is increasing interest in developing nicotine releasing materials as potent

tumoricidal agents in which high and localized concentrations of nicotine may

be directly released in a sustained manner to the tumor site. Therefore, a

suggested activity that increases your metabolic rate can help speed up the

clearance of nicotine.

Acknowledgments
No available.

Conflicts of interests
Author discloses no conflicts of interest including any financial.

References

1. Powan, P.; Chanvorachote P. Nitric oxide mediates cell aggregation and mesenchymal to epithelial transition in anoikis-resistant lung cancer cells. *Mol Cell Biochem.* **2014**, 393, 237-245.

2. Kyte, S.L.; Gewirtz, D.A. The Influence of Nicotine on Lung Tumor Growth, Cancer Chemotherapy, and Chemotherapy-Induced Peripheral Neuropathy. *J Pharmacol Exp Ther.* **2018**, 366, 303-313.

3. Bao, N.; Ou, J.; Xu, M.; Guan, F.; Shi, W.; Sun, J.; Chen, L. Novel NO-releasing plumbagin derivatives: Design, synthesis and evaluation of antiproliferative activity. *Eur J Med Chem.* **2017**, 137, 88-95.

4. Muto, S.; Takagi, H.; Owada, Y.; Inoue, T., Watanabe, Y.; Yamaura, T. et al. Serum Nitric Oxide as a Predictive Biomarker for Bevacizumab in Non-small Cell Lung Cancer Patients. *Anticancer Res.* **2017**, 37, 3169-3174.

5. Yamamoto, Y.; Kiyohara, C.; Suetsugu-Ogata, S.; Hamada, N.; Nakanishi, Y. Biological interaction of cigarette smoking on the association between genetic polymorphisms involved in inflammation and the risk of lung cancer: A case-control study in Japan. *Oncol Lett.* **2017**, 13, 3873-3881.

6. Du, X.; Qi, F.; Lu, S.; Li, Y.; Han, W. Nicotine upregulates FGFR3 and RB1 expression and promotes non-small cell lung cancer cell proliferation and epithelial-to-mesenchymal transition via downregulation of miR-99b and miR-192. *Biomed Pharmacother.* **2018**, 101, 656-662.

7. Wang, C.; Gu, W.; Zhang, Y., Ji, Y.; Wen, Y.; Xu, X. Nicotine promotes

cervical carcinoma cell line HeLa migration and invasion by activating PI3k/Akt/NF-κB pathway in vitro. *Exp Toxicol Pathol.* **2017**, 69, 402-407.

8. Schaal, C., Chellappan, S. Nicotine-Mediated Regulation of Nicotinic Acetylcholine Receptors in Non-Small Cell Lung Adenocarcinoma by E2F1 and STAT1 Transcription Factors. *PLoS One.* **2016**, 11, e0156451.

9. Nair, S.; Bora-Singhal, N.; Perumal, D., Chellappan, S. Nicotine-mediated invasion and migration of non-small cell lung carcinoma cells by modulating STMN3 and GSPT1 genes in an ID1-dependent manner. Mol Cancer. **2014**, 13, 173.

10. Zhang, J.; Kamdar, O.; Le, W.; Rosen, G.D.; Upadhyay, D. Nicotine induces resistance to chemotherapy by modulating mitochondrial signaling in lung cancer. *Am J Respir Cell Mol Biol.* 2009, 40, 135-146.

11. Grozio, A.; Catassi, A.; Cavalieri, Z.; Paleari, L.; Cesario, A.; Russo, P. Nicotine, lung and cancer. *Anticancer Agents Med Chem.* **2007,** 7, 461-466.

12. Jensen, K.P.; DeVito, E.E.; Sofuoglu, M. How Intravenous Nicotine Administration in Smokers Can Inform Tobacco Regulatory Science. *Tob Regul Sci.* **2016**, 2, 452-463.

13. Bordas, A.; Cedillo, J.L.; Arnalich, F.; Esteban-Rodriguez, I.; Guerra-Pastrián, L.; de Castro J. et al. Expression patterns for nicotinic acetylcholine receptor subunit genes in smoking-related lung cancers. *Oncotarget.* **2017**, 8, 67878-67890.

14. Tsimberidou AM. Targeted therapy in cancer. *Cancer Chemother Pharmacol.* **2015**, 76, 1113-1132.

15. Zhang, C.; Yu, P.; Zhu, L.; Zhao, Q.; Lu, X.; Bo, S. Blockade of α7 nicotinic acetylcholine receptors inhibit nicotine-induced tumor growth and vimentin expression in non-small cell lung cancer through MEK/ERK signaling way. *Oncol Rep.* **2017**, 38, 3309-3318.

16. Zhang, C.; Ding, X.P.; Zhao, Q.N.; Yang, X.J.; An, S.M.; Wang, H. et al. Role of α7-nicotinic acetylcholine receptor in nicotine-induced invasion and epithelial-to-mesenchymal transition in human non-small cell lung cancer cells. *Oncotarget.* **2016**, 7, 59199-59208.

17. Hahn, S.S.; Tang, Q.; Zheng, F., Zhao, S.; Wu, J. GW1929 inhibits α7 nAChR expression through PPARγ-independent activation of p38 MAPK and inactivation of PI3-K/mTOR: The role of Egr-1. *Cell Signal.* **2014**, 26, 730-739.

18. Kyte, S.L.; Gewirtz, D.A. The Influence of Nicotine on Lung Tumor Growth, Cancer Chemotherapy, and Chemotherapy-Induced Peripheral Neuropathy. *J Pharmacol Exp Ther.* **2018**, 366, 303-313.

19. Yuge, K.; Kikuchi, E.; Hagiwara, M.; Yasumizu, Y.; Tanaka, N.; Kosaka, T et al. Nicotine Induces Tumor Growth and Chemoresistance through Activation of the PI3K/Akt/mTOR Pathway in Bladder Cancer. *Mol Cancer Ther.* **2015,** 14, 2112-2120.

20. Nishioka, T.; Tada, H.; Ibaragi, S.; Chen, C.; Sasano, T. Nicotine exposure induces the proliferation of oral cancer cells through the α7 subunit of the nicotinic acetylcholine receptor. *Biochem Biophys Res Commun.* **2019**, 509, 514-520.

21. Nakayama, H.; Numakawa, T.; Ikeuchi, T. Nicotine-induced phosphorylation of Akt through epidermal growth factor receptor and Src in PC12h cells. *J Neurochem.* **2002**, 83, 1372-9.

22. Simakajornboon, N.; Kuptanon, T.; Jirapongsuwan, P. The effect of prenatal nicotine exposure on PDGFR-mediated anti-apoptotic mechanism in the caudal brainstem of developing rat. *Neurosci Lett.* **2010**, 478, 46-50.

23. Liu, X.; Shao, Y.; Zhou, J.; Qian, G.; Ma, Z. Nuclear Factor κB Signaling and Its Related Non-coding RNAs in Cancer Therapy. *Mol Ther Nucleic Acids.* **2020**; 19, 208-217.

24. Yang, S.H.; Lee, T.Y.; Ho, C.A. Yang, C.Y. Huang, W.Y.; Lin, Y.C. et al. Exposure to nicotine-derived nitrosamine ketone and arecoline synergistically facilitates tumor aggressiveness via overexpression of epidermal growth factor receptor and its downstream signaling in head and neck squamous cell carcinoma. PLoS ONE. **2018**, 13, 1-16.

25. Lam, D.C.; Girard, L.; Ramirez, R.; Chau, W.S.; Suen, W.S.; Sheridan, S. et al. Expression of nicotinic acetylcholine receptor subunit genes in non-small-cell lung cancer reveals differences between smokers and nonsmokers. Cancer Res. **2007**; 67, 4638-47.

26. Grozio, A.; Paleari, L.; Catassi, A.; Servent, D.; Cilli, M.; Piccardi, F. et al. Natural agents targeting the alpha7-nicotinic-receptor in NSCLC: a promising prospective in anti-cancer drug development. Int J Cancer. **2008**; 122,1911-5.

Figure Legends

Figure 1. A dose-response curve of nicotine concentration by MTT analysis. Statistical analysis of cell viability (%) with the concentration of nicotine at 0, 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 uM for designed doses received for 6, 12, 24, 48, 72, 96 and 120 h using MTT assay. All data was expressed mean ± SEM, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.01$ significant difference compared with control without treatment nicotine.

Figure 2. Duration-dependent effects of nicotine exposure on H520 NSCLC cancer cell growth.
Statistical analysis of cell viability (%) with the different times at 6, 12, 24, 48, 72, 96 and 120 h effects on concentration of nicotine at 0, 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 uM for cancer cell growth using MTT assay. All data were expressed mean ± SEM, $^{*}p<0.05$, $^{**}p<0.01$ significant difference compared with control without treatment nicotine.

Figure 3. The relation of α7nAchR, cell growth and inflammation factors molecular mechanisms with nicotine concentration in H520 NSCLC squamous lung cancer cells.
(A). Statistical analysis of cell viability (%) with nicotine concentration at 0, 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 uM for 36 h in the H520 NSCLC cell using MTT assay. Data was expressed mean ± SEM, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.01$ significant difference compared with control without treatment nicotine. (B). Immunofluorescence staining of HDAC2 protein expression levels at 0, 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 uM concentration of nicotine. DAPI is in blue. HDAC2 is in green. (C).Protein expression of α7nAchR and EGFR relative with nicotine treatment using western blotting analysis. Quantification of western blot analysis. All data was expressed mean ± SEM, $^{*}p<0.05$, $^{**}p<0.01$ significant difference compared with control without treatment nicotine. (D). Nicotine dose concentration curve relative cell growth protein expression levels of HDAC2, HDAC4 and HDAC5 in the H520 NSCLC cancer cells using western blotting analysis. Quantification of western blot analysis. All data was expressed mean ± SEM, $^{*}p<0.05$, $^{**}p<0.01$ significant difference compared with control without treatment nicotine. (D). Nicotine dose-dependent curve relative inflammation protein expression of COX2 and NF-KB using western blotting analysis in the

H520 NSCLC cancer cells. Quantification of western blot analysis. All data was expressed mean ± SEM, $^*p$<0.05, $^{**}p$<0.01 significant difference compared with control without treatment nicotine.

Figure 4. Nicotine dose-response curve relative cell growth and apoptosis mechanisms using western blotting analysis.

(A). Nicotine dose-dependent curve relative cell growth protein expression of Bcl2 and p-Akt1 using western blotting analysis in the H520 NSCLC cancer cells. Quantification of western blot analysis. All data was expressed mean ± SEM, $^*p$<0.05, $^{**}p$<0.01 significant difference compared with control without treatment nicotine. (B). Nicotine dose-dependent curve relative cell cycle protein expression of Cyclin D1 and Cyclin E using western blotting analysis in the H520 NSCLC cancer cells. Quantification of western blot analysis. All data was expressed mean ± SEM, $^*p$<0.05, $^{**}p$<0.01 significant difference compared with control without treatment nicotine. (C) Nicotine dose-dependent curve relative cell cycle suppress protein expression of p27 and p21 using western blotting analysis in the H520 NSCLC cancer cells. Quantification of western blot analysis. All data was expressed mean ± SEM, $^*p$<0.05, $^{**}p$<0.01 significant difference compared with control without treatment nicotine. (D). Nicotine dose-dependent curve relative apoptosis protein expression of c-jun and p38α protein expression using western blotting analysis in the H157 NSCLC cancer cells. Quantification of western blot analysis. All data was expressed mean ± SEM, $^*p$<0.05, $^{**}p$<0.01 significant difference compared with control without treatment nicotine.

Figure 5. Nicotine induced inflammation and cell cytotoxicity in the H520 NSCLC cell growth.

(A) Concentration of nicotine induced inflammation factor, NOS2, protein levels at 0, 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 uM in the H520 NSCLC cell growth by western blotting analysis. Quantification of western blot analysis. All data was expressed mean ± SEM, $^*p$<0.05, $^{**}p$<0.01 significant difference compared with control without treatment nicotine. (B). Nicotine concentration induced cell cytotoxicity at 0, 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 uM in the H520 NSCLC cell growth by western blotting analysis. Quantification of western blot proteins, CYP2A6, CYP2A13 and CYP1B1 analysis. All data was expressed mean ± SEM, $^*p$<0.05, $^{**}p$<0.01 significant difference compared with control without treatment nicotine.
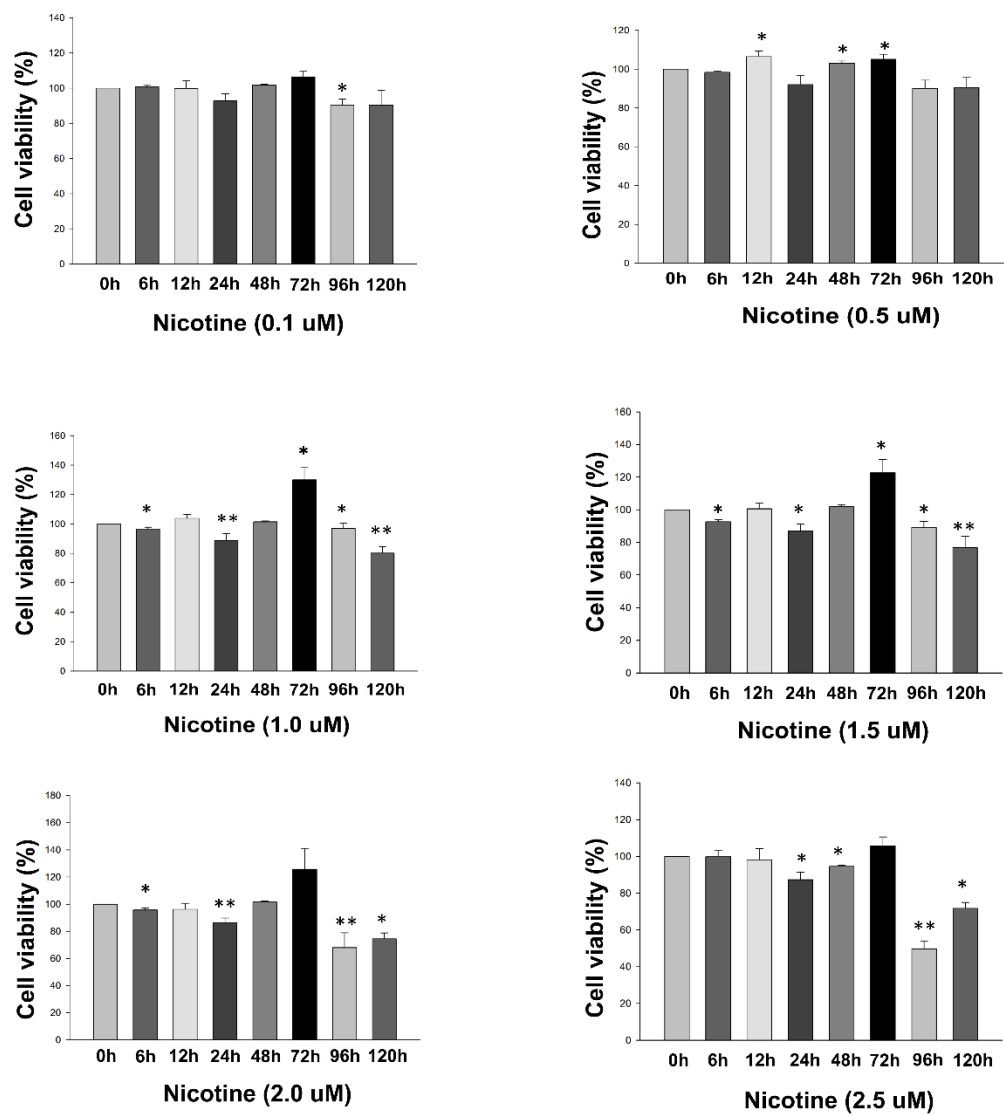
Figure 1.

Figure 2.

Figure 3.

A

B

C

D

E

Figure 4.

Figure 5.

A

B
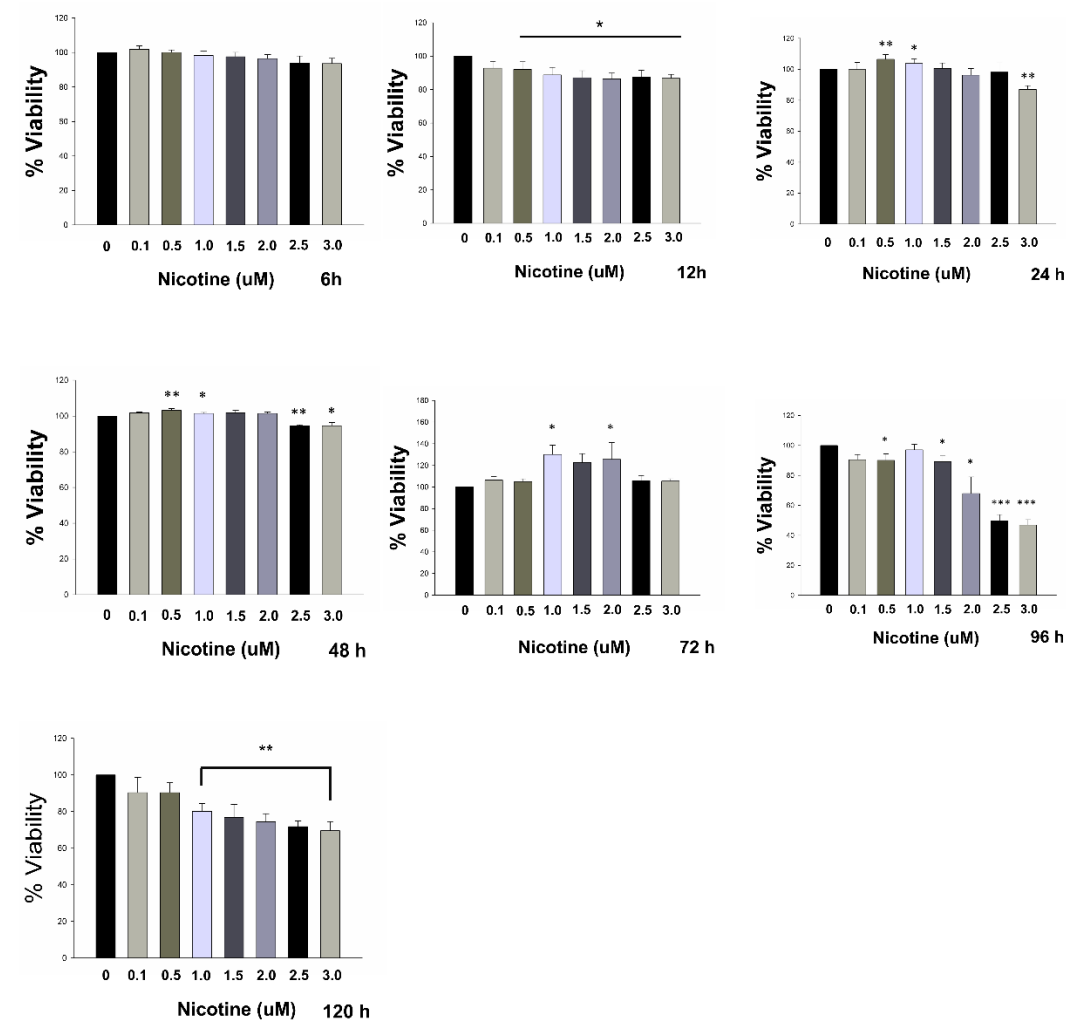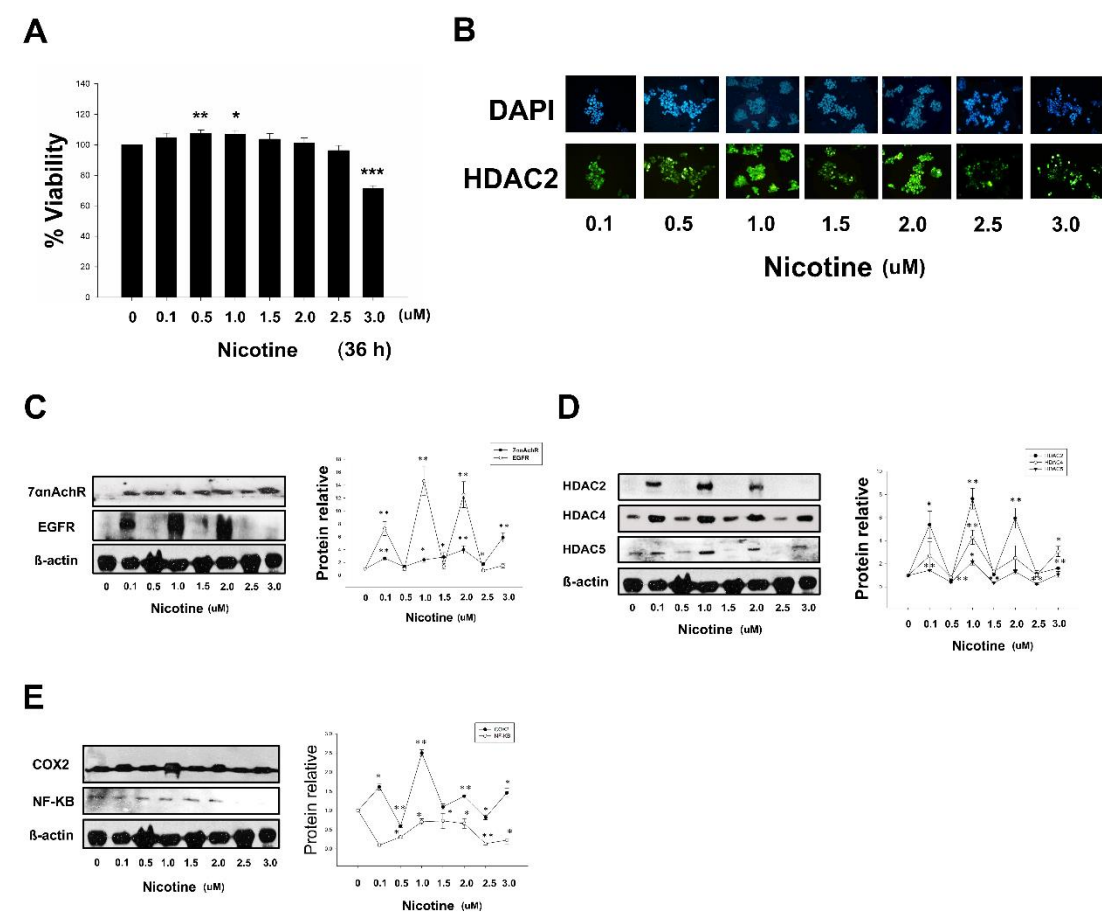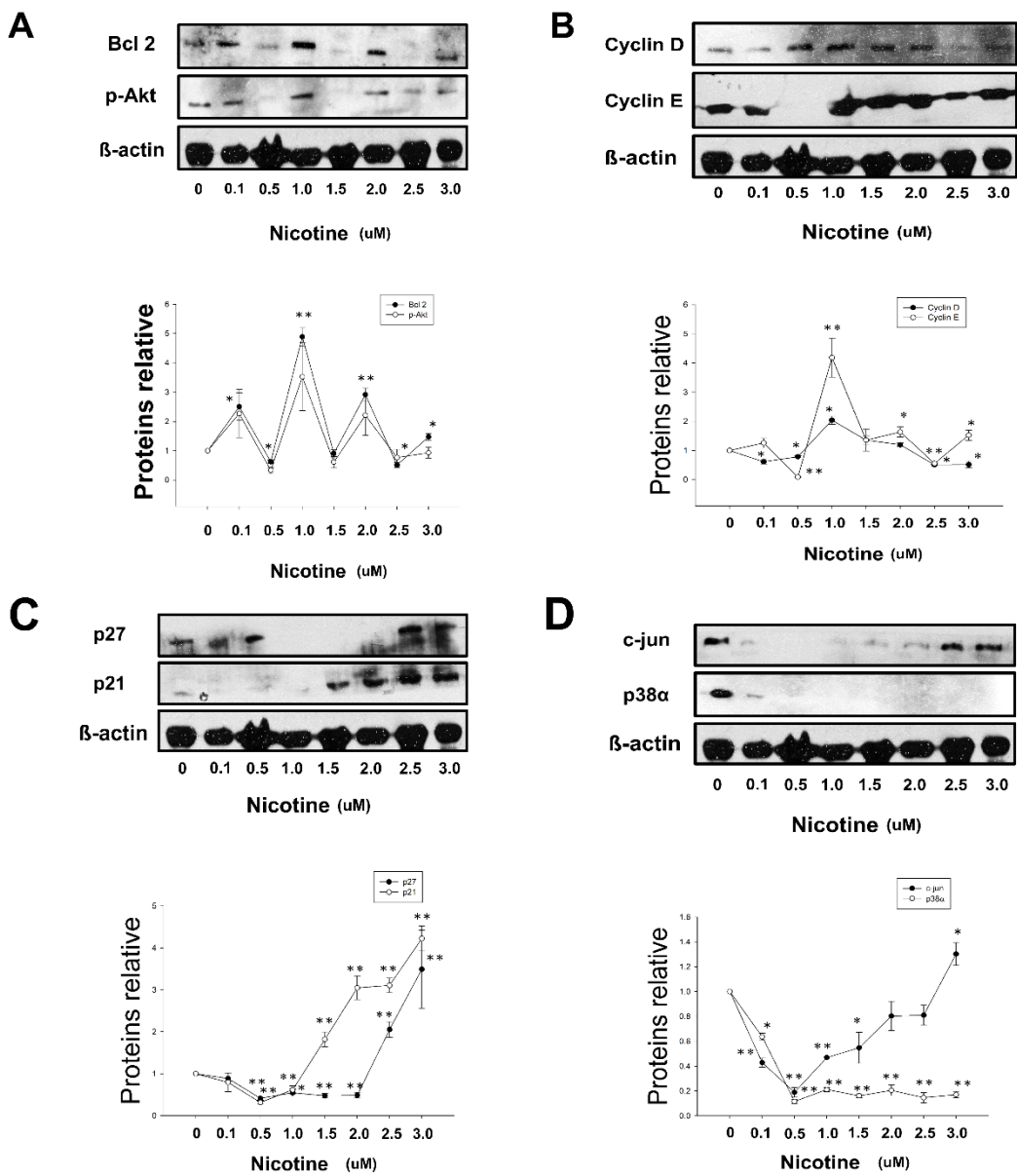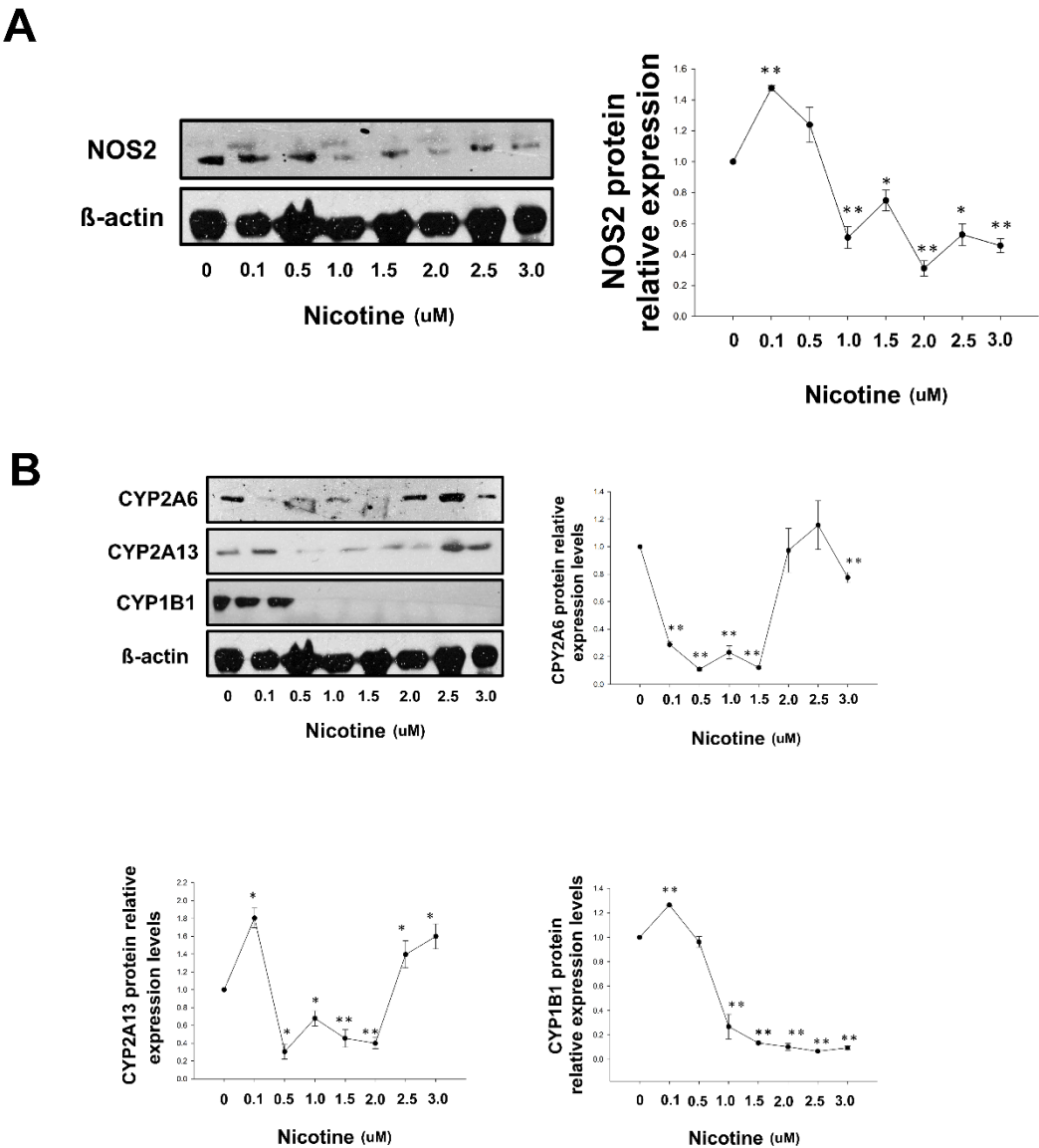
Graphical abstract:

Scheme summarizing the results. Concentration expression of nicotine induce NSCLC carcinogens from inflammation to growth and cell death. Lower 1.0 uM nicotine concentration induce NSCLC cell inflammation led to COX2, NF-KB and NOS2 increases. Cell growth related proteins including α7nAchR, EGFR, HDAC2, HDAC4, HDAC5, Bcl2, p-Akt, Cyclin D and Cyclin E were increased by 1.0 uM nicotine concentration. Above 1.0 uM nicotine concentration, at high levels concentration induce cell cytotoxicity led to CYP2A6, CYP2A13 and CYP1B1 increases. Nicotine induces cancer cell inflammation and growth.